Diaphanous formin mDia2 regulates CENP-A levels at centromeres

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Centromeres of higher eukaryotes are epigenetically defined by centromere protein A (CENP-A), a centromere-specific histone H3 variant. The incorporation of new CENP-A into centromeres to maintain the epigenetic marker after genome replication in S phase occurs in G1 phase; however, how new CENP-A is loaded and stabilized remains poorly understood. Here, we identify the formin mDia2 as essential for stable replenishment of new CENP-A at centromeres. Quantitative imaging, pulse-chase analysis, and high-resolution ratiometric live-cell studies demonstrate that mDia2 and its nuclear localization are required to maintain CENP-A levels at centromeres. Depletion of mDia2 results in a prolonged centromere association of holiday junction recognition protein (HJURP), the chaperone required for CENP-A loading. A constitutively active form of mDia2 rescues the defect in new CENP-A loading caused by depletion of male germ cell Rac GTPase-activating protein (MgcRacGAP), a component of the small GTPase pathway essential for CENP-A maintenance. Thus, the formin mDia2 functions downstream of the MgcRacGAP-dependent pathway in regulating assembly of new CENP-A containing nucleosomes at centromeres.

Introduction

The epigenetic landscape of the chromosome is well inherited independent of underlying DNA sequences. In mammals, centromeres, the fundamental unit for chromosome segregation during mitosis, are defined epigenetically by nucleosomes containing the histone H3 variant centromere protein A (CENP-A; Cleveland et al., 2003; Nechemia-Arbely et al., 2012; Fukagawa and Earnshaw, 2014). To maintain centromere identity against CENP-A dilution as DNA replicates and cell divides, newly synthesized CENP-A proteins are deposited at centromeres during early G1 of each cell cycle (Jansen et al., 2007). This process is initiated by Plk1-mediated (McKinley and Cheeseman, 2014) centromeric recruitment of the Mis18 complex at anaphase onset (Hayashi et al., 2004; Fujita et al., 2007; Maddox et al., 2007) and involves the recruitment of holiday junction recognition protein (HJURP), the CENP-A chaperone (Dunleavy et al., 2009; Foltz et al., 2009).

The process to incorporate new CENP-A at centromeres remains poorly understood. A small GTPase molecular switch has been shown to stabilize newly loaded CENP-A. Depletion of Cdc42 or Rac1 in human cells leads to a decrease of CENP-A level at centromeres (Lagana et al., 2010). The downstream effectors of this small GTPase activity remain unidentified. Mammalian diaphanous-related (mDia) formins nucleate and assemble unbranched actin structures downstream of Rho family GTPase signaling (Xu et al., 2004). Recent studies have revealed potential nuclear roles for formins (Baarlink et al., 2013; Belin et al., 2015). Among mDia formin proteins (mDia1-3), only mDia2 shuttles between the cytoplasm and the nucleus (Miki et al., 2009; Baarlink et al., 2013). By affinity purification and mass spectrometry analysis, histones and topoisomerases have been identified as binding partners of mDia2, but neither mDia1 nor mDia3 (Daou et al., 2014).

Using quantitative imaging, we now provide direct evidence that the formin mDia2 is a novel cytoskeleton protein required for maintaining CENP-A levels at centromeres. As a constitutively active form of mDia2 rescues centromeric CENP-A levels caused by depletion of male germ cell Rac GTPase-activating protein (MgcRacGAP), a component of the small GTPase pathway essential for CENP-A maintenance, we additionally uncover mDia2 as the downstream effector of the GTPase activity for epigenetic centromere maintenance.

