SUPPLEMENTAL FIGURE AND TABLE LEGENDS

Figure S1. Example of raw data from a re-replication microarray experiment. For most experiments, two independent sets of genomic DNA were prepared and each set was competitively hybridized in duplicate. The ratio of signal intensity of Cy5 to Cy3 was calculated for each sequence element on the array and normalized such that the average ratio of all elements was set to the median DNA content of the re-replicating cells. Normalized raw ratios of the four hybridizations from the experiment described in Figure 2B are shown for chromosome XIV (top four panels). These normalized ratios were subjected to local averaging and Fourier convolution smoothing to generate a smoothed profile. The four smoothed profiles were then merged (see Supplemental Methods) to generate a composite re-replication profile (bottom panel).

Figure S2. Replication profiles generated by comparative genomic hybridization. CGH replication assay was performed on YJL5038**,** a wild-type yeast strain in the S288c background, in an experiment described in Figure 1B. G1 phase genomic DNA was isolated from cells arrested in alpha factor. S phase genomic DNA was isolated from cells released from an alpha factor arrest in the presence of 100 mM hydroxyurea (HU) for 120 min (DNA content was 1.4 C). The composite replication profiles (blue lines) plus and minus one standard deviation (light gray bands, see Methods) are shown for all sixteen chromosomes. Positions of the 212 origins identified by application of a peak finding algorithm are shown (blue diamonds). Positions of ARSs annotated in the Saccharomyces Genome Database (SGD, (Balakrishnan)) (black open triangles), locations of pro-ARSs mapped by Wyrick *et al.* (Wyrick *et al.*, 2001) (red triangles) and the centromeres (black circles) are marked along the X-axis. Replication profiles derived from Raghuraman *et al.* (Raghuraman *et al.*, 2001) (violet lines) and Yabuki *et al.* (Yabuki *et al.*, 2002) (orange lines) are shown for comparison.

Figure S3. The S phase replication profile of the re-replication competent OMC strain and the congenic wild-type strain are similar. S phase replication profiles were generated for the OMC strain YJL3248 (*MCM7-2NLS orc2-cdk6A orc6-cdk4A pGAL1-Δntcdc6 pMET3-HA3-CDC20*) and YJL5834 (*pGAL1*), a wild-type yeast strain in the A364a background, in an experiment described in Figure 1D. S phase OMC cells were harvested 180 min after alpha factor release into HU (DNA content was 1.4 C). S phase A364a cells were harvested 135 min after alpha factor release into HU (DNA content was 1.35 C). The S phase replication profiles for the OMC strain (green lines), S phase replication profiles for the A364a strain (red lines), the positions of the 193 origins identified in the OMC strain (green diamonds), and the positions of the 231 origins identified in the A364a strain (red diamonds) are shown for all sixteen chromosomes. Positions of origins annotated in SGD, (Balakrishnan) (black open triangles), locations of pro-ARSs mapped by Wyrick *et al.* (Wyrick *et al.*, 2001) (red triangles) and the centromeres (black circles) are marked along the X-axis.

Figure S4. Replication timing in the OMC re-replication competent mutant correlates with replication timing in the A364a background. Application of a peak finding algorithm to the S phase replication profiles in Figure S3 identified 193 origins in the OMC strain YJL3248 (*MCM7-2NLS orc2-cdk6A orc6-cdk4A pGAL1-Δntcdc6 pMET3-HA3-CDC20*), and 231 origins in the A364a strain YJL5834 (*pGAL1*). 166 (86%) of the origins identified from the OMC strain had a corresponding origin within 10kb in the wild-type A364a strain. For each of the shared origins, the S phase copy number in the A364a replication profile from Figure S3 was plotted against that for the OMC strain in Figure S3. A linear regression line fitted to these points showed a good correlation, with an R^2 value of 0.58.

Figure S5. Different strain backgrounds have very similar replication timing profiles. The replication profiles (red lines) and identified origins (red diamonds) from the congenic wild-type A364a strain YJL5834 (*pGAL1*) from Figure S3 are compared to the replication profiles (blue lines) and identified origins (blue diamonds) from the S288c strain in Figure S2. Positions of origins annotated in SGD (Balakrishnan) (black open triangles), locations of pro-ARSs mapped by Wyrick *et al.* (Wyrick *et al.*, 2001) (red triangles) and the centromeres (black circles) are marked along the X-axis.

Figure S6. Replication timing in the S288c background strongly correlates with replication timing in the A364a background. Application of a peak finding algorithm to the S phase replication profiles in Figure S5 identified 212 origins in the S288c strain, YJL5038 and 231 origins in the A364a strain YJL5834 (*pGAL1*). Origin usage during S phase was closely matched between the two strain backgrounds; 193 (92%) of

origins identified in the S288c background were within 10kb of origins identified in the A364a background. The mean distance between these corresponding origins was 2.1 kb. For each shared origin, the S phase copy number in the A364a background was plotted against that for the S288c background. A linear regression line fitted to these points showed very strong correlation, with an R^2 value of 0.92.

