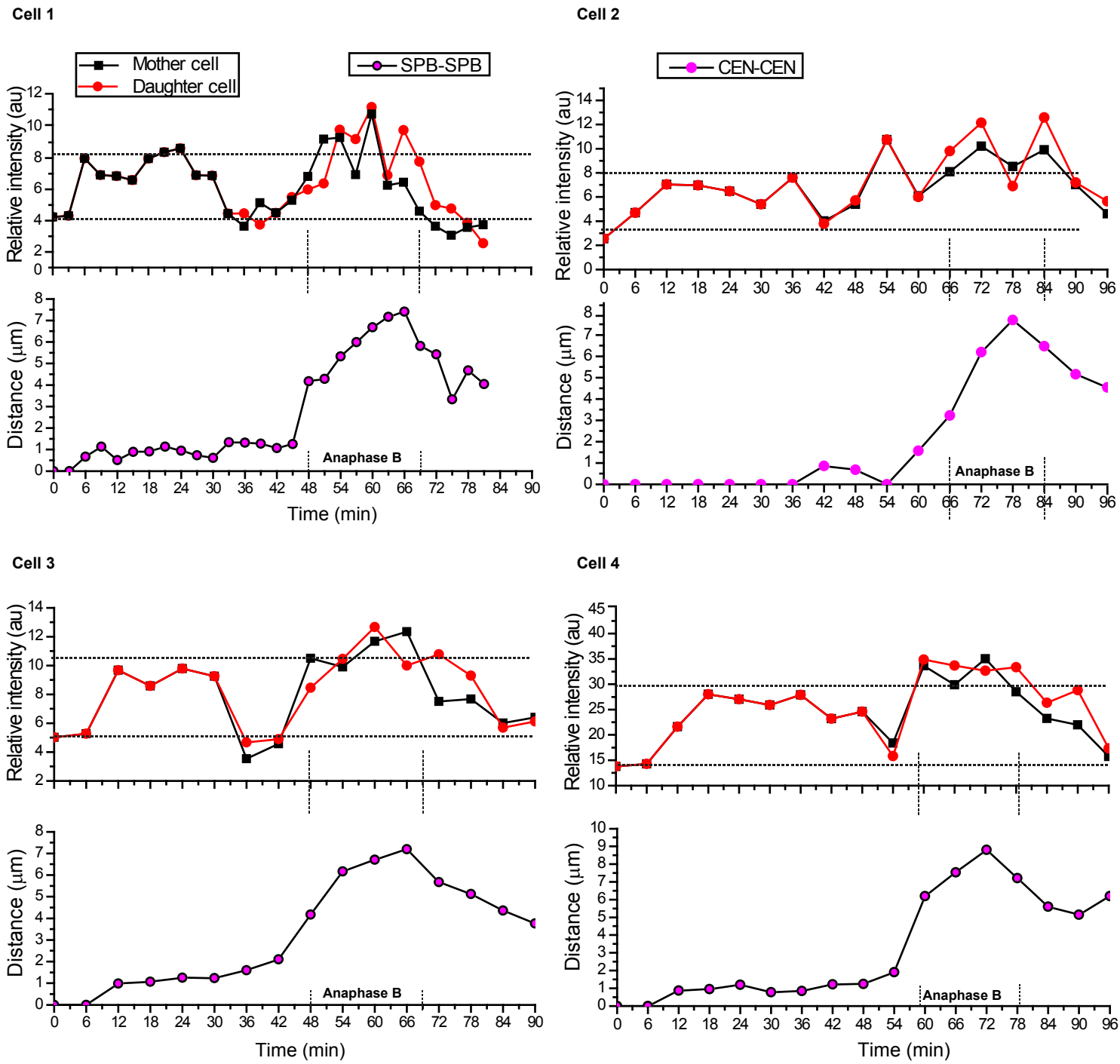
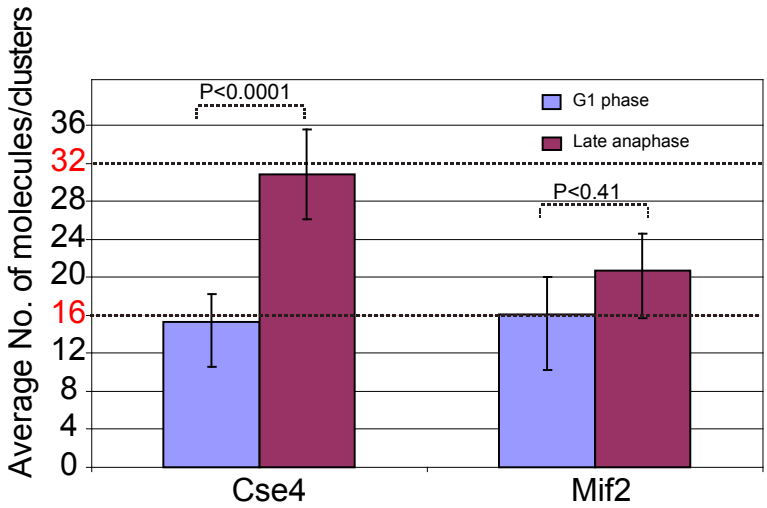
Supplemental figure 1

