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UNIVERSITY OF CALIFORNIA, SAN DIEGO

**PHF8 Mediates Histone H4 Lysine 20 Demethylation Events Involved in
Cell Cycle Progression**

**A dissertation submitted in partial satisfaction of the requirements for
the degree Doctor of Philosophy**

in

Biology

by

Wen Liu

Committee in charge:

**Professor Michael G. Rosenfeld, Chair
Professor Steven Briggs
Professor Christopher K. Glass
Professor William McGinnis
Professor Cornelis Murre**

2011

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University of California, San Diego

2011

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ABSTRACT OF THE DISSERTATION

PHF8 Mediates Histone H4 Lysine 20 Demethylation Events Involved in Cell
Cycle Progression

by

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Doctor of Philosophy in Biology

University of California, San Diego, 2011

Professor Michael G. Rosenfeld, Chair

While reversible histone modifications are linked to an ever-expanding range of biological functions, the demethylases for histone H4 lysine 20 and their potential regulatory roles remain unknown. Here, we report that the PHD and Jumonji C (JmjC) domain-containing protein, PHF8, while using multiple substrates, including H3K9me1/2 and H3K27me2, also functions as an H4K20me1 demethylase. PHF8 is recruited to promoters by its PHD domain based on interaction with H3K4me2/3 and controls G1–S transition in conjunction with E2F1, HCF-1 (also known as HCFC1) and SET1A (also

known as SETD1A, at least in part, by removing the repressive H4K20me1 mark from a subset of E2F1-regulated gene promoters. Phosphorylation-dependent PHF8 dismissal from chromatin in prophase is apparently required for the accumulation of H4K20me1 during early mitosis, which might represent a component of the condensin II loading process. Accordingly, the HEAT repeat clusters in two non-structural maintenance of chromosomes (SMC) condensin II subunits, N-CAPD3 and N-CAPG2 (also known as NCAPD3 and NCAPG2, respectively, are capable of recognizing H4K20me1, and ChIP-Seq analysis demonstrates a significant overlap of condensin II and H4K20me1 sites in mitotic HeLa cells. Thus, the identification and characterization of an H4K20me1 demethylase, PHF8, has revealed an intimate link between this enzyme and two distinct events in cell cycle progression.

INTRODUCTION

The discovery of lysine and arginine methylation in histones and other proteins and the enzymes that carry out these posttranslational modifications has added a new dimension to the transcription field. In particular, there has been a huge progress in our understanding of how methylation of nucleosomal histones at specific lysine or arginine residues affects chromatin conformations and either facilitates or inhibits transcription from neighboring genes¹.

The nitrogens of arginine within polypeptides can be posttranslationally modified to contain methyl groups, a process termed arginine methylation. Protein arginine methylation results in the addition of one or two methyl groups to the guanidino nitrogen atoms of arginine². Protein arginine methylation is catalyzed by a family of enzymes called protein arginine methyltransferases (PRMTs). The PRMT family has been shown to include at least nine methyltransferases, designated as PRMT 1-9 based on differences in primary sequences and substrate specificity³. PRMTs are classified into two groups: type I can form NG-mono and asymmetric dimethyl-L-arginine and type II forms mono- and symmetric dimethyl-L-arginine³. While PRMT1, PRMT3, PRMT4/CARM1, PRMT6 and PRMT8 belong to Type I methyltransferases,

PRMT5, PRMT7, PRMT9 and PRMT2 belong to type II methyltransferases³. Many proteins, among them abundant ones such as histones and RNA-binding proteins, have shown to contain methylarginines⁴. Three PRMTs have been reported to catalyze histone methylation: CARM1 methylates Arg 2, Arg 17 and Arg 26 of histone H3^{5,6}; PRMT1 methylates Arg 3 of histone H4⁷; and PRMT5 methylates Arg 8 of histone H3 and Arg 3 of histone H4^{8,9}. In conjunction with the expanding number of proteins that are known to be arginine methylated, a growing list of biological processes is being shown to involve arginine methylation, which include transcription, RNA processing and transport, translation, signal transduction, DNA repair and apoptosis^{1,10}. Arginine methyltransferases have been linked to diseases, including prostate and breast cancers, cardiovascular diseases, viral pathogenesis and spinal muscular atrophy, suggesting these enzymes might be promising drug targets¹⁰. Small molecule inhibitors of PRMT are currently under investigation¹¹.

