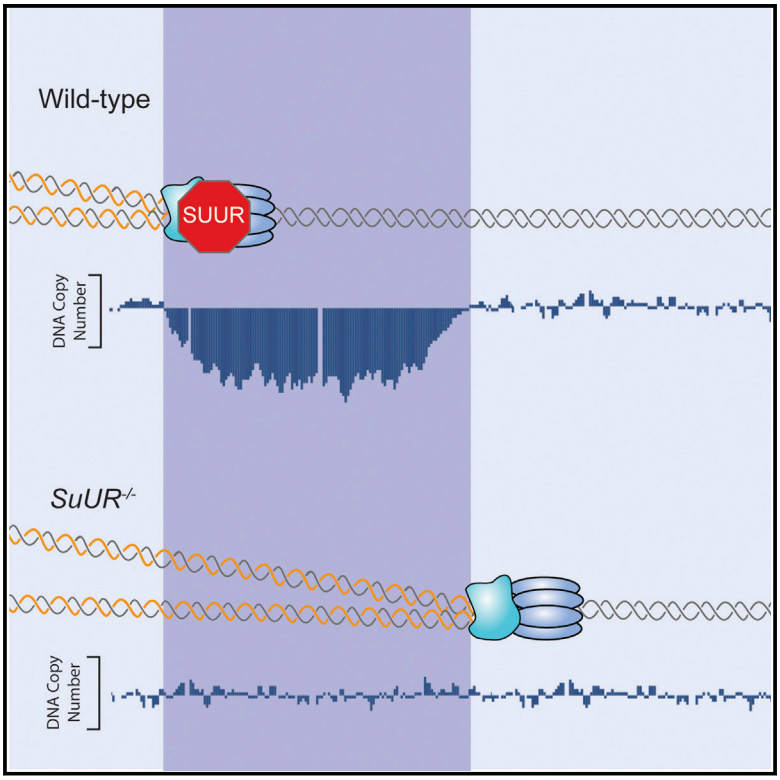
Report

Cell Reports

DNA Copy-Number Control through Inhibition of Replication Fork Progression

Graphical Abstract



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In Brief

Proper genome duplication relies on both initiation and elongation phases of DNA replication, and regulation of DNA replication is thought to occur predominantly at the level of initiation. By studying developmentally programmed repression of DNA replication in *Drosophila*, Nordman et al. now find that a metazoan protein can control DNA replication and copy number through direct inhibition of replication fork progression.

Highlights

Replication fork progression is subject to developmental control

The SUUR chromatin protein localizes to active replication forks

SUUR inhibits replication fork progression in specific developmental contexts

DNA copy number can be controlled through modulation of replication fork progression

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Report

DNA Copy-Number Control through Inhibition of Replication Fork Progression

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SUMMARY

Proper control of DNA replication is essential to ensure faithful transmission of genetic material and prevent chromosomal aberrations that can drive cancer progression and developmental disorders. DNA replication is regulated primarily at the level of initiation and is under strict cell-cycle regulation. Importantly, DNA replication is highly influenced by developmental cues. In *Drosophila*, specific regions of the genome are repressed for DNA replication during differentiation by the SNF2 domain-containing protein SUUR through an unknown mechanism. We demonstrate that SUUR is recruited to active replication forks and mediates the repression of DNA replication by directly inhibiting replication fork progression instead of functioning as a replication fork barrier. Mass spectrometry identification of SUUR-associated proteins identified the replicative helicase member CDC45 as a SUUR-associated protein, supporting a role for SUUR directly at replication forks. Our results reveal that control of eukaryotic DNA copy number can occur through the inhibition of replication fork progression.

INTRODUCTION

Proper genome duplication is essential for the accurate transmission of genetic information in all organisms, as errors can result in mutation, copy-number variations, and multiple genomic abnormalities implicated in cancer progression and developmental disorders (Jackson et al., 2014). DNA replication is largely regulated at the level of initiation when the origin recognition complex (ORC) binds to *cis*-acting origins of replication and together with Cdc6 and Cdt1/Dup loads the replicative helicase (Bell and Kaguni, 2013). Subsequent activation of the helicase results in the formation of two independent bidirectional replication forks that travel outward from the origin of replication (Boos et al., 2012). In metazoans, replication origins lack a consensus sequence, and epigenetic and structural factors likely influence their determination (Aggarwal and Calvi, 2004; Cayrou et al., 2011; Eaton et al., 2011; Mesner et al., 2011; Remus et al., 2004). One key feature of replication origins is that they are not uniformly distributed throughout the genome. This can result in large regions of the genome that are devoid of replication origins and dependent on replication forks emanating from distal origins for their replication. These regions are associated with genome instability and chromosome fragility (Debatisse et al., 2012; Durkin and Glover, 2007; Letessier et al., 2011; Norio et al., 2005), which makes it critical to define the mechanisms controlling replication fork progression and stability.

One factor that could influence replication fork progression and genome stability is the structure of chromatin itself. Pericentric heterochromatin and histone H1-containing chromatin represent two types of chromatin that are more compact than the rest of the genome (Woodcock and Ghosh, 2010). How replication forks stably progress through chromatin with different compaction states is not understood. It has been shown that a chromatin-remodeling complex consisting of ACF1-SNF2H (ATP-utilizing chromatin assembly and remodeling factor 1/sucrose nonfermenting-2 homolog) is recruited to pericentric heterochromatin to facilitate replication of these regions (Collins et al., 2002). Recently, SNF2H has been shown to associate with replication forks, suggesting that chromatin-remodeling activity could be important for replication fork progression (Lopez-Contreras et al., 2013; Sirbu et al., 2013). Histone H1 is phosphorylated throughout S phase, and this phosphorylation is thought to decondense histone H1-containing chromatin (Gurley et al., 1978; Lu et al., 1994). Cdc45, a key component of the replicative helicase, may function to recruit Cdk2 to replication forks to phosphorylate histone H1 and decondense chromatin, thereby facilitating replication of histone H1-containing regions (Alexandrow and Hamlin, 2005).