Results and discussion

Diaphanous formin mDia2 is essential to maintain CENP-A levels at centromeres

To test if the formin mDia2 is required for CENP-A level maintenance at centromeres, mDia2 protein levels were reduced in human cells (0.47 ± 0.11 relative to control, P < 0.0001) by the transfection of siRNA duplexes targeting mDia2 for 48 h (Fig. 1A). The mDia2 depletion resulted in a decreased level, but...
not elimination, of CENP-A at centromeres (Fig. 1 B) without affecting total CENP-A protein levels (Fig. 1 A) compared with control cells (transfected with GAPDH siRNA). Significantly, the loss of CENP-A at centromeres could be rescued by the co-expression of the siRNA-resistant full-length mDia2 (Fig. 1 B), excluding the possibility of an off-target effect from mDia2 siRNA. CENP-A levels at centromeres from large numbers of cells were quantified using an automatic image-analysis algorithm (Fig. S1), designed in this study, without human bias. This confirmed the partial reduction in CENP-A levels at centromeres in mDia2-depleted cells (Fig. 1 C). The decrease of CENP-A level was not caused by loss of centromere numbers in individual cells, judging by counting the immunostaining of CENP-B (Fig. 1 D), which localizes to centromeres independently of CENP-A (Masumoto et al., 1989). In contrast to mDia2, knockdown of mDia3, a formin protein that has been shown to associate with kinetochores and to be important for kinetochore-microtubule attachment (Yasuda et al., 2004; Cheng et al., 2011), did not result in loss of CENP-A at centromeres (Fig. 1, B and C). These results support a role for the formin mDia2 in CENP-A level maintenance at centromeres.

To exclude the possibility that the CENP-A loss in mDia2-depleted cells could be caused by cell cycle–dependent transcriptional regulation of CENP-A, YFP-CENP-A levels were measured in fixed cells stably expressing a YFP-tagged CENP-A. Despite that the YFP-CENP-A fusion is controlled by the 5′ long terminal repeat of the virus (Foltz et al., 2009), a similar CENP-A loss at centromeres was observed upon mDia2 depletion (Fig. 1, E and F). Reduced CENP-A levels in mDia2-depleted cells is reminiscent of the depletion of the CENP-A chaperon HJU RP (Fig. 1, E and F). These results are consistent with a role for mDia2 in regulating CENP-A levels at centromeres.

The mDia2 protein is specifically required for loading of new CENP-A

To determine if mDia2 is required for cell cycle–dependent incorporation of new CENP-A into centromeres of duplicated...
sister chromatids after mitotic exit into G1, YFP-CENP-A levels at individual centromeres were followed by high-resolution ratiometric live-cell imaging designed in this study (Fig. S2). In control cells, the increase of centromeric YFP-CENP-A levels began shortly after anaphase onset and continued for several hours (Fig. 2, A and B; and Video 1), which is consistent with previous observations (Jansen et al., 2007; Lagana et al., 2010). In contrast, the increase of YFP-CENP-A levels at centromeres in mDia2-depleted cells could not be maintained despite an initial slight increase within 2 h after anaphase onset (Fig. 2, A and B; and Video 2). The defect of YFP-CENP-A loading in mDia2-depleted cells was also manifested with a significantly shorter apparent half-time (Fig. S2, B and C), consistent with attempted yet failed loading events. These results clearly demonstrate that mDia2 is essential for replenishing CENP-A levels during early G1 phase, when new CENP-A is loaded onto centromeres marked with preexisting CENP-A.

Whereas cell cycle distribution was not significantly altered upon depletion of mDia2 (Fig. S2 G), live-cell imaging analysis showed reduced levels of CENP-A recruitment at centromeres upon mDia2 depletion. Cells with fully loaded CENP-A at centromeres inherits 50% “old” CENP-A during S phase and recruit 50% “new” CENP-A at early G1. To test which population of CENP-A was affected by mDia2 depletion, old and new CENP-A levels were measured by the SNAP pulse-chase method (Jansen et al., 2007; Lagana et al., 2010). The old inherited CENP-A was pulse-labeled (15 min) with a fluorescent mark, whereas new CENP-A was chased with a nonfluorescent label (Fig. 2 C). This analysis revealed that mDia2 depletion resulted in 37% reduction of total CENP-A levels, but unchanged levels of old CENP-A compared with control cells (Fig. 2, D–F). Thus, the formin mDia2 functions in the recruitment of new CENP-A onto centromeres in G1 cells.
The formin mDia2 is a downstream effector of the MgcRacGAP-dependent small GTPase pathway required to maintain CENP-A levels at centromeres

