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Maintenance of Silent Chromatin through Replication Requires SWI/SNF-like Chromatin Remodeler SMARCAD1

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SUMMARY

Epigenetic marks such as posttranslational histone modifications specify the functional states of underlying DNA sequences, though how they are maintained after their disruption during DNA replication remains a critical question. We identify the mammalian SWI/SNF-like protein SMARCAD1 as a key factor required for the re-establishment of repressive chromatin. The ATPase activity of SMARCAD1 is necessary for global deacetylation of histones H3/H4. In this way, SMARCAD1 promotes methylation of H3K9, the establishment of heterochromatin, and faithful chromosome segregation. SMARCAD1 associates with transcriptional repressors including KAP1, histone deacetylases HDAC1/2 and the histone methyltransferase G9a/GLP and modulates the interaction of HDAC1 and KAP1 with heterochromatin. SMARCAD1 directly interacts with PCNA, a central component of the replication machinery, and is recruited to sites of DNA replication. Our findings suggest that chromatin remodeling by SMARCAD1 ensures that silenced loci, such as pericentric heterochromatin, are correctly perpetuated.

INTRODUCTION

Functional chromatin domains in eukaryotic genomes are characterized by distinct patterns of histone modifications, critical for specifying cell identity, dictating growth, and directing development. Chromatin organization is continuously challenged, locally by DNA repair and transcription and genome wide when DNA is replicated. Nucleosome disassembly ahead of the replication fork necessitates the rebuilding of chromatin domains after every S phase. A fundamental but poorly understood aspect of chromatin assembly on newly replicated DNA is the re-establishment of the original epigenetic marks (Groth et al., 2007; Margueron and Reinberg, 2010; Probst et al., 2009). Defects in the maintenance of specific chromatin domains can lead to aberrant gene expression and chromosome instability (Peters et al., 2001; Taddei et al., 2001).

A paradigm for understanding transmission of transcriptionally silent chromatin is pericentric heterochromatin, which flanks centromeres and is essential for chromosome segregation. Pericentric heterochromatin is enriched in methylated DNA, hypoacetylated histones, and trimethylated lysine 9 on histone H3 (H3K9me3), the preferred binding site for heterochromatin protein 1 (HP1), which together are involved in heterochromatin formation and maintenance (Kwon and Workman, 2008; Maison and Almouzni, 2004). Studies on the machinery required for heterochromatin replication have focused on histone chaperones (Corpet and Almouzni, 2009), but the role of chromatin modifying enzymes and nucleosome remodelers has yet to be fully investigated.

ATP-dependent nucleosome-remodeling enzymes are key mediators of chromatin dynamics (Clapier and Cairns, 2009). They share a catalytic subunit with homology to the yeast SWI/SNF ATPase and use ATP hydrolysis to alter histone-DNA interactions to regulate access to DNA. Few remodeling complexes have been implicated in replication in higher eukaryotes. The WICH-remodeling complex has been linked to maintaining an open chromatin state (Poot et al., 2004). The ACF1-SNF2H complex is required for efficient replication through heterochromatin (Collins et al., 2002), and the Mi-2/NuRD complex has been implicated in a heterochromatin assembly pathway specific to lymphoid cells (Helbling Chadwick et al., 2009). Whether a SWI/SNF-like factor is generally required for the propagation of heterochromatin has not been addressed. SMARCAD1 (ETL1 or HEL1), a widely expressed SWI/SNF-like protein conserved from yeast to man, has emerged as a regulator of pluripotency and self renewal (Adra et al., 2000; Hong et al., 2009; Schoor et al., 1993). Its deletion is associated with developmental defects but its precise function is unknown (Schoor et al., 1999). The budding yeast ortholog of SMARCAD1, Fun30, has been linked to silencing and remodels nucleosomes in vitro and in vivo (Awad et al., 2010; Neves-Costa et al., 2009).

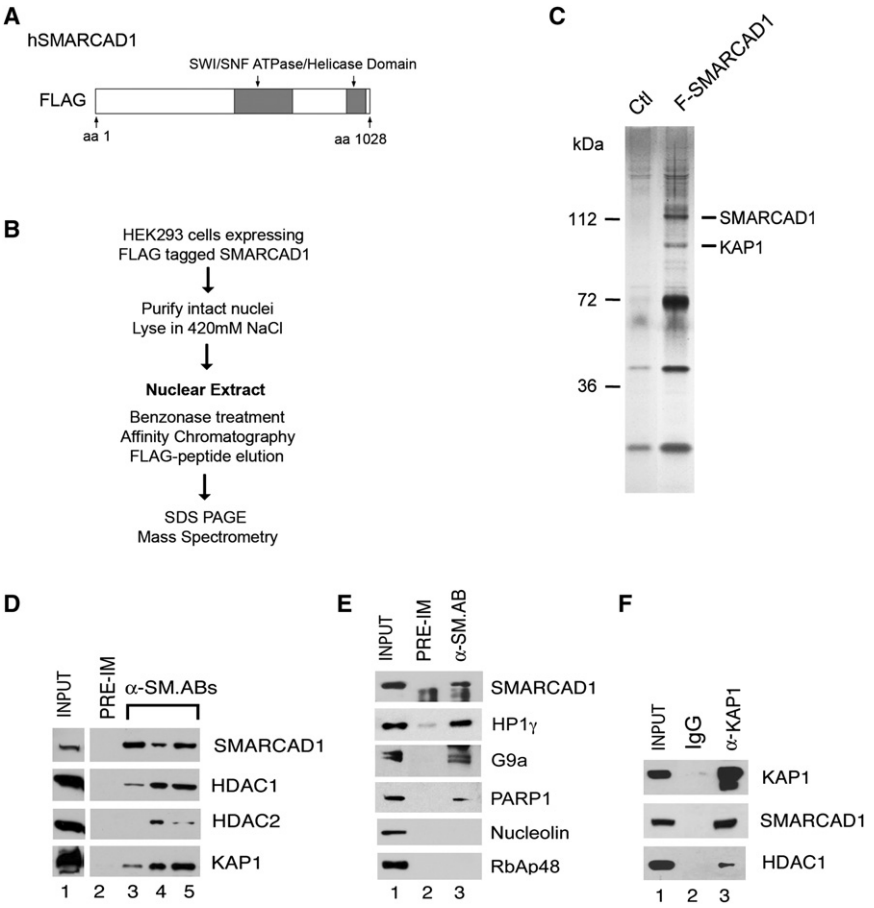


Figure 1. SMARCAD1 Interacts with Proteins Involved in Transcriptional Repression

(A) Human SMARCAD1 domain organization highlighting the ATPase domain (SNF2 motifs I–IV, aa 517–734; SNF2 motifs V–VI, aa 927–999) and an N-terminal FLAG tag.

(B) Purification procedure outline.

(C) Silver stained SDS PAGE of a typical FLAG-SMARCAD1 purification. The high intensity of the band ~70 kDa is due to the presence of BSA.

(D and E) KAP1, HDAC1-2, G9a, PARP1, and HP1γ stably associate with SMARCAD1 in the presence of Benzonase and ethidium bromide (EtBr). Proteins coimmunoprecipitated with endogenous SMARCAD1 from HeLa nuclear extracts were detected with indicated antibodies; RbAp48 and nucleolin are negative controls. Lane 1, input; lane 2, preimmune serum.

