Research

Conservation of replication timing reveals global and local regulation of replication origin activity

Carolin A. Müller and Conrad A. Nieduszynski[1]

Centre for Genetics and Genomics, The University of Nottingham, Medical School, Queen's Medical Centre, Nottingham NG7 2UH, United Kingdom

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. arboricolus*, 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 (Sclafani 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: centromere 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 (Sclafani 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

[1]**Corresponding author**
E-mail Conrad.Nieduszynski@nottingham.ac.uk

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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

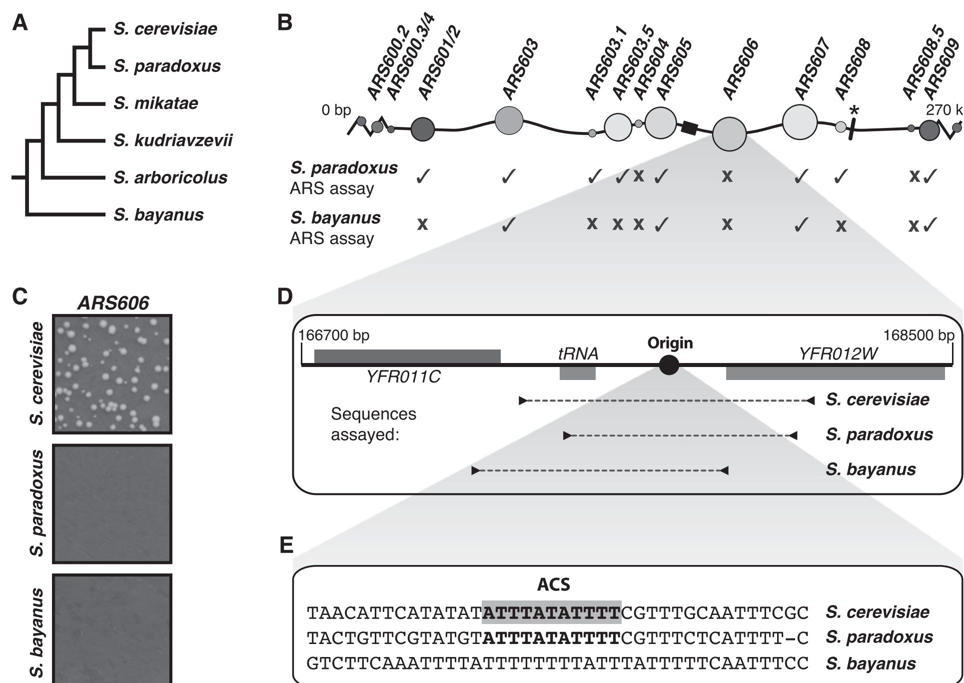

Figure 1. Functional conservation of replication origins on chromosome 6. (*A*) Phylogenetic relationship between *sensu stricto* yeast species (not to scale). (*B*) Schematic of *S. cerevisiae* chromosome 6 illustrating the location of replication origins (origins represented by circles; circle area 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 *FAR7/GCN20* and *YFL040W/ACT1* intergenic spaces. Intriguingly, in *S. cerevisiae* the *FAR7/GCN20* intergenic space was identified as an ORC- and Mcm2-binding site by ChIP (Xu et al. 2006; Shor et al. 2009) and as a site of DNA synthesis in checkpoint mutants subjected to HU (Crabbe et al. 2010), but does not support plasmid replication (Xu et al. 2006; Shor et al. 2009).

At some origin locations there is only limited phylogenetic sequence conservation, and we did not anticipate functional origin conservation. For example, at *ARS604* there are sequence differences between the species that result in only *S. cerevisiae* having a match to the ACS motif. However, overall, the low level of functional conservation surprised us given the success of phylogenetic footprinting to identify the ACS (Nieduszynski et al. 2006). Intriguingly, the ACS at *ARS606* is identical in *S. paradoxus* (although not conserved in *S. bayanus*), yet we did not detect activity for this origin in other species (Fig. 1B–E). The four origins that are functionally conserved in all three species are all highly active origins (efficiencies between 29% and 88% in W303) (Yamashita et al. 1997), raising the possibility that functional conservation is related to activity. To directly test this we measured the location and activity of replication origins throughout the genome in related *sensu stricto* species.

