DNA replication initiates from defined locations called replication origins; some origins are highly active, whereas others are dormant and rarely used. Origins also differ in their activation time, resulting in particular genomic regions replicating at characteristic times and in a defined temporal order. Here we report the comparison of genome replication in four budding yeast species: Saccharomyces cerevisiae, S. paradoxus, S. arboricola, and S. bayanus. First, we find that the locations of active origins are predominantly conserved between species, whereas dormant origins are poorly conserved. Second, we generated genome-wide replication profiles for each of these species and discovered that the temporal order of genome replication is highly conserved. Therefore, active origins are not only conserved in location, but also in activation time. Only a minority of these conserved origins show differences in activation time between these species. To gain insight as to the mechanisms by which origin activation time is regulated we generated replication profiles for a S. cerevisiae/S. bayanus hybrid strain and find that there are both local and global regulators of origin function.

[Supplemental material is available for this manuscript.]

The replication of eukaryotic genomes starts at multiple sites, called replication origins, and is completed during a discrete cell cycle phase, the synthesis or S phase. The budding yeast genome is replicated from hundreds of origins (Nieduszynski et al. 2007) and metazoan genomes from thousands of origins (Mechali 2010). Failure to activate sufficient or appropriately distributed origins can delay genome replication and may be the underlying cause of some human diseases (Bicknell et al. 2011a,b; Guernsey et al. 2011; Letessier et al. 2011).

Saccharomyces cerevisiae replication origins are called autonomously replicating sequences (ARS) and are primarily defined by sequence (Nieduszynski et al. 2006). Each origin contains an essential sequence element (the ARS consensus sequence or ACS) that is responsible for recruiting the Origin Recognition Complex (ORC). In turn, ORC recruits the other proteins required for origin function (Scafani and Holzen 2007). A match to the ACS motif is necessary, but not sufficient for origin function. Additional factors involved in the recruitment of ORC and subsequent origin activity include proximal nucleosomes (Lipford and Bell 2001; Nieduszynski et al. 2006; Berbenetz et al. 2010; Eaton et al. 2010; Müller et al. 2010) and a region of helical instability (Umek and Kowalski 1988).

Replication origins activate at characteristic times, with some activated early in the S phase and others later. Origins also have different efficiencies, that is, the proportion of cells in which the origin activates, with some origins active in the majority of cells while other origins are dormant and rarely used (Friedman et al. 1997; Yamashita et al. 1997). The interplay between origin location, efficiency, and activation time determines the distribution of active origins within each cell (de Moura et al. 2010).

Genome-wide measurements of S. cerevisiae replication dynamics suggest that there are broad chromosomal zones replicated from origins with similar activity; for example, clusters of late-activating origins (Raghuraman et al. 2001; Yabuki et al. 2002; McCune et al. 2008). Particular chromosomal regions have characteristic replication times: centromeric proximal regions replicate early and telomere proximal regions replicate late. The replication time of telomere proximal regions is regulated by telomere length with shorter telomeres replicated earlier (Bianchi and Shore 2007; Lian et al. 2011). Chromatin modifications have also been implicated in the regulation of replication origin activation time (Vogelauer et al. 2002; Knott et al. 2009); however, no clear correlations between specific chromatin modifications and replication time have been identified. In summary, the molecular mechanisms responsible for differences in replication time remain elusive (Scafani and Holzen 2007).

Comparative genomic approaches in the sensu stricto group of Saccharomyces yeasts revealed phylogenetic conservation of the ORC-binding site and facilitated the identification of functional ACS motifs (Theis et al. 1999; Nieduszynski et al. 2006; Chang et al. 2008). Other studies have investigated genome replication in a wider range of Saccharomycotina species. These included the plasmid-based identification of autonomously replicating sequences (ARS) in Kluyveromyces lactis (Liachko et al. 2010) and Lachancea kluyveri (Liachko et al. 2011) and the temporal order of genome replication in L. kluyveri (Payen et al. 2009) and Candida albicans (Koren et al. 2010b). These studies extend the scope of what has been learned in S. cerevisiae, confirming the existence of essential sequence elements in K. lactis and L. kluyveri origins and the role of centromeres in defining early origin activation times (Koren et al. 2010b).

Although these examples have been informative, the species examined are sufficiently divergent from S. cerevisiae to make direct comparisons difficult (Nieduszynski and Liti 2011). Here, we directly address the evolution of replication origin location and activity by comparing origin function and activity across the genomes of multiple sensu stricto species. Comparisons between species show conservation in the location and activation time of chromosomally active origins, but divergence in dormant origin location. There is significant conservation in the temporal order of genome replication, revealing that the signals responsible for directing
origin activity must be conserved. Finally, we examine the dynamics of genome replication in a hybrid between two sensu stricto yeasts and demonstrate that there are both local and global regulators of replication origin function.

