Report

The Histone-Fold Protein CHRAC14 Influences Chromatin Composition in Response to DNA Damage

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SUMMARY

Chromatin reorganization and the incorporation of specific histone modifications during DNA damage response are essential steps for the successful repair of any DNA lesion. Here, we show that the histone-fold protein CHRAC14 plays an essential role in response to DNA damage in *Drosophila*. *Chrac14* mutants are hypersensitive to genotoxic stress and do not activate the G2/M cell-cycle checkpoint after damage induction. Even though the DNA damage repair process is activated in the absence of CHRAC14, lesions are not repaired efficiently. In the absence of CHRAC14, the centromere-specific histone H3 variant CENP-A localizes to sites of DNA damage, causing ectopic kinetochore formation and genome instability. CENP-A and CHRAC14 are able to interact upon damage. Our data suggest that CHRAC14 modulates chromatin composition in response to DNA damage, which is required for efficient DNA damage repair in *Drosophila*.

INTRODUCTION

Chromatin is a highly dynamic structure that is actively remodeled during intrinsic changes such as cell cycle or developmental progression and responds to external cues, such as DNA damage. In response to DNA damage, chromatin-remodeling factors regulate the access of repair proteins to DNA, participate in checkpoint signaling, and contribute to the actual DNA repair process (Kusch et al., 2004; Osley et al., 2007; Soria et al., 2012). For instance, the noncatalytic subunit of the nucleosome remodeling complex *chr*omatin *a*ccessibility *c*omplex (CHRAC), ACF1, is required for DNA double-stranded break (DSB) repair in human cells and regulates the G2-M checkpoint (Lan et al., 2010; Sánchez-Molina et al., 2011). Another subunit of CHRAC is the histone-fold protein CHRAC14 that has subsequently been found associated with other multisubunit protein complexes involved in chromatin remodeling and regulation of DNA polymerase activity (Corona et al., 2000; Li et al., 2000; Poot et al., 2000; Suganuma et al., 2008). Within these complexes,

CHRAC14 typically forms homo- or heterodimers with other histone-fold proteins (Eberharter et al., 2001; Hartlepp et al., 2005; Ito et al., 1997; Kukimoto et al., 2004; Varga-Weisz et al., 1997).
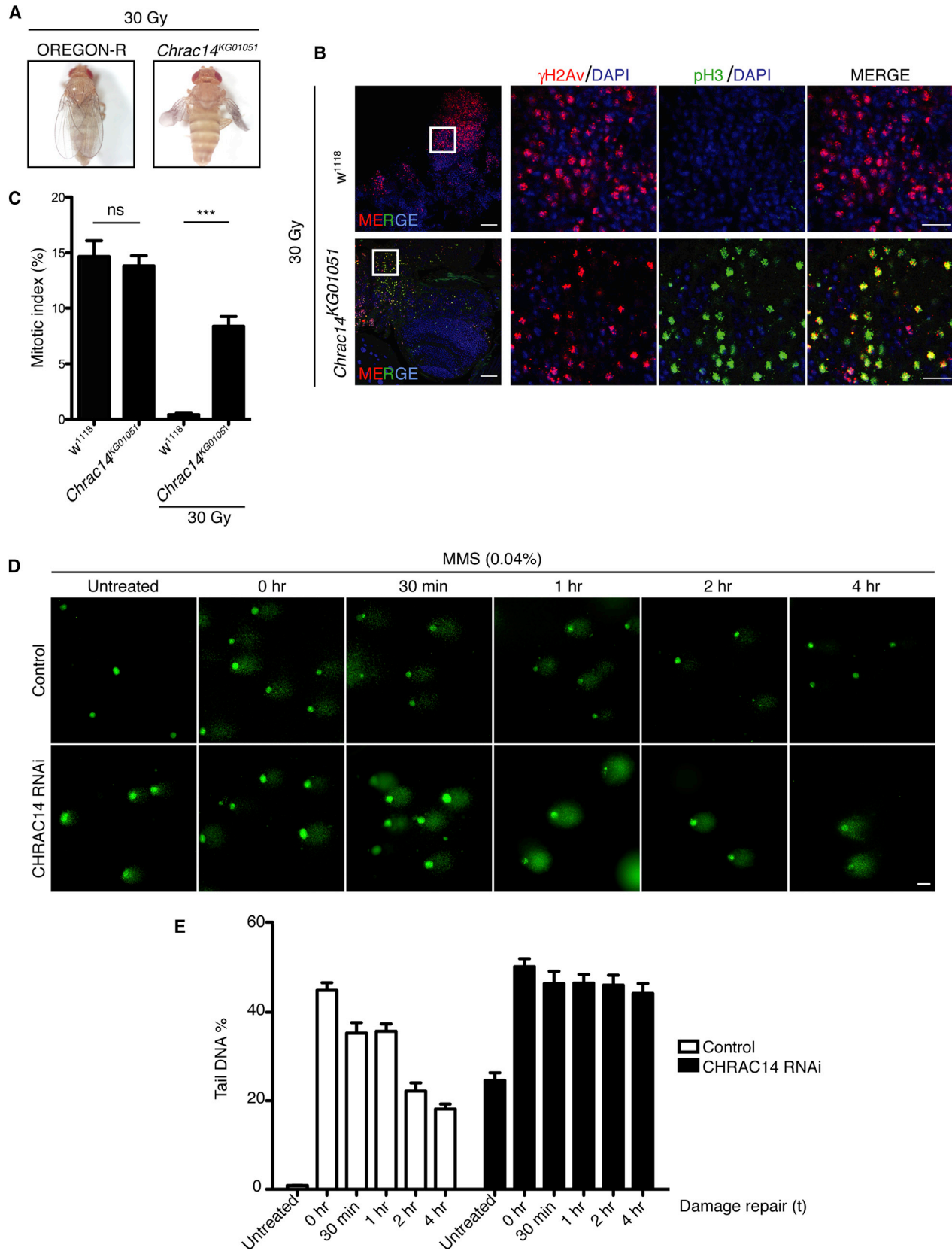
Chromosome segregation requires specialized centromeric chromatin structures that are characterized by the presence of the histone H3 variant CENP-A (also known as CID in *Drosophila melanogaster*) (Allshire and Karpen, 2008). The formation and maintenance of centromeric chromatin is essential for faithful attachment of spindle microtubules to kinetochores, and loss of centromere identity leads to aneuploidy and cancer (Kops et al., 2005). Tight regulation of CENP-A is necessary for chromosome segregation as both overexpression and depletion of CENP-A are deleterious for cells and organisms (Stellfox et al., 2013).