Drosophila provides a powerful system to understand how chromatin influences DNA replication. Most tissues in *Drosophila*

are polyploid, having multiple copies of the genome per cell (Edgar and Orr-Weaver, 2001; Lilly and Duronio, 2005; Zielke et al., 2013). Copy number, however, is not uniform throughout the genome of polyploid cells. Heterochromatin is repressed for DNA replication in *Drosophila* polyploid cells (Rudkin, 1969; Spradling and Orr-Weaver, 1987). More recently, it was demonstrated that specific euchromatic regions of the genome also are repressed for replication in a developmentally programmed manner (Nordman et al., 2011). Importantly, these euchromatic regions of the genome share several key properties with common fragile sites: they are devoid of replication origins, prone to DNA damage, and display cell-type specificity (Andreyeva et al., 2008; Nordman et al., 2011; Sher et al., 2012). Although the underlying molecular mechanism resulting in repression of DNA replication during development has remained elusive, the gene *Suppressor of UnderReplication* (*SuUR*) directly mediates repression of DNA replication at all known sites (Belyaeva et al., 1998; Makunin et al., 2002; Nordman et al., 2011).

The SUUR protein may provide an opportunity to understand how replication is influenced by chromatin. The N terminus of SUUR has a recognizable SNF2 chromatin-remodeling domain, but residues critical for ATP binding and hydrolysis are not conserved (Makunin et al., 2002). Based on DamID studies in cell culture, SUUR together with histone H1, Lamin, and other proteins have been proposed to form a repressive chromatin subtype, termed "BLACK" chromatin, which occupies 48% of the *Drosophila* genome (Filion et al., 2010). SUUR function is specific for DNA replication, as loss of SUUR function has no significant effect on gene expression or RNA polymerase II recruitment (Sher et al., 2012).

Previous studies of SUUR function have suggested that SUUR could influence replication fork progression. In salivary glands, SUUR binding to pericentric heterochromatin is constant throughout the endocycle, but its association with chromosome arms is dynamic and S phase dependent (Kolesnikova et al., 2013). SUUR has no effect on ORC binding sites in salivary gland chromosomes, indicating that SUUR-mediated repression of DNA replication occurs independently of ORC binding (Sher et al., 2012). Rather, *SuUR* mutants show enhanced replication fork progression, although it was not clear if the effect of SUUR on fork progression is direct and effects of overexpression were not examined (Sher et al., 2012). These studies raised the possibility that SUUR functions as a replication fork barrier (RFB), preventing replication forks from entering specific chromosomal domains. Alternatively, SUUR could act directly at replication forks to inhibit their progression within specific regions of the genome. Elucidating the mechanism by which SUUR influences replication fork progression could serve as a valuable tool in understanding how replication fork progression is regulated throughout the genome, as no eukaryotic protein is known to inhibit fork progression and DNA copy number directly. Here, we demonstrate that SUUR modulates the DNA replication program through inhibition of replication fork progression. This provides a mechanism through which copy-number control can be achieved independently of initiation of DNA replication.

RESULTS

The SNF2 Domain-Containing Protein SUUR Localizes to Active Replication Forks

To test if SUUR acts directly at active replication forks, we utilized the well-characterized gene amplification system in the follicle cells of the *Drosophila* ovary, which permits direct visualization of replication forks (Calvi et al., 1998; Claycomb et al., 2002). At a specific stage in follicle cell differentiation, genomic replication ceases and six sites in the genome become amplified through a re-replication-based mechanism with bidirectional fork movement from an origin region (Claycomb and Orr-Weaver, 2005; Kim et al., 2011). Sites of amplification can be visualized by monitoring the incorporation of a nucleotide analog such as 5-ethynyl-2′-deoxyuridine (EdU), providing a direct method to observe site-specific DNA replication (Calvi et al., 1998; Claycomb et al., 2002). During the initial stages of gene amplification at the major amplification locus, *DAFC-66D*, both initiation and elongation phases of DNA replication are coupled, giving rise to a single replication focus (Figure 1A). In late stages of gene amplification, origin firing is inhibited at *DAFC-66D*, thus active replication forks are visible as a distinct double-bar structure, in which each bar represents a series of replication forks traveling outward from the replication origin (Figure 1A). It was previously shown that replication forks at amplification loci progress farther in *SuUR* mutants than in wild-type (Sher et al., 2012).

If SUUR functions as an RFB, we would expect SUUR to localize to sites distal to amplification foci, prior to the arrival of replication forks, only overlapping replication forks late during gene amplification when forks reach these sites. Alternatively, if SUUR is targeted to active replication forks, it would localize to, and track with, replication forks during gene amplification. To distinguish between these two distinct mechanisms, SUUR localization was monitored in amplifying follicle cells using an affinity purified anti-SUUR antibody throughout all stages of gene amplification at *DAFC-66D* (Figure 1). We noticed two patterns of SUUR localization. First, SUUR constitutively localized to heterochromatin, consistent with previous studies (Makunin et al., 2002; Zhimulev et al., 2012). Second, SUUR dynamically localized to active replication forks at *DAFC-66D* even prior to their resolution into double-bar structures (Figures 1B–1D). No signal was observed when *SuUR* mutant ovaries were stained with the same antibody, and this localization pattern was recapitulated using a functional *GFP-SuUR* transgene under the control of its own promoter (Figure S1). Thus, SUUR localizes to, and tracks with, active replication forks and does not act as an RFB.