Arginine methylation has been considered to be an irreversible post-translational modification until recently. Two groups showed that arginine methylation of histones can be reduced in vitro by deimination with PADI4 (Peptidyl-arginine deiminase 4), which converts both unmodified arginine and monomethylarginine, but not dimethylarginine, to citrulline, an amino acid that is not transcriptionally incorporated into proteins^{12,13}. However, PADs are not real demethylases because citrulline lacks the positively charged guanidine group and, thus, can not functionally substitute for arginine. Recently, a

jumonji-domain containing protein, JMJD6, was shown to demethylate Histone H3 arginine 2 and Histone H4 arginine 3. However, whether JMJD6 functions as an arginine demethylase has been very controversial as shown by several reports (reference). The methylated arginines can be detected by in vitro or in vivo labeling with S-adenosyl-L-[methyl-3H]-methionine ([3H]-AdoMet), by western blotting using methyl-arginine-specific antibodies and by mass spectrometry (MS)³.

Similar to arginine methylation, lysine methylation has also been observed on a variety of proteins associated with gene regulation, including histones and non-histone proteins. Histone lysine methylation occurs on histone H3 at lysine 4, 9, 27, 36 and 79 and histone H4 at lysine 20 and 59^{14,15}. All of the lysine-specific histone methyltransferases (HMTs) except Dot1 share a SET [Su(var), Enhancer of zeste, trithorax] domain that is responsible for catalysis and binding of cofactor S-adenosyl-L- methionine (AdoMet). HMTs then add one or more methyl groups to the ϵ -amino group of lysine residues, resulting in mono-, di-, or trimethylated lysine. Histone lysine methylation plays a pivotal role in a wide array of cellular processes including heterochromatin formation, X-chromosome inactivation and transcription regulation¹⁶. Particularly, it is now clear that histone lysine methylation plays a major role in regulating the state of chromatin compaction, and thus the establishment and maintenance of heterochromatic and euchromatic regions in chromatin. In addition, histone lysine methylation also apparently plays central roles in regulating activation and repression of gene transcription within

euchromatin. In general, methylation of histone H3 at Lys-4, 36, and 79 is correlated with euchromatin and transcriptional activation, whereas methylation of histone H3 at lys-9 and 27 and histone H4 at Lys-20 is associated with heterochromatin and transcriptional repression. However, for methylation at many lysine residues, there are indications of its involvement in both activation and repression, either in different regulatory situations or due to differences in the histone code among different species. Aberrant histone lysine methylation has been linked to a number of developmental disorders and human disease¹⁷. For example, suppression of SMYD3 protein expression significantly inhibits the growth of colorectal and hepatocellular carcinoma cells¹⁸; hDot1L is shown to be involved in leukemogenesis¹⁹; and EZH2 is involved in progression of prostate cancer²⁰. Future development of small molecule inhibitors of HMTs could lead to novel anticancer therapies.

Until recently, it was unclear whether enzymes capable of antagonizing histone lysine methylation existed. LSD1 was the founding member of demethylases, which directly reverse histone H3K4 or H3K9 modifications by an oxidative demethylation reaction in which flavin is a cofactor^{21,22}. In addition, the largest classes of demethylases enzymes, which contain a Jumonji C (JmjC) domain and catalyze lysine demethylation of histones through an oxidative reaction that requires iron Fe (II) and α -ketoglutarate (α KG) as cofactors, have been identified²³. While LSD1 can only remove mono- and dimethyl lysine modifications, the JmjC-domain-containing histone demethylases (JHDMS) can remove all three histone lysine methylation states.

For example, JHDM1 specifically demethylates H3 mono- and dimethylated Lys36²³; JHDM2A demethylates H3 mono- and dimethylated Lysine 9²⁴; and JMJD2 family demethylates H3 trimethylated Lysine 9 or Lysine 36²⁵⁻²⁸ to give rise to dimethylated counterparts; Jarid1 family demethylates H3 di- and trimethylated lysine 4²⁹; JMJD3 family demethylates H3 di- and tri-methylated lysine 27³⁰⁻³³; JMJD5 demethylates H3 di-methylated lysine 36³⁴.

