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The HP1α–CAF1–SetDB1-containing complex provides H3K9me1 for Suv39-mediated K9me3 in pericentric heterochromatin

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Trimethylation of lysine 9 in histone H3 (H3K9me3) enrichment is a characteristic of pericentric heterochromatin. The hypothesis of a stepwise mechanism to establish and maintain this mark during DNA replication suggests that newly synthesized histone H3 goes through an intermediate methylation state to become a substrate for the histone methyltransferase Suppressor of variegation 39 (Suv39H1/H2). How this intermediate methylation state is achieved and how it is targeted to the correct place at the right time is not yet known. Here, we show that the histone H3K9 methyltransferase SetDB1 associates with the specific heterochromatin protein 1α (HP1α)–chromatin assembly factor 1 (CAF1) chaperone complex. This complex monomethylates K9 on non-nucleosomal histone H3. Therefore, the heterochromatic HP1α–CAF1–SetDB1 complex probably provides H3K9me1 for subsequent trimethylation by Suv39H1/H2 in pericentric regions. The connection of CAF1 with DNA replication, HP1α with heterochromatin formation and SetDB1 for H3K9me1 suggests a highly coordinated mechanism to ensure the propagation of H3K9me3 in pericentric heterochromatin during DNA replication.

Keywords: CAF1; H3K9 methylation; HP1α; pericentric heterochromatin; SetDB1

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INTRODUCTION

Histone proteins are heavily modified post-translationally, and patterns of these modifications mark specific functional nuclear domains. For example, trimethylation of lysine 9 in histone H3 (H3K9me3) is highly enriched in pericentric heterochromatin, the constitutive heterochromatin domain that flanks centromeres. In addition to H3K9me3, this domain is enriched in H4K20me3, hypoacetylated histones, DNA methylation, heterochromatin protein 1α (HP1α) and HP1β, and an RNA component (Maison & Almouzni, 2004; Grewal & Jia, 2007). A current model for the propagation of H3K9 methylation and HP1 binding in this region through cell division proposes a self-sustaining loop in which HP1 binds to H3K9me3 through its chromodomain, which, in turn, recruits more of the H3K9–histone methyltransferase (HMTase) Suv39H1/H2. However, in this scheme, some level of H3K9me3 is necessary to nucleate heterochromatin formation. In addition, it is unclear whether Suv39H1/H2 is responsible for only H3K9me3 or whether it affects other intermediate K9 methylation states.

Interestingly, in Suv39H1/H2 double null cells, accumulation of both H3K9me3 and HP1α in pericentric heterochromatin is lost with a concomitant enrichment of H3K9me1 (Peters *et al*, 2001; Loyola *et al*, 2006). These results indicate that Suv39H1/H2 is not involved in the monomethylation of H3K9 in these regions and instead suggest that H3K9me1 might be used as a substrate for trimethylation. In support of this view, transfection of the Suv39H1/H2 double null cells with recombinant wild-type Suv39H1/H2 is sufficient to restore the transition from H3K9me1

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towards H3K9me3 (Loyola *et al*, 2006). Moreover, *in vitro* methylation assays show that H3K9me1 is an optimal substrate for Suv39H1/H2-mediated trimethylation of H3K9 (Loyola *et al*, 2006). Therefore, the question that arises is how H3K9me1 is directed to pericentric heterochromatin.

The above self-sustaining model for the inheritance of heterochromatin marks would work at any time during the cell cycle, assuming that some level of H3K9me3 is available for the nucleation of HP1 recruitment. However, a particular situation arises during DNA replication in which nucleosome disruption occurs ahead of the replication fork and newly synthesized plus recycled histones are deposited behind the fork onto newly synthesized DNA (Groth *et al*, 2007a). Parental H3K9me3 might provide nucleation events to maintain HP1 and Suv39H1/H2 in the pericentric domain; however, a replication-coupled mechanism of H3K9 methylation might be needed to promote the deposition of this histone mark onto newly synthesized histones at the time of incorporation into chromatin.

One attractive model is that the chromatin assembly factor CAF1—which functions in a manner coupled with DNA synthesis and is present at all replication foci—might promote the loading of H3.1K9me1 and HP1α in heterochromatin, thereby facilitating the subsequent trimethylation of H3.1K9me1 by Suv39H1/H2 and HP1α binding. Indeed, CAF1 might act as a platform to integrate and coordinate a series of activities necessary for the inheritance of both H3K9 and DNA methylation. CAF1 interacts with the methyl-CpG-binding domain protein MBD1 and the H3K9–HMTase SetDB1 during S phase (Sarraf & Stancheva, 2004). However, how SetDB1 functions in conjunction with Suv39H1/H2 to promote H3K9me3 at pericentric heterochromatin during DNA replication is not known. Furthermore, no studies have yet addressed how these two lysine methyltransferases (KMTs) contribute to H3K9 mono-, di- and trimethylated states to ultimately establish the characteristic H3K9me3 pattern found in pericentric heterochromatin.