(D) Immunoprecipitation with anti-SMARCAD1 N-terminal antibody (lane 3), anti-peptide rabbit 1 (lane 4), and rabbit 2 (lane 5).

(E) Immunoprecipitation with anti-peptide SMARCAD1 antibody, lane 3.

(F) A KAP1 specific antibody coimmunoprecipitates SMARCAD1 and HDAC1 from HeLa cells in the presence of Benzonase and EtBr. Lane 1, input; lane 2, IgG; lane 3, IP.

See also Figure S1 and Table S1.

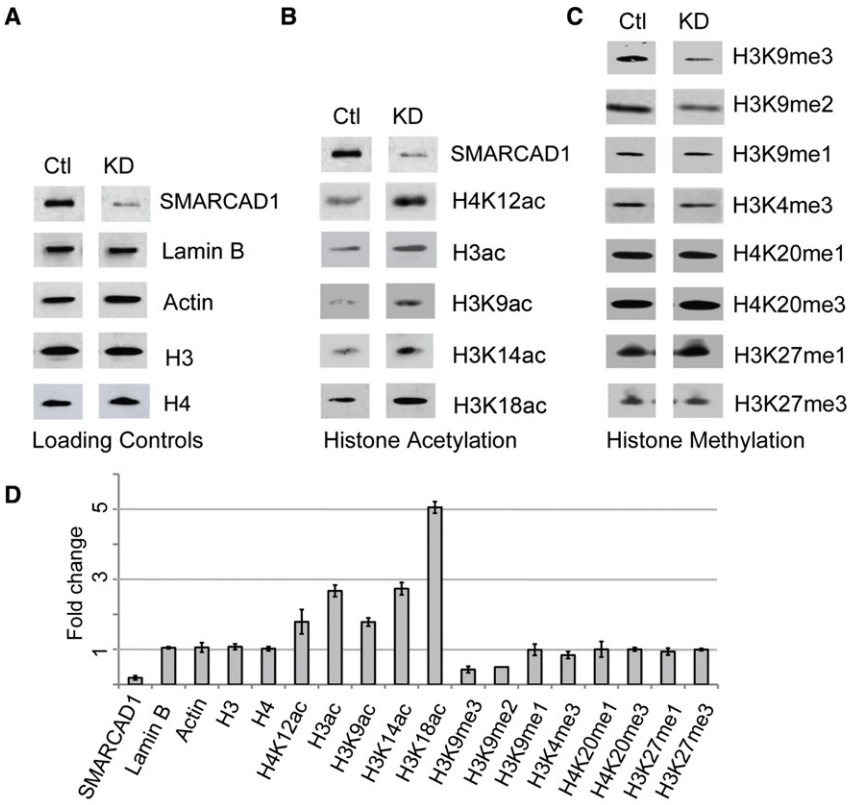
Here, we provide evidence that SMARCAD1 facilitates the maintenance of heterochromatin by directing histone deacetylation during the cell cycle. Our results highlight a key role for SWI/SNF-like factors in allowing efficient transmission of epigenetic information.

RESULTS

SMARCAD1 Interacts with Proteins Involved in Transcriptional Repression, DNA Replication, and Repair

SWI/SNF proteins often comprise the catalytic core of multisubunit complexes in which distinct subunits contribute to targeting, regulation of remodeling, and interactions with other chromatin proteins (Clapier and Cairns, 2009; Ho and Crabtree, 2010). To dissect the mechanism of SMARCAD1 function and understand how its activity is integrated with cellular processes, we identified SMARCAD1 interaction partners in HEK293 cells using FLAG-affinity protein purification and proteomic analysis (Figures 1A and 1B and Figure S1A available online). Analysis of immunoprecipitated proteins revealed two specific, prominent bands (Figure 1C). These were identified by mass spectrometry as SMARCAD1 and KRAB-associated protein KAP1 (TRIM28 or TIF1β) and confirmed by immunoblotting (Figures S1B and S1C). This interaction is consistent with similar observations in other cell lines (Okazaki et al., 2008; Seki et al., 2010). Recombinant GST-SMARCAD1 also robustly associates with KAP1 from nuclear extracts (Figure S1D). Therefore, KAP1 emerges as a major interacting partner of SMARCAD1.

The presence of multiple low-intensity bands in the FLAG-SMARCAD1 purification indicates that SMARCAD1 interacts substoichometrically with several other proteins (Figure 1C). Mass spectrometry analysis revealed that these interacting proteins fall predominantly into three major functional categories (Table S1): (1) gene silencing/heterochromatin formation, (2) DNA replication, and (3) repair, providing clues to the biological roles of SMARCAD1. KAP1 has been linked to all these processes; it acts as a transcriptional corepressor for Krüppel-associated box domain-containing zinc-finger proteins (Abrink et al., 2001; Friedman et al., 1996; Le Douarin et al., 1996) and has been implicated in replication and repair of heterochromatin (Goodarzi et al., 2008; Loyola et al., 2009; White et al., 2006; Ziv et al., 2006). We detected proteins that bind posttranslationally modified histones and contribute to silencing (HP1γ, L3MBTL2) (Guo et al., 2009; Kwon and Workman, 2008; Yoo et al., 2010), as well as enzymes that catalyze histone modifications linked to silenced chromatin. The latter include the histone deacetylases HDAC1 and HDAC2 and the histone H3K9 methyltransferase (HMT) complex G9a/GLP, which binds methylated H3K9 and catalyzes mono- and dimethylation of H3K9 (Collins and Cheng, 2010).



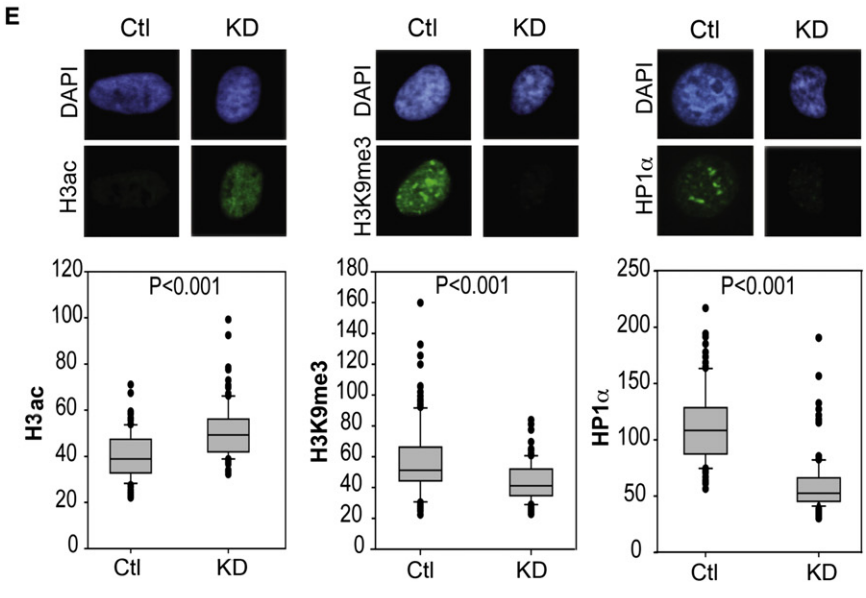


Figure 2. Depletion of SMARCAD1 Causes Global Changes in Histone Modifications and HP1 Binding

SMARCAD1 depletion results in upregulation of histone acetylation and downregulation of H3K9 methylation.

