Biphasic chromatin binding of histone chaperone FACT during eukaryotic chromatin DNA replication

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ABSTRACT

The facilitates chromatin transcription (FACT) complex affects nuclear DNA transactions in a chromatin context. Though the involvement of FACT in eukaryotic DNA replication has been revealed, a clear understanding of its biochemical behavior during DNA replication still remains elusive. Here, we analyzed the chromatin-binding dynamics of FACT using *Xenopus* egg extract cell-free system. We found that FACT has at least two distinct chromatin-binding phases: (1) a rapid chromatin-binding phase at the onset of DNA replication that did not involve origin licensing and (2) a second phase of chromatin binding that initiated after origin licensing. Intriguingly, early-binding FACT dissociated from chromatin when DNA replication was blocked by the addition of Cdc6 in the licensed state before origin firing. Cdc6-induced removal of FACT was blocked by the inhibition of origin licensing with geminin, but not by suppressing the activity of DNA polymerases, CDK, or Cdc7. Furthermore, chromatin transfer experiments revealed that impairing the later binding of FACT severely compromises DNA replication activity. Taken together, we propose that even though FACT has rapid chromatin-binding activity, the binding pattern of FACT on chromatin changes after origin licensing, which may contribute to the establishment of its functional link to the DNA replication machinery.

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1. Introduction

The initiation of eukaryotic DNA replication is a multistep process starting with the assembly of the pre-replicative complex (pre-RC) during the G_1 phase of the cell cycle by sequential recruitment of the Orc1–6, Cdc6, Cdt1, and Mcm2–7 onto DNA origins [1–3]. Pre-RC is later converted into the pre-initiation complex (pre-IC) by the recruitment of Cdc45 and the Sld5–Psf1–Psf2–Psf3 complex (GINS; Go-Ichi-Ni-San, i.e., 5-1-2-3 in Japanese), DNA polymerase α (Pol α) and other replication factors by the combined actions of two S-phase-promoting kinases: Cdc7, which is a Dbf4- and Drf1-dependent kinase (DDK), and the cyclin-dependent kinase (CDK) [4].

During the replication of eukaryotic chromosomes, chromatin structure must change dynamically to overcome the barrier posed by nucleosomes and to assemble nascent DNA strands into chromatin. Despite extensive analyses that revealed the modular organization

and temporal regulation of DNA replication factors, the influence of chromatin structure and the roles of chromatin-modifying proteins in DNA replication remain largely elusive [4,5]. FACT is an essential chromatin reorganizing factor [6–8], which has been thought to act in conjunction with pathway-specific cofactors, thereby mediating chromatin transactions during the progression of different DNA-metabolizing events, such as DNA replication, transcription, and repair [9–11]. The FACT complex is composed of two subunits, SPT16/CDC68 and SSRP1, which are highly conserved among eukaryotes; however, orthologs of SSRP1 in budding yeast are separated into two proteins, Pob3 and Nhp6 [12–14]. In *Xenopus*, FACT was originally identified as DNA-unwinding-factor (DUF) complex consisting of 140 kDa (DUF140) and 87 kDa (DUF87) polypeptides, orthologs of Spt16 and SSRP1, respectively [15].

It has been proposed that FACT has a functional link with DNA replication in human cells [16]. Furthermore, FACT has been shown to be a part of the replication machinery; FACT directly binds to Pol α and replication protein A (RPA) and genetically interacts with several replication factors in yeast [7,17–22]. Furthermore, a subset of FACT mutations increases sensitivity of cells to hydroxyurea [7,8,19]. In

higher eukaryotes, FACT has been shown to promote DNA replication in *Xenopus* egg extracts [15]. In HeLa cells, the involvement of FACT in DNA replication is partly mediated by the stable association with the replicative helicase Mcm2–7, especially with the Mcm4 subunit [16]. Despite these findings, the behavior and molecular function of FACT during eukaryotic DNA replication have not yet been completely substantiated.

In this study, we explored the chromatin-binding dynamics of FACT during the process of DNA replication using *Xenopus* egg extract cell-free system, by which genomic DNA replication can be analyzed without the influence of transcription [23]. We found that FACT associates with chromatin in at least two distinct phases: an initial binding phase unrelated to DNA replication events and a second phase of chromatin binding that initiated after origin licensing. Initially loaded FACT is susceptible to removal from licensed chromatin but not from unlicensed chromatin, and the later association of FACT is inhibited if DNA replication is blocked before origin firing by the addition of Cdc6 [24]. Our results also suggest that FACT binding after origin licensing is necessary for the efficient replication of eukaryotic chromatin.

2. Materials and methods

2.1. Preparation of Xenopus egg extracts

Interphase extracts were prepared as described previously [3,24,25]. *Xenopus* egg extracts were supplemented with 250 µg/ml cycloheximide, 25 mM phosphocreatine, and 15 µg/ml creatine phosphokinase before use. *Xenopus* sperm nuclei were prepared after demembranation with lysolecithin as described previously [25].

2.2. Antibodies and recombinant proteins

Glutathion-S-transferase (GST)-tagged wild-type *Xenopus laevis* Cdc6 was purified as described previously [24]. N-terminal hexahistidine-tagged *Xenopus* geminin H lacking a destruction box and hexahistidine-tagged p21 were expressed and purified as described previously [3].