Here, we report that the HP1α–CAF1 complex associates with SetDB1, and that this complex monomethylates non-nucleosomal H3K9. Furthermore, we show that SetDB1 localizes to pericentric heterochromatin, correlating with the replication of these sites. On the basis of these results, we propose a model for how the CAF1 chromatin assembly pathway coupled with DNA replication affects the establishment of H3K9 methylation patterns.

RESULTS AND DISCUSSION

The HP1α–CAF1 complex is associated with SetDB1

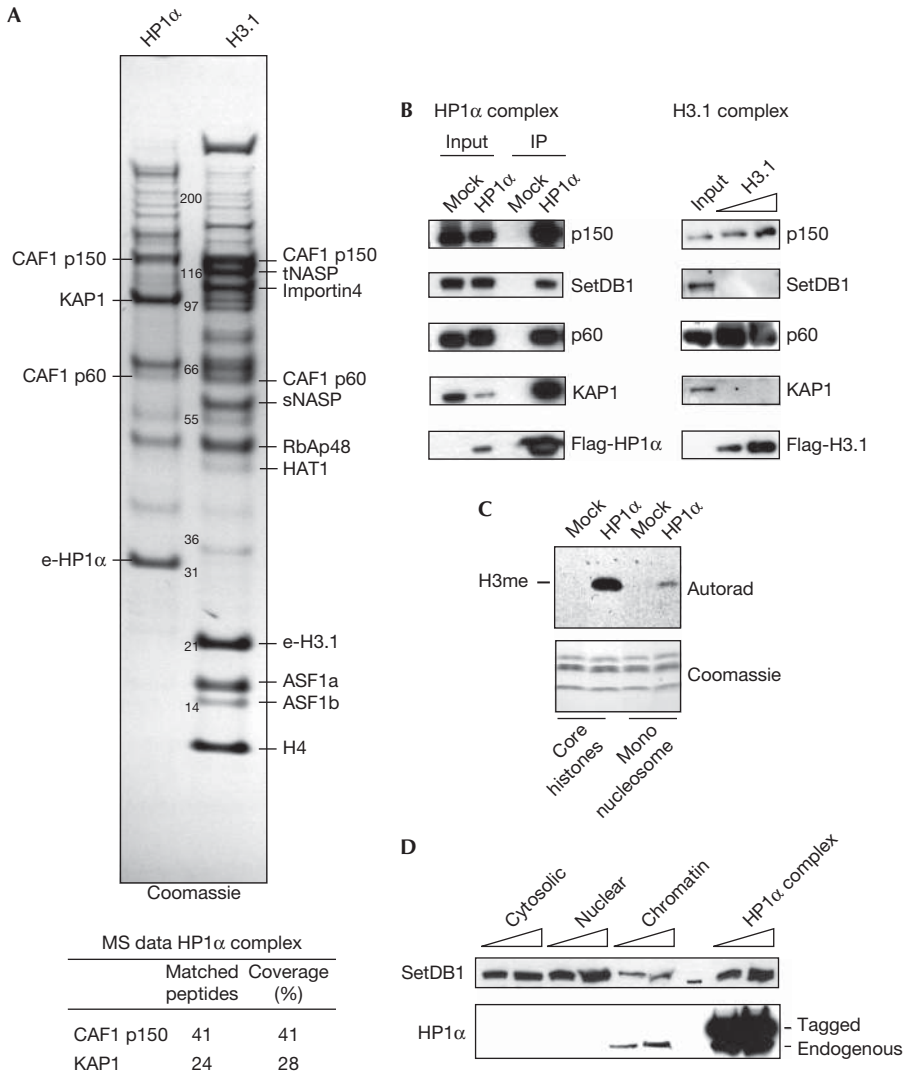
Previously, using an HeLa cell line expressing Flag and haemagglutinin (HA) epitope-tagged HP1α, we found that HP1α associates with HP1γ and the three subunits of CAF1, namely p150, p60 and RbAp48 (Quivy *et al*, 2004; Fig 1A). This HP1 complex, which contains CAF1, is distinct from the H3.1 complex (Tagami *et al*, 2004; Fig 1A,B; supplementary Fig S1A online). It is noted that the H3K9 methyltransferase SetDB1 is present in this HP1 complex (as shown by immunoblotting, Fig 1B) and is also found when retrieving CAF1-associated proteins (supplementary Fig S1B online). These data support the hypothesis that the three proteins could be associated in a common complex. Interestingly, additional mass spectrometric and immunoblot analyses identified the transcriptional repressor KRAB-ZFP-associated protein 1 (KAP1) as another component of the HP1α–CAF1 complex, but not of the H3.1–CAF1 complex (Fig 1A,B). Given that KAP1 is known to interact directly with HP1 (Nielsen *et al*, 1999; Ryan *et al*, 1999) and SetDB1 (Schultz *et al*, 2002), its presence in the HP1–CAF1 complex might act to promote the association of SetDB1 with this complex.