Measuring relative replication time by deep sequencing

We measured the relative replication time of each sequence in the genome using a two-step approach (adapted from Koren et al. 2010a). First, we sorted S phase and G2 phase cells from asynchronous cultures of *S. cerevisiae*, *S. paradoxus*, *S. arboricolus*, and *S. bayanus* (Supplemental Fig. S1). This method removes the need for genetic manipulation of the strains and allowed us to work with wild-type diploid cells. The use of diploids has the additional benefit of maximizing the absolute difference between G1 and G2 DNA content as measured by the cell sorter, thus facilitating the sorting of S phase cells. We selected the more abundant G2 cells as the nonreplicating control. Second, we precisely measured the relative copy number of each sequence using quantitative deep sequencing. In each experiment we mapped >17,000,000 short sequence reads to unique regions of the genome (Supplemental Table S3). Biological replicates demonstrated that we can measure relative copy number with a standard deviation of ~5% (data not shown). We normalized the S phase signal using the G2 phase signal to generate a ratio where we defined the minima to be 1 (see Methods; Supplemental Fig. S2; Supplemental Table S3). This approach allowed us to measure the replication dynamics of wild strains in a completely unperturbed cell cycle.

Sequences that replicate very early in S phase are present at twice the relative copy number of sequences that replicate very late 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-

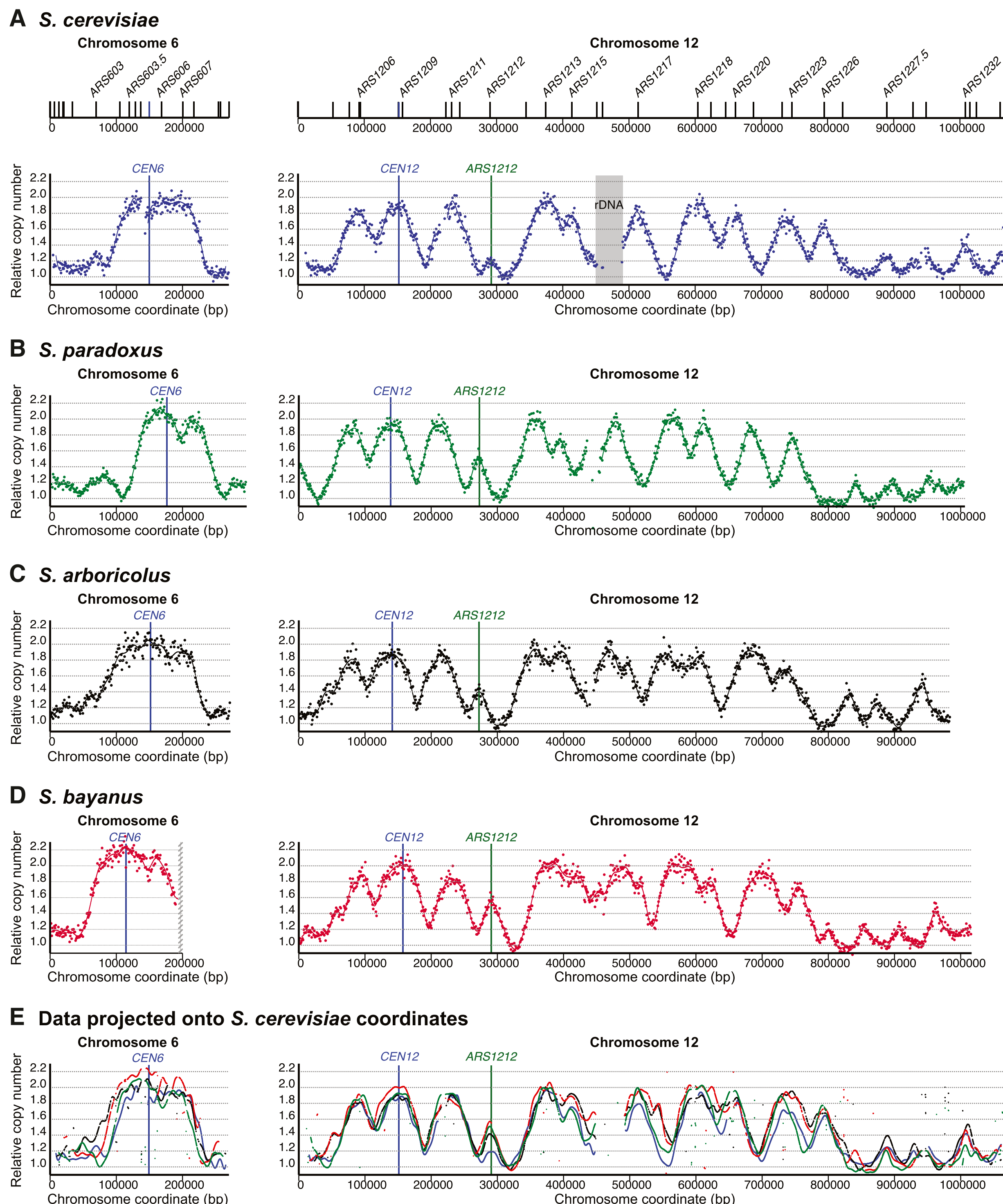

Figure 2. Replication "timing" profiles for chromosomes 6 and 12 from diploid *sensu stricto* species. Each profile shows the chromosomal position on the *x*-axis and the normalized relative copy number (as a proxy for replication time) on the *y*-axis. Individual points represent raw data with the line indicating smoothed data (see Methods). Profiles are shown for chromosomes 6 and 12 from *S. cerevisiae* (*A*), *S. paradoxus* (*B*), *S. arboricolus* (*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).

