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KAP1 facilitates reinstatement of heterochromatin after DNA replication

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ABSTRACT

During cell division, maintenance of chromatin features from the parental genome requires their proper establishment on its newly synthetized copy. The loss of epigenetic marks within heterochromatin, typically enriched in repetitive elements, endangers genome stability and permits chromosomal rearrangements via recombination. However, how histone modifications associated with heterochromatin are maintained across mitosis remains poorly understood. KAP1 is known to act as a scaffold for a repressor complex that mediates local heterochromatin formation, and was previously demonstrated to play an important role during DNA repair. Accordingly, we investigated a putative role for this protein in the replication of heterochromatic regions. We first found that KAP1 associates with several DNA replication factors including PCNA, MCM3 and MCM6. We then observed that these interactions are promoted by KAP1 phosphorylation on serine 473 during S phase. Finally, we could demonstrate that KAP1 forms a complex with PCNA and the histone-lysine methyltransferase Suv39h1 to reinstate heterochromatin after DNA replication.

INTRODUCTION

Heterochromatin is paramount to the stability of eukaryotic genomes, which abound in repetitive units such as satellite repeats and transposable elements (1,2). Loss of control over these regions can lead to transcriptional perturbations, abnormal splicing and DNA recombination, events all at the root of oncogenic transformation (3–8). Heterochromatin is largely transcriptionally inactive and typically described as either constitutive or facultative. Facultative heterochromatin is made up of locus- and cell type-specific domains, the epigenetic features of which may be regulated, whereas its constitutive counterpart is traditionally more extensive, ubiquitous and permanent, such as in pericentromeric regions of chromosomes (9). In mammalian genomes, constitutive heterochromatic regions are typically marked with post-translational modifications (PTM) of histones such as tri-methylation of histone H3 on lysine 9 (10) and 64 (11) (H3K9me3 and H3K64me3 respectively), and of histone H4 on lysine 20 (H4K20me3) (12–14). Furthermore, they are enriched in non-histone proteins such as Heterochromatin Protein 1 (HP1) (15) and KAP1 (KRAB-associated protein 1, also known as TRIM28 or Tif1β) (16), and the underlying DNA is generally methylated (17). H3K9 tri-methylation plays a pivotal role in the organization of this heterochromatin, as it promotes the binding of HP1 (15) and of the histone-lysine methyltransferases (KMT) Suv39h1 (Suppressor of Variegation 3–9 Homolog 1) (18) and Suv420h1 (Suppressor of Variegation 420 Homolog 1) (12). Moreover, heterochromatin-associated non-coding RNAs (ncRNAs) play an important role in the regulation and formation of constitutive heterochromatin by stabilizing Suv39h1, which can instate H3K9me3 (19–21), and KAP1 itself can associate with all five KMTs so far identified in mammals, namely, SETDB1 (SET Domain Bifurcated 1), GLP, and G9a in addition to Suv39h1/h2. These enzymes act differentially on their substrate, with
GLP/G9a and SETDB1 capable of mediating mono, di and tri-methylation of H3K9, whereas SuV39h1/b2 can only deposit a third methyl group on previously mono- and di-methylated H3K9 (22–27). Heterochromatic regions are mostly replicated during late S phase and chromatin marks present on the parental DNA must be recapitulated in the daughter cell at the outset of this process. KAP1 was previously found to participate in these events by ensuring the maintenance of H3K9me3 as part of a complex also comprising CAF1 (chromatin-associated factor 1) and HP1, which recruits the KMT SETDB1 responsible for mono-methylating histone H3 before its association with the newly synthesized DNA. Moreover, SMARCAD1 (a SWI/SNF-like protein) and CHD can be associated with KAP1 in order to maintain H3K9 methylation and heterochromatin integrity during cell division at pericentricromeric and subtelomeric heterochromatin (27–29). However, how these heterochromatin marks are maintained during cell division is not known. Here, we reveal that KAP1 becomes phosphorylated on serine 473 (to generate pS473KAP1) during late S phase, triggering the formation of a PCNA-KAP1-Suv39h1 complex that plays an essential role in the maintenance of heterochromatin-associated histone modifications across cell division.