Figure S7. Re-replication induced during G2/M phase when ORC, Mcm2-7 and Cdc6 are deregulated. Genomic DNA was purified from the OMC strain YJL3248 (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1-Δntcdc6 pMET3-HA3-CDC20)* and the control OM strain YJL3244 (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1 pMET3-HA3-CDC20*) after 3 hr of galactose induction from G2/M arrest in an experiment described in Figure 2 (DNA content for the OMC strain was 2.7 C at 3 hr). The OMC G2/M phase re-replication profiles (blue lines, right axis), the positions of the 106 re-replicating peaks identified by application of a peak finding algorithm (blue diamonds), the OMC S phase replication profile (green line, left axis) and identified origins (green diamonds) replotted from Figure S3 are shown for all sixteen chromosomes. The locations of pro-ARSs mapped by Wyrick *et al.* (Wyrick *et al.*, 2001) (red triangles) and the centromeres (black circles) are marked along the X-axis. In the course of these experiments, we observed that the control OM strain YJL3244 (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1 pMET3-HA3-CDC20*) contained a duplication of a region of chromosome IV (515kb to 645 kb) and that the OMC strain YJL3248 (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1-Δntcdc6 pMET3-HA3-CDC20)* had an extra copy of chromosome XI in much of the population. Shown for chromosomes IV and XI are data from a replicate experiment using an isogenic OM strain YJL5493 (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1 pMET3-HA3-CDC20*) and an isogenic OMC strain YJL3249 (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1-Δntcdc6 pMET3-HA3-CDC20*), lacking these genomic alterations (yellow lines).

Figure S8. The observed mean distance from re-replication peaks to pro-ARSs is highly significant. When re-replication was induced in G2/M, 106 re-replicating origins were identified (Figure S7). The mean distance from those origins to the closest potential S phase origin defined by Wyrick *et al.* (Wyrick *et al.*, 2001) (pro-ARSs) was 7.0 kb. To determine the significance of this value, 106 random chromosomal loci were selected and the mean distance to the closest pro-ARS was calculated. This was repeated 100,000 times and a histogram was generated showing the percent of the random samples with the indicated mean distances. The actual observed mean, which is greatly below the expected random mean, is indicated with an arrow.

Figure S9. OMC cells can re-initiate and re-replicate within S phase. The OMC strain YJL3249 (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1-Δntcdc6 pMET3-HA3-CDC20*) was induced to re-replicate while still in S phase in an experiment described in Figure 3. The cells were arrested in G1 phase with alpha factor, induced to express Δntcdc6 by the addition of 2% galactose, then released from the arrest into YEPGal containing 100 mM hydroxyurea (HU) to delay cells from exiting S phase. Genomic DNA from the OMC strain was isolated at the 0 hr (G1 phase) and 4 hr (S phase, DNA content 1.4 C) time points and competitively hybridized against each other. The resulting profiles shown for all sixteen chromosomes reflect copy number increases due to both replication and re-replication. The locations of pro-ARSs mapped by Wyrick *et al.* (Wyrick *et al.*, 2001) (red triangles) and the centromeres (black circles) are plotted along the X-axis.

Figure S10. Re-replication induced upon release from a G1 arrest when ORC, Mcm2-7 and Cdc6 are deregulated. The OMC strain YJL3248 (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1-Δntcdc6 pMET3-HA3-CDC20)* was induced to re-replicate during G1 release in an experiment described in Figure 4. Genomic DNA was purified from the OMC strain and the OM strain YJL3244 (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1 pMET3-HA3-CDC20*) after 3 hr of galactose induction while cells were released from G1 into G2/M phase (DNA content for the OMC strain was 3.2 C at 3 hr). The two DNA preparations were labeled and competitively hybridized against each other to generate the OMC G1 release re-replication profiles shown for all sixteen chromosomes (blue lines) and to identify 87 re-replicating peaks (blue diamonds). The locations of pro-ARSs mapped by Wyrick *et al.* (Wyrick *et al.*, 2001) (red triangles) and the centromeres (black circles) are plotted along the X-axis. In the course of these experiments, we observed the same genomic alterations of Chromosome IV and XI described in Figure S7. Shown for chromosomes IV and XI are data from a replicate experiment using an isogenic OM strain YJL5493 (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1 pMET3-HA3-CDC20*) and an isogenic OMC strain YJL3249 (*orc2-cdk6A orc6-cdk4A MCM7-2NLS pGAL1-Δntcdc6 pMET3-HA3-CDC20*), lacking these genomic alterations (yellow lines).

