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HBO1 Histone Acetylase Activity Is Essential for DNA Replication Licensing and Inhibited by Geminin

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SUMMARY

HBO1, an H4-specific histone acetylase, is a coactivator of the DNA replication licensing factor Cdt1. HBO1 acetylase activity is required for licensing, because a histone acetylase (HAT)-defective mutant of HBO1 bound at origins is unable to load the MCM complex. H4 acetylation at origins is cell-cycle regulated, with maximal activity at the G1/S transition, and coexpression of HBO1 and Jade-1 increases histone acetylation and MCM complex loading. Overexpression of the Set8 histone H4 tail-binding domain specifically inhibits MCM loading, suggesting that histones are a physiologically relevant target for licensing. Lastly, Geminin inhibits HBO1 acetylase activity in the context of a Cdt1-HBO1 complex, and it associates with origins and inhibits H4 acetylation and licensing *in vivo*. Thus, H4 acetylation at origins by HBO1 is critical for replication licensing by Cdt1, and negative regulation of licensing by Geminin is likely to involve inhibition of HBO1 histone acetylase activity.

INTRODUCTION

In eukaryotes, DNA replication is controlled so that the genome is replicated only once per cell cycle. The first step of DNA replication is the formation of the pre-replication complex (pre-RC) on origins of replication distributed throughout the genome. The pre-RC contains the origin recognition complex (ORC), Cdt1, Cdc6, and the MCM (minichromosome maintenance) complex that are sequentially assembled onto replication origins in the context of chromatin. ORC associates with DNA replication origins throughout the entire cell cycle. When cells exit mitosis, the Cdt1 and Cdc6 licensing factors are loaded onto origins, followed by the MCM complex, the putative replicative helicase. The resulting pre-RC is “licensed” for replication that will occur in the subsequent S phase (Thömmes and Blow, 1997; Bell and Dutta, 2002). After replication origins fire and DNA synthesis is initiated, the pre-RC disassembles, and new pre-RC formation is prevented in S phase, thereby restricting DNA synthesis to once per cell cycle.

Regulation of Cdt1 is the key event in replication licensing that permits the ordered assembly and disassembly of the pre-RC (Arias and Walter, 2007). Cdt1 function is inhibited in S phase by ubiquitin-dependent proteolysis, thereby restricting expression to the G1 phase of the cell cycle (Nishitani et al., 2001; Zhong et al., 2003). In addition, Cdt1 activity is inhibited by Geminin in S phase via a direct interaction between these two proteins (Wohlschlegel et al., 2000; Tada et al., 2001). These mechanisms of Cdt1 regulation permit licensing and, hence, subsequent DNA replication to occur only once per cell cycle. Misregulation of Cdt1, by overexpression or by a mutant derivative that is insensitive to proteolysis, results in rereplication and genome instability (Vaziri et al., 2003; Saxena and Dutta, 2005; Tatsumi et al., 2006).

Histone acetylation is linked to pre-RC assembly and the control of initiation of DNA replication. Early-firing origins are typically localized in genomic regions that are transcribed and contain hyperacetylated chromatin, whereas late-firing origins lie in silenced heterochromatic domains (Kemp et al., 2005; Zhou et al., 2005; Kamani et al., 2007; Lucas et al., 2007; Goren et al., 2008). In addition, histone acetylation is involved in origin activation during early development in *Xenopus* (Danis et al., 2004) and at the chorion gene loci in *Drosophila* follicle cells (Aggarwal and Calvi, 2004; Hartl et al., 2007). More generally, diverse chromatin-modifying activities can associate with pre-RC components in protein-binding assays and/or genetically alter replication initiation (Takei et al., 2001, 2002; Vogelauer et al., 2002; Aggarwal and Calvi, 2004; Danis et al., 2004; Pappas et al., 2004; Doyon et al., 2006; Iizuka et al., 2006; Sugimoto et al., 2008; Crampton et al., 2008; Yin et al., 2008). For example, ORC-dependent chromatin remodeling contributes to optimal loading of the MCM complex onto origins in yeast (Lipford and Bell, 2001), and alteration of the ordered nucleosome arrangement at the human c-Myc replicator selectively decreases MCM complex loading (Ghosh et al., 2006).

HBO1 (human acetylase binding to ORC1; also known as KAT7 and MYST2) is an H4-specific histone acetylase that interacts with transcriptional activator proteins (Georgiakaki et al., 2006; Miotto et al., 2006; Miotto and Struhl, 2006), mRNA coding regions (Saksouk et al., 2009), and MCM2 and ORC1 (Iizuka and Stillman, 1999; Burke et al., 2001). HBO1 is required for licensing and DNA replication (Doyon et al., 2006; Iizuka et al., 2006), and in *Drosophila* follicle cells it increases origin activity when artificially recruited to a synthetic replication origin (Aggarwal and Calvi, 2004). In previous work, we showed that HBO1 associates

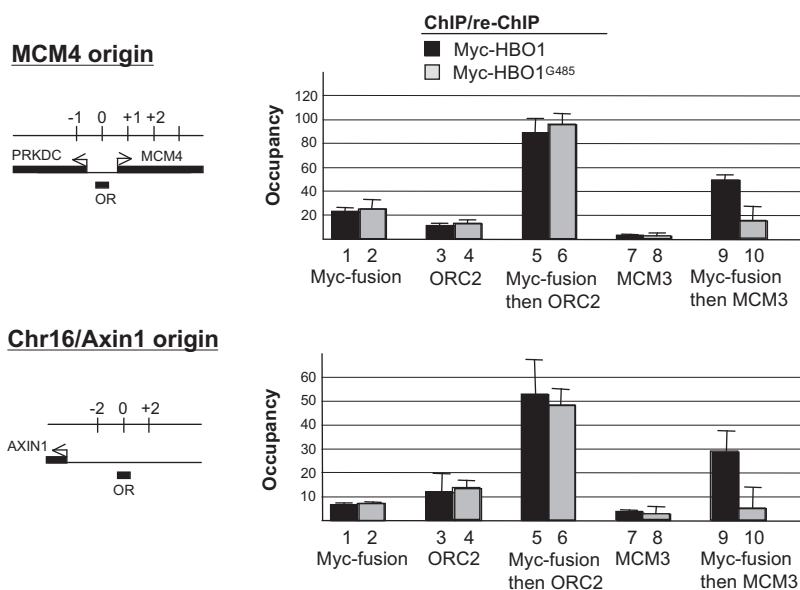


Figure 1. HBO1 Acetylase Activity Is Essential for DNA Licensing

DNA fragments bound by Myc-HBO1 and HAT-inactive Myc-HBO1^{G485} were analyzed by sequential ChIP analysis for ORC and MCM complexes co-occupancy at the MCM4 and Chr16/Axin origin ($n = 2$). The genomic regions around these replication origins (OR) are shown with neighboring genes (arrows), and positions of primer pairs (black bars) are indicated (coordinates flanking the origin are in kb). Values are expressed as relative occupancy over the control background region (mean \pm SD).

with replication origins specifically during the G1 phase of the cell cycle (Miotto and Struhl, 2008). HBO1 association with origins depends on Cdt1, but is independent of Geminin. HBO1 directly interacts with Cdt1, and it enhances Cdt1-dependent rereplication. Thus, HBO1 plays a direct role at replication origins as a coactivator of the Cdt1 licensing factor, although the mechanism is unknown.