Here, we identify CHRAC14 as an essential component of the *Drosophila* DNA damage response. *Chrac14* mutant flies are viable and fertile but display developmental defects when treated with doses of DNA-damaging agents that are tolerated by wild-type animals. Even though DNA repair processes are initiated, the lesions remain unrepaired for an extended period of time. We further found that CHRAC14 and CENP-A interact and that, in the absence of CHRAC14, CENP-A localizes to sites of damage, causing severe mitotic defects. We therefore hypothesize that CHRAC14 prevents inappropriate CENP-A incorporation at sites of damaged chromatin. Our data show that CHRAC14 plays a hitherto unappreciated role in DNA repair at times when the replication-independent assembly of histone variants prevails.

RESULTS AND DISCUSSION

Chrac14 Mutant Flies Are Defective in Response of DNA Damage

Misexpression of CENP-A leads to chromosome segregation defects and aneuploidy (Blower et al., 2006; Heun et al., 2006). However, the mechanisms of CENP-A regulation are still only partially understood. We used a genetic screen to identify factors that modulate overexpressed CENP-A misincorporation into chromatin and identified CHRAC14 as one potential candidate (Figure S1A). CHRAC14 is a subunit of the ATP-dependent CHRAC complex (Corona et al., 2000; Poot et al., 2000), and

A

30 Gy

OREGON-R   Chrac14^{KG01051}

B

30 Gy

w^{1118}

Chrac14^{KG01051}

γH2Av/DAPI   pH3/DAPI   MERGE

MERGE

MERGE

C

Mitotic index (%)

ns   ***

w^{1118} Chrac14^{KG01051} w^{1118} Chrac14^{KG01051}

30 Gy

D

MMS (0.04%)

Untreated   0 hr   30 min   1 hr   2 hr   4 hr

Control

CHRAC14 RNAi

E

Tail DNA %

Untreated 0 hr 30 min 1 hr 2 hr 4 hr Untreated 0 hr 30 min 1 hr 2 hr 4 hr

□ Control
■ CHRAC14 RNAi

Damage repair (t)

(legend on next page)

the large subunit of the complex, ACF1, has been implicated in G2-M checkpoint activation and repair in human (Lan et al., 2010; Sánchez-Molina et al., 2011). *Chrac14* mutant flies are viable, fertile, and do not show any obvious developmental defects but have not been tested for defects in DNA damage repair. To analyze a potential function in DNA damage repair, we exposed third instar larvae from control and *Chrac14* mutants to γ irradiation. After irradiation, wild-type flies (Oregon-R) hatched with no obvious defects except a slight rough eye phenotype in some flies, whereas *Chrac14* mutant flies were barely able to hatch, and virtually all flies displayed major developmental defects, including wing deformation and tumor formations (Figure 1A). Moreover, most irradiated *Chrac14* mutant flies were unable to balance their bodies or fly and died within 24 hr of irradiation treatment (data not shown). This indicates that *Chrac14* mutants are hypersensitive to DNA-damaging agents.

To analyze potential defects in DNA damage response, we dissected brains from irradiated control and *Chrac14* mutant larvae and used immunofluorescence (IF) for phosphorylated H2A.X (γH2Av in flies), a well-described modification at sites of DNA damage (Rogakou et al., 1998; Madigan et al., 2002). Mitotic cells were marked with serine 10-phosphorylated histone H3 (pH3) -specific antibodies. Upon damage, control flies showed drastically reduced numbers of mitotic cells due to G2-M checkpoint activation, from about 15% in untreated cells to below 1% in irradiated cells. In contrast, about 8% of pH3-positive cells were still present in *Chrac14*[KG01051] flies after damage (Figures 1B and 1C), indicating a failure to arrest damaged cells before entering mitosis. Additionally, anaphase figures in irradiated *Chrac14* mutant tissues suggested that cells do not arrest in G2/M but actually proceed through mitosis and therefore do not have a functional G2-M checkpoint (Figure S1B). This conclusion was substantiated by fluorescence-activated cell sorting analysis of irradiated larval tissue. Whereas control cells displayed a G2-M arrest, *Chrac14* mutant cells showed an almost normal cell-cycle profile with only slightly higher G2-M counts and a normal number of cells in S phase (Figure S1C) after irradiation. These checkpoint defects of *Chrac14* mutants were independent of the altered CENP-A overexpression phenotype that we observed genetically because overexpression of CENP-A does not interfere with the G2-M checkpoint after DNA damage (Figure S1D). Our data thus reveal a function of CHRAC14 in the DNA damage response. In contrast to human ACF1 (Sánchez-Molina et al., 2011), *Drosophila Acf1* mutant flies maintained a functional G2-M

checkpoint (Figure S1E), suggesting a function of CHRAC14 in G2-M checkpoint activation independent of the CHRAC complex that may involve alternative interacting partners of CHRAC14 (Iida and Araki, 2004; Suganuma et al., 2008).

