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[1]Nuclear Dynamics and Function
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SUMMARY

Epigenetic marks such as posttranslational histone modiÞcations 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 Þndings 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 modiÞcations, critical for specifying cell identity, dictating growth, and directing development. Chromatin organization is continuously challenged,

Here, we provide evidence that SMARCAD1 facilitates the maintenance of heterochromatin by directing histone deacetyla-

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 2

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

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

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, puriÞed 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 25^{th} and 75^{th} 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 signiÞcant ($p < 0.001$, t test).

accumulated in replicating pericentric heterochromatin (Figure S5C), and, signiÞcantly, 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 efÞciently 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 ßuorescent-labeled PCNA and SMARCAD1 were coexpressed in MRC-5 cells, colocalization with replica-

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

(AÐD) 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 efÞciencies 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 Þdelity. 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 Þve experiments ($p < 0.001$; PearsonÕs chi square test).

As SMARCAD1 localizes to replicating pericentric heterochromatin, we assessed whether these structures are among the loci where histone modiÞcations 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 modiÞcations 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 modiÞcations were less pronounced on SMARCAD1 KD, pointing to the existence of additional regulatory pathways for telomeric histone modiÞcations.

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 signiÞcant 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

We have elucidated a role for the SWI/SNF-like factor SMARCAD1 in the maintenance of epigenetic patterns. A key Þnding 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-deÞcient 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 predeposition 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-deÞcient 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 Þnding 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 modiÞcations 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 identiÞed 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).

See the Supplemental Experimental Procedures.

Extracts, Immunoprecipitation, and Protein PuriÞcation

Whole-cell lysates were prepared (Bozhenok et al., 2004); quantiÞcation 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 (50Ð88 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%Ð18% of the quantity of extract used for immunoprecipitation. PuriÞcation 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).

Immunoßuorescence and FRET

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

SUPPLEMENTAL INFORMATION

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

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