(A) SMARCAD1 knockdown cells (KD) show reduced SMARCAD1 protein levels compared to the control (Ctl). Western blot with indicated antibodies confirm equal loading.

(B and C) Whole-cell lysates were immunoblotted for histone modifications. Similar results were obtained in repeat experiments and independently derived cell lines.

(D) Quantification of panels (A)–(C) and one independent experiment showing fold change of levels in KD over Ctl. Error bars represent the standard deviation.

(E) Control (Ctl) and SMARCAD1 knockdown (KD) cells immunostained for H3ac, H3K9me3, or HP1α after extraction of soluble proteins. Box plots show quantification by measuring pixel intensity of ≥100 individual cells from two independent experiments with ImageJ software (y axis). The median value (line within box) of the KD cells lies outside the box (75^{th}/25^{th} percentile) of the Ctl population. Wilcoxon rank-sum test revealed that all differences observed are significant.

See also Figure S2.

While we anticipate that the composition of SMARCAD1 complexes may vary according to physiological signals and dependent on the genomic locus, the presence of histone modifying enzymes suggests that SMARCAD1 modulates histone modification states. We therefore focused on this group of interactors in addition to KAP1. We verified their association with SMARCAD1 by coimmunoprecipitations with endogenous SMARCAD1 in HeLa cells and by reciprocal immunoprecipitation with KAP1, HDAC1 and G9a specific antibodies (Figures 1D–1F and Figures S1E and S1F). Our results indicate that KAP1, HDAC1, HDAC2, G9a, PARP1, and HP1γ are bona fide SMARCAD1-associated factors in multiple human cell lines.

SMARCAD1 Regulates Global Histone H3/H4 Acetylation and H3K9 Methylation

The presence of HDAC1-2 and G9a/GLP in the SMARCAD1 complex prompted us to assess whether SMARCAD1 regulates deacetylation and methylation of lysine residues in histones H3 and H4. We generated HeLa cell lines stably depleted for SMARCAD1 (Figure 2A and Figures S2A and S2B). Total histones from SMARCAD1 and mock-depleted cells were screened by western blot with a panel of histone modification-specific antibodies. SMARCAD1 depletion resulted in a striking upregulation of histone H3 and H4 acetylation (Figures 2B–2D), suggesting that SMARCAD1 knockdown (KD) impacts on a large fraction of the genome. Transient SMARCAD1 KD led to a similar increase in H3 and H4 acetylation (Figure S2C). Individual residues whose acetylation was affected include H3K9, H3K14, H3K18, H4K12, and H4K16. Increase in acetylation of

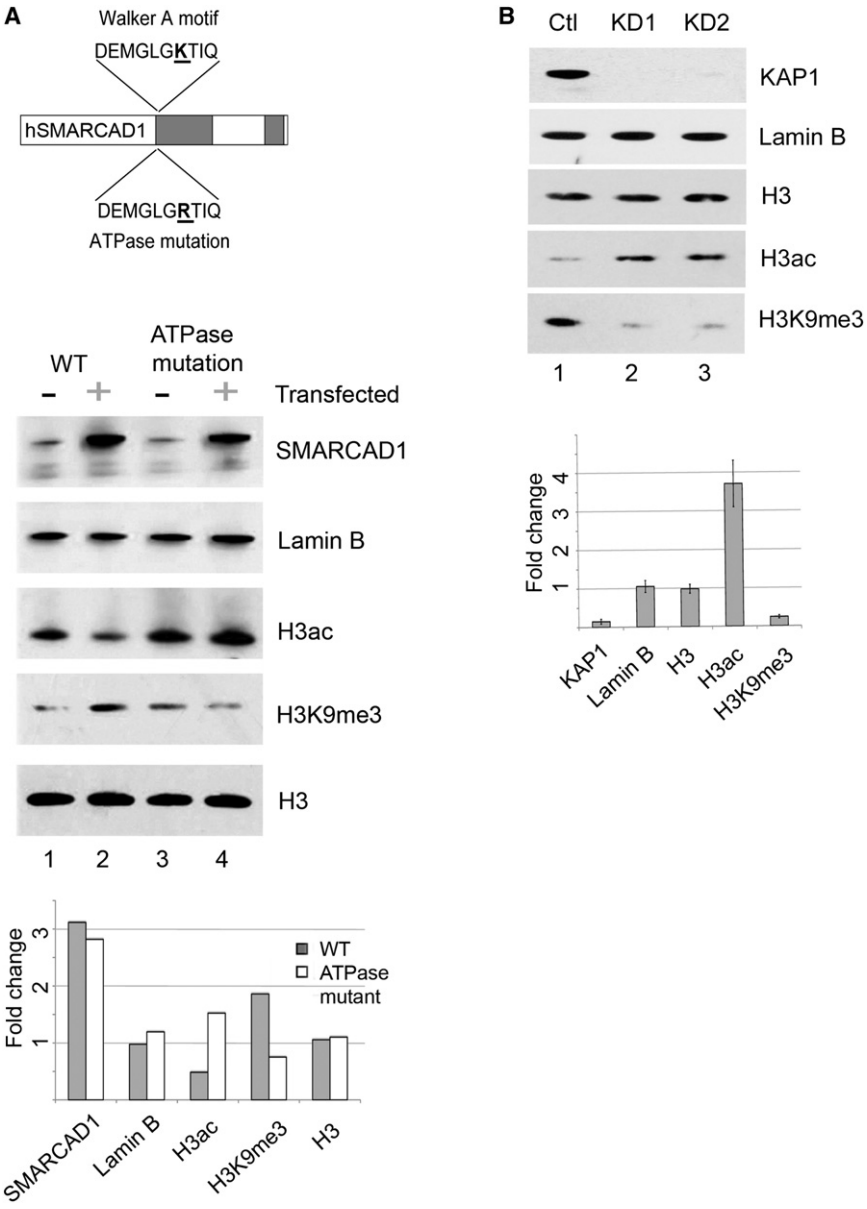


Figure 3. Regulation of Global Histone Modifications Involves KAP1 and the ATPase Function of SMARCAD1

(A) An intact ATPase domain of SMARCAD1 is required to modulate histone modifications. SMARCAD1 KD cells were cotransfected with plasmids encoding GFP and either wild-type (WT) or ATPase mutant SMARCAD1. After 48 hr, cells were FACS sorted into transfected (+) and nontransfected (–) populations using GFP fluorescence. Whole-cell lysates of sorted cells were immunoblotted for indicated proteins and modifications. Quantification of observed changes in transfected over nontransfected cells is shown. Identical results were obtained with two independent cell lines. See also Figures S3A and S3B.

(B) KAP1 depletion results in global increase in H3 acetylation and decrease of H3K9me3. Stable depletion of KAP1 by shRNA in HeLa cells (KD1 and KD2) compared to cells expressing a nonspecific shRNA (Ctl). Whole-cell lysates were analyzed; Lamin B and H3 are loading controls. Quantification from two Ctl and two KAP1 KDs are shown. Error bars represent standard deviation. See also Figure S3C.