The mDia2 protein contains functional nuclear localization (Fig. 3 A) and nuclear export signals, and shuttles between the nucleus and the cytoplasm through importin-α/β– and CRM1-mediated nuclear transport mechanisms (Miki et al., 2009; Shao et al., 2015). Depletion of mDia2 did not change CENP-A distributions between the cytoplasm and the nucleus, though resulted in less CENP-A bound to chromatins, as expected (Figs. 3 B and S3 A). Full-length mEmerald-mDia2 proteins were accumulated in the nucleus upon treatment with leptomycin B (LMB) to block CRM1. In contrast, the K35A/R36A mutation within the nuclear localization signal (NLS) abolished nuclear accumulation of mEmerald-mDia2 upon LMB treatment (Fig. 3 C). Replacing endogenous mDia2 with this mutant resulted in significantly reduced levels of CENP-A at centromeres (Fig. 3, D and E), demonstrating that the nuclear function of mDia2 is required for its role in CENP-A maintenance.

A small GTPase switch including MgcRacGAP, a Rho family GTPase activating protein, is involved in CENP-A maintenance at centromeres (Lagana et al., 2010). The mDia2 formin proteins are autoinhibited through an intramolecular interaction between N-terminal GBD-DID domains and C-terminal DAD domain (Alberts et al., 1998). The autoinhibition is relieved upon the binding of the small GTPase at the GBD domain (Watanabe et al., 1999). The mDia2 construct lacking the regulatory domains (FH1FH2-mDia2), but not FH1FH2-mDia2 constructs with actin assembly defective point mutations (K853A, I704A, and W630A; Bartolini et al., 2008), was able to restore CENP-A levels at centromeres as well as the full-length mDia2 in cells depleted of endogenous mDia2 (Fig. 4, A and B). Although lacking the known NLS, the EGFP-fused FH1FH2-mDia2 localized to the nucleus in G1 cells (Fig. 4 C). Further, the constitutively active form of mDia2 was very efficient in restoring CENP-A levels at centromeres in MgcRacGAP-depleted cells (Fig. 4 D). This is consistent with the formin mDia2 as a downstream effector of the MgcRacGAP-dependent GTPase pathway to play a role in centromeric CENP-A maintenance.

Depletion of mDia2 results in a prolonged centromere association of HJURP

CENP-A loading at G1 centromeres requires the CENP-A histone chaperone HJURP, which also has the CENP-A nucleosome assembly activity (Barnhart et al., 2011). A subset of early G1 cells has HJURP colocalized to centromeres (Fig. 4 C). Further, the constitutively active form of mDia2 was very efficient in restoring CENP-A levels at centromeres in MgcRacGAP-depleted cells (Fig. 4 D). This is consistent with the formin mDia2 as a downstream effector of the MgcRacGAP-dependent GTPase pathway to play a role in centromeric CENP-A maintenance.
mDia2 is required for CeNP-A loading • Liu and Mao

control cells (Fig. 5 D). These results indicate a prolonged attempting of new CeNP-A nucleosome assembly by HJU RP in the absence of mDia2. Stochastic simulation demonstrated that prolonged HJU RP dwelling (i.e., reduced HJU RP turnover) not only suffices to cause a higher percentage of HJU RP positive cells as observed experimentally, but also contributes to the inability of CeNP-A accumulation on centromeres, which is in good quantitative agreement with ratiometric live cell measurement over time (Fig. S3, B–E). These results are consistent with a role of mDia2 in regulating CeNP-A levels at centromeres (Fig. 5 E).