Anti-DUF140/Spt16, anti-DUF87/SSRP1, anti-Orc1, anti-Cdc6, and anti-Cdc7 antibodies were prepared as described previously [2,3,15,26,27]. Anti-Mcm4 antibody was generously provided by Yukio Ishimi (Ibaraki University), anti-Sld5 and anti-Cdc45 by Yumiko Kubota and Haruhiko Takisawa (Osaka University), and anti-Pol α by Fumiko Hirose (University of Hyogo). Anti-histone H3 (ChIP grade) and anti-PSTAIR (CDK) antibodies were purchased from ABcam and Sigma-Aldrich Co. (St. Louis, MO), respectively.

2.3. Measurement of DNA synthesis in Xenopus egg extracts

To measure DNA replication, $[\alpha\text{-}^{32}\text{P}]$dATP was added to the reaction mixture, and total DNA synthesized after incubation at 23 °C was measured as the radioactivity incorporated into a fraction insoluble in 10% TCA as described previously [25].

2.4. Isolation of chromatin fraction and immunodepletion of proteins from Xenopus egg extracts

Isolation of chromatin fractions and immunodepletion were performed as described previously [24]. The efficiency of depletion was confirmed by immunoblot analyses or measurements of DNA replication activity.

2.5. Immunoblotting

Samples for immunoblotting were electrophoresed on a 10% SDS-polyacrylamide gel and electrically transferred onto a PVDF membrane (BioRad). The membrane was blocked with 0.5% skim milk in phosphate-buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 8.04 mM Na_2HPO_4, and 1.47 mM KH_2PO_4) and incubated with a primary antibody diluted 1000- to 5000-fold with PBS containing 0.05% Tween-20 and 3% BSA. After washing with 0.05% Tween-20–PBS three times and incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG, protein bands that reacted with the primary antibody were visualized with an ECL Western Blotting Detection System (GE Healthcare).

2.6. Data presentation

All figures in this article indicate representative results obtained from at least three independent experiments to verify their reproducibility.

3. Results

3.1. FACT binds chromatin at an early stage of eukaryotic DNA replication independent of Orc1 or origin licensing

Previous reports have shown that FACT is essential for DNA replication in *Xenopus* egg extracts [15]. To study the involvement of FACT with different stages of DNA replication, we investigated its binding to *Xenopus* sperm chromatin during the course of DNA replication with a time-course analysis (Fig. 1A). Consistent with previous reports, Mcm4 bound to chromatin at 5 min, indicating the licensing of origins, followed by Cdc7 binding. Chromatin binding of Sld5, a subunit of GINS complex, and Pol α was observed at 30 min, and reached a peak at 45 min concomitant with DNA replication, which starts 30–45 min after sperm DNA addition and continues at a maximal rate for a further 20–30 min in these extracts (Fig. 1B). Chromatin binding of FACT subunits, DUF140/Spt16 and DUF87/SSRP1 in *Xenopus*, was observed within 5 min, and reached saturation at 45 min, as expected from the doubling of DNA content during S phase. This result suggests that FACT binds chromatin rapidly after the addition of sperm nuclei to egg extracts (Fig. 1A).

Because chromatin binding of FACT was observed at an early stage of DNA replication, we investigated whether chromatin loading of FACT was dependent on origin licensing. After a 30-min incubation, chromatin was isolated from egg extracts supplemented with or without geminin, an inhibitor of Cdt1 that blocks origin licensing [28,29]. Fig. 1C shows that Mcm4 loaded onto chromatin in control extracts, which was completely blocked in the presence of geminin. Intriguingly, both DUF140/Spt16 and DUF87/SSRP1 were recruited to chromatin irrespective of the presence of geminin, indicating that origin licensing is not involved in the rapid binding of FACT onto chromatin.