Although SUUR was localized to replication forks, it was not always present at *DAFC-66D*. SUUR localization to *DAFC-66D* was first observed in a subset of late stage 10B follicle cells, staged based on egg chamber morphology and their pattern of EdU incorporation. In contrast, during the initial stage of amplification, in early stage 10B follicle cells, SUUR was not detectable at *DAFC-66D* (Figure 1D). Taken together, these results demonstrate that SUUR is recruited to active replication forks after an initial period of gene amplification.

To independently verify these results, we localized SUUR more precisely at the molecular level. To this end, egg chambers were dissected from oogenesis stages corresponding to early

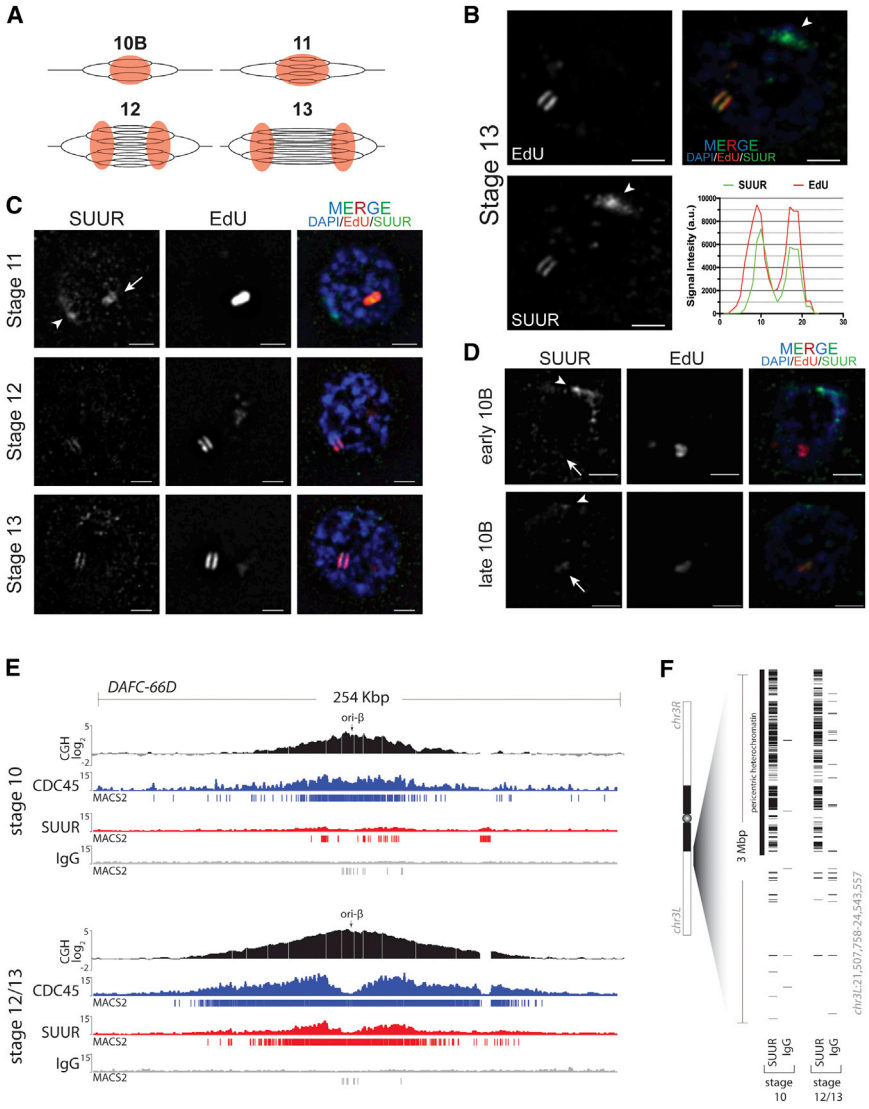


Figure 1. SUUR Is Localized to, and Tracks with, Active Replication Forks

(A) Representation of copy-number changes and replication fork localization (orange) during all stages of gene amplification at the major follicle cell amplification locus *DAFC-66D*.

(B) Localization of SUUR in a stage 13 follicle cell relative to active replication forks at *DAFC-66D*. Replication forks are marked by EdU incorporation (red), SUUR by immunostaining (green), and DNA by DAPI staining (blue). Individual channels are shown as labeled. The arrowhead marks SUUR localized to heterochromatin. Scale bar, 2 μm. The graph shows the intensity profiles of SUUR and EdU signals through a perpendicular line relative to the double-bar structure.

(C) Localization of SUUR during gene amplification in stage 11–13 follicle cells. Labels are as in (B). Arrowhead in stage 11 shows SUUR localized to heterochromatin and arrow shows SUUR at the *DAFC-66D* amplicon. Each panel shows a single representative follicle cell nucleus. All images were taken with equal exposure times, and brightness was scaled linearly for clarity of presentation. Scale bar, 2 μm

(D) SUUR localization in stage 10B follicle cells. Labels are as in (C). Each panel shows a single representative follicle cell nucleus. Images were taken at equal exposure times, and brightness was scaled linearly for presentation. Arrowhead shows SUUR localized to heterochromatin, and arrow shows the *DAFC-66D* amplicon. Scale bar, 2 μm

(E) CDC45 (blue), SUUR (red), and IgG (gray) ChIP enrichment at the major amplification locus *DAFC-66D*. Copy-number profiles are shown in black. Top: stage 10 egg chambers (early amplification). Bottom: pooled stage 12 and 13 egg chambers (late amplification). Enrichment values are scaled equally for clarity of presentation (0–15). MACS2 peak calls are included for reference. The gap in the right arm of the CGH profiles is due to a repetitive region that is devoid of array-based comparative genomic hybridization (aCGH) probes. Localization of the predominant origin, ori-β, is indicated.