MATERIALS AND METHODS

Cell culture, antibodies and reagents

Cell lines were maintained at 37°C in a 7% CO2 incubator in medium containing 100U/ml penicillin-streptomycin, for NIH3T3, MEF, HeLa and 293T cells Dulbecco’s modified Eagle’s medium (DMEM, Gibco BRL), for K562 cells RPMI-1640, all with 10% fetal bovine serum (FBS), and for HeLa and 293T cells Dulbecco’s modified medium containing 100U/ml penicillin-streptomycin, for Tris–HCl pH 8, 5 mM EDTA, 0.5% SDS, protease inhibitors) transferred to TC 12 x 12 tubes (Corvaris) and sonicated (Corvaris settings: 20 min, 5% duty cycle, 140 W, 200 cycles). Sonication was assessed by reverse cross-linking (37°C RNase A at 1 μg/ml 1 h, then 65°C, Proteinase K at 400 μg/ml, overnight), followed by DNA extraction. Fragment size (between 200 and 400 bp) was checked on a Bioanalyzer (Agilent 2100). Immunoprecipitations were performed in IP buffer (10 mM Tris at pH 8, 1 mM EDTA, 0.1% SDS, 150 mM NaCl, 10% Triton X-100 and protease inhibitors) overnight. Chromatin was reverse cross-linked (65°C, Proteinase K at 400 μg/ml, overnight) and DNA was further extracted for analysis. ChIP samples were used for SYBER Green qPCR (Applied Biosystems) or library preparation for sequencing as previously described (31).

RNA extraction and RT-qPCR

RNA was extracted with High Pure RNA Isolation Kit from Roche. All RT-qPCR reactions were performed with independent biological duplicates using random hexamers, and each cDNA was tested in triplicate with SYBR Green mix (Applied Biosystems) and primers listed in Supplementary Table S2. Negative controls without reverse transcriptase were processed in parallel.

Protein and histone extraction

Whole-cell lysates were extracted in 8 M urea lysis buffer. Histone extraction was achieved according Abcam’s histone extraction protocols (http://www.abcam.com/protocols/histone-extraction-protocol-for-western-blot). Harvest cells were resuspended and lysed in Triton Extraction Buffer (0.5% Triton X100; 2 mM PMSF; 0.02% NaN₃). Histone extraction was achieved with 0.2N HCl over night at 4°C. Protein estimation was determined using a ND-1000 spectrophotometer (Nanodrop, Thermo Fisher Scientific).

Lentiviral vector production and transduction

Production and titration lentiviral vectors were achieved as previously described (32). Transduction was performed as previously described (33) (see http://tronolab.epfl.ch/lentivectors for details).

Cell fractionation

KAP1 KO MEFs were lysed in soluble buffer (0.1% NP40, 10 mM Tris–HCl pH 8, 5 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, protease inhibitor) during 3 min at 4°C, then centrifuged 30 s at 6000 rpm at 4°C (supernatant = soluble fraction). The pellet was re-incubated with nucleus buffer (250 mM NaCl, 50 mM Tris–HCl pH 8, 5 mM EDTA, 0.1% NP40, 1 mM PMSF, protease inhibitor) during 30
and Supplementary Figure S1D). Moreover, this interaction was found by cell fractionation to occur mainly within the nuclear fraction and weakly on chromatin fraction (Figure 1E), and not to be mediated by DNA since it resisted DNase treatment (Supplementary Figure S1E).

S phase-specific KAP1 phosphorylation on serine 473 promotes interaction with DNA replication factors

