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Supplemental figure 1--Analysis of additional cell cycle movies showing that Cse4-EGFP intensity doubles at anaphase B. Since the timing is different in each time course we could not overlay the data from multiple movies.

Supplemental figure 2--Comparison of Mif2-EGFP and Cse4-EGFP

Along with Cse4-EGFP we counted Mif2-EGFP/CENP-C copy number at different stages of the cell cycle; specifically we looked at G1 and anaphase. In G1, Mif2 is present at one copy per centromere. The difference in the anaphase measurements is not statistically significant ($p=0.41$). Copy number doubling at anaphase is specific to Cse4.

Supplemental figure 3--Centromere cluster size

Centromere clusters of similar size were used for counting Cse4 copy number. The graph shows the standard deviation of cluster size in microns as a function of Cse4 copy number. For our copy number analysis we used clusters with size below 0.19 micron.

Supplemental figure 4--Analysis of Cse4-EGFP and aneuploidy in additional strains

A) Karyotype analysis by quantitative PCR of the strains used for microscopy. Because the UCSF Cse4-GFP library strain was aneuploid for three chromosomes (Chromosome II, IX and XI) we generated a new haploid Cse4-EGFP and diploid (Cse4-EGFP and Cse4-EGFP) strain in the S288C background. Error bars represent ± the average deviation of technical replicates. B) The average number of Cse4 copies per cluster was counted for the haploid strain (black), the UCSF strain (red), and the diploid strain (blue). Error bars represent ± the standard deviation for each data set. P values are calculated by standard student's t-test.

Supplemental Figure 5--Analysis of Cse4-EGFP and aneuploidy in published strains

A) Karyotype analysis by qPCR of the strains used for microscopy in Lawrimore et al., and Coffman et al. Error bars represent ± the average deviation of technical replicates. B) The average number of Cse4 copies per cluster was counted for our haploid strain, strain A and strain B. Error bars represent ± the standard deviation for each data set. P values are calculated by standard student's t-test.

Supplemental Figure 6--Analysis of GFP-MotB

The fluorescent lifetime measurements shows shorter lifetime for GFP-MotB compared to Cse4-EGFP. The upper panel shows the bar plot for fluorescent lifetimes from single exponential fits of Cse4-EGFP centromere clusters and GFP-MotB. The lower panel shows the normalized average fluorescence decay for Cse4-EGFP centromere clusters (red), and GFP-MotB. Error bars represents ± the standard error of the mean for each data set.

Supplemental Figure 7--Analysis of Nup49 in NPCs

We used our unique FCS calibration method to count the copy number of Nup49-EGFP in a single NPC. To confirm that we were quantifying the signal from single NPC's, we have collected data for several clearly separated NPC clusters in the anaphase bridge during anaphase. We could detect distribution of clusters that had signal in multiples of 16, demonstrating our ability to identify single NPCs. This data establishes that the FCS method can count EGFP tagged proteins with reasonable accuracy and provides further support for the claim that the copy number of Cse4-EGFP oscillates between 16 and 32 molecules per cluster.

Movie S1

Cse4-EGFP intensity doubles at anaphase B. Time-lapse movie assembled from

fluorescent images of cells which expresses Cse4-EGFP as the sole copy of Cse4. The cell at the center of the screen shows G1 to telophase/G1. The intensity doubled at anaphase B compared to G1/telophase. The images shown are a single focal plane selected from a z-stack every 5 min. A C-Apochromat 40x 1.2 NA water objective was used for imaging, and image scale is 125 nm/pixel. Duration of this movie is 75 min in real time.

Movie S2

Cse4-EGFP can be deposited at early anaphase. Time-lapse movie assembled from Cse4-EGFP fluorescent images after photobleaching (0 min). The recovery of fluorescence was monitored at 15, 25, 30, and 40 mins. The starting fluorescence (before bleach) intensity was compared to the recovered intensity. All the imaging parameters were the same as in movie S1.

Movie S3

Doubling of Cse4-GFP intensity in anaphase in *Candida albicans*. Time-lapse movie assembled from CaCSE4-GFP fluorescent images from metaphase to late anaphase. The intensity doubled during late anaphase. The numbers in the movie represents the distance between the centromeric clusters in micrometers (μm). All the imaging parameters were the same as in movie S1.