(F) MACS peak calls from a region of chromosome *3L* reveals SUUR binding to pericentric heterochromatin. The black bar represents pericentric heterochromatin from chromosome *3L* (*chr3L*:22955576-24543557; Smith et al., 2007). A schematic representation of chromosome *3* is included for reference, with centric heterochromatin shown in black.

and late stages of gene amplification, and SUUR localization was monitored with high resolution at *DAFC-66D* by chromatin immunoprecipitation sequencing (ChIP-seq). As a marker of replication forks, ChIP-seq was performed with an affinity-purified antibody specific to CDC45, a member of the CMG complex (CDC45/MCM/GINS) that is the active form of the replicative helicase (Moyer et al., 2006).

During the earliest stage of gene amplification (stage 10), CDC45 enrichment was highest at, and proximal to, the replication origin and decreased as forks progressed away from the origin (Figure 1E). In contrast, SUUR was not significantly enriched at, or proximal to, the replication origin (Figure 1E). A modest amount of SUUR was enriched at sites distal to the replication origin that were also occupied by CDC45. In late stages of amplification, SUUR continued to show no significant enrichment at, or immediately proximal to, the replication origin (Figure 1E). SUUR enrichment significantly increased, however, at sites distal to the replication origin, where CDC45 also showed the most significant enrichment (Figure 1E). These data indicate that SUUR is recruited to active replication forks after amplification of an initial domain surrounding the origin of replication. Importantly, these data rule out the possibility that SUUR acts through an RFB-type mechanism, as this mode of replication fork inhibition would require SUUR to associate with chromatin prior to the arrival of replication forks. Rather, by both immunofluorescence and ChIP, SUUR localization to sites of amplification appears replication dependent.

ChIP-seq also was used to monitor the association of SUUR with pericentric heterochromatin during gene amplification. Unlike the dynamic association of SUUR at *DAFC-66D*, SUUR

Table 1. Mass Spectrometry Identification of SUUR-Associated Proteins

		Rabbit Anti-SUUR IP		Guinea Pig Anti-SUUR IP		Mock	
Protein	Size (Da)	Mascot Score	Coverage (%)	Mascot Score	Coverage (%)	Mascot Score	Coverage (%)
SUUR	108,072	1,672	26.3	1,714	26.9	0	–
HP1	23,228	533	37.9	571	41.7	0	–
CDC45	66,419	890	32.3	223	10.6	0	–

Immunoprecipitations (IPs) were performed from soluble nuclear extracts derived from 0–24 hr embryos.

was localized constitutively to pericentric heterochromatin for the duration of gene amplification (Figure 1F). This molecular analysis of SUUR localization during gene amplification recapitulates the SUUR localization pattern obtained by immunofluorescence.

SUUR Associates with the CMG Complex Member CDC45

Given that SUUR is localized to replication forks in amplifying follicle cells, we wanted to determine if SUUR associates with replication forks in other cell types. To this end, SUUR was immunoprecipitated from embryonic nuclear extracts using affinity-purified rabbit and guinea pig antibodies specific for SUUR, and associated proteins were identified by mass spectrometry. One of the top SUUR-associated proteins we identified was HP1, which is known to associate with SUUR, validating our approach to identify SUUR-associated proteins (Table 1) (Pindyurin et al., 2008). Intriguingly, we detected an association between SUUR and CDC45 (Table 1). Together with the cytological localization and ChIP analysis of SUUR, these results strongly indicate that SUUR acts directly at replication forks.

To test the significance of the association between CDC45 and SUUR, we examined SUUR localization on salivary gland chromosomes after genetic ablation of CDC45 function through RNA interference (RNAi) with the driver *da-GAL4*. RNAi against *cdc45* resulted in a significant reduction in CDC45 protein levels and disrupted endocycling (Figures 2A and 2B). The percentage of nuclei in S phase was significantly lower in *da-GAL4 cdc45* RNAi salivary glands (57.75%; 502/871) compared with the *da-GAL4* driver alone (92.11%; 747/811) ($p = 2.314215 \times 10^{-63}$, Fisher's exact test; Figure 2B). Depletion of CDC45 resulted in a concomitant loss of SUUR localization along the arms of polytene chromosomes during both S and G phases (Figures 2C and 2D). Loss of SUUR localization is specific to a reduction in CDC45 levels, as decreased proliferating cell nuclear antigen (PCNA) levels do not alter SUUR localization (Kolesnikova et al., 2013). Thus, CDC45 is crucial for proper SUUR binding to chromosomes, as is HP1 (Pindyurin et al., 2008). The fact that localization of SUUR is dependent on CDC45, but not PCNA, likely reflects their distinct biochemical roles in the process of DNA replication.

SUUR Affects Replication Fork Progression

Having demonstrated that SUUR localizes to active replication forks, we tested whether SUUR has functional consequences on replication fork progression. A previous study demonstrated that loss of SUUR function resulted in increased fork progression using mixed-stage follicle cells (Sher et al., 2012). We extended this analysis by measuring the effect SUUR overexpression has on fork progression using follicle cells from a defined stage.

To overexpress SUUR, we utilized transgenic flies that harbor two or four additional copies of the *SuUR* gene under the control of its own promoter (*4X-SuUR* and *6X-SuUR*, respectively) (Makunin et al., 2002). DNA was extracted from dissected stage 13 egg chambers, fluorescently labeled, and hybridized together with fluorescently labeled embryonic control DNA to microarrays. To quantify the effect loss of SUUR function or SUUR overexpression has on replication profiles at each site of amplification, we defined the point on each arm of the amplicon corresponding to half the maximum copy number and determined the distance between these two positions.