Results

Comprehensive S. cerevisiae replication origin map
Phylogenetic comparisons between species benefit from a reference species with a well-annotated genome. In S. cerevisiae approximately half of the proposed origin sites (351/740) have been functionally confirmed (Siow et al. 2011). We sought to increase the number of confirmed origin sites and estimate the proportion of reported sites that are false positives. Potential origin sites that have been proposed by one or more systematic genome-wide studies (catalogued at the Replication Origin Database, OriDB) were selected to experimentally test ARS function. Our strategy utilized an established plasmid-based assay (a recombinational ARS assay) to test for ARS activity in 392 large (>4 kb) DNA clones that span potential origin sites (Liti et al. 2009a; Shor et al. 2009). We found that 183 clones tested positive and a subset (62) were cloned as smaller fragments (<500 bp) to precisely confirm the location of the origin. The remaining 209 large DNA clones tested negative, allowing us to remove the majority of the false positives currently in the literature. For example, we tested 46 sites that were proposed to be replication origins by the first chromatin-immunoprecipitation (ChIP) analysis of pre-RC proteins (Wyrick et al. 2001) but not by more recent studies. Of these sites we confirmed eight as true positives and 34 as false positives. By comparison, origin sites proposed from a more recent ChIP study (Xu et al. 2006), but not the earlier study, were found to be true positives at 57 out of 87 sites tested. These data (Supplemental Table S1) will be deposited in the DNA replication origin database (OriDB) to allow community access to this near-complete genome annotation.

Evolutionary gain and loss of replication origins in Saccharomyces sensu stricto species
We examined the conservation of replication origin location between S. cerevisiae, its nearest relative, S. paradoxus, and the most distant sensu stricto species, S. bayanus (Fig. 1A). Phylogenetic sequence conservation allowed the precise identification of functional ACS motifs. Although the majority of ACS elements were found to be conserved in at least one other sensu stricto species, we found that only 18% were conserved in the five species investigated (Nieduszynski et al. 2006). The lack of sequence conservation could reflect genuine divergence between the species or could be a consequence of limited sequence data, poor sequence alignment, or the definition of sequence conservation. To distinguish between these alternatives, we sought to directly test the degree of origin conservation. Initially we focused on the origins from chromosome 6, since these have been comprehensively mapped in S. cerevisiae, and the activation time and efficiency of each origin has been measured (Shirahige et al. 1993; Friedman et al. 1997; Yamashita et al. 1997). We selected 11 intergenic spaces that contain an origin in S. cerevisiae and identified the syntenic intergenic spaces from the other species to test for origin activity.

Figure 1. Functional conservation of replication origins on chromosome 6. (A) Phylogenetic relationship between sensu stricto yeast species (not to scale). (B) Schematic of S. cerevisiae chromosome 6 illustrating the location of replication origins (origins represented by circles; circle area corresponds to origin efficiency in W303; later origin activation time is represented by darker shading) (Yamashita et al. 1997), the centromere (black box), and a translocation relative to S. bayanus (black bar marked *). Locations equivalent to S. cerevisiae replication origins were assayed for ARS activity in S. paradoxus and S. bayanus. Ticks indicate that the replication origin is functionally conserved in the related species; crosses, that it is not. (C) Example assay plates for S. cerevisiae ARS606 and equivalent locations from S. paradoxus and S. bayanus. (D) Schematic of the gene structure around ARS606 and the fragments assayed for ARS activity in the indicated species. (E) Alignment of the ARS consensus sequence (ACS) from the indicated species.
(Supplemental Table S2). This approach ensures that any failure to detect functional conservation of origin activity would not be a consequence of limited or biased sampling. The origin activity of each region was then assessed using a plasmid-based ARS assay. In S. paradoxus we found that eight out of 11 of the sites syntenic to S. cerevisiae origins show origin activity. In S. bayanus only four of 11 tested syntenic sites showed origin activity (Fig. 1B). Additionally, in S. paradoxus we selected two regions spanning a total of ~90 kb and identified two origins that are not syntenic to S. cerevisiae origins. Positive ARS assay results were obtained for fragments that spanned chromosome 6 regions 83,364–88,948 bp and 183,584–189,865 bp. Based on negative results for overlapping regions (Supplemental Table S2) and the overwhelming tendency for origins to be located intergenically, we propose that these origins lie in the Table S2) and the overwhelming tendency for origins to be located intergenically, we propose that these origins lie in the

in S phase. Therefore, in resulting plots of relative copy number across a chromosome (Fig. 2; Supplemental Figs. 3–7) we find that early replicating sequences (i.e., origins) give rise to peaks and later replicating sequences to valleys. Mathematically, we can show that relative copy number is proportional to the mean replication time or Trep (R Retkute, AP de Moura, and CA Nieduszynski, unpubl.). Therefore, the relative copy number is a proxy for the replication time with high copy numbers corresponding to early replication times. Here we present replication profiles as relative copy numbers, but these values can easily be transformed to give the relative replication time in minutes (Koren et al. 2010a).

We compared our deep-sequencing measure of S. cerevisiae replication time with published mean replication timing (Trep) data from haploids (Raghuraman et al. 2001; Yabuki et al. 2002) and find correlation coefficients of 0.73 and 0.87, respectively (Supplemental Fig. S8); this compares to a correlation coefficient of 0.74 between the two haploid data sets. We visually compared our replication profiles with these published data sets and did not identify origins with altered replication time. Therefore, S. cerevisiae haploids and diploids replicate with similar dynamics.