H3K9 upon SMARCAD1 depletion correlated with a decrease in di-and trimethylation of this lysine but was not accompanied by a change in monomethylation of H3K9 (Figures 2C and 2D). Global changes in H3K27me1/3, H4K20me1/3, or H3K4 trimethylation were not observed (Figures 2C and 2D). Importantly, levels of proteins implicated in these modifications (HDAC1, HDAC2, G9a, and the H4K16 acetyltransferase, hMOF) or of KAP1 were unaltered by SMARCAD1 depletion (Figure S2D and data not shown).

Increase in H3 acetylation was apparent throughout the nucleus of SMARCAD1-depleted cells (Figure 2E). The pattern of H3K9me3 in control cells consists of a general, diffuse staining and small foci corresponding to pericentric heterochromatin (Figure 2E). Cells depleted for SMARCAD1 showed a reduction of both diffuse and focal H3K9me3 staining, suggesting that euchromatic and heterochromatic loci are affected. The observed upregulation of acetylation, a characteristic of active chromatin, together with a downregulation of H3K9me3, a hallmark of heterochromatin, is consistent with a role for SMARCAD1 in silencing and points to a function in the establishment or maintenance of heterochromatin. HP1α is a marker of centromeric heterochromatin due to its affinity for methylated H3K9 (Bannister et al., 2001; Lachner et al., 2001; Maison et al., 2002). While HP1 levels were not affected by depletion of SMARCAD1 (Figure S2D), HP1α became readily extractable (Figure 2E and Figure S2E). This is in line with the previously reported dissociation of HP1 proteins from hyperacetylated chromatin (Bártová et al., 2005; Taddei et al., 2001) and suggests that SMARCAD1 depletion perturbs heterochromatin.

Global Regulation of Histone Modifications Requires SMARCAD1 ATPase Function

To confirm that histone modification changes in knockdown cells are due to the action of SMARCAD1, we carried out complementation experiments. When SMARCAD1 was reintroduced into KD cells, H3 acetylation levels were reduced and levels of H3K9me3 increased compared to when SMARCAD1 protein levels are depleted (Figure 3A, lanes 1 and 2). SMARCAD1 contains a signature motif of SNF2 enzymes, an ATP binding pocket encompassing Walker A and B motifs (Flaus et al., 2006).

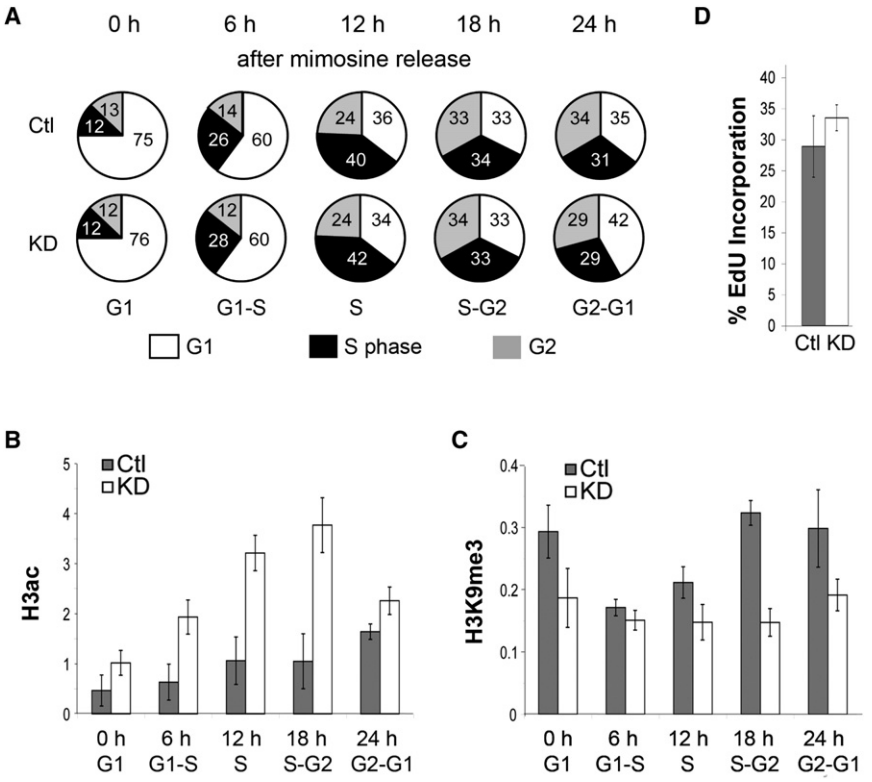


Figure 4. Global Upregulation of Histone Acetylation and Reduction in H3K9me3 Observed in SMARCAD1 Knockdown Cells Is Linked to Progression through S Phase

(A) SMARCAD1 depletion has no effect on cell cycle progression. Control (Ctl) and SMARCAD1 knockdown (KD) HeLa cells were synchronized in late G1 with mimosine. After release, cells were harvested at 6 hr intervals and analyzed by FACS. The percentage of cells in each cell-cycle phase is depicted; data are averaged from three experiments with two control and three KD cell lines.

(B and C) Lysates from synchronized cells collected at indicated time points were immunoblotted for Lamin B and either H3ac (B) or H3K9me3 (C) and quantified with chemifluorescent imaging. Values represent the 2D fluorescence density of the histone modification band (fluorescence − background/area [LAU/mm^2]), normalized to Lamin B. Error bars represent the standard deviation from three cell lines. See also Figure S4.

(D) EdU pulse labeling (30 min) of asynchronous cultures reveals that the fraction of cells in S phase is similar in Ctl (n = 703) and KD (n = 1040) (p = 0.209, t test). Standard deviation is for three experiments with five cell lines.

Substitution of a conserved lysine with arginine within this domain results in catalytically inactive SWI/SNF proteins (Deuring et al., 2000; Richmond and Peterson, 1996; Shen et al., 2000). Therefore, we examined the ability of an ATPase mutant SMARCAD1 to restore histone modifications (Figure 3A). Mutant SMARCAD1 was expressed and localized similarly to the wild-type protein (Figure S3A), but was unable to complement the aberrant levels of H3 acetylation or H3K9me3 observed when SMARCAD1 is depleted (Figure 3A, lanes 3 and 4, and Figure S3B). This result demonstrates that the ATPase function of SMARCAD1 is essential for its role in modulating histone modifications.

Based on our observation that KAP1 is a stoichiometric component of a SMARCAD1 complex, we investigated the contribution of KAP1 to the regulation of global histone modifications. Depletion of KAP1 did not affect SMARCAD1 levels but resulted in a global increase of H3 acetylation concomitant with a decrease in H3K9me3 (Figure 3B and Figure S3C and data not shown), similar to the changes in SMARCAD1 depletion experiments.

We conclude that SMARCAD1 directs histone deacetylation in the genome, possibly through its interactions with HDAC1 and HDAC2. Our results imply that SMARCAD1 and KAP1 function together to maintain normal levels of histone H3 acetylation and H3K9 methylation, as depletion of either component leads to aberrant levels of H3 modifications. Moreover, our experiments indicate a causative role for the ATPase activity of SMARCAD1, suggesting that ATP-dependent remodeling is involved in this reaction.

