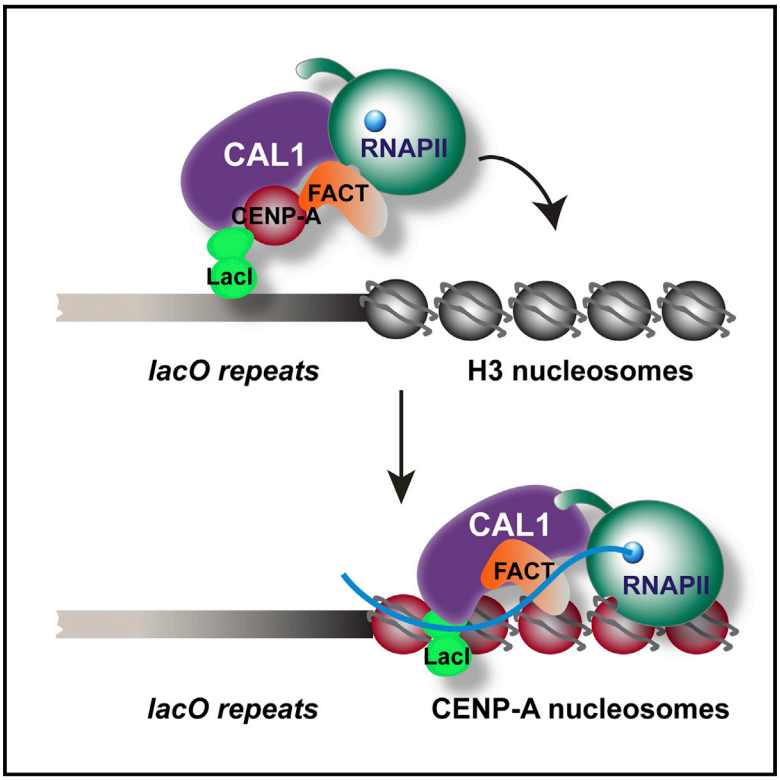
Article

Developmental Cell

Establishment of Centromeric Chromatin by the CENP-A Assembly Factor CAL1 Requires FACT-Mediated Transcription

Graphical Abstract



Authors

Chin-Chi Chen, Sarion Bowers, Zoltan Lipinszki, ..., David M. Glover, Rachel J. O'Neill, Barbara G. Mellone

Correspondence

barbara.mellone@uconn.edu

In Brief

Centromeres are specified epigenetically by chromatin containing the histone H3 variant CENP-A. Chen et al. shed light on CENP-A deposition mechanisms, showing that the CENP-A chaperone CAL1 recruits FACT and RNAPII to CENP-A assembly sites, where they trigger transcription. In the absence of FACT, transcription ceases and CENP-A deposition is defective.

Highlights

- CENP-A deposition is coupled with transcription
- CAL1 recruits RNAPII onto DNA during CENP-A deposition
- CAL1 interacts directly with the histone chaperone FACT
- FACT depletion causes loss of transcription and defective CENP-A deposition

Chen et al., 2015, Developmental Cell *34*, 73–84
July 6, 2015
http://dx.doi.org/10.1016/j.devcel.2015.05.012

CellPress

Developmental Cell
Article

Establishment of Centromeric Chromatin by the CENP-A Assembly Factor CAL1 Requires FACT-Mediated Transcription

Chin-Chi Chen,[1] Sarion Bowers,[1,5] Zoltan Lipinszki,[2,3] Jason Palladino,[1] Sarah Trusiak,[1] Emily Bettini,[1] Leah Rosin,[1] Marcin R. Przewloka,[2] David M. Glover,[2] Rachel J. O'Neill,[1,4] and Barbara G. Mellone[1,4,*]
[1]Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269, USA
[2]Department of Genetics, University of Cambridge, Cambridge CB2 3EH, UK
[3]Biological Research Centre of the Hungarian Academy of Sciences, Institute of Biochemistry, P.O. Box 521, 6701 Szeged, Hungary
[4]Institute for Systems Genomics, University of Connecticut, Storrs, CT 06269, USA
[5]Present address: Wellcome Trust Sanger Institute, Cambridge CB10 1SA, UK
*Correspondence: barbara.mellone@uconn.edu
http://dx.doi.org/10.1016/j.devcel.2015.05.012

SUMMARY

Centromeres are essential chromosomal structures that mediate accurate chromosome segregation during cell division. Centromeres are specified epigenetically by the heritable incorporation of the centromeric histone H3 variant CENP-A. While many of the primary factors that mediate centromeric deposition of CENP-A are known, the chromatin and DNA requirements of this process have remained elusive. Here, we uncover a role for transcription in *Drosophila* CENP-A deposition. Using an inducible ectopic centromere system that uncouples CENP-A deposition from endogenous centromere function and cell-cycle progression, we demonstrate that CENP-A assembly by its loading factor, CAL1, requires RNAPII-mediated transcription of the underlying DNA. This transcription depends on the CAL1 binding partner FACT, but not on CENP-A incorporation. Our work establishes RNAPII passage as a key step in chaperone-mediated CENP-A chromatin establishment and propagation.

INTRODUCTION

Accurate chromosome segregation during cell division is dependent on the correct assembly and propagation of a distinct region of the chromosome known as the centromere. The centromere forms the structural basis for the assembly of the kinetochore, a multi-protein complex to which spindle microtubules attach during mitosis and meiosis. In most eukaryotes, the position of the centromere is defined epigenetically through the heritable incorporation of the histone H3 variant CENP-A, which is both necessary and sufficient for centromere activity (De Rop et al., 2012).

Centromeric chromatin displays a conserved organization composed of interspersed blocks of CENP-A and H3 nucleosomes (Blower et al., 2002). During DNA replication in human cells, no new CENP-A deposition occurs (Jansen et al., 2007), and histone H3.3 and H3.1 are deposited as placeholders (Dunleavy et al., 2011). CENP-A deposition occurs during or after mitosis in *Drosophila* and humans, respectively (Hemmerich et al., 2008; Jansen et al., 2007; Mellone et al., 2011; Schuh et al., 2007) and is mediated by specialized histone chaperones known as Scm3 in fungi (Camahort et al., 2007; Pidoux et al., 2009; Stoler et al., 2007), HJURP in tetrapods (Barnhart et al., 2011; Bernad et al., 2011; Dunleavy et al., 2009; Foltz et al., 2009; Sanchez-Pulido et al., 2009; Shuaib et al., 2010), and CAL1 in flies (Chen et al., 2014). Each of these chaperones has been shown to selectively bind CENP-A, and not canonical H3, and to mediate the formation of CENP-A nucleosomes in vitro. However, how placeholder nucleosomes are reorganized to incorporate CENP-A/H4 tetramers is unknown. Additional histone chaperones have been found to either bind to CENP-A or contribute to proper CENP-A localization in vertebrate cells (Foltz et al., 2006; Okada et al., 2009; Perpelescu et al., 2009), but whether or not they are involved in this reorganization is unknown.

Mounting evidence points to a functional interplay between the transcription of centromeric repeats and centromere function across species. For instance, manipulation of a human artificial chromosome (HAC) revealed that targeting a transcriptional silencer to alpha-satellite repeats caused loss of CENP-A (Nakano et al., 2008). Remarkably, transcripts emanating from centromeric DNA have been identified in yeast, human, wallabies, and plants (Carone et al., 2009; Chan et al., 2012; Choi et al., 2011; Ohkuni and Kitagawa, 2011; Quénet and Dalal, 2014; Topp et al., 2004) and have been shown to be important for centromere integrity. However, the idea that specific RNAs play a role in centromere integrity is inconsistent with the notion that centromeres can form independently of centromeric DNA (Marshall et al., 2008). Additionally, the functional significance of transcription in the CENP-A assembly cascade remains poorly defined.

Here, we identify RNA polymerase II (RNAPII)-dependent transcription as a key requirement for *Drosophila* CENP-A deposition. Using an inducible ectopic centromere system, which allows for the direct comparison of chromatin states in the

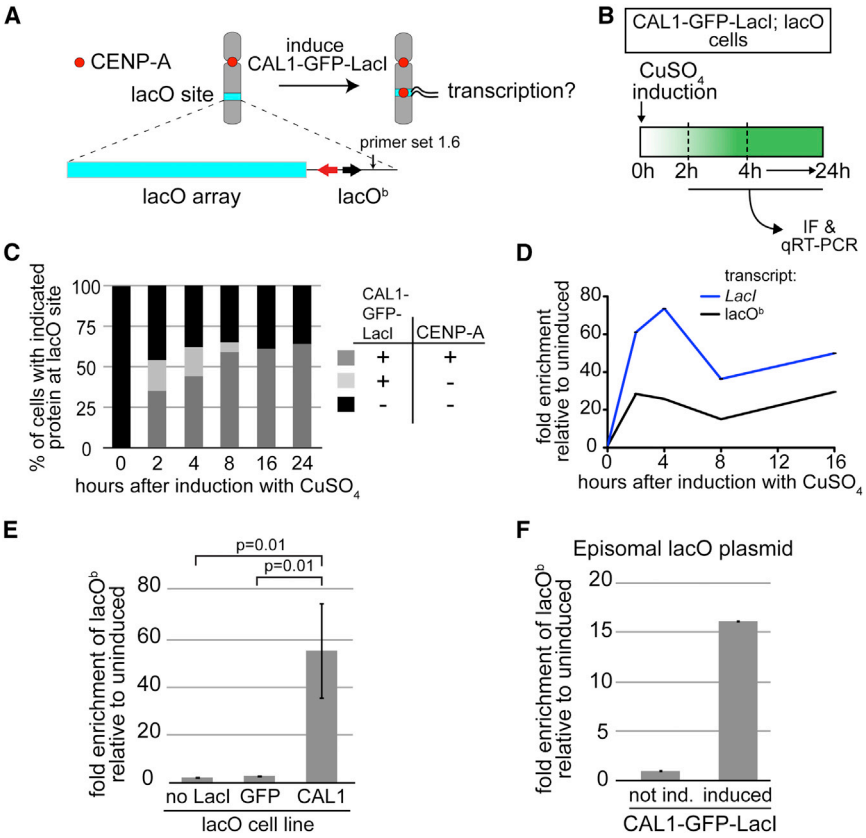


Figure 1. CENP-A Deposition at the Ectopic lacO Site Is Associated with Transcription

(A) Experimental approach to determine if transcription is coupled with CENP-A deposition. The lacO vector is stably inserted in S2 cells and contains 256 lacO repeats (lacO array; blue bar), the bacterial Amp resistance gene (black arrow), and the yeast TRP1 gene (red arrow). Primer set 1.6 (arrow) is within the lacO vector backbone ($lacO^b$).