A novel nuclear function of formin mDia2 in centromere epigenetic regulation

Centromeres are epigenetically marked by the conserved histone H3 variant CeNP-A. Each sister centromere inherits one half the number of CeNP-A molecules upon DNA duplication. To maintain centromere identity, new CeNP-A is added to double the number of CeNP-A molecules at centromeres during G1. Loading of new CeNP-A into centromeres includes the following steps (Fig. 5 E, left): (1) the assembly of licensing factors, the Mis18 complex, at anaphase onset (Hayashi et al., 2004; Fujita et al., 2007; Maddox et al., 2007), (2) a maintenance step involving a small GTPase molecular switch (Lagana et al., 2010). Here, our results reveal a critical role for the formin mDia2 in regulating epigenetic maintenance of centromere identity. Quantitative imaging and high resolution ratiometric live cell studies have demonstrated that knockdown of mDia2 results in reduced levels of CeNP-A at centromeres. In contrast, depletion of mDia2 does not affect the recruitment of H2A.Z, another histone variant in human cells (Fig. S3, F and G).

The formin mDia2 is not likely to play a role in centromere licensing or the recruitment of centromere components, as we have not been able to detect mDia2 centromere localization at any point in the cell cycle. This is further supported by the normal level of HJU RP associated with centromeres in mDia2-depleted cells. In contrast, the increased percentage of HJU RP positive cells upon mDia2 depletion indicates a role of mDia2 in regulating nucleosome assembly for new CeNP-A incorporation (Fig. 5 E, right). Expressing a constitutively active form of mDia2 is able to rescue the CeNP-A deposition defect caused by knockdown of MgcRacGAP, a phenotype that is consistent with the formin mDia2 being the downstream effector of the MgcRacGAP-dependent GTPase pathway during the maintenance step to stabilize newly incorporated CeNP-A.

The CeNP-A chaperone HJU RP at early G1 (Barnhart et al., 2011), and (3) a maintenance step involving a small GTPase molecular switch (Lagana et al., 2010). Here, our results reveal a critical role for the formin mDia2 in regulating epigenetic maintenance of centromere identity. Quantitative imaging and high resolution ratiometric live cell studies have demonstrated that knockdown of mDia2 results in reduced levels of CeNP-A at centromeres. In contrast, depletion of mDia2 does not affect the recruitment of H2A.Z, another histone variant in human cells (Fig. S3, F and G).

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Figure 4. The formin mDia2 is a downstream effector of the MgcRacGAP-dependent GTPase pathway to regulate epigenetic centromere maintenance. (A) HeLa cells 48 h after transfection of indicated siRNAs along with expression vectors were fixed and stained with DAPI (DNA), CeNP-B, and CeNP-A. Transfected cells were identified by fluorescence markers. Bar, 10 µm. (B and D) Quantifications of normalized CeNP-A integrated intensity per nucleus plotted as means ± SD overlaid with scatterplot. The p-value was computed using a two-tailed t test. Control siRNA: n = 425, mDia2 siRNA: n = 260, mDia2 siRNA + FL-mDia2: n = 136, mDia2 siRNA + WT FH1FH2-mDia2: n = 383, mDia2 siRNA + K853A-FH1FH2-mDia2: n = 151, mDia2 siRNA + I704A-FH1FH2-mDia2: n = 158, and mDia2 siRNA + W630A-FH1FH2-mDia2: n = 128. Control shRNA: n = 135, MgcRacGAP shRNA: n = 117, and MgcRacGAP shRNA + WT FH1FH2-mDia2: n = 88 from at least three independent experiments. (C) Live-cell imaging stills showing EGFP-FH1FH2-mDia2 nuclear localization during G1 phase upon anaphase onset (0 min). Bar, 10 µm.
formin mDia2 could be involved in this process by, at least, two different mechanisms: (1) assisting chromatin-remodeling for new CENP-A incorporation, as the FH2 region of mDia formins has been shown to interact with CENP-A using a yeast two-hybrid assay (Yasuda et al., 2004); and (2) altering mobility or organization of chromatin. The latter possibility will require mDia2-mediated nuclear actin activity. Recent studies have demonstrated nuclear actin network assembly mediated by formin proteins in regulating the MAL/SRF (megakaryocytic acute leukemia/serum response factor) transcription function (Baarlink et al., 2013) and in DNA damage response (Belin et al., 2015). Although it has been shown that latrunculin A or cytochalasin D treatment does not affect CENP-A levels at centromeres (Lagana et al., 2010), actin in nucleus could form short oligomers or other forms of structures that are less sensitive to drug treatment than actin polymers (Gonsior et al., 1999; Schoenenberger et al., 2005; McDonald et al., 2006; Belin et al., 2013). It will be important to understand whether actin dynamics is important for epigenetic centromere maintenance in future studies.