Here, we show that H4 acetylation by HBO1 is critical for replication licensing and that Geminin inhibits HBO1 acetylase activity in the context of a Cdt1-HBO1 complex. Thus, by analogy with activator proteins targeting histone acetylases to enhancers to stimulate transcription, our results suggest that targeted histone acetylation at replication origins is a crucial and regulated step for DNA replication.

RESULTS

HBO1 Acetylase Activity Is Essential for Licensing of Replication Origins

HBO1 plays a critical role in replication licensing (Iizuka et al., 2006; Miotto and Struhl, 2008), but the importance of the histone acetylase activity is unknown. To address this issue, we analyzed human cells expressing HBO1^{G485}, which contains a mutation of an invariant glycine in the histone acetyltransferase (HAT) domain that abolishes enzymatic activity (Iizuka et al., 2008). HBO1^{G485} association with replication origins is comparable to that of wild-type HBO1 (Figure 1), indicating that the histone acetylase activity is not important for HBO1 association with origins. In contrast, overexpression of HBO1^{G485} impairs BrdU incorporation, indicating a defect in DNA replication (Figure S1).

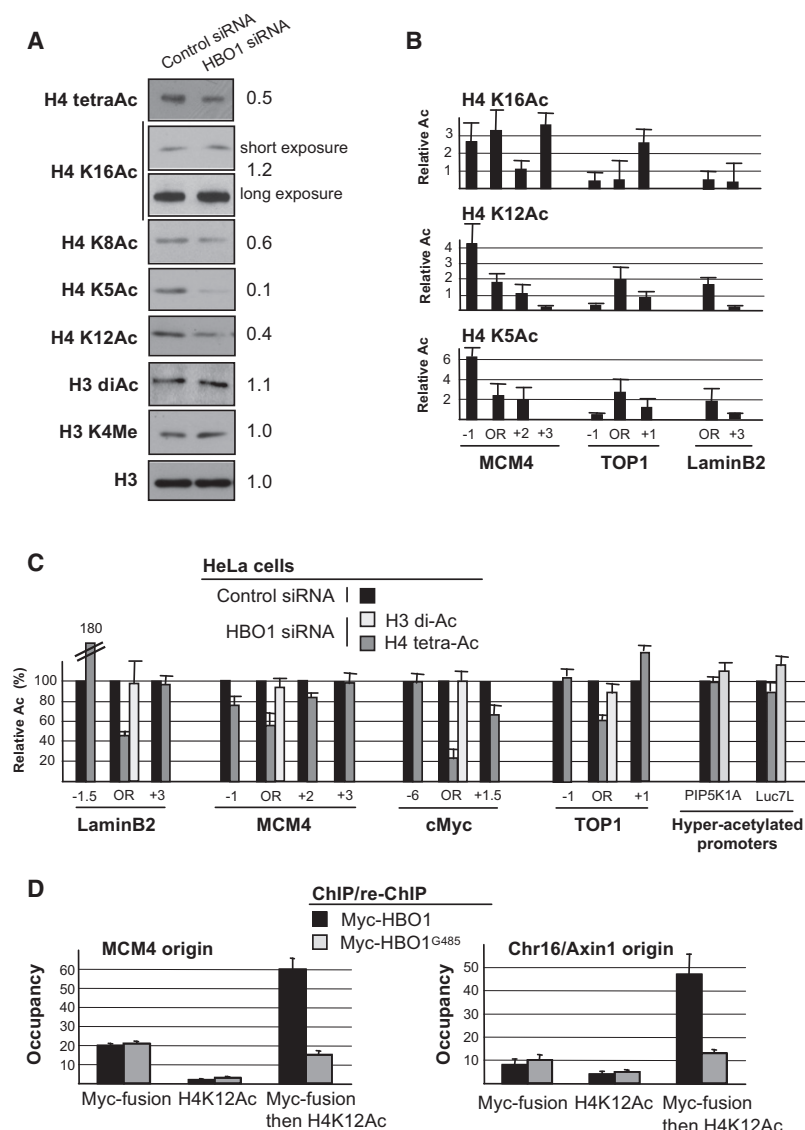
To directly measure replication licensing, we used sequential chromatin immunoprecipitation (ChIP) to determine the association of ORC2 and MCM3 at HBO1- or HBO1^{G485}-bound origins (Figure 1). Unlike the standard approach of monitoring licensing in crude chromatin fractions (Iizuka et al., 2006; Miotto and Struhl, 2008), sequential ChIP directly monitors MCM loading at a repli-

cation origin as a function of HBO1 activity. As HBO1 selectively associates with origins during G1 (Miotto and Struhl, 2008), MCM and ORC association detected in the second immunoprecipitation reflects MCM and ORC binding at the time of DNA licensing. As expected, HBO1-bound origins show significant coassociation of ORC2 (compare lane 5 with lanes 1 and 3) and MCM3 (compare lane 9 with lanes 1 and 7); i.e., the fold enrichments of the sequential ChIP samples are significantly higher than the fold enrichment for HBO1 alone. In contrast, while HBO1^{G485}-bound origins show comparable levels of ORC complex co-occupancy (lanes 5 and 6), the level of MCM complex co-occupancy is clearly reduced in comparison to that observed at HBO1-bound origins (lanes 9 and 10). The fold enrichment of the HBO1^{G485} + MCM3 sequential ChIP sample is comparable to that of the individual HBO1^{G485} ChIP sample, indicating that little or no MCM3 associates with HBO1^{G485}-bound origins. Therefore, recruitment of a HAT-inactive HBO1 derivative at origins selectively blocks MCM complex loading in G1, demonstrating that HBO1 acetylase activity is directly involved in DNA licensing prior to MCM complex loading.

