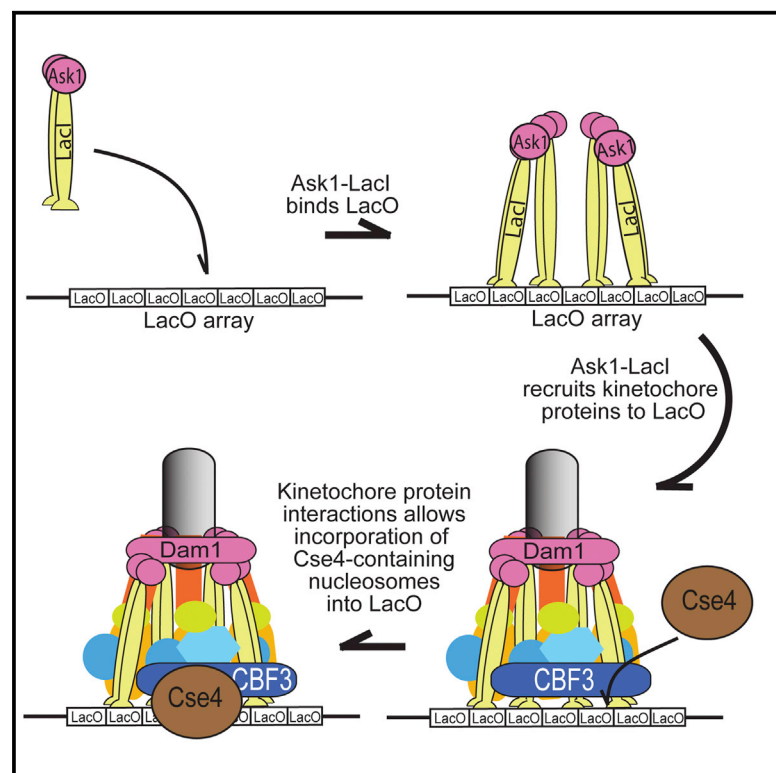


# Cell Reports

## Localization and Function of Budding Yeast CENP-A Depends upon Kinetochores Protein Interactions and Is Independent of Canonical Centromere Sequence

### Graphical Abstract



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### In Brief

In budding yeast, recruitment of CENP-A (Cse4) to the centromere was thought to require a defined centromere sequence. Ho et al. demonstrate that Cse4 localizes and functions at a synthetic kinetochore assembly site that lacks centromere sequence. Outer and inner kinetochore complexes are required for Cse4 localization to that site.

### Highlights

- Budding yeast CENP-A localizes to a synthetic kinetochore without a CEN sequence
- Synthetic kinetochore localization of Cse4 requires CBF3 and Dam1-DASH complexes
- The function of the synthetic kinetochore requires Cse4
- The Dam1-DASH complex is important for Cse4 localization at the natural centromere



# Localization and Function of Budding Yeast CENP-A Depends upon Kinetochores Protein Interactions and Is Independent of Canonical Centromere Sequence

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## SUMMARY

In many eukaryotes, the centromere is epigenetically specified and not strictly defined by sequence. In contrast, budding yeast has a specific 125 bp sequence required for kinetochore function. Despite the difference in centromere specification, budding yeast and multicellular eukaryotic centromeres contain a highly conserved histone H3 variant, CENP-A. The localization of budding yeast CENP-A, Cse4, requires the centromere DNA binding components, which are not conserved in multicellular eukaryotes. Here, we report that Cse4 localizes and functions at a synthetic kinetochore assembly site that lacks centromere sequence. The outer kinetochore Dam1-DASH and inner kinetochore CBF3 complexes are required for Cse4 localization to that site. Furthermore, the natural kinetochore also requires the outer kinetochore proteins for full Cse4 localization. Our results suggest that Cse4 localization at a functional kinetochore does not require the recognition of a specific DNA sequence by the CBF3 complex; rather, its localization depends on stable interactions among kinetochore proteins.