Materials and methods

siRNA sequences, constructs, and antibodies
The siRNAs used in this study include GAPDH (NM_002046.4, 5′-UGG UUUACAUUGUCCAAUUA-3′), DIAPH3/mDia2 (AB244756.1, 5′-CUCGGGCACAUCAGGUCAA-3′), DIAPH2/mDia3 (BC117414, 5′-CACCGTCTCAATGACATTGCA-3′), and HJURP (NM_018410.4, 5′-CUACUGGGCUACUGCAAU-3′).

The constructs used in this study include H2B-YFP, H2B-RFP (Foltzetal., 2009), FL-mEmerald-mDia2, FL-ΔNLS-mEmerald-mDia2, pEGFP-FH1FH2mDia2-WT, pEGFP-FH1FH2mDia2-K853A, pEGFP-FH1FH2mDia2-I704A, pEGFP-FH1FH2mDia2-W630A, pmCherry-C1 (catalog number 632524; Takara Bio Inc.), and MgcRacGAP MISON shRNA plasmid (NM_013277.3-2165s21c1 and NM_013277.2-456s1c1; Sigma-Aldrich). The pEGFP-FH1FH2mDia2-based constructs were gifts from F. Bartolini (Columbia University Medical Center, New York, NY). The construct of mEmerald-mDia2-C-14 was a gift from M. Davidson (National High Magnetic Field Laboratory, Tallahassee, FL; plasmid 54158; Addgene). Site-directed mutagenesis was performed to generate the K35AR36A mutant, using QuikChange Lightning following the manufacturer’s instructions.

Primary antibodies used in this study include rabbit anti-mDia2 (Watanabe et al., 2008), rabbit anti-mDia3 (LS-C19007; Lifespan), mouse anti–CENP-A (Ab13939; Abcam), mouse antitubulin (T6199; Sigma-Aldrich), rabbit anti-HJU RP (Ab100800; Abcam), and rabbit anti-H2A.Z (Ab4174; Abcam).

Cell culture, transfection, and drug treatment
HeLa cells were used for most of the quantitative imaging experiments in this study. A HeLa cell line stably expressing YFP-CENP-A was used for high-resolution ratiometric live-cell imaging. A HeLa cell line...
was performed with Hiperfect (QIA  GEN) following the manufactur-
37°C), fixed in cold MeOH at
20°C for 5 min, and then permeablized
−
on poly-d-lysine–coated coverslips were washed in PBS (prewarmed at
For indirect immunofluorescence and fixed-cell imaging, cells grown
Quantitative fixed-cell imaging and data analysis using the INCA
CENP-A images within the nuclear masks were eventually measured
cost proteins. Control cells were transfected with GAPDH siRNA. All
knockdowns were confirmed by immunoblotting analysis. Cells were
fixed following a slightly different protocol to preserve YFP's fluores-
identically across groups. Cells stably expressing YFP-CENP-A were
exposure time. Representative images presented in figures are scaled
with appropriate controls were collected on the same day with identical
measurements were performed using the INCA method
ana et al., 2010). Imaging experiment with or without cotransfection
markers were both performed and analyzed using the INCA method
with the same conclusions.