KAP1 can be subjected to several post-translational modifications (PTM) including sumoylation, ubiquitination and phosphorylation. Among these, phosphorylation on serine 473 (S473) was observed in the setting of DNA damage (35), metabolic stress (36), T cell receptor activation (37,38) and KSHV (Kaposi’s sarcoma-associated herpesvirus) infection (39), and to be mediated by PKCδ (Protein Kinase C δ) during S phase (40). We thus examined the influence of the cell cycle on this KAP1 PTM by performing IF with a pS473KAP1-specific antibody on NIH3T3 cells synchronized by 3 days of serum starvation followed by serum re-addition. This revealed that KAP1 became phosphorylated on S473 two hours after serum addition and stayed so for about 5 h, yielding an IF pattern that overlapped with that of PCNA (Supplementary Figure S2A). This prompted us to ask if KAP1 association with PCNA and other components of the DNA replication machinery was influenced by this PTM. To this aim, we used phospho-null (S473A) and phospho-mimetic (S473E) GST-KAP1 mutants. GST-KAP1(S473E) interacted more strongly than the other two derivatives with PCNA and, to a lesser extent, MCM proteins, whether human or murine cellular extracts were used as capture substrates, whereas all three variants associated equally well with HP1γ, which served as a positive control (Figure 2A and Supplementary Figure S2B). The S473A mutation did not abrogate PCNA recruitment in this system, but this might have been due to oligomerization of the mutant protein with its wild type counterpart, with secondary recruitment of PCNA. We next performed co-immunoprecipitation (co-IP) studies with both endogenous PCNA and KAP1 in mouse embryonic fibroblasts (MEFs) synchronized/ arrested in S phase following treatment with hydroxyurea (HU). We observed that KAP1 increasingly associated with PCNA between 90 and 150 min of HU treatment, then decreased. Interestingly, this interaction coincided with KAP1 phosphorylation on serine 473 (Figure 2B). We then performed co-IP studies on synchronized Kap1 KO MEFs complemented with either HA-KAP1(WT), HA-KAP1(S473A) or HA-KAP1(S473E). After serum addition, PCNA increasingly associated with HA-KAP1(WT), peaking at 22 h, again remarkably coinciding with KAP1 S473 phosphorylation in these cells, which have a significantly longer cycle compared with NIH3T3 fibroblasts (Figure 2C). Similar results were obtained when using extracts from NIH3T3 cells overexpressing HA-KAP1(WT) as substrates for the co-IP (Supplementary Figure S2C). In contrast, the association of the phospho-null HA-KAP1(S473A) with PCNA was weak and restricted to 22–28 h after serum addition (Figure 2D, left panel), whereas the phospho-mimetic HA-KAP1(S473E) constitutively interacted with PCNA (Figure 2D, right panel). These results were confirmed by deter-
Figure 1. KAP1 interacts with replication factors. (A) Main KAP1-interacting proteins detected by mass spectrometry in hESC and K562 cells, with indication of some over-represented biological processes. Data were generated with a Scaffold software with option quantitative values calculating P-value with unpaired, two tailed t-test. (B and C) Recombinant GST, GST-KAP1 and mutants were incubated for pull-down assays with (B) NIH3T3 cell extracts or (C) recombinant HIS-PCNA. Western blotting (WB) was performed with indicated antibodies. (D) Immunofluorescence microscopy was performed with indicated antibodies in NIH3T3 cells. Representative of 159 cells examined, 53.5% of which were in S-phase (27.7% early, 15.7% mid, 10% late). (E) KAPI KO MEFs complemented with HA-KAP1(WT) were subjected to fractionation to yield soluble, nucleus and chromatin fractions, before immunoprecipitation (IP) and WB with indicated antibodies.
Figure 2. Phosphorylation-stimulated interaction of KAP1 with DNA replication factors. (A) Recombinant GST, GST-KAP1 and mutants were incubated with NIH3T3 cell extracts for pull down assays, and WB performed with indicated antibodies. Results representative of 3 independent experiments. (B) MEFs were treated with HU for indicated numbers of minutes and cell extracts subjected to co-IP/WB with antibodies against indicated proteins. (C and D) Kap1 KO MEFs were complemented with HA-tagged KAP1 WT, S473A or S473E and synchronized by serum starvation for 72 hrs, before performing IP/WB at indicated number of hrs post-serum re-addition. (B, C and D) are each representative of two independent experiments.
mining the fraction of total input HA-KAP1 that was co-immunoprecipitated with PCNA (Supplementary Figure S2D). Of note, levels of all KAP1 forms (WT, S473A and S473E) were significantly decreased at the end of the serum starvation period and 30 h after serum re-addition, suggesting their regulation by the cell cycle (Figure 2C and D). The temporal pattern of KAP1-PCNA co-immunoprecipitation remarkably coincided with that of KAP1 S473 phosphorylation and this association preferentially occurred during S phase as demonstrated by FACS analysis (Supplementary Figure S2E). Confirming this result, PCNA interacted with transfected HA-KAP1 (WT) and (S473E) but much less with the phospho-null S473A mutant in wild type MEFs synchronized/arrested in S phase by HU treatment (Supplementary Figure S2F). Confirming the stimulation of PCNA-KAP1 association by phosphorylation, levels of HA-KAP1 co-immunoprecipitated with PCNA were considerably decreased by phosphatase treatment (Supplementary Figure S2G). Taken together, these results demonstrate that KAP1 phosphorylation on S473, an S phase-specific modification, governs its interactions with components of the DNA replication complex.