**Functional conservation of replication timing in sensu stricto species**

Replication origins activate continuously throughout S phase (Yamashita et al. 1997); however, origins can be experimentally divided into “early” origins that are not subject to the intra-S phase checkpoint/ do not require Clb5-CDK activity and “late” origins (checkpoint and Clb5 dependent) (Yabuki et al. 2002; Feng et al. 2006; McCune et al. 2008). The S. cerevisiae genome is replicated from zones of “early” and “late” activating origins. We observe that these zones, including the approximate boundaries, are conserved between the sensu stricto Saccharomyces species (Fig. 2A–D; Supplemental Figs. S7, S9). For example, in S. cerevisiae centromeric zones are among the first sequences to replicate and telomere-proximal zones are among the last (Raghuraman et al. 2001). The S. paradoxus, S. arboricolus, and S. bayanus centromeres are also early replicating and the telomeres late replicating.

We compared the replication time of chromosomes 6 and 12 in the four sensu stricto species. Note that in S. bayanus the right end of chromosome 6 has been involved in a reciprocal translocation (Fischer et al. 2000), but chromosome 12 has no structure rearrangements in any of these species (although there are differences in chromosome length). Despite the changes in replication origin repertoire on chromosome 6 (see above), globally the temporal order of replication is highly conserved between the species (Fig. 2A–D, left panel). One of the S. paradoxus origins that we identified as not syntenic with an S. cerevisiae origin (in the FAR7/GCN20 intergenic space) gives a clear peak in relative copy number consistent with this origin being chromosomally active (Supplemental Fig. S10).

To allow a more direct comparison between the species we projected the data from S. paradoxus, S. arboricolus, and S. bayanus onto the S. cerevisiae chromosomal coordinates. Figure 2E highlights how replication time is remarkably similar in these organisms, despite some changes in origin (peak) location and significant sequence divergence (S. cerevisiae—S. bayanus nucleotide identity is 80% in coding regions and 62% in noncoding regions). In these replication timing profiles we only detect active replication origins, therefore these data are consistent with the location of active origins being frequently conserved (as we observed in our sys-
Figure 2. Replication “timing” profiles for chromosomes 6 and 12 from diploid *sensu stricto* species. Each profile shows the chromosomal position on the x-axis and the normalized relative copy number (as a proxy for replication time) on the y-axis. Individual points represent raw data with the line indicating smoothed data (see Methods). Profiles are shown for chromosomes 6 and 12 from *S. cerevisiae* (A), *S. paradoxus* (B), *S. arboricola* (C), and *S. bayanus* (D). The locations of experimentally confirmed *S. cerevisiae* origins are shown above the profile (only a selection of active origins are named for clarity). (D) The position of the reciprocal translocation on *S. bayanus* chromosome 6 is marked by a vertical bar. (E) The smoothed data from all three species are shown projected onto the *S. cerevisiae* coordinates to aid comparison. Note that chromosome 12 includes the rDNA and associated nonunique loci to which we have not mapped reads (*S. cerevisiae* 450–490 kb).
A minority of origins have evolved different activities

We examined the replication profiles to identify examples of replication origins with different activities between the species. The six possible pairwise comparisons between the four species revealed a total of 88 origins with a difference in activity in at least one pairwise comparison (difference in relative copy number >0.3; Supplemental Table S4). In the comparison between *S. cerevisiae* and *S. bayanus* (the most divergent pair of species that we have looked at) we identify 10 origins that are earlier in *S. cerevisiae* and 14 origins that are earlier in *S. bayanus* from a genomic repertoire of >300 chromosomally active origins (Raghuraman et al. 2001). When our *S. bayanus* replication profiles are projected onto *S. cerevisiae* coordinates we see discontinuities in the profiles at some of the sites of reciprocal translocations (Fischer et al. 2000). For example, in the projected *S. bayanus* profile on chromosome 2 (~300 kb) and chromosome 4 (~465 kb) there are clear discontinuities resulting from the associated reciprocal translocation (Supplemental Fig. S11). The translocations change the environment of some origins (including the proximity to other origins) and this may be responsible for changes in peak height (e.g., *ARS211*).

In addition, we identified changes in peak height and, therefore, changes in origin activity that are not associated with translocations. We find examples of origins that are more active (or activate earlier) in *S. cerevisiae* than *S. bayanus* and vice versa (Fig. 3B). For example, the *S. cerevisiae* origin *ARS1320* gives a significantly higher peak than the corresponding origin in *S. bayanus* (difference in relative copy number of 0.4). In *S. cerevisiae* *ARS1320* has been found to be both active and early activating; for example, it is not inhibited by the intra-S phase checkpoint (Feng et al. 2006; Crabbe et al. 2010). Conversely, the *S. cerevisiae* origin *ARS1212* activates late (and is checked by the intra-S phase checkpoint), but the corresponding origin in *S. bayanus* activates earlier (or is more active). For both of these examples we observe that the corresponding origins in *S. arboricola* have intermediate peak heights (and therefore origin activity; Supplemental Fig. S7). These examples offer the possibility to investigate the mechanisms involved in regulating origin activity. As a first step we sought to address whether the mechanisms involved act locally or globally.