tematic analysis of chromosome 6). Furthermore, the similarity of the chromosome 6 replication profiles between *S. cerevisiae* and *S. bayanus* suggests that there are examples of *S. bayanus* origins that, although not syntenic with *S. cerevisiae* origins, have similar activity. Genome wide we found significant correlation between the temporal patterns of replication in these species. Each data set was projected onto the *S. cerevisiae* chromosomal coordinates and then Pearson's correlation coefficients were calculated for each pairwise comparison (Fig. 3A). Resulting correlation coefficients were in the range of 0.69 to 0.88; values that are comparable to the correlation coefficients between different published *S. cerevisiae* replication timing profiles (see above).

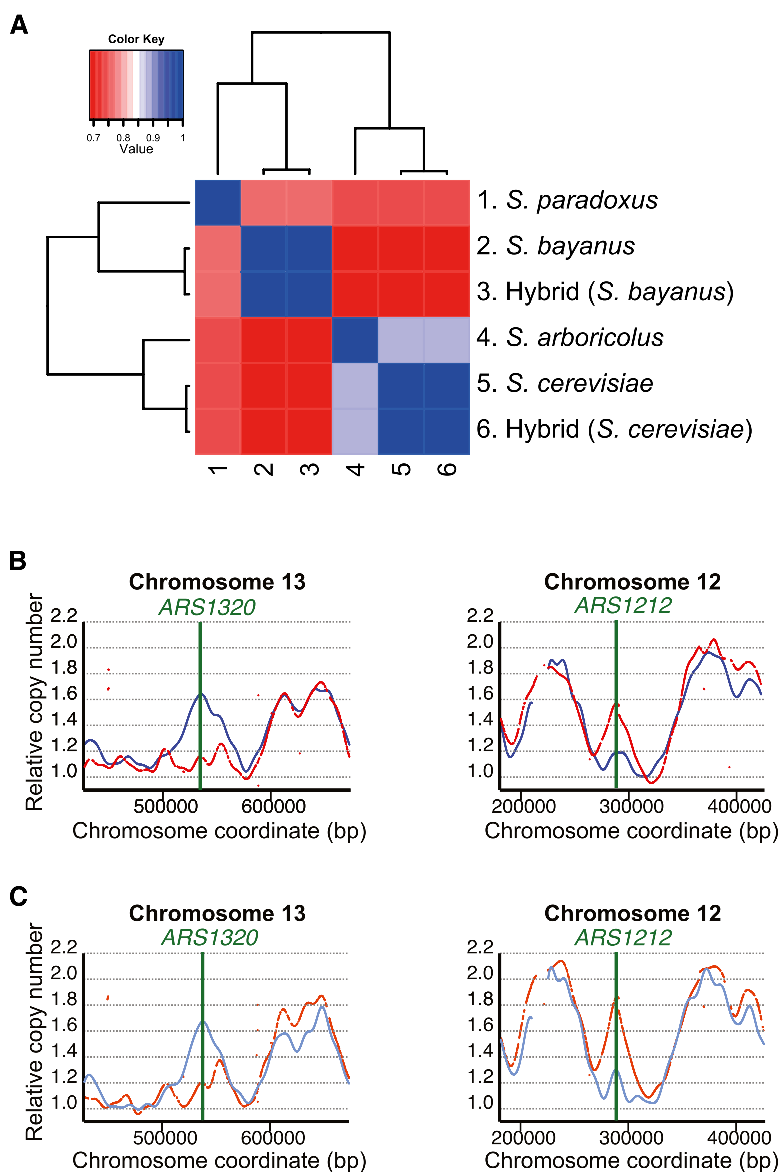

Figure 3. Comparison of replication timing profiles identifies global similarities and local differences. (*A*) Clustered correlation matrix showing the genome-wide Pearson's correlation coefficients between the different data sets. Color shading represents correlation coefficients of 0.7 (red) to 1 (blue), with blue indicating the most highly correlated data sets. (*B*) Replication profiles identify differences in the activity of *ARS1320* and *ARS1212* between *S. cerevisiae* (blue) and *S. bayanus* (red). (*C*) Differences in origin activity remain in an *S. cerevisiae* (blue)/*S. bayanus* (red) hybrid. Sections of replication profiles are drawn as described in the legend to Figure 2.