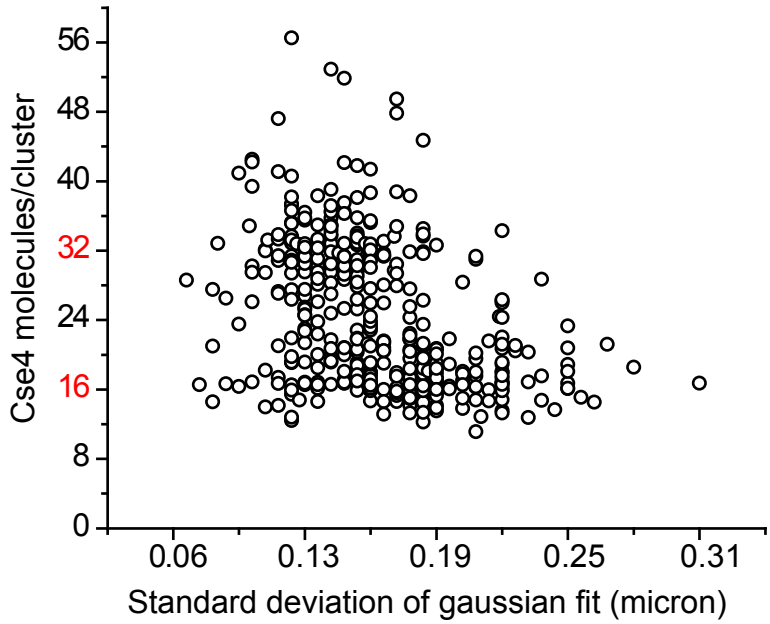


Supplemental Figure 2

Supplemental figure 2

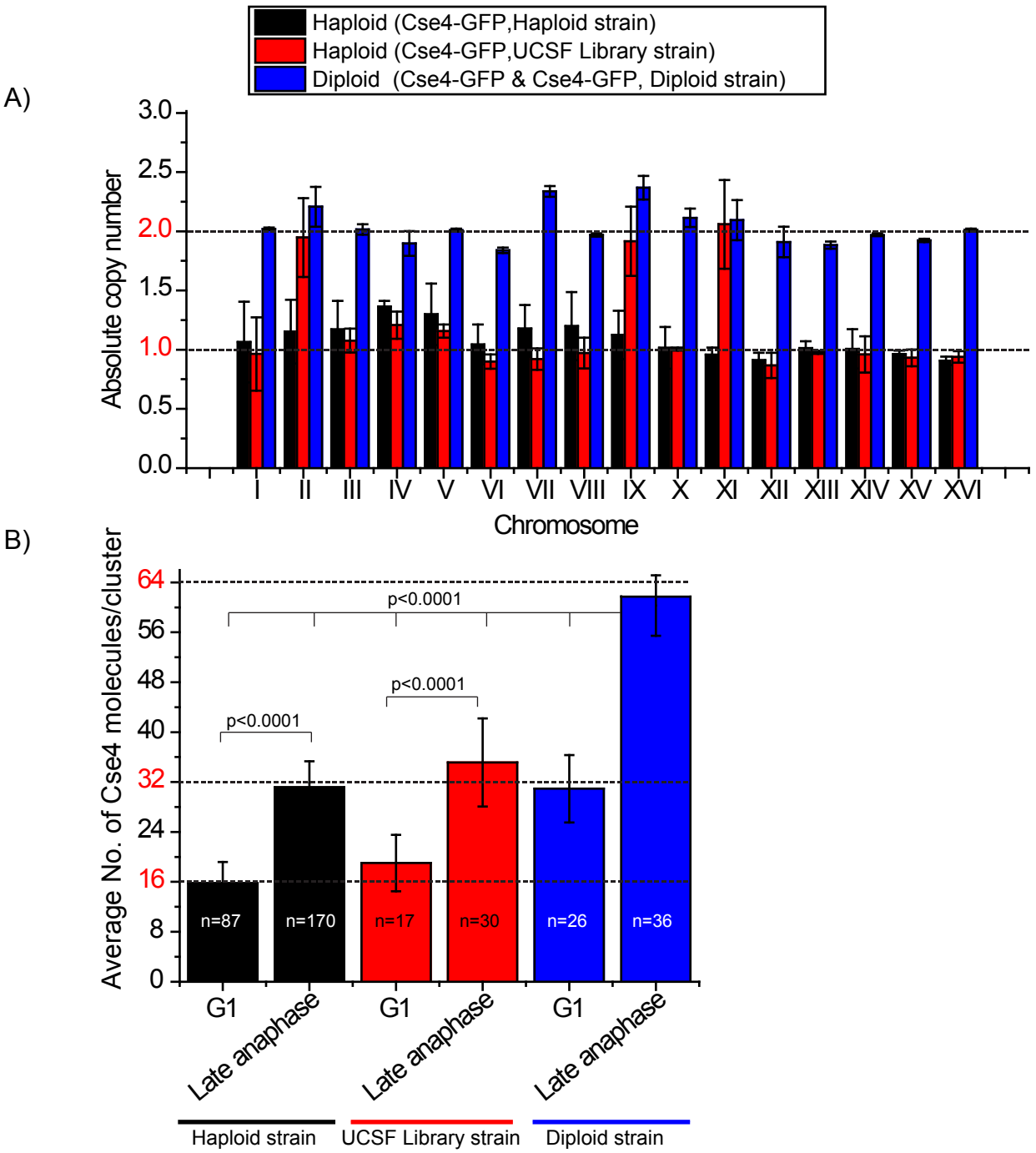


Supplemental figure 3



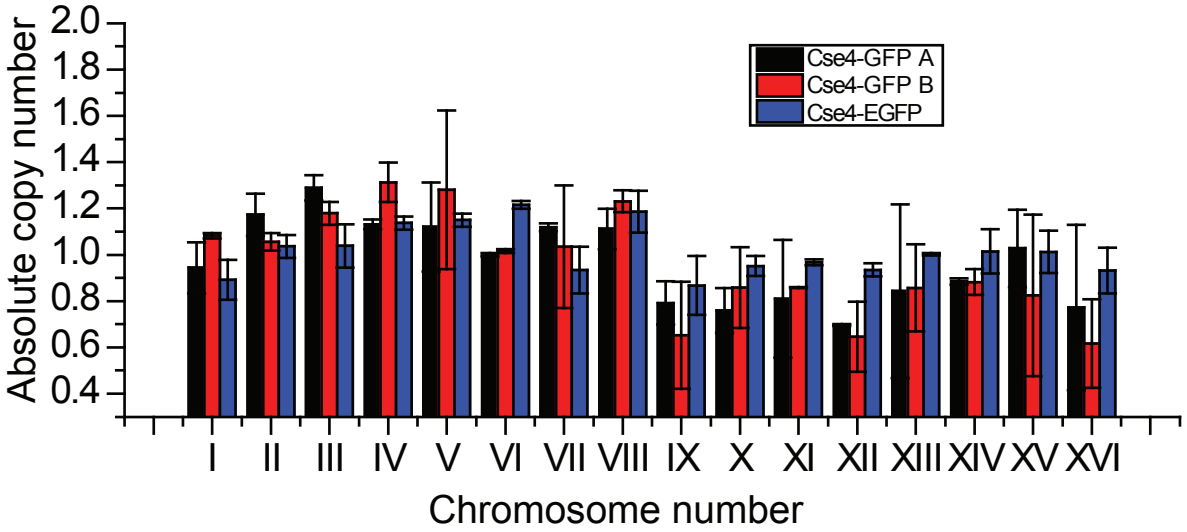
Supplemental Figure 4

Supplemental figure 4

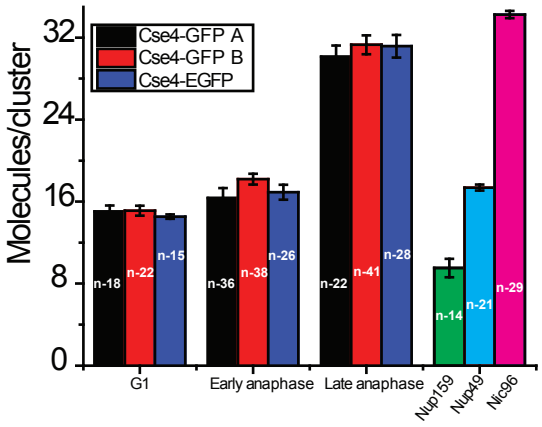


Supplemental Figure 5

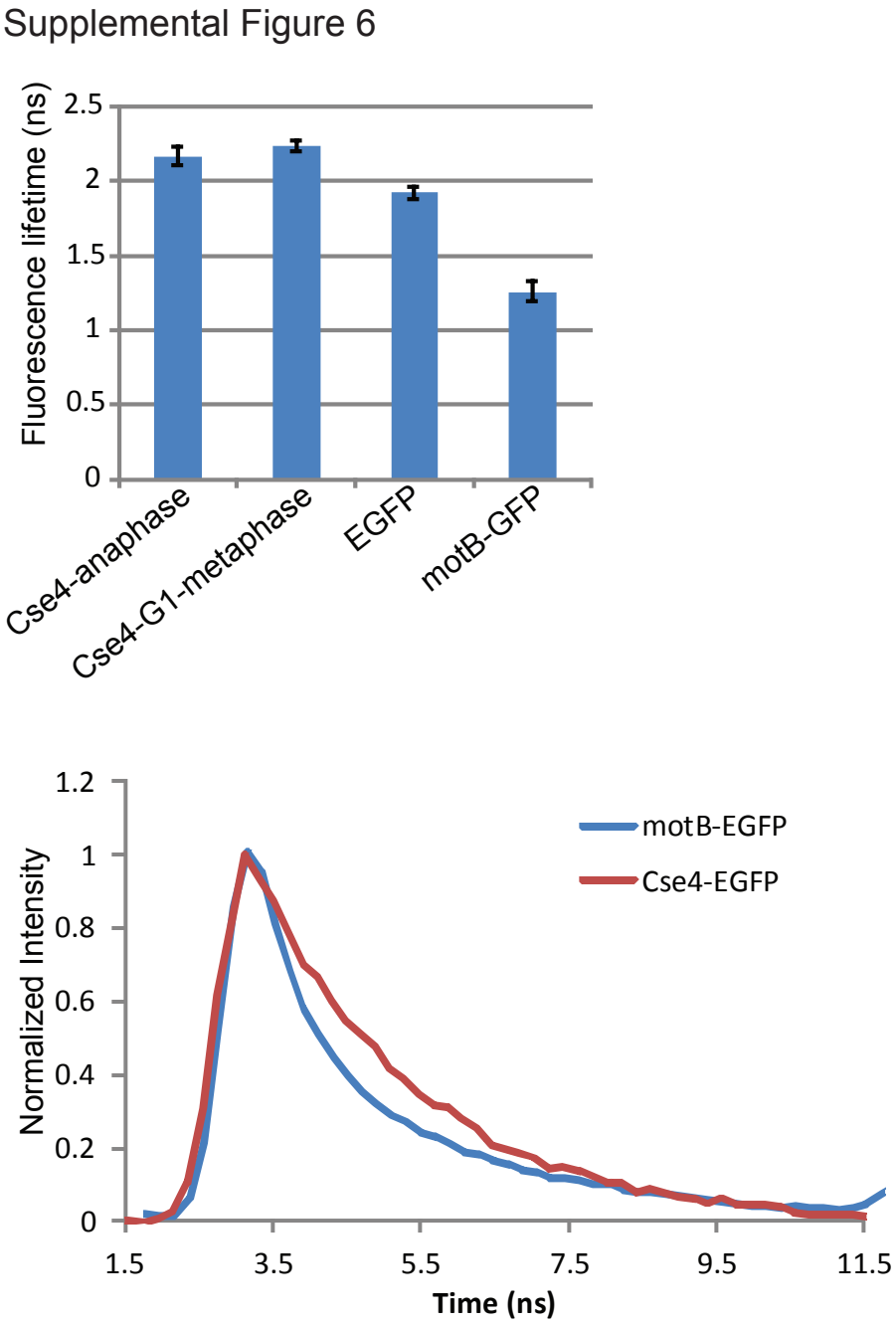
A



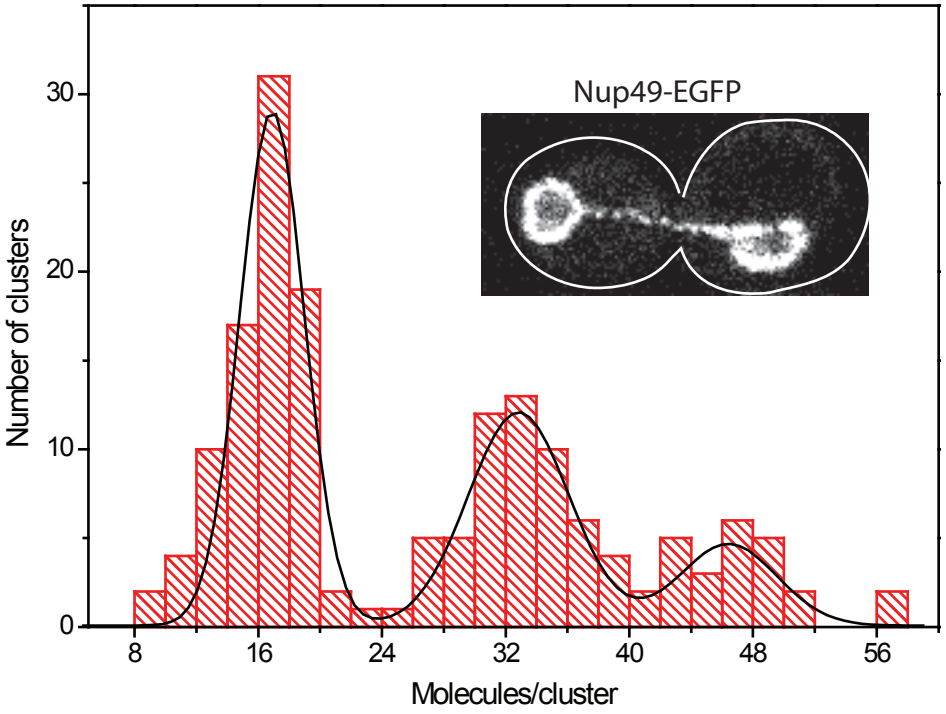
B



Supplemental Figure 6



Supplemental Figure 7

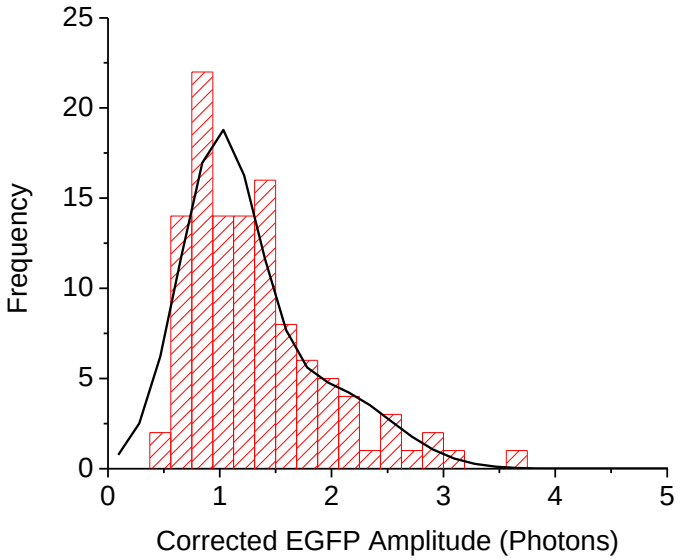


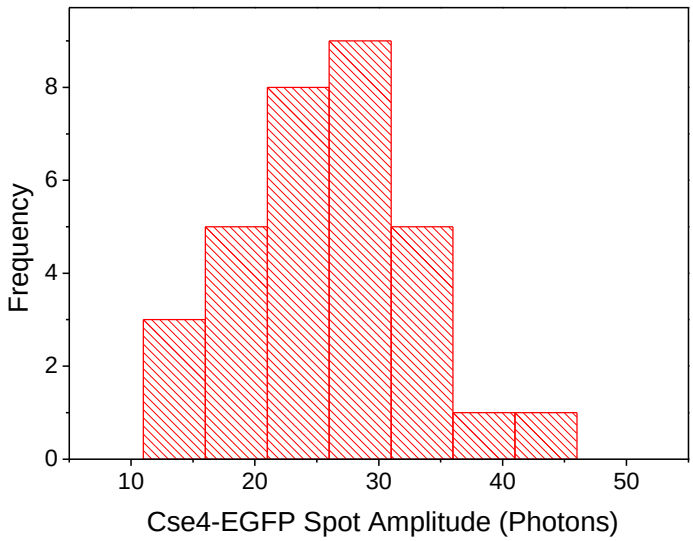
Supplemental Figure for reviewers

In order to assess the relationship between our methods of quantitating centromere occupation number and the methods of Coffman et al and Lawrimore et al, we analyzed MotB-EGFP in bacteria cells and EGFP on a surface. These two controls were relied on