Movie S4

Deposition of Cse4-GFP during early anaphase in *Candida albicans*. Time-lapse movie assembled from Cse4-GFP fluorescent images after photobleaching (0 min). The recovery of fluorescence was monitored at 20 and 60 minutes. The starting fluorescence (before bleach) intensity was compared to the recovered intensity. All the imaging

parameters were the same as in movie S1.

Supplemental Table 1

	Strain Name	**Genotype**
Budding yeast Cse4 EGFP	MS171	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 BZZ1::GFP::SpHIS5*
	MS 195	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Cse4-GFP::SpHIS3 pSpc42-mCherry::LEU2*
	MS 189.1	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0 Cse4-GFP::SpHIS5/Cse4-GFP::URA3*
	MS191.1	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0 Cse4-GFP::SpHIS5/Cse4-mCherry::URA3*
	MS 56	*Cse4-GFP strain from UCSF(Library)*
	MS 275.1	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nup49-GFP::SpHIS3*
	MS 278	*Nup159-GFP strain from UCSF(Library)*
	MS 277	*Nic96-GFP strain from UCSF(Library)*
	MS 268(Cse4-GFP A)	*SBY8584 (W 303a leu2-3,112his3-11, trp1-1,can1-100,ade2-1,bar1-1,lys2$^{\Lambda}$* ,cse4::KANMX,ura3-1:Cse4-XbaI(GFP):URA3,Dsn1-HIS-FLAG:URA3
	MS 269(Cse4-GFP B)	*KBY 7006 473a CSE4-GFP:Kan (C terminal)*
	MS 274 (GFP-MotB)	*JPA750 (E.coli GFP-MotB)*
Scm3 ChIP	RC82	*MATa ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 CSE4-12myc::URA3 SCM3-3HA::KAN*
Cse4 sequential ChIP	MM118	*MATa ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 CSE4-12myc::URA3 pRS413-2NFLAG-Cse4*
H2A ChIP	RC177	*MATa ura3-1 lys2Δ::hisG trp1-1 his3-11,15 leu2-3,112 can1-100 HTA1-Flag:LoxP/HTA2-3FLAG:KAN*
Candida CaCse4-GFP	MS203	*ura3Δ::λimm434/ Ura3::Δimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG CSE4-GFP-CSE4/ CSE4-GFP-CSE4*

Supplemental Experimental Procedures

Fluorescence Correlation Spectroscopy (FCS) calibrated intensity measurement of number of Cse4-EGFP molecules in the centromere.

In order to quantify the number of Cse4-EGFP molecules in the yeast centromere cluster, we took advantage of the unique ability of FCS to determine the average intensity per 1X-EGFP and average number of 1X-EGFP molecules in the focal volume for a mobile, diffuse protein (Figure 3). Once this was determined, calibrated imaging was performed to compare the spot intensity of centromeric Cse4-EGFP to the intensity obtained for this diffuse protein using identical imaging parameters (Figure 3). The maximum intensity of a single immobile fluorophore (also defined as the molecular brightness, ε) can be determined from the intensity of a solution of known concentration: $\varepsilon = I/N$ (Skinner et al., 2008). N is the number of particles in a predefined volume (e.g. the focal volume) which can be accurately determined using FCS measurements. Once the intensity of a single particle is determined, it is straightforward to determine the number of particles in a diffraction limited puncta: $N_{puncta} = I_{puncta}/ \varepsilon$. Note that all immobile intensities here refer to the maximum intensity of the observed diffraction limited spot. We emphasize that this method calculates Cse4-EGFP molecules in the centromere from first principles, without the need to make any assumptions about concentration of any control samples or proteins.

For this method, we used as a control cytosolic EGFP under the control of the endogenous *BZZ1* promoter in yeast (Figure 3A) (Slaughter et al., 2008). As it is driven under an endogenous promoter at one gene copy per cell, concentration on a cell by cell basis is remarkably consistent. Previous analysis has shown that cytosolic EGFP in

yeast is diffuse, relatively uniform over the yeast cell, and mobile (Slaughter et al., 2008). Using the Zeiss ConfoCor 3, FCS was performed on cytosolic EGFP proteins using the identical imaging set-up described above. A pinhole of 1.0 airy units was used, with an excitation intensity of approximately 5 μW at the sample. Autocorrelations were calculated from raw data with a bin time of 50 μs. Data processing was performed using custom written plugins for the ImageJ software package. Correlation functions were fit to the following formula using non-linear least squares: $G(\tau) = \gamma \Big/ N\left(1+\tau/\tau_D\right)\sqrt{1+r^2\,\tau/\tau_D}$.