In eukaryotic DNA replication, Orc1, a subunit of the Orc1–6 complex, is one of the earliest players to load onto chromatin [30,31]. Although chromatin binding of FACT was independent of origin licensing, it was still possible that FACT binding depends on Orc1 loading onto chromatin. Thus, we examined whether chromatin binding of FACT was dependent on Orc1. Sperm chromatin was added to egg extracts immunodepleted of Orc1 and isolated after incubating for 2 or 20 min. As shown in Fig. 1D, Orc1 and Cdc6 loaded onto chromatin at 2 min after the start of incubation in mock-treated extracts. At 20 min, chromatin binding of Cdc6 was observed to be lower, consistent with "licensing-dependent origin inactivation," as has been proposed previously [26,32]. Immunodepletion of Orc1 from extracts resulted in chromatin without Orc1 or Cdc6. However, comparable amounts of both DUF140/Spt16 and DUF87/SSRP1 were loaded onto chromatin both in mock- or Orc1-depleted extracts. We further confirmed that immunodepletion of Cdc6 from egg extracts did not affect chromatin loading of DUF140/Spt16 and DUF87/SSRP1 (data not shown). These results indicate that the recruitment of FACT
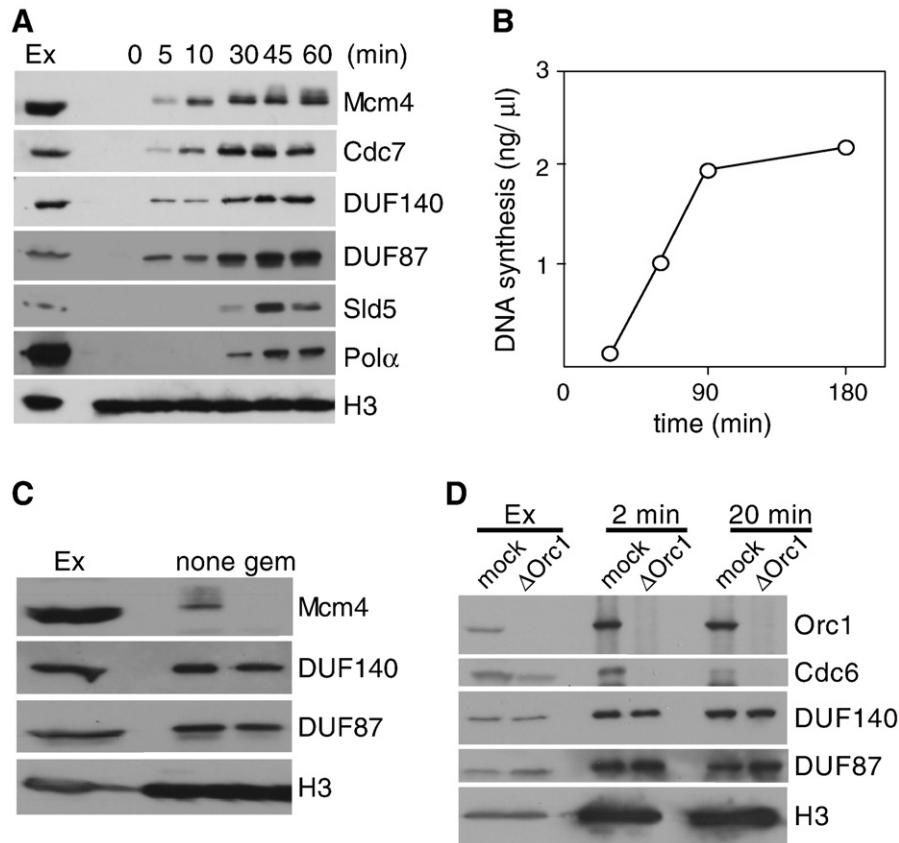
Fig. 1. FACT rapidly binds chromatin independently of origin licensing. (A) Sperm nuclei (7.5 ng DNA/μl) were incubated in extracts and isolated at indicated time points for immunoblotting. Egg extracts (Ex; 2 μl) were also applied. (B) Synthesized DNA at indicated time points was measured after incubation of sperm nuclei (3 ng DNA/μl) with *Xenopus* egg extracts. (C) Chromatin was isolated after incubation of sperm nuclei (7.5 ng DNA/μl) with extracts in the presence (gem) or absence (none) of geminin (100 nM). Isolated chromatin was subjected to immunoblotting. Egg extracts (Ex; 2 μl) were also applied. (D) Sperm nuclei (6 ng DNA/μl) were incubated for 2 or 20 min in mock-treated (mock) or Orc1-depleted (ΔOrc1) extracts. Isolated chromatin was subjected to immunoblotting. Mock-treated (mock) and Orc1-depleted (ΔOrc1) extracts (Ex; 2 μl) were also applied.

to chromatin is independent of earlier steps of DNA replication, including chromatin loading of Orc1 or Cdc6.

3.2. Stalling DNA replication by supplementing Cdc6 affects the amount of FACT on chromatin

FACT is found in a complex with the Mcm2–7 complex and RPA during eukaryotic DNA replication [16,33]. Therefore, even though chromatin loading of FACT does not depend on origin licensing, relationships between FACT and other DNA replication events might exist. To address this issue, chromatin loading of FACT was analyzed under conditions in which the stages of DNA replication were dissected by several DNA replication inhibitors that block specific stages of DNA replication (Fig. 2A). Chromatin was isolated to obtain these intermediates after incubating for 45 min, when DUF140/Spt16, DUF87/SSRP1, Mcm4, Sld5 (Fig. 2B), and Pol α (data not shown) bind to chromatin in control extracts. Chromatin loading of Mcm4 and subsequent steps were inhibited in geminin-supplemented extracts. Consistent with our previous report, Cdc6 inhibited phosphorylation of Mcm4, observed as upper-shifted bands of the immunoblot signal, and chromatin loading of Sld5 [24]. A Cdk2 inhibitor, p21, inhibited both chromatin loading of Sld5 and the subsequent loading of Pol α (data not shown) [34], but not phosphorylation of Mcm4. An inhibitor of DNA polymerases, aphidicolin, inhibited synthesis of the nascent DNA strand, but not chromatin loading of Pol α [35,36] after Cdk2-dependent loading of Sld5 onto chromatin. Intriguingly, the amount of chromatin-bound DUF140/Spt16 and DUF87/SSRP1 was markedly low in Cdc6-supplemented extracts, whereas very little change, if any, was observed in geminin-, p21-, or aphidicolin-treated extracts.

Although Cdc6 is an essential protein for origin licensing during DNA replication, it is readily destabilized from chromatin after Mcm2–7 loading [32,37]. We have previously proposed that forced accumulation of Cdc6 on chromatin after origin licensing suppresses the initiation of DNA replication [24]. We further analyzed whether chromatin binding of FACT was affected by Cdc6 in a dose-dependent manner. Chromatin binding of DUF140/Spt16 and DUF87/SSRP1 was suppressed as the amount of Cdc6 increased, which correlates well with the suppression of Mcm4 phosphorylation and chromatin loading of Sld5 (Supplementary Fig. S1) [24].

Because Cdc6 halts DNA replication after chromatin loading of Cdc7 and before Cdc7-mediated phosphorylation of Mcm2–7 complex [24], we assessed whether chromatin binding of DUF140/Spt16 and DUF87/SSRP1 was directly affected by Cdc7 kinase activity. Sperm chromatin was incubated in egg extracts immunodepleted of Cdc7 in the presence or absence of supplemented Cdc6 (Fig. 2C). Chromatin binding of DUF140/Spt16 and DUF87/SSRP1 in Cdc7-depleted extracts was not significantly different from that in mock-depleted extracts, which were severely compromised by the addition of Cdc6. In addition, phosphorylation of chromatin-bound Mcm4 was not observed in the Cdc7-depleted extracts, indicating efficient removal of Cdc7 [24,38]. Therefore, Cdc7 kinase was not directly linked to this effect even though chromatin binding of FACT was lowered by the addition of Cdc6, which inhibits DNA replication before Cdc7-mediated phosphorylation of Mcm4.