SMARCAD1 Regulation of Histone Modifications Is Linked to S Phase Progression

In proliferating cells increased acetylation of lysine residues in the tails of H3 and H4 is a hallmark of replication dependent nucleosome deposition during S phase (Annunziato and Seale, 1983; Scharf et al., 2009; Sobel et al., 1995; Taddei et al., 1999). To test the possibility that SMARCAD1 regulates replication-associated histone deacetylation, we addressed whether the observed histone modification changes are linked to S phase. Using synchronized SMARCAD1 KD cells we found that depletion of SMARCAD1 had no notable effect on cell-cycle progression (Figure 4A) but altered the dynamics of histone acetylation and methylation during the cell cycle (Figures 4B and 4C and Figure S4). Previous work has shown that lysine acetylation coupled with histone deposition is transient (Annunziato and Seale, 1983; Scharf et al., 2009; Sobel et al., 1995; Taddei et al., 1999). Consistent with this, global histone acetylation levels in control cells did not fluctuate significantly throughout the cell cycle (Figure 4B and Figure S4). However, in SMARCAD1 KD cells, the acetylation of H3 (Figure 4B and Figure S4A) and H4 (Figure S4B) increased progressively throughout the cell cycle, peaking in S-G2. Levels of H4K12ac were elevated throughout the cell cycle, including G1, suggesting that this mark persists in the absence of SMARCAD1 (Figure S4C). Moreover, SMARCAD1 depletion resulted in a failure to restore H3K9me3 after S phase (Figure 4C). Thus, SMARCAD1-mediated deacetylation of H3 requires progression through S phase. To verify that there is no S phase defect upon SMARCAD1 knockdown we pulse labeled newly synthesized

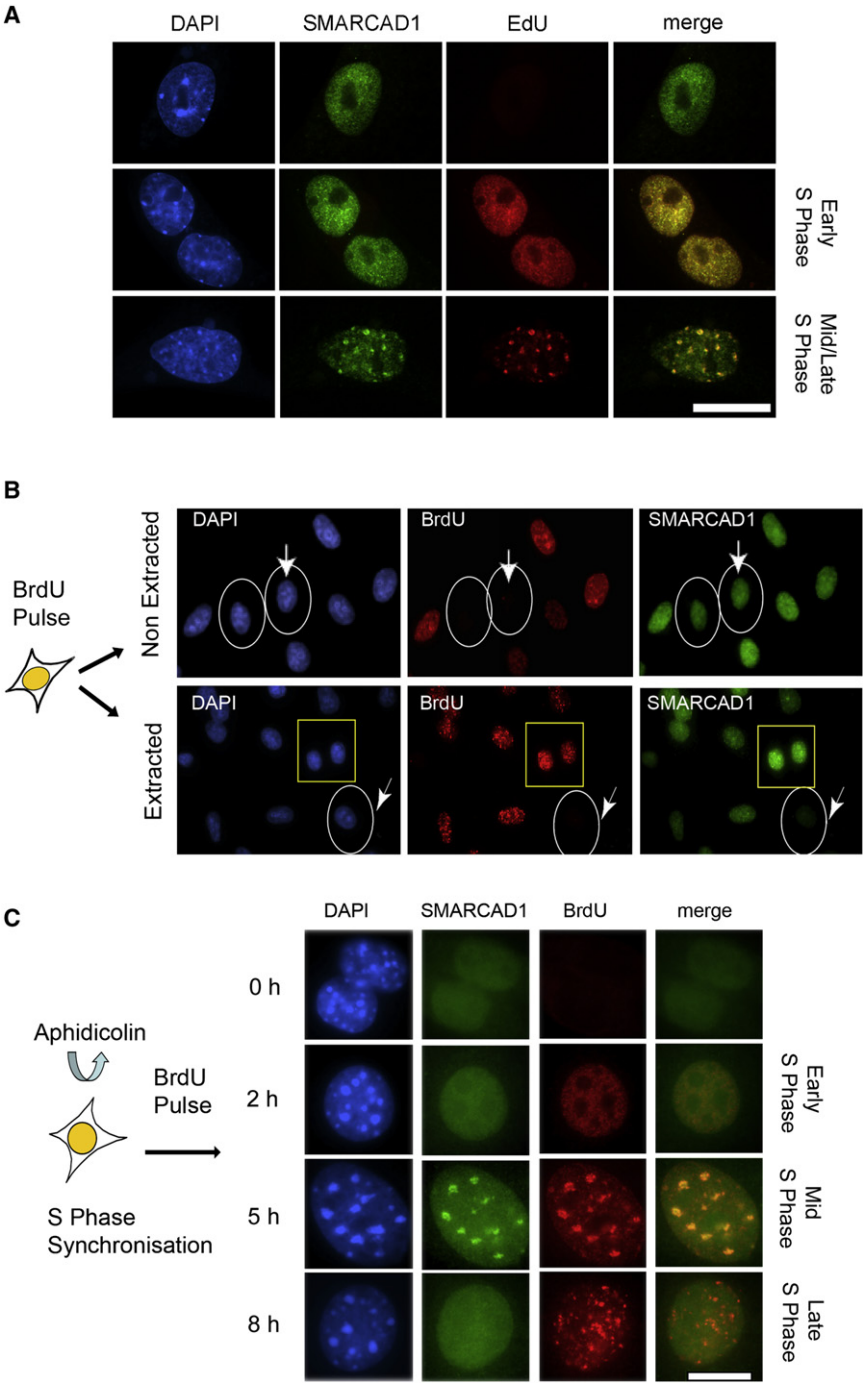


Figure 5. SMARCAD1 Localizes to Sites of DNA Replication

(A) In mouse primary fibroblasts SMARCAD1 shows a general nuclear distribution in nonreplicating cells and associates with replication sites labeled with EdU (15 min pulse) in early and mid- to late-replicating cells. DAPI bright foci mark pericentric heterochromatin. Scale bar represents 10 μm.

(B) SMARCAD1 is retained readily in S phase cells. HeLa cells pulse labeled with BrdU were stained for SMARCAD1 either directly or after extraction of soluble proteins. DNA was counterstained with DAPI. Oval, example of SMARCAD1 localization in cells not incorporating BrdU. Square, example of SMARCAD1 retention in cells incorporating BrdU.

(C) SMARCAD1 localization to pericentric heterochromatin correlates with their replication. NIH 3T3 cells were synchronized at the G1/S boundary with aphidicolin and released into S phase by wash-out, pulsed with BrdU at times indicated and stained for BrdU incorporation and SMARCAD1. Scale bar represents 10 μm.

See also Figure S5.

DNA and found an equal number of S phase cells in normally cycling control cells and SMARCAD1 depleted cells (Figure 4D).

SMARCAD1 Localizes to Sites of Replication

To investigate whether SMARCAD1 associates with sites of replication, we stained for SMARCAD1 and visualized newly replicated DNA by pulse-labeling with nucleotide analogs. In mouse primary fibroblasts and in HeLa cells, SMARCAD1 localizes to EdU-labeled, DAPI-dense foci characteristic of mid- to late-replicating heterochromatin (Figure 5A and Figure S5A). We also observed colocalization of SMARCAD1 with early-replicating euchromatin marked by uniform nuclear distribution of EdU or BrdU (Figure 5A and Figure S5A). SMARCAD1 protein was readily detectable in all nonextracted cells, independent of their cell-cycle stage (Figure 5B). Protein extraction removes soluble SMARCAD1, except in S phase cells, which retain SMARCAD1 (Figure 5B). These observations indicate that a pool of SMARCAD1 is tightly bound to chromatin in S phase, supporting a role for SMARCAD1 in chromatin replication. HeLa cells that retained SMARCAD1 after extraction also retained HDAC1, HDAC2, G9a, and KAP1 (Figure S5B), consistent with our hypothesis that these proteins function together in vivo. Interestingly, previous reports have implicated these factors in replication (Estève et al., 2006; Loyola et al., 2009; Milutinovic et al., 2002; Rountree et al., 2000).