The roles of diverse histone modifications in control of gene transcription is now clearly established^{16,35-37}, with specific factors mediating the methylation or demethylation of specific residues on histones³⁸⁻⁴¹. More interestingly, it has been suggested that the mono-, di-, and tri- methylated states exert distinct functional roles based on binding of different protein complexes⁴²⁻⁴⁵. For example, the histone H3K4me³ mark is largely localized to the nucleosome immediately downstream of the transcription CAP site recruiting specific activation complexes, while the H3K4me¹ mark is characteristically located on enhancers⁴⁶⁻⁴⁸. In contrast, the H3K9me² and H3K9me³ marks are associated with repressive gene transcription events⁴⁹⁻⁵¹. Similarly, functional roles have been established for H3K27, H3K36, H3K79 and H4K20 marks^{16,52}. While histone demethylases have been identified for H3K4, H3K9, H3K27 and H3K36^{38,53}, the enzymes responsible for demethylation of H3K79 and H4K20 residues have remained unknown. Here, we report the identification and characterization of the first H4K20me¹ demethylase, PHF8.

CHAPTER 1

PHF8 could function as a histone H4K20me¹ demethylase in vitro and in cells

We evaluated potential substrates for the putative histone demethylase, PHF8, on mononucleosomes. Flag-tagged PHF8 immunoprecipitated from HEK293T cells was capable of demethylating H4K20me¹, H3K9me^{1/2} and H3K27me², but had no effects on other histone methylation marks tested. Mutation of histidine 247, predicted to be part of the Fe (II) binding site⁵, impaired these activities (Fig. 1a), which were similarly confirmed using bacterially expressed PHF8 (Fig. 1b, left panels) and in a dose- and time-dependent manner (Supplementary Fig. 2a). Surprisingly, when using core histones as substrates, whereas the activities towards H3K9me² and H3K27me² were preserved, only minimal activities were detected towards H4K20me¹ and H3K9me¹ (Fig. 1b, right panels), as similarly found for PHF8 immunoprecipitated from HEK293T cell lysates (Supplementary Fig. 2b).

To gain insight into biological substrates of PHF8, we performed ChIP-Seq in HeLa cells, finding that ~72% of PHF8 peaks localized on promoters, with the most statistically significant predicted binding sites being those for Ets (also known as ETS) and E2F1 (Fig. 1c). The great majority of PHF8-bound promoters overlapped with H3K4me³⁴⁸- and H3K4me²-positive promoters, whereas very few harboured H4K20me¹, H3K9me^{1/2}, or H3K27me² marks (Fig. 1d and Supplementary Fig. 2c). The PHF8 peak summit distribution

relative to transcription start site corresponded to that of the H3K4me2 mark (Fig. 1e), indicating that PHF8 might be recruited to promoters by H3K4me2/3 on the basis of interaction with its PHD finger, a known methyl lysine binding motif⁵⁴. In vitro peptide pull-down showed that PHF8 bound specifically to H3K4me2 and H3K4me3 histone tails, but not to others tested (Fig. 1f). Direct interaction of PHD finger of PHF8 with H3K4me2/3 histone tails was confirmed using bacterially expressed PHF8 PHD finger (Fig. 1g), whereas PHF8 Δ PHD failed to interact (Supplementary Fig. 2d). Consistent with these data, PHF8 Δ PHD retained its activities on mononucleosomes towards H3K9me2 and H3K27me2, but not towards H4K20me1 or H3K9me1 (Fig. 1h). These results indicate a required PHD finger-mediated targeting of PHF8 to H3K4me2/3-containing nucleosomes to efficiently demethylate H4K20me1 or H3K9me1.

To evaluate PHF8 enzymatic activities in vivo, a PHF8-specific siRNA was transfected into HeLa cells, which led to significantly increased H4K20me1 levels and slightly increased H4K20me2 and H3K9me1, without changes in other histone marks (Fig. 2a). This H4K20me1 increase was dependent on PHF8 enzymatic activity (Supplementary Fig. 2e), perhaps also partially attributed to PHF8 depletion interference with cell cycle progression (see below). Other known H3K9/K27 demethylases might compensate for the loss of PHF8, therefore minimizing PHF8 siRNA effects. Immunofluorescence microscopy analysis in HeLa cells overexpressing Flag-tagged full-length PHF8 showed that ~25% of cells had significantly decreased H4K20me1