(B) Experimental strategy used to follow ectopic CENP-A deposition and transcription from $lacO^b$ after induction of CAL1-GFP-LacI.

(C) Quantification of the presence (+) or absence (–) of CAL1-GFP-LacI and CENP-A foci at the lacO site during a time course. n = 100 cells for each time point.

(D) qRT-PCR analysis of $lacO^b$ (black) and CAL1-GFP-LacI transcripts (blue) in induced CAL1-GFP-LacI cells at the indicated times. Error bars, SD of three technical replicates.

(E) qRT-PCR measuring $lacO^b$ transcription after 24 hr induction in cell lines: lacO only (no LacI), lacO with GFP-LacI (GFP), and lacO with CAL1-GFP-LacI (CAL1). The means ± SD of three experiments are shown. p = 0.01, unpaired t test.

(F) Transcription from $lacO^b$ determined by qRT-PCR in CAL1-GFP-LacI cells (induced 24 hr), where the lacO plasmid is episomal. Error bars, 95% confidence interval (CI) of three technical replicates.

See also Figure S1.

presence or absence of active CENP-A deposition, we find that CENP-A assembly by its loading factor CAL1 is coupled with transcription of the underlying DNA. We identify facilitates chromatin transcription (FACT; Orphanides et al., 1998) as a central molecular player in this process and show that its role in centromere integrity is that of driving DNA sequence-independent RNAPII transcription through centromeric chromatin via a direct interaction with CAL1. Thus, current models for centromere transcription must take into account the transcriptional requirements for CENP-A recruitment by its assembly factor.

RESULTS

De Novo CENP-A Incorporation Temporally Coincides with Transcription of the Underlying DNA

Transcription of centromeric DNA has been described in several species (Chan and Wong, 2012), but whether or not it is directly linked to CENP-A deposition has remained elusive. One limitation of studying transcription at endogenous centromeres is the inability to precisely compare the same genomic locus in the presence and absence of active CENP-A deposition without interfering with cell-cycle progression or global transcription, which can result in reciprocal perturbation (Adolph et al., 1993; Whitfield et al., 2002). Furthermore, the endogenous CENP-A-bound DNA sequences of *Drosophila* are unknown, making an assessment of their transcription unfeasible. To overcome these limitations, we employed an ectopic centromere strategy based on the LacI/lacO system (Straight et al., 1996). This system utilizes a stably inserted lacO vector (10 kb of lacO repeats and 3 kb of vector backbone inserted within one arm of chromosome 2 or 3; Mendiburo et al., 2011), coupled with the inducible expression of the *Drosophila* CENP-A chaperone CAL1 fused to the lac repressor LacI (CAL1-GFP-LacI; Figure 1A). A GFP-LacI protein is used as a negative control. lacO-tethered CAL1-GFP-LacI induces the formation of fully functional and epigenetically propagated ectopic centromeres at the lacO site (Chen et al., 2014), allowing the direct comparison of the transcriptional status between lacO DNA with or without ongoing CENP-A incorporation.

CAL1-GFP-LacI is under the control of a metallothionein (MT) promoter, which can be induced by addition of $CuSO_4$ to the growth medium. First, we investigated how long after induction of CAL1-GFP-LacI CENP-A foci become visible at the lacO site by immunofluorescence (IF) on metaphase chromosome spreads. In parallel, we assessed transcription from the lacO backbone ($lacO^b$) by qRT-PCR (primer set 1.6; Figures 1A and 1B; Mendiburo et al., 2011). Transcription of the lacO array portion of the vector could not be assessed by this method due to its repetitive nature. 2 hr after induction, 54% of metaphase spreads displayed CAL1-GFP-LacI foci on the lacO-containing chromosome arm, with ~65% of these foci also containing CENP-A, showing that the recruitment of CENP-A at the lacO site occurs soon after CAL1-GFP-LacI induction (Figure 1C). Strikingly, at the 2 hr time point, a 28-fold change in transcription from $lacO^b$ was detected by qRT-PCR. Furthermore, $lacO^b$ transcript abundance persisted throughout the remainder of the time course (Figure 1D). *LacI* and $lacO^b$

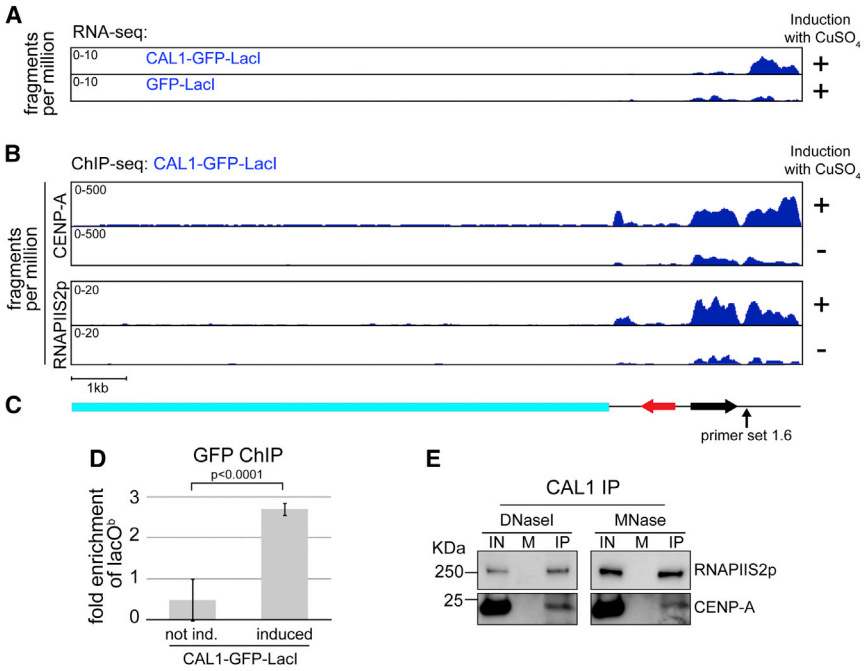


Figure 2. Transcription of lacO^b Correlates with CENP-A and RNAPII Distribution

(A) Coverage tracks of paired-end RNA-seq from induced (24 hr) CAL1-GFP-LacI (top) and GFP-LacI cells (bottom), mapped to the lacO vector. The x axis represents the position along the vector, and the y axis represents the fragments per million reads (normalized to the sequencing depth of each library). $p < 0.05$ (see the Supplemental Experimental Procedures).

(B) Coverage tracks of CENP-A and RNAPIIS2p paired-end ChIP-seq from induced (24 hr) and uninduced CAL1-GFP-LacI cells, mapped to the lacO vector. The x axis represents the position along the vector, and the y axis represents the fragments per million reads (normalized to the sequencing depth of each library).

(C) Schematic of the lacO vector as in Figure 1A.

(D) GFP ChIP-qPCR in CAL1-GFP-LacI uninduced and induced cells (24 hr). Error bars, 95% CI. p value, unpaired t test.

(E) Western blots with indicated antibodies of CAL1 IPs from chromatin extracts digested with either DNase or MNase. IN, input; M, mock (IP with beads only).

transcript levels displayed a decrease at 8 hr post-induction that was also observed in an independent time course experiment (Figure S1A). This transient dip likely reflects the kinetics of induction of the MT promoter (Bunch et al., 1988) driving CAL1-GFP-LacI. Detection of nascent lacO^b transcripts showed they are produced continuously, reaching a peak from 24–30 hr after induction (Figures S1B and S1C), a time during which it is expected that most lacO cells have acquired CENP-A at the lacO site (Figure 1C). Thus, we reveal a striking correlation between transcription and CENP-A incorporation at the lacO site.

To ensure that transcription of lacO^b is not due to the addition of $CuSO_4$ or is not somehow linked to the binding of the GFP-LacI fusion protein to the lacO array, we carried out qRT-PCR comparisons in the presence or absence of $CuSO_4$ between the following cell lines: CAL1-GFP-LacI cells, GFP-LacI cells, and cells completely lacking any *LacI* transgene, yet still harboring the integrated lacO plasmid (lacO). These experiments showed that transcription from lacO^b was only observed in induced CAL1-GFP-LacI cells (Figure 1E). No lacO^b transcription was detected in induced cells containing CAL1-GFP-LacI or GFP-LacI without lacO (data not shown).

The expression of a control gene, *actin*, which is transcribed by RNAPII, was unaffected, suggesting that addition of $CuSO_4$ does not cause non-specific transcriptional upregulation (Figure S1D). An increase in transcription from lacO^b was also observed when the lacO plasmid was introduced episomally, along with the CAL1-GFP-LacI plasmid, via transient transfection in S2 cells (Figure 1F). These transient transfections displayed low efficiency (~12%, as estimated by IF with anti-GFP antibodies, data not shown), resulting in fewer transcripts being detected by qRT-PCR compared to stable cells. Nonetheless, they demonstrate that transcription of lacO^b occurs independently of its chromosomal insertion.

Transcription of lacO^b Correlates with CENP-A and RNAPII Distribution

To gain more insight into the relationship between transcription and CENP-A occupancy across the lacO locus, we performed paired-end RNA sequencing (RNA-seq) and chromatin immunoprecipitation sequencing (ChIP-seq) experiments. RNA-seq of induced CAL1-GFP-LacI cells revealed that 28.5 fragments per million (fpm) mapped to lacO^b, whereas only 0.45 fpm mapped to the lacO array itself, where CAL1 is tethered. This could be due to blockage of RNAP passage by LacI bound to lacO repeats (Jacob and Monod, 1961). Thus, upon CAL1-GFP-LacI induction, most of the transcription originates from lacO^b sequences. Consistent with our qRT-PCR results (Figures 1D–1F), induced GFP-LacI cells displayed fewer reads mapping to lacO^b (12.8 fpm; $p < 0.05$; Figure 2A).