Mouse NIH 3T3 cells have prominent pericentric heterochromatin foci, allowing us to visualize the association of SMARCAD1 with these regions. Pericentric heterochromatin replicates in mid-S phase (Dimitrova and Berezney, 2002; O'Keefe et al., 1992) (Figure 5C, "5 h") and by pulse-labeling synchronized NIH 3T3 cells we found that SMARCAD1 accumulated at pericentromeric structures only when they were labeled with BrdU. In asynchronous cells SMARCAD1 also

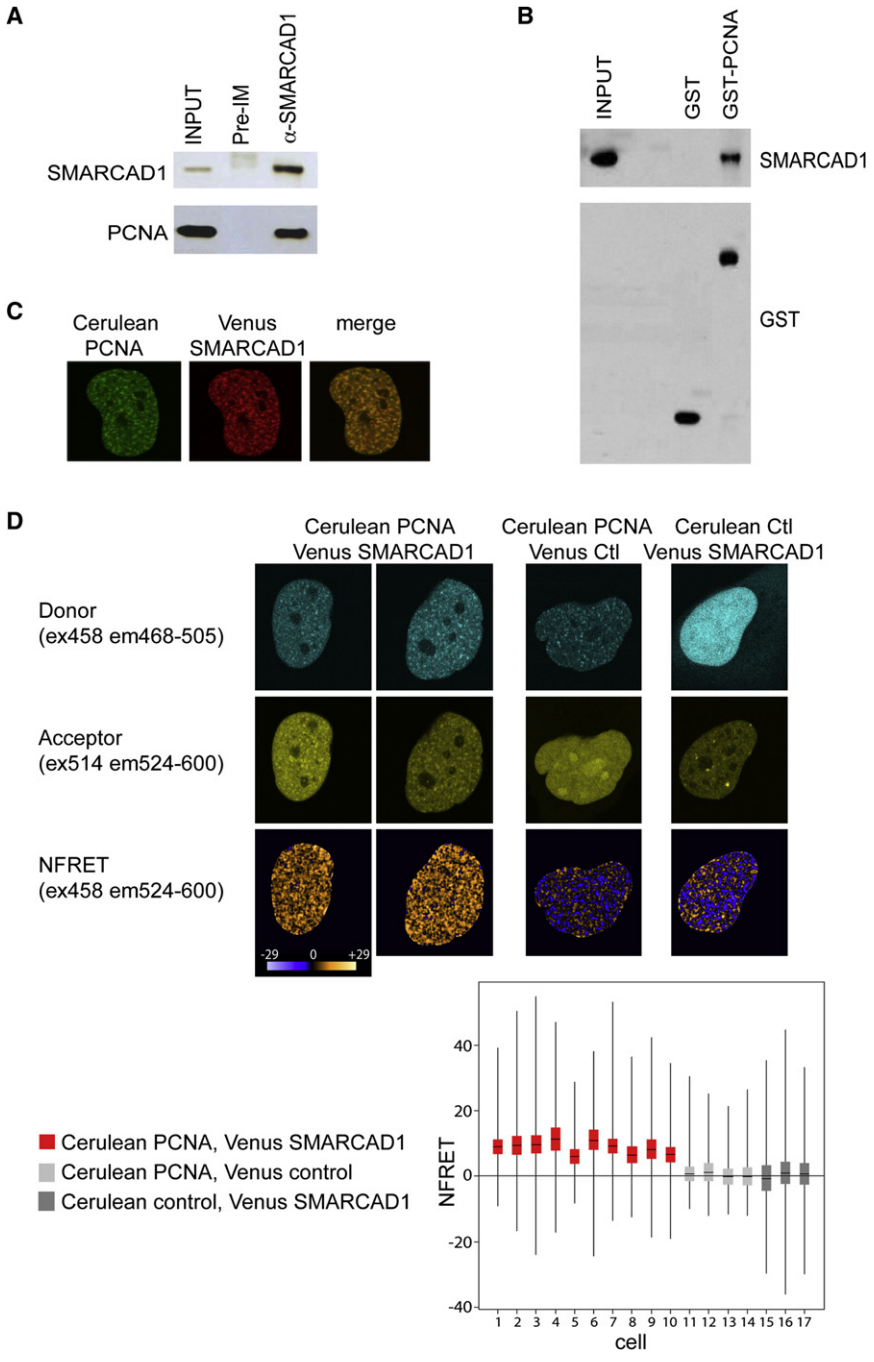


Figure 6. SMARCAD1 Directly Interacts with PCNA

(A) HeLa cell nuclear extract immunoprecipitated with SMARCAD1 antibody and preimmune serum; analyzed by immunoblotting.

(B) GST pulldown analysis shows that recombinant, purified SMARCAD1 binds to GST-PCNA but not GST.

(C) PCNA and SMARCAD1 colocalize in replication foci. MRC5 cells were transiently transfected with Cerulean-PCNA and Venus-SMARCAD1 vectors. Merge reveals regions of colocalization.

(D) PCNA and SMARCAD1 interact in vivo. FRET between Cerulean-PCNA and Venus-SMARCAD1, and indicated controls. Images were acquired in the donor channel (cyan), the acceptor channel (yellow) and the FRET condition and normalized using the ImageJ PixFRET plugin. The corresponding NFRET image was pseudocolored (scale bar). Box and whisker plots corresponding to the NFRET signal in 17 cells. The box boundaries mark the 25th and 75th percentiles; the bar is the median value of all nonzero pixels of each image. The differences between control (cell 11) and Cerulean PCNA, Venus SMARCAD1 (cell 10) are highly significant ($p < 0.001$, t test).

accumulated in replicating pericentric heterochromatin (Figure S5C), and, significantly, all cells that showed replicated pericentric heterochromatin had SMARCAD1 in these foci (Figure S5D).

SMARCAD1 Interacts with PCNA at Replication Sites

To understand the molecular basis of SMARCAD1 association with replication sites, we explored the involvement of the proliferating cell nuclear antigen (PCNA), which recruits several DNA replication and chromatin assembly factors to the replication fork (Moldovan et al., 2007; Naryzhny, 2008). A SMARCAD1 antibody efficiently coprecipitated endogenous PCNA from HeLa extracts (Figure 6A) and pure recombinant SMARCAD1 bound GST-PCNA fusion protein (Figure 6B), demonstrating a direct interaction in vitro. When fluorescent-labeled PCNA and SMARCAD1 were coexpressed in MRC-5 cells, colocalization with replication foci was detected by live imaging (Figure 6C), consistent with endogenous SMARCAD1 colocalizing with replication sites. When analyzed for fluorescence resonance energy transfer (FRET) (Feige et al., 2005), all cells coexpressing SMARCAD1 and PCNA gave strong FRET values compared to controls, independent of cell-cycle stage (Figure 6D). FRET only occurs when fluorophores are <10 nm apart, indicating direct contact (Sekar and Periasamy, 2003). These experiments establish a direct interaction between SMARCAD1 and PCNA in vivo, including at replication sites. While this interaction appears insufficiently stable to persist through the steps of the SMARCAD1 purification, replication factor C proteins involved in loading PCNA onto DNA are present in the SMARCAD1 interactome (Table S1). Thus, we envisage a model in which the SMARCAD1-PCNA interaction promotes targeting of SMARCAD1 specifically to newly replicated DNA.