γ is a shape factor reported to be ~ 0.35 for Gaussian focal volumes (Thompson, 1991). Here, we explicity measured γ using diffraction limited (100 nm) fluorescence beads by collecting three dimensional confocal images of the bead and integrating the signal from the bead as follows:

$$\gamma = \int PSF^2(\vec{r})dr \Big/ \int PSF(\vec{r})dr$$

We found γ to be 0.27 on our microscope with our acquisition conditions. N is the average number of particles in the focal volume, τ_d is the average diffusion time through the focal volume per particle, and *r* is the radial to axial size ratio of the focal volume reported to be approximately 5 for systems with a pinhole close to 1 Airy unit (Hess and Webb, 2002). Note the only parameter needed for our calibrated imaging procedure is N, the average number of molecules in the focal volume.

The fluorescence of freely diffusing cytosolic EGFP was derived from the FCS measurements. Next we performed calibrated imaging comparing the yeast strain

expressing cytosolic EGFP and the yeast strain expressing Cse4-EGFP as its sole copy (Figure 3B). The karyotype of the strain was confirmed by qPCR (Supplemental figure 3). We note that the Cse4-GFP strain from the UCSF collection has some anueploidy. Identical pixel dwell times of 6.3 μs were used, and emission photons were collected on single-photon counting avalanche photodiodes. A 3-dimensional z-stack was acquired with 8 total slices, each with 0.5 μM spacing. A pinhole of 1 airy unit was used to reject out of focus light. Data was spatially binned 2x2 prior to processing. Great care was taken to calibrate the system each day and to take images of Cse4-EGFP and cytosolic EGFP with identical imaging parameters, and only to compare data taken on the same day. For cytosolic EGFP, the fluorescent intensity was calculated as the average over approximately 5 square pixels in the most intense region of the cell. As the centromere may be most in focus at any of the z-slices, we took Cse4-EGFP intensity in the slice where the centromere was most intense.

The centromeric Cse4-EGFP signal has two parts. The first part coincided with the intensity from the EGFP in the diffraction limited or near-diffraction limited point source of the centromere. As the centromere is a diffraction limited spot, a single z slice at the best focus will accurately portray the intensity of the centromere, plus surrounding nuclear background from above, below, and beside the centromere cluster, since the typical z-resolution of a confocal slice is on the order of ~1μm. The second contribution is due to the nuclear, non-centromeric fraction of Cse4-EGFP that resides on top of, below, and next to the centromere. Due to the size of the focal volume, especially in the z-dimension, it is not feasible to collect fluorescence emission from solely the centromere, and exclude the nuclear pool directly above or below the point

centromere, with standard confocal techniques. To separate fluorescence intensity of centromeric Cse4-EGFP from non-centromeric, nuclear Cse4, we fit the intensity residing at the centromere to a 3-dimensional Gaussian (Figure 3C) with non-zero background outside the peak. The fit was performed using a grid-search algorithm over the x and y coordinates as well as the standard deviation with linear least squares determination of the best fit amplitude and background at each point in the grid. This algorithm ensured robust convergence to the absolute best fit for even the noisiest peaks. The non-zero background was subtracted from the peak intensity to give a maximum intensity emanating from the Cse4-EGFP in the centromere. This intensity was compared to the intensity of cytosolic EGFP taken with identical imaging parameters. This comparison, with the knowledge of the number of cytosolic EGFP particles per focal volume, gives us number of Cse4-EGFP molecules in the yeast centromere. These calculations were done also as a function of spindle pole distance. For spindle pole measurements, Spc42-mCherry was recorded using 561 nm excitation and emission was collected through a LP 580 nm filter. Distance between the spindle poles was measured in 3D using ImageJ.

Fluorescence Recovery After Photobleaching (FRAP)

FRAP measurements were performed to examine the ability of centromeric Cse4-EGFP to exchange with the non-centromeric pool. Yeast cells expressing Cse4-EGFP as the only copy of Cse4 were grown to mid-log phase in synthetic complete media, spun down, and sandwiched between a slide and cover slip in a 10% agarose solution made with medium. Long time lapse imaging demonstrated yeast cells were

alive and divided at a normal rate in the agar pad for up to 4 hours. We have taken time points with intervals that minimize bleaching.

