SUPPLEMENTAL INFORMATION for Knott et al.

SUPPLEMENTAL DATA LEGENDS

Data S1, related to Figure 1. Analysis of early S-phase BrdU incorporation showing all chromosomes.

Data S2, related to Figure 2. Temporal analysis of DNA replication by BrdU pulse-labeling of *WT* and *fkh1Δ fkh2ΔC* cells showing all chromosomes.

SUPPLEMENTAL TABLE LEGENDS

Table S1. List of genes identified as differentially regulated in *fkh1*Δ *fkh2*Δ*C* cells.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1, related to Figure 1. A. Suppression of pseudohyphal growth of *fkh1Δ fkh2Δ* cells by expression of Fkh2ΔC. Phase-contrast images of the indicated strains grown in liquid culture and sonicated mildly to disrupt cell aggregates. **B.** Origins deregulated in *fkh1Δ*, *fkh1Δ fkh2Δ*, and *fkh1Δ fkh2Δ + fkh2ΔC* cells. Venn diagrams showing overlap of deregulated origins identified as Fkh-activated and Fkh-repressed.

Figure S2, related to Figure 2. A. FACScan analysis of DNA content of *WT* and *fkh1Δ fkh2ΔC* cells synchronized in G1-phase with α–factor and released synchronously into S-phase. **B.** Two-dimensional gel electrophoresis analysis of *ARS305* (Fkh-activated) and

ARS1520 (Fkh-repressed) in unsynchronized *WT* and *fkh1Δ fkh2ΔC* cells. Genomic DNA was digested with *NcoI* and *SalI*.

Figure S3. Non-random distribution of Fkh-regulated origins. **A.** Chromosomal positions of Fhk-activated and –repressed origins are plotted. **B.** Histogram displaying the frequency of "Cut" counts observed in the 10^5 simulations as well as the experimentally observed "Cut" count. "Cuts" refers to the number of times a Fkh-activated origin is followed by a Fkh-repressed origin, or vice-versa, given a random distribution (see Methods).

Figure S4, related to Figure 6. 4C analysis of *ARS305* interactions. **A.** Scheme of the 4C method showing relevant *XbaI* (X1-X4) and *MseI* (M1-M4) restriction sites surrounding *ARS305* (Bait) and a hypothetical interacting locus (Prey), and primers (P1-P4) used to amplify captured loci for identification by microarray. The tethering agent represents cross-linked protein(s) mediating interaction between the bait and prey. **B.** Statistical analysis of *ARS305* interacting sites by chromosome showing the expected preference for intrachromosomal interactions (i.e., with chromosome III). The *p* value is based on the number of observed versus expected interactions for each chromosome (the expected number of interactions is directly proportional to the number of *XbaI* fragments per individual chromosome).

EXPERIMENTAL PROCEDURES

Yeast strain and plasmid constructions

W303-derived, BrdU-incorporating strains CVy43 (*Mat**a** ade2-1, bar1::hisG, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1::BrdU-Inc::URA3*) or CVy63 (*Mat**a** ade2-1, bar1::hisG, can1-*

100, his3-11,15, leu2-3,112, trp1-1, leu2::BrdU-Inc::LEU2) were the *WT* parents for all strain constructions (Viggiani and Aparicio, 2006). *FKH1* and *FKH2* were deleted in CVy43 as described (Longtine et al., 1998), yielding strains: ZOy1 (*fkh1*Δ*::kanMX6*), CVy138 (*fkh2*Δ*::His3MX6*), and CVy139 (*fkh1*Δ*::kanMX6 fkh2*Δ*::His3MX6*); only differences in genotype from CVy43 are indicated. Plasmid pfkh2ΔC contains a C-terminally truncated *NotI-KpnI* fragment of *FKH2* (truncated at the native *KpnI* site in *FKH2*, deleting amino acids 624-862; this maintains the entire DNA binding domain and all homology with Fkh1) into pRS424 digested with the same enzymes; pfkh2ΔC was transformed into CVy139 yielding strain SKy1. *CDC45-HA3* (*LEU2*) was introduced into strains CVy43 and CVy139 + pfkh2ΔC using p405-CDC45-HA/C as described (Aparicio et al., 1997), yielding strains CVy46 and T2y3, respectively. *FKH1-MYC9* replaced *FKH1* in CVy138 using plasmid pTOPO-Fkh1-Myc9, yielding strain ZOy22. pTOPO-Fkh1-Myc9 was constructed using Phusion High-Fidelity PCR kit (New England Biolabs, M0530) to amplify *FKH1-MYC9-TRP1* from genomic DNA of strain Z1448 (Harbison et al., 2004), and inserting it into pCR2.1-TOPO vector (Invitrogen).