To visualize potentially altered DNA damage response or repair kinetics, we performed a comet assay on control or CHRAC14-depleted S2 cells that had been treated with methylmethane sulfonate (MMS; 0.04%), an effective DNA-damaging agent (Tercero and Diffley, 2001). Untreated control cells did not have any significant comet tails. After MMS treatment, a large tail of DNA fragments was visible that was mostly repaired within 2 hr (Figures 1D and 1E). Untreated CHRAC14-depleted cells already displayed a significant comet, indicating that CHRAC14 depletion alone either causes DNA damage or normal levels of damage are not repaired efficiently in the absence of CHRAC14 (Figures 1D and 1E). When we treated CHRAC14-depleted cells with MMS, we did not only observe a larger comet indicating more breaks but also found that the repair process was very ineffective, never reaching completion. S phase, however, was equally effected by MMS in controls and CHRAC14-depleted cells (Schwartz, 1989) (Figure S1F). We conclude that cells lacking CHRAC14 recognize DNA damage, but their response does not suffice to significantly repair damaged DNA.

CHRAC14 Depletion Causes Ectopic CENP-A Localization and Chromosome Segregation Defects

To gain insight into the relationship between CHRAC14 and CENP-A, we analyzed the effects of CHRAC14 depletion or mutation on endogenous CENP-A in S2 cells and embryos. Depletion of CHRAC14 in S2 cells led to a significant increase in CENP-A spots in the nucleus (Figures 2A and 2B). Likewise, endogenous CENP-A protein levels increased by about 1.8-fold in CHRAC14 knockdown cells compared to control cells (Figure 2C). Furthermore, inner and outer kinetochore proteins colocalized to virtually all CENP-A spots, indicating that ectopic CENP-A caused kinetochore assembly with inner (CENP-C) and outer (Spc105) components (Figure 2D). We excluded that aberrant genome duplication or an increase in the DNA content caused this increase of CENP-A foci by analyzing the nuclear size and cell-cycle profiles of CHRAC14-depleted cells (Figures S2A and S2B). We extended our analysis to embryo and assessed endogenous CENP-A in control (*w*[1118]) and *Chrac14* mutant (*Chrac14*[KG01051]) embryos. Increased numbers of CENP-A spots and changes in chromatin composition were

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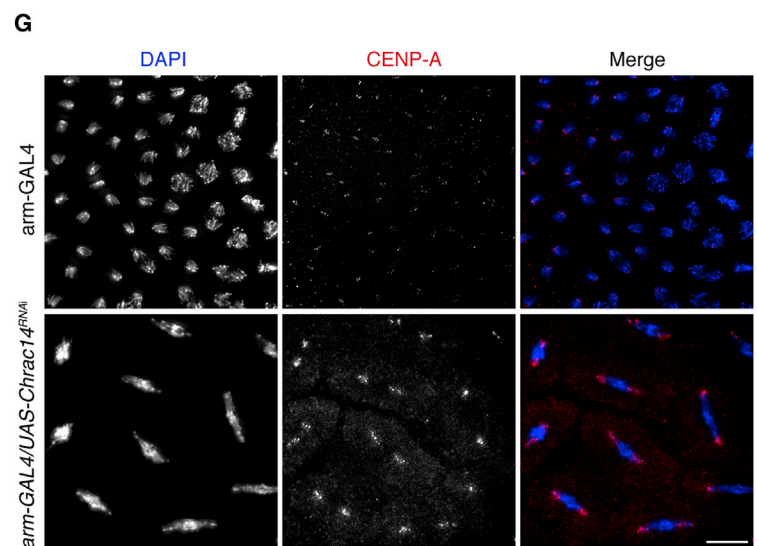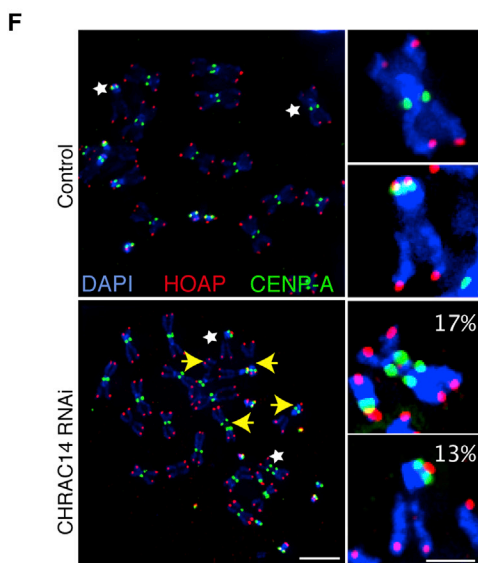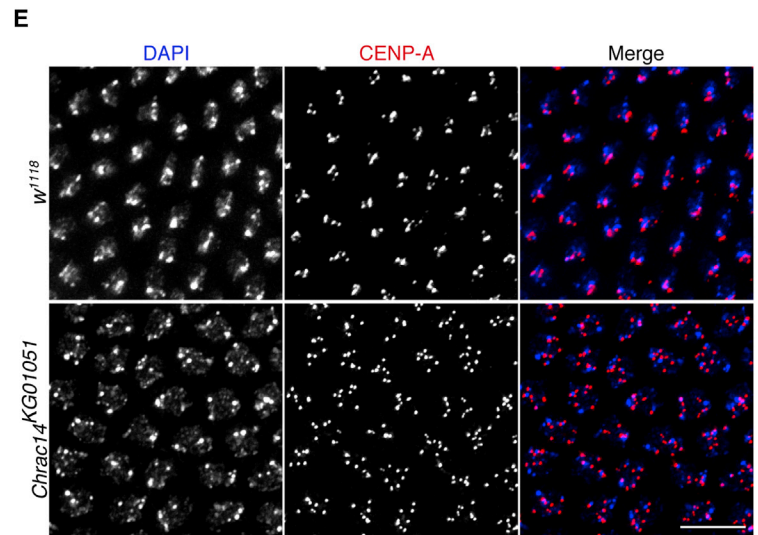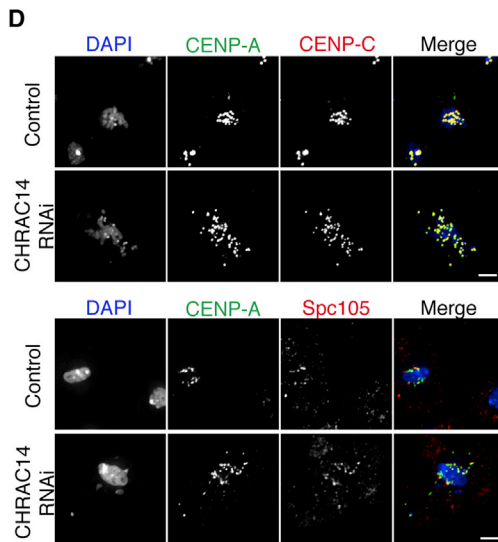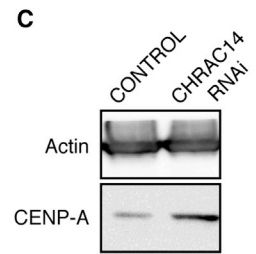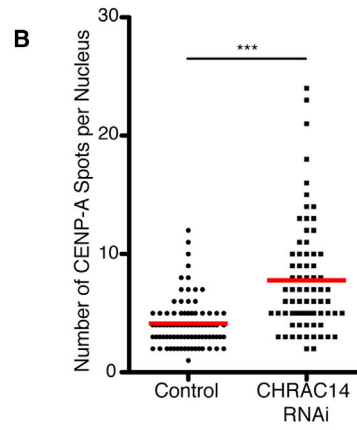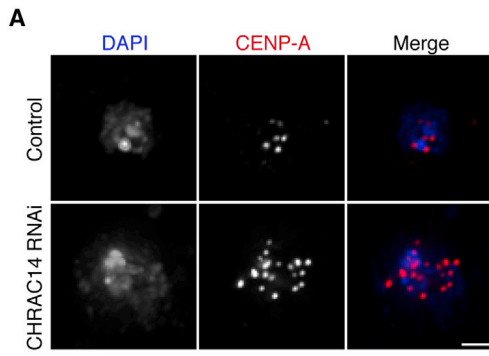**Figure 1. *Chrac14* Mutant Flies Are Defective in Response of DNA Damage**