The presence of SetDB1 in the HP1α–CAF1 complex prompted us to assess whether the complex has histone H3 methyltransferase activity. Indeed, we found associated with the HP1α–CAF1 complex an H3-specific HMTase activity (Fig 1C) that co-fractionates with the HP1α–CAF1 complex on a glycerol gradient (supplementary Fig S2A,B online). The H3.1–CAF1 complex did not show any HMTase activity when tested under similar conditions (supplementary Fig S2C online). Interestingly, this HMTase activity preferentially methylated free-core histones rather than nucleosomes (Fig 1C), suggesting that the HMTase activity modifies histones that have not yet been incorporated into chromatin. Cellular fractionation analysis showed that SetDB1 localized mainly in the cytosolic and nuclear extracts rather than in the chromatin fraction (Fig 1D). This distribution is in contrast with that of other H3K9 methyltransferases, including Eu-HMTase, G9a and Suv39H1, which are localized mainly in the nuclear and chromatin fractions (Loyola *et al*, 2006). The localization of SetDB1 further suggests that it modifies non-nucleosomal histones, which is consistent with the observation that free histones are better substrates for the HP1α–CAF1 complex than nucleosomes (Fig 1C).

The HP1α–CAF1 complex contains the HMTase SetDB1

Next, we investigated further the specificity of the HMTase activity associated with the HP1α–CAF1 complex. Mass spectrometric analysis of the products of the methylation reaction obtained with recombinant histone H3 and the HP1α–CAF1 complex detected the formation of H3K9me1 (Fig 2A, middle panel left). Interestingly, we were unable to detect the formation of either H3K9me2 or H3K9me3 by the HP1α–CAF1 complex, although these products were detected in a control reaction using recombinant Suv39H1 (Fig 2A, bottom panel left). In addition, no other methylated lysines were found, as shown by the analysis of the (27–40) H3 peptide (Fig 2A, right panel). To confirm these results, we performed HMTase filter peptide assays with the HP1α–CAF1 complex and a peptide containing amino acids (1–19) of H3 (Fig 2B). We observed methylation of H3 by the HP1α complex, but not by the mock Flag immunoprecipitate. To show that the activity was specific for H3K9, we repeated the assay using a peptide containing trimethylated K9 (Fig 2B). As expected, the complex was not active towards the H3K9me3 peptide (Fig 2B). Taken together, these results indicate that the HP1α–CAF1 complex contains H3K9me1-specific activity.

To determine whether SetDB1 is responsible for the HMTase activity in the HP1 complex, we sought to knock down SetDB1. However, SetDB1 knockout mice are embryonic lethal (Dodge *et al*, 2004), and attempts to impair SetDB1 function *in vivo* in stable cell lines have proven difficult owing to high levels of cell death (Wang *et al*, 2003; Sarraf & Stancheva, 2004). Therefore, we selected partial RNA interference (small interfering RNA (siRNA)) conditions to reduce SetDB1 protein while allowing the growth of enough cells for our analysis (Fig 2C). These conditions were met with a reduction in SetDB1 to about 40% in the HP1α–CAF1 complex when compared with the complex isolated from control



MS data HP1α complex

	Matched peptides	Coverage (%)
CAF1 p150	41	41
KAP1	24	28
CAF1 p60	27	44
HP1α	13	52