Figure 1: Histone demethylation mediated by PHF8. **a**, Demethylase activity of Flag-tagged full-length wild-type and mutant (H247A) PHF8 immunoprecipitated from HEK293T cell lysates was assessed using mononucleosomes (Mono) as substrates. Expression of PHF8 proteins was visualized by Coomassie blue staining (CBS). Asterisk denotes potential substrate. **b**, Demethylase activity of purified bacterially expressed His-tagged full-length wild-type and mutant (H247A) PHF8 proteins assessed using mononucleosomes (left panels) or core histones (right panels) as substrates. Expression of PHF8 proteins was visualized by CBS (bottom left panel). Asterisk denotes potential substrate. **c**, Genomic distribution and top enriched motifs of PHF8 ChIP-Seq peaks ($n = 14,490$) in HeLa cells. **d**, Venn diagrams showing overlap between PHF8-bound and H3K4me3- and H3K4me2-marked promoters. **e**, Tag density plots displaying PHF8 and H3K4me2 tags distribution relative to the transcriptional start site (TSS). **f**, **g**, Peptide pull-down assays mixing HeLa nuclear extracts (**f**) or purified bacterially expressed His-tagged PHF8 PHD finger (amino acids 1–54) (**g**) with biotinylated histone tails. Pull-downs were analysed by immunoblotting. **h**, Demethylase activity of purified bacterially expressed His-tagged wild-type and Δ PHD finger (54–1024) PHF8 proteins assessed using mononucleosomes as substrates. Expression of PHF8 proteins was visualized by CBS.

signal intensity ($n = 200$), whereas PHF8 Δ PHD or PHF8(H247A) had no effects (Fig. 2b and Supplementary Fig. 2f). PHF8 overexpression caused no significant differences for other substrates (Supplementary Fig. 2g–n). Similar results were obtained with PHF8 (1–447) (Supplementary Fig. 3a), consistent with recent studies^{55,56}, except that we additionally observed PHF8 activity towards H4K20me1. U2OS cells overexpressing Flag-tagged full-length PHF8 showed a decrease in H4K20me1 (~20%) as well as H3K9me2 (~60%), but not in H3K9me1 or H3K27me2 signal intensity ($n = 200$) (Supplementary Fig. 3b). Thus, PHF8 activity may be regulated both in a cell-type-dependent manner and by specific modifications^{56–58}. To evaluate PHF8 enzymatic activities in HeLa cells further, ChIP-Seq analysis of H4K20me1, H3K9me1, H3K9me2 and H3K27me2 was performed in control or PHF8 siRNA-transfected HeLa cells, finding that H4K20me1 and H3K9me1, but not H3K9me2 or H3K27me2, increased significantly on PHF8-bound promoter regions upon PHF8 depletion (Fig. 2c, d and Supplementary Fig. 3c, d). The difference in H4K20me1 and H3K9me1 levels between PHF8 siRNA-transfected and control cells correlated with the number of PHF8 ChIP-Seq tags on promoters (Supplementary Fig. 3e, f).

Figure 2: Characterization of PHF8 protein. **a**, HeLa cells transfected with control or PHF8 specific siRNAs were analysed by immunoblotting. **b**, Immunohistochemical analysis of HeLa cells transfected with Flag-tagged wild type, Δ PHD or mutant (H247A) PHF8. Cells were stained with anti-flag (red), anti-H4K20me¹ (green) and DAPI (blue). White arrows indicate cells transfected with PHF8. **c**, **d**, H4K20me¹ (**c**) or H3K9me¹ (**d**) ChIP-Seq tags distribution over PHF8 promoter regions in control and PHF8 siRNA-transfected HeLa cells. **e**, HeLa nuclear extracts immunoprecipitated with control IgG or PHF8 antibody and analysed by immunoblotting. **f**, Flow-cytometry analysis of HeLa cells transfected with control or PHF8 siRNAs and stained with propidium iodide. **g**, Chromatin-bound fractions from HeLa cells transfected with control or PHF8 siRNAs and synchronized to different cell cycle phases as indicated were analysed by immunoblotting. **h**, **i**, ChIP analysis of H4K20me¹ (**h**) and L3MBTL1 (**i**) on selected promoter regions in HeLa cells transfected with control or PHF8 siRNA ($\pm$ s.e.m., *P < 0.05, **P < 0.01).