Loss of SUUR function resulted in extended gene amplification gradients with no significant effect on copy number at the origin of replication at all amplicons (Figure 3; Figure S2; Table S1) (Sher et al., 2012). At the *DAFC-66D* locus, loss of SUUR function resulted in a 32% increase (75.6 kb to 99.8 kb) in the size of the replication gradient (Figure 3). In contrast, the presence of only two additional copies of SUUR reduced the size of the replication gradient by 48% (75.6 kb to 39 kb; Figure 3; Figure S2; Table S1). The presence of four additional copies did not have a further effect, but we do not know whether there is a linear increase in protein levels or activity. Importantly, overexpression of SUUR did not reduce the copy number at the origin of replication or the flanking ~25 kb surrounding the peak of amplification, suggesting that SUUR affects replication fork progression at specific chromosomal regions, which could be accomplished by modulating SUUR activity as a function of replication timing or follicle cell differentiation state.

SUUR Affects Replication Fork Stability Rather Than Fork Rate

Increased replication fork progression associated with loss of SUUR function could be due to an increase in replication fork speed and/or stability. If loss of SUUR function results in increased replication fork rate, then we would expect to see changes in fork progression during all stages of gene amplification. Previous copy-number analysis, however, indicated that loss of SUUR function does not affect fork progression during the early stages of gene amplification (Sher et al., 2012). We confirmed this using an independent cytological analysis as a measure of fork progression (Figure S3A). Together, these results indicate that SUUR does not affect the rate of replication fork progression.

Given that loss of SUUR function results in extended replication gradients at all amplicons, we asked if this is due to a prolonged period of gene amplification by quantifying the fraction of amplifying follicle cells at each stage of gene amplification. Starting in stage 10B, all follicle cells synchronously enter the

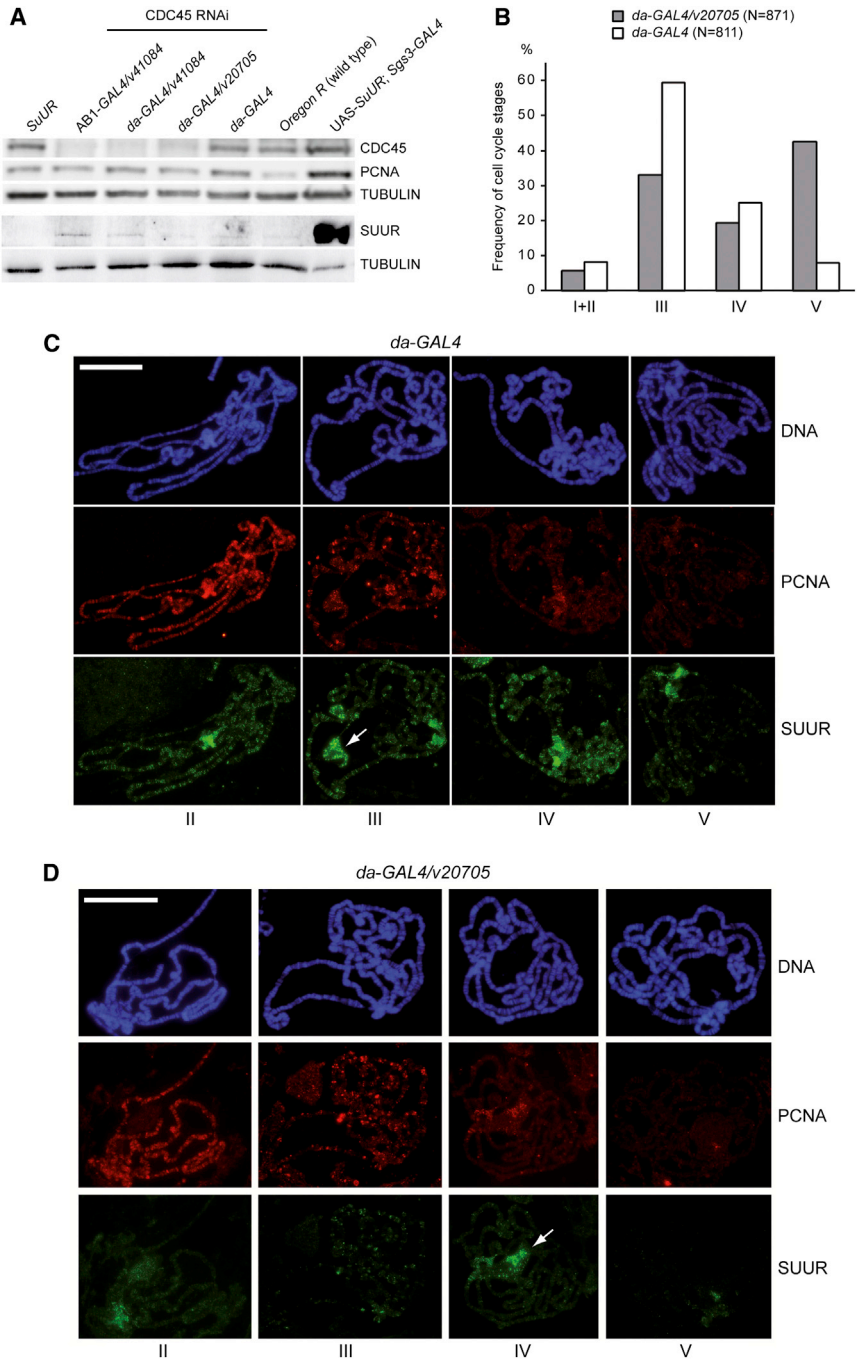


Figure 2. CDC45 Depletion Suppresses the Endocycle and Inhibits SUUR Binding to Polytene Chromosomes

(A) Western blot analysis of salivary glands of different genetic backgrounds with anti-CDC45, anti-PCNA, and anti-SUUR. TUBULIN was used as a loading control.