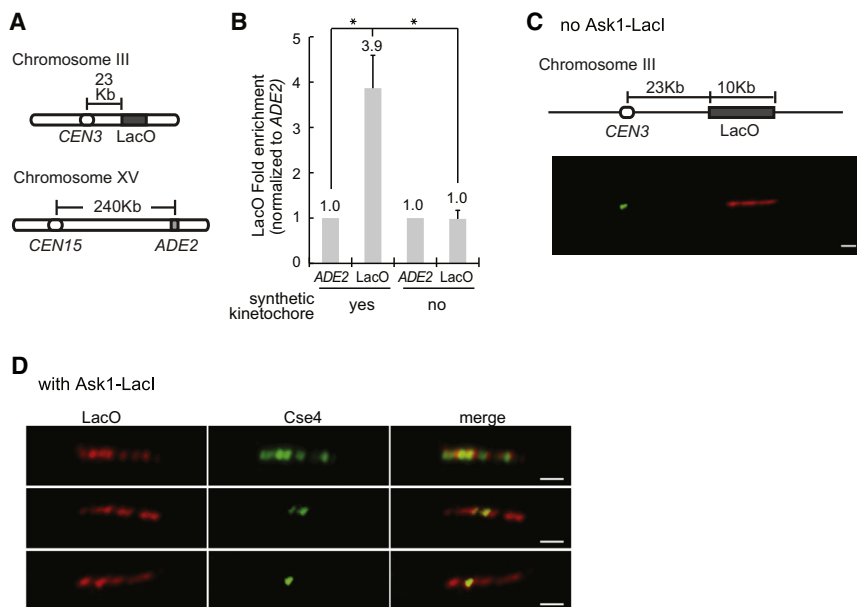
## INTRODUCTION

The accurate segregation of chromosomes depends on the kinetochore, a large protein complex that links spindle microtubules to chromatin. Kinetochores assemble on centromeres and are regulated to ensure that sister chromatids attach to opposite spindle poles in metaphase and travel along depolymerizing microtubules in anaphase (Biggins, 2013). Many kinetochore proteins are conserved among eukaryotic organisms; however, the underlying centromere sequence is highly variable. Centromeres of multicellular eukaryotes are epigenetically maintained and embedded in highly repetitive AT-rich DNA lacking strict sequence identity (De Rop et al., 2012). In contrast, the budding yeast centromere is a defined ~125 bp sequence consisting of three elements (*CDEI*, *CDEII*, and *CDEIII*), sufficient for centromere specification (Biggins, 2013).

Despite the intrinsic differences between the epigenetically maintained centromeres of multicellular eukaryotes and sequence-specified centromeres of budding yeast, both types of centromeres incorporate a histone H3 variant called CENP-A (De Rop et al., 2012). The incorporation of nucleosomes with CENP-A (Cse4 in budding yeast) is required for proper kinetochore assembly. CENP-A integrates at the centromere with the assistance of a histone chaperone, known as HJURP in vertebrates and Scm3 in budding and fission yeast (De Rop et al., 2012). HJURP/Scm3 has a highly conserved domain that binds CENP-A. In budding yeast, the inner kinetochore CBF3 complex facilitates the deposition of Cse4 at the centromere. A mutation in *CDEIII* eliminates Cse4 localization, suggesting that the *CEN* sequence is needed for Cse4 deposition (Ortiz et al., 1999). Because the CBF3 complex binds *CDEIII* and also binds Scm3, the current model is that CBF3 binds to the centromere and recruits Scm3, which deposits Cse4 in centromere chromatin (Camahort et al., 2007; Lechner and Carbon, 1991).

Here, we address the question of whether recognition of the centromere by the CBF3 complex is essential for Cse4 localization and function. Others and we previously demonstrated that recruiting a microtubule binding complex to DNA allows the assembly of a functional kinetochore at a noncentromeric locus (Kiermaier et al., 2009; Lacefield et al., 2009). By fusing Ask1, a member of the Dam1-DASH microtubule binding complex, to the lactose repressor (Ask1-LacI) and by placing tandem repeats of the lactose operator (LacO) on a chromosome, we created a “synthetic kinetochore”; Ask1-LacI binds LacO and recruits other kinetochore proteins (Lacefield et al., 2009). The synthetic kinetochore can replace an endogenous kinetochore on a chromosome and segregate sister chromatids in mitosis. Using the synthetic kinetochore, we tested whether Cse4 incorporates into a noncentromeric DNA locus.

We find that Cse4 localizes and functions at the synthetic kinetochore, independent of centromere sequence. The full localization of Cse4 to the synthetic kinetochore requires the Dam1-DASH and CBF3 complexes, suggesting that binding of CBF3 to *CDEIII* is not required for Cse4 incorporation. Rather, recruitment of CBF3 through kinetochore protein interactions allows Cse4 incorporation. The Dam1-DASH complex is also required for full Cse4 localization at the natural centromere, suggesting that outer microtubule binding components stabilize the inner kinetochore for Cse4 localization. Our results suggest that the localization and function of Cse4 at the centromere are not



**Figure 1. Cse4 Localizes to the Site of Synthetic Kinetochore Assembly**

(A) The three loci analyzed by ChIP. (B) Quantification of ChIP-qPCR averaged from three experiments  $\pm$  SD, showing the LacO enrichment over nonspecific binding at the *ADE2* locus. The asterisk represents statistical significance.

(C and D) Representative images of chromatin fiber immunofluorescence. Strains have the LacO array and express GFP-Cse4 and mCherry-LacI. Cse4 and LacI were detected using anti-GFP and anti-mCherry antibodies. The scale bar represents 1  $\mu$ m.

(C) Chromatin fiber from a strain without Ask1-LacI showing Cse4 at *CEN3* and LacO  $\sim$ 23 kb away. (D) Images of chromatin fibers from a strain with Ask1-LacI with Cse4 along the entire array (top), part of the array (middle), and in one focus (bottom).

strictly specified by centromere sequence but rely on the interactions of kinetochore protein complexes.