High-resolution ratiometric live-cell imaging
HeLa cells stably expressing YFP-CENP-A (Foltz et al., 2009) were
plated onto poly-o-lysine–coated 35-mm glass-bottom dishes (MatTek
Corporation) and maintained in CO2 independent medium supple-
mented with 4 mM L-glutamine and 10% FBS, with an environmen-
tally controlled chamber at 37°C during imaging. Images were acquired
every 20 min for a total duration of ~13 h, with 11 z-sections span-
ing 10 µm (1 µm apart each optical section) being acquired at each
time point. Exposure time was kept constant throughout the duration
of live imaging (200 ms for YFP after being optimized against photo-
bleaching). All live imaging was performed using a motorized inverted
microscope (IX81; Olympus) with a 60x NA 1.42 Plan Apochromat
oil-immersion objective (Olympus) and a interline transfer cooled
dergative exit and during G1. Only cells staying in focus were subjected
to measurement (mCherry-C1 was used as a cotransfection marker for
identifying siRNA transfected cells). Measurements were performed
on maximum z-projections with all 16-bit depth preserved. Ratiometric
analysis was performed by normalizing the time course of each cell’s
centromeric CENP-A intensities with a “reference frame,” which rep-
resents the averaged centromeric CENP-A level for that particular cell
before CENP-A loading occurs. Because of resolution constraints (i.e.,
more than one centromeres are inevitably overlapped during anaphase/
telophase), to ensure it is the single centromere’s intensity that was
measured, the reference frame (against which the “loading curve” was
normalized) was selected to be late prometaphase or early metaphase,
where a single sister centromere can be identified and measured to ob-
tain an internal reference intensity before any new CENP-A loading
has occurred. During the course of telophase/G1 phase, only well-
separated single centromeres, whose intensity distributions are circu-
ar-symmetric on z-projection images, were randomly selected and
measured using ImageJ (National Institutes of Health). Because pixel
intensities on each centromere approximately follow 2D-Gaussian
distribution, local maxima were considered as a reasonable measure-
ment of CENP-A levels per centromere. The measurements were fol-
lowed by normalization: each data point throughout the time course
was normalized against the host cell’s mean measurement in the
“reference frame.” After normalization, data were plotted as mean ±
SD. For visual comparison, representative maximum z-projection im-
age were demonstrated, with linear intensity transformation function
being applied (Inoué, 1986) and identical dynamic range/LUT being
used for all images of each cell throughout the time course.
Regarding photobleaching, our protocol for long term live cell imaging has been optimized such that only minimal photobleach occurred by 10 h after imaging started (Fig. S2, D–F; and Video 3).

**SNAP-tag pulse chase assay**

The SNAP-tag pulse chase assay was performed based on published protocols (Jansen et al., 2007; Lagana et al., 2010). Essentially, HeLa cells stably expressing CENP-A-SNAP-3×HA were synchronized using double thymidine block (2 mM thymidine, 17 h each) with 9-h release in between (in medium supplemented with 24 µM deoxyctidine). During the second round of thymidine arrest, siRNAs were transfected to allow for ~48-h knockdown time before fixation whereas only one round of CENP-A deposition during the immediate next G1 was affected. Upon releasing from the second round of thymidine block, cells were pulse-labeled with TMR-Star, a fluorescent SNAP substrate (3 µM), for 15 min, followed with complete washes and block. Cells were fixed and stained for total CENP-A (anti-HA tag). Quantitative imaging and image processing were performed using the INCA method.