KAP1 participates in the maintenance of heterochromatin across cell division

Given the roles previously proposed for KAP1 in the maintenance of H3K9 tri-methylation (27–29), we next explored the impact of mutations affecting its interaction with PCNA on the maintenance of this heterochromatin mark. For this, we carried out histone extraction in NIH3T3 cells overexpressing wild type or mutant versions of HA-KAP1. We first verified that steady-state levels of H3K9me3 were not modified in the presence of these proteins in asynchronous cell populations. Then, following addition of nocodazole, a compound that blocks the cell cycle in G2/M, we found that overexpression of wild type, S473A and S473E KAP1 increased levels of H3K9me3 (Figure 3A, left panel), although with a stronger effect for the phospho-mimetic mutant. Similar observations were made in 293T cells (Supplementary Figure S3A). These results suggest that the gain of H3K9me3 induced by KAP1 overexpression might occur after S phase or in G2. To explore further the influence of KAP1 on H3K9 mono- and di-methylation, we used WT and Kap1 KO MEFs. A decrease of all three methylated forms of H3K9 was observed in the knockout cells. Complementation with KAP1(WT) and KAP1(S473E) restored wild type levels of di- and tri-methylation, although effects on H3K9me1 levels were only modest. In contrast, the phospho-null KAP1(S473A) and plant homeodomain deletion (ΔPHD) mutants rescued H3K9 mono- and dimethylation but only very weakly affected levels of trimethylation (Figure 3A, middle and right panel). We confirmed H3K9me3 reduction in NIH3T3 cells treated with a short hairpin RNA targeting KAP1 (shKAP1) (Supplementary Figure S3B). Moreover, in these cells as well, complementation with the phospho-null KAP1 mutant only weakly increased H3K9me3 (Figure 3A, right panel), consistent with previous data demonstrating that KAP1 is required to provide H3K9me1/2 as substrate for Suv39h1 (27). We next asked whether this reduction of H3K9me3 was genome wide or a local effect restricted to the surroundings of KAP1 binding sites. For this, we first performed KAP1 chromatin immunoprecipitation followed by deep sequencing (ChIP-Seq) in WT MEFs. As expected (41,42), KAP1 was detected at the 3′ end of zinc finger protein genes (e.g. ZFP316) and over imprinting control regions (e.g. Grb10 and MEST) (Figure 3B and Supplementary Figure S3C). We then compared by ChIP-qPCR the distribution of several heterochromatin marks at these loci in WT and Kap1 KO MEFs. H3K9me3, H4K20me3 and H3K64me3, a modification within the globular domain of H3 found at pericentromeric heterochromatin (11,43), were all enriched over KAP1-binding regions in wild type cells and markedly decreased at these sites in their Kap1 KO counterparts (Figure 3C). The KMTs Suv39h1 and Suv420h1, involved in tri-methylation of respectively H3K9 and H4K20, were also less enriched at these loci in the absence of KAP1 (Figure 3D). HP1 proteins recognize H3K9me3 via their chromodomain (15). In Kap1 KD NIH3T3 cells, HP1α was also delocalized from pericentromeric heterochromatin domains (Supplementary Figure S3D), consistent with the observed decrease of H3K9me3 (Supplementary Figure S3B). Importantly, total levels of a large series of heterochromatin regulators were not significantly altered in Kap1 KO MEFs (Supplementary Figure S3E). This indicated that the decreased DNA association of Suv39h1, Suv420h3, H3K9me3, H4K20me3 and HP1α resulted primarily from a defect in the genomic recruitment of these proteins. Therefore, KAP1 contributes to the maintenance of all forms of H3K9 methylation (mono-, di-, and tri-), with pS473KAP1 specifically required to preserve H3K9me3. Moreover, at genomic loci normally bound by KAP1, absence of this regulator results in loss of both writers (KMT) and readers (HP1) of these heterochromatin histone marks.