## RESULTS

### Cse4 Localizes to a Synthetic Kinetochore Assembly Site Independent of Centromere Sequence

We investigated whether Cse4 is recruited to a noncentromeric sequence, the synthetic kinetochore assembly site at the LacO array. We performed chromatin immunoprecipitation (ChIP), comparing two strains: one expressing Ask1-LacI (assembling a synthetic kinetochore at LacO) and one that does not express Ask1-LacI (no synthetic kinetochore). Both strains have 256 repeats of LacO integrated at the *LEU2* locus, which serves as the site of kinetochore assembly because Ask1-LacI binds LacO and recruits kinetochore proteins (Lacefield et al., 2009). Cse4 is internally tagged with GFP (GFP-Cse4), making a fully functional protein that can be immunoprecipitated with anti-GFP antibody (Chen et al., 2000). Due to the repetitive sequence of the LacO array, we used primers that amplify a unique region at one end of the array to avoid the amplification of different numbers of repeats, which interferes with quantification (Figure S1A). As a positive control, we used primers that amplify *CEN15* because GFP-Cse4 localizes at centromeres. As a negative control, we used primers that amplify *ADE2*, a locus in which Cse4 is not enriched (Figure 1A). The minimal level of Cse4 at *ADE2* measures nonspecific binding. As expected, there was an enrichment of Cse4 at *CEN15* when compared to the *ADE2* locus (Figure S1B).

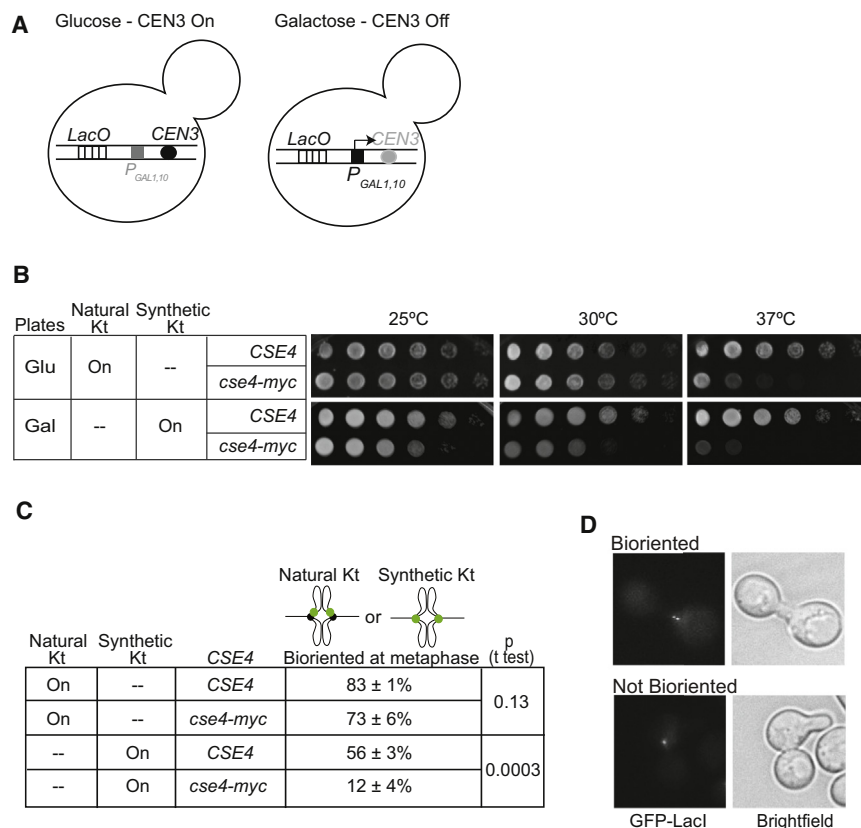
ChIP-quantitative PCR (qPCR) showed a 3.9-fold increase of Cse4 at LacO in cells with the synthetic kinetochore compared to cells without the synthetic kinetochore (Figure 1B). The enrichment of GFP-Cse4 at LacO versus *ADE2* is statistically significant (t test;  $p < 0.0001$ ). The level of Cse4 at LacO shown is likely an underestimate due to the necessity of having ChIP primers

that amplify one end of the LacO array. In the strain without the synthetic kinetochore, the level of LacO in the immunoprecipitation (IP) is not statistically different from that of *ADE2* (t test;  $p = 0.8$ ), suggesting that only background levels of Cse4 are present at LacO (Figure 1B). In summary, the ChIP demonstrates that Cse4 localizes to a noncentromeric sequence, LacO, when the synthetic kinetochore is present.

To further test Cse4 localization to the site of synthetic kinetochore assembly, we performed chromatin fiber immunofluorescence. Chromatin fibers were spread from strains with and without the synthetic kinetochore. Both strains have 256 repeats of LacO  $\sim$ 23 kb from *CEN3* and express GFP-Cse4 and mCherry-LacI. Immunostaining with anti-GFP and anti-mCherry antibodies detect Cse4 and LacO, respectively. Chromatin fibers from a strain without the synthetic kinetochore showed a stretch of mCherry signal, representing LacO and Cse4 positioned at the centromere (Figure 1C). From a strain with the synthetic kinetochore, 51% of LacO arrays showed Cse4 at the array. We are likely underestimating the percent of LacO arrays with Cse4 due to loss of some nucleosomes in the preparation of stretched chromatin. Of those LacO arrays with Cse4 signal, Cse4 was along the array, in multiple foci, or in one focus ( $n = 64$ ; Figure 1D). In contrast, in a strain without the synthetic kinetochore, only 6% of LacO arrays showed Cse4 at LacO, mostly in one focus, likely representing immunofluorescence background ( $n = 59$ ). These results demonstrate that Cse4 localizes to a noncentromeric sequence with an assembled synthetic kinetochore.