(A) *Chrac14* mutant and control third instar larvae were treated with γ irradiation (30 Gy). After hatching, irradiated *Chrac14* mutant flies displayed massive developmental defects, whereas irradiated control flies (Oregon-R) did not show any developmental defects.

(B) *Chrac14* mutant embryos lack a functional G2-M checkpoint. Images of whole third instar larval brain of control (*w*[1118]) and *Chrac14* mutant flies after γ irradiation treatment (30 Gy) stained for γH2Av (red) to monitor DNA damage, pH3 (green) to mark mitotic cells, and DAPI (blue). The scale bar represents 40 μm. The inlets on the right show a close up of cells marked in the white square. The scale bar represents 20 μm.

(C) The mitotic index (%) of cells in control (*w*[1118]) and *Chrac14* mutant larval brains untreated and following irradiation is shown from seven brains ± SEM with at least 1,000 cells analyzed for each brain. ns, not significant.

(D and E) Alkaline comet assay. CHRAC14-depleted cells and control cells were treated with MMS (0.04%), and the recovery time of the induced DNA breaks were monitored by the tail length and quantified by measuring the percentage of DNA within the comet tail (tail DNA %). Representative comet figures for without MMS treatment and immediately (0 hr) and 30 min–4 hr after treatment of control and CHRAC14 RNAi cells are shown. The scale bar represents 20 μm. The tail DNA % from untreated cells and cells up to 4 hr after treatment was measured, and the mean values of three independent experiments with SEs are shown in the graph (n = 100 cells per condition).

See also Figure S1.

A

DAPI | CENP-A | Merge

Control

CHRAC14 RNAi

B

Number of CENP-A Spots per Nucleus

***

Control | CHRAC14 RNAi

C

CONTROL | CHRAC14 RNAi

Actin

CENP-A

D

DAPI | CENP-A | CENP-C | Merge

Control

CHRAC14 RNAi

DAPI | CENP-A | Spc105 | Merge

Control

CHRAC14 RNAi

E

DAPI | CENP-A | Merge

w^1118

Chrac14^KG01051

F

Control

CHRAC14 RNAi

DAPI HOAP CENP-A

17%

13%

G

DAPI | CENP-A | Merge

arm-GAL4

arm-GAL4/UAS-Chrac14^RNAi

(legend on next page)

evident in *Chrac14* mutant embryos (Figure 2E), recapitulating our observations in cultured cells.

Next, we prepared mitotic chromosome spreads and performed IF using α-CENP-A and α-HOAP antibodies, the latter marking telomeres (Cenci et al., 2003). Whereas control cells exhibited properly condensed chromosomes with two bright CENP-A spots, one on each centromeric region (Figure 2F, upper panel, see zoom), spreads from CHRAC14-depleted cells displayed more than two CENP-A spots (usually four) forming dicentric chromosomes (17%) or multiple CENP-A spots along chromosome arms (Figures 2F, lower panel, see zoom, and S2C). This prominent phenotype of dicentric chromosomes was only observed on a subset of chromosomes after CHRAC14 RNAi treatment, usually about one or two of any of the chromosomes per mitotic spread, but never in control cells. In addition, 13% of CHRAC14-depleted cells also displayed chromosome breaks (Figure 2F). Interestingly, however, chromosomes or chromosome fragments did not fuse because α-HOAP staining was only observed at chromosomes ends. These chromosome aberrations may consequently lead to defects during mitosis. Indeed, especially late blastoderm embryos depleted of CHRAC14 displayed mitotic defects, including anaphase bridges and lagging chromosomes (Figures 2G, S2D, and S2E). We conclude that CHRAC14 depletion causes endogenous CENP-A to localize to noncentromeric sites forming ectopic kinetochores that can cause chromosome segregation errors.