CENP-A ChIP-seq of induced CAL1-GFP-LacI cells revealed a preferential association of CENP-A with the lacO^b versus the lacO array (4,751.6 versus 2,462.5 fpm; Figures 2B and 2C). Since GFP ChIP-qPCR showed that CAL1-GFP-LacI is also enriched at lacO^b (Figure 2D), we concluded that CENP-A and CAL1-GFP-LacI spread to lacO^b from the lacO array, where CAL1-GFP-LacI is initially tethered.

Active RNAPII localizes to the centromeres of metaphase chromosomes in *Drosophila* (Rošić et al., 2014), raising the possibility that RNAPII may mediate transcription of lacO^b upon CAL1-GFP-LacI tethering. ChIP-seq with antibodies specific for the elongating form of RNAPII (RNAPIIS2p) showed a marked increase in RNAPIIS2p occupancy at the lacO^b after CAL1-GFP-LacI induction (178.3 fpm versus 75.5 fpm), suggesting that RNAPII mediates lacO^b transcription. Furthermore, the distribution of RNAPIIS2p closely resembled that of CENP-A in induced cells, with low occupancy on the lacO array and higher occupancy on the lacO^b array (32.3 versus 178.3 fpm; Figures 2B–2C), consistent with a

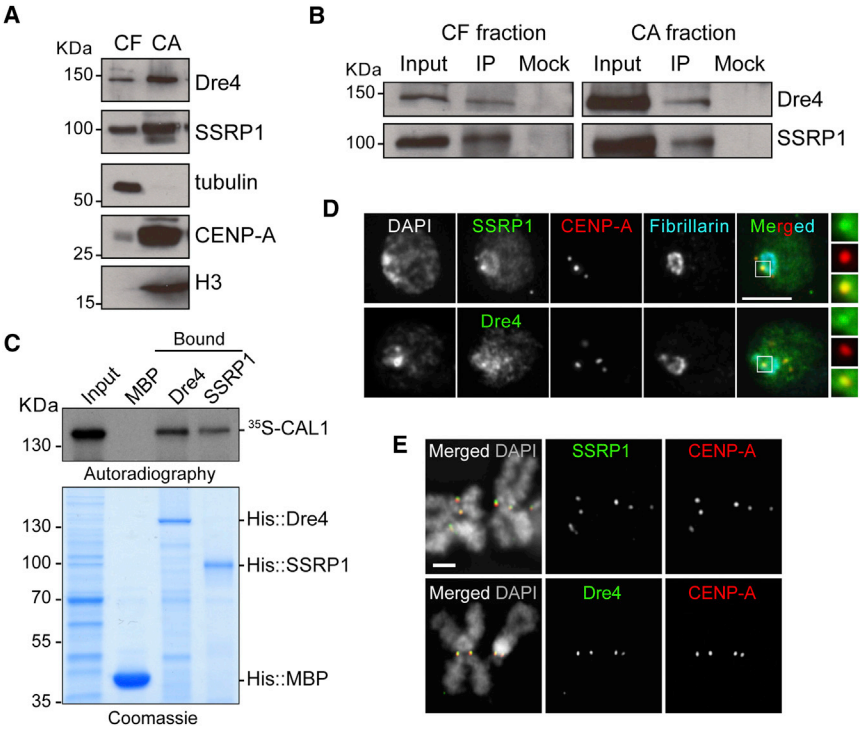


Figure 3. FACT Interacts with CAL1 and Localizes to the Centromere in S2 Cells

(A) Western blots of chromatin-free (CF) and chromatin-associated (CA) extracts from S2 cells with indicated antibodies. Tubulin and histone H3 antibodies are positive controls for their respective fractions.

(B) Western blots of IPs with anti-CAL1 antibodies from CF and CA extracts. Mock are IPs with rabbit immunoglobulin G (IgG).

(C) Direct interaction between in-vitro-translated ^{35}S-methionine-labeled CAL1 with recombinant His::Dre4 or His::SSRP1 bound to Ni-NTA beads. His::MBP, negative control.

(D) IF with anti-SSRP1 or anti-Dre4 (green), anti-CENP-A (red), and anti-fibrillarin (blue) antibodies. DAPI shown in gray. Insets show 3× magnifications of boxed centromere. Scale bar, 5 μm.

(E) IF on metaphase chromosomes with anti-SSRP1 or anti-Dre4 (green) and anti-CENP-A (red) antibodies. DAPI shown in gray. Scale bar, 1 μm.

See also Figure S2 and Table S1.

functional interplay between CENP-A assembly and transcriptional activity.

Low levels of CENP-A and RNAPIIS2p were observed in uninduced CAL1-GFP-LacI cells (Figure 2B), possibly due to leaky expression of CAL1-GFP-LacI, but these were found to be significantly lower than in induced samples ($p < 0.001$ and $q < 0.001$ for both CENP-A and RNAPIIS2p ChIPs; see the Supplemental Experimental Procedures; ChIP-seq and RNA-seq mapping).

Interestingly, immunoprecipitation (IP) of chromatin-associated CAL1 revealed a physical association between CAL1 and RNAPIIS2p (Figure 2E). This interaction and our ChIP-seq results indicate that CAL1 recruits RNAPIIS2p onto chromatin, in turn stimulating transcription. Why only a subset of the RNAPIIS2p-associated sequences produced transcripts by RNA-seq remains unclear. It is possible that some of these transcripts are unstable and cannot be detected by this type of assay.

Isolation of FACT, a CAL1 Interactor

Having shown that targeting of CAL1 to lacO triggers accumulation of RNAPIIS2p and transcription, we sought to identify the key components of this process by isolating CAL1-interacting factors. We performed IPs of FLAG-tagged CAL1 (Chen et al., 2012) from chromatin-free (CF) and chromatin-associated (CA) cell extracts with anti-FLAG- M_2 agarose beads, using *Drosophila* S2 cells (no FLAG tag) as a negative control. Mass spectrometric analysis (see Table S1; data not shown) identified among the highest scoring unique hits Spt16 (called Dre4 in *Drosophila*) and SSRP1, the two subunits of the heterodimeric FACT complex (Orphanides et al., 1999). FACT allows the progression of the transcriptional machinery through chromatinized templates (Belotserkovskaya et al., 2003; Orphanides et al., 1999), by a mechanism involving nucleosome destabilization (Hondele and Ladurner, 2013; Hondele et al., 2013), and was found to be associated with human CENP-A (Foltz et al., 2006), and to be important for CENP-A localization in chickens (Okada et al., 2009). Thus, FACT is a strong candidate for mediating CENP-A deposition-coupled transcription.

In order to confirm the association between CAL1 and the FACT complex, CF and CA fractions were prepared from S2 cells, and CAL1 IPs were performed using anti-CAL1 antibodies. Dre4 and SSRP1 were present in both fractions (Figure 3A) and associated with CAL1 in both cases (Figure 3B), confirming our proteomic results. Reciprocal IPs were performed using anti-FLAG antibodies to precipitate FLAG-Dre4 or FLAG-SSRP1; however, CAL1 was not detected (Figure S2A), suggesting that only a small fraction of FACT interacts with CAL1.

Next, we sought to determine if the association between CAL1, Dre4, and SSRP1 is direct by analyzing protein-protein interactions between recombinant His-Dre4 or His-SSPR1 and in-vitro-translated ^{35}S-methionine-labeled CAL1. The His-Dre4 and His-SSRP1 proteins heterodimerized in vitro (Figure S2B), suggesting that they are properly folded, and both pulled down CAL1 (Figure 3C), demonstrating direct interaction.

FACT is involved in RNAPII (Belotserkovskaya et al., 2003; Krogan et al., 2002; LeRoy et al., 1998; Orphanides et al., 1998), RNAPI, and RNAPIII transcription (Birch et al., 2009); therefore, it is expected to be broadly distributed throughout chromatin. To determine if FACT displays any centromeric enrichment, we used IF with anti-Dre4 and anti-SSRP1 antibodies. After extraction with detergent pre-fixation, a treatment expected to remove loosely chromatin-bound proteins, FACT was enriched at interphase centromeres (identified by CENP-A staining) and at the nucleolus (identified by Fibrillarin staining; Figure 3D). Examination of FACT localization on metaphase chromosome spreads revealed an even more striking centromeric accumulation of Dre4 and SSRP1, demonstrating that during mitosis, when active deposition of newly synthesized

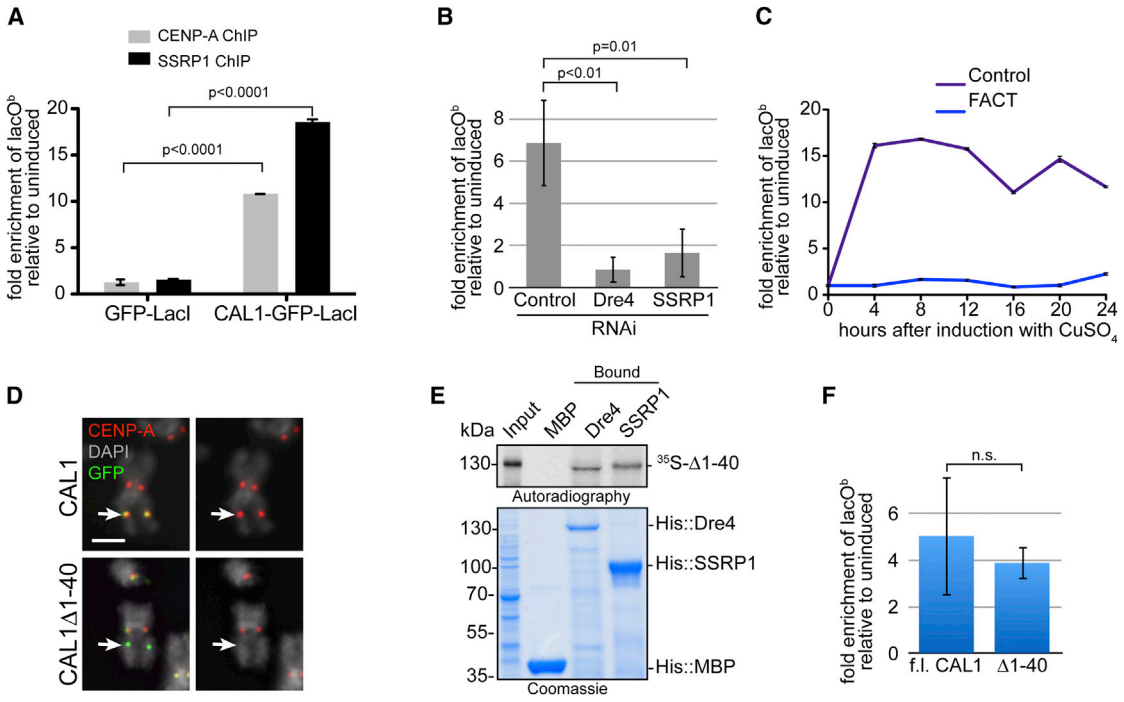


Figure 4. FACT Is Required for CENP-A Deposition-Coupled Transcription

(A) CENP-A and SSRP1 ChIP-qPCR in CAL1-GFP-LacI and GFP-LacI lacO cells. The graph shows the enrichment of induced cells (24 hr) relative to uninduced cells. Error bars, 95% CI of three technical replicates. Significant p values (unpaired t test) are shown.