Fig 1 | The HP1α–CAF1 complex associates with the histone H3 methyltransferase SetDB1. (**A**) Coomassie blue-stained gel of the HP1α–CAF1 and H3.1 complexes isolated by Flag immunoprecipitation (IP). The subunits of each complex, as identified by mass spectrometry (MS), are shown. Below is the mass spectrometric data for the HP1α complex. (**B**) Western blots of the HP1α–CAF1 and H3.1 complexes showing selected components as indicated. Mock IP refers to the IP using nuclear extract derived from the same cells, but that are not expressing a Flag-tagged protein. (**C**) Comparison of free-core histones and mononucleosomes as substrates for the HMTase activity associated with the HP1α–CAF1 complex. We incubated the HP1 complex with histones in the presence of ^{3}H-SAM. Reaction products resolved by gel electrophoresis and revealed by autoradiography showed the presence of methylated histones (H3me; top). Coomassie staining provides a loading control (bottom). (**D**) SetDB1 and HP1α Western blots, using 15 and 30 μg of cytosolic, nuclear and chromatin extracts and two dilutions of the HP1α–CAF1 complex. ASF1, anti-silencing function 1 protein; CAF1, chromatin assembly factor 1; e-HP1α, epitope-tagged HP1α; HAT1, histone acetyltransferase 1; HMTase, histone methyltransferase; HP1α, heterochromatin protein 1α; ^{3}H-SAM, tritiated S-adenosyl-L-methionine; KAP1, KRAB-ZFP-associated protein 1; RbAp48, pRB-associated proteins p46 and p48; sNASP, somatic nuclear autoantigenic sperm protein (NASP); tNASP, testicular NASP.

siRNA-treated cells (immunoblot, Fig 2C, left panel). Knockdown of SetDB1 did not affect the stability of the complex, as other subunits remained associated with HP1 (Fig 2C, left panel). HMTase activity towards the H3 (1–19) peptide was diminished when SetDB1 was depleted (Fig 2C, right panel). These data show that the H3K9me1 HMTase activity in the complex is largely accounted for by SetDB1, arguing for its crucial role in this function. To confirm that our observations were not due to off-target effects, we used a different SetDB1 siRNA (supplementary Fig S3 online). Interestingly, we observed a reduction in the levels of both nucleosomal and non-nucleosomal H3K9me1 after a similar SetDB1 knockdown (supplementary Fig S4A,B online). The