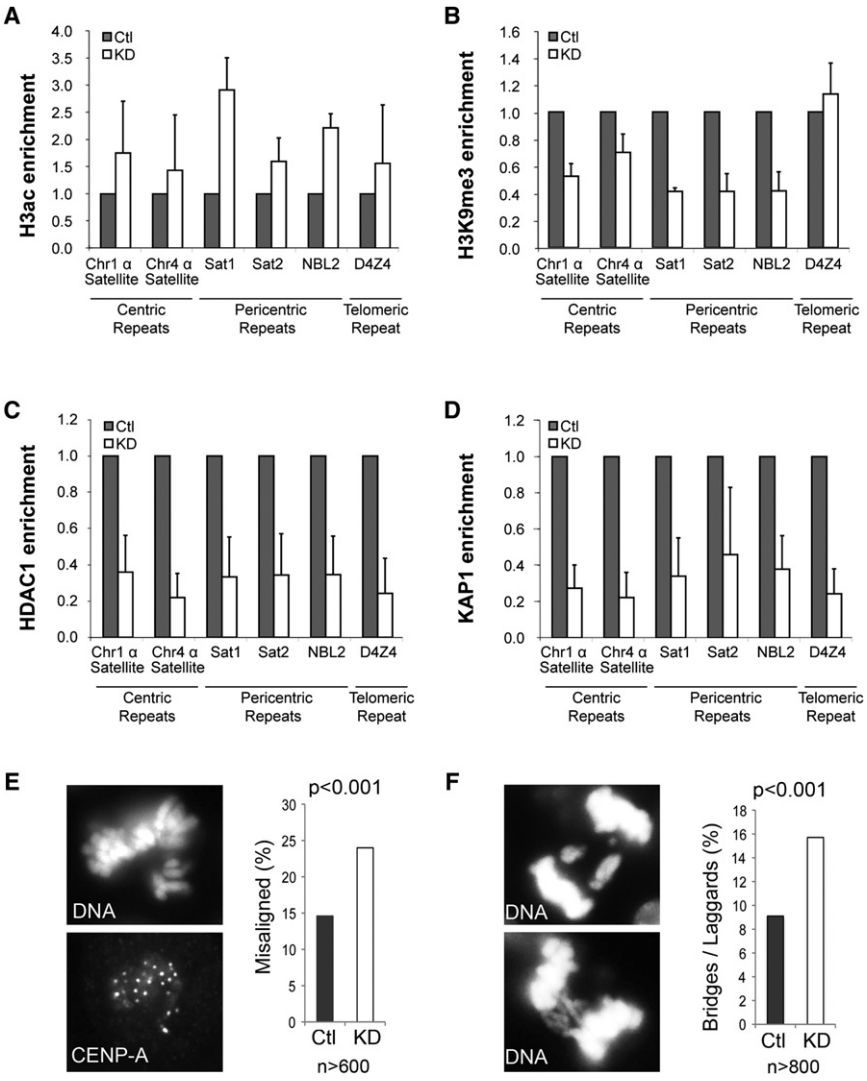


Figure 7. SMARCAD1 Depletion Impacts on Pericentric Heterochromatin and Impairs Chromosome Segregation

(A–D) Depletion of SMARCAD1 causes an increase in H3ac at constitutive heterochromatin, correlating with a decrease in H3K9me and a decrease in HDAC1 and KAP1 occupancy. Comparison of relative CHIP efficiencies of H3ac (A), H3K9me3 (B), HDAC1 (C), and KAP1 (D) at different repeat regions in control (Ctl) and SMARCAD1 knockdown cells (KD). See also Figure S6. Error bars represent the standard deviation from three experiments with two independent cell lines.

(E and F) Depletion of SMARCAD1 compromises mitotic fidelity. Analysis of mitotic HCT116 cells transfected with control (Ctl) or SMARCAD1 (KD) siRNA oligos reveals an increase in the frequency of the misalignement of centromeres at the metaphase plate (E) and of lagging chromosomes (top) and DNA bridges (bottom) during anaphase and telophase (F). DNA was visualized with DAPI, centromeres by centromere protein A (CENP-A) staining (see also Figures S6F and S6G). Bar charts show the percentage of mitotic defects from five experiments ($p < 0.001$; Pearson's chi square test).

SMARCAD1 Regulates Histone Modifications and Occupancy of HDAC1 and KAP1 at Pericentric Heterochromatin and Ensures Mitotic Fidelity

As SMARCAD1 localizes to replicating pericentric heterochromatin, we assessed whether these structures are among the loci where histone modifications are affected when SMARCAD1 is depleted. Immunoprecipitations with anti-H3ac antibodies revealed that levels of acetylated H3 were markedly increased at constitutive heterochromatin repeat sequences in SMARCAD1 knockdown cells (Figure 7A). This was especially pronounced at pericentric repeats Sat1 and NBL2 (Figure S6A). Similarly, reduction of H3K9me3 was seen in centric and pericentric repeats in SMARCAD1-depleted cells (Figure 7B and Figure S6B). Thus, changes in histone modifications in constitutive heterochromatin correlate with the localization of SMARCAD1 protein, linking SMARCAD1 localization and function. Furthermore, when SMARCAD1 levels were reduced, levels of HDAC1 and KAP1 were markedly diminished at pericentic and centric repeats (Figures 7C and 7D and Figures S6C and S6D). This was also observed at the D4Z4 telomeric repeat where changes in histone modifications were less pronounced on SMARCAD1 KD, pointing to the existence of additional regulatory pathways for telomeric histone modifications.

Since pericentric heterochromatin underpins centromere function, we examined whether SMARCAD1 depletion impairs chromosome segregation in mitosis. HCT116 cells have a relatively stable karyotype and knockdown of SMARCAD1 in these cells resulted in an increase in H3ac levels similar to KD in HeLa cells (Figure S6E). We observed a significant increase in the frequency of misalignement of centromeres at the metaphase plate (23%) compared to control cells (14%, $p < 0.001$) (Figure 7E and Figure S6F). The mitotic spindle appeared unaffected as judged from a normal α tubulin pattern (data not shown). Cytogenetic indicators of defective sister chromatid separation include lagging chromosomes and DNA bridges (Figure 7F and Figure S6G), which represent incompletely segregated chromosomal DNA connecting the daughter nuclei. In a sample of over 800 anaphase and telophase cells, we found a significant increase in the average number of these mitotic errors in SMARCAD1 KD cells (15%) compared to control cells (9%, $p < 0.001$) (Figure 7F). Together, these data reveal that SMARCAD1 function impacts on centromere integrity and has a crucial role in ensuring correct chromosome segregation.

DISCUSSION

We have elucidated a role for the SWI/SNF-like factor SMARCAD1 in the maintenance of epigenetic patterns. A key finding is that SMARCAD1 and KAP1 are critical components of the machinery that establishes and maintains heterochromatin structures characterized by histone hypoacetylation and H3K9 methylation. We propose that SMARCAD1 controls removal of histone acetylation marks and subsequent replacement by methylation marks during replication-coupled chromatin assembly, which is essential for the inheritance of the heterochromatic state and genome integrity. In direct support for this proposal, we have shown that SMARCAD1 is required for centromere function and faithful chromosome segregation, as SMARCAD1-deficient cells display increased mitotic defects.