### Cse4 Functions at the Synthetic Kinetochore

If Cse4 functions at the synthetic kinetochore, a Cse4 loss-of-function mutant would disrupt synthetic kinetochore activity. We characterized a temperature-sensitive allele of Cse4, with a Myc tag at the C terminus (*cse4-Myc*), to determine if the C-terminal tag creates a conditional loss-of-function allele of Cse4. The temperature sensitivity of *cse4-Myc* cells can be rescued by GFP-Cse4, with GFP internally integrated into Cse4 (Chen et al., 2000; Wisniewski et al., 2014; Figure S2A). At 37°C,



**Figure 2. Cse4-Myc Disrupts Synthetic Kinetochores Function**

(A) Depiction of natural kinetochores function in cells with the *GAL1,10* promoter at *CEN3* ( $P_{GAL1,10}CEN3$ ) and grown in glucose versus galactose-containing medium. (B) Serial dilutions of cultures spotted on glucose and galactose plates at 25°C, 30°C, and 37°C. (C) The percent biorientation of natural or synthetic kinetochores in metaphase-arrested cells grown at 37°C (average of three experiments ± SD; 100 cells counted per experiment). (D) Images show a *cse4-Myc* cell with (top) and without (bottom) bioriented synthetic kinetochores.

and 30°C but grow worse at 37°C, suggesting that Cse4-Myc does not interfere with the function of the natural kinetochores at 25°C and 30°C (Figures 2B and S2A). On galactose plates, the natural kinetochores is off and Cse4-Myc localizes to LacO when the synthetic kinetochores is present (Figure S2E). The *cse4-Myc* cells grow worse than CSE4 cells at 25°C, 30°C, and 37°C when segregation of chromosome III depends on the synthetic kinetochores (Figure 2B). The growth defect of *cse4-Myc* cells compared to CSE4 cells at 25°C and 30°C on galactose

*cse4-Myc* cells arrest with large buds. The arrest depends on spindle checkpoint protein Mad2, suggesting that *cse4-Myc* cells have defective kinetochores-microtubule attachments and signal the spindle checkpoint (Figure S2B). By ChIP with anti-Myc antibody, Cse4-Myc localizes to *CEN15* at 25°C and 37°C, albeit at somewhat reduced levels at 37°C (Figure S2C). The Cse4 protein levels are similar in *cse4-Myc*, *GFP-Cse4*, and CSE4 cells at 25°C and 37°C (Figure S2D). Therefore, the mutant phenotype is due to altered protein function and not decreased protein levels. We conclude that *cse4-Myc* is a conditional partial loss-of-function allele of CSE4 that can be used to test the role of Cse4 at the synthetic kinetochores.

To determine if Cse4 functions at the synthetic kinetochores, we first analyzed growth of *cse4-Myc* cells dependent on the synthetic kinetochores for segregation of a chromosome. As previously described, to make chromosome III segregation dependent on the synthetic kinetochores, we integrated the *GAL1,10* promoter upstream of *CEN3* ( $GAL-CEN3$ ; Lacefield et al., 2009). *CEN3* is repressed when cells are grown in galactose medium (*CEN3* off; Figure 2A; Hill and Bloom, 1987). Our previous experiments showed that cells with the synthetic kinetochores grow in galactose medium, but cells without the synthetic kinetochores grow poorly (Lacefield et al., 2009). We tested whether Cse4-Myc disrupted growth of cells dependent on the synthetic kinetochores for survival. We spotted dilutions of a yeast culture onto glucose and galactose plates and grew them at 25°C, 30°C, and 37°C. On glucose plates, the natural kinetochores is on and *cse4-Myc* cells grow similarly to CSE4 cells at 25°C

plates suggests that functional Cse4 is required for the synthetic kinetochores to segregate a chromosome.

To determine if Cse4-Myc directly alters synthetic kinetochores function, we analyzed the synthetic kinetochores biorientation at metaphase. We monitored *GAL-CEN3* LacO cells that express both Ask1-LacI and GFP-LacI to mark a synthetic kinetochores with GFP. We previously found that the synthetic kinetochores biorients at metaphase, as shown by the separation of two sister GFP foci ~0.5 μm apart (Lacefield et al., 2009). To determine if Cse4-Myc disrupts biorientation, we allowed CSE4 and *cse4-Myc* cells to reach metaphase arrest (by depletion of Cdc20) in galactose to turn off *CEN3*. We counted the percent of cells with separated GFP foci at 37°C (Figures 2C and 2D). With Cse4, 56% ± 3% of cells bioriented synthetic kinetochores. In contrast, only 12% ± 4% of *cse4-Myc* cells bioriented synthetic kinetochores. We monitored biorientation of a natural kinetochores in cells with a LacO array at *CEN15* and expressing GFP-LacI and found that 83% ± 1% of CSE4 cells and 73% ± 6% of *cse4-Myc* cells had bioriented natural kinetochores at 37°C (Figure 2C). Our data suggest that Cse4-Myc more severely disrupts synthetic kinetochores function than natural kinetochores function. We conclude that Cse4 is required for synthetic kinetochores function.