3.3. Rapidly loaded FACT is destabilized from chromatin in Cdc6-supplemented extracts

Next, we examined whether Cdc6 treatment limited the rapid loading of FACT that we have observed at the earliest stages of the
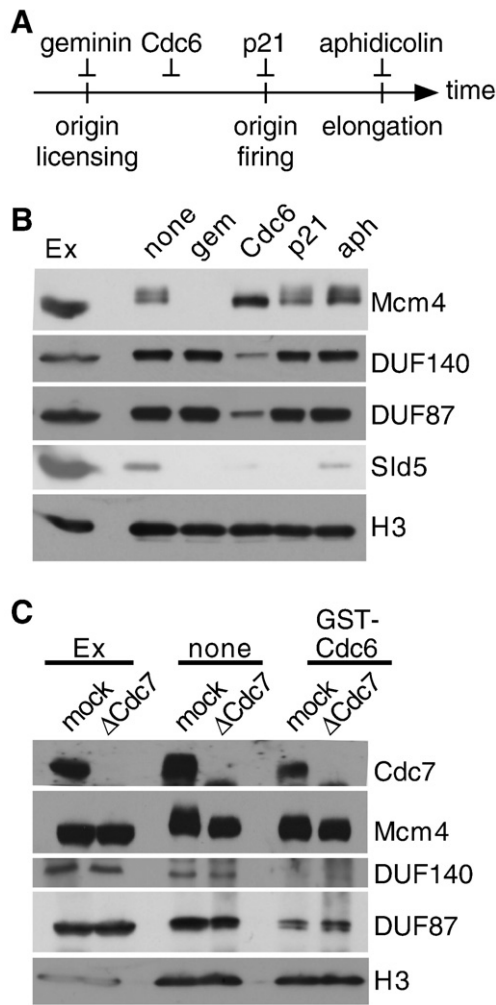
Fig. 2. Chromatin binding of FACT is affected in Cdc6-induced replication block. (A) A schematic representation of the stages of DNA replication inhibited by geminin, Cdc6, p21, or aphidicolin. (B) Chromatin was isolated after a 45 min incubation of sperm nuclei (7.5 ng DNA/µl) with extracts supplemented with buffer (none), geminin (gem; 100 nM), GST-Cdc6 (Cdc6; 700 nM), p21 (5 ng/µl) or aphidicolin (aph; 40 ng/µl) and subjected to immunoblotting. Egg extracts (Ex; 2 µl) were also applied. (C) Chromatin was isolated after a 45 min incubation of sperm nuclei (15 ng DNA/µl) with mock-treated (mock) or Cdc7-depleted (ΔCdc7) extracts in the absence (none) or presence of GST-Cdc6 (700 nM). Isolated chromatin was then subjected to immunoblotting. Mock-treated (mock) and Cdc7-depleted (ΔCdc7) extracts (Ex; 2 µl) were also applied.

Fig. 3. Initially loaded FACT is destabilized in Cdc6-induced replication block. Sperm nuclei (7.5 ng DNA/µl) were incubated in extracts supplemented with buffer (none) or GST-Cdc6 (700 nM). Chromatin was isolated at indicated time points and subjected to immunoblotting. Egg extracts (Ex; 2 µl) were also applied.

reaction, or FACT that had already been loaded onto chromatin was destabilized in Cdc6-supplemented extracts. To distinguish between these possibilities, we analyzed the time course of chromatin binding of FACT in the presence or absence of Cdc6 (Fig. 3). Intriguingly, chromatin loading of DUF140/Spt16 and DUF87/SSRP1 in Cdc6-supplemented extracts was comparable to that in control extracts until 10 min; however, chromatin-bound DUF140/Spt16 and DUF87/SSRP1 started to dissociate from chromatin at 30 min and almost disappeared later in Cdc6-supplemented extracts, in which phosphorylation of Mcm4 and chromatin binding of Sld5, Cdc45, and Pol α were severely inhibited [24]. The effect of Cdc6 on chromatin binding of FACT highly contrasted with its effect on the amount of chromatin-bound Mcm4, Cdc7, Dbf4, and Cdk2, which did not significantly differ from that in control extracts. This result suggests that the initial chromatin binding of FACT was not inhibited by Cdc6 treatment, but instead FACT that had already been loaded onto chromatin at an earlier stage was destabilized from chromatin at a later stage.
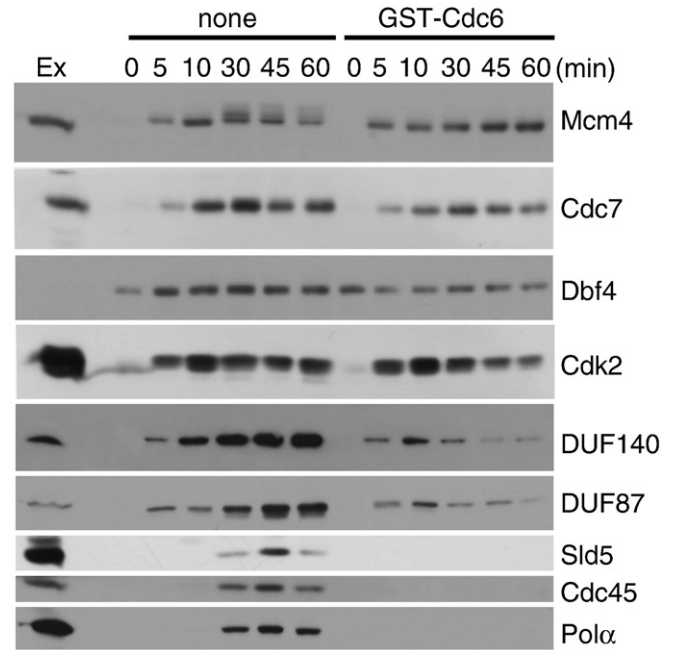
3.4. Initially loaded FACT is susceptible to removal from licensed chromatin, but not from unlicensed chromatin