**Cell fractionation**

Cell fractionation analysis was performed using an adapted protocol based on established methods (Szentirmay and Sawadogo, 2000; Baarlink et al., 2013; Bodor et al., 2014). In brief, cells (5 × 10⁶) were harvested and washed with cold PBS. Cell pellet was resuspended in 500 µl 1× hypotonic buffer (20 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 1 mM PMSF, and 1× protease inhibitor cocktail from Roche) by pipetting several times, followed by 15-min incubation on ice to allow for swelling. Then, 25 µl of 40% NP-40 was added into the system before vortexing for 10 s. The homogenate was then centrifuged (3,000 rpm for 10 min at 4°C) to separate the cytoplasmic fraction (supernatant) from the nuclear pellet. Nuclear pellet was rewarshed with hypotonic buffer (without NP-40) and centrifuged and was then resuspended in 50 µl of relatively low-salt extraction buffer (10 mM Tris, pH 7.4, 2 mM Na₃VO₄, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 1 mM EGTA, 0.1% SDS, 1 mM NaF, 0.5% deoxycholate, 20 mM Na₃P₂O₇, 1 mM PMSF, and 1× protease inhibitor cocktail) and incubated on ice for 30 min, with vortexing every 10 min. The mixture was then centrifuged for 30 min at 14,000 g at 4°C. Supernatant is nucleoplasm and pellet is chromatin-associated materials. For immunoblotting, tubulin was used as the cytoplasmic marker (Baarlink et al., 2013), RNA polymerase II as the chromatin-associated materials. For immunoblotting, tubulin was used as the cytoplasmic marker (Baarlink et al., 2013), RNA polymerase II as the chromatin-associated materials. For immunoblotting, tubulin was used as the cytoplasmic marker (Baarlink et al., 2013), RNA polymerase II as the chromatin-associated materials. For immunoblotting, tubulin was used as the cytoplasmic marker (Baarlink et al., 2013), RNA polymerase II as the chromatin-associated materials.

**Immunoblotting analysis**

Immunoblotting was performed as previously described (Liu et al., 2015). In brief, cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, and 0.5% Na-deoxycholate acid) and then denatured using SDS sample buffer. Cell lysates were subsequently subjected to 10% SDS-PAGE followed by membrane transfer (Immobilon-P and Towbin transfer buffer, pH 8.3; EMD Millipore). Immunoblots on the membrane were blocked with 5% nonfat milk dissolved in Tris-buffered saline with Tween (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween) and then probed with primary antibodies diluted in Tris-buffered saline with Tween. Primary antibodies were visualized using Alexa Fluor 680–conjugated secondary antibodies (Thermo Fisher Scientific) together with the LI-COR imaging system (LI-COR Biosciences).

**Cell cycle analysis**

Control HeLa cells or mDia2 knockdown cells (confluent) were trypanotized from six-well plates, fixed with MeOH (~20°C), and stained with DAPI. The BD LSRII Cell Analyzer was used for FACS experiments. The FlowJo was used for FACS data analysis with Gaussian fitting based on univariate cell cycle model (Watson et al., 1987).

**Statistical analysis and plotting**

All statistical analyses were performed with GraphPad Prism 5 (GraphPad Software) using an unpaired, two-tailed t test between groups unless noted otherwise (e.g., z-test with MATLAB to compare two sample proportions). All plots were prepared in MATLAB (R2013a; MathWorks), Prism (GraphPad Software), Origin 8.6 (OriginLab), or Excel (Microsoft). Control groups and mDia2-depleted groups in all experiments were pooled together after normalization and presented.

**Stochastic simulation of HJU RP turnover at centromeres**

To test if extended dwelling time of HJU RP molecules on centromere can contribute to the change in the observed percentage of HJU RP–positive cells, we applied the Gillespie next reaction algorithm to numerically simulate the stochastic association/dissociation events of HJU RP molecules on the centromere (Gillespie, 1977). Parameteric choices were based on reported numbers (e.g., Table S1) or realistic assumptions when no parameters are available (numbers of docking site per centromere). In brief, a realistic number of docking sites (30, which is smaller than the total number of CENP-A nucleosomes per centromere) were assigned for each single centromere and there is no HJU RP associated on any docking site at the beginning. Each round of simulation starts with generating a series of random numbers (random probability [P_rand]) for each specific step (t_i) and compare the P_rand(t_i) with the actual probability of either association (P_assoc) or a dissociation event (P_dissoc) given the current docking site status is either unoccupied or occupied, respectively, at time t_i. P_assoc and P_dissoc are calculated using equations P_assoc = 1 − e^(-kon*t_i) and P_dissoc = 1 − e^(-koff*t_i), where k_off (dissociation rate constant, min⁻¹) and k_on (pseudoassociation rate constant, min⁻¹) are derived from parameters listed in Table S1.

