SUPPLEMENTAL MATERIALS AND METHODS

Plasmids

All plasmids are described in Table 1. Only pJL1488 (*pGAL1-Δntcdc6-cdk2A*) was constructed in this study. It contains the sequence 5′-TATGAGCGGCCGC-3′ followed by *CDC6* from +139 to +1983 inserted in the *Sma*I site of pJL806 downstream of the *GAL1* promoter. This plasmid expresses a truncated Cdc6 with amino acids 2-47 replaced by amino acids S-G-R and with S354A and S372A alanine substitutions at the two remaining CDK consensus phosphorylation sites (S/T-P-X-K/R). The S354A mutation was marked with an *Nhe*I restriction site by introducing silent nucleotide substitutions T1059a and T1060g. The S372A mutation was marked with a *Nar*I restriction site by introducing silent nucleotide substitutions T1113g, T1114g, and T1116g. Amino acid and base substitutions are listed relative to the first amino acid and nucleotide, respectively, of the wild type *CDC6* ORF (+1); the starting amino acid or nucleotide is on the left, and the substitution is on the right.

Strain construction

All strains (Table 2) with the exception of YJL5038 were derived from YJL1737 (*MAT*a *orc2-cdk6A orc6-cdk4A leu2 ura3-52 trp1-289 ade2 ade3 bar1Δ::LEU2*) (Nguyen *et al.*, 2001). The *orc2-cdk6A* and *orc6-cdk4A* alleles encode mutant proteins in which alanine is substituted for the phosphoacceptor serines or threonines at all full CDK consensus phosphorylation sites (residues 16, 24, 70, 174, 188, and 206 for *orc2-cdk6A*, and residues 106, 116, 123, and 146 *orc6-cdk4A*). Plasmids pMP933 (*ORC2, URA3*/EcoNI), pJL737 (*ORC6, URA3*/SphI), pJL1206 (*MCM7-2NLS, URA3*/AspI), pKI1260 (*MCM7-2nls3A*/AspI) (Nguyen *et al.*, 2001) and pPP117 *(cdc7-1, URA3*/ClaI) (Hollingsworth *et al.*, 1992) were used in 2-step gene replacements at their respective chromosomal loci. YIp22 (*pMET3-HA3-CDC20, TRP1*/MscI) (Uhlmann *et al.*, 2000) was used in a one-step gene replacement at the CDC20 locus. Plasmids pJL806 (*pGAL1, URA3*/StuI), pJL1488 (*pGAL1-Δntcdc6-cdk2A, URA3*/StuI), and pJL1489 (*pGAL1-Δntcdc6, URA3*/StuI) (Nguyen *et al.*, 2001) were inserted at the *URA3* locus by one step integration.

ARS316, *ARS317* and *ARS318* were deleted using PCR fragments containing KanMX6 or NatMX4 that were amplified, respectively, from pFA6a (Wach *et al.*, 1994)) or pAG25 (Goldstein and McCusker, 1999) using oligonucleotide primers shown in Table 3. The *ars316*Δ removes a 1.19 kb sequence containing *ARS316* and replaces it with a KanMX6 cassette (Poloumienko *et al.*, 2001). The *ars317*Δ removes a 99 bp sequence containing the ARS consensus sequence (ACS) and the ABF1 binding site and replaces it with a KanMX6 cassette. The *ars318*Δ removes an 89 bp sequence containing the ARS consensus sequence (ACS) and the ABF1 binding site and replaces it with a NatMX6 cassette.

YJL5038 (*MAT*a *his3Δ::KanMX leu2Δ0 met15Δ0 ura3Δ0 bar1Δ::NatMX4 can1Δ::pMFA1-HIS3::pMFα1-LEU2*) was derived from a cross between YJL4161 (YD02458, *MAT*a *his3Δ::KanMX4 leu2Δ0 met15Δ0 ura3Δ0*, from the Saccharomyces Genome Deletion Project) (Winzeler *et al.*, 1999) and YJL4954 (*MATα bar1Δ::NatMX4 can1Δ::pMFA1-HIS3::pMFα1-LEU2 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 met15Δ0*). YJL4954 was generated by deleting *BAR1* in Y3655 (*MATα can1Δ::pMFA1-HIS3::pMFα1-LEU2 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 met15Δ0*) (Tong *et al.*, 2004) using a PCR fragment containing NatMX4 amplified from pAG25 (Goldstein and McCusker, 1999) using oligonucleotide primers shown in Table 3.