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. arboricolus* 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

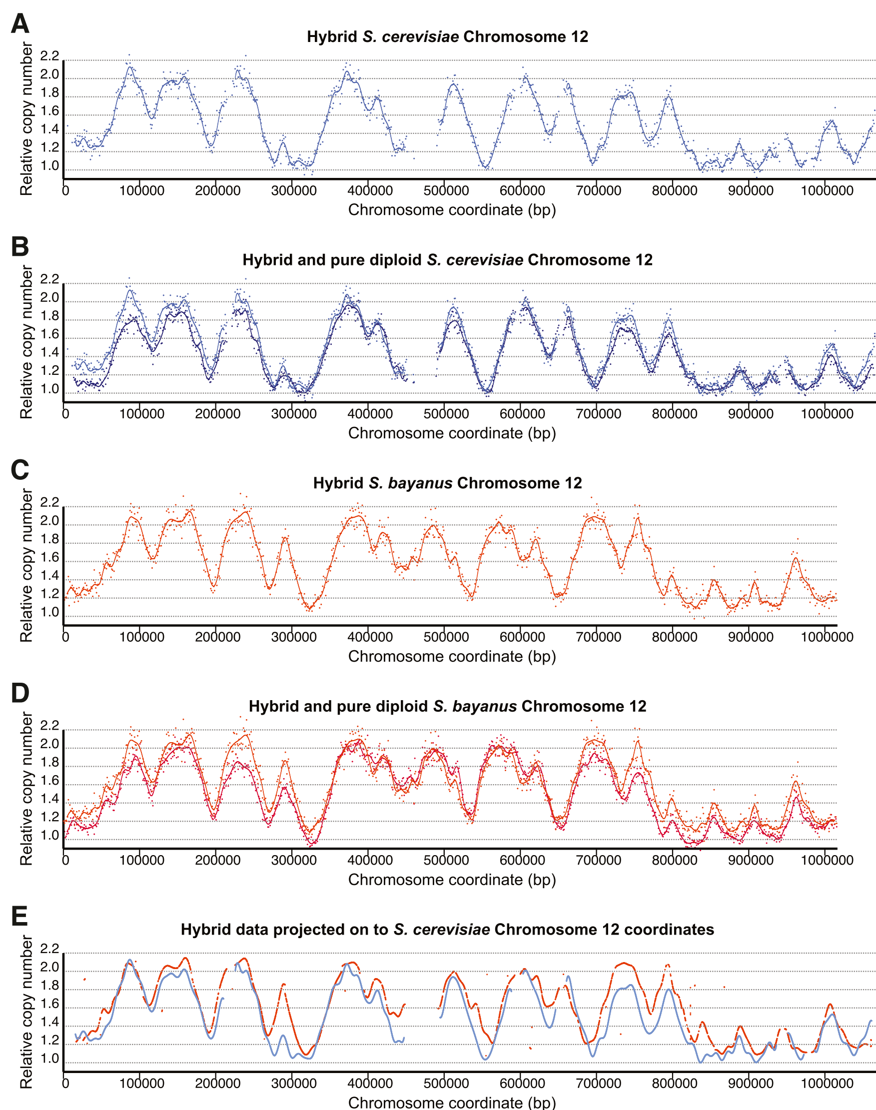

Figure 4. Replication "timing" profiles for chromosome 12 from an *S. cerevisiae*/*S. bayanus* hybrid. Replication profiles are drawn as described in the legend to Figure 2. (*A*,*C*) Profiles are shown for the *S. cerevisiae* and *S. bayanus* chromosomes from the hybrid. (*B*,*D*) Hybrid data are shown superimposed on data from the respective nonhybrid diploids. (*E*) Smoothed data from the *S. cerevisiae* and *S. bayanus* chromosomes from the hybrid are shown projected onto the *S. cerevisiae* coordinates to aid comparison.

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 the chromosome 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 *yKu* 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 genome-wide timing study of a *yku70* mutant (Lian et al. 2011). Telomere length has been reported to be shorter in *S. cerevisiae*/*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 *Saccharomyces 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 location 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 *FAR7*/*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-mL YPD culture were fixed in 70% Ethanol, washed with 50 mM Sodium Citrate, sonicated, and treated with RNaseA 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 Zymolyase 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. arboricolus* 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 (Raghuraman 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.

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