(B) qRT-PCR of lacO^b transcripts in CAL1-GFP-LacI cells induced (24 hr) 6 days after the indicated RNAi treatments. p values (unpaired t test) are shown. The means ± SD of three experiments are shown.

(C) qRT-PCR of lacO^b transcripts in control (purple) and SSRP1/Dre4 RNAi (blue) cells at the indicated times. Error bars, SD of three technical replicates.

(D) IF with anti-CENP-A (red) and anti-GFP (green) antibodies in lacO cells expressing full-length CAL1-GFP-LacI (top) or CAL1Δ1-40-GFP-LacI (bottom). DAPI is shown in gray. Arrow points to the lacO site. Scale bar, 1 μm.

(E) Direct interaction between in-vitro-translated ^{35}S-methionine-labeled CAL1Δ1-40 (^{35}S-Δ1-40) with recombinant His::Dre4 (Dre4) or His::SSRP1 (SSRP1) bound to Ni-NTA beads. His::MBP (MBP) is a negative control.

(F) qRT-PCR of lacO^b transcripts in induced cells (24 hr) transiently expressing full-length (fl) CAL1-GFP-LacI or CAL1Δ1-40-GFP-LacI. Means ± SEM of three experiments are shown. p = 0.68 (not significant; unpaired t test).

CENP-A takes place (Mellone et al., 2011), FACT is more strongly associated with the centromere than with other regions of the genome (Figure 3E). These results were confirmed with epitope tagged SSRP1 and Dre4 (data not shown) and are consistent with a previous study in chicken DT-40 cells (Okada et al., 2009).

The Transcription Associated with CENP-A Deposition Requires FACT

Given that FACT enables RNAP progression, we next investigated whether FACT is required for the transcription we observed during CAL1-mediated CENP-A assembly at the lacO site. First, we investigated if CAL1-GFP-LacI recruits FACT to lacO^b using ChIP-qPCR with anti-SSRP1 antibodies (Nakayama et al., 2007). CENP-A ChIPs were performed in parallel. CAL1-GFP-LacI or GFP-LacI (negative control) cells were induced for 24 hr. CENP-A and SSRP1 were both found to be enriched in induced CAL1-GFP-LacI cells (Figure 4A). We concluded that FACT is recruited by CAL1-GFP-LacI to lacO^b.

Next, we measured lacO^b transcription by qRT-PCR after induction of CAL1-GFP-LacI cells, in which Dre4 and SSRP1 had been knocked down by RNAi for 6 days. Control cells displayed, on average, a 6.8-fold increase in lacO^b transcript levels 24 hr after induction with $CuSO_4$. In contrast, cells lacking FACT showed virtually no increase (Figure 4B). This result was also confirmed in a time course experiment, in which, after 6 days of FACT RNAi, CAL1-GFP-LacI was induced and qRT-PCR was performed on RNA extracted every 4 hr for 24 hr (Figure 4C). Together, these data demonstrate that FACT is required for the transcription observed upon CAL1 targeting.

CAL1-directed transcription could be a by-product of CENP-A incorporation, or it could occur independently of CENP-A deposition through the recruitment of RNAPII and FACT onto chromatin. To distinguish between these two possibilities, we used a CAL1 mutation lacking a short Scm3-like domain (Phansalkar et al., 2012; CAL1Δ1-40), which is defective in recruiting CENP-A to the lacO (Figure 4D; Chen et al., 2014). Importantly, CAL1Δ1-40 can interact directly with Dre4 and SSRP1 (Figure 4E). When we tethered CAL1Δ1-40-GFP-LacI to the lacO, we observed levels of lacO^b transcription indistinguishable from those initiated by CAL1-GFP-LacI (Figure 4F). We concluded that lacO^b transcription depends on CAL1 and FACT, but it does not require CENP-A incorporation.

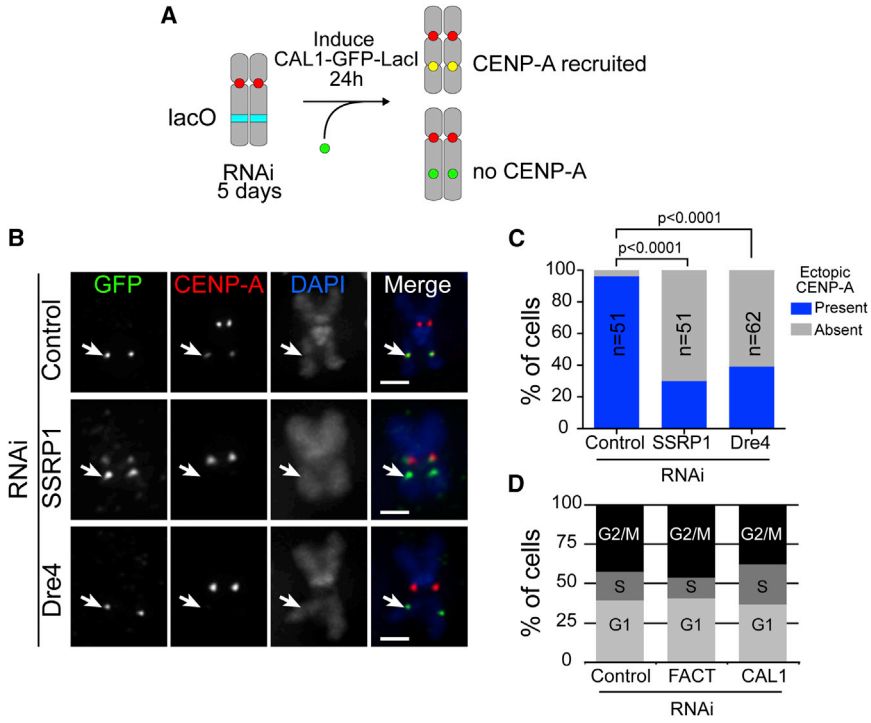


Figure 5. FACT Is Required for the De Novo Incorporation of CENP-A at an Ectopic Locus

(A) Cartoon depicting the experimental strategy to assess de novo CENP-A recruitment in the absence of FACT.

(B) lacO CAL1-GFP-LacI cells were subjected to RNAi to deplete Dre4, SSRP1, or a control for 5 days, followed by induction with $CuSO_4$ for 24 hr. Metaphase chromosome spreads were stained with anti-GFP (green) and anti-CENP-A antibodies (red). DNA was stained with DAPI (blue).

(C) Graph showing the percentage of CAL1-GFP-LacI-positive cells in which ectopic CENP-A signal was present or absent. Bars, 1 μm. $p < 0.0001$ (chi-square test).

(D) FACS profile of control, CAL1, and SSRP1/Dre4 (FACT) RNAi cells.

See also Figure S3 and Table S2.

FACT-Mediated Transcription Is Required for De Novo CENP-A Incorporation

CAL1 binds directly to FACT and recruits FACT and RNAPII to sites of CENP-A assembly. To determine if the absence of lacO^b transcription caused by knockdown of FACT affects deposition of CENP-A at the lacO site, we depleted Dre4 or SSRP1 by RNAi for 5 days, induced CAL1-GFP-LacI for 24 hr, and performed IF with anti-CENP-A and anti-GFP antibodies on metaphase spreads (Figure 5A). Ectopic targeting of CAL1 via LacI/lacO leads to efficient de novo incorporation of CENP-A (Chen et al., 2014). In contrast, depletion of either SSRP1 or Dre4 resulted in a significant reduction in the percentage of CENP-A-positive lacO sites (Figures 5B and 5C). Since FACT depletion did not affect the formation of the CENP-A/CENP-C/CAL1 complex (Erhardt et al., 2008; Figure S3A), a defect in CENP-A incorporation is the most likely explanation for this reduction in ectopic CENP-A. Thus, these experiments demonstrate that efficient recruitment of CENP-A by CAL1 requires FACT and imply that CAL1 is not sufficient to assemble CENP-A into nucleosomes when chromatin is the substrate, as opposed to when naked DNA is the substrate (Chen et al., 2014).

Given the ubiquitous role of FACT in DNA metabolism, we investigated possible pleiotropic effects that could account for the CENP-A incorporation defect seen after FACT RNAi. Fluorescence-activated cell sorting (FACS) analysis showed no change in the distribution of cells in G1, S, or G2-M upon FACT RNAi (Figure 5D), suggesting that the CENP-A incorporation defect is not due to a cell-cycle defect. Additionally, qRT-PCR analyses of *cenp-a*, *cal1*, or *cenp-c* transcripts (Figures S3B and S3C) and western blot analyses from total protein extracts (Figure S3D) demonstrated that FACT depletion did not decrease the expression of these essential centromere genes. Similarly, expression of eight handpicked genes that are bound to Dre4 based on ChIP-chip data (Kharchenko et al., 2011; Table S2), as well as that of the FACT-associated gene Hsp70 (Saunders et al., 2003), did not decrease upon FACT RNAi. This suggests that general transcription involves redundant chromatin remodeling activities, at least in *Drosophila*-cultured cells. Altogether, these results demonstrate that FACT plays a specific function in CENP-A deposition.