Evolutionary differences in origin activity are locally regulated

We measured the replication dynamics of a stable *S. cerevisiae*/*S. bayanus* hybrid.
strain. This hybrid allows us to test whether differences in replication origin activity are a consequence of sequences local to the origin or a result of diffusible factors (assuming cross-functionality of the factors involved, which we test below). If sequences local to origins are responsible for differences in activity, we would anticipate that the differences would be equally apparent in the hybrid. Conversely, if diffusible factors are responsible, then we would anticipate that differences would be lost in the hybrid. Furthermore, measuring the replication time in the hybrid offers the opportunity to control for any differences between profiles that might have arisen due to differences in the cell sorting.

Deep-sequence data from the hybrid samples were mapped to a hybrid reference genome sequence (combined S. cerevisiae and S. bayanus genome sequences), and only those reads that mapped uniquely within the combined hybrid genome were analyzed further. Despite regions of identity between the two genome sequences, we were able to uniquely map a high proportion of the reads (75% of total reads mapped uniquely within the hybrid genome compared with 77% and 69% mapping to the S. cerevisiae and S. bayanus diploid samples, respectively). These mapped reads were then used to generate replication profiles for the hybrid genome as described above.

Resulting replication profiles from the S. cerevisiae chromosome set in the hybrid are virtually superimposable with those from the pure S. cerevisiae diploid (correlation coefficient 0.99) (Figs. 3A, 4; Supplemental Figs. S12, S13). Similarly, the replication profiles of the S. bayanus chromosome set from the hybrid genome and the S. bayanus diploid are also nearly identical (correlation coefficient 0.99) (Figs. 3A, 4; Supplemental Figs. S14, S15). When we project the hybrid replication profile from the S. bayanus chromosome set onto the data from the S. cerevisiae chromosome set we found that differences apparent in the individual diploids are also apparent in the hybrid (Figs. 3B,C, 4; Supplemental Fig. S16). For example, ARS1320 replicates earlier (or is more active) on the S. cerevisiae chromosome than the corresponding origin from the S. bayanus chromosome (Fig. 3C). Likewise, ARS1212 replicates later (or is less active) on the S. cerevisiae chromosome than the corresponding origin from the S. bayanus chromosome (Fig. 3C). These data allow us to exclude experimental differences between the S. cerevisiae and S. bayanus samples (e.g., differences in the fraction of cells sorted) as an explanation for differences in origin activity. Furthermore, the results from the hybrid are consistent with sequences local to the origins being responsible for the observed differences in activity.

We tested various possible mechanisms that could account for our observation that origin activity is regulated locally. First, we sought to exclude the possibility that the replication machinery proteins function in a species-specific manner in the S. bayanus/S. cerevisiae hybrid, since this would give a false impression of local regulation. To test this we generated hybrid strains in which the S. cerevisiae copy of essential replication genes (MCM4, MCM5, ORC1, CDC7, and CDC9) were deleted. In each case the deletion strains were viable and had a normal DNA content (Supplemental Fig. S17), indicating that the S. bayanus replication machinery can complement the S. cerevisiae deletions and replicate the S. cerevisiae half of the hybrid genome. Second, we noted that one of the genes adjacent to ARS1212 (GAL2/YLR081W) is duplicated in S. bayanus, raising the possibility that ARS1212 is also duplicated (Supplemental Fig. S18). The presence of two close origins could then

![Figure 4](https://example.com/figure4.png)
account for the observed (locally regulated) difference in replication time. However, we noted that this gene is not duplicated in either *S. paradoxus* or *S. arboricolus*, which replicate this region early, and therefore this hypothesis could not account for the changed replication time in these species. To exclude the presence of a second active origin in *S. bayanus*, we performed ARS assays across this region. We detected ARS activity in the *S. bayanus* intergene that is syntenic with *S. cerevisiae* ARS1212, but not in either of the adjacent intergenes (Supplemental Fig. S18). Therefore, there has not been a duplication of this origin and this hypothesis cannot account for the altered replication time. Finally, we looked to see whether differences in gene expression between *S. cerevisiae* and *S. bayanus* (Tsankov et al. 2010) correlated with changes in replication time. We found no correlation between differences in gene expression and differences in replication time either genome wide or close to origins with altered replication dynamics (data not shown). We conclude that, as yet unknown, local sequence-based mechanisms can have a substantial impact on replication origin activity.