To determine the step at which initially loaded FACT was destabilized, we added Cdc6 to egg extracts at various time points and investigated the chromatin-bound state of DUF140/Spt16 and DUF87/SSRP1 (Fig. 4A). We have previously shown that DNA replication is severely inhibited when Cdc6 is added at 10 min after the start of incubation or earlier, whereas the addition of Cdc6 later than 20 min has no major effects on the subsequent DNA replication (Supplementary Fig. S2) [24]. Under these conditions, chromatin binding of DUF140 and DUF87 was markedly suppressed by Cdc6 when added before 20 min, whereas adding Cdc6 later than 20 min did not have any effect, strongly correlating with the inhibition of DNA replication by Cdc6 (Fig. 4A; Supplementary Fig. S2). This result suggests that the timing of destabilization of FACT on chromatin by Cdc6 treatment is concomitant with the suppression of the initiation of DNA replication by Cdc6. Thus, we speculated that destabilization of FACT from chromatin may reflect a physiological event at an earlier stage of DNA replication that is observed with the timely addition of Cdc6.

We then sought to assess the specific stage(s) of DNA replication that trigger(s) changes in chromatin binding of FACT. Chromatin was isolated from extracts supplemented with or without Cdc6 in the presence of buffer, geminin, Cdk2-inhibitor p21, or DNA polymerase-inhibitor aphidicolin and subjected to immunoblotting (Fig. 4B). Chromatin binding of DUF140/Spt16 and DUF87/SSRP1 at 45 min was observed in control extracts, but was markedly compromised in Cdc6-treated extracts. In contrast, chromatin-bound FACT was restored when geminin was added together with Cdc6 (Fig. 4B; Supplementary Fig. S3). In contrast, p21 or aphidicolin could not recover the loss of FACT from chromatin induced by the Cdc6 addition, suggesting that destabilization of FACT from chromatin occurred after origins were licensed, but before Cdk2-dependent events occurred.
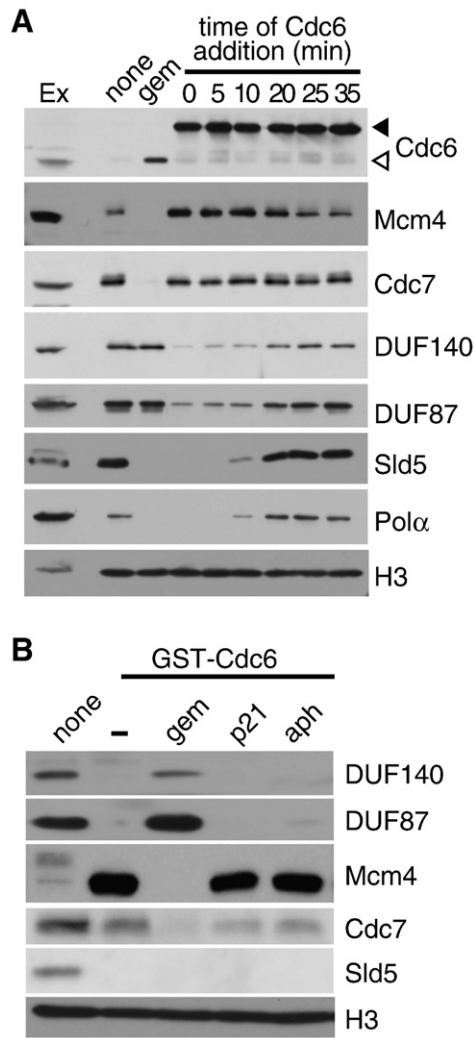
Fig. 4. FACT is destabilized from chromatin after origin licensing. (A) GST-Cdc6 (700 nM) was added to the reaction mixture at indicated times after the initiation of incubation of sperm nuclei (7.5 ng DNA/μl) with egg extracts. Chromatin was isolated at 45 min after the addition of sperm nuclei and subjected to immunoblotting. Open and closed arrowheads represent endogenous Cdc6 and GST-Cdc6, respectively. Egg extracts (Ex; 2 μl) and chromatin fractions isolated after 45 min incubations with buffer-supplemented (none) or geminin-supplemented (gem) extracts were also applied. (B) Chromatin was isolated after a 45 min incubation of sperm nuclei (7.5 ng DNA/μl) with extracts in the presence of buffer (none) or GST-Cdc6 (700 nM). None (−), geminin (gem; 100 nM), p21 (5 ng/μl), or aphidicolin (aph; 40 ng/μl) was also added to the GST-Cdc6-supplemented extracts as indicated. Isolated chromatin was subjected to immunoblotting.