Differential KAP1 requirements for H3K9me3 and H4K20me3 maintenance

We found KAP1 to be required for maintaining H3K9me3 and H4K20me3 at its genomic targets (Figure 3C). However, histone extraction and IF studies detected a global decrease in levels of the former but not the latter histone mark following Kap1 KD in NIH3T3 cells (Supplementary Figure S4A and B). This suggested that the KAP1-dependent accumulation of H3K9me3, but not H4K20me3, might extend beyond KAP1 binding sites. We thus performed H3K9me3 and H4K20me3-specific ChIP-Seq in WT and Kap1 KO MEFs and could identify and characterize three patterns (Figure 4A): (i) KAP1 binding regions with loss of H3K9me3 and H4K20me3 upon KAP1 deletion; (ii) H3K9me3 and H4K20me3 enriched sites without detectable KAP1 at baseline, but losing these marks in the absence of the scaffold protein and (iii) domains with only H4K20me3, for which KAP1 depletion had no effect. Thus, the accumulation of H3K9me3 seemed to be globally KAP1-dependent, whereas that of H4K20me3 appeared to require this protein only at some sites, where it was coupled with H3K9me3. To explore this point further, we mapped all H3K9me3- and H4K20me3-enriched regions in WT and Kap1 KO MEFs. This confirmed an overall reduction of H3K9me3 in the absence of KAP1, not limited to...
Figure 3. KAP1 maintains heterochromatin-associated histone modifications and factors. (A) Left, NIH3T3 cells expressing indicated KAP1 variants were treated or not with nocodazole for 10 h before performing WB on histone extracts with indicated antibodies. Right, same experiment on WT and Kap1 KO MEFs without nocodazole treatment. (B) Screenshot of selected KAP1 ChIP-Seq peaks in WT MEFs. (C and D) ChIP-qPCR with indicated antibodies on pre-mapped KAP1-binding regions. Data are means ± s.e.m. from three independent experiments. Significance of the differences was estimated using Student’s t-test. ***P < 0.01, 0.01 < **P < 0.05 and 0.05 < *P < 0.1.
Figure 4. Differential KAP1-mediated maintenance of H3K9me3 and H4K20me3. (A) KAP1, H3K9me3 and H4K20me3 ChIP-Seq peaks in WT and Kap1 KO MEFs, pointing on top to regions (1, 2 and 3 asterisk) with differential patterns. (B–D) Heatmaps representing the raw data –/+10 kb around the middle of histone mark peaks in WT and Kap1 KO MEFs. H3K9me3/H4K20me3-positive regions were defined with a stringent P-value (10e–8), and specific loci as positive according to this criterion in one dataset and negative even with low stringency P-value in the other data set (<10e–8 versus >10e–3).
which contrasted with H4K20me3 (Figure 4A and C). We then
defined 723 regions enriched both with H3K9me3 and
H4K20me3 (Supplementary Figure S4C), and could deter-
mine that H4K20me3 was lost only at these loci upon KAP1
KO (Figure 4A and D). H4K20me3 is a mark previously de-
scribed as enriched within pericentromeric heterochromatin
and generally associated with H3K9me3. However, we
found this modification also present downstream of tran-
scription start sites (TSS), where KAP1 depletion had no
effect (Supplementary Figure S4D). The TSS-association of
H4K20me3 was previously reported, and appears to regu-
late pol II release (44). Taken together, our results indi-
cate that KAP1 plays a critical role for the overall mainte-
nance of H3K9me3, and indirectly that of H4K20me3 at
H3K9me3-positive loci.

KAP1 partners up with Suv39h1 to maintain heterochromatin
during DNA replication

We next asked which histone KMT is recruited by KAP1
to maintain H3K9me3 during DNA replication. In mamma-
lian cells, five such enzymes have been identified: SETDB1, GLP, G9a and Suv39h1/h2, all of which have been
detected in complexes with KAP1 by mass spectrom-
ometry (30). G9a was unlikely to be the KMT involved in
the phenomenon described here, because we previously
observed that KAP1 phosphorylation on S473 prevents its
recruitment, at least within MyoD transcription com-
plexes (45). Still, overexpression each one of these methyl-
transferases resulted in increased levels of H3K9 methyla-
tion in 293T cells, although Suv39h1 had the strongest ef-
ect (Supplementary Figure S5A). Moreover, pull-down as-
says with WT, S473A or S473E GST-KAP1 on NIH3T3
or HeLa protein extracts expressing HA-tagged Suv39h1
revealed that this enzyme interacted most strongly with
the phospho-mimetic KAP1 mutant (S473E), and most
weakly with its phospho-null S473A counterpart. (Fig-
ure 5A and Supplementary Figure S5B). Recently, both
Suv39h1 and KAP1 were described as potential compo-
ments of nascent chromatin (46,47). This prompted us to
ask whether Suv39h1 might also interact with DNA replica-
tion factors. Co-localization of Suv39h1 and PCNA at
DAP1-intense loci was first documented by immunofluo-
rescence microscopy (Figure 5B). We next performed co-
IP studies on NIH3T3 overexpressing HA-Suv39h1 syn-
cronized by HU treatment. Expression of Cyclin E and
Cyclin D ascertained that these cells were well synchro-
nized and in S phase. PCNA and HA-Suv39h1 were found
to interact increasingly after HU addition, with a peak at
300 min (Figure 5C). Similar results were obtained in HA-
Suv39h1-overexpressing NIH3T3, and HeLa cells synchro-
nized by serum starvation/re-addition (Supplemental Fig-
ure S5C and D). We next examined the expression pro-
file of Suv39h1 during the cell cycle in KAP1 KO MEFs com-
plemented with HA-KAP1(WT). This revealed that
Suv39h1 levels peaked during S phase (24 hrs and 22 hrs
after serum addition for mRNA and protein, respectively),
when KAP1 and PCNA interact (Figure 5D). These results
suggest that Suv39h1, when expressed in S phase, might
associate with PCNA. Furthermore, knocking down Kap1 in
NIH3T3 cells overexpressing HA-Suv39h1 resulted in re-
ducing, albeit not abrogating, the Suv39h1-PCNA interac-
tion (Figure 5E). In contrast, GST pull-down assays per-
formed with Suv39h1/2 KO MEFs demonstrated that the
interaction of PCNA or MCM6 with KAP1 is independent of
these KMTs (Supplementary Figure S5E). These data sug-
gest that p473KAP1 serves as a bridge between Suv39h1
and PCNA. We next investigated if KAP1 and Suv39h1
were recruited on newly synthesized DNA. For this, we per-
formed ChiP-qPCR on control and Kap1 KO NIH3T3 cells
synchronized by serum starvation, using antibodies against
KAP1, Suv39h1 and H3K9me3, focusing on strong KAP1
targets (e.g. Dlk1, Supplementary Figure S5F). In control
cells, KAP1 genomic recruitment was increased shortly (2
h) after serum addition, Suv39h1 enrichment was sustained
for several hours and H3K9me3 levels remained robust. In
contrast, in KAP1-depleted cells, Suv39h1 recruitment was
only transient, dropping 4–5 h after serum addition, and
H3K9me3 levels were lower than in controls cells through-
out the several hour-long experiment. Together, these data
are consistent with a model whereby phosphorylation of
serine 473 of KAP1 during S phase fosters the recruit-
ment of Suv39h1 by PCNA.