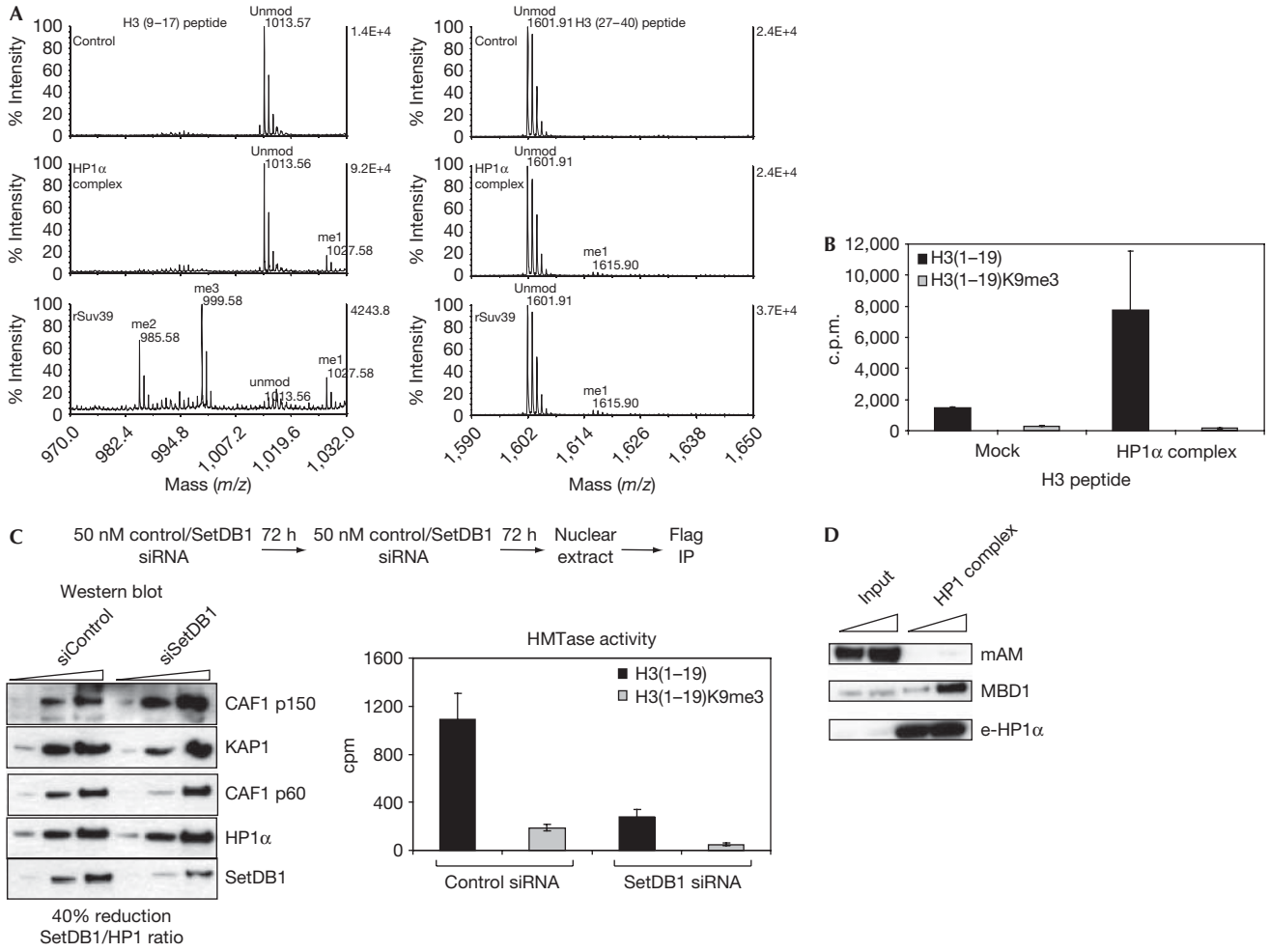


Fig 2 | The HP1α–CAF1 complex contains a SetDB1-dependent H3K9me1 HMTase activity. (**A**) Peptide mass fingerprints of the products of an HMTase assay using histone H3 and no enzyme (top), HP1α–CAF1 complex (middle) and recombinant Suv39 (bottom). The graphs correspond to enlarged areas of the spectra of peptides (9–17) (left) and (27–40) (right) of the histone H3. (**B**) The graph represents a peptide HMTase assay using the HP1α–CAF1 or mock complex with the H3 peptide (1–19) unmodified (black) and K9me3 (grey). We derived error bars from a duplicate assay, which is a representation of three independent experiments. (**C**) On top, a scheme to illustrate the control and SetDB1 siRNA. (Left panel) Western blots on increasing amounts of the HP1α–CAF1 complex subunits as labelled. Quantification of the SetDB1 bands normalized to HP1α bands is presented underneath the blot. (Right panel) Peptide HMTase assay using the HP1α–CAF1 complex derived from control and SetDB1 siRNA-treated cells. H3 peptides (1–19) were unmodified (black) or K9me3 (grey). Error bars are derived from two experiments. (**D**) Western blots of the HP1α–CAF1 complex subunits, as indicated. CAF1, chromatin assembly factor 1; e-HP1α, epitope-tagged HP1α; HMTase, histone methyltransferase; HP1α, heterochromatin protein 1α; KAP1, KRAB-ZFP-associated protein 1; mAM; murine ATFa-associated modulator; MBD1, methyl-CpG binding protein 1; siRNA, small interfering RNA.

fact that the HMTase activity associated with the HP1α–CAF1 complex that we attribute to SETDB1 is specific for H3K9me1 might seem surprising given that other studies have shown that SetDB1 can modify H3K9me2 to H3K9me3 in the presence of the protein murine ATFa-associated modulator (mAM; Wang *et al*, 2003). Interestingly, we were unable to detect H3K9me2 or H3K9me3 in our assays using the HP1α–CAF1–SetDB1 complex (Fig 2A). Given that mAM is not part of the HP1α–CAF1–SetDB1complex (Fig 2D), SetDB1 activity might be regulated by association with different co-factors; for example, HP1α–CAF1 versus mAM.

SetDB1 localizes at HP1-enriched domains in S phase

To investigate whether SetDB1 associates with heterochromatin during DNA replication, we performed immunofluorescence analysis of SetDB1 in mouse 3T3 cells (Fig 3A). We carried out dual staining for SetDB1 and proliferating cell nuclear antigen (PCNA) as markers for replication foci. PCNA labelling allowed us to distinguish non-S-phase cells from early, mid—when pericentromeric regions replicate (Guenatri *et al*, 2004; Quivy *et al*, 2004)—and late S-phase cells. We observed two cell populations: the first group showed diffuse SetDB1 nuclear staining that did not coincide with pericentric heterochromatin (64%; Fig 3A);