Depletion of FACT Causes Defective CENP-A Recruitment at Endogenous Centromeres

To determine if FACT is required for the recruitment of newly synthesized CENP-A at endogenous centromeres, we performed quench-chase-pulse experiments in cells stably expressing SNAP-tagged CENP-A (Jansen et al., 2007; Mellone et al., 2011). FACT was knocked down by simultaneous RNAi of Dre4 and SSRP1 for 6 days, after which time pre-existing SNAP-tagged CENP-A was irreversibly quenched using the BG-blocking agent (T_0; quench). RNAi of CAL1 was used as a positive control. After a chase that lasted until cells had divided once, newly synthesized SNAP-tagged CENP-A was labeled using TMR* (T_1; pulse) and cells were fixed and processed for IF (Figure 6A). Immediately after quenching SNAP-CENP-A, no TMR*-labeled CENP-A signal was observed, as expected, while labeling with an anti-CENP-A antibody showed that low levels of CENP-A were still present in both FACT and CAL1 RNAi (Figure 6B, T_0). After one cell division, cells were incubated with TMR* to label newly synthesized SNAP-CENP-A. Newly synthesized SNAP-CENP-A was clearly visible at the centromeres of control cells (Figure 6B, T_1, top). In contrast, there was a significant drop in the TMR*-CENP-A intensity levels of FACT RNAi cells, consistent with defective CENP-A recruitment (Figures 6B, T_1, and 6C).

To determine if FACT is also required to retain pre-existing centromeric CENP-A through one cell division, we quantified the total centromeric CENP-A IF signal at T_0 and T_1. In control cells, retention and recruitment of CENP-A are intact; therefore, no change in total CENP-A intensity occurs over one cell

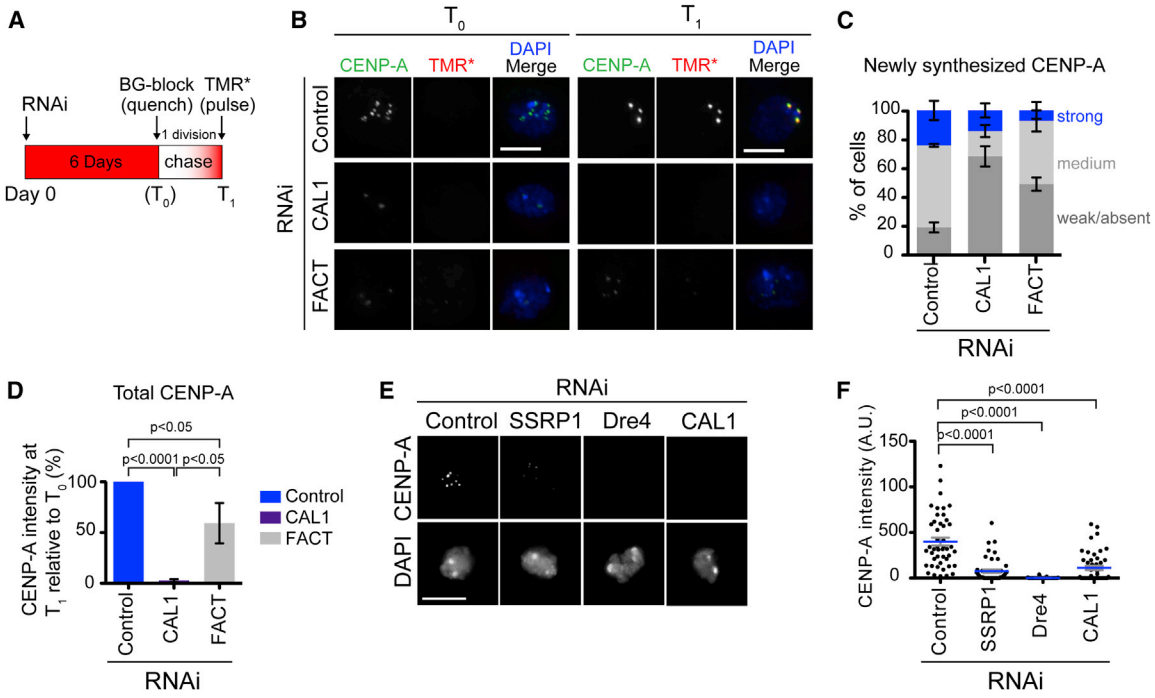


Figure 6. FACT Is Required for CENP-A Recruitment at Endogenous Centromeres

(A) Diagram of the quench-chase-pulse experiment.

(B) IF with anti-CENP-A antibodies (green) in control and Dre4/SSRP1 RNAi SNAP-CENP-A cells pulsed with TMR* (red) immediately after BG-block (T_0, left) or after having completed one cell division (T_1, right). DAPI is shown in blue. Scale bars, 5 μm.

(C) Quantification of the signal intensity of TMR*-labeled CENP-A foci. The means ± SEM of three experiments (100 cells quantified per RNAi treatment) are shown. $p < 0.0001$ for control versus SSRP1/Dre4 RNAi (unpaired t test).

(D) The total CENP-A centromeric intensity for control cells, CAL1 RNAi cells, and SSRP1/Dre4 RNAi (FACT) was quantified at T_0 and T_1. The mean change in CENP-A intensity at T_1 relative to T_0 ± SEM is shown. n = 3 experiments (150 cells each RNAi treatment). p values from an unpaired t test are shown.

(E) IF with anti-CENP-A antibodies of S2 cells subjected to the indicated RNAi treatments. DNA is stained with DAPI. Scale bar, 5 μm.

(F) Scatter plot showing total centromeric CENP-A signal intensity per cell from the experiment in (E). n = 50 cells per condition. p values from an unpaired t test are shown.

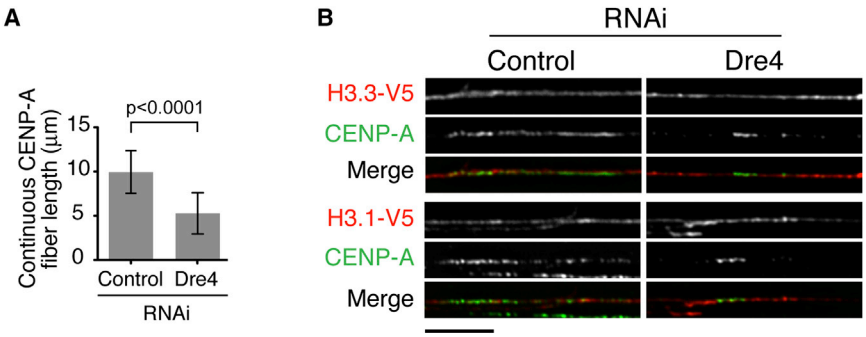
See also Figure S4.

division (T_1/T_0 = 100%). In contrast, in cells lacking FACT, centromeric CENP-A signal displayed a decrease in intensity consistent with a loading defect (T_1/T_0 = ~59%; Figure 6D; a ratio lower that 50% would be expected if the retention of pre-existing CENP-A were also affected). These results also explain why CENP-A is lost at a relatively slower rate in the absence of FACT (6 days): its loading is compromised but its retention is not. In contrast, loss of CENP-A from the centromere in the absence of CAL1 is much more rapid (Figure 6D), consistent with the dual role of CAL1 in CENP-A loading and stabilization from degradation (Chen et al., 2014). Collectively, our data demonstrate that FACT is required for the centromeric recruitment of newly synthesized CENP-A at the centromere.

To examine if FACT depletion can lead to complete loss of CENP-A from centromeres, we knocked down Dre4 or SSRP1, transfecting S2 cells twice with double-stranded RNA (dsRNA) over 6 days and examined the intensity of centromeric CENP-A by IF. We observed a dramatic decrease in the intensity of CENP-A foci upon Dre4 or SSRP1 RNAi compared to control cells (Figures 6E and 6F), demonstrating that two consecutive RNAi lead to a nearly complete loss of CENP-A from centromeres. Consistent with defective CENP-A recruitment, we observed a significant increase in chromosome missegregation in mitosis in cells lacking FACT (Figure S4).

H3.1 and H3.3 Accumulate within Centromeric Chromatin upon FACT RNAi

In human cells, histone H3 nucleosomes are deposited in S phase as temporary placeholders that need to be replaced by CENP-A in order to maintain centromere identity (Dunleavy et al., 2011). Whether CENP-A chaperones or other factors perform this exchange is unknown. Transcription at the centromere could mediate the eviction of the placeholder H3 during CAL1-mediated CENP-A deposition, analogously to H3.3 deposition at active genes (Schwartz and Ahmad, 2005). To determine if, in the absence of FACT and transcription, histone H3.1 or H3.3 accumulate at centromeres, we depleted Dre4 (which causes loss of SSRP1 as well; Figure S5) in S2 cells transiently transfected with plasmids expressing V5-tagged H3.1 and H3.3 and inspected centromeric chromatin by IF on stretched chromatin fibers. In Dre4-depleted cells, the average length of continuous CENP-A fibers was about one-half that of control cells (Figure 7A) and the CENP-A signal became less contiguous, suggesting that



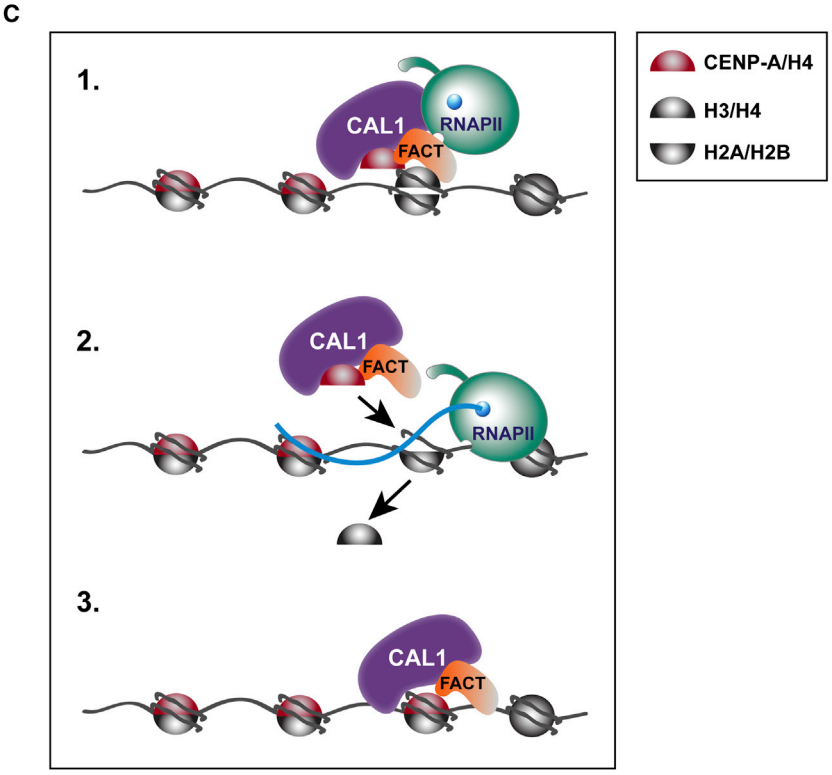


Figure 7. FACT Depletion Results in Accumulation of Histone H3-Containing Nucleosomes within Centromeric Chromatin

(A) Quantification of CENP-A fiber length from three experiments (n = 74 total fibers per condition). The means ± SD are shown. $p < 0.0001$ for control versus each RNAi (unpaired t test).