Differential effects of KAP1 and Suv39h1 at major satellite
repeats

Even though MEFs stably deleted for Kap1 could be gener-
ated, acute KAP1 depletion was highly toxic in several cell
lines including NIH3T3, where it induced cell death within
less than 10 days (Supplementary Figure S6A). We rea-
soned that if KAP1 and Suv39h1 were functionally linked,
elevating levels of Suv39h1 might attenuate this effect. We
thus induced HA-Suv39h1 overexpression in Kap1 KO
NIH3T3 cells, and remarkably observed a rescue of their
toxic phenotype (Supplementary Figure S6A), with cells
further displaying wild type levels of H3K9me3 (Figure 6A
and Supplementary Figure S6B). To determine whether this
effect was maintained throughout the cell cycle, we cul-
tured HA-Suv39h1 overexpressing NIH3T3 cells depleted
for Kap1 during 3 weeks and monitored H3K9me3 lev-
els every three days. H3K9me3 levels were maintained up
to 15 days after Kap1 KO, but gradually decreased there-
after (Supplementary Figure S6C). These results indicate
that Suv39h1 overexpression can compensate for KAP1
depletion in global maintenance of heterochromatin, al-
though this effect is progressively lost as cell divisions accu-
mulate. We next examined the relative KAP1-dependence
and Suv39h1-mediated rescue of H3K9 tri-methylation at
different subsets of genomic loci. For this, we compared
WT and Kap1 KD NIH3T3 cells overexpressing or not
Suv39h1. We found a marked drop in H3K9me3 at Kap1
binding sites upon Kap1 KD, which was only partly rescued
by Suv39h1 overexpression, at least at the time point ex-
amined (15 days post-KD) (Figure 6B). The recent anal-
ysis of MEFs stably deleted for Suv39h1/h2 served as a
basis for implicating these histone methyltransferases in
the establishment and maintenance H3K9me3 at constitu-
tive heterochromatin, including major satellite repeats (48).
When we analyzed H3K9me3 ChIP-Seq data in WT and
Suv39h1/h2 KO MEFs (H3K9me3 ChIP-Seq from (48)), we
Figure 5. pS473KAP1 dependent Suv39h1-replication factors complex formation. (A) Recombinant GST, GST-KAP1 and mutants were incubated with NIH3T3 cell extracts overexpressing HA-Suv39h1. WB was performed with indicated antibodies. (B) Immunofluorescence with DAPI or indicated antibodies in NIH3T3 cells overexpressing HA-Suv39h1. (C) NIH3T3 cells overexpressing HA-Suv39h1 were synchronized/arrested by HU treatment. Co-IP experiments and WB were performed with indicated antibodies at indicated minutes post-HU addition. (D) Nuclear protein extraction and RNA extractions were performed in KAP1 KO MEFs complemented with HA-KAP1 (WT), arrested by serum starvation for 72 h and released to cell cycle with serum addition then WB were performed with indicated antibodies. Levels of mRNA for the indicated genes were measured by RT-qPCR at indicated times post-serum addition. Two different primers for Suv39h1 were used in this experiment. (E) Co-IP experiments and WB were performed in NIH3T3 cells overexpressing HA-Suv39h1 and transduced with shE or shKAP1 with indicated antibodies.
Figure 6. Suv39h1 overexpression overcomes cell death and restores H3K9me3 depletion induced by KAP1 depletion at major satellite regions. (A) NIH3T3 cells overexpressing or not HA-Suv39h1 were transduced with lentiviral vectors expressing (shKAP1) or not (shE) KAP1-targeting small hairpin RNAs. Histones were extracted and tested by WB with indicated antibodies. (*) Corresponds to lower exposure time. (B–D) H3K9me3-specific ChIP-qPCR in indicated cell lines. Data are means ± s.e.m. from two independent experiments. Student’s t-test, ***P < 0.01, **P < 0.05 and *P < 0.1 (three different primers used for major satellite). (E) RT-qPCR measurement of indicated transcripts in WT and KAP1 KO MEFs. (F) H3K64me3 (left) and H4K20me3 (right) -specific ChIP-qPCR at indicated loci. Data are means ± s.e.m. from three independent experiments. Student’s t-test, ***P < 0.01, **P < 0.05 and *P < 0.1.
observed that depletion of Suv39h1/h2 did not lead to an overall reduction in H3K9me3, contrasting with what was found in Kap1 KO MEFs (Supplementary Figure S6D and Figure 4B). Furthermore, while KAP1 depletion reduced H3K9me3 levels at major satellite repeats, Suv39h1 overexpression completely abrogated this phenomenon (Figure 6C), consistent with a major role for this KMT in the maintenance of constitutive heterochromatin (48). When we repeated ChIP-qPCR experiments in stable Kap1 KO MEFs, there was a decrease in H3K9me3 at sites normally bound by the regulator (as illustrated in Figure 3C), but not at major satellite repeats (Figure 6D). Correspondingly, the presence HP1α and HP1β was dramatically decreased at the former, but not at the latter type of loci (Supplementary Figure S6E). Together, these data indicate that cell death and loss of heterochromatin integrity induced by KAP1 depletion can be rescued by Suv39h1 overexpression. Noteworthy, in spite of their persistent association with H3K9me3, the expression of major satellite transcripts increased in Kap1 KO MEFs (Figure 6E). It suggests that this chromatin mark does not primarily influence transcription from these loci. We then examined by ChIP-qPCR the enrichment in H3K64me3 and H4K20me3, two other marks of constitutive heterochromatin. While H4K20me3, as H3K9me3, was lost at Kap1 binding sites but preserved at major satellite repeats in Kap1 KO MEFs, H3K64me3 was reduced at both places (Figures 3C and 6F). Thus, the H3K64me3 histone mark appears to play an important role in repressing transcription at major satellite repeats.