2-D Gel Electrophoresis

Neutral-neutral two-dimensional (2D) gel analysis was performed essentially as described at http://fangman-brewer.genetics.washington.edu. The DNA preparation described there is a slight modification of the one used in Huberman *et al.* (Huberman *et al.*, 1987). The following modifications to the previous protocols were made. Thirty micrograms of DNA was digested with *Cla*I and *Bgl*II for analysis of ARS317. Digested DNA was then enriched for replication intermediates with BND cellulose as follows. 4 g BND cellulose (Sigma B6385) was boiled in 20 ml water in a 50 ml conical tube for 5 min then spun at 2,000 rpm for 2 min in a SX4750 rotor using a GS-6 centrifuge (Beckman). The BND cellulose was washed once with 20 ml water and twice with NET (1 M NaCl, 1 mM EDTA, 10 mM Tris, pH 8.0) buffer. 1 ml packed column volume of BND cellulose suspension was placed in a disposable chromatography column (BioRad 731-1550) for each sample and washed with 5 ml of NET buffer. 5 M NaCl was added to each DNA digest to a final concentration of 1 M and the DNA was loaded on the column by passing the sample through twice. The column was washed with 5 ml NET and eluted with 3 ml 50 ˚C NET plus 1.8% caffeine. 3 ml isopropanol was added to the eluate, mixed by inversion and placed on ice for 30 min. The samples were spun for 30 min at 10,000 rpm at 4 ˚C, and the pellet was washed with ice cold 70% ethanol before being air-dried and resuspended in 40 μl TE. Loading dye (final

concentrations: 2% w/v Ficoll 400, 0.01 M EDTA, 2% w/v SDS, 0.025% w/v bromophenol blue, 0.025% xylene cyanol) was added to the pellet and the entire sample was loaded on the gel.

For direct comparison, up to four samples were electrophoresed in the second dimension in quadrants of a single large gel and transferred using the high-salt downward capillary transfer method (Ausubel *et al.*, 2000) together to a single membrane GeneScreen Plus nitrocellulose membrane (NEN) and cross-linked with 0.12 J of UV light in a UV Stratalinker 1800 (Stratagene). The ARS317 probe was generated by PCR amplification of yeast genomic DNA using primers OJL1607 and OJL1608 (Table 3). This probe was labeled with the MegaPrime DNA labeling kit (Amersham Pharmacia), hybridized with ExpressHyb (Clontech) per the manufacturer's instructions and detected on a Storm 860 PhosphorImager (Molecular Dynamics).

Array Design and Fabrication

PCR products representing every ORF and intergenic region were designed and amplified as previously described (DeRisi *et al.*, 1997; Iyer *et al.*, 2001). Intergenic regions larger than 1.5 kb were amplified in segments of at most 1.5 kb. Each of the PCR products was resuspended in 3X SSC and robotically arrayed onto poly-L-lysine coated glass slides as previously described (DeRisi *et al.*, 1997). The remaining poly-L-lysine was then blocked as previously described (DeRisi *et al.*, 1997) (protocol is available at http://derisilab.ucsf.edu/core/resources/index.html) with the following modifications. The hydration step was omitted and instead slides were incubated in 3X SSC 0.2% SDS at 65˚C for 5 min. Slides were washed successively with H_2O and 95% ethanol, and then dried by centrifugation for 2 min at 500 rpm in a SX4750 rotor using a GS-6 centrifuge (Beckman) and processed as described previously.