Prior to photobleaching, a z-series was taken with 0.5 μM step size and 6.4 μs pixel dwell time. Due to the mobility of the centromere in living yeast cells, acquisition of a z-stack was essential to ensure proper quantitation of centromere intensity. After the initial acquisition, either centromeric Cse4-EGFP, or Cse4-EGFP in the entire cell were irreversibly photobleached by 4 rapid scans with high 488 nm laser power. The ability of the cells to continue to grow and divide ensured that photobleaching did not grossly damage the cells. After photobleaching, movies were acquired to examine for recovery of the Cse4-GFP at the centromere. In most cases, cells were used that also expressed Spc42-mCherry from a centromeric plasmid to mark the cell cycle. Recovery of centromeric Cse4-GFP was observed as a re-appearance of a punctuate spot centered in the nucleus.

Fluorescence resonance energy transfer (FRET) measurements

FRET between EGFP and mCherry labeled proteins was performed using the acceptor photobleaching method (Gu et al., 2004). A diploid yeast strain expressing Cse4-EGFP and Cse4-mCherry as the only copies from the endogenous loci was used. The fluorescence intensity of the Cse4-EGFP was measured at the centromere from the most intense focus of a z-stack with 0.5 μm spacing, as discussed above. Immediately following the initial z-stack, the Cse4-mCherry in the entire cell was irreversibly photobleached using 561 nm excitation. The intensity of Cse4-EGFP at the centromere focus was re-measured after acceptor photobleaching. In the scenario where the donor

is undergoing FRET, irreversible photobleaching of the acceptor probe will result in one less pathway for relaxation of the donor, and hence the fluorescence of the donor will increase.

Apparent FRET efficiencies for each centromere cluster was calculated as follows: $E(\%) = 1 - {I_{before}}/{I_{after}} X100$. Here I_{before} and I_{after} denotes the average fluorescence intensity of the donor before and after acceptor photobleaching.

The chromophore for the mCherry acceptor is estimated to mature fully about 45% of the time. In the fully matured EGFP-mCherry pairs, the FRET efficiency is estimated to be 58% due to the intrinsic size of the fluorophores (Padilla-Parra et al., 2009). These numbers together give maximum FRET efficiency of 26%. Furthermore, for FRET to occur, Cse4-mCherry and Cse4-EGFP must co-occur in the same nucleosome. Assuming the efficiency of this is 50%, we estimate the maximum efficiency at 13%, suggesting that the observed 10-14% is close to the maximum rate of FRET. In terms of distance, the beta barrel cylinder containing the EGFP and mCherry fluorophores are on the order of 3 nm, meaning the two fluorescent molecules must lie within 4 nm for significant FRET to be observed. FRET efficiency falls dramatically as distance increases, therefore significant FRET is likely to occur only when two proteins are physically interacting.

Cell synchronization and release experiments

To localize Scm3-HA at centromere and Cse4 sequential ChIP's cells were grown to logarithmic (LOG) phase at 30°C, treated with 0.2M hydroxyurea (Sigma) for 90 minutes to arrest cells in G1, washed, and released into media containing nocodazole for 60

minutes to arrest cells in metaphase. Samples were taken at 20, 30, 38 and 45 minutes time points after release from nocodazole arrest and used for FACS and ChIP analysis.

FACS and nuclear morphology analyses

FACS analysis was performed to confirm cell-cycle arrest on cells fixed in 70% ethanol. Cells were washed with FACS buffer (50 mM Na Citrate), treated with RNase, stained with Sytox Green (1 mM final), and analyzed by using a Cyan cytometer (Dako Cytomation). Cell cycle stages were determined by examining cell morphology and nuclear position DAPI-stained cells under the Axiovert 451D inverted widefield microscope (Carl Zeiss Inc., Thornwood, USA). Cell cycle stages were defined as follows: G1, single round/unbudded cells with undivided nuclei; S-phase, cells showing a small bud with undivided nuclei; metaphase, large budded cells with nucleus at the neck; early anaphase, elongated nuclei (within the 4µm length) and late anaphase; large budded cells with elongated/separated nuclei; and telophase, large budded cells with nuclei separated and nuclei centered in mother and daughter cells (Calvert and Lannigan, 2001).