### The CBF3 and Dam1-DASH Complexes Are Important for Cse4 Localization at the Synthetic Kinetochores

The current model for targeting Cse4 to the centromere depends on the interaction of the inner kinetochores CBF3 complex with

the *CDEIII* DNA element. With CBF3 bound to *CDEIII*, the histone chaperone Scm3 binds to CBF3 component Ndc10 and mediates the deposition of Cse4 at the centromere (Camahort et al., 2007; Figure 3A). Despite the absence of *CDEIII* at the synthetic kinetochore assembly site, we tested the role of the CBF3 complex in Cse4 localization. We first analyzed Ndc10 localization to the synthetic kinetochore. We tagged Ndc10 with GFP and performed ChIP-qPCR with antibody against GFP. There was a 9-fold enrichment of Ndc10 at LacO in cells with the synthetic kinetochore compared to cells without the synthetic kinetochore (Figure 3B). This result shows that Ndc10 does not bind LacO independently but localizes to LacO through the assembled synthetic kinetochore.

We analyzed the localization of Cse4 to LacO in *ndc10-1* and *ctf13-30* temperature-sensitive mutants. If the kinetochore protein has a role in Cse4 localization at LacO, Cse4 localization should be reduced in the mutant strains. At 37°C, Cse4 localization at LacO is reduced 77% in *ndc10-1* cells and 53% in *ctf13-30* cells compared to wild-type cells (Figure 3C). These results suggest that the CBF3 complex, which normally binds *CDEIII*, is important for Cse4 localization at a synthetic kinetochore assembly site, even in the absence of the *CDEIII* element.

Next, we tested the role of other kinetochore components in Cse4 localization to the synthetic kinetochore. Because some kinetochore mutations activate the spindle checkpoint and cause an enrichment of metaphase cells, we compared the level of Cse4 localization in a wild-type asynchronously growing strain to that of a *MET25*-promoted *CDC20* strain, which arrests in metaphase with methionine addition due to the depletion of Cdc20. The results showed that asynchronous and metaphase-enriched cultures had similar levels of GFP-Cse4 at LacO (Figure 3D).

Because Ask1 is a component of the Dam1-DASH outer kinetochore complex, we tested whether recruitment of other Dam1-DASH components was important for Cse4 localization to LacO. In the *dad1-13* strain, Cse4 levels at LacO were decreased 93% in comparison to the wild-type strain (Figure 3E). In the *dam1-1* and the *duo1-2* strains, Cse4 levels at LacO were decreased 73% and 53% relative to that of a wild-type strain, respectively. The difference in the reduction of Cse4 in the *dad1-13*, *dam1-1*, and *duo1-2* mutants likely reflects the severity of the temperature-sensitive mutations. To ensure that the decreased Cse4 localization was not due to differences in cell cycle stage, we repeated the ChIP in cells enriched in metaphase (due to the depletion of Cdc20). The metaphase-enriched cells mutant for Dam1-DASH components also show a significantly decreased level of Cse4 at LacO when compared to wild-type cells (Figure S3A).

Because *dad1-13* mutant cells have a defect in chromosome-microtubule attachments (Janke et al., 2002), we tested whether the loss of microtubule attachments causes decreased Cse4 localization at the synthetic kinetochore. We added microtubule depolymerizing drugs to *dad1-13* and wild-type cells (Figure S3B). The level of Cse4 at LacO was not reduced in wild-type cells with nocodazole, suggesting that disruption of microtubules does not decrease Cse4 levels at LacO (Figures S3A and S3B). With nocodazole, *dad1-13* cells have an 88% reduction of Cse4 levels at LacO compared to wild-type cells. These results suggest that the Dam1-DASH complex is required

for Cse4 localization at LacO, likely due to its role in recruiting other kinetochore proteins to the synthetic kinetochore.

We tested proteins in two highly conserved microtubule binding kinetochore complexes, the Mis12/Mtw1 and Ndc80 complexes, for their role in Cse4 localization at the synthetic kinetochore assembly site. Cse4 localization at LacO was reduced between 33% and 56% in temperature-sensitive mutants of the Mis12/Mtw1 complex (*dsn1-7*, *nnf1-77*, *nsl1-5*, and *mtw1-11*) compared to wild-type (Figure 3F). Cse4 localization at LacO was reduced between 19% and 51% in temperature-sensitive mutants of the Ndc80 complex (*ndc80-1*, *nuf2-61*, *spc24-1*, and *spc25-1*) compared to wild-type (Figure 3G). Although the reduction of Cse4 localization in mutants of the Mis12/Mtw1 and Ndc80 complexes is not as severe as that of the Dam1-DASH complex, the results suggest that the Mtw1 and Ndc80 complexes contribute to Cse4 localization.