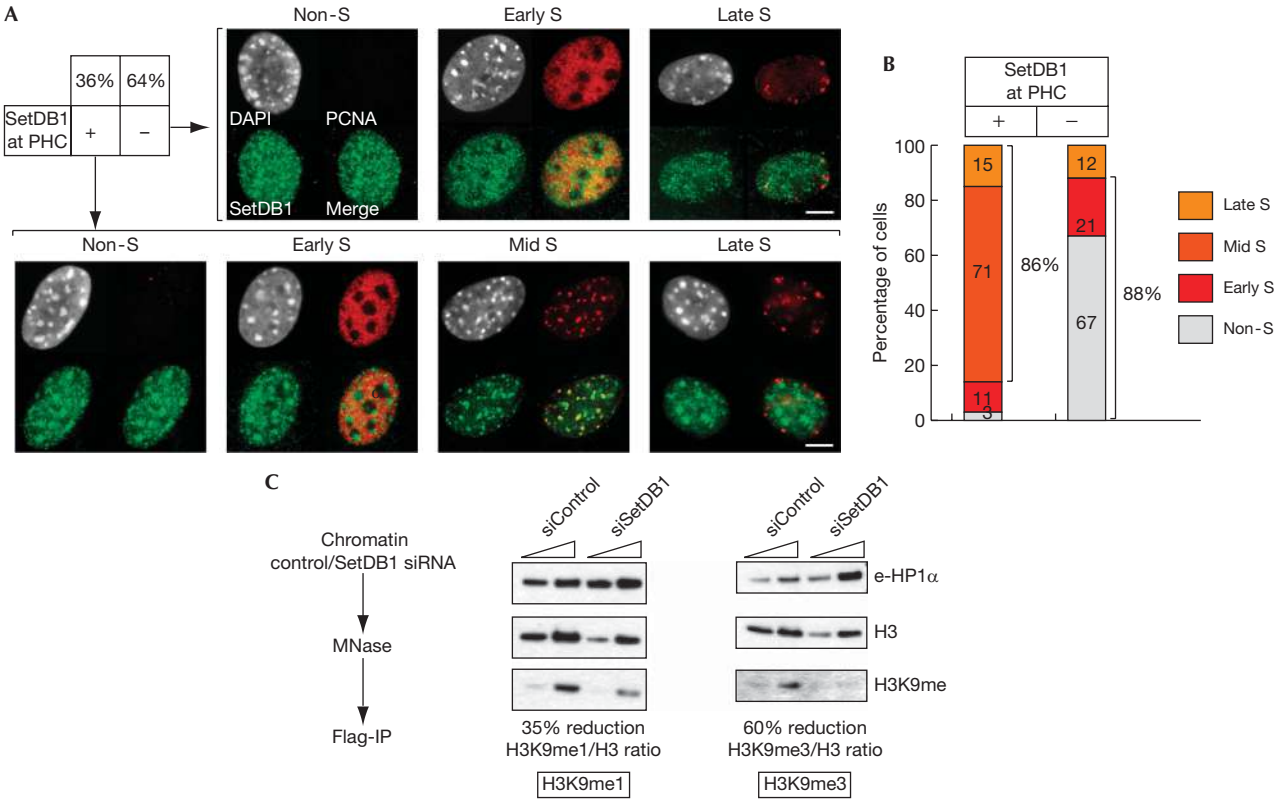


Fig 3 | SetDB1 localization during S phase. (**A**) Dual staining of SetDB1 (green) and PCNA (red) in mouse embryonic fibroblast cells during S phase. The early, mid and late S phase profiles based on PCNA staining are shown. (**B**) Quantitation analysis of the percentage of cells that are positive for SetDB1 staining in non-S phase, early, mid and late S phase, based on PCNA staining. (**C**) On the left, a scheme to illustrate the isolation of HP1α-enriched nucleosomes. We analysed increasing amounts of the HP1α-enriched nucleosomes by Western blot, as indicated. We quantified the bands by densitometry analysis of scanned autoradiography, and normalized the H3K9me1 and H3K9me3 bands, respectively, with their corresponding H3 band to obtain the H3K9me1/H3 and H3K9me3/H3 ratios. We compared this number between SetDB1 knockdown and control conditions to calculate the percentage reduction relative to control conditions. DAPI, 4′,6-diamidino-2 phenylindole; e-HP1α, epitope-tagged HP1α; HP1α, heterochromatin protein 1α; IP, immunoprecipitation; MNase, micrococcal nuclease; PCNA, proliferating cell nuclear antigen; PHC, pericentric heterochromatin; si, small interfering.

the second group showed SetDB1 staining at pericentric heterochromatin (36%; Fig 3A). PCNA staining showed that 67% of cells with diffuse SetDB1 staining were not in S phase and 21% were in early S phase; thus, 88% of these cells were in a stage before the replication of pericentric heterochromatin. By contrast, 71% of cells with SetDB1 localized at pericentric heterochromatin were in mid S phase, and therefore replicating pericentric heterochromatin, and 15% were in late S phase. Thus, 86% of these cells were at a stage after pericentric heterochromatin replication. Taken together, these data indicate that SetDB1 localization at pericentric heterochromatin correlates with the replication of these sites. We would expect this localization if SetDB1 is important in the inheritance of the heterochromatic H3K9 methylation patterns in a replication-dependent (CAF1/PCNA driven) mechanism.