**Early telomere replication in a hybrid**

We found that replication profiles from hybrid and nonhybrid diploids are virtually identical; however, one global difference was apparent. On almost every chromosome, we observed earlier telomere replication in the hybrid than the nonhybrid diploids (Fig. 4B,D; Supplemental Fig. S13). To quantify this effect we determined the difference in copy number (as a proxy for difference in replication time) between the *S. cerevisiae* diploid and the *S. cerevisiae* half of the hybrid. Plotting these differences in replication time against distance from the nearest telomere shows that the largest difference in replication time is observed at chromosomes ends, decreasing with distance from the telomere (Supplemental Fig. S19). The replication time of loci >100 kb from a telomere end are almost identical in the *S. cerevisiae* diploid and the *S. cerevisiae* half of the hybrid. This result is reminiscent of the changes in replication time observed in *yku70* mutants and other strains with shorter telomeres (Cosgrove et al. 2002; Bianchi and Shore 2007; Lian et al. 2011). Both the magnitude and extent of the effect is almost identical to that recently reported in a genomewide timing study of a *yku70* mutant (Lian et al. 2011). Telomere length has been reported to be shorter in *S. cerevisiae* than in *S. bayanus* hybrids (Martin et al. 2009) and we confirmed this observation for our hybrid strain (Supplemental Fig. S20). Negative epistasis between genes involved in maintaining telomere length has previously been reported to result in short telomeres in highly diverged *S. paradoxus* lineages (Liti et al. 2009b) and a similar effect may be responsible for the shorter telomeres that we observe. In summary, the shorter telomeres in the hybrid offer an explanation for our observation of globally earlier replication of telomere proximal sequences in the hybrid strain.

**Discussion**

We report the first analysis of genome replication in multiple species within a single clade: the *Saccharomycys sensu stricto* clade. Comparison of the replication profiles from *S. cerevisiae*, *S. paradoxus*, *S. arboricolus*, and *S. bayanus* reveal a high degree of conservation in the replication timing program (Fig. 2). Therefore, in addition to replication origin sequences showing phylogenetic conservation (Nieduszynski et al. 2006), the functional activities of origins have also been conserved across this evolutionary distance. Our study provides evidence for the strong conservation of the three established global features of *S. cerevisiae* genome replication among the *sensu stricto* clade: early centromere replication, late telomere replication, and clusters or zones of early (or late) activating origins.

To allow analysis of replication profiles in multiple different species, we combined a FACS enrichment for replicating and nonreplicating cells (Koren et al. 2010a) with deep sequencing to measure DNA copy number. We find that the resulting replication profiles are reproducible and have low noise (standard deviation of ∼5%). This methodology has allowed us to measure replication dynamics in wild diploid cells that would otherwise require manipulation to allow cell cycle synchronization. Our approach therefore allows us to analyze the replication profiles from hybrid strains. In the future this approach could be used to measure replication dynamics in almost any culturable organism with a reference genome.

Replication timing profiles allow the analysis of chromosomally active replication origins; however, approximately half of all origins in *S. cerevisiae* are dormant—that is, they are not normally active in their chromosomal context (Nieduszynski et al. 2007). In yeast the activity of these dormant origins can be assayed by their ability to support plasmid replication. Here we report two systematic plasmid-based assays for origin activity. First, we assayed for ARS activity at the majority of *S. cerevisiae* origin sites that have been proposed, but not yet confirmed. These data contribute to a community effort to confirm the localization of all *S. cerevisiae* origins and provide a reference for comparisons between species. Second, we systematically assayed for origin function in *S. paradoxus* and *S. bayanus* at sites syntenic to *S. cerevisiae* origins on chromosome 6. This analysis revealed that chromosomally active origins are predominantly conserved in location, whereas the dormant origins are poorly conserved between these species. Surprisingly, we discovered that despite complete conservation of the ARS606 ORC-binding site sequences between *S. cerevisiae* and *S. paradoxus*, we were unable to detect ARS activity at this site in *S. paradoxus*. We propose that in *S. cerevisiae*, ARS606 has evolved origin activity since the divergence from *S. paradoxus*. In the future it will be interesting to determine whether ORC binds at the syntenic location of ARS606 in *S. paradoxus*. In an analogous manner we find that the EAR7/GCN20 intergenic space is associated with origin activity in *S. paradoxus*, but not in *S. cerevisiae*, despite the observation that this intergenic space recruits ORC and Mcm2-7 in *S. cerevisiae*. Sites such as these will allow investigation of the mechanisms by which origin function evolves and may help explain why only a fraction of all sequence matches to the ORC-Binding motif (the ACS) have origin function.

Although there is extensive conservation of the replication timing program between *sensu stricto* species, we did identify a minority of origins that differ in activity between the species. These origins offer insight into the mechanisms that regulate origin activity, such as the time during S phase when the origin activates. Long-range global mechanisms that are involved in the regulation of origin activity include chromatin modifications (Friedman et al. 1996; Vogelauer et al. 2002; Aparicio et al. 2004) and proximity to a telomere or a centromere (Raghuraman et al. 2001; Pohl et al. 2012). Consistent with these findings, we observe that shorter telomeres in a *S. cerevisiae/S. bayanus* hybrid correlate with a global advancement of telomere replication time. In addition to these global regulators of origin activity, we provide evidence that there are local regulators of origin function that influence the activity of individual origins. The differences in origin activity that we observe between *S. cerevisiae* and *S. bayanus* are also apparent in a hybrid of these two species (Fig. 4). This allows us to conclude that sequences local (cis-acting) to the origins, rather than long-
range or diffusible factors (trans-acting), are responsible for these evolutionary differences in origin activity.