CHRAC14 Influences CENP-A Localization in Response to DNA Damage

Studies in *Xenopus* and mammalian cells showed that CENP-A is recruited to sites of DNA damage (Zeitlin et al., 2005, 2009). Additionally, vertebrate-specific histone-fold proteins that localize to kinetochores are also found at sites of DNA damage in U2OS cells (Helfricht et al., 2013). We did not observe CENP-A recruitment to sites of DNA damage caused by lasers, γ irradiation, or treatment with DNA break-inducing drugs in flies (data not shown). However, because we saw a clear role of CHRAC14 in DNA damage responses and ectopic localization of CENP-A in CHRAC14-deficient cells, we hypothesized that CHRAC14 may function by regulating the histone complement at DNA damage sites, for instance, by retaining or removing his-

tone variants like CENP-A and γH2Av. We therefore measured γH2Av and CENP-A protein levels in CHRAC14-depleted cells. Both γH2Av and CENP-A protein levels increased upon depletion of CHRAC14 in S2 cells (Figure 3A). Misincorporated CENP-A is proteolytically removed from chromatin to prevent ectopic centromere formation (Moreno-Moreno et al., 2006, 2011). Inhibition of the proteasome by MG132 led to an increase of CENP-A in S2 cells independent of the presence of CHRAC14 (Figure S3A). This indicates that a defect in CENP-A protein degradation is unlikely to be the cause of ectopic CENP-A incorporation in the absence of CHRAC14. When we treated cells stably expressing GFP-CHRAC14 with the DNA break-inducing drug bleomycin (Burger et al., 1982), we saw an expected increase in γH2Av levels. Interestingly, the levels of GFP-CHRAC14 also increased upon bleomycin treatment (Figure 3B). Conceivably, these increased CHRAC14 levels may be due to protein stabilization, a notion to be addressed in the future.

We next tested whether CENP-A is recruited to sites of DNA damage in CHRAC14-depleted cells after treatment with bleomycin (1 μg/ml) or MMS (0.04%) (Tercero and Diffley, 2001). CHRAC14-depleted cells showed elevated numbers of γH2Av and CENP-A foci, but bleomycin or MMS treatment led to a further increase (Figures 3C, 3D, S3B, and S3C). By quantitative colocalization analysis (Mander's coefficient) (Bolte and Cordelières, 2006) of CENP-A and γH2Av, we found a statistically significant increase of CENP-A spots in close proximity to γH2Av foci in drug-treated CHRAC14-depleted cells (Figure 3E). This colocalization is not attributable to the increased number of CENP-A and γH2Av spots, which is accounted for in our statistical analysis. We conclude that, in *Drosophila*, DNA damage induction alone is not sufficient to recruit detectable amounts of CENP-A to sites of damage. However, DNA damage coupled with the loss of CHRAC14 leads to detectable amounts of CENP-A nearby DNA lesions. Evidently, CHRAC14 may prevent CENP-A localization at DNA lesions.

CENP-A and CHRAC14 are both histone-fold proteins, suggesting the possibility of a direct interaction. Indeed, both recombinant proteins can interact in a glutathione S-transferase (GST) pull-down assay (Figure 3F). The genetic interaction and mislocalization of CENP-A in CHRAC14-depleted cells may therefore be caused by a lack of a direct interacting partner of

Figure 2. CHRAC14 Affects Endogenous CENP-A Distribution and Causes Mitotic Defects

(A) CHRAC14 depletion increases endogenous CENP-A levels. Representative images of S2 cells treated with CHRAC14 RNAi (lower panel) and control (upper panel) stained for CENP-A (red) and DAPI (blue). The scale bar represents 5 μm.

(B) Single-cell quantification of number of CENP-A spots per nucleus for cells depicted in (A). Shown are the mean values from three independent experiments ± SD with at least 100 cells each. The increase in number of CENP-A spots in CHRAC14-depleted cells is significant (p < 0.0001 t test).

(C) Western blot analysis of CENP-A protein amount in cells depleted of CHRAC14, normalized to endogenous CENP-A levels in untreated cells. Actin serves as loading control.

(D) CHRAC14 depletion causes ectopic kinetochore formation. IF of CHRAC14-depleted and control cells stained for CENP-A (green) and the inner kinetochore protein CENP-C (red, upper panel) or the outer kinetochore protein Spc105 (red, lower panel), DAPI in blue. The scale bar represents 2 μm.

(E) *Chrac14* mutant embryos show an elevated CENP-A level. IF of wild-type (*w*[1118]) and *Chrac14*[KG01051] embryos stained for CENP-A (red) and DAPI (blue). The scale bar represents 10 μm.

(F) CHRAC14 depletion causes chromosomal abnormalities. IF on mitotic chromosomes from control (upper panel) and CHRAC14 RNAi-treated (lower panel) cells stained for CENP-A (green) and HOAP (red). The scale bar represents 5 μm. Inlets on the right show zooms of chromosomes marked with white asterisks and the percentage of chromosomes in CHRAC14-depleted cells with dicentrics and breaks (n = 68).

(G) CHRAC14-depleted embryos show mitotic defects. Arm-Gal4: dicer-UAS induced CHRAC14 RNAi (lower panel) and control arm-GAL4 flies (control; upper panel) stained for CENP-A (red) and DAPI (blue). The scale bar represents 10 μm.