3.5. Chromatin binding of FACT after origin licensing is critical to promote DNA replication

Next, we assessed whether chromatin loading of FACT can be retrieved upon release from Cdc6-mediated replication arrest by transferring the isolated chromatin into fresh extracts without supplemented Cdc6. To achieve this, chromatin was isolated from extracts supplemented with or without GST-Cdc6 after an incubation of 20 min when binding of FACT on chromatin isolated from Cdc6-supplemented extracts is lower, yet not completely out of FACT, compared to control chromatin (Fig. 5A and refer to Fig. 3). The isolated chromatin was then transferred to fresh extracts with geminin that prevents de novo origin licensing, and chromatin binding of FACT, Mcm4, and other proteins in the second incubation was monitored (Fig. 5A). Consistent with our previous report [24], phosphorylation of Mcm4 and chromatin binding of Sld5 that had been suppressed in Cdc6-supplemented extracts started to rapidly

recover with a time lag allowing the removal of excess Cdc6 from chromatin when compared to control chromatin. Chromatin binding of both DUF140/Spt16 and DUF87/SSRP1 was also retrieved in a similar kinetics correspondingly. This result indicates that the inhibition of chromatin binding of FACT in the presence of Cdc6 is reversible and also that FACT binds to chromatin after origin licensing.

Finally, the chromatin transfer experiments were carried out to assess the significance of FACT binding to chromatin after origin licensing (Fig. 5B). Chromatin isolated from Cdc6-supplemented extracts (Cdc6 chromatin), which allows initial binding of FACT before licensing but causes almost complete dissociation later at 45 min (see Fig. 3), was prepared and incubated with extracts that had been immunodepleted of DUF140/Spt16 (Fig. 5C). After further incubation, the amount of synthesized DNA was measured (Fig. 5D). DNA replication of Cdc6 chromatin or untreated sperm chromatin was severely compromised in DUF140/Spt16-immunodepleted extracts, while replication of chromatin isolated after incubation with control extracts (control chromatin) was well replicated in these immunodepleted extracts. In mock-treated extracts, replication of Cdc6 chromatin was comparable to that of untreated sperm chromatin or control chromatin. This result suggests that chromatin binding of FACT after origin licensing is required to support DNA replication.

4. Discussion

Several lines of evidence suggest that the conversion of chromatin structure plays a critical role in the initiation and progression of S phase [39–43]. Despite extensive studies that have investigated the temporal regulation and coordination of DNA replication, a clear understanding of the cellular factors responsible for the conversion of chromatin structure to facilitate initiation or progression of S phase remains largely elusive. The involvement of histone chaperone FACT during DNA replication was previously documented by genetic and biochemical studies on yeast, Xenopus and human FACT homologs [15–17,20,21]. However, how FACT behaves during DNA replication together with its links with DNA replication related events has not yet been fully substantiated.

Here, we reveal the chromatin-binding behavior of FACT and its relationship with various DNA replication events. We found that FACT rapidly bound chromatin without the involvement of origin licensing and gradually reached a plateau as DNA replication proceeds. This finding is consistent with a previous report of chromatin proteome analysis showing residual early FACT binding in the presence of geminin [44]. The presence of FACT on chromatin throughout the course of DNA replication raised the question as to the stage(s) when FACT functions for the efficient replication of eukaryotic chromatin. By dissecting several steps of DNA replication, we found that FACT rapidly bound to chromatin, but dissociated in the later phase when DNA replication was blocked between origin licensing and origin firing steps by the addition of Cdc6 to extracts at earlier time points. These results suggest the presence of a specific fragile period of FACT on chromatin in the earlier stages of DNA replication. In contrast, the addition of Cdc6 after 20 min was not sufficient to destabilize FACT from chromatin, implying the establishment of a new interaction with chromatin, which is different from the rapid association of FACT.

In search of the step at which FACT starts dissociating from chromatin, we found that FACT kept on binding with chromatin when DNA replication was halted before origin licensing by cotreatment of geminin with Cdc6. Therefore, we speculated that initially loaded FACT is susceptible to destabilization after origin licensing, which can be visualized by making time-specific use of Cdc6 as an inhibitor of DNA replication initiation. From this point of view, FACT should reassociate with chromatin after a step sensitive to the additive Cdc6. Indeed, FACT regained its ability to load onto chromatin after the Cdc6-induced inhibition of DNA replication was terminated. We also propose that the second chromatin-binding mode of FACT after origin