Local sequence determinants of origin activity might include changes to ORC affinity (Shor et al. 2009) or recruitment of additional proteins such as transcription factors that might alter the affinity of the pre-replication complex for replication initiation factors present in limited abundance (Mantiero et al. 2011; Tanaka et al. 2011). Recently the proximal binding of Forkhead transcription factors (Fkh1 and Fkh2) has been implicated in determining early origin activation time (Knott et al. 2012). We find that of the 24 origins that replicate early in *S. cerevisiae*, but later in at least one other sensu stricto species, only seven are reported to be associated with Fkh1 and/or Fkh2 in *S. cerevisiae* (Supplemental Table S4; Venters et al. 2011). Therefore, evolutionary loss of Fkh1/2 association cannot explain the observed difference in replication time for the majority of these origins. Consequently, further experiments, such as chimeric origins (Nieduszynski et al. 2005), will be required to isolate the sequences responsible for these differences. However, we have already been able to exclude a number of possible explanations for the differences in activity, including differences in the number of origins and differences in gene expression.

Temporal regulation of genome replication has been reported in many eukaryotes (Mechali 2010). The late replication of telomeres may be involved in feedback control of telomere length (Bianchi and Shore 2007), and the early replication of centromeres may be important for proper chromosome segregation (Feng et al. 2009). Furthermore, the elevated mutation rates observed in late replicating regions might exert a selective pressure for particular regions to replicate at specific times (Stamatoyannopoulos et al. 2009; Chen et al. 2010; Lang and Murray 2011; Agier and Fischer 2012; Marsolier-Kergoat and Goldar 2012). However, it remains unclear why particular chromosomal zones replicate at particular times during S phase. The remarkable conservation of these replication timing zones in the sensu stricto yeast species illustrates the potential importance of tight temporal regulation of genome replication.

**Methods**

**Yeast strains and methods**

All yeast strains and growth temperatures are listed in Supplemental Table S5. Cells were grown in standard rich or selective media as appropriate. To delete *S. cerevisiae* genes in a hybrid strain we transferred the appropriate deletion cassette from the *S. cerevisiae* gene deletion collection to the hybrid by PCR and transformation. Diagnostic PCRs and Southern blotting were used to verify gene deletions in these hybrid strains. Oligonucleotide sequences are available on request.

All ARS assays were performed in the same species as the origin DNA was isolated from. ARS assays for *S. cerevisiae* and *S. paradoxus* replication origins were performed as described previously using a recombination-based strategy (Nieduszynski and Donaldson 2009). *S. bayanus* replication origins were assayed using a conventional plasmid-based ARS assay due to the lower transformation efficiency of *S. bayanus*.

For cell sorting, exponentially growing cells from a 100-mLYPD culture were fixed in 70% Ethanol, washed with 50 mM Sodium Citrate, sonicated, and treated with RNase A and Proteinase K. DNA was stained with Sytox Green at ten times the manufacturers’ recommended concentration. Diploid cells were sorted using the MoFlo Cell Sorter (Beckman-Coulter), simultaneously taking S and G2 phase cells, to obtain between 30 and 40 million S phase cells and between 50 and 60 million G2 phase cells. The cell cycle phase of each sorted fraction was confirmed by taking an aliquot, restaining with Sytox Green and analyzing by flow cytometry (Supplemental Fig. S1). Cells obtained from FACS were resuspended in 500 µL of 1.2 M sorbitol, 200 mM Tris-HCl (pH7.5), 20 mM EDTA, 0.1% β-mercaptoethanol. Cells were spheroplasted with Zymolase and then treated with SDS, Proteinase K and RNase A. DNA was purified by phenol chloroform extraction.

**Deep sequencing**

Deep sequencing was performed on the AB SOLiD 4 analyzer platform. Sequencing libraries were made using the NEB Next kit (New England Biolabs) as advised by the manufacturer. Each sequencing sample was assigned 1/16 of an AB SOLiD sequencing slide. Resulting reads were mapped to reference genomes (Supplemental Table S3) using Bioscope 1.3.1 (LifeTechnologies). The genome sequence of *S. arboricolas* will be published elsewhere.

**Data analysis**

To generate replication timing profiles we calculated the ratio of uniquely mapped reads in the replicating (S phase) sample to the nonreplicating (G2 phase) sample. Custom Perl scripts (available on request) were used to independently calculate this ratio for every 1-kb window. We excluded windows where fewer than 250 reads were mapped in either sample. The resulting absolute ratios reflect the read numbers; therefore, we normalized data by dividing by an empirically determined factor. This resulted in >95% of the data points lying between 1 and 2 (a biologically imposed restraint). Resulting replication profiles were subjected to smoothing using a Fourier transformation, essentially as described previously (Raghuaraman et al. 2001), but excluding regions close to chromosome ends and regions with low data density (e.g., nonunique repeat units).

To compare replication profiles between species we used the liftOver tool to project smoothed data from the genome assembly of one species to the *S. cerevisiae* genome. Pearson correlation coefficients were calculated for all pairwise species combinations, limiting comparisons to genomic positions where data are available from all four species. Intersections between data sets were performed using the program closestBed (from BEDtools). The difference in replication time between species was calculated by subtracting the relative copy number in one species by that from the other (at those points where data was available for both species as determined using liftOver). Differences in gene expression level (Tsankov et al. 2010) were calculated for 4547 genes for which data was available in both species.

**Data access**

All deep sequencing data have been submitted to the NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) under accession no. GSE36045.