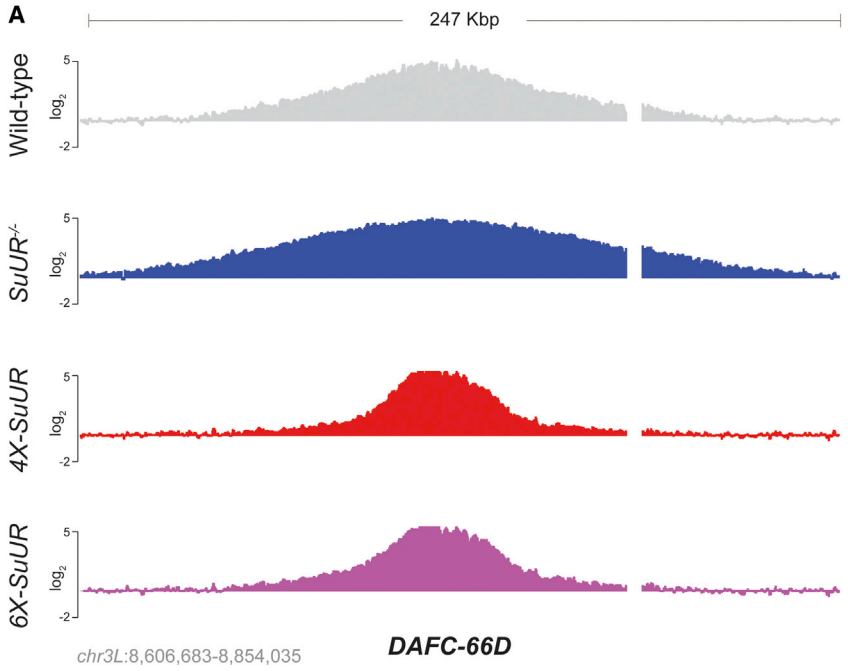
(B) Frequencies (%) of salivary gland nuclei at different S phase stages. Endocycle stages were determined according to Kolesnikova et al. (2013): (I–II) the early S phase and early to late S phase transition; (III) "typical" late S phase; (IV) the end of S phase; and (V) G phase.

(C and D) Salivary gland polytene chromosomes were coimmunostained with SUUR (green) and PCNA (red), and DNA was detected by DAPI. *da-GAL4* control (C) and *da-GAL4/cdc45*-RNAi-*v20705* (D). SUUR signal intensity was significantly reduced upon CDC45 depletion; n = 871 for *da-GAL4/cdc45*-RNAi-*v20705* and n = 811 for the *da-GAL4* control. Arrows indicate SUUR binding to the nucleolus. Scale bar, 50 μm.

gene amplification program (Calvi et al., 1998). After ~7.5 hr (stage 13 of egg chamber development), follicle cells cease amplification as judged by their lack of detectable nucleotide incorporation (Calvi et al., 1998). It has been shown that loss of SUUR function does not affect the timing of egg chamber development (Sher et al., 2012). In wild-type and *SuUR* mutant follicle cells, nearly 100% of all nuclei had visible replication foci during stages 10B, 11, and 12 with little variance (Figure S3B). In stage 13 egg chambers, however, only 37% of wild-type follicle cells displayed clearly visible amplification foci with substantial variance even within the same biological replicate (SD = 0.41). In contrast, 99% of *SuUR* mutant stage 13 follicle cell nuclei had visible amplification foci with very little variance (SD = 0.02). This indicates that loss of *SuUR* function results in prolonged EdU incorporation, and this accounts for the increased size of amplified domains. These cytological data are consistent with loss of SUUR protein causing increased fork stability rather than increased rate of fork progression in the context of gene amplification.

SUUR Inhibits Replication Fork Progression throughout Underreplicated Domains

We wanted to monitor the DNA damage profile relative to underreplicated domains to determine if SUUR could promote replication fork stalling within underreplicated domains. Replication fork stalling can trigger the DNA damage response (DDR) and influence replication fork stability (Branzei and Foiani, 2010; Cimprich and Cortez, 2008). We utilized the *Drosophila* DNA damage-specific marker γH2Av, the equivalent of mammalian γH2AX (Madigan et al., 2002), to localize precisely sites of DNA damage relative to underreplicated domains. If SUUR acts as an RFB at sites of underreplication, we would expect γH2Av only at the edges of the underreplicated domain, given that the barrier would prevent replication forks from entering these domains. In contrast, if SUUR stalls replication forks within underreplicated domains, then γH2Av should be enriched throughout



B

	log2 copy number at peak	Left arm (bp)	Right arm (bp)	half max to half max (bp)
Wild-type	4.2808	36300	39340	75640
SuUR$^{-/-}$	4.4111	53660	46100	99760
4X-SuUR	5.0383	17630	21410	39040
6X-SuUR	4.9185	20880	23660	44540

Figure 3. SUUR Affects Replication Fork Progression in a Dosage-Dependent Manner

(A) aCGH of the major follicle cell amplification locus, *DAFC-66D*. DNA extracted from stage 13 egg chambers of the indicated genotypes was compared to diploid 0–2 hr embryonic DNA. The height of the copy-number gradient reflects the number of rounds of initiation, and the width reflects fork progression. The gap in the right arm of the CGH profiles is due to a repetitive region that is devoid of CGH probes.

(B) Quantitative analysis of aCGH profiles at *DAFC-66D*.

the entire domain. Immunofluorescence studies of *Drosophila* polytene chromosomes have shown that DNA damage is associated with underreplicated domains, and this damage correlates with level of SUUR expression (Andreyeva et al., 2008). These studies, however, lacked the resolution to determine where the damage occurs relative to an underreplicated domain.