H4 Acetylation at Replication Origins Depends on HBO1 and Is Cell-Cycle Regulated

H4 acetylation plays an important role in controlling chorion origin activity in *Drosophila* follicle cells (Aggarwal and Calvi, 2004; Hartl et al., 2007), and HBO1 is recruited to origins by Cdt1 (Miotto and Struhl, 2008). We therefore monitored the profile of H4 acetylation on genomic regions encompassing well-characterized origins. A peak of H4 acetylation is observed on all origins tested, with lower levels of acetylation at flanking regions. Consistent with the selectivity of HBO1 acetylation for lysine residues (Figure 2A) (Doyon et al., 2006), H4 acetylation at K5 and K12, but not K16, is specifically enriched at origins (Figure 2B).

HBO1 is responsible for most H4 acetylation in human cells, because depletion of HBO1 substantially reduces the overall level of H4 acetylation (Figure 2A) (Doyon et al., 2006). This loss of H4 but not H3 acetylation upon HBO1 depletion is also observed at origins (Figures 2C and S2A). HBO1 depletion does not affect the expression of genes localized in the vicinity of origins (Figure S2B), indicating that loss of H4 acetylation at origins is not an indirect effect of transcription. Interestingly, some promoter regions hyperacetylated at H4 are not affected by HBO1 depletion (Figure 2C), presumably due to targeted

**Figure 2. HBO1 Controls H4 Acetylation at Origins**

(A) Bulk histones from control and HBO1-depleted HeLa cells were analyzed by western blots with antibodies directed against the indicated modifications of H4 and H3. Total H3 serves as a loading control. The relative acetylation level in HBO1-depleted cells as compared to control cells is indicated to the right of each panel.

(B) Acetylation levels (mean \pm SD) of the indicated H4 lysine at origins and surrounding regions ($n = 3$).

(C) Acetylation levels (mean \pm SD) of H3 and H4 at the indicated origins and constitutively hyperacetylated promoters in HeLa cells depleted of HBO1 expressed as a percent of the levels in control cells ($n = 3$).

(D) DNA fragments bound by Myc-HBO1 and HAT inactive Myc-HBO1^{G485} were analyzed by sequential ChIP analysis for H4-K12 acetylation (H4-K12Ac) co-occupancy at the MCM4 and Chr16/Axin origin ($n = 2$).

G1/S is significantly reduced in comparison to G1 (Miotto and Struhl, 2008). However, these origins are in close proximity to an active promoter or coding sequence (Figure 2A), suggesting that transcriptional activity may maintain H4 acetylation during S phase. Indeed, H4 acetylation at isolated origins not near annotated promoter and coding sequences (Cadoret et al., 2008) (Figure S3) rises during G1 and decreases when cells are staged in S phase (Figure 3B). Thus, on isolated origins, G1-specific H4 acetylation concomitant with HBO1 binding is required for MCM complex loading. In addition, H4 deacetylation is not required for initiation of replication and MCM helicase activation, as some origins have significant level of H4 acetylation during S phase.

Histone H4 Acetylation at Origins Influences MCM Complex Loading

HBO1 mediates targeted H4 acetylation at origins, and its HAT activity is required for

recruitment of an H4 acetylase distinct from HBO1. Thus, recruitment of HBO1 to origins results in a peak of H4 acetylation.

We confirmed that HBO1 acetylase activity is responsible for H4 acetylation at origins by performing a sequential ChIP experiment (Figure 2D). As expected, HBO1-bound origins show significant coassociation of H4-K12 acetylation. In contrast, at HBO1^{G485}-bound origins, the level of H4-K12 acetylation co-occupancy is clearly reduced in comparison to that observed at HBO1-bound origins. Thus, the peak of H4 acetylation at origins is induced by HBO1 activity.

The above results and the fact that HBO1 associates with origins in a cell-cycle-dependent manner (Miotto and Struhl, 2008) suggest that licensing is associated with a transient increase in H4 acetylation at origins. Indeed, H4 acetylation at all origins tested is 3-fold lower in cells staged in G2/M than in G1 (Figure 3A). Unexpectedly, H4 acetylation levels at the TOP1, LaminB2, and MCM4 origins are similar in cells staged in G1 and G1/S (Figure 3A), even though HBO1 association at

licensing, suggesting that H4 acetylation is required for loading of the MCM complex. However, as is typical for histone acetylases, HBO1 acetylates nonhistone substrates in vitro, including ORC2, Geminin, MCM2, and Cdc6 (Iizuka et al., 2006). To establish that H4 acetylation is important for MCM complex loading, we utilized two independent approaches in which proteins presumed to specifically affect acetylation of H4 but not nonhistone substrates were introduced into cells.

First, we analyzed the HBO1 cofactor Jade-1/PHF17. Jade-1 and HBO1 are components of two ING complexes implicated in DNA replication (Doyon et al., 2006), and Jade-1 stabilizes HBO1 in the nucleus and increases HBO1 association with chromatin through its PHD domains (Foy et al., 2008). Overexpression of HBO1 alone barely increases H4 acetylation, whereas coexpression of HBO1 and Jade-1 increases H4 acetylation levels (Foy et al., 2008). Coexpression of Jade-1 and HBO1 strongly enhances MCM loading, whereas overexpression of HBO1 alone does not (Figure 4A). Importantly, the synergy of HBO1 and