**Acknowledgments**

We thank Gianni Liti, Ed Louis, and Oliver Zill for the kind gift of yeast strains and species. All of the deep sequencing was performed with DeepSeq, University of Nottingham. We thank members of the Nieduszynski lab, Gianni Liti, Ed Louis, Sunir Malla, Martin Blythe, Anne Donaldson, Nick Rhind, Catherine Fox, and the University of Nottingham Flow Cytometry Facility for help, advice, and comments. Thanks to Alessandro de Moura for assistance with Fourier transformations and Cheuk Siow for assistance with...
References

Agier N, Fischer G. 2012. The mutational profile of the yeast genome is shaped by replication. *Mol Biol Evol* 29: 905–913.

Aparicio JG, Viggiano CJ, Gibson DG, Aparicio OM. 2004. The Rpd3-Sin3 histone deacetylase regulates replication timing and enables S phase origin control in *Saccharomyces cerevisiae*. *Mol Cell Biol* 24: 4769–4780.

Birnbaum, KB, Bongers EM, Leitch A, Brown S, Schoots J, Harley ME, Aftimos S, Al-Aama JY, Bober M, et al. 2011a. Mutations in ORC1, encoding the largest subunit of the origin recognition complex, cause Meier-Gorlin syndrome. *Cell* 145: 205–218.

Birnbaum, KB, Bongers EM, Leitch A, Brown S, Schoots J, Harley ME, Aftimos S, Al-Aama JY, Bober M, et al. 2011b. Mutations in the pre-replication complex binding site of ORC6, encoding the largest subunit of the origin recognition complex, cause Meier-Gorlin syndrome. *Nat Genet* 43: 355–359.

Chang J, Theis JF, Miller J, Nieduszynski CA. 2012. Novel features of ARS selection in budding yeast. *PLoS Genet* 8: e1002677. doi: 10.1371/journal.pgen.1002677.

D’Aubenton-Carafa Y, Arneodo A, Hyrien O, et al. 2010. Impact of compositional biases in yeast. *Mol Biol Evol* 27: 5071–5081.

Friedman KL, Diller JD, Ferguson BM, Nyland SV, Brewer BJ, Fangman WL. 2001. Replication initiation programs set fragility of the FRA3B fragile site. *Nature* 407: 129–133.

Giovanni Agier N, Fischer G. 2012. The mutational profile of the yeast genome is shaped by replication. *Mol Biol Evol* 29: 905–913.

Aparicio JG, Viggiano CJ, Gibson DG, Aparicio OM. 2004. The Rpd3-Sin3 histone deacetylase regulates replication timing and enables S phase origin control in *Saccharomyces cerevisiae*. *Mol Cell Biol* 24: 4769–4780.

Birnbaum, KB, Bongers EM, Leitch A, Brown S, Schoots J, Harley ME, Aftimos S, Al-Aama JY, Bober M, et al. 2011a. Mutations in ORC1, encoding the largest subunit of the origin recognition complex, cause Meier-Gorlin syndrome. *Cell* 145: 205–218.

Birnbaum, KB, Bongers EM, Leitch A, Brown S, Schoots J, Harley ME, Aftimos S, Al-Aama JY, Bober M, et al. 2011b. Mutations in the pre-replication complex binding site of ORC6, encoding the largest subunit of the origin recognition complex, cause Meier-Gorlin syndrome. *Nat Genet* 43: 355–359.

Chang J, Theis JF, Miller J, Nieduszynski CA. 2012. Novel features of ARS selection in budding yeast. *PLoS Genet* 8: e1002677. doi: 10.1371/journal.pgen.1002677.

D’Aubenton-Carafa Y, Arneodo A, Hyrien O, et al. 2010. Impact of compositional biases in yeast. *Mol Biol Evol* 27: 5071–5081.

Friedman KL, Diller JD, Ferguson BM, Nyland SV, Brewer BJ, Fangman WL. 2001. Replication initiation programs set fragility of the FRA3B fragile site. *Nature* 407: 129–133.

Giovanni Agier N, Fischer G. 2012. The mutational profile of the yeast genome is shaped by replication. *Mol Biol Evol* 29: 905–913.

Aparicio JG, Viggiano CJ, Gibson DG, Aparicio OM. 2004. The Rpd3-Sin3 histone deacetylase regulates replication timing and enables S phase origin control in *Saccharomyces cerevisiae*. *Mol Cell Biol* 24: 4769–4780.

Birnbaum, KB, Bongers EM, Leitch A, Brown S, Schoots J, Harley ME, Aftimos S, Al-Aama JY, Bober M, et al. 2011a. Mutations in ORC1, encoding the largest subunit of the origin recognition complex, cause Meier-Gorlin syndrome. *Cell* 145: 205–218.

Birnbaum, KB, Bongers EM, Leitch A, Brown S, Schoots J, Harley ME, Aftimos S, Al-Aama JY, Bober M, et al. 2011b. Mutations in the pre-replication complex binding site of ORC6, encoding the largest subunit of the origin recognition complex, cause Meier-Gorlin syndrome. *Nat Genet* 43: 355–359.