(B) IF on stretched chromatin fibers from control and Dre4 RNAi S2 cells expressing H3.1-V5 or H3.3-V5. V5 shown in red and CENP-A in green (n = 10–17 fibers per condition). Scale bar, 5 μm.

(C) Model for the role of RNAPII transcription in centromere propagation. FACT is recruited to the centromere along with CAL1 and CENP-A/H4; here, it destabilizes H3-containing nucleosomes, allowing the passage of RNAPII through chromatin (1). RNAPII transcribes through the region causing the eviction of H3/H4 tetramers (2), thereby allowing deposition of new CENP-A/H4 tetramers (3).

CENP-A is lost throughout CENP-A centromeric chromatin stretches, as well as from their edges. IF with anti-CENP-A and anti-V5 antibodies showed that H3.1 and H3.3 were continuously present across CENP-A fibers in control and RNAi cells, indicating that upon loss of CENP-A, no "gaps" were left at the centromere (Figure 7B). These results, which are consistent with a previous study that looked at centromeric fibers upon CENP-A depletion (Blower et al., 2002), suggest defective exchange between the placeholders H3.1/H3.3 and CENP-A in the absence of FACT and transcription.

Collectively, our data suggest a model in which FACT is recruited to the centromere by interacting directly with CAL1 in a pre-nucleosomal complex. Once at the centromere, FACT destabilizes nucleosomes (Hondele and Ladurner, 2013; Hondele et al., 2013), allowing transcription through the region via RNAPII. Finally, transcription by RNAPII causes the eviction of the placeholders H3.1 and H3.3, allowing the deposition of CENP-A by CAL1 (Figure 7C).

DISCUSSION

The epigenetic maintenance of centromeres through faithful CENP-A deposition is a process crucial for genome stability. Much of the recent advances in understanding this process in metazoans have focused on the dissection of the specific proteins involved in CENP-A recruitment. In contrast, the roles of DNA and chromatin in CENP-A deposition have remained largely elusive. In this study, we have uncovered a key role for transcription in *Drosophila* CENP-A deposition and have identified FACT as a central player in this process. This mechanism of nucleosome reorganization—combining RNAPII passage with CENP-A/H3 exchange—is analogous to other paradigms seen during transcription and development. For example, FACT is recruited to specific genomic loci by the GAGA factor, where it destabilizes nucleosomes, allowing replacement of histone H3.1 with H3.3 by the chaperone HIRA and thereby modulating the expression of *Hox* genes (Nakayama et al., 2007; Shimojima et al., 2003).

To ensure the fidelity of centromere propagation, CENP-A chromatin must be replenished after each round of DNA replication. In human cells, newly synthesized CENP-A is recruited to centromeric chromatin along with newly synthesized histone H4, indicating that CENP-A and H4 form a sub-nucleosomal core, which is assembled simultaneously (Bodor et al., 2013). As such, it is conceivable that CENP-A/H4 deposition involves the eviction of pre-existing H3/H4 tetramers.

To determine if CENP-A assembly is coupled to transcription, we used an inducible ectopic centromere system in *Drosophila* S2 cells. We discovered that a remarkable change in transcription occurs rapidly upon CAL1-GFP-LacI targeting at the lacO site. The same DNA that is transcribed is enriched in RNAPII,

suggesting that this polymerase is the one mediating this transcription. The interaction between CAL1 and RNAPII supports this idea, although the involvement of additional RNAPs cannot be ruled out.

In order to characterize this phenomenon mechanistically, we biochemically isolated the CAL1 partner FACT and demonstrated that it is necessary for the transcription of the lacO site. Despite its function in global RNAP elongation, the depletion of FACT did not cause a decrease in expression of FACT-associated genes, suggesting a redundancy of mechanisms directing general transcription in *Drosophila* cells. In contrast, upon FACT RNAi, transcription at the lacO site was impaired, resulting in defective de novo CENP-A deposition, demonstrating a specific disruption of centromere chromatin assembly.

Surprisingly, we found that transcription at the lacO site is independent of CENP-A assembly, revealing that CENP-A chaperones can initiate local chromatin reorganization through the recruitment of FACT and RNAPII.

The discovery that chromatin poses a barrier to CENP-A deposition by its chaperone and the involvement of FACT-mediated transcription in overcoming this barrier is likely to be relevant to other complex eukaryotes. In budding yeast, FACT allows Phs1 to access misincorporated CENP-A/Cse4 nucleosomes, allowing the ubiquitylation and subsequent degradation of CENP-A/Cse4 (Deyter and Biggins, 2014). However, our studies in *Drosophila* demonstrate that FACT is directly implicated in CENP-A deposition. The finding that FACT is required for CENP-A localization in chicken (Okada et al., 2009) and interacts with human CENP-A (Foltz et al., 2006) raises the possibility that the mechanism by which FACT promotes chromatin reorganization during CENP-A deposition by its chaperone may be conserved from flies to vertebrates.

In budding yeast, FACT increases nucleosome accessibility to nucleases in the absence of H2A-H2B dimer displacement, suggesting that it can reorganize nucleosomes in a more open configuration, while maintaining their original composition (Xin et al., 2009). Consistent with this, the crystal structure and mutational analyses of Spt16/Dre4 showed that FACT allows a gradual invasion of the nucleosome, breaking strong octamer-DNA contacts and allowing the passage of polymerases (Hondele et al., 2013). Thus, FACT is likely to function as a nucleosome destabilizer (Hondele and Ladurner, 2013), allowing the passage of RNAPII, which in turn interacts with CAL1 (Figure 7C).

A question that remains unanswered is whether the transcripts produced during CENP-A deposition are simply a by-product of the ongoing chromatin reorganization or if they are necessary components of centromere structure and identity. Specific RNAs emanating from centromeres do appear to play a role in centromere/kinetochore integrity (Carone et al., 2013; Quénet and Dalal, 2014; Rošić et al., 2014; Topp et al., 2004). However, the sequence requirements of these RNAs remain poorly defined. Our work demonstrates a requirement for transcription in CENP-A deposition as a means to reorganize nucleosomes and suggests the dispensability of specific centromeric RNA sequences in this process. Either there is a generic, non-sequence specific role for RNA at the centromere or specific sequences emanating from the centromere possess additional structural properties. Further work is needed to elucidate the functional relationship between CENP-A deposition-coupled transcription and structural centromeric RNAs.

EXPERIMENTAL PROCEDURES

Large-Scale Immunoprecipitation and Mass Spectrometry

FLAG-CAL1 complexes were purified from chromatin-free extracts generated from 2×10^9 S2 cells, as described previously (Chen et al., 2012; Mellone et al., 2011). FLAG-CAL1 complexes from chromatin-associated complexes were generated by homogenization, nuclear extraction, and digestion with benzonase. Extracts were added to anti-FLAG M2 beads (Sigma-Aldrich). After washing, complexes were eluted with FLAG peptide (Sigma-Aldrich) and sent for mass spectrometric analysis (see the Supplemental Experimental Procedures for details).

Small-Scale Immunoprecipitations

Extracts from chromatin-free and chromatin-associated fractions were prepared from 10^8 cells, as described before (Mellone et al., 2011). Extracts were added to Dynabeads-protein A beads (Life Technologies) coupled with anti-CAL1 or anti-FLAG antibodies (Sigma-Aldrich) and incubated for 10 min at room temperature, followed by a 30 min incubation at 4°C with rotation. Beads were washed three times with PBS-T (PBS; 0.1% Triton). 6% of the input and 50% of the IP was analyzed by 10% SDS-PAGE, followed by western blot. See the Supplemental Experimental Procedures for CAL1/RNAPII IPs.

In Vitro Protein Binding Assay

All steps were performed at room temperature. ~5 μg of purified His::MBP (negative control) and His::Dre4 or His::SSRP1 immobilized on Ni-NTA agarose (QIAGEN) were equilibrated in binding buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.1% Triton X-100, 1× EDTA-free protease inhibitor cocktail (Roche), 20 mM imidazole, and 0.5 mg/ml BSA, mixed with ^{35}S-methionine-labeled proteins expressed by a coupled in vitro transcription translation system (IVTT), and incubated for 1 hr. Beads were then washed in binding buffer (without BSA); proteins were eluted by boiling in Laemmli sample buffer and subjected to SDS-PAGE, followed by autoradiography. See also the Supplemental Experimental Procedures.

Cell Culture and RNAi

Stable S2 cells containing an integrated lacO plasmid (pAFS52; Straight et al., 1996) were described before (Chen et al., 2014; Mendiburo et al., 2011). Additional stable S2 cells were generated by transfection with Cellfectin reagent (Life Technologies) and selection with 450 μg/ml hygromycin. Stable lacO S2 cells were re-thawed after 1 month in culture due to loss of the lacO array over time. Transient transfections were performed by treating cells with FuGENE HD (Promega) for 2 days. Cells were induced with 0.5 mM $CuSO_4$ for 24 hr.

Stable CAL1-GFP-LacI or GFP-LacI cells were induced with 0.5 mM $CuSO_4$ at 25°C for 1–48 hr or left uninduced. RNAi was performed using DOTAP and 10 μg of dsRNA (see the Supplemental Experimental Procedures).

Total RNA Extraction and qRT-PCR

Total RNA was isolated from 1×10^7 cells using TRI-reagent (Sigma-Aldrich). 10 μg of RNA was treated with 1 μl of Turbo DNase (Life Technologies) for 30 min at 37°C. RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad), and 2 μl were used in qPCR using SYBR-green (Bio-Rad) on a CFX96 Real-Time System (Bio-Rad). Transcription from the $lacO^b$ (using primer pairs 1.6 or 3; Mendiburo et al., 2011) was normalized to uninduced samples. Values were calculated using the Pfaffl method (Pfaffl, 2001), with Rp49 (unaffected by FACT; Nakayama et al., 2007) as a reference gene. Some variability in the fold increase in $lacO^b$ transcription between experiments was observed due to instability of the lacO array during cell culture over time. See the Supplemental Experimental Procedures for primer sequences.