If P_assoc(t_i) > P_rand(t_i), it suggests that compared with random probability, the association event is more likely to happen given an unoccupied docking site. One molecule of HJU RP will jump on the docking site. If P_dissoc(t_i) > P_rand(t_i), it suggests that compared with random probability, the dissociation event is more likely to happen given an occupied docking site. One molecule of HJU RP will jump off and leave the docking site available for the next round of possible association event.

In the case of incomplete or failed incorporation, the increased level of CENP-A as a result of HJU RP association will be removed after HJU RP dissociation, giving rise to unsuccessful association and the inability of new CENP-A nucleosomes to build up. Meanwhile, k_off is lowered by half compared with ordinary conditions to manifest on altered HJU RP dwelling time and thus turnover rate (Fig. S3 A).

Extended dwelling time shouldn’t affect the number of dwelling events per docking site per centromere. To estimate the total numbers of time steps in case of lowered k_off and failed incorporation to get similar numbers of dwelling events, we plotted the number of dwelling event per docking site with empirical increment of time steps. It turns out that 3,500 time steps under this circumstance is the minimum requirement to achieve similar numbers of total dwelling event per docking site (P = 0.0923). Total dwelling time per docking site therefore has the mean value of 333.384 min as compared with 185.06 min under ordinary conditions. Δt is therefore 2.4721 h longer under the condition of “failed incorporation + lower k_off.”

Next, to assess the influence of temporal changes on the percentage of observable HJU RP–positive cells, we initiated another matrix to simulate thousands of cells’ collective behavior. It is assumed that a cell shares the same temporal property of the docking site regarding HJU RP’s presence on its centromeres. Instructed by experimental observations
and practical experiences (Table S1), here we assumed 50% cells are synchronized around the G1/S boundary upon being released from single round of thymidine arrest. The exact position of each cell’s time line at the start of simulation (upon thymidine release) is stochastically distributed within a normal time window ($\mu = 0$ and $SD = 2$). As a reference, a cell starts right at G1/S boundary (0 h) will proceed for 9 h to start having HJU RP on its centromeres. After 3 h for this cell to be an HJURP-positive cell, HJURP will stop being associated with centromeres, therefore making 12 h the last time point for it to be an HJURP-positive cell (Fig. S3 C). “Green point” and “red point” will be used hereafter to name the start and end of HJURP association, respectively. Despite the intrinsic uncertainties associated with cell synchronization, all cells are fixed at 11 h after thymidine release for imaging (invariant observational point). Consequently, all cells with their red point before the observational point, and all cells with their green point after the observational point, will not be documented as HJURP-positive cells. The percentage of HJURP-positive cells can then be calculated based on these criteria (Fig. S3 D).

To test if the number of HJURP molecules per centromere is altered at any given time during the period of being an HJURP-positive cell, 10 random position inside the HJURP-positive time window were chosen (per simulation) to count how many docking site are occupied at that particular time point. The number of occupied docking sites on that centromere reflects the number of HJURP molecules per centromere at that time. This process is repeated three times for plotting the simulated HJURP level per centromere (Fig. S3 D).

Finally, to visualize the time-dependent “loading” of CENP-A nucleosomes on the centromere, results from stochastic simulations were summed up over time to create loading curves of the accumulated number of CENP-A nucleosomes per centromere. Ordinary conditions with “failed incorporation + lower $k_{on}$” were processed, respectively, and plotted to compare with experimental measurements (Fig. S3 E).

Online supplemental material

Fig. S1 shows the INCA measurement method developed in this study. Fig. S2 shows the method used for quantifying high-resolution ratiometric live cell imaging data and details of nonlinear regression. Fig. S3 shows the stochastic simulation of HJU RP turnover at G1 centromeres. Video 1 shows live-cell imaging movies of a control cell and an mDia2 knockdown cell stably expressing YFP-CENP-A undergoing G1 phase. Video 2 shows the ratiometric live-cell imaging of YFP-CENP-A signals in a control cell and an mDia2 knockdown cell. Video 3 shows a representative cell expressing YFP-CENP-A arrested through G1 phase. Video 2 shows the ratiometric live-cell imaging data and details of nonlinear regression.

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