See also Figure S1 and S2.
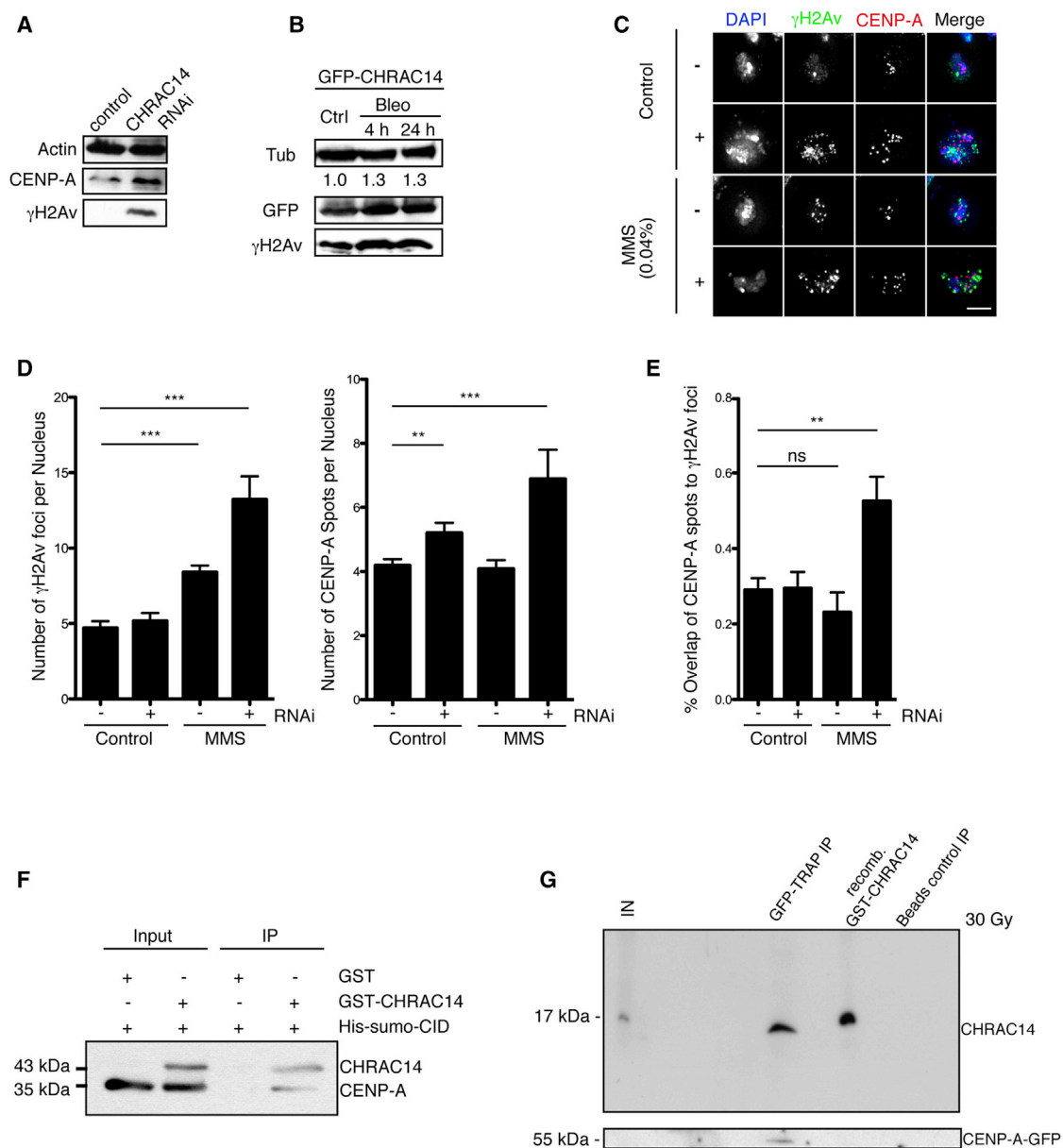
Figure 3. CHRAC14 Influences CENP-A Localization upon DNA Damage Induction

(A) Western blot analysis of control and CHRAC14 RNAi-treated cells. The blot was probed for CENP-A, γH2Av, and actin (loading control).

(B) GFP-CHRAC14 levels increase upon DNA damage induction (numbers are GFP signal intensities where control intensities were normalized to one). Control (untreated) and bleomycin-treated GFP-CHRAC14 cells were probed for GFP (GFP-CHRAC14 protein levels), γH2Av antibody (DNA damage response), and tubulin (loading control).

(C) Localization of γH2Av and CENP-A in CHRAC14-depleted cells with and without damage induction. IF images of S2 cells after treatment with the DNA-damage-inducing drug MMS. The cells were stained for CENP-A (red), γH2Av (green), and DAPI. The scale bar represents 5 μm.

(D) Single-cell quantification of the number of γH2Av foci (left graph) and the number of CENP-A spots (right graph) in the nucleus of cells as depicted in (A) from three independent experiments ± SD with at least 100 cells analyzed for each condition.

(E) The combination of CHRAC14 RNAi and MMS treatment causes a significant overlap of CENP-A and γH2Av foci. Single-cell quantification of percentage overlap of CENP-A spots with γH2Av foci in DNA-damage-induced cells by MMS, CHRAC14 RNAi cells, and the combination as indicated and compared to untreated control cells. Depicted are the mean values from three independent experiments ± SD with at least 25 cells per condition each. The percentage overlap (Mander's colocalization coefficient) of CENP-A spots to γH2Av foci was found significantly increased in CHRAC14-depleted cells treated with MMS ($p < 0.005$).

(F) In vitro interaction of CHRAC14 and CENP-A. GST-CHRAC14 fusion protein or control GST was bound to glutathione-sepharose and incubated with purified His-sumo-CENP-A. Bound proteins were resolved by SDS-PAGE and immunoblotted with GST and CENP-A antibodies. Input denotes the starting material. IP, immunoprecipitation.

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CENP-A. We prepared protein extracts from CENP-A-GFP embryos and detected an interaction of CHRAC14 and CENP-A in irradiated CENP-A-GFP embryos (Figures 3G and S3D). This suggests that the interaction of CHRAC14 and CENP-A occurs when DNA damage repair pathways are activated. The molecular context for this previously uncharacterized function of CHRAC14 is unknown. Among the candidate complexes are the DNA polymerase epsilon machinery or ATAC, an acetyltransferase complex (Iida and Araki, 2004; Suganuma et al., 2008). In support of this notion, we observed mislocalization of CENP-A when DNA polymerase epsilon or the ATAC subunit WDS was depleted in S2 cells (Figures S4A–S4C).