Genomic DNA preparation for CGH

450 ml of culture was mixed with 2.25 ml of 20% sodium azide and added to 50 ml of frozen, –80 ˚C, 0.2 M EDTA, 0.1% sodium azide. Cells were pelleted, washed with 50 ml 4 ˚C TE (10 mM TrisCl 1 mM EDTA pH 7.5) and stored frozen at –80˚C. Pellets were resuspended in 4 ml Lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl, 1 mM EDTA pH8.0) and mixed with 4 ml of phenol:$CHCl_3$:isoamyl alcohol (25:25:1) and 8 ml 0.5 mm glass beads (BioSpec Products, Inc., Bartlesville, OK). The suspension was vortexed seven times for 2 min separated by 2 min intervals at room temperature until greater than 95% of the cells lysed. The lysate was diluted with 8 ml phenol:$CHCl_3$:isoamyl alcohol and 8 ml TE, and then centrifuged at 18,500 x g for 15 min at RT. After collecting the aqueous phase, the interphase was re-extracted with 8 ml TE, and the second aqueous phase from this re-extraction pooled with the first. The combined aqueous phases were extracted with an equal volume of $CHCl_3$. The bulk of the RNA in the extract was selectively precipitated by addition of 0.01 volume 5 M NaCl to 50 mM and 0.4 volumes isopropanol and centrifugation at 12,000 x g for 15 min at RT. The RNA pellet was discarded and an additional 0.4 volumes of isopropanol was added to the supernatant. The sample was pelleted, washed with 70% ethanol, dried, and resuspended with 5.3 ml 10 mM Tris-Cl, (pH 8) 1 mM EDTA 1 M NaCl. RNase A (Qiagen, Valencia, CA) was added to 225 μg/ml followed by incubation at 37ºC for 30 min. Proteinase K was then added to 350 μg/ml followed by incubation at 55 ˚C for 30 min. Finally, 0.6 ml of 10% (w/v) Cetyltrimethylammonium Bromide (CTAB) in 1 M NaCl (prewarmed to 65 °C) was added and the sample was incubated for 20 min at 65 ˚C before being extracted with 8 ml $CHCl_3$ and centrifuged at 6000 x g for 15 min at RT. The DNA in the aqueous phase was precipitated with 0.8 volumes isopropanol at RT, washed with 70% ethanol, dried, and resuspended in 10 ml Qiagen buffer QBT. DNA was loaded and purified on a Qiagen Genomic-tip 100/G column as per the manufacturer's instructions (Qiagen, Valencia, CA). The eluted DNA was precipitated with 0.8 volumes isopropanol at 4 ˚C, washed with 70% ethanol, dried, and resuspended in 250 μl 2 mM Tris pH 7.5. This highly purified genomic DNA (OD 260/280 1.82-1.86) was sheared by sonication with a Branson Sonifier 450 to an average fragment size of 500 bp. Isolating DNA of this purity is important for generating reproducible replication profiles.

Labeling and Hybridization

5 μg of sheared genomic DNA was randomly primed with 10 μg of N_9 nonomer by boiling for 5 min, then cooling on ice for 5 min. 5-(3-Aminoallyl)-2'-deoxyuridine 5'-triphosphate (Sigma A0410, St. Louis, MO) was incorporated into the primed genomic DNA in a 50 μl reaction containing 10 mM TrisHCl pH 7.5, 5 mM MgCl2, 7.5 mM dithiothreitol, 120 μM dATP, 120 μM dCTP, 120 μM dGTP, 20 μM dTTP, 100 μM 5-(3-Aminoallyl)-2'-deoxyuridine 5'-triphosphate, and 5 U Klenow fragment. The reaction was incubated at 37˚C for 4 hr, and the DNA was purified using the DNA Clean and Concentrator kit (Zymo Research, Orange, California). 15-40 nmol of Cy3 and Cy5 (Amersham, Piscataway, NJ) were then

separately coupled to the appropriate DNA with 0.1 M $NAHCO_3$, pH 9.0 for 1 hr (Bozdech *et al.*, 2003), and the fluorescently labeled DNA purified using the DNA Clean and Concentrator kit (Zymo Research, Orange, California). For most hybridizations, the replicating or re-replicating DNA was labeled with Cy5 and the non-replicating control DNA was labeled with Cy3.