Chang J, Theis JF, Miller J, Nieduszynski CA. 2012. Novel features of ARS selection in budding yeast. *PLoS Genet* 8: e1002677. doi: 10.1371/journal.pgen.1002677.

D’Aubenton-Carafa Y, Arneodo A, Hyrien O, et al. 2010. Impact of compositional biases in yeast. *Mol Biol Evol* 27: 5071–5081.

Friedman KL, Diller JD, Ferguson BM, Nyland SV, Brewer BJ, Fangman WL. 2001. Replication initiation programs set fragility of the FRA3B fragile site. *Nature* 407: 129–133.

Giovanni Agier N, Fischer G. 2012. The mutational profile of the yeast genome is shaped by replication. *Mol Biol Evol* 29: 905–913.

Aparicio JG, Viggiano CJ, Gibson DG, Aparicio OM. 2004. The Rpd3-Sin3 histone deacetylase regulates replication timing and enables S phase origin control in *Saccharomyces cerevisiae*. *Mol Cell Biol* 24: 4769–4780.

Birnbaum, KB, Bongers EM, Leitch A, Brown S, Schoots J, Harley ME, Aftimos S, Al-Aama JY, Bober M, et al. 2011a. Mutations in ORC1, encoding the largest subunit of the origin recognition complex, cause Meier-Gorlin syndrome. *Cell* 145: 205–218.

Birnbaum, KB, Bongers EM, Leitch A, Brown S, Schoots J, Harley ME, Aftimos S, Al-Aama JY, Bober M, et al. 2011b. Mutations in the pre-replication complex binding site of ORC6, encoding the largest subunit of the origin recognition complex, cause Meier-Gorlin syndrome. *Nat Genet* 43: 355–359.

Chang J, Theis JF, Miller J, Nieduszynski CA. 2012. Novel features of ARS selection in budding yeast. *PLoS Genet* 8: e1002677. doi: 10.1371/journal.pgen.1002677.

D’Aubenton-Carafa Y, Arneodo A, Hyrien O, et al. 2010. Impact of compositional biases in yeast. *Mol Biol Evol* 27: 5071–5081.

Friedman KL, Diller JD, Ferguson BM, Nyland SV, Brewer BJ, Fangman WL. 2001. Replication initiation programs set fragility of the FRA3B fragile site. *Nature* 407: 129–133.
Siow CC, Nieduszynska SR, Müller CA, Nieduszynski CA. 2011. OriDB, the DNA replication origin database updated and extended. Nucleic Acids Res 40: D682–D686.

Stamatoyannopoulos JA, Adzhubei I, Thurman RE, Kryukov GV, Mirkin SM, Sunyaev SR. 2009. Human mutation rate associated with DNA replication timing. Nat Genet 41: 393–395.

Tanaka S, Nakato R, Katou Y, Shirahige K, Araki H. 2011. Origin association of Sld3, Sld7, and Cdc45 proteins is a key step for determination of origin-firing timing. Curr Biol 21: 2055–2063.

Theis JF, Yang C, Schaefer CB, Newlon CS. 1999. DNA sequence and functional analysis of homologous ARS elements of Saccharomyces cerevisiae and S. carlsbergensis. Genetics 152: 943–952.

Tsankov AM, Thompson DA, Socha A, Reges A, Rando OJ. 2010. The role of nucleosome positioning in the evolution of gene regulation. PLoS Biol 8: e1000414. doi: 10.1371/journal.pbio.1000414.

Umek RM, Kowalski D. 1988. The ease of DNA unwinding as a determinant of initiation at yeast replication origins. Cell 52: 559–567.

Venters BJ, Wachi S, Mavrich TN, Andersen BE, Jena P, Sinnamon AJ, Jain P, Rollier NS, Jiang C, Hemeryck-Walsh C, et al. 2011. A comprehensive genomic binding map of gene and chromatin regulatory proteins in Saccharomyces. Mol Cell 41: 480–492.

Vogelauer M, Rubbi L, Lucas I, Brewer BJ, Grunstein M. 2002. Histone acetylation regulates the time of replication origin firing. Mol Cell 10: 1223–1233.

Wyrick JJ, Aparicio JG, Chen T, Barnett JD, Jennings EG, Young RA, Bell SP, Aparicio OM. 2001. Genome-wide distribution of ORC and MCM proteins in S. cerevisiae: High-resolution mapping of replication origins. Science 294: 2357–2360.

Xu W, Aparicio JG, Aparicio OM, Tavare S. 2006. Genome-wide mapping of ORC and Mcm2p binding sites on tiling arrays and identification of essential ARS consensus sequences in S. cerevisiae. BMC Genomics 7: 276. doi: 10.1186/1471-2164-7-276.

Yabuki N, Terashima H, Kitada K. 2002. Mapping of early firing origins on a replication profile of budding yeast. Genes Cells 7: 781–789.

Yamashita M, Hori Y, Shinomiya T, Obuse C, Tsurimoto T, Yoshikawa H, Shirahige K. 1997. The efficiency and timing of initiation of replication of multiple replicons of Saccharomyces cerevisiae chromosome VI. Genes Cells 2: 655–666.

Received February 23, 2012; accepted in revised form June 19, 2012.