For RNA-seq, libraries were generated using the Tru-Seq kit (Illumina) and ran on a HiSeq. See the Supplemental Experimental Procedures for mapping information.

Total Protein Extraction and Western Blotting

Total cell extracts were obtained from 1 × 10^6 cells resuspended in 15 μl of RIPA buffer (150 mM NaCl, 50 mM Tris [pH 8], 1% NP40, and 0.1% SDS), incubated on ice for 10 min, and digested with 1 μl of benzonase (Novagen) for 20 min at 37°C. Extracts were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. After 30 min incubation in blocking buffer (TBS, 0.1% Tween 20, 5% powder non-fat milk), membranes were incubated overnight at 4°C with anti-CAL1 (rabbit, 1:1,000; from A. Straight), anti-CENP-A (rabbit, 1:1,000; Active Motif), anti-FLAG (mouse, 1:1,000; Sigma-Aldrich), anti-Dre4 and anti-SSRP1 (rabbit, 1:1,000; from S. Hirose), and anti-RNAPIIS2p (mouse, 1:1,000; Abcam); anti-CENP-C (guinea pig, 1:3,000; Mellone et al., 2011), anti-tubulin (mouse, 1:1,000; Sigma-Aldrich), or anti-histone H3 (rabbit, 1:5,000; Abcam) antibodies were used as a loading control.

Immunofluorescence

IF on settled cells and metaphase spreads was performed as described (Chen et al., 2014). For pre-extraction with detergent, settled cells were immersed in 100 μl of PBS-T for 5 min, followed by the addition of 11 μl of 37% formaldehyde for 10 min. Stretched chromatin fibers were performed essentially as described (Sullivan, 2010), using twice the amount of primary antibodies than conventional IF. Only extensively stretched fibers (DAPI nearly undetectable) were used for our analyses. The antibodies used were anti-CENP-A (chicken, 1:1,000; Blower and Karpen, 2001), anti-GFP (rabbit-488 conjugated, 1:100; Life Technologies), anti-CENP-C (guinea pig, 1:1,000; Mellone et al., 2011), anti-fibrillarin (mouse, 1:500; Cytoskeleton), and anti-V5 (mouse, 1:50; Life Technologies). Secondary antibodies (Life Technologies Alexa-Fluor 488 or 546 conjugated; Santa Cruz biotechnology CY5 conjugated; 1:500) were used as appropriate. Slides were mounted in Slowfade (Life Technologies) containing DAPI.

Quench-Pulse-Chase Assay

RNAi of both Dre4 and SSRP1 was simultaneously performed for 6 days in a 12-well plate. Quench-chase-pulse, followed by IF, was performed as described (Mellone et al., 2011), making sure the cells had divided once (~24 hr for control and 24–48 hr for FACT RNAi) between BG-block (quench) and TMR* labeling (chase).

Fluorescence-Activated Cell Sorting

After 6 days of RNAi (Bw for control and SSRP1/Dre4 for FACT), 1 × 10^6 S2 cells were washed in PBS with 2% BSA and then incubated in PBS containing 50 μg/ml propidium iodide, 200 μg/ml RNase A, and 0.1% Triton X-100 for 15 min at 25°C in the dark. Samples were analyzed on a BD FACSCaliber Flow Cytometer and analyzed using FloJo.

Imaging

All images were taken at 25°C on an Olympus Fluorescence Microscope (PersonalDV; Applied Precision) equipped with a 60× 1.42 NA or a 100× 1.40 NA oil-immersion objective (Olympus) and a CoolSnap HQ_2 Camera (Photometrics), keeping exposure conditions constant between all samples. Images were acquired and deconvolved using softWoRx (Applied Precision), maintaining the scaling constant between samples, and all images were saved as Photoshop files. Figures were assembled in Adobe Illustrator. See the Supplemental Experimental Procedures for image quantifications.

Chromatin Immunoprecipitation

ChIP was performed using the MAGnify Kit (Life Technologies). 10^6 cells (~10 μg DNA) was used for each IP, and chromatin was sheared to fragments 100–300 bp long. 1 μl of anti-CENP-A (rabbit, Active Motif), anti-SSRP1 (Nakayama et al., 2007), anti-GFP (Abcam), or anti-RNAPIIS2p (Abcam) were coupled to 10 μl beads for 2 hr and mixed with chromatin overnight at 4°C. Immunoprecipitated DNA was eluted in 50 μl of elution buffer and analyzed by qPCR. Normalization was performed using the following formula: $100 \times AE^{(averageCT\ INPUT\ -\ averageCT\ IP)}$, where AE is the amplification efficiency calculated by the formula $AE = 10^{(-1/slope)}$. The values obtained for induced cells were normalized by those for uninduced cells to calculate enrichment. For ChIP-seq, DNA from three independent ChIP experiments were pooled and made into libraries with the TruSeq ChIP Kit (Illumina). Samples were run on a MiSeq using the Reagent Kit (v. 3). See the Supplemental Experimental Procedures for mapping information.

Statistical Methods

SE, SD, and CI were calculated using Numbers (Apple). Unpaired t test and chi-square tests were performed in Prism (GraphPad). See the Supplemental Experimental Procedures for statistical analysis of next-generation sequencing data.

ACCESSION NUMBERS

The accession number for the ChIP-seq and RNA-seq raw data reported in this paper is NCBI: SRP059507.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.05.012.

ACKNOWLEDGMENTS

We thank Bo Reese (CGI) for sequencing support, G. Karpen, S. Hirose, A. Straight, and P. Heun for reagents, I. Cheeseman for critically reading the manuscript, L. Core for suggestions and help with ChIP-chip data analysis, and the Drosophila RNAi Screening Center (DRSC) for resources. This work was supported by awards from the NSF (1024973) (to B.G.M.) and the NIH (GM108829) (to B.G.M.). D.M.G. is supported by grants from Cancer Research UK (C3/A11431) and the Medical Research Council (G1001696); Z.L. is supported by a long-term FEBS Fellowship; and R.J.O. is supported by an NSF award (1244146).

Received: October 7, 2014
Revised: April 9, 2015
Accepted: May 18, 2015
Published: July 6, 2015

REFERENCES

Adolph, S., Brüsselbach, S., and Müller, R. (1993). Inhibition of transcription blocks cell cycle progression of NIH3T3 fibroblasts specifically in G1. J. Cell Sci. *105*, 113–122.

Barnhart, M.C., Kuich, P.H., Stellfox, M.E., Ward, J.A., Bassett, E.A., Black, B.E., and Foltz, D.R. (2011). HJURP is a CENP-A chromatin assembly factor sufficient to form a functional de novo kinetochore. J. Cell Biol. *194*, 229–243.

Belotserkovskaya, R., Oh, S., Bondarenko, V.A., Orphanides, G., Studitsky, V.M., and Reinberg, D. (2003). FACT facilitates transcription-dependent nucleosome alteration. Science *301*, 1090–1093.

Bernad, R., Sánchez, P., Rivera, T., Rodríguez-Corsino, M., Boyarchuk, E., Vassias, I., Ray-Gallet, D., Arnaoutov, A., Dasso, M., Almouzni, G., and Losada, A. (2011). Xenopus HJURP and condensin II are required for CENP-A assembly. J. Cell Biol. *192*, 569–582.

Birch, J.L., Tan, B.C., Panov, K.I., Panova, T.B., Andersen, J.S., Owen-Hughes, T.A., Russell, J., Lee, S.C., and Zomerdijk, J.C. (2009). FACT facilitates chromatin transcription by RNA polymerases I and III. EMBO J. *28*, 854–865.

Blower, M.D., and Karpen, G.H. (2001). The role of Drosophila CID in kinetochore formation, cell-cycle progression and heterochromatin interactions. Nat. Cell Biol. *3*, 730–739.

Blower, M.D., Sullivan, B.A., and Karpen, G.H. (2002). Conserved organization of centromeric chromatin in flies and humans. Dev. Cell *2*, 319–330.

Bodor, D.L., Valente, L.P., Mata, J.F., Black, B.E., and Jansen, L.E. (2013). Assembly in G1 phase and long-term stability are unique intrinsic features of CENP-A nucleosomes. Mol. Biol. Cell *24*, 923–932.

Bunch, T.A., Grinblat, Y., and Goldstein, L.S. (1988). Characterization and use of the Drosophila metallothionein promoter in cultured Drosophila melanogaster cells. Nucleic Acids Res. *16*, 1043–1061.

Camahort, R., Li, B., Florens, L., Swanson, S.K., Washburn, M.P., and Gerton, J.L. (2007). Scm3 is essential to recruit the histone h3 variant cse4 to centromeres and to maintain a functional kinetochore. Mol. Cell *26*, 853–865.

Carone, D.M., Longo, M.S., Ferreri, G.C., Hall, L., Harris, M., Shook, N., Bulazel, K.V., Carone, B.R., Obergfell, C., O'Neill, M.J., and O'Neill, R.J. (2009). A new class of retroviral and satellite encoded small RNAs emanates from mammalian centromeres. Chromosoma *118*, 113–125.

Carone, D.M., Zhang, C., Hall, L.E., Obergfell, C., Carone, B.R., O'Neill, M.J., and O'Neill, R.J. (2013). Hypermorphic expression of centromeric retroelement-encoded small RNAs impairs CENP-A loading. Chromosome Res. *21*, 49–62.

Chan, F.L., and Wong, L.H. (2012). Transcription in the maintenance of centromere chromatin identity. Nucleic Acids Res. *40*, 11178–11188.

Chan, F.L., Marshall, O.J., Saffery, R., Kim, B.W., Earle, E., Choo, K.H., and Wong, L.H. (2012). Active transcription and essential role of RNA polymerase II at the centromere during mitosis. Proc. Natl. Acad. Sci. USA *109*, 1979–1984.

Chen, C.C., Greene, E., Bowers, S.R., and Mellone, B.G. (2012). A role for the CAL1-partner Modulo in centromere integrity and accurate chromosome segregation in Drosophila. PLoS ONE *7*, e45094.

Chen, C.C., Dechassa, M.L., Bettini, E., Ledoux, M.B., Belisario, C., Heun, P., Luger, K., and Mellone, B.G. (2014). CAL1 is the Drosophila CENP-A assembly factor. J. Cell Biol. *204*, 313–329.