Cy3 and Cy5 labeled DNA were pooled in a 40 μl mixture containing 3X SSC, 25 mM HEPES pH 7.0, and 0.25% SDS. Samples denatured for 2 min at 100˚C and hybridized under a glass mSeries Lifterslip (Erie Scientific 25x40I-M-5227, Portsmouth, NH) to a microarray for 18-24 hours at 63˚C. Microarrays were washed successively in 0.85X SSC, 0.02%SDS and 0.035X SSC immediately before scanning. The microarrays were spun dry and scanned with a GenePix 4000B scanner (Axon Instruments Union City, California) in an enclosed chamber where atmospheric ozone was maintained below 10 ppb using two OI-45 Ozone Interceptors (Ozone solutions, Sioux Center, Iowa). Reducing Cy5 exposure to atmospheric ozone during the final drying and scanning is essential for obtaining reproducible replication profiles.

Data analysis

Genepix Pro 4.0 software (Axon Instruments, Union City, CA) was used for micorarray image analysis and quantification. Data were filtered to remove features that had (1) obvious defects, (2) saturated pixels, (3) regression R^2 values less than 0.5, or (4) fewer than 55% of their pixels with fluorescence intensity greater than 2 standard deviations above background. Data was also filtered to remove 1572 features that contain repetitive sequences from the analysis. The median of the ratios for each element was used for the raw Cy5/Cy3 value.

The raw ratios were normalized by multiplying each value by a scalar normalization factor chosen so that the average of the normalized values was equal to the DNA content of the cells. DNA content was calculated from the median of the flow cytometry profile after correcting for signal increase due to mitochondrial replication (detailed information on the calculation of DNA content is provided below). The raw data were then binned and smoothed essentially as described (Raghuraman *et al.*, 2001). In short, a moving median was calculated over a 10 kb window for every 0.5 kb location along the genome. If a given 10 kb window did not contain any raw data points after filtering it was defined as a no data zone and the binned value from the previous window was used for smoothing purposes. The binned data were then smoothed using Fourier Convolution Smoothing essentially as described (Raghuraman *et al.*, 2001). However, the equation for k(S) was incorrectly provided in part II.3 of the supplemental information of that paper. The correct equation is as follows (personal communication, Collingwood D.):

$$k(S) = \{\exp(-2^{-S} n^2) : \text{n is an integer satisfying } -\left\lceil \frac{T}{2} \right\rceil \le n \le T - 1 - \left\lceil \frac{T}{2} \right\rceil\}$$

In Raghuraman *et al.* (Raghuraman *et al.*, 2001) the optimal value for S was computationally determined for each chromosome for each experiment. While this was effective for replication profiles, we found that predetermined values for S resulted in better re-replication profiles. Thus, for all G2/M and G1 release re-replication profiles, the following values for S were used for each chromosome: I: 8, II: 9.75, III: 8.25, IV: 12, V: 9, VI: 8, VII: 10.5, VIII: 9, IX: 8.75, X: 9.5, XI: 9.25, XII: 10.5, XIII: 10.25, XIV: 9.75, XV: 10.75, XVI: 10.25.

In most cases, two hybridizations were performed from each of two independent genomic DNA preparations. For presentation purposes, the resulting four replication profiles were averaged into one composite profile. Table S2 contains the value at each chromosomal locus for each of the composite profiles in this manuscript. In the final replication profiles, no data regions as described above are presented as gaps in the profiles.

Peak Finding

In order to identify potential origins, all local maxima in the smoothed data were identified and filtered based on two parameters, slide and drop. The maxima that satisfy our slide and drop parameters define the origin list. Due to the very different nature of replication and re-replication, some peak finding parameters were different between the two types of profiles. Solely for peak finding purposes, the re-replication data were normalized to half of their DNA content, in order to use the same range of parameters as are used during replication.

The drop value is a semi global measure of peak height. For each maximum, drop is the difference between the Cy5/Cy3 ratio of that maximum and the lowest Cy5/Cy3 value within 15kb (replication) or 200kb (re-replication) on either side of that maximum. The slide value (Glynn *et al.*, 2004)

is a local measure of peak height and is the difference between the Cy5/Cy3 ratio of the maxima being considered and the closest local minima on either side of the maxima. The total slide is the sum of the left slide and the right slide. For replication profiles, a local maxima was identified as a potential origin if the following conditions all apply: 1) the drop value was greater than 0.05, 2) the left slide was greater than 0.005, 3) the right slide was greater than 0.005 and 4) the total slide was greater than 0.05 (replication) or 0.02 (re-replication). .