**DISCUSSION**

This work demonstrates a central role for KAP1 in the maintenance of heterochromatin during DNA replication. In contrast to previous work focused on the role of KAP1 in the maintenance of H3K9me3 during cell division at pericentromeric and subtelomeric heterochromatin (27–29), we demonstrate here that KAP1 phosphorylation on serine 473 is required to maintain H3K9me3 globally and that this modification also promotes H4K20me3 and H3K64me3. Furthermore, we reveal that KAP1 is not required for maintenance of only H3K9me3 but of all three methylated forms of H3K9. Based on our result and previous report, we propose a model for heterochromatin maintenance across cell division whereby, during the late phase of DNA synthesis, KAP1 is phosphorylated on serine 473, which triggers its association with PCNA and other components of the DNA replication machinery within heterochromatic regions. The Suv39h1 KMT is in turn recruited, enabling the tri-methylation on lysine 9 of newly incorporated histone H3 molecules, after they have been mono-methylated by the CAF1–KAP1–HP1–SETDB1 complex (27). The H3K9me3 mark is then recognized by HP1, and Suv420h1 is recruited, which establishes H4K20me3 (12).

Finally, H3K64 tri-methylation ensues, through the action of an enzyme that remains to be identified, which results in transcriptional silencing notably at satellite repeats (Figure 7). We also observed that Suv39h1 overexpression could rescue cell death induced by KAP1 depletion by preserving heterochromatin integrity.

Previously, Fritsch et al. found KAP1 associated with H3K9 KMT such as GLP/G9a, SETDB1 and Suv39h1/h2 (30). However, we found only EHMT1 (G9a) in our mass spectrometry data. The difference could stem from our immune-precipitating endogenous KAP1 for our affinity purification / mass spectrometry, whereas they used an overexpressed and FLAG-tagged form of the KMT to recover KAP1.