Next, we tested the inner kinetochore Ctf19 complex proteins, Cnn1, and Mif2 for their role in Cse4 localization at LacO. Deletions of nonessential proteins Ctf19 or Cnn1 or a temperature-sensitive mutation in Okp1 did not reduce Cse4 enrichment at LacO when the synthetic kinetochore was present (Figure 3H). In *mif2-3* cells, Cse4 localization was reduced 37% (Figure 3I). Therefore, Mif2 may contribute but Cnn1 and the Ctf19 complex are not important for Cse4 localization to the synthetic kinetochore.

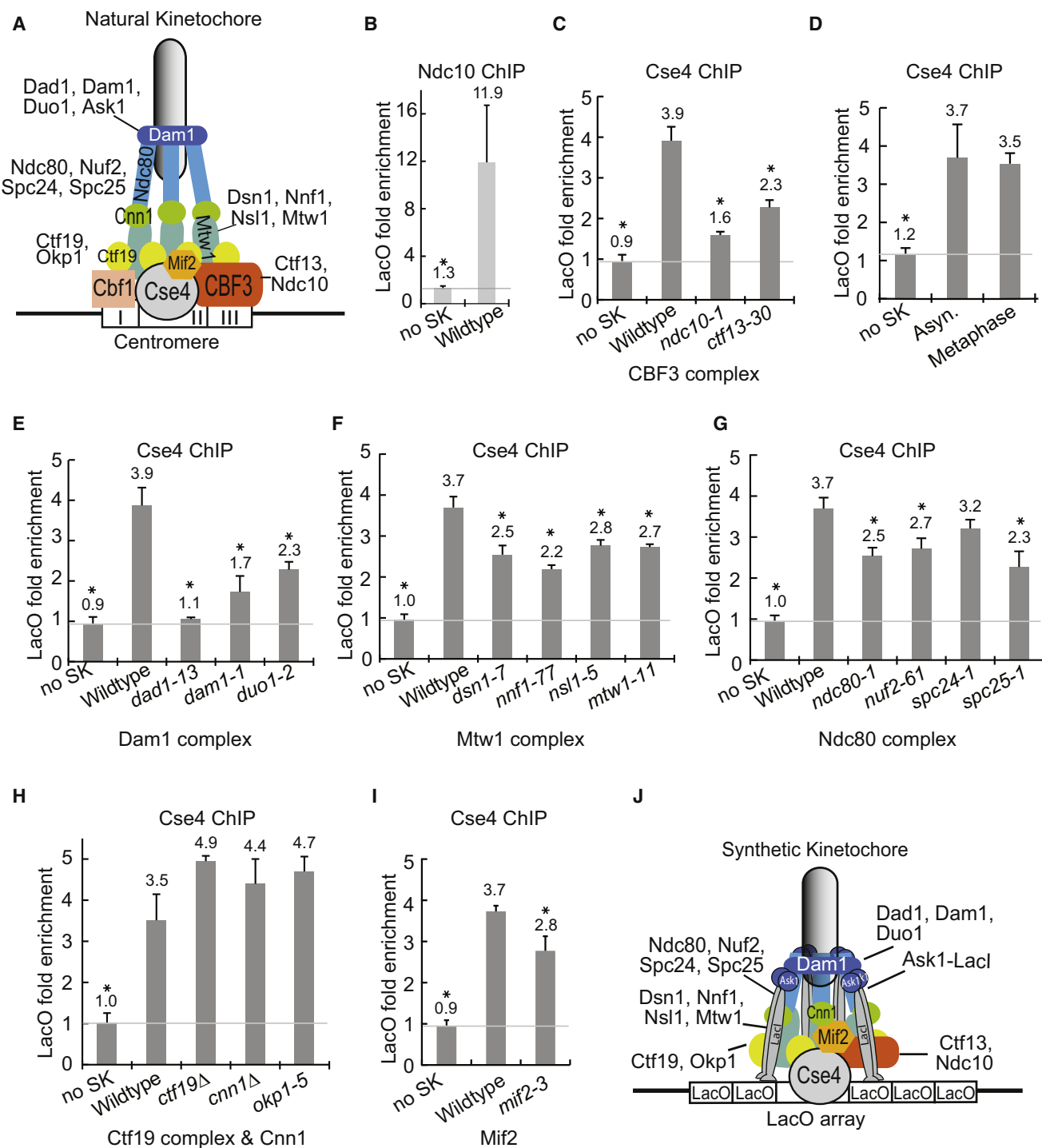
We further tested the localization of GFP-Cse4 in Dam1-DASH and CBF3 complex mutants by chromatin fiber immunofluorescence. We prepared chromatin fibers from wild-type, *dad1-13*, and *ndc10-1* cells (Figure S3C). In wild-type cells with the synthetic kinetochore, 51% have Cse4 at the LacO array ( $n = 64$ ). In contrast, in *ndc10-1* and *dad1-13* cells, only 27% and 26% of chromatin fibers have Cse4 at the LacO array, respectively, suggesting that fewer cells have Cse4 at LacO ( $n = 72$  for *ndc10-1*;  $n = 113$  for *dad1-13*).

In summary, our results suggest that the Dam1-DASH and CBF3 complexes have an important role in Cse4 incorporation to the chromatin underlying the synthetic kinetochore. The Mis12/Mtw1 and Ndc80 complexes and Mif2 also contribute to Cse4 localization. Because CBF3 is not known to directly interact with Dam1-DASH, we propose that the Mis12/Mtw1 and Ndc80 complexes have overlapping roles linking the two complexes to recruit Cse4 to the synthetic kinetochore. A model of the synthetic kinetochore is depicted in Figure 3J.