To identify a list of origins for a given experimental condition, duplicate microarrays were performed for each of two independent genomic DNA preparations, and the sets of potential origins from the four individual microarrays were merged as follows. Hierarchical clustering (average linkage) was used to identify locations where several individual microarray experiments had potential origins. If three of the four microarray experiments recorded a potential origin in a 15 kb region (replication) or two of the four within 20 kb (re-replication), the locations of those potential origins were averaged and reported as the origin position for the merged dataset. Table S3 contains the list of identified origins for all experiments for which peak finding was performed.

Scatter Plot

The locations of 351 pro-ARSs from all the budding yeast chromosomes except chromosomes IV and XI were obtained from Wyrick *et al.* (Wyrick *et al.*, 2001). These chromosomes were excluded because some of the strains used in our study have duplications of portions of these chromosomes. These genomic alterations do not have any effect on the extent or origin usage of either replication or re-replication (data not shown). For each pro-ARS, the normalized Cy5/Cy3 ratio of that chromosomal locus for replication or re-replication was plotted against the ratio at that locus of the other profile being compared. The linear regression formula and R^2 value are shown on the plot.

Normalization of Replication and Re-replication Profiles by Quantification of Flow Cytometry Data

Flow cytometry was used to calculate the DNA content in each experiment. The genomic replication profiles and re-replication profiles were then normalized to the calculated DNA content. Quantification of absolute DNA content in *Saccharomyces cerevisiae* is complicated by fact that roughly 10% of the total DNA in a yeast cell is mitochondrial. Furthermore, cell cycle independent mitochondrial DNA replication causes the peak of flow cytometry profile to gradually increases in cell cycle arrested yeast (Pichler *et al.*, 1997). This complicates the quantification of the absolute DNA content by flow cytometry in synchronized yeast cultures. The following calculations were used to correct for the increase in the flow cytometry peak due to mitochondrial replication and thus determine the actual DNA content from the observed flow cytometry peak.

Replication: G1 into 0.1M HU

First, the absolute increase in fluorescent intensity due to the duplication of the yeast genome was calculated for each strain. The fluorescence intensity for the G1 peak (p_{Asyn}^{1C}) and the G2 peak (p_{Asyn}^{2C}) were determined from the asynchronous sample collected for each strain at the beginning of each experiment. The values for the G1 and G2 peaks in asynchronous samples were determined by applying the cell cycle model described by Watson *et al.* (Watson *et al.*, 1987) using the computer software FlowJo (Tree Star, Inc., Ashland, OR). The peak value for cell cycle synchronized samples was determined by taking the median of the flow cytometry graph. The difference (Δp) between these two peaks was the signal increase due to a single round of replication.

$$\Delta p = p_{Asyn}^{2C} - p_{Asyn}^{1C} \qquad (1)$$

When cells are cell cycle arrested, mitochondria continue to replicate their DNA. Thus, the fluorescent intensity continues to increase in the absence of ongoing genomic DNA replication. Thus, the peak of the G1 synchronized cells (p_0^{1C}) is greater than the G1 peak in the asynchronous population (p_{Asyn}^{1C}). Therefore, when all the cells in a population reach the G1 arrest, the subsequent G2 peak will no longer be twice value of the G1 peak. The subsequent G2 peak will be G1 plus the increase due to duplication of the genomic DNA (Δp). For cells arrested in G1 with a factor the calculated 2C value at time 0 (c_0^{2C}) was:

$$c_0^{2C} = p_0^{1C} + \Delta p \quad (2)$$

Cells were subsequently released from the G1 arrest into G2 in the presence of hydroxyurea to slow S phase and nocodazole to arrest cells in the subsequent G2. The calculated value of the G2 peak was confirmed by the measured position of peak when the cells reached the nocodazole arrest. The rightward shift in the flow cytometry peak due to genomic independent (mitochondrial) replication was reduced in the presence of hydroxyurea. It has previously been shown that mitochondrial DNA copy number is sensitive to nucleotide levels, which could explain the lack of further shift (Taylor *et al.*, 2005).