Strain ARy23 containing mutations of two Fkh1/2 binding sites at *ARS305* was constructed by pop-in/pop-out of plasmid p306-ARS305-Δ2BS into strain CVy63 and confirmed by sequencing of PCR-amplified genomic DNA. Plasmid p306-ARS305Δ2BS was constructed as follows: Two ~1kb fragments covering *ARS305* with overlapping ends were amplified from genomic DNA (using primers: 5´-gtcaagcttggcaatgtcaagagcagagc with 5´-gtcctcgaggaatacataacaaaaatataaaaacc for one fragment and 5´-tgagaattcaggcatcagtttgatgttgg with 5´-gtcctcgaggtccctttaattttaggatatgaaaac for the second fragment), digested with *EcoRI +XhoI* and with *XhoI + HindIII*, respectively, and three-way ligated into pRS306 digested with *EcoRI +*

HindIII. The *XhoI* site changes the first predicted Fkh1/2 binding site (chr III coordinates 39,563-39,570) without deleting or inserting additional sequence. The resulting plasmid, p306-ARS305Δ1BS was sequenced to confirm that only the desired sequence changes were introduced. This plasmid was mutagenized using QuikChange Lightning Multi Site mutagenesis kit (Agilent# 210515-5) using primer (5´-caaagaaaaaaatcttagctttaagaactacaaagtcctcgaggaataataaatcacaccggacagtacatg) to change the second predicted Fkh1/2 binding site (chr III coordinates 39,483-39,490) to an *XhoI* site without deleting or inserting additional sequence. The resulting plasmid p306-ARS305Δ2BS was sequenced to confirm that only the desired sequence changes were introduced.

Yeast methods and molecular procedures.

Cell cycle block-and-release, DNA content analysis, and two-dimensional gel electrophoresis analysis have been described (Aparicio et al., 2004). For BrdU and chromatin IPs we used: anti-BrdU at 1:1000 (GE Healthcare, RPN202), anti-Fkh1 at 1:200 (Casey et al., 2008), anti-ORC at 1:500 (Wyrick et al., 2001), anti-Mcm2 at 1:50 (Santa Cruz Biotech., SC-6680), anti-Mcm4 at 1:50 (Santa Cruz Biotech., SC-33622), anti-Ha 16B12 at 1:200 (Covance, MMS101R), and anti-Rpb3 at 1:500 (Neoclone, W0012). We used anti-Myc 9E10 at 1:100 and 1:2000 (Covance, MMS150P), and anti-ORC at 1:100 and 1:1000, for co-IP and immunoblotting, respectively. Co-IP was performed as described (Hu et al., 2008), except Dynabeads Protein G (Invitrogen, 100-04D) was used.

BrdU-labeled DNA was isolated as described (Viggiani et al., 2010), except salmon sperm DNA was omitted for sequencing analysis. 80ng of BrdU-IPed DNA was prepared for single-end

sequencing according to Illumina ChIP-Seq protocol or 10ng of BrdU-IPed DNA was prepared for hybridization to microarrays as described (Viggiani et al., 2010). ChIP-chip was performed and analyzed as described (Knott et al., 2009; Viggiani et al., 2009). ChIP-Seq was performed identically except that culture size was scaled-up four-fold to generate 5-10ng of IP material for single-end sequencing by Illumina ChIP-Seq protocol. RNA was isolated from 20mL cultures using the RiboPure Yeast Kit (Ambion, AM1926). rRNA was depleted with Ribominus Beads (Invitrogen, A10837-08), and purified RNA was prepared for strand-specific RNA-Seq as described in (Parkhomchuk et al., 2009). We used a custom microarray design (Nimblegen) that tiles one ~60bp oligonucleotide for every ~80bp of unique genomic sequence. For hybridization and washing we followed Nimblegen protocols, and for image capture used a Genepix Personal 4100A Scanner (Axon Instruments).