### Dam1-DASH Complex Components Contribute to Cse4 Localization to the Natural Centromere

To test if kinetochore proteins involved in Cse4 localization at the synthetic kinetochore also contribute to Cse4 localization at the natural centromere, we analyzed centromere localization of Cse4 in kinetochore temperature-sensitive mutants. We verified that Cse4 localization was substantially reduced in an *ndc10-1* strain at the restrictive temperature (Ortiz et al., 1999; Figure 4A).

Interestingly, Cse4 levels at the centromere are reduced in Dam1-DASH complex mutants. Cells were metaphase arrested (by Cdc20 depletion) to ensure the comparison of cells at the same cell cycle stage. Cse4 enrichment at *CEN15* was reduced 60% and 59% in *dam1-1* and *duo1-2* mutants compared to wild-type cells, respectively (Figure 4B). Because the *dad1-13*



**Figure 3. The CBF3 Complex and the Dam1-DASH Complex Are Required for Cse4 Localization at LacO**

(A) Model of the natural kinetochore.

(B–I) The quantification of ChIP-qPCR averaged from at least three experiments  $\pm$  SD. The asterisk represents statistical significance (t test;  $p < 0.05$ ).

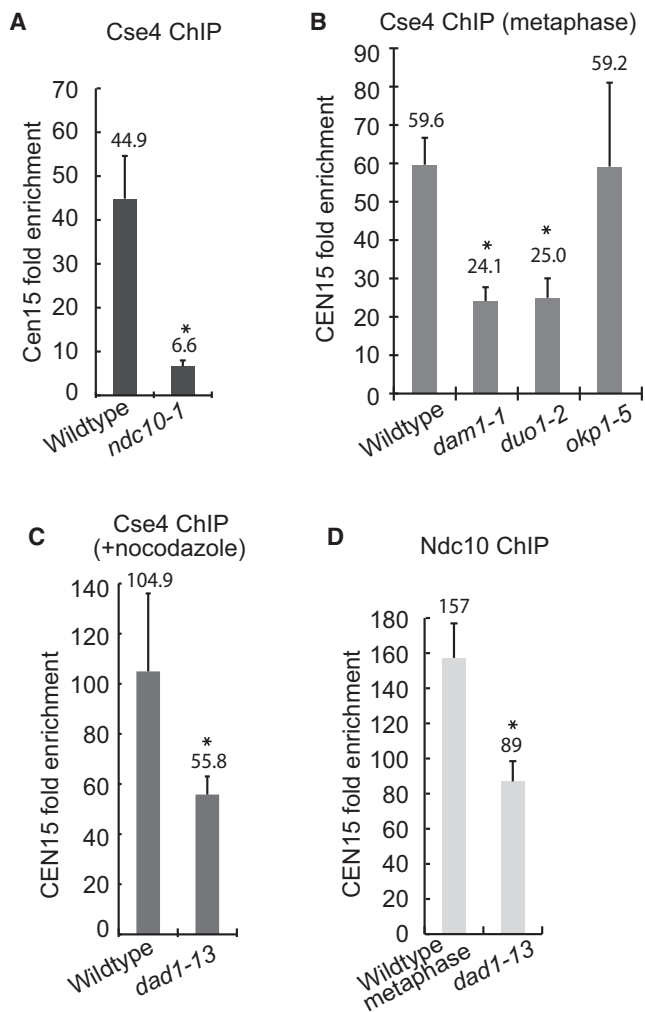
(B) The ChIP of Ndc10-GFP with anti-GFP antibody.

(C) The ChIP of GFP-Cse4 with anti-GFP antibody, comparing asynchronous cells with kinetochore mutations at 37°C.

(D) The ChIP of GFP-Cse4 with anti-GFP antibody, comparing asynchronous to metaphase-enriched cells.

(E–I) The ChIP of GFP-Cse4 with anti-GFP antibody, comparing asynchronous cells with kinetochore mutations at 37°C. SK, synthetic kinetochore.

(J) Model of the synthetic kinetochore.



**Figure 4. The CBF3 and Dam1-DASH Complexes Are Required for Cse4 Localization at the Natural Centromere**

(A–C) The quantification of ChIP-qPCR amplifying *CEN15* DNA (average of three experiments  $\pm$  SD). Anti-GFP antibody was used to IP GFP-Cse4 in cells with kinetochore mutations at 37°C. The asterisk represents statistical significance (t test:  $p < 0.05$ ).

(B) Cells were arrested in metaphase.

(C) Nocodazole and benomyl were added to the cells.