heavily by the above groups as calibrations for the intensities of single EGFP molecules. Firstly, we performed fluorescence lifetime measurements on both of these samples to assess whether the fluorescence quantum yield in these samples is identical to that in yeast centromeres. As we mentioned in our original submission, all EGFP samples measured thus far in live yeast cells (EGFP, Cse4-EGFP, and Nup49-EGFP) show essentially identical fluorescence lifetimes. To our surprise, MotB-EGFP bacterial cells showed strong quenching relative to all EGFP samples in live yeast with a reduction in fluorescence lifetime of approximately 50%. This indicates that the quantum yield of EGFP in this sample is dramatically lower than that in yeast cells and that it is not an appropriate control for the intensity of single EGFP molecules in live yeast.

EGFP molecules on a surface did, however, show a similar fluorescence lifetime to those in live yeast. As a result, we undertook single molecule surface measurements on our microscope in a manner similar to that of Lawrimore et al. Not surprisingly, it is quite difficult to accurately measure single EGFP spot intensities given issues of background, noise, and poor surface binding affinity. To demonstrate the feasibility of our measurements we decided to set up the microscope with high laser power, large exposure time (summing multiple confocal images), and a large confocal pinhole so as to increase the certainty in measuring single EGFP molecules. For comparison, Cse4-EGFP intensities were measured with the same pinhole but no averaging and half the laser power. This setup has the