Preprocessing of sequence data

All sequencing was carried out with an Illumina GAII. BrdU-IP-Seq and ChIP-Seq were analyzed with 36bp single-end reads, while RNA-Seq was analyzed with 36bp paired-end reads. All reads were aligned to the *S. cerevisiae* genome (release r.64) with the mapping algorithm PerM (Chen et al., 2009), allowing only unique alignments with a maximum of two mismatches per end. BrdU-IP-Seq and Rpb3 ChIP-Seq reads were binned into non-overlapping 50bp bins. Bin-counts were median-smoothed (1000bp and 500bp windows, respectively) and then quantile-normalized across all experiments. Following quantile-normalization, a second smoothing step was applied. For Figure 4, RNA-Seq reads were normalized as described for Rpb3 ChIP-Seq. For all other gene expression analysis, each RNA-Seq read was assigned to a gene only when at least one of its paired-ends was fully contained within the gene's ORF and

when the read's orientation corresponded to the gene's transcriptional direction. Reads whose pair-ends mapped to two or more genes were not considered further. Gene read-counts were quantile-normalized prior to differential expression analysis.

BrdU-IP-Seq analysis

To identify an initial set of peaks in each experiment, a set of apices (bins whose count was higher than any neighboring bin within 500bp) were detected. We assigned a magnitude to these peaks equal to the number of reads mapping to within 500bp of the apex; only peaks with a magnitude >10 were considered further. For each strain we aligned replicate apex chromosomal locations using the dynamic programming algorithm as described (Knott et al., 2009), with a gap penalty of 1000bp. Apices that did not align across all replicates were removed from consideration. Next, for each strain we aligned peaks (387) with the set of previously annotated origins listed in OriDB (Nieduszynski et al., 2007); peaks (35) that did not align to an annotated origin were not considered further.

Origins that were not detected to incorporate BrdU within a given strain were assigned a count equal to the number of reads that mapped to within 500bp of the average of its corresponding detected apices. To test for differential BrdU-incorporation across strains, we employed DESeq (Anders and Huber, 2010). Origin counts were normalized using DESeq's internal size and variance normalization strategies and were called as different between two strains with a significance cutoff of FDR<0.005.

BrdU-IP-chip time-course data analysis

Due to the high proportion of enriched probes in BrdU-IP-chip experiments, within-array normalization methods designed for ChIP-chip are not suitable (Knott et al., 2009). To compensate for this, we developed a procedure and tested it on BrdU-IP-chip experiments performed in the presence of HU. This method requires that un-enriched probes form a dense cluster in the M=log(IP/Total) vs. A=(log(IP)+log(Total))/2 plane (Knott et al., 2009). However, in BrdU incorporation experiments without HU (where the percentage of enriched probes can reach 80%), this requirement is sometimes not met. To account for this, we developed a technique specifically for such experiments. This method requires a mock control, for which we hybridized BrdU-IP material obtained from a 12min BrdU pulse using G1-arrested (non-replicating) cells against genomic DNA. First, we identified the best axes on which to transform the experimental data by applying our previous method on the control data (Knott et al., 2009). After transforming the control and experimental data onto these axes, the median absolute deviations of both datasets were normalized to 1. Then, the M values of the experimental data were location-normalized such that mean of the lowest 20% of probes were equal to mean of the lowest 20% of control probes. Subsequently, we followed our previous method (Knott et al., 2009).

Analysis of linear clustering of Fkh-regulated origins

To test whether Fkh-activated and –repressed origins cluster in separate groups linearly along chromosomes, we defined a clustering metric equal to the number of "cuts" required to separate Fkh-repressed and Fkh-activated origins (this is equivalent to the number of instances where a Fkh-activated origin neighbors a Fkh-repressed origin, ignoring non-Fkh-regulated origins). A low "cut" count indicates higher clustering of like-regulated origins. We obtained a "cut" count

of 65 in the experimental data. To test if this was significantly low, we performed 10^6 simulations on the 352 origins that were detected in *WT* or *fkh1*Δ *fkh2*Δ*C* cells. In each simulation we randomly assigned 95 origins as Fkh-activated, 80 as Fkh-repressed, and the remaining as Fkh-unregulated. Fewer than 1% of the simulations resulted in a "cut" count <65 (Fig. S3B).

Analysis of Fkh1 and Fkh2 binding sites

To determine whether Fkh1 and Fkh2 were bound significantly at Fkh-regulated versus non-regulated origins we used the Position Weight Matrices (PWMs) defined in (Morozov and Siggia, 2007)) to identify all putative Fkh1/2 binding sites near origins (PWM-score cutoff =5.5). We defined Fkh1/2 bound origins as those with a putative site within 500bp of its BrdU-peak apex. To determine the distribution of Fkh1/2 binding sites in relation to origin ACSs, for each Fkh1/2 bound origin with a previously defined ACS, we calculated the distance from the ACS to the highest scoring binding site (ACS locations from (Eaton et al., 2010)). We then applied a kernel density function to these distances to define the probability curves displayed in Fig. 3C.