We found that SMARCAD1 directs deacetylation of H4K12, H4K16, and of several lysines on H3. Newly synthesized histones are acetylated before deposition on chromatin; H4 diacetylation on K5 and K12 is an evolutionary conserved pre-deposition mark while acetylation of H3 is variable between species (Loyola et al., 2006; Sobel et al., 1995). H4K12 acetylation, normally turned over within 2 hr (Scharf et al., 2009), is increased throughout the cell cycle in SMARCAD1-deficient cells. H4K16 acetylation has previously been shown to peak in S phase in mammals and has been suggested to be acetylated upon deposition (Vaquero et al., 2006; Scharf et al., 2009). Acetylation of H3K9 has been linked to S phase in *S. cerevisae* (Berndsen et al., 2008; Kuo et al., 1996; Unnikrishnan et al., 2010), and our study establishes that it accumulates during S-G2 phase in higher eukaryotes unless removed by a mechanism involving SMARCAD1. Given that SMARCAD1 is tightly associated with chromatin in S phase and colocalizes with sites of replication, a likely explanation is that it contributes to the adjustment of acetylation states during S phase progression. SMARCAD1 depletion did not cause an S phase defect, indicating that SMARCAD1 is dispensable for progression of the replication fork, supporting a role for SMARCAD1 behind the replication fork. We demonstrate a physical interaction between SMARCAD1 and PCNA in vitro and in vivo. This suggests a mechanism whereby the association of SMARCAD1 with replication sites is promoted by its interaction with PCNA. Future analysis will reveal whether other components contribute to the targeting of SMARCAD1 to replicating chromatin.

Steady-state acetylation levels of histones are maintained by a balance between opposing activities of acetyl transferases and HDACs. We demonstrate that SMARCAD1 associates with HDAC1 and HDAC2, providing a straightforward model for how deacetylation of H3/H4 is achieved. A key finding is that SMARCAD1 impacts on the occupancy of HDAC1 and KAP1 in heterochromatin. Loss of chromatin-bound HDAC1 is consistent with the hyperacetylated chromatin state observed upon depletion of SMARCAD1 and suggests that SMARCAD1 contributes to the regulation of HDAC1 function. A potential role of SMARCAD1 could be to ensure that HDAC1 and KAP1 are delivered to sites of chromatin assembly after DNA replication or control their access to target sites.

How might SMARCAD1 stimulate histone deacetylation? SMARCAD1 and its yeast ortholog Fun30 share extensive sequence similarity with the Ino80/SWR1 class of ATP-dependent nucleosome-remodeling enzymes active in histone exchange (Flaus et al., 2006). Fun30 has nucleosome-remodeling activity (Awad et al., 2010; Neves-Costa et al., 2009), and while we have been unable to show remodeling activity using recombinant SMARCAD1, we demonstrate that an intact ATPase domain is essential for mediating global histone modifications in vivo, suggesting that deacetylation requires prior or concurrent chromatin remodeling. Collectively, our results lead us to propose that deacetylation of histones H3/ H4 is the result of direct nucleosome remodeling by SMARCAD1. In vitro studies have shown ATP-dependent nucleosome deacetylation by the NuRD complex, indicating that nucleosome remodeling can facilitate histone deacetylation (Tong et al., 1998; Zhang et al., 1998). In *S. pombe*, the HDAC-containing chromatin-remodeling complex SHREC is required for heterochromatin maintenance (Sugiyama et al., 2007). We postulate that ATP-dependent nucleosome remodeling is a more general requirement for HDAC function than previously appreciated.

Removal of acetylation is essential for the formation of repressive chromatin domains as it permits the setting of silent marks (Annunziato and Seale, 1983; Taddei et al., 1999); acetylated H3K9 and H3K14 are unfavorable substrates for the methylation of H3K9. Indeed, depletion of SMARCAD1 or KAP1 results in a global decrease of H3K9 methylation. HDAC1, HDAC2, and HMTases could act cooperatively to coordinate the removal of active marks with the perpetuation of H3K9 methylation, allowing recruitment of heterochromatin factors such as HP1. In line with this, KAP1 is also in a complex with HP1α-CAF1 (chromatin assembly factor 1) and the HMTase SetDB1 (Loyola et al., 2009). We identified the HMTase complex G9a/GLP and HP1γ as stable interactors of SMARCAD1. As these proteins function in gene silencing and constitutive heterochomatin formation (Lomberk et al., 2006; Minc et al., 2000; Tachibana et al., 2002), SMARCAD1 likely operates in both facultative and constitutive heterochromatin.

The SMARCAD1 interactome contains repair proteins (Table S1) and a large-scale screen for proteins phosphorylated in response to DNA damage links SMARCAD1 to the DNA damage response (Matsuoka et al., 2007). PCNA participates in both replication and repair and these processes share several factors, also with transcription. SMARCAD1 has been shown to bind to transcriptional start sites and the majority of HDACs in the human genome contribute to transcriptional activation (Okazaki et al., 2008; Wang et al., 2009). Together, these observations raise the possibility that SMARCAD1 has a broader role in chromatin assembly during transcription and repair. In this study, we reveal a role for SMARCAD1 in the maintenance of epigenetic modification to pericentric heterochromatin. This extends the repertoire of activity associated with this chromatin remodeler, which may be functionally connected to the suggested role as a pluripotency factor (Hong et al., 2009). We are currently developing a genetic model to address these questions and extend our understanding of the functions of SMARCAD1 during development and in cancer.

EXPERIMENTAL PROCEDURES

Plasmids, Cell Culture, Antibodies, and Complementation Experiments

See the Supplemental Experimental Procedures.

Extracts, Immunoprecipitation, and Protein Purification

Whole-cell lysates were prepared (Bozhenok et al., 2004); quantification of western blots is described in the Supplemental Experimental Procedures. HeLa nuclear extract (CIL, Belgium) was treated with Benzonase (Novagen, 125 U-222 U/ml)/ethidium bromide (50–88 μg/ml) to avoid nucleic acid-mediated interactions. Immunoprecipitations and GST pulldowns were performed as described (Poot et al., 2004) and in the Supplemental Experimental Procedures. Inputs correspond to 1%–18% of the quantity of extract used for immunoprecipitation. Purification of SMARCAD1 complexes is described in the Supplemental Experimental Procedures. Chromatin immunoprecipitations were carried out as described (Umlauf et al., 2004) (Supplemental Experimental Procedures).

Cell-Cycle Analysis

BrdU /EdU labeling was as described (Collins et al., 2002) or with the Click-iT Kit (Invitrogen). Synchronization of cells with aphidicolin was as in (Bozhenok et al., 2004). Cell-cycle analysis used a mimosine block-and-release protocol (see the Supplemental Experimental Procedures).

Immunofluorescence and FRET

Immunohistochemistry was as in (Poot et al., 2004; Santos et al., 2005) and the Supplemental Experimental Procedures, which also details image processing, immunofluorescence quantification and FRET (Feige et al., 2005).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at doi:10.1016/j.molcel.2011.02.036.

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