To calculate the extent of replication following synchronous release from a factor an equation relating DNA content to the flow cytometry peak was generated using the measured G1 peak (p_0^{1C}) and calculated G2 peak (c_0^{2C}) as endpoints. In other words, since it is possible to correlate flow cytometry peak value to DNA content at 1C and 2C, a line connecting those points would enable us to determine the DNA content represented by any measured flow cytometry peak value between those two points. Thus, a linear regression line of the form $d = mp + b$ was generated where d is the DNA content, p is the median of the flow cytometry peak for a sample, m is the slope and b is the intercept. For a given time point (t) the median of the flow cytometry data (p_t) was used to calculate the DNA content (d_t). This value was then used as the normalization factor for the corresponding genomic replication profile. The DNA content of the G1 peak (d_{1C}) was one and the G2 peak (d_{2C}) was two.

$$d_t = \overbrace{\left(\frac{d_{2C} - d_{1C}}{c_0^{2C} - p_0^{1C}}\right)}^{m} p_t + \overbrace{\left[d_{1C} - \left(\frac{d_{2C} - d_{1C}}{c_0^{2C} - p_0^{1C}}\right) p_0^{1C}\right]}^{b} \quad (3)$$

Re-replication: G2/M

A similar approach was used to correct for the shift in the flow cytometry peak due to mitochondrial replication during G2 arrest and induction of re-replication. For each re-replicating strain containing *pGAL1*-Δ*ntcdc6* there was a complementary *pGAL1* control strain. This *pGAL1* control did not re-replicate but did experience the genomic re-replication independent shifting of the flow cytometry peak. Thus, its shift could be used to correct for re-replication independent shift in the re-replicating strain. At a given time point this shift (s_t^{pGAL1}) was the difference of the median of the flow cytometry data of the *pGAL1* control at the start the experiment ($p_0^{2C,pGAL1}$) and the median at the time of interest (p_t^{pGAL1}):

$$s_t^{pGAL1} = p_t^{pGAL1} - p_0^{2C,pGAL1} \quad (4)$$

The corrected value of the re-replicating strain's flow cytometry peak (f_t^{rerep}) at a given time was calculated by subtracting s_t^{pGAL1} from the median of the measured flow cytometry peak in the re-replicating strain (p_t^{rerep}).

$$f_t^{rerep} = p_t^{rerep} - s_t^{pGAL1} \quad (5)$$

As in the S phase experiments, the absolute increase in fluorescent intensity due to the duplication of the genome (Δp^{rerep}) was calculated using the G1 peak ($p_{Asyn}^{1C,rerep}$) and G2 peak ($p_{Asyn}^{2C,rerep}$) from the asynchronous sample of the *pGAL1*-Δ*ntcdc6* re-replicating strains.

$$\Delta p^{rerep} = p_{Asyn}^{2C,rerep} - p_{Asyn}^{1C,rerep} \quad (6)$$

Next, the value of a hypothetical 4C peak ($c_0^{4C,rerep}$) was calculated by adding twice Δp^{rerep} to the measured value of the G2/M arrested peak ($p_0^{2C,rerep}$).

$$c_0^{4C,rerep} = p_0^{2C,rerep} + 2(\Delta p^{rerep}) \tag{7}$$

A linear regression line $d = mf + b$ was generated similar to the S phase experiment above where d is the DNA content, f is the corrected median of the FACS peak for a sample, m is the slope and b is the intercept. For a given time point (t) the corrected median of the flow cytometry data (f_t^{rerep}) was used to calculate the DNA content (d_t). This value was then used as the normalization factor for the corresponding genomic re-replication profile. The DNA content of the G2 peak (d_{2C}) was two and the hypothetical 4C peak (d_{4C}) was four.