licensing is critical for efficient DNA replication, which further validates previous reports showing the involvement of FACT during DNA replication [15–17]. This bimodal chromatin binding of FACT suggests that FACT has two functions, a major one during S phase, and an earlier (and possibly less important) one executed soon after DNA addition to the extracts.
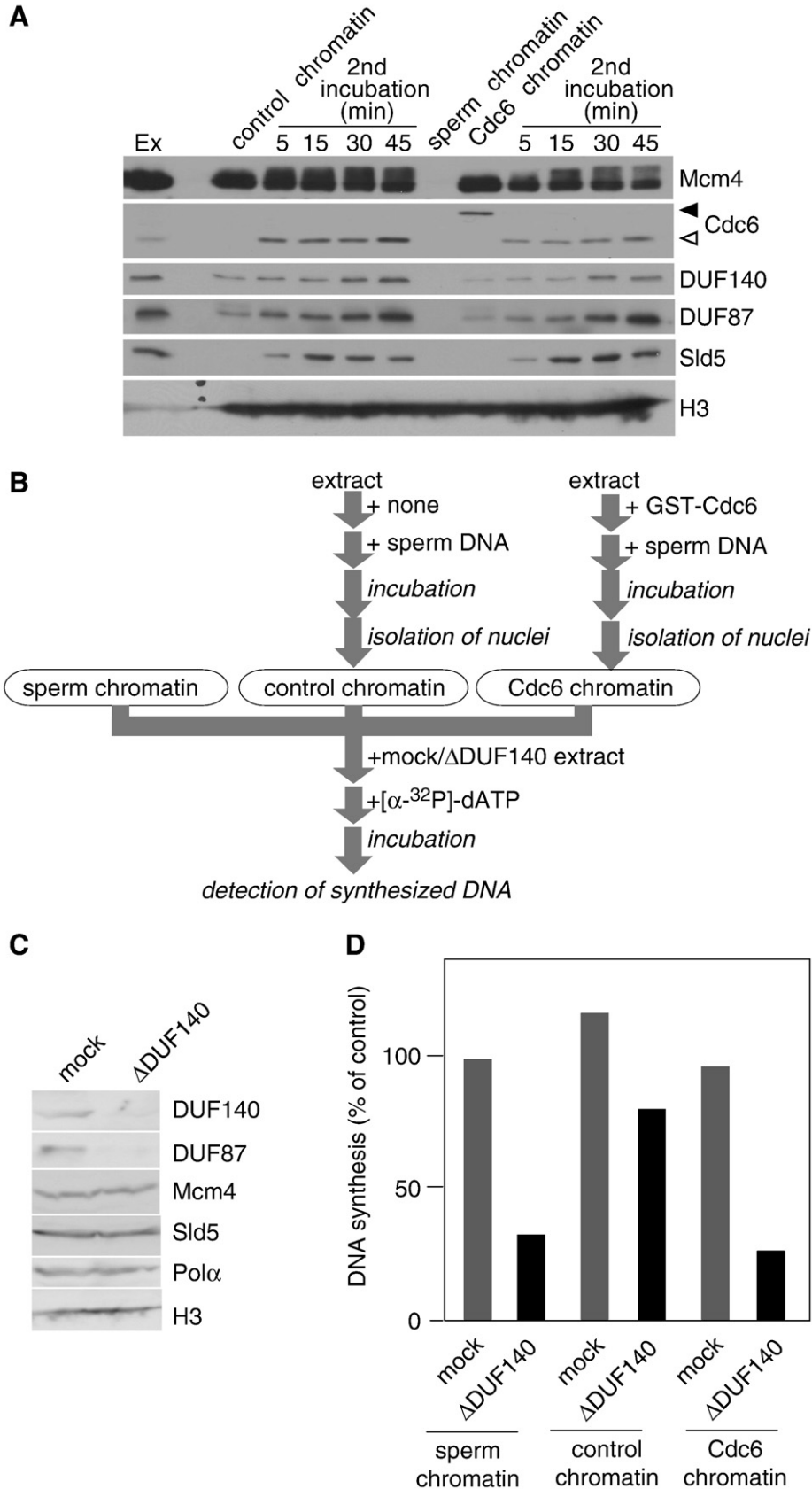
In this study, we found that immunodepletion of Orc1 (Fig. 1D), Cdc6 (data not shown), or Cdc7 (Fig. 2C) from egg extracts did not alter chromatin loading of FACT. Therefore, it is likely that the rapid loading of FACT onto chromatin is dependent on other protein(s) that is (are) not directly involved in known origin licensing or early DNA replication events. Recently, a study in budding yeast has shown that Rtt101-dependent ubiquitylation of FACT targets it for replication-dependent recruitment to chromatin [45]. Thus, post-translational modification(s) of FACT may also contribute to link dynamic changes of chromatin binding of FACT during DNA replication in *Xenopus* egg extracts.

At least two interpretations exist for the nature of this bimodal chromatin binding of FACT. One is that FACT accumulating on chromatin gradually undergoes a change from fragile to stable mode, and the second is that the rapidly associated FACT dissociates from chromatin after origin licensing, followed by another recruitment of FACT in a different mode for the efficient replication of chromatin. In the former case, a possible explanation for the Cdc6 effect is that excess Cdc6 prevents the progression from the rapid and fragile mode of FACT binding to a secondary and stable mode. However, we hypothesize that the second possibility is more likely because FACT stably associates with chromatin even in the excess Cdc6 condition in which geminin prevents licensing, suggesting that rapid chromatin binding of FACT is not fragile or sensitive to Cdc6. As Cdc6 halts DNA replication between origin licensing and origin firing steps [24], this effect resultantly prevents the progression of chromatin from the primary state, where the rapidly associated FACT is dissociating after its early function has been executed, into the secondary mode after origin licensing. An outline of our hypothesis is depicted in Fig. 6; however, other interpretations may explain the results, and further studies will be needed to clarify the detailed dynamics and functions of FACT during DNA replication.

Because FACT has a strong affinity for histones, we hypothesize that the rapid and licensing-independent binding of FACT is more likely to be universal for the DNA transactions. A previous report demonstrated that the yeast Mcm2–7, with the assistance of the GINS complex, interacts with the replisome progression complex that includes the yeast FACT heterodimer [17]. In HeLa cells, FACT has been shown to interact directly with Mcm4 and facilitate the helicase activity of Mcm2–7 complex *in vitro* [16]. In light of these reports and our current observations, the binding mode of FACT on chromatin may undergo a change upon Mcm2–7 loading to establish its functional link to Mcm2–7. In this case, there should be at least a subset of FACT on chromatin which (re)localizes near Mcm2–7 helicase during replication initiation to facilitate its helicase activity during DNA replication. Interestingly, the interaction domain of human Mcm4 with FACT lies between 120 and 250 amino acids [16], and its homologous region of yeast Mcm4 overlaps with the locus to bind with and to be phosphorylated by DDK [46]. Given that Cdc6 inhibits Cdc7-mediated hyperphosphorylation of Mcm4, Cdc6 most probably conceals the N-terminal domain of Mcm4 from interacting with FACT as well as Cdc7, which results in the inhibition of FACT binding with Mcm4 that takes place after origin licensing. Indeed, we observed that addition of recombinant Mcm4 to egg extracts tended to sequester chromatin binding of FACT, although not as drastically as Cdc6 (Supplementary Fig. S4). Furthermore, the effect of the addition of Mcm4 seemed synergistic with the effect of Cdc6.
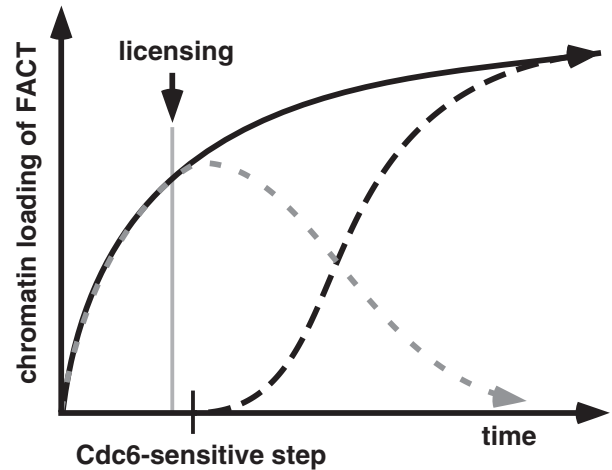