(D) The quantification of ChIP-qPCR using anti-GFP antibody to IP Ndc10-GFP.

mutant causes severe microtubule attachment errors (Janke et al., 2002), we depolymerized microtubules with nocodazole in wild-type and *dad1-13* cells to compare strains without microtubule attachments. The Cse4 levels at *CEN15* were reduced 47% in *dad1-13* cells compared to wild-type cells (Figure 4C). ChIP against Ndc10-GFP shows a 44% reduction of Ndc10 at *CEN15* in the *dad1-13* strain (Figure 4D). Because the loss of Dam1-DASH components moderately disrupts centromere clustering (Anderson et al., 2009; Richmond et al., 2013; Figure S4), we were concerned that disruption of clustering could decrease Cse4 levels in ChIP assays. However, in *okp1-5* mutant cells, centromere clustering is severely disrupted, but the levels of

Cse4 at the centromere are not reduced in the ChIP assay (Figures 4B, S4A, and S4B). The results suggest that the decreased level of Cse4 at the centromere in the Dam1-DASH mutants is not simply a consequence of the loss of centromere clustering. We conclude that kinetochore complexes important for Ndc10 and Cse4 localization to the synthetic kinetochore also contribute to Cse4 localization at the natural kinetochore.

## DISCUSSION

In budding yeast, a 125 bp sequence specifies the centromere; the widely accepted view was that this sequence is required for CENP-A deposition (Biggins, 2013). In other eukaryotes, such as *S. pombe*, *D. melanogaster*, and mammals, the centromere is inherited epigenetically through maintenance of CENP-A-containing chromatin (De Rop et al., 2012). Interestingly, HJURP, the histone chaperone required for CENP-A deposition, is also conserved from yeast to humans. Therefore, we tested whether CENP-A integration requires a specific DNA sequence in budding yeast. We demonstrate that CENP-A homolog Cse4 is stably recruited and functions at a noncentromeric sequence, the synthetic kinetochore assembly site. Localization of Cse4 to the synthetic kinetochore assembly site requires the CBF3 complex. Because CBF3 normally binds the centromere to facilitate Cse4 deposition (Camahort et al., 2007), the results suggest that centromere binding of the CBF3 complex is not strictly required for Cse4 localization; instead, the stable interaction of CBF3 with kinetochore proteins allows Cse4 recruitment to DNA.

The role of the CBF3 complex in Cse4 localization seems contradictory to our previous finding that CBF3 components are not required for synthetic kinetochore biorientation (Lacefield et al., 2009). However, the results are consistent when considering the incomplete depletion of Cse4 from LacO in the CBF3 mutants. Although Cse4 levels are reduced by 77% in the *ndc10-1* mutant, the remaining Cse4 is likely sufficient for synthetic kinetochore biorientation.

The localization of Cse4 through kinetochore protein interactions is comparable to CENP-A recruitment to epigenetic centromeres in other eukaryotic organisms. In *S. pombe* and vertebrate cells, the Mis18 complex is required for CENP-A incorporation in the centromere (Barnhart et al., 2011; Fujita et al., 2007; Hayashi et al., 2004; Pidoux et al., 2009). Targeting the kinetochore proteins CENP-C and CENP-I allows the integration of CENP-A to a noncentromeric locus and recruits a fully functioning heritable kinetochore (Hori et al., 2013). Furthermore, Cse4 incorporates into centromeric histones and rescues CENP-A depletion phenotypes in human cells, suggesting that Cse4 can utilize the CENP-A localization pathways of human cells (Wieland et al., 2004). These studies, combined with our results, suggest that, although centromere sequence and DNA binding components of the kinetochore have rapidly changed throughout eukaryotic evolution, many features of the histone recruitment pathway remain largely the same.

Interestingly, the inner CBF3 complex and outer Dam1-DASH complex contribute to Cse4 localization at both the synthetic and natural kinetochores. Therefore, a fully assembled kinetochore contributes to stable Cse4 localization at the natural centromere. Conversely, past studies showed that depletion of Cse4 reduces

the localization of most kinetochore proteins at the centromere (Camahort et al., 2007; Collins et al., 2005). Combined, the results suggest that the fully assembled kinetochore and Cse4-containing chromatin reciprocally stabilize each other. We propose that the formation of the assembled kinetochore and the interaction between kinetochore proteins facilitate the stable localization of Cse4 at the centromere.

## EXPERIMENTAL PROCEDURES

### Strains

Strains are listed in Table S1. Synthetic kinetochore strains were previously described (Lacefield et al., 2009). The  $P_{CSE4}GFP-CSE4$  construct, with GFP integrated at residue 83, was integrated at *URA3*, and the endogenous *CSE4* was deleted (Chen et al., 2000). The C-terminally tagged *CSE4-Myc* was made through PCR integration of the 13Myc epitope (Longtine et al., 1998).

### ChIP and qPCR

ChIP was performed as described, with growth conditions listed in the Supplemental Information (Strahl-Bolsinger et al., 1997). Oligo sequences for qPCR are listed in Table S2. The qPCR was performed as described (Haring et al., 2007). The results shown are the average of at least three ChIP experiments for each genotype  $\pm$  SD. The percent reduction of *CSE4* enrichment in the mutants was calculated relative to the recruitment to the synthetic kinetochore in comparison to the no synthetic kinetochore control. Statistical significance was determined using the t test.

### Chromatin Fiber Immunofluorescence

Chromatin fibers were prepared from protoplasts as described (Tsuchiya and Taga, 2001).

Other experimental procedures are listed in the Supplemental Information.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.11.037>.

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