Choi, E.S., Strålfors, A., Castillo, A.G., Durand-Dubief, M., Ekwall, K., and Allshire, R.C. (2011). Identification of noncoding transcripts from within CENP-A chromatin at fission yeast centromeres. J. Biol. Chem. *286*, 23600–23607.

De Rop, V., Padeganeh, A., and Maddox, P.S. (2012). CENP-A: the key player behind centromere identity, propagation, and kinetochore assembly. Chromosoma *121*, 527–538.

Deyter, G.M., and Biggins, S. (2014). The FACT complex interacts with the E3 ubiquitin ligase Psh1 to prevent ectopic localization of CENP-A. Genes Dev. *28*, 1815–1826.

Dunleavy, E.M., Roche, D., Tagami, H., Lacoste, N., Ray-Gallet, D., Nakamura, Y., Daigo, Y., Nakatani, Y., and Almouzni-Pettinotti, G. (2009). HJURP is a cell-cycle-dependent maintenance and deposition factor of CENP-A at centromeres. Cell *137*, 485–497.

Dunleavy, E.M., Almouzni, G., and Karpen, G.H. (2011). H3.3 is deposited at centromeres in S phase as a placeholder for newly assembled CENP-A in G_1 phase. Nucleus *2*, 146–157.

Erhardt, S., Mellone, B.G., Betts, C.M., Zhang, W., Karpen, G.H., and Straight, A.F. (2008). Genome-wide analysis reveals a cell cycle-dependent mechanism controlling centromere propagation. J. Cell Biol. *183*, 805–818.

Foltz, D.R., Jansen, L.E., Black, B.E., Bailey, A.O., Yates, J.R., 3rd, and Cleveland, D.W. (2006). The human CENP-A centromeric nucleosome-associated complex. Nat. Cell Biol. *8*, 458–469.

Foltz, D.R., Jansen, L.E., Bailey, A.O., Yates, J.R., 3rd, Bassett, E.A., Wood, S., Black, B.E., and Cleveland, D.W. (2009). Centromere-specific assembly of CENP-a nucleosomes is mediated by HJURP. Cell *137*, 472–484.

Hemmerich, P., Weidtkamp-Peters, S., Hoischen, C., Schmiedeberg, L., Erliandri, I., and Diekmann, S. (2008). Dynamics of inner kinetochore assembly and maintenance in living cells. J. Cell Biol. *180*, 1101–1114.

Hondele, M., and Ladurner, A.G. (2013). Catch me if you can: how the histone chaperone FACT capitalizes on nucleosome breathing. Nucleus *4*, 443–449.

Hondele, M., Stuwe, T., Hassler, M., Halbach, F., Bowman, A., Zhang, E.T., Nijmeijer, B., Kotthoff, C., Rybin, V., Amlacher, S., et al. (2013). Structural basis of histone H2A-H2B recognition by the essential chaperone FACT. Nature *499*, 111–114.

Jacob, F., and Monod, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. *3*, 318–356.

Jansen, L.E., Black, B.E., Foltz, D.R., and Cleveland, D.W. (2007). Propagation of centromeric chromatin requires exit from mitosis. J. Cell Biol. *176*, 795–805.

Kharchenko, P.V., Alekseyenko, A.A., Schwartz, Y.B., Minoda, A., Riddle, N.C., Ernst, J., Sabo, P.J., Larschan, E., Gorchakov, A.A., Gu, T., et al. (2011). Comprehensive analysis of the chromatin landscape in Drosophila melanogaster. Nature *471*, 480–485.

Krogan, N.J., Kim, M., Ahn, S.H., Zhong, G., Kobor, M.S., Cagney, G., Emili, A., Shilatifard, A., Buratowski, S., and Greenblatt, J.F. (2002). RNA polymerase II elongation factors of Saccharomyces cerevisiae: a targeted proteomics approach. Mol. Cell. Biol. *22*, 6979–6992.

LeRoy, G., Orphanides, G., Lane, W.S., and Reinberg, D. (1998). Requirement of RSF and FACT for transcription of chromatin templates in vitro. Science *282*, 1900–1904.

Marshall, O.J., Chueh, A.C., Wong, L.H., and Choo, K.H. (2008). Neocentromeres: new insights into centromere structure, disease development, and karyotype evolution. Am. J. Hum. Genet. *82*, 261–282.

Mellone, B.G., Grive, K.J., Shteyn, V., Bowers, S.R., Oderberg, I., and Karpen, G.H. (2011). Assembly of Drosophila centromeric chromatin proteins during mitosis. PLoS Genet. *7*, e1002068.

Mendiburo, M.J., Padeken, J., Fülöp, S., Schepers, A., and Heun, P. (2011). Drosophila CENH3 is sufficient for centromere formation. Science *334*, 686–690.

Nakano, M., Cardinale, S., Noskov, V.N., Gassmann, R., Vagnarelli, P., Kandels-Lewis, S., Larionov, V., Earnshaw, W.C., and Masumoto, H. (2008). Inactivation of a human kinetochore by specific targeting of chromatin modifiers. Dev. Cell *14*, 507–522.

Nakayama, T., Nishioka, K., Dong, Y.X., Shimojima, T., and Hirose, S. (2007). Drosophila GAGA factor directs histone H3.3 replacement that prevents the heterochromatin spreading. Genes Dev. *21*, 552–561.

Ohkuni, K., and Kitagawa, K. (2011). Endogenous transcription at the centromere facilitates centromere activity in budding yeast. Curr. Biol. *21*, 1695–1703.

Okada, M., Okawa, K., Isobe, T., and Fukagawa, T. (2009). CENP-H-containing complex facilitates centromere deposition of CENP-A in cooperation with FACT and CHD1. Mol. Biol. Cell *20*, 3986–3995.

Orphanides, G., LeRoy, G., Chang, C.H., Luse, D.S., and Reinberg, D. (1998). FACT, a factor that facilitates transcript elongation through nucleosomes. Cell *92*, 105–116.

Orphanides, G., Wu, W.H., Lane, W.S., Hampsey, M., and Reinberg, D. (1999). The chromatin-specific transcription elongation factor FACT comprises human SPT16 and SSRP1 proteins. Nature *400*, 284–288.

Perpelescu, M., Nozaki, N., Obuse, C., Yang, H., and Yoda, K. (2009). Active establishment of centromeric CENP-A chromatin by RSF complex. J. Cell Biol. *185*, 397–407.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. *29*, e45.

Phansalkar, R., Lapierre, P., and Mellone, B.G. (2012). Evolutionary insights into the role of the essential centromere protein CAL1 in Drosophila. Chromosome Res. *20*, 493–504.

Pidoux, A.L., Choi, E.S., Abbott, J.K., Liu, X., Kagansky, A., Castillo, A.G., Hamilton, G.L., Richardson, W., Rappsilber, J., He, X., and Allshire, R.C. (2009). Fission yeast Scm3: A CENP-A receptor required for integrity of subkinetochore chromatin. Mol. Cell *33*, 299–311.

Quénet, D., and Dalal, Y. (2014). A long non-coding RNA is required for targeting centromeric protein A to the human centromere. eLife *3*, e03254.

Rošić, S., Köhler, F., and Erhardt, S. (2014). Repetitive centromeric satellite RNA is essential for kinetochore formation and cell division. J. Cell Biol. *207*, 335–349.

Sanchez-Pulido, L., Pidoux, A.L., Ponting, C.P., and Allshire, R.C. (2009). Common ancestry of the CENP-A chaperones Scm3 and HJURP. Cell *137*, 1173–1174.

Saunders, A., Werner, J., Andrulis, E.D., Nakayama, T., Hirose, S., Reinberg, D., and Lis, J.T. (2003). Tracking FACT and the RNA polymerase II elongation complex through chromatin in vivo. Science *301*, 1094–1096.

Schuh, M., Lehner, C.F., and Heidmann, S. (2007). Incorporation of Drosophila CID/CENP-A and CENP-C into centromeres during early embryonic anaphase. Curr. Biol. *17*, 237–243.

Schwartz, B.E., and Ahmad, K. (2005). Transcriptional activation triggers deposition and removal of the histone variant H3.3. Genes Dev. *19*, 804–814.

Shimojima, T., Okada, M., Nakayama, T., Ueda, H., Okawa, K., Iwamatsu, A., Handa, H., and Hirose, S. (2003). Drosophila FACT contributes to Hox gene expression through physical and functional interactions with GAGA factor. Genes Dev. *17*, 1605–1616.

Shuaib, M., Ouararhni, K., Dimitrov, S., and Hamiche, A. (2010). HJURP binds CENP-A via a highly conserved N-terminal domain and mediates its deposition at centromeres. Proc. Natl. Acad. Sci. USA *107*, 1349–1354.

Stoler, S., Rogers, K., Weitze, S., Morey, L., Fitzgerald-Hayes, M., and Baker, R.E. (2007). Scm3, an essential Saccharomyces cerevisiae centromere protein required for G2/M progression and Cse4 localization. Proc. Natl. Acad. Sci. USA *104*, 10571–10576.

Straight, A.F., Belmont, A.S., Robinett, C.C., and Murray, A.W. (1996). GFP tagging of budding yeast chromosomes reveals that protein-protein interactions can mediate sister chromatid cohesion. Curr. Biol. *6*, 1599–1608.

Sullivan, B.A. (2010). Optical mapping of protein-DNA complexes on chromatin fibers. Methods Mol. Biol. *659*, 99–115.

Topp, C.N., Zhong, C.X., and Dawe, R.K. (2004). Centromere-encoded RNAs are integral components of the maize kinetochore. Proc. Natl. Acad. Sci. USA *101*, 15986–15991.

Whitfield, M.L., Sherlock, G., Saldanha, A.J., Murray, J.I., Ball, C.A., Alexander, K.E., Matese, J.C., Perou, C.M., Hurt, M.M., Brown, P.O., and Botstein, D. (2002). Identification of genes periodically expressed in the human cell cycle and their expression in tumors. Mol. Biol. Cell *13*, 1977–2000.

Xin, H., Takahata, S., Blanksma, M., McCullough, L., Stillman, D.J., and Formosa, T. (2009). yFACT induces global accessibility of nucleosomal DNA without H2A-H2B displacement. Mol. Cell *35*, 365–376.