Fig. 6. An outline of the chromatin-binding patterns of FACT. FACT rapidly binds chromatin without the involvement of DNA replication events and gradually reaches peak at origin licensing (broken line in gray). Following licensing, initially loaded FACT is destabilized or converted into a secondary mode of binding that is gradually increased as DNA synthesis proceeds (broken line in black). The total amount of FACT on chromatin is depicted as black continuous line. Cdc6 inhibits a step after origin licensing but before the initiation of the later phase of FACT binding, thereby allowing observation of dissociation of FACT in the first phase of chromatin binding.

These observations support the idea that Cdc6 does not directly act upon FACT to halt its chromatin binding; rather, it conceals the interaction domain of Mcm4 with FACT.

Furthermore, a recent report has suggested the existence of two distinct FACT-MCM subassemblies, FACT Mcm2/3/4/5 and FACT-Mcm2/4/6/7, which act in distinct and possibly sequential steps during origin establishment and replication initiation [47]. Interestingly, they showed that FACT-Mcm2/3/4/5 subassembly coprecipitated with Cdc6 and Orc1, whereas FACT-Mcm2/4/6/7 did not. This finding supports our observation that chromatin binding of FACT undergoes a change after origin licensing when Cdc6 is mostly absent from chromatin. However, the interaction of other proteins involved in DNA replication initiation with these subassemblies still remains unclear.

The presence of FACT on chromatin throughout the course of DNA replication is in contrast with most other replication factors that load onto chromatin only at stages to execute their function, thereby making it difficult to address the functional involvement of FACT with specific stages of DNA replication. While previous studies did not dissect the behavioral aspect of chromatin binding of FACT, the present study further indicates that the chromatin binding of FACT after origin licensing plays an important role during DNA replication, which is distinct from its association at earlier stages by the unique approach of combining *Xenopus* egg extract cell-free system with the usage of Cdc6 as a novel tool to halt the initiation of DNA replication.

Based on these results, we propose that FACT binds to chromatin in at least two distinct ways and establishes equilibrium in its chromatin binding before and after origin licensing, which is reflected as a constant binding onto chromatin throughout the process of DNA replication. Primarily bound FACT, which undergoes a rapid and drastic change upon origin licensing, might play its well-known role

Fig. 5. DNA replication is dependent on chromatin binding of FACT after origin licensing. (A) Sperm nuclei (15 ng DNA/μl) were incubated for 20 min in extracts supplemented without or with GST-Cdc6 (700 nM) and were isolated (control chromatin and Cdc6 chromatin, respectively). The isolated nuclei were incubated with fresh extracts supplemented with geminin (100 nM). FACT, Mcm4, Cdc6, Sld5, and histone H3 on the chromatin at indicated time points were detected by immunoblotting. Open and closed arrowheads represent endogenous Cdc6 and GST-Cdc6, respectively. Egg extracts (Ex; 2 μl) and untreated sperm chromatin (sperm chromatin) were also applied. (B) A schematic representation of the experiment in (C) and (D). (C) Mock-treated (mock) and DUF140-depleted (ΔDUF140) extracts (Ex; 2 μl) used in (D) were subjected to immunoblotting. (D) DNA synthesis on sperm nuclei was measured after incubation for 90 min with mock-treated (mock) or DUF140/Spt16-depleted (ΔDUF140) extracts (sperm chromatin). Sperm nuclei (15 ng DNA/μl) were incubated in extracts supplemented with buffer (control chromatin) or 700 nM of GST-Cdc6 (Cdc6 chromatin) for 45 min, followed by nuclear isolation. DNA synthesis was assayed after a 90 min incubation of control or Cdc6 chromatin with mock or ΔDUF140 extracts containing 100 nM of geminin. DNA synthesis is represented as a percentage of DNA synthesis in the samples of interest to that after incubation with normal extracts for 90 min.

as a histone chaperone followed by DNA replication-coupled mode of chromatin binding. This "reorganization" of FACT binding may thus contribute to the establishment of its functional link to the DNA replication machinery.

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Author contributions

L.R.K. and S.T. designed the study. L.R.K. and N.W. performed experiments. H.M. contributed DUF140 and DUF87 antibodies, J.J.B./A.J.S. contributed Orc1 antibody, and S.W./A.F. contributed Cdc7 antibody. L.R.K. and S.T. discussed and wrote the manuscript with M.S., H.M., J.J.B., M.H., and T.E.

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