It has been recently suggested that histone-fold proteins can form nucleosome-like structures at centromeric sites (Nishino et al., 2012). Our finding of genetic, functional, and physical interactions of CHRAC14 and CENP-A suggest a heterodimer of two histone-fold proteins that forms only during the DNA damage response. Our data are consistent with the idea that CHRAC14 prevents CENP-A localization to sites of DNA repair. Whether this interaction occurs in chromatin or in the form of soluble dimers remains to be explored.

CENP-A Accumulates at Sites of DNA Damage in the Absence of CHRAC14

To confirm that CENP-A is recruited to sites of DNA damage, we depleted the telomere-capping component HIPHOP. This causes unprotected chromosome ends, which are recognized as DSBs and repaired by chromosome fusion (Gao et al., 2010). In cells depleted of HIPHOP alone, 78% of the chromosomes were fused by their telomeres as reported previously (Gao et al., 2010). When we codepleted HIPHOP and CHRAC14, however, we detected only 22% of fused chromosome ends, indicating that these unprotected telomeres are not repaired efficiently anymore (Figures 4A and 4B). Importantly, we detected clear CENP-A accumulation at about 23% of unprotected telomeres if both HIPHOP and CHRAC14 were depleted (Figure 4A). HIPHOP depletion alone did not cause ectopic CENP-A localization, presumably because chromosome ends are efficiently repaired and fused (Figure 4A). The finding of CENP-A localization to dysfunctional telomeres in CHRAC14-depleted cells provides strong support to the earlier notion that CHRAC14 prevents CENP-A mislocalization at sites of DNA damage. Unprotected telomeres are repaired by telomere fusion in a Ku70-dependent nonhomologous end joining (NHEJ) repair mechanism (Celli et al., 2006). We observe defective double strand repair at telomeres when CHRAC14 was depleted, suggesting that CHRAC14 influences NHEJ. This, however, does not exclude that CHRAC14 is also required for homologous recombination repair.

Chromatin needs to be restored after repair, and errors in reestablishing its integrity can be detrimental to the cell (Soria et al., 2012). Based on our observations, we propose the following model for the role of CHRAC14 in the DNA damage response (Figure 4C). Upon repair of DNA lesion, chromatin integrity needs to be re-established, which involves de novo nucleosome assembly and the reversal of damage signals on existing nucleosomes (Soria et al., 2012). Nucleosomes are formed from the available histone pool, which may lead to incorporation of histone variants, such as CENP-A. This bears the risk of ectopic kinetochore formation and requires mechanisms to prevent or correct CENP-A incorporation. Our data suggest that CHRAC14 plays an important role in preventing the mistargeting of CENP-A. Why do CENP-A and its orthologs in other organisms incorporate into sites of damage when it bears the risk of genome instability? We imagine that the ectopic incorporation of histone variants rather than canonical histones at break sites may be of advantage for a cell: histone variants are incorporated in a replication-independent mechanism and can therefore be used at any time during the cell cycle (Schuh et al., 2007; Sullivan and Karpen, 2001). Variants can be distinguished from canonical histones and are targets for subsequent eviction during later stages of chromatin restoration. Lastly, ectopic incorporation of CENP-A stimulates neocentromere formation that may be advantageous from an evolutionary point of view by ensuring that a centromeric region resides on every chromosome and chromosome segregation can take place in the next round of cell division (Burrack and Berman, 2012).

EXPERIMENTAL PROCEDURES

DNA Damage Assays

Gamma Irradiation on Larvae

Third instar larvae were irradiated with 30 Gy ($\sim$3,000 rads) of gamma irradiation for 5 min at a dose rate of 6 Gy/min using a GammaCell cesium source followed by recovery for 30–60 min at 25°C. For dissection and for screening development defects, the larvae were incubated further at 25°C after irradiation until hatching. After recovery, brains were dissected and stained as described in Supplemental Experimental Procedures. Larval brains were imaged using the Zeiss LSM 780 confocal microscope. The images were processed with ImageJ.

Bleomycin and MMS Treatment

S2 cells were treated with bleomycin (Burger et al., 1982; Morel et al., 2008) for 24 hr or MMS (Nakayama et al., 2006) for 4 hr (day 4 of CHRAC14 RNAi) as described in Supplemental Experimental Procedures. The cells were recovered for 30–60 min followed by IF (see Supplemental Experimental Procedures).

Comet Assay

DNA damage recovery times were quantified using alkaline comet assay (Olive and Banáth, 2006). After treatment with 0.04% MMS for 4 hr (on day 4 of an RNAi regime), cells were washed and incubated in fresh serum medium for the time indicated. At each time point, 10^5 cells were suspended in PBS and mixed with 0.7% of low-melting agarose and pipetted onto a slide precoated with 1% agarose, followed by comet assay using an alkaline electrophoresis buffer. The percentage of DNA within the comet tail (tail DNA %) calculated from 100 cells per sample was obtained using an Image J plugin developed for manual comet analysis (available upon request), and data are shown as mean values with SEs from three independent experiments.