$$d_t = \overbrace{\left(\frac{d_{4C} - d_{2C}}{c_0^{4C,rerep} - p_0^{2C,rerep}}\right)}^{m} f_t^{rerep} + \overbrace{\left[d_{2C} - \left(\frac{d_{4C} - d_{2C}}{c_0^{4C,rerep} - p_0^{2C,rerep}}\right) p_0^{2C,rerep}\right]}^{b} \tag{8}$$

Re-replication: G1 into G2/M

As in the S phase experiments, the absolute increase in fluorescent intensity due to the duplication of the genome (Δp) was calculated using the G1 peak (p_{Asyn}^{1C}) and G2 peak (p_{Asyn}^{2C}) from asynchronous samples of both the *pGAL1* control and *pGAL1*-Δ*ntcdc6* re-replicating strains.

$$\Delta p^{pGAL1} = p_{Asyn}^{2C,pGAL1} - p_{Asyn}^{1C,pGAL1} \tag{9}$$

$$\Delta p^{rerep} = p_{Asyn}^{2C,rerep} - p_{Asyn}^{1C,rerep} \tag{10}$$

In this type of experiment the flow cytometry peak for the *pGAL1* control will increase for two reasons: genomic replication and genomic independent (mitochondrial) replication. The flow cytometry peaks in the *pGAL1*-Δ*ntcdc6* re-replicating strains will increase for up to three reasons: genomic replication, genomic independent (mitochondrial) replication and potentially re-replication. The value of the subsequent G2 peak ($c_0^{2C,pGAL1}$), which accounts for genomic replication, was calculated for the *pGAL1* control and *pGAL1*-Δ*ntcdc6* re-replicating strain.

$$c_0^{2C,pGAL1} = p_0^{1C,pGAL1} + \Delta p^{pGAL1} \tag{11}$$

$$c_0^{2C,rerep} = p_0^{1C,rerep} + \Delta p^{rerep} \tag{12}$$

The value of the fluorescence increase due to genomic replication independent (mitochondrial) shifting (s_t^{pGAL1}) was calculated from the *pGAL1* control strain by subtracting the calculated value of G2 peak ($c_0^{2C,pGAL1}$) from the median of the flow cytometry data at the cells were harvested (p_t^{pGAL1}).

$$s_t^{pGAL1} = p_t^{pGAL1} - c_0^{2C,pGAL1} \tag{13}$$

The shift (s_t^{pGAL1}) was then used to correct the observed flow cytometry peak of the re-replicating strain such that the peak value would reflect only the increases due to genomic replication and re-replication. The corrected value of the flow cytometry peak (f_t^{rerep}) was determined by subtracting s_t^{pGAL1} from the measured median of the flow cytometry data in the re-replicating strain (p_t^{rerep}).

$$f_t^{rerep} = p_t^{rerep} - s_t^{pGAL1} \quad (14)$$

Next, the value of a hypothetical 4C peak ($c_0^{4C,rerep}$) was calculated by adding three times Δp^{rerep} to the measured value of the G1 arrested peak ($p_0^{1C,rerep}$).

$$c_0^{4C,rerep} = p_0^{1C,rerep} + 3(\Delta p^{rerep}) \quad (15)$$

Finally, a linear regression line $d = mf + b$ was generated similar to the above experiments where d is the DNA content, f is the corrected median of the FACS peak for a sample, m is the slope and b is the intercept. For a given time point (t) the corrected median of the flow cytometry data (f_t^{rerep}) was used to calculate the DNA content (d_t). This value was then used as the normalization factor for the corresponding genomic re-replication profile. The DNA content of the G2 peak (d_{2C}) was two and the hypothetical 4C peak (d_{4C}) was four.

$$d_t = \overbrace{\left(\frac{d_{4C} - d_{2C}}{c_0^{4C,rerep} - c_0^{2C,rerep}}\right)}^{m} f_t^{rerep} + \overbrace{\left[d_{2C} - \left(\frac{d_{4C} - d_{2C}}{c_0^{4C,rerep} - c_0^{2C,rerep}}\right) c_0^{2C,rerep}\right]}^{b} \quad (16)$$

Re-replication: G1 into HU

The DNA content for re-replication induced from G1 into hydroxyurea was calculated as in the S phase experiments (Replication: G1 into HU) in the absence of induction of re-replication.

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