As SetDB1 localizes to pericentric heterochromatin domains enriched in HP1, it seems plausible that it could be involved in the methylation of H3K9 in these domains. Therefore, we evaluated the impact of SetDB1 depletion on the levels of H3K9me1 in chromosomal sites enriched with HP1α. We isolated HP1α-enriched di- and trinucleosomes (Loyola *et al*, 2006) derived from SetDB1-depleted cells to determine the extent of H3K9me1 present on this heterochromatin-derived material (Fig 3C). A careful quantification of the data, by comparison with the corresponding loading controls, Flag-HP1α and histone H3, allowed us to estimate that there is a reduction of about 35% in the amount of H3K9me1 levels at HP1-enriched nucleosomes in the SetDB1-depleted cells compared with control cells (Fig 3C). It should be pointed out that SetDB1 is depleted by only about 40%, which explains the relatively modest reduction observed in the H3K9me1 levels. Interestingly, the level of H3K9me3 (Fig 3C), but not H3K9me2 (supplementary Fig S4C online), was also reduced in these sites, further supporting the idea that H3K9me1 is the substrate used to reach H3K9me3. These data show that SetDB1 associated with the HP1α–CAF1 complex contributes significantly to the presence of H3K9me1 within oligonucleosomes associated with HP1.

Establishment of the heterochromatic H3K9me3 pattern

During S phase, CAF1 can form a specific complex with both SetDB1 and MBD1, leading to the hypothesis that these associations link both H3K9 and DNA methylation with

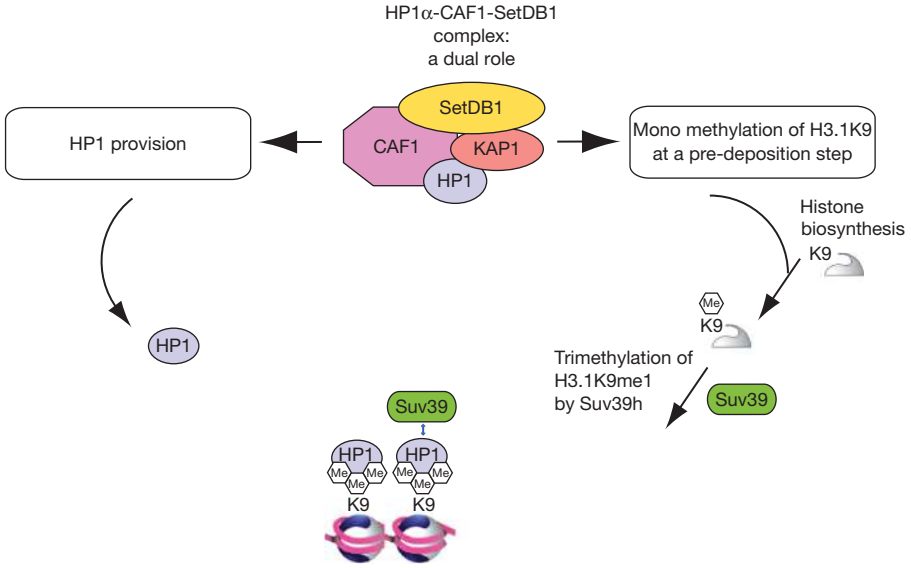


Fig 4 | Iterative model for the maintenance of pericentric heterochromatin marking during DNA replication. During DNA replication, CAF1 deposits H3.1 into newly replicated DNA and helps to target SetDB1 and HP1α to sites of heterochromatin formation, promoting both the monomethylation of H3.1K9 and the loading of HP1α in these regions. We propose a model in which the HP1α–CAF1–SetDB1 complex has a dual role to (i) monomethylate H3.1K9 in a pre-deposition state and (ii) provide HP1 to bind to H3.1K9me3. For simplicity of the scheme, the histone chaperone of newly synthesized histone H3.1 is not drawn. CAF1, chromatin assembly factor 1; HP1, heterochromatin protein 1; KAP1, KRAB-ZFP-associated protein 1.