Analysis of Fkh-regulated transcription versus Fkh-regulated origin function

To determine whether proximal genes show co-regulation with Fkh-regulated origins, we performed a permutation test on the distances between Fkh-regulated origins and the nearest Fkh-regulated genes. Fkh-regulated genes were identified as those that showed differential expression (DESeq FDR<0.01) between *WT* and *fkh1*Δ *fkh2*Δ*C* cells in the same condition (unsynchronized or G1-synchronized). This analysis was performed using both the RNA-Seq and Rpb3-ChIP-Seq datasets, (genes detected as differentially expressed in each of the

experiments are listed in Table S1). For each experiment we calculated the distance from each Fkh-regulated origin to the nearest Fkh-regulated gene's promoter. Next, 10^5 simulated origins sets were identified by randomly selecting 172 origins, and randomly assigning 95 as Fkh-activated and 82 as Fkh-repressed. For each of these sets, the minimum distances to the nearest Fkh-regulated genes were calculated. With this analysis we determined for all possible pair-wise combinations (e.g. up-regulated gene and Fkh-activated origin, down-regulated gene and Fkh-activated origin, etc.) that Fkh-regulated origins are not significantly clustered with Fkh-regulated genes along the chromosome.

To test for correlation of Fkh-regulated origins with flanking gene expression, we performed regression analysis separately on Fkh-regulated origins lying within intergenic regions flanked by diverging, converging, and tandemly oriented genes. For converging and diverging intergenic regions, we used two covariates representing the unsynchronized and G1-phase *fkh1*Δ *fkh2*Δ*C-WT* RNA-Seq read count differences of the closest transcript (as measured in bp between the origin's ARS-consensus sequence (ACS) and the gene's nearest end) and two covariates representing the same difference measure in the farther of the two transcripts. For tandem intergenic regions, two covariates represented unsynchronized and G1-phase *fkh1*Δ *fkh2*Δ*C-WT* RNA-Seq read count differences for the converging gene and another two covariates represented the differences for the diverging gene. In this analysis the only covariate that showed significant correlation with origin regulation was the gene farthest away from origins within converging intergenic regions in unsynchronized cells ($p<0.05$). A closer inspection revealed that this correlation was due to four outlying data points, and when these were removed, the same analysis found no covariate to be significantly correlated with origin regulation. Furthermore,

the application of this same analysis to read count differences in the Rpb3 ChIP-Seq data showed no covariate to be significantly predictive of origin regulation.

Analysis of global 4C (Duan et al., 2010)

226 origins whose defined regions (as listed in OriDB) were fully contained within an *EcoRI* restriction fragment and a *HindIII* restriction fragment were analyzed. The fragment interaction map developed in (Duan et al., 2010) was used to build two-dimensional interaction matrices for each restriction fragments set containing the 226 origins. The matrix value represents the interaction distance between two origin containing restriction fragments, a value between 0 and 4, defined in (Duan et al., 2010). Next, the two matrices were summed and the two-dimensional clustering algorithm defined in (Duan et al., 2010) was applied. 17 clusters containing fewer than ten origins each (45 total) were not analyzed further.

Chromosome conformation capture on chip (4C)

Chromatin isolation: 50mL of G1-sychronized cells were crosslinked and harvested as described for ChIP-chip (Viggiani et al., 2009). Cells were suspended in 9.5mL Buffer Z (0.7M Sorbitol, 50mM Tris (pH 7.4), heat sterilized) plus freshly added 2-mercaptoethanol (20mM final) and protease inhibitor cocktail (Roche, Mini Complete). 0.5mL Zymolyase 100T (ICN, 10 mg/mL freshly made in Buffer Z) was added and the suspension was incubated at 30°C with gentle agitation, 35 min. The suspension was split into six 2mL microcentrifuge tubes and centrifuged at 16,000g, 20 min at 4°C. The supernatants were discarded, each pellet was suspended in 300µL NP buffer (1M Sorbitol, 100mM Tris (pH 7.4), 50mM NaCl, 5mM $MgCl_2$, 1mM $CaCl_2$,

heat sterilized) containing 0.5mM Spermidine (freshly added from 250mM stock) by gently pipetting with a wide-bore pipet tip, and the samples were pooled in a 2mL microcentrifuge tube.