Figure S11. Re-replication can be induced when only ORC and Cdc6p are deregulated. The OC strain YJL3240 (*orc2-cdk6A orc6-cdk4A pGAL1-Δntcdc6 pMET3-HA3-CDC20)* and the control O strain YJL4832 (*orc2-cdk6A orc6-cdk4A pGAL1 pMET3-HA3-CDC20*) were induced to re-replicate in G2/M phase or during a G1 release in experiments described in Figures 5A and 5B, respectively. The DNA content of the OC strain was 2.0 C for the G2/M induction and 2.6 C for the G1 release. For each induction protocol, OC and O strain genomic DNA were prepared and competitively hybridized against each other as described in Figure1A. Shown for all sixteen chromosomes are OC G2/M phase re-replication profiles (blue lines), OC G1 release re-replication profiles (green lines), locations of pro-ARSs mapped by Wyrick *et al.* (Wyrick *et al.*, 2001) (red triangles), and centromeres (black circles).

Figure S12. Re-replication occurs primarily on a single chromosome when Mcm2-7 and Cdc6 are deregulated. Re-replication in the MC_{2A} strain occurs primarily on chromosome III. The MC_{2A} strain YJL4489 (*MCM7-NLS pGAL1-Δntcdc6-cdk2A pMET3-HA3-CDC20*) and the control M strain YJL4486 (*MCM7-2NLS pGAL1 pMET3-HA3-CDC20*) were induced to re-replicate in G2/M phase or during a G1 release in experiments described in Figures 6A and 6B, respectively. The DNA content of the MC_{2A} strain was 2.0 C for both the G2/M induction and for the G1 release. For each induction protocol, MC_{2A} and M strain genomic DNA were prepared and competitively hybridized against each other as described in Figure1A. Shown for all sixteen chromosomes are MC_{2A} G2/M phase re-replication profiles (blue lines), MC_{2A} G1 release re-replication profiles (green lines), locations of pro-ARSs mapped by Wyrick *et al.* (Wyrick *et al.*, 2001) (red triangles) and the centromeres (black circles).

Figure S13. MC_{2A}-*cdc7* strain is competent to re-replicate at the permissive temperature. The MC_{2A} strain YJL4489 (*MCM7-2NLS pGAL1-Δntcdc6-2A pMET3-HA3-CDC20*), the congenic MC_{2A}-*cdc7* strain YJL5821 (*MCM7-2NLS pGAL1-Δntcdc6-2A pMET3-HA3-CDC20 cdc7-1*) and their respective controls, the M strain YJL4486 (*MCM7-2NLS pGAL1 pMET3-HA3-CDC20*) and the M-*cdc7* strain YJL5816 (*MCM7-2NLS pGAL1 pMET3-HA3-CDC20 cdc7-1*) were induced with galactose as described in Figure 7C, except that following the initial arrest at 23° C, the arrested cells were maintained at 23° C for 1 hr, before the addition of galactose. Genomic DNA was isolated 4 hr after galactose addition and competitively hybridized (MC_{2A} versus M and MC_{2A}-*cdc7* versus M-*cdc7*) as described in Figure 1A. Re-replication profiles for the MC_{2A} (blue line) and MC_{2A}-*cdc7* (green line) strains are shown for chromosome III. The locations of pro-ARSs mapped by Wyrick *et al.* (Wyrick *et al.*, 2001) (red triangles), and the centromere (black circle) are plotted along the X-axis.

Table S1. CGH on spotted microarrays accurately identifies S phase replication origins.
Our analysis of the replication of a wild type yeast in the S288c background identified 212 replication origins throughout the genome, which is roughly comparable to the numbers obtained by Rhaguraman *et al.* (Raghuraman *et al.*, 2001) (332) and Yabuki *et al.* (Yabuki *et al.*, 2002) (260). Origins on chromosome III (Greenfeder and Newlon, 1992; Poloumienko *et al.*, 2001), VI (Yamashita *et al.*, 1997), V (Tanaka *et al.*, 1996) and X (Wyrick *et al.*, 2001) have been systematically mapped by 2-D gel electrophoresis and and/or ARS plasmid assay, and annotated in the Saccharomyces Genome Database (SGD) (Balakrishnan). For each origin that was identified on chromosome III, V, VI, and X, the distance to the closest annotated origin in SGD was determined and the mean of these distances was calculated (This study, four hybridizations). A similar comparison was performed for the other origins identified by three previously published genome-wide analyses of budding yeast origins (Raghuraman *et al.*, 2001; Yabuki *et al.*, 2002) or potential origins (Wyrick *et al.*, 2001). We note that for screening purposes, our assay can be streamlined even further by using replication profiles from a single microarray (This study, single hybridization.)

Table S2. Copy number values and chromosomal coordinates for all replication profiles. The smoothed and merged value for each chromosomal locus is listed for each experiment described in this manuscript.

Table S3. List of identified origins for all experiments. For each experiment for which peak finding was performed the chromosomal coordinate and copy number for each peak is listed.

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