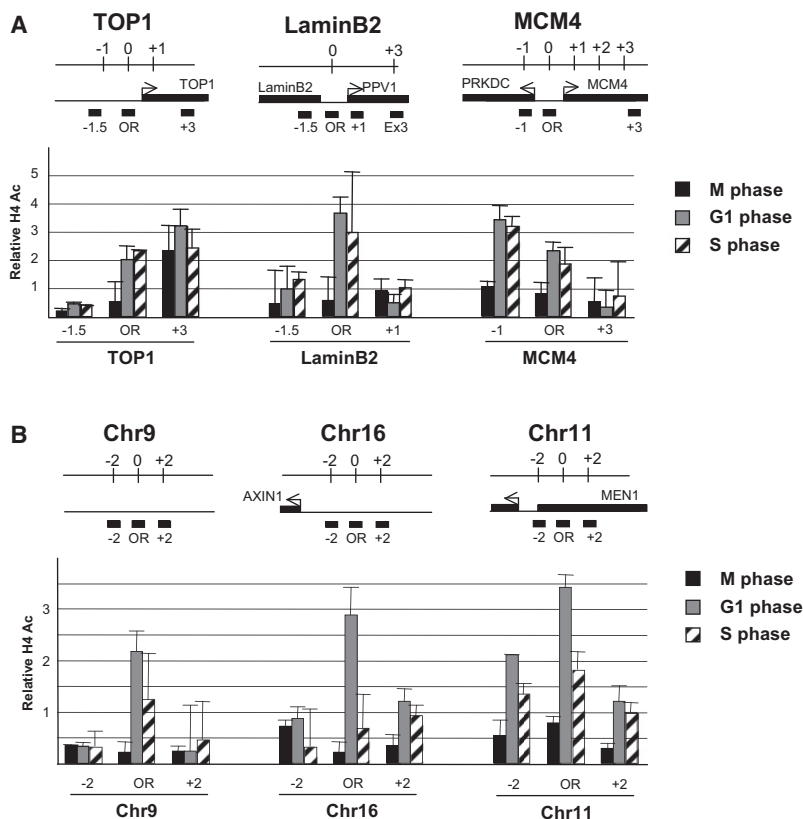


Figure 3. H4 Acetylation at Origins is Cell-Cycle Regulated

(A) H4 acetylation levels (mean \pm SD) at replication origins and flanking regions (indicated in kb from the origin) in cells at the indicated stages of the cell cycle ($n = 3$). The genomic regions around these replication origins (OR) are shown with neighboring genes (arrows), and positions of primer pairs (black bars) are indicated (coordinates flanking the origin are in kb).

(B) Same as above, except that the origins were isolated from annotated promoters.

Jade-1 for MCM complex loading is not observed in parallel experiments involving the catalytically inactive HBO1^{G485} derivative (Figure 4B). Therefore, increased HBO1 association with chromatin and H4 hyperacetylation is sufficient to promote excess MCM loading. Nevertheless, cells overexpressing Jade-1 and HBO1 show no detectable rereplication (data not shown), as also observed for cells treated with HDAC inhibitors (data not shown) or expressing HBO1 alone (Miotto and Struhl, 2008).

Second, we analyzed MCM complex loading in cells where H4 acetylation is presumed to be specifically blocked in G1 via the histone-binding domain (HBD) of the Set8 H4-K20 histone methylase. The Set8 HBD interacts with H4 tails, lacks proteolytic destruction sites, and accumulates in G1 (Yin et al., 2008). As previously reported (Yin et al., 2008), overexpression of Set8-HBD but not Set8 during G1 reduces bulk H4 acetylation on histone H4 residues K5, K8, and K12, but not K16, and blocks the cell-cycle progression prior to S phase entry (Figure 5A). Importantly, overexpression of Set8-HBD but not full-length Set8 also blocks MCM complex association at origins (Figure 5B) without affecting loading of HBO1, Cdt1, Cdc6, and the ORC complex or expression of MCM components (Figure 5C). As overexpression of Set8-HBD is unlikely to inhibit acetylation of nonhistone substrates, this observation strongly argues that histones are the physiological substrate for HBO1 that is required for efficient MCM complex loading.

To exclude the possibility that Set8-HBD indirectly affects H4 acetylation and MCM complex recruitment due to the block in

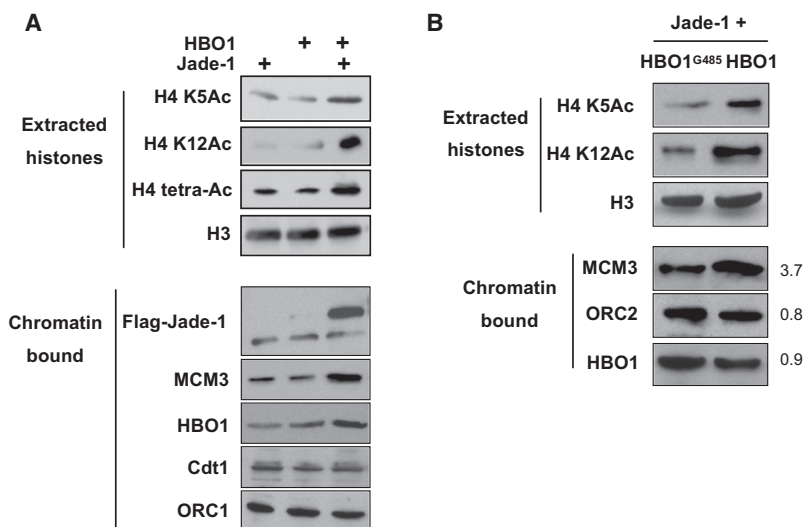


Figure 4. Coexpression of HBO1 and Jade-1 Stimulates H4 Acetylation and MCM Complex Loading

(A) Western blot analysis showing H4 acetylation status (extracted histones; H3 level serves as the internal control) and presence of the indicated proteins within chromatin in cells overexpressing Jade-1 or HBO1 or both.

(B) Similar experiment in cells coexpressing Jade-1 with either HBO1 or HAT-defective derivative HBO1^{G485}.