For high-resolution analysis of γH2Av localization, chromatin was isolated from dissected wandering third-instar salivary glands and ChIP-seq was performed using an anti-γH2Av antibody and immunoglobulin G (IgG) antibody as a negative control. We found γH2Av enriched throughout underreplicated domains, and this enrichment was dependent on SUUR (Figures 4A and 4B; Figure S4). The γH2Av signal is unlikely due to spreading in response to double-strand breaks, given that spreading occurs bidirectionally from the break site and would extend beyond underreplicated boundaries (Berkovich et al., 2007; Iacovoni et al., 2010; Kim et al., 2007; Rogakou et al., 1999; Savic et al., 2009). Therefore, the DNA damage profile relative to underreplicated domains is consistent with SUUR acting to promote replication fork stalling and the DDR throughout repressed domains, rather than acting as a RFB.

DISCUSSION

By studying the mechanism by which the SNF2 domain-containing chromatin protein SUUR mediates repression of replication, we have uncovered an example of a eukaryotic protein that controls DNA copy number through direct inhibition of replication fork progression. We have provided several independent lines of evidence that SUUR functions by targeting and inhibiting active replication forks. First, SUUR is associated directly with active replication forks as evidenced by immunofluorescence, ChIP, and association with CDC45. Second, whereas loss of SUUR function results in increased replication fork progression, overexpression of SUUR drastically inhibits replication fork progression. Third, SUUR function results in replication fork stalling and DNA damage within underreplicated domains. Unlike proteins that function to promote replication fork progression through specific DNA structures or chromatin domains (Branzei and Foiani, 2010; Collins et al., 2002; Paeschke et al., 2011), SUUR has the opposite function in that it inhibits replication fork progression within specific regions of the genome.

Our results demonstrate that SUUR functions to stall replication forks, resulting in induction of the DDR as judged by the presence of γH2Av (Andreyeva et al., 2008). γH2Av signal could represent double-strand breaks (DSBs) and/or stalled replication forks. The fact that DNA alterations have been shown to be associated with underreplicated domains suggests that SUUR-mediated inhibition of replication forks leads to fork instability (Andreyenkova et al., 2009; Glaser et al., 1997; Yarosh and Spradling, 2014). How SUUR stalls replication forks remains an open question. SUUR could inhibit the factors necessary to decondense specific regions of the genome to facilitate their replication. In fact, even in the absence of SUUR, several regions of the genome remain condensed and contain DNA damage, suggesting that replication forks struggle to progress through these regions (Andreyeva et al., 2008; Nordman et al., 2011; Sher et al., 2012).

SUUR is not detectable at replication forks from the onset of gene amplification, but rather appears to have a discrete time of association. This is similar to observations in salivary gland chromosomes where SUUR association with euchromatic regions of the genome occurs late in S phase (Kolesnikova et al.,

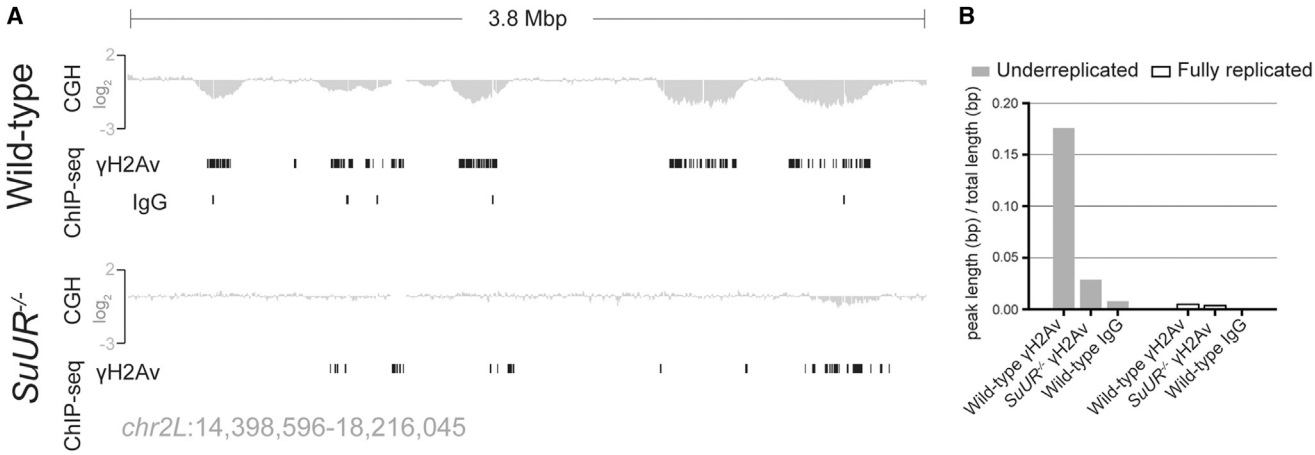


Figure 4. γH2Av Is Localized throughout Underreplicated Domains and Is Dependent on SUUR Function

(A) aCGH and γH2Av ChIP-seq profiles from wild-type and *SuUR* mutant wandering third-instar larval salivary glands. A region of the left arm of *chr2L* is shown. ChIP-seq peaks were called by MACS relative to input DNA. aCGH data are from Sher et al. (2012).

(B) Peak enrichment within the euchromatic regions of salivary-gland-specific underreplication (Nordman et al., 2011) compared to the fully replicated portion of the genome. The length (bp) of each peak within a particular region was summed and divided by the total length (bp) of the appropriate region.