How KAP1 is specifically targeted to heterochromatic regions during DNA synthesis is unclear since PCNA is found within both euchromatin and heterochromatin. In our IF experiment, we never observed PCNA-KAP1 co-localization at early S phase, when euchromatin is known to be replicating. It could be that during heterochromatin replication the kinase responsible for phosphorylating KAP1 on S473 is activated, since this modification enhances KAP1 association with the DNA replication complex. KAP1 phosphorylation on S473 and S824 prevents its interaction with the KMTs G9a and SETDB1, respectively (49,50). During DNA damage, KAP1 is first phosphorylated on S824, and then on S473 by ATM-Chk2 or ATR-Chk1 (35,51,52). It could be that a phospho-switch mechanism is at play following double strand break (DSB), with phosphorylation first on S824 to relax chromatin and facilitate the recruitment of DNA repair factors (53,54), and secondly on S473 to re-establish heterochromatin marks via the PCNA-pS473KAP1-Suv39h1 complex (Figure 7).

Our demonstration that pS473KAP1 acts as bridge between Suv39h1 and PCNA fits with the detection of both KAP1 and Suv39h1 at the replication fork (46,47). Furthermore, our finding that H3K9 tri- but not mono- or di-methylation requires KAP1 phosphorylation on S473, a modification needed to recruit Suv39h1, is consistent with results indicating that CAF1-HP1-KAP1-SETDB1 can induce mono-methylation of newly synthesized histone H3 on lysine 9 (27) and implicates KAP1 at several steps of the H3K9 methylation process through two and perhaps three distinct KMT partners, G9a being apparently involved at some loci (28).

KAP1 depletion was previously noted to be toxic in a number of cells (31,55–57), and here Kap1 KO caused the death of NIH3T3 cells. This effect could be avoided at least temporarily by overexpressing Suv39h1, which further prevented loss of H3K9me3 at major satellite repeats, consistent with a major role for this KMT in the maintenance of H3K9me3 at constitutive heterochromatin (48). More surprisingly, stable Kap1 knockout MEFs cell lines could be developed, which displayed normal levels of H3K9me3, H4K20me3 and HP1 at major satellite repeats, indicating possible compensatory mechanism. Nevertheless, transcription from these regions was aberrantly activated in these cells, coinciding with a decrease in H3K64me3. Acetylation at this histone residue (H3K64ac) was recently reported to facilitate transcription (58) and to provide the underlying DNA with enhancer activity (59). The modulation of major satellite transcription likely plays an important role in the establishment, formation and spreading of heterochromatin. RNAs emanating from heterochromatin can guide HP1 proteins to these regions (60–62). Moreover, in fission yeast, expression of long noncoding RNA (LncRNA) transcribed from constitutive heterochromatin is required
Figure 7. A model for heterochromatin maintenance across cell division. During S phase, the complex SETDB1-KAP1-CAF1 induces the mono-methylation of lysine 9 on histone H3 (1), which is incorporated into nucleosomes via CAF1 (2). During late S phase (3), an unknown kinase phosphorylates KAP1 on serine 473, which promotes interaction with PCNA and other replication factors. At the replication fork, KAP1 recruits Suv39h1, which mediates the formation of H3K9me3 (4). During Interphase, nucleosomal H3K9me3 is recognized by HP1 (5), which in turn tethers Suv420h1, responsible for the tri-methylation of H4K20 (6), whereas an unknown KMT mediates the formation of H3K64me3 in an H3K9me3-dependant manner (7).

for the establishment and spreading of repressive marks via the RNAi pathway (63). On the contrary, Borderline, a lncRNA transcribed at the border between heterochromatin and euchromatin, inhibits the spreading of the former into the latter in Schizosaccharomyces pombe (64). Supporting our model, it was very recently proposed that major satellite repeat transcripts, by forming RNA:DNA hybrids, can stabilize the chromatin retention of Suv39h (20,21). Together, these results warrant future experiments exploring the relationship between KAP1, H3K64me3 and major satellite repeats expression.

KAP1 contributes to genome stability through a variety of effects, whether by silencing the endovirome (3–6,56,57), participating in DNA repair (35,51–54) or ensuring the maintenance of heterochromatin across cell division (the present work and (27–29)), which likely minimizes recombination between repetitive sequences (65). As such, KAP1 truly represents a key factor in the preservation of genome integrity.

DATA AVAILABILITY

All next-generation sequencing data have been submitted to the NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) database under the accession number GEO: GSE 86391.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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