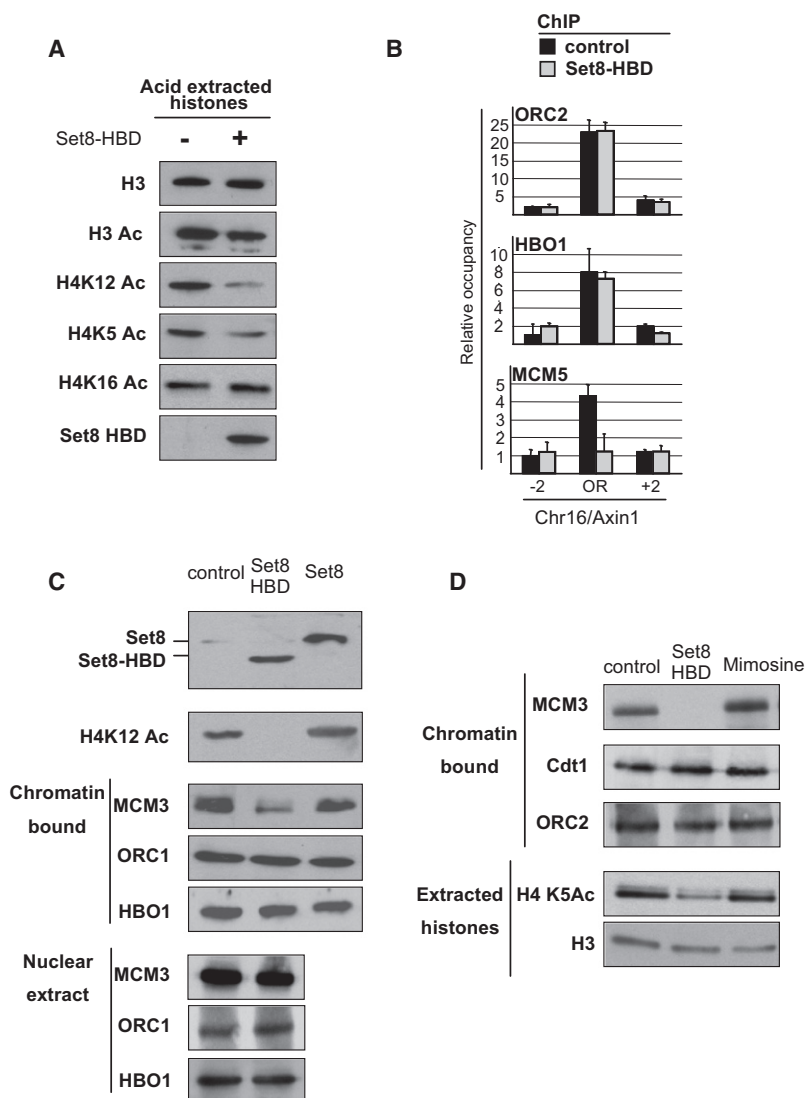


Figure 5. Expression of the Set8 Histone Tail-Binding Domain Inhibits H4 Acetylation and MCM Complex Loading

(A) Western blot analysis to measure acetylation at the indicated residues in acid-extracted histones prepared from HeLa cells that do or do not express the HBD of Set8. (B) Association of ORC2, HBO1, and MCM5 (mean \pm SD) at the Chr16/Axin1 origin detected by ChIP in cells that do or do not express Set8-HBD ($n = 3$).

(C) Comparison of full-length Set8 and Set8-HBD effects on H4 acetylation and levels of MCM3, ORC1, and HBO1 in chromatin and nuclear extract.

(D) Western blot analysis to examine the effect of mimosine and Set8-HBD (blocks in early S phase) on H4 acetylation and levels of MCM3, Cdt1, and ORC2 in the chromatin.

cells (Iizuka et al., 2006; Miotto and Struhl, 2008), Set8-HBD expression does not alter the accumulation of pre-RC subunits (Figure 5C). Second, in contrast to MCM complex loading, chromatin association of Pol II, TBP, TBP-associated factors (TAFs), TFIIIB, TFIIH (CCNH subunit), and Mediator (Med26 subunit) are not affected by Set8-HBD expression (Figure S4A) or HBO1 depletion (Figure S4B). Third, Set8-HBD expression and HBO1 depletion do not affect mRNA levels (Figure S2B and data not shown) or Pol II occupancy in the coding sequence of all genes tested (Figure S4C), indicating that low levels of H4 acetylation do not have a general effect on Pol II transcription. These observations are consistent with transcription-independent replication assays in *Xenopus* extracts showing the involvement of HBO1 in pre-RC assembly prior to MCM complex loading (Iizuka et al., 2006). Therefore, it is highly likely that targeted HBO1-dependent H4 acetylation controls MCM complex loading at origins, independently of global effect at the transcriptional level.

G1, we examined cells treated with mimosine, a drug used to block the cell cycle in late G1 (Miotto and Struhl, 2008). Cells staged in G1 by mimosine have an equivalent amount of H4 acetylation and association of ORC, Cdt1, and the MCM complex as compared to untreated cells (Figure 5D). The observation that Set8-HBD and mimosine treatment affect different steps during replication initiation, even though they both block the cell cycle in late G1, strongly emphasizes the functional connection between H4 acetylation and MCM complex loading during G1.

Importance of H4 Acetylation for Licensing Is Not an Indirect Consequence of Transcriptional Effects

Set8-HBD-expressing or HBO1-depleted cells have a global defect in H4 acetylation, and such low levels of H4 acetylation could indirectly affect MCM complex loading via an effect on transcription by RNA polymerase (Pol) II. Three lines of evidence exclude this possibility. First, as observed in HBO1-depleted

Geminin, a Cdt1 Repressor, Inhibits HBO1 Acetylase Activity in a Cdt1-Dependent Manner

Geminin interacts directly with Cdt1, and it is a potent inhibitor of Cdt1 licensing activity (Wohlschlegel et al., 2000; Tada et al., 2001). However, Geminin does not block the interaction of Cdt1 with HBO1 in vitro or Cdt1-dependent recruitment of HBO1 to replication origins in vivo (Miotto and Struhl, 2008). In addition, the enzymatic activity of HBO1 immunoprecipitates is enhanced from mitosis to late G1 phase (Iizuka et al., 2006), suggesting the possibility that HBO1 acetylase activity is regulated by a repressor. As our results indicate that H4 acetylation by HBO1 is required for licensing, we considered the possibility that Geminin inhibits HBO1 histone acetylase activity.

Geminin does not inhibit acetylation of H4-K5 or H4-K12 by the purified yeast piccolo NuA4 complex (Figure 6A), indicating