2013). There are several possible explanations for these observations. One is that SUUR is recruited to replication forks once they encounter a specific chromatin subtype. Another possibility is that SUUR activity is inhibited by high cyclin E/CDK2 activity present at the beginning of S phase and early gene amplification (Calvi et al., 1998). Therefore, only when cyclin E/CDK2 activity is reduced below a certain threshold late in S phase or gene amplification would SUUR be able to associate with replication forks. Previously, it was shown that a specific mutation in *cyclin E* could restore replication of heterochromatin in polyploid nurse cells of the *Drosophila* ovary (Lilly and Spradling, 1996). Finally, SUUR activity could change as a function of S phase independently of cyclin E/CDK2 activity, resulting in its association with replication forks. Overexpression of SUUR could counteract this regulation, resulting in earlier activation of SUUR with respect to S phase progression.

Our results beg the question: why have a protein to stop replication forks? One reason could be that replication fork inhibition is an extension of the replication-timing program, albeit an extreme one. Replication fork inhibition would serve to delay further the replication of genomic regions that are devoid of replication origins and thus cannot be regulated at the level of initiation. Replication timing is a conserved feature of genome duplication from yeast to humans, yet its purpose remains largely unknown. It is possible that coordinating the number of replication forks throughout S phase serves to moderate the supply limiting substrates such as deoxynucleotide triphosphates and histones to maximize genome stability (Mantiero et al., 2011). Another possibility is the proposal that regulated replication timing spreads termination events across the genome (Hawkins et al., 2013). Replication timing is correlated with genome structure and highly influenced by development, but the molecular mechanisms that regulate the replication timing remain unclear. We propose that inhibition of replication fork progression provides a mechanism to modulate replication timing.

EXPERIMENTAL PROCEDURES

Drosophila Strains

Wild-type: *Oregon R*, *SuUR*: *w*; *SuUR^{ES}*, *GFP-SuUR*: *w*; *PBac{w^{+}GFP-SuUR} attP40*; *SuUR^{ES}*. *4X-SuUR* and *6X-SuUR* have been previously described (Andreyeva et al., 2008; Makunin et al., 2002).

Cytological Analysis and Microscopy

Ovaries were dissected in Ephrussi-Beadle ringers (EBR) solution (Beadle and Ephrussi, 1935) or Grace's unsupplemented medium from females fattened for 2 days on wet yeast and pulse labeled with 50 μM EdU for 30 min. Ovaries were prepared for antibody staining as indicated in Royzman et al. (1999), with modifications detailed in Supplemental Experimental Procedures.

Image Processing and Quantification

All images were captured on a Nikon Eclipse Ti microscope using either a Nikon Plan Apo 60× or a Nikon Apo TIRF 100× oil objective with a Hamamatsu camera. Images were processed and deconvolved using NIS-Elements AR 3.2 software.

CDC45-RNAi Analysis

Immunostaining of polytene chromosome squashes was performed as described previously (Kolesnikova et al., 2013), with modifications detailed in Supplemental Experimental Procedures. The *UAS-SuUR*; *Sgs3-GAL4* strain has been described (Andreyeva et al., 2005). Fly stocks v20705 and v41084 with transgenic RNAi constructs against the *cdc45* gene were obtained from the Vienna *Drosophila* RNAi Center (Dietzl et al., 2007). Fly stocks with *AB1-GAL4* and *da-GAL4* drivers were obtained from Bloomington Stock Center.

Comparative Genomic Hybridization

One hundred stage 13 egg chambers were dissected for each genotype from fattened females, and control DNA was isolated from 0–2 hr embryos. Genomic DNA was phenol-chloroform extracted and labeled with Cy3-dUTP or Cy5-dUTP by random priming as described (Blitzblau et al., 2007). Labeled DNA was hybridized to custom tiling arrays. Array information and bioinformatics analysis are detailed in Supplemental Experimental Procedures. For the comparative genomic hybridization (CGH) profile in Figure 1, 150 10B egg chambers were dissected from wild-type females and processed as described, with the exception that labeled DNAs were hybridized to whole-genome tiling arrays at 125 bp resolution.

The half-maximum determination of copy-number profiles was used to quantify the size of the replication gradients. Smoothed data were used to extract the point in each gradient with the maximum copy number. Next, the

distance from the maximum copy number to the half-maximum copy number was determined for each side of the replication gradient.

ChIP-Seq

Egg Chambers

Ovaries were dissected from females fattened for 2 days on wet yeast in EBR and fixed in 2% formaldehyde for 12 min. Stage 10 egg chambers were isolated from fixed ovaries for the early amplification sample, and stage 12 and 13 egg chambers were collected and pooled for the late-stage amplification sample. Six hundred egg chambers were used for each individual ChIP reaction. Egg chambers were resuspended in LB3 and dounced using a Kontes B-type pestle. Sonication was done in a Bioruptor300 (Diagenode) using 30 cycles of 30 s on 30 s off at maximal power. Antibody information can be found in Supplemental Experimental Procedures.

Salivary Glands

Salivary glands were dissected in EBR from 200 wandering third-instar larvae per ChIP reaction and fixed for 12 min in 2% formaldehyde. Larvae were a mixture of both males and females. Salivary glands were dounced in LB3 (MacAlpine et al., 2010) and sonicated as for egg chambers, except 40 cycles were used. Rabbit anti-γH2Av (Rockland) or rabbit IgG (Abcam) was added to the chromatin extract and incubated at 4°C for 3 hr. Library construction, sequencing information and bioinformatics analysis are described in Supplemental Experimental Procedures.

Immunoprecipitation and Mass Spectrometry

The 0–24 hr embryos were collected and soluble nuclear extracts were prepared as described previously (Shao et al., 1999). SUUR complexes were immunoprecipitated using both rabbit and guinea pig anti-SUUR sera that were affinity purified. Additional details can be found in Supplemental Experimental Procedures.

ACCESSION NUMBERS

The CGH and ChIP-seq data sets have been deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE56056.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.10.005.

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