drawback of significantly underestimating EGFP molecular brightness due to premature bleaching (molecules bleach within the exposure time) and molecular saturation (inability to excite a molecule after it has absorbed a photon and before it has released a fluorescence photon). As a result, the ratio between Cse4-EGFP intensity and surface EGFP intensity represents an upper limit on that value and the true value is likely much lower. Nevertheless, if this value is significantly lower than the 90 molecules previously reported it would support our claims.

The histograms of EGFP spot amplitudes and Cse4-EGFP spot amplitudes as determined by a Gaussian fit are shown below. The EGFP spot amplitudes have been corrected to account for the increased exposure time and laser power used to collect those measurements. The EGFP distribution was fit to two Gaussians for monomer and dimer molecules. The intensity for the monomer fits to 0.99 photons with SEM of 0.05. The average intensity of the early metaphase Cse4-EGFP spots is 25.5 photons with a standard error of 1.3 photons. The ratio is 25.7 with an SEM value of 1.9. The ratio between these is far below the value of 90 molecules determined by Lawrimore et al and significantly below the value of 32 molecules required for the octasome model at this cell cycle stage. The p value according to the t-test for the ratio being greater than or equal to 32 is 1x10e-5. Therefore surface EGFP measurements are consistent with our results.


Frequency
25
20
15
10
5
0
0
1
2
3
4
5
Corrected EGFP Amplitude (Photons)


Frequency
8
6
4
2
0
10
20
30
40
50
Cse4-EGFP Spot Amplitude (Photons)