(G) Coimmunoprecipitation (coIP) of CENP-A-GFP with CHRAC14 after DNA damage induction. Protein extracts from CENP-A-GFP embryos with γ irradiation with a final dose of 30 Gy after 1 hr recovery were incubated with GFPtrap. The bound material was then analyzed by western blot using CHRAC14 antibody. IN, input (20%). Beads control IP, blocked agarose beads (used as binding control for the IP reaction). Recombinant GST-CHRAC14 fusion protein as in (F) was loaded as a control.
See also Figure S3.
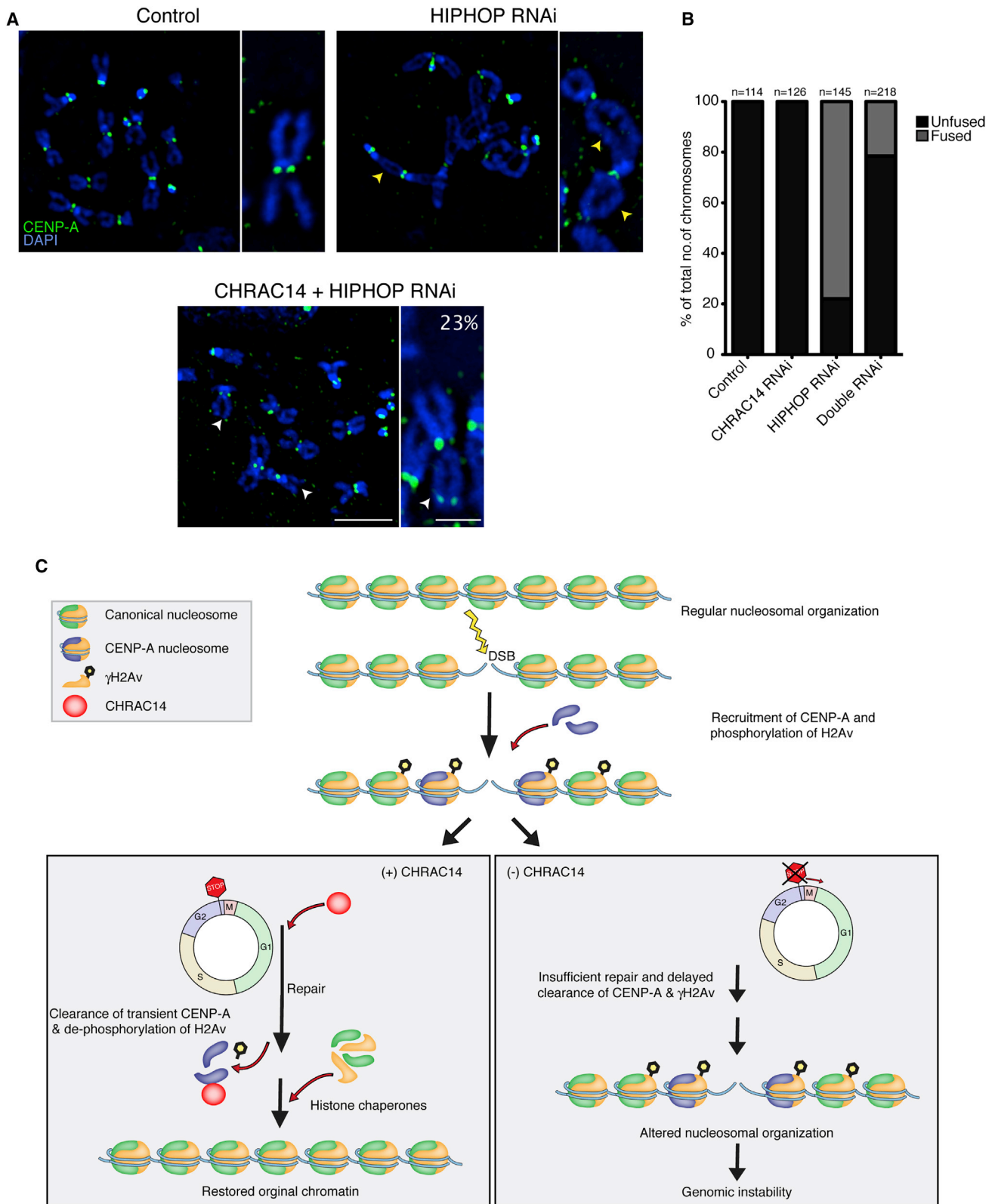
Figure 4. CENP-A Localizes Specifically to Sites of DNA Damage in CHRAC14-Depleted Cells
(A) CENP-A specifically localizes to unprotected telomeres when CHRAC14 is depleted. Mitotic chromosome spreads of S2 cells depleted for HIPHOP or HIPHOP and CHRAC14 in combination. White arrows indicate sites of ectopic CENP-A incorporation at chromosome ends in the double-depleted cells.
(legend continued on next page)

In Vitro Interaction Assay

A direct interaction between CENP-A and CHRAC14 was addressed by adding 4 μl (0.8 mg/μl) of His-sumo-CENP-A to the GST-CHRAC14/bead mixture (mixed and 10% immediately retracted for input). Four microliters (0.8 mg/μl) of GST-CHRAC14 and 5 μl (0.6 mg/μl) of GST alone (control) were preincubated for 30 min at 4°C with equilibrated glutathione sepharose beads (Amersham Biosciences). The interaction buffer contained 50 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 10% NP-40, and 2 mM phenylmethanesulfonylfluoride. After incubating for 1 hr at 4°C, the beads were washed 3× using interaction buffer, then eluted with 10 mM glutathione/50 mM Tris (pH 8.0), and later analyzed by western blot.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.03.008.

AUTHOR CONTRIBUTIONS

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Magnifications on the right show additional chromosomes of each condition. The inlet shows the percentage of chromosomes in double-depleted cells with CENP-A at telomeres (n = 140). Cells were stained for CENP-A (green) and DAPI. The scale bar represents 2 μm.

(B) Quantification of fused and unfused chromosomes as depicted in (A).

(C) Model: Induction of DNA damage stops cell-cycle progression at checkpoints and changes chromatin composition best exemplified by the phosphorylation of H2Av (γH2Av). Further chromatin changes may occur at the break sites, for instance, the recruitment of other histone variants such as CENP-A. During or after repair, CENP-A and γH2Av are cleared and histone chaperones further facilitate chromatin restoration (left panel). The absence of CHRAC14 might delay or fail these processes, leading to a G2-M checkpoint override with unrepaired breaks and failed clearance of CENP-A, resulting in ectopic kinetochore formation and genome instability (right panel).

See also Figure S4.

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