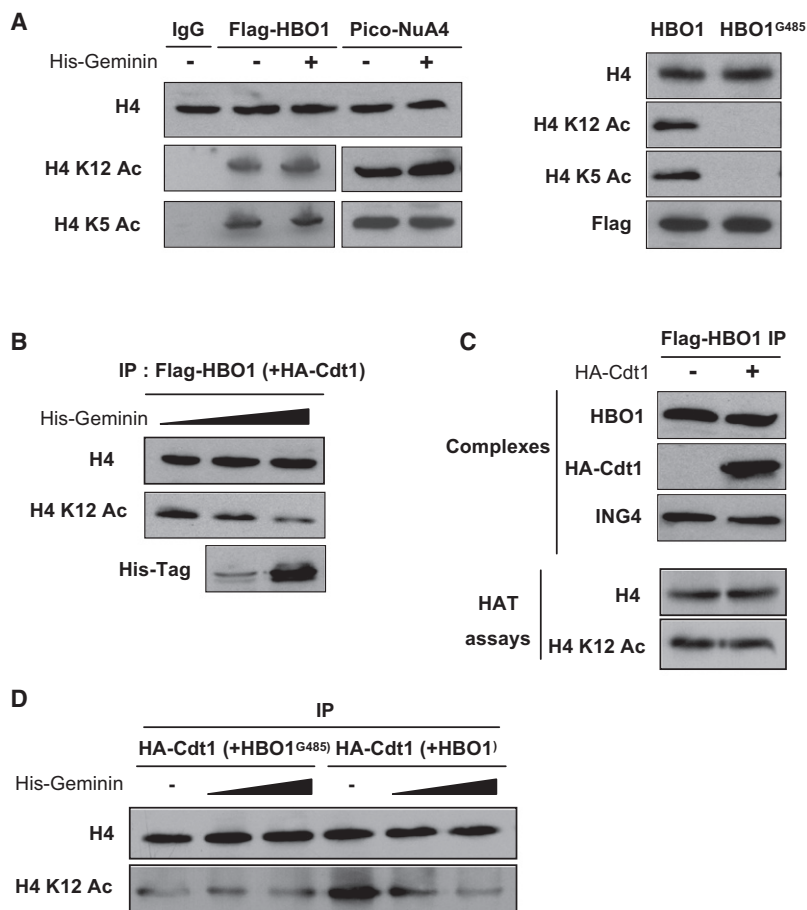


Figure 6. Geminin Inhibits H4 Acetylase Activity of HBO1 In Vitro

(A) Effect of recombinant full-length Geminin on the H4 acetylase activity of immunoprecipitated Flag-HBO1, Flag-HBO1^{G485}, and purified yeast piccolo NuA4 complex. (B) Same experiment as in (A), except that Flag-HBO1 was purified from cells overexpressing Flag-HBO1 and HA-Cdt1.

(C) Flag-HBO1 immunoprecipitates prepared from cells that do or do not coexpress HA-Cdt1 were analyzed by western blotting with antibodies against HBO1, HA-Cdt1, and ING4. HBO1 complexes in the presence or absence of Cdt1 were also tested for their HAT activity on H4-K12. (D) Effect of Geminin on the H4 activity of HA-Cdt1 complexes purified from cells coexpressing either HA-Cdt1 + Flag-HBO1 or HA-Cdt1 + Flag-HBO1^{G485}. Numbers under the panels indicate the relative amount of acetylated H4-K12 HAT as compared to cells in the absence of Geminin. Note that the weak H4-K12 acetylase activity observed in the HA-Cdt1 + Flag-HBO1^{G485} immunoprecipitates is insensitive to Geminin.

Geminin Associates with Replication Origins and Inhibits H4 Acetylation In Vivo

The above biochemical observations predict that Geminin interferes with HBO1-dependent H4 acetylation at origins in vivo. To test this hypothesis, we overexpressed a nondegradable derivative of Geminin, Gem^{L26A} (Wohlschlegel et al., 2002), that blocks MCM complex loading but does not interfere with binding of ORC, Cdt1, Cdc6, and HBO1 (Miotto and Struhl, 2008). As shown in Figure 7A, Gem^{L26A} inhibits

that Geminin does not interact with the histone H4 tail and nonspecifically mask H4 acetylation sites. In addition, acetylation of an H4 peptide by immunoprecipitated Flag-HBO1 is not inhibited significantly by Geminin (Figure 6A). The observed activity is due to HBO1, because immunoprecipitated Flag-HBO1^{G485} has no detectable HAT activity. In contrast, when Flag-HBO1 is immunoprecipitated from cells also expressing HA-Cdt1, the bound material is enriched in HBO1/Cdt1 complex, whose ability to acetylate the H4 tail is inhibited by Geminin in a concentration-dependent manner (Figures 6B and 6C).

To verify that Geminin inhibits H4 acetylation by the Cdt1-HBO1 complex, we purified HA-Cdt1 from cells coexpressing either Flag-HBO1 or Flag-HBO1^{G485} and assessed the activity of the resulting complexes in presence of recombinant Geminin (Figure 6D). The Cdt1-HBO1^{G485} complex has weak H4 activity and acetylates H4 in the presence of recombinant Geminin, presumably due to weak association of a histone acetylase other than HBO1. Importantly, the Cdt1-HBO1 complex has strong H4 acetylase activity that is inhibited by recombinant Geminin in a concentration-dependent manner (Figure 6D). Therefore, Geminin inhibits HBO1 HAT activity in a Cdt1-dependent manner, consistent with the observation that Geminin interacts with HBO1 only in the presence of Cdt1 (Miotto and Struhl, 2008).

H4 acetylation at the Myc and Chr16 replication origin, but not at flanking regions or at control hyperacetylated loci. Furthermore, Gem^{L26A} associates with all four replication origins tested (2- to 4-fold enrichments), but not with control hyperacetylated regions (Figure 7B), suggesting that Geminin directly affects H4 acetylation at origins via its interaction with Cdt1 and HBO1. Taken together, these biochemical and genetic observations further support the crucial role of HBO1 acetylase activity during replication licensing, and they strongly suggest a mechanism (not necessarily exclusive) for how Geminin represses licensing.

DISCUSSION

HBO1 Acetylase Activity Is Important for Licensing

Although the Cdt1 licensing factor directly interacts with the MCM complex (Tanaka and Diffley, 2002; You and Masai, 2008), recruitment of HBO1 to origins by Cdt1 is required for MCM complex loading in human cells. Here, we demonstrate that HBO1 function at origins requires its acetylase activity, indicating that MCM loading is enhanced by a Cdt1-dependent acetylation event. Specifically, as assayed by sequential ChIP, a replication origin simultaneously bound by ORC and HBO1^{G485} is defective in recruiting the MCM complex (Figure 1). Thus, HBO1 acetylase activity is required prior to MCM complex

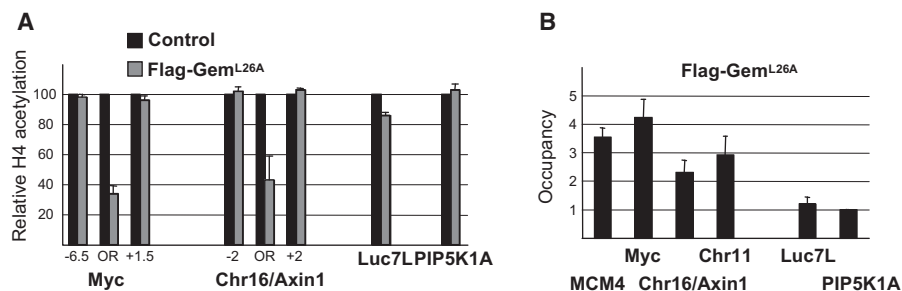


Figure 7. Geminin Associates with Replication Origins and Inhibits H4 Acetylation In Vivo

(A) Relative H4 tetra-acetylation level (mean ±SD) at origins in cells expressing the nondegradable Flag-Gem^{L26A} derivative (n = 3). (B) Association of Flag-Gem^{L26A} (mean ±SD) at origins (MCM4, Myc, Chr16, Chr11) and control regions (Luc7L, PIP5K1A) (n = 3).

loading for pre-RC assembly at origins. The importance of HBO1 acetylase activity for licensing is also supported by the observation that modulators of this enzymatic activity, such as Geminin (Figure 7), Jade-1 (Figure 4), p53 (Iizuka et al., 2008), and Polo-like kinase 1 (Wu and Liu, 2008), are linked to MCM loading under specific conditions. More generally, regulation of chromatin structure regulation can affect MCM complex loading (Lipford and Bell, 2001; Ghosh et al., 2006), and our results provide an additional mechanism by which this occurs.