Digestion and ligation I: The suspension was centrifuged as above and the pellet was suspended in 500µL ice-cold 1X NEB (New England Biolabs) digestion buffer II, and centrifuged again. This wash step with digestion buffer was repeated and the pellet was suspended in 50µL 1X NEB digestion buffer II. 42µL 1% SDS was added, mixed gently, incubated at 60°C, 15 min. 328µL of ice-cold 1X NEB digestion buffer II was added and the resulting suspension was centrifuged at 600g, 1 min at 4°C. 400µL of the supernatant was transferred to a fresh microcentrifuge tube (the remainder was discarded), and 44µL 10% Triton-X100 was added and mixed gently by pipetting with a wide-bore pipet tip. This suspension was placed on ice for 15 min, after which 58.4µL of H_2O, 16µL 10X NEB digestion buffer II, and 1.6µL BSA (NEB, 10mg/mL) were added.

4µL *XbaI* (NEB, 100 U/µL) was added, mixed gently, and incubated at 37°C for a minimum of 8hr while shaking at 275 rpm. 10µL H_2O, 50µL 10% SDS, and 9µL 0.5 M EDTA was added, mixed, and incubated at 65°C for 10 min, followed by 60°C for 10 min, and on ice for 5 min. The sample was transferred to a 15mL conical screw-cap tube on ice and 3554µL H_2O, 250µL 10X T4 DNA ligase buffer (NEB), 50µL BSA (10mg/mL), 500µL 10% Triton-X100, and 125µL 1M Tris (pH 7.5) were added, mixed gently, and incubated on ice, 15 min. While on ice, 2µL T4 DNA ligase (NEB, 400U/µL) was added, mixed gently, and incubated at 16°C for 4 hr, after which 60µL 0.5 M EDTA was added.

To the ligated sample, 50µL 5M NaCl and 5µL RNAase A (20mg/mL) were added, mixed, and incubated at 37°C, 1 hr. 25µL Proteinase K (20mg/mL) was added, mixed, and incubated overnight at 65°C. The sample was transferred to a 15mL phase-lock tube (5-Prime, 2302850) and the DNA was purified by extraction with 6mL phenol:chloroform:isoamyl alcohol (25:24:1) and centrifugation according to the manufacturer's instructions. To the 4.2mL of aqueous solution recovered, 225µL 5M NaCl and 6µL glycoblue were added and mixed, and 11mL of ice-cold ethanol was added, mixed, and incubated at -20°C, 8 hr. The sample was aliquoted into eight 2mL microcentrifuge tubes and centrifuged at ~16,000g, 30 min at 4°C. After discarding the supernatant, each pellet was dissolved in 50µL 1X TE and the samples were pooled. 30µL 3M NaOAc (pH 5.2) and 825µL of ice-cold ethanol were added, mixed, and incubated at -80°C, 2 hr. The precipitate was recovered by centrifugation at 16,000g, 30 min at 4°C, and after discarding the supernatant, the pellet was dissolved in 50µL TE.

Digestion and ligation II: To 25µL (~100ng) of the ligated sample, 64µL H_2O, 10µL 10X NEB digestion buffer II, 1µL BSA (10mg/mL, NEB) were added and mixed, and 2µL of *MseI* (10 U/µL, NEB) was added, mixed, and incubated at 37°C, 2 hr. 1µL 20% SDS was added and incubated at 65°C, 20 min; 30µL 10% Triton-X100 was added and incubated on ice for 15 min. 757µL H_2O, 100µL T4 DNA ligase buffer, and 10µL BSA (10mg/mL) were added and incubated on ice for 15 min. While still on ice, 2µL T4 DNA Ligase (400U/µL) was added, mixed by pipetting gently, and incubated overnight at 16°C. The sample was split into two 500µL aliquots (in 2mL microcentrifuge tubes) and 25µL 5M NaCl, 2µL glycoblue, and 1.2mL ice-cold ethanol was added to each, mixed and incubated at -20°C, 2 hr. The precipitate was

recovered by centrifugation at 16,000g, 30 min at 4°C; the supernatant was discarded and each pellet was dissolved in 25µL TE and pooled.

Amplification and microarray analysis: 5µL was amplified by standard PCR (25 cycles) with the following primers: 5'CTAAGTGTCCTGTTTCGGAAC, and 5'CAGGCCGCTCTTATAAAATGA. 1µg amplified DNA was labeled with Cy5 and hybridized against Cy3-labeled reference DNA (G1-synchronized total genomic DNA) as described for BrdU-IP-chip (Viggiani et al., 2010). Analysis was performed as described in (Knott et al., 2009) to identify enriched probes, and *Xba1* fragments containing >3 enriched probes immediately adjacent to either cut site were deemed to be interacting.

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