Crosslinked and MNase ChIP

Crosslinked and Mnase ChIP performed as previously described (Camahort et al., 2009). Cultures were fixed with formaldehyde (1% final) for 10 minutes and chromatin was harvested by beadbeating in the presence of lysis buffer (100 mM Tris pH 7.5, 150

mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1% NP-40, 10% glycerol, protease inhibitors). For sheared DNA, chromatin was sonicated to obtain fragments ~200 bps - 1 kb in size. For mononucleosome sequential Cse4 ChIPs, chromatin fractionation was performed as previously described (Zhang et al., 2005). $CaCl_2$ (3 mM final) was added to the chromatin. Micrococcal nuclease was added (500 units, Worthington), and chromatin was incubated for 30 minutes at 37°C. The MNase reaction was stopped by addition of EDTA and EGTA to a final concentration of 25 mM. For sequential ChIP, eluates from the αMyc ChIP were diluted to 0.05% SDS with lysis buffer and an αFLAG ChIP was performed. DNA recovered from XChIPs and "no antibody" controls was tested for enrichment of CEN3 sequence. The antibodies used for ChIP are as follows and were all used at 1:500: αMyc (Santa Cruz, 9E10), αFLAG M2 (Sigma), αHA (Roche, 12CA5), Controls omitting antibody were done for all ChIPs.

Quantitative PCR

Quantitative PCR was performed on an iCycler real-time PCR machine using IQ Sybr Green Supermix (Bio-Rad). Previously a used primer set that amplifies 125-bp of CEN3 sequence was used (Krassovsky et al., 2012). PCR of ChIP DNA was quantified for biological replicates by comparing immunoprecipitated samples against a standard curve established with PCRs of serial 10-fold dilutions of a DNA standard. Occupancy level was determined by dividing the average of the ChIP DNA by the relative abundance of a control total chromatin sample. This ratio represents the enrichment of immunoprecipitated DNA over the input DNA for a specific target.

Reconstitution of protein complexes and *in vitro* chromatin assembly

Cse4-containing histone octamers were reconstituted using established protocols (Luger et al., 1999). Briefly, equal molar amounts of the four purified recombinant histones (H2A, H2B, Cse4 and H4) were dissolved in unfolding buffer (7M guanidine-HCl, 20mM Tris-HCl, pH7.5, 10mM DTT) at 2 mg/ml. This histone mixture was dialyzed against four changes of two liters each of refolding buffer (10 mM Tris-HCl, pH7.5, 1 mM EDTA, 5 mM β-mercaptoethanol, 0.1mM PMSF) containing 2M NaCl overnight at 4°C. The mixture was transferred to 1.5ml Eppendorf tubes and centrifuged at 15,000 rpm in a micro-centrifuge to remove any insoluble material. Reconstituted octamers were separated from H2A-H2B dimers and Cse4-H4 tetramers by size fractionation on a Superdex 200 column (Amersham Biosciences, Inc.) and an AKTA FPLC (GE Healthcare) in refolding buffer containing 2M NaCl.

The assembly of chromatin was performed as previously described (Camahort et al., 2009; Ito et al., 1997; Shivaraju et al., 2011). Briefly, 200 ng of plasmid was relaxed with Topoisomerase I. Purified Cse4 containing octamers and 6xHIS-Scm3 or Nap1 were added and incubated for 2 hr at room temperature in the presence of Topoisomerase I with 8.3 mM HEPES pH 7.4, 0.5 mM EGTA, 0.65 mM $MgCl_2$, 1.7% glycerol, 0.005% NP-40, 33 mM KCl, 0.33 mM DTT, and 0.02 mg/ml BSA. Plasmid DNA was deproteinized and purified by standard methods, and topoisomers were resolved in 1% agarose gels. 0.4μm/ml chloroquine was included in the gel to determine the directionality of the DNA wrap. The value of 0.4μm/ml was determined for the plasmids used to obtain maximal resolution of all topoisomers. Recombinant topoisomerase I was a kind gift from S. Venkatesh, Stowers Institute. Two plasmid

were used: 1) pG5E4-5S contains five repeats of 5S flanking each side of an E4 core promoter downstream of five Gal4-binding sites (gift from the Workman lab, Stowers Institute) and 2) pCEN1-10X contains 10 tandem repeats of Centromere 1 sequence.

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