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

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
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
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
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–SetDB1 complex (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);
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 HP1x. We isolated HP1x-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-HP1x and histone H3, allowed us to estimate that there is a reduction of about 35% in the amount of H3K9me1 levels at HP1x-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 HP1x–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) mono-methylate H3.1K9 in a pre-deposition state and (ii) provide HP1α 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 HP1α 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 HP1α ahead of the fork, one complex would allow the disruption of the parental nucleosome. Second, the deposition of both recycled and new HP1α would leave CAF1 available to deposit H3–H4 (recycled or new). Interestingly, in this scheme, the recycled parental H3 would become available immediately for HP1α 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’: GGGUGUUUUCAUUAAACACAtt), 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](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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