H4 Acetylation by HBO1 Is Critical for Licensing

Several observations suggest the importance of H4 acetylation for licensing. First, HBO1 is the predominant H4 acetylase in human cells (Figure 2A) (Iizuka et al., 2008), and it is recruited to origins at the time of licensing, where it is important for loading the MCM complex (Miotto and Struhl, 2008). Second, treatments of cells that either increase (overexpression of HBO1 and Jade-1) or decrease (Set8-HBD overexpression) H4 acetylation reveal a correlation between levels of bulk H4 acetylation and MCM complex loading (Figures 4 and 5). Third, in accord with the HBO1 association profile, H4 acetylation peaks at replication origins in comparison to most flanking sequences (Figure 2B), and it is cell-cycle regulated (Figure 3).

The strong correlation between H4 acetylation and replication licensing does not necessarily indicate that histones are the physiologically relevant substrate for HBO1. As is typical for histone acetylases, HBO1 acetylates nonhistone substrates in vitro, including ORC2, Geminin, MCM2, and Cdc6 (Iizuka et al., 2006). Thus, the requirement for the HBO1 acetylase activity might involve a nonhistone substrate, and the increased H4 acetylation at origins might be a consequence of recruitment, rather than having a direct effect on licensing. Establishing that histones are the physiological substrate is difficult in human cells, unlike in yeast, where it is possible to examine strains with genetically modified histones.

Although formal proof is lacking, two observations strongly suggest that H4 is the physiologically relevant substrate for MCM complex loading. First, overexpression of Jade-1 and wild-type (but not mutant) HBO1 increases H4 acetylation and MCM complex loading (Figure 4). The PHD fingers of Jade-1 are required for histone binding and stimulation of H4 acetylation, but not for the association of Jade-1 and HBO1

(Foy et al., 2008). The requirement for the PHD fingers suggests that Jade-1 can stimulate acetylation of H4, but not nonhistone substrates. Second, the histone tail-binding domain of Set8, a histone methylase, blocks H4 acetylation and MCM complex loading (Figure 5). As this Set8 domain is unlikely to interact with nonhistone substrates, the observation strongly suggests that the observed effects on MCM complex loading involve an interaction with H4.

In accord with HBO1 association with origins being restricted to G1, isolated origins far from annotated promoters show preferential H4 acetylation at G1. However, H4 acetylation at other origins remains high throughout S phase, as also observed at the *Drosophila* chorion gene origin (Hartl et al., 2007) and the Epstein-Barr oriP origin (Zhou et al., 2005). This difference between H4 acetylation and HBO1 association at some origins during S phase likely reflects transcriptional-related events in the vicinity of the origin that involve other H4 acetylases, such as Tip60. The effects of H4 acetylation may vary among origins, and the histone deacetylase inhibitor TSA alters DNA replication patterns in human cells (Kemp et al., 2005). Similarly, mutation of the Sir2 histone deacetylase affects licensing in yeast, but only 4 of the 25 origins tested show reduced levels of H4-K16 acetylation, possibly due to a conserved DNA element bound by a well-positioned nucleosome (Crampton et al., 2008).

While our results indicate that Cdt1-dependent H4 acetylation by HBO1 is important for MCM complex loading, the precise role of H4 acetylation during replication licensing is unknown. One possibility is that H4 acetylation increases accessibility or fluidity of chromatin, thereby facilitating association of the MCM complex. In this regard, Cdt1 interacts with SNF2H and WSTF (Sugimoto et al., 2008), which are components of nucleosome remodeling complexes that preferentially associate with acetylated histones in chromatin (Hakimi et al., 2002; Fujiki et al., 2005). Alternatively, the MCM complex or an associated factor might recognize the acetylated lysines on H4, and this interaction would stabilize the interaction of the MCM complex with chromatin. Although an HBO1 homolog does not exist in yeast, HAT1 histone acetylase interacts with the ORC complex and affects ORC function in vivo (Suter et al., 2007). Like HBO1, HAT1 preferentially acetylates H4 at K5, K8, and K12 (but not K16), suggesting that it might play an analogous role at origins.

Geminin Inhibits HBO1 Histone Acetylase Activity in the Context of a Cdt1 Complex

Geminin plays a key role in the downregulation of Cdt1 activity when cells enter S phase. Although Geminin directly interacts with Cdt1, it does not block the interaction of Cdt1 and HBO1 but rather forms a ternary complex *in vitro* (Miotto and Struhl, 2008). This ternary complex is mediated by independent interactions of Geminin or HBO1 with different surfaces of Cdt1, because Geminin and HBO1 do not interact with each other. In accord with these observations, forcing Geminin expression in G1 does not inhibit HBO1 association with origins *in vivo*, even though it inhibits licensing (Miotto and Struhl, 2008). It has been suggested that Geminin binding to Cdt1 inhibits the interaction between Cdt1 and the MCM complex (Yanagi et al., 2002; Cook et al., 2004; Lee et al., 2004), although Cdt1 lacking the evolutionarily conserved region that interacts with the MCM complex is capable of rereplication (Teer and Dutta, 2008).

Here, we show that Geminin inhibits HBO1-dependent acetylation of H4 *in vitro* (Figure 6). This inhibition requires Cdt1 and presumably occurs in the context of the Cdt1-HBO1-geminin complex. Geminin-mediated inhibition of HBO1 histone acetylase activity might also account for the observation that enzymatic activity of HBO1 immunoprecipitates is enhanced from mitosis to late G1 phase (Iizuka et al., 2006). Most importantly, H4 acetylation is specifically impaired at origins in cells expressing the Gem^{L26A} derivative, and this is likely to be a direct effect because Gem^{L26A} associates with origins (Figure 7). Given the importance of H4 acetylation in MCM complex loading at origins, our results strongly suggest that Geminin inhibition of HBO1 histone acetylase activity contributes to replication licensing and the blockage of rereplication.