replication-coupled chromatin assembly in heterochromatic regions (Sarraf & Stancheva, 2004). Remarkably, CAF1 can exchange between a histone-containing complex, the H3.1 complex, and an HP complex, the HP1α complex. An attractive hypothesis is that these two separate complexes function sequentially to chaperone successive histones and HP1 at the replication fork, either in the context of disruption or reassembly of heterochromatin, through the synergistic action of the histone chaperone Anti-silencing function 1 protein (ASF1; Groth *et al*, 2007b). First, by removing and/or accepting HP1 ahead of the fork, one complex would allow the disruption of the parental nucleosome. Second, the deposition of both recycled and new HP1 would leave CAF1 available to deposit H3–H4 (recycled or new). Interestingly, in this scheme, the recycled parental H3 would become available immediately for HP1 binding, whereas the new histone would gain progressively mono- and then trimethylation by the coordinated actions of the SetDB1 and Suv39 HMTases. The connection between HMTases to guarantee the reassembly of heterochromatin behind the fork would thus ensure an iterative mechanism for histone deposition and/or modification. Interestingly, the HP1α–CAF1 complex associates with the H3K9 HMTase SetDB1, and a significant fraction of SetDB1 accumulates in pericentric heterochromatin at the time of its replication (Fig 3A,B). Furthermore, SetDB1 prefers free histones rather than nucleosomes as substrates for methylation *in vitro* (Fig 1C), which suggests that SetDB1 might modify histones before their incorporation into chromatin. Consistently, the level of non-nucleosomal H3K9me1 decreases when the level of SetDB1 is reduced (supplementary Fig S4B online). Therefore, during DNA replication, CAF1 might have a function beyond deposition of the replicative histone H3.1 onto the DNA, and might help to target SetDB1 and HP1α to sites of heterochromatin formation, promoting both the monomethylation of H3.1K9 and the loading of HP1α. Interestingly, we have recently shown that the interaction between p150, the largest subunit of CAF1, and HP1α is necessary for the replication of pericentric heterochromatin, in a manner that is independent of its histone H3.1 deposition activity (Quivy *et al*, 2008). Given the known interaction between HP1α and Suv39H1 (Schotta *et al*, 2002; Stewart *et al*, 2005), we propose that, once methylated by the HP1α–CAF1–SetDB1 complex, H3.1K9me1 subsequently becomes trimethylated by Suv39H1/H2, as shown in Fig 4. We are only just beginning to unravel the complex dynamics of the interactions and choreography of events that help to propagate particular histone post-translational modifications associated with pericentric heterochromatin during replication. We anticipate that this model will prove useful in considering how other modifications in other domains might be transmitted during replication, an issue of fundamental interest in epigenetics.

METHODS

Complex purification. We grew Flag–HA-tagged HP1α- and H3.1-expressing HeLa S3 cell lines as described previously (Quivy *et al*, 2004; Tagami *et al*, 2004). We purified H3.1, HP1α–CAF1 and p150–CAF1 complexes from nuclear extracts by immunoprecipitation using anti-Flag antibody conjugated agarose beads.

Histone methyltransferase assay. We performed the assays as described by Eskeland *et al* (2004), using either 4 μg of recombinant histones or nucleosomes, or 1 μg of histone H3 peptides containing the first 19 amino acids either unmodified or trimethylated at K9.

MALDI-mass spectrometry. We acquired and analysed matrix-assisted laser desorption/ionization (MALDI) spectra as described previously (Bonaldi *et al*, 2004; Loyola *et al*, 2006).

Immunofluorescence. We performed immunofluorescence after extraction with Triton-X100 to remove soluble proteins on

mouse 3T3 cells as described previously (Taddei *et al*, 2001; Maison *et al*, 2002).

SetDB1 siRNA. The assay is shown in Fig 2C. In brief, we used two distinct SetDB1 siRNAs, 50 nM of SetDB1 siRNA duplex (Silencer®Pre-designed siRNA, Ambion, Austin, TX, USA; #1: ID: 138242, sequence 5′-3′: GGGCAGUGACUAAUUGUGAtt, #2: ID 3897, sequence 5′-3′: GGGUGUUUUCAUUAACACAtt), to ensure that our observations were not due to off-target effects. As a negative control, we used 50 nM of negative control siRNA (Silencer®Negative Control #1 siRNA, Ambion), a sequence designed without any significant sequence similarity with mouse, rat or human transcripts sequences. Two rounds of transfections were necessary to effectively knock down SetDB1 present in both the soluble fraction and in the HP1α–CAF1 complex.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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