While likely to be important, the ability of Geminin to inhibit the acetylase activity of HBO1 might not fully account for how it regulates Cdt1 activity. This model is not mutually exclusive with the idea that Geminin blocks the interaction between Cdt1 and the MCM complex. Geminin also protects Cdt1 from proteasome-mediated degradation by inhibiting its ubiquitination, and inhibition of Geminin during M phase impairs pre-RC formation during the following cell cycle (Ballabeni et al., 2004). In addition, a Geminin-Cdt1 complex can license origins, but block rereplication *in vitro*, and it has been suggested that this switch is due to increased stoichiometry of Geminin with respect to Cdt1 (Lutzmann et al., 2006). As inhibition of HBO1 histone acetylase activity by Geminin is concentration dependent, perhaps this mechanism is more important for preventing rereplication as opposed to blocking licensing. In any event, as replication licensing and the prevention of rereplication is of paramount importance for long-term genome stability, it would not be surprising that Geminin functions by multiple mechanisms.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents

Expression vectors for Myc-HBO1^{G485}, YFP-Set8, YFP-Set8 histone-binding domain (Set8-HBD), and Flag-Jade-1 (long isoform) were described elsewhere (Contzler et al., 2006; Foy et al., 2008; Yin et al., 2008). Other materials not listed here have been previously described (Miotto and Struhl, 2008).

Antibodies

Antibodies were obtained from the following sources: Santa Cruz Biotechnology (Santa Cruz, CA) for HBO1, CCNH, MED26, HA-Tag (F7), and Myc-Tag (9E10); Abcam (Cambridge, MA) for anti-H4 K16Ac, H3 K4Met, ORC1, ORC2, TFIIB, and Histone H3; Upstate Biotechnology (Billerica, MA) for anti-H4 tetra-Ac (K5, K8, K12, and K16), H3 di-Ac (K9 and K14), H4 K12Ac, H4 K8Ac, H4 K5Ac, and H4 K16Ac; Bethyl Laboratories (Montgomery, TX) for MCM proteins and Cdt1; Covance (Princeton, NJ) for Pol II; Novus Biologicals (Littleton, CO) for HBO1; and Sigma-Aldrich (St. Louis) for Flag-Tag (M2), Actin β , and Cdc6. TBP antibody was obtained from Arnie Berk. Protein A and protein G Sepharose beads as well as control IgG-Sepharose beads used in coimmunoprecipitation and ChIP assays were purchased from Amersham Biosciences.

Western Blot Analysis of Histone Modifications

Histones were acid-extracted from cells, electrophoretically separated, blotted, and assayed for H4 and H3 modifications using the appropriate antibodies.

H4 Acetylation Assays

In vitro acetylation reactions were performed with minor modifications from a previously described protocol (Ait-Si-Ali et al., 1998). Acetylation of the histone H4 tail peptide (Abcam) was detected with antibodies specific for histone H4 acetylated on K5 or K12. Purified piccolo NuA4 complex was kindly provided by Danesh Moazed, and recombinant Geminin was produced in *E. coli*. Flag-HBO1 derivatives were immunopurified from HeLa cells 48 hr following transfection. Cells were lysed in RIPA buffer, and material from 5×10^6 cells was immunoprecipitated overnight with an anti-Flag Sepharose resin. The resulting material was extensively washed in buffer (500 mM KCl, 1% Triton X-100, 0.1% SDS), and the amount of Flag-fusion proteins retained on the beads was monitored by western blot and normalized for *in vitro* acetylation activity. When Flag fusions were coexpressed with HA-Cdt1 and copurified on anti-HA beads, the KCl concentration during the washing steps was 240 mM to avoid HBO1 dissociation from Cdt1.

Immunofluorescence and Flow Cytometry

BrdU incorporation tests were performed as previously described (Miotto and Struhl, 2008). BrdU incorporation was detected with an anti-BrdU (Abcam) antibody, and the percentage of the total number of stained cells was determined on random fields for a total of ~ 1000 nuclei scored under a microscope. For flow cytometry analyses, cells were stained with 20 $\mu\text{g}/\text{ml}$ propidium iodide (Sigma) in the presence of 50 $\mu\text{g}/\text{ml}$ of RNase H for 30 min at RT in the dark and extensively washed in PBS. Cells (1×10^6) were subsequently analyzed for each condition using a FACSCalibur flow cytometer, and data were processed using the CellQuest software (Becton Dickinson; Billerica, MA).

ChIP and Sequential ChIP Assays

Asynchronously growing cells (2×10^9) were treated with formaldehyde to 1% final concentration to create protein-DNA crosslinks. Crosslinked chromatin was extracted in 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 3 mM MgCl₂, 0.5% NP-40 and then sheared by sonication (Misonix Sonicator 3000) on ice to an average length of 500 bp. After preclearing with protein A Sepharose beads (4°C for 1 hr), chromatin from an equivalent of 5×10^7 cells was used for immunoprecipitation with adequate primary antibodies or IgG as a control. After 4°C overnight incubation, beads were washed, eluted in 25 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.5% SDS, and crosslinks were reversed at 65°C with Proteinase K for 6 hr. The resulting DNAs were purified using the QIAquick PCR Purification Kit (QIAGEN) and eluted in 100 μl distilled water. For sequential ChIP analysis, chromatin-bound material after the first immunoprecipitation was treated for 20 min at 65°C, and the eluted DNA was adjusted to 150 mM NaCl, 0.1% Triton X-100. The second immunoprecipitation was performed overnight at 4°C, and DNA was purified as described above. Quantitative real-time PCR was performed in triplicate using SYBR Green I, and enrichment for a specific DNA sequence was calculated using the comparative Ct method, as previously described (Miotto and Struhl, 2008). PCR primer pairs are available on request.

Cellular and Chromatin Fractionation

Cells (2×10^7) were scraped in ice-cold PBS, resuspended in lysis buffer (10 mM HEPES [pH 7.9], 100 mM NaCl, 300 mM sucrose, 0.1% Triton X-100) containing protease inhibitors (Roche; Indianapolis, IN), and incubated on ice for 10 min. After centrifugation at 1000 rpm for 5 min, the pellet was washed twice in the same buffer. Cell remnants were then resuspended in extraction buffer (100 mM HEPES [pH 7.9], 200 mM NaCl, 300 mM sucrose, 0.1% Triton X-100, 5 mM $MgCl_2$) containing 100 units of DNase I (Promega; Madison, WI). Following incubation at 25°C for 30 min, the DNA-bound proteins (soluble) and the nuclear matrix-bound protein (insoluble) were isolated by centrifugation at 2500 rpm for 5 min.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at doi:10.1016/j.molcel.2009.12.012.

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