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CHAPTER 2

PHF8 functions as a cell cycle regulator

We next focused on investigating the PHF8 activity towards H4K20me1, a histone mark that has been implicated in cell cycle regulation⁵⁹⁻⁶³. Interestingly, gene ontology analysis of PHF8-bound promoters identified by ChIP-Seq (Fig. 1c and Supplementary Fig. 4a) showed that one of the most statistically significant terms was ‘cell cycle’ (Supplementary Fig. 4b), as similarly found in RNA profiling analysis for genes positively regulated by PHF8 (Supplementary Fig. 4c, d), with ~72% of which proving to be PHF8 ChIP-Seq targets. To gain further insight into PHF8 function, its associated proteins were purified, which included critical G1–S transition regulators, E2F1, HCF-1 and SET1A^{64,65}. Interactions between PHF8 and these proteins were confirmed by immunoprecipitation (Fig. 2e). Gel filtration chromatography revealed co-fractionation of native PHF8, E2F1, HCF-1 and SET1A (Supplementary Fig. 4e). Furthermore, E2F1 ChIP-Seq analysis in HeLa cells indicated that >79% of E2F1-bound promoters corresponded to those binding PHF8 (Supplementary Fig. 5a–c), indicating a role of PHF8 in regulating E2F1 target genes.

Consistent with potential roles of PHF8 in cell cycle regulation, PHF8 siRNA-treated HeLa cells exhibited a strikingly decreased cell proliferation (Supplementary Fig. 6a). Flow-cytometry analysis showed a delay in G1–S transition and revealed a slight increase in M phase population upon PHF8

knockdown (Fig. 2f). In addition, there was a decrease in cell size after PHF8 siRNA treatment (Supplementary Fig. 6b). BrdU incorporation in control or PHF8 siRNA-treated HeLa cells released from mitotic arrest confirmed that PHF8 depletion led to S phase entry delay (Supplementary Fig. 6c, d). To assess further PHF8 enzymatic activity at different stages during cell cycle, chromatin-bound fractions were isolated from control or PHF8 siRNA-treated HeLa cells that were synchronized following the protocols in Supplementary Fig. 6e, finding that PHF8 siRNA treatment led to a significant and distinct increase in H4K20me1 at G1–S and S phases, but exhibited minimal effects at G2–M (Fig. 2g and Supplementary Fig. 6f, g), which was confirmed by immunofluorescence microscopy analysis (Supplementary Fig. 6h). Minimal effects at G2–M might be due to PHF8 dissociation from chromatin at this stage (see below). ChIP analysis on G1–S transition-regulated promoters bound by PHF8 and E2F1, including RBL1 (p107), CDC25A, CCNE1 and E2F1, showed an increase in H4K20me1 levels and a decrease in PHF8 binding upon PHF8 knockdown (Fig. 2h and Supplementary Fig. 7a). The levels of H4K20me3, H3K9me1/2/3 or H3K27me1/2/3 were not significantly altered, except for an increase of H4K20me3 on the CCNE1 promoter (Supplementary Fig. 7b–h). L3MBTL1, which has been shown to associate with H4K20me1 and function in gene repression⁶⁶, showed increased binding (Fig. 2i), whereas H3K4me3 decreased upon PHF8 knockdown (Supplementary Fig. 7i). ChIP analysis on promoters exhibiting ChIP-Seq changes in H3K9me1 and/or H4K20me1 confirmed subsets exhibiting an

Figure 3: PHF8 regulates E2F target genes in conjunction with HCF-1 and SET1A during G1-S transition. **a**, Venn diagram showing overlapping between PHF8 ChIP-Seq peaks in G1 and G1-S phases (n = 9,211 and 20,846, respectively). **b-d**, ChIP analysis of PHF8 (**b**), H4K20me1 (**c**) and L3MBTL1 (**d**) on selected promoter regions in HeLa cells synchronized to G1 or G1-S phases. **e**, Cell extracts from Flag-PHF8 and GFP-HCF-1 co-transfected HEK293T cells were immunoprecipitated (IP) and analysed by immunoblotting (IB) as indicated. **f**, Cell extracts from Flag-PHF8 and haemagglutinin-conjugated HA-HCF-1 (N) (N terminus of HCF-1) or HA-HCF-1 (C) (C terminus of HCF-1) co-transfected HEK293T cells were immunoprecipitated and analysed by immunoblotting as indicated. **g, h**, ChIP analysis of HCF-1 (**g**) or SET1A (**h**) on selected promoter regions in HeLa cells transfected with control, PHF8 or HCF-1 siRNAs. ($\pm$ s.e.m., * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

increase of only H4K20me1 or H3K9me1, or both marks upon PHF8 knockdown (Supplementary Fig. 8). Similarly, a change in H3K9me2 on

ribosomal RNA gene promoter regions in PHF8 siRNA-treated HEK293T cells has been reported⁵⁸. Therefore, PHF8 uses distinct substrates on different subsets of genes to exert its function.

To further elucidate the role of PHF8 in G1–S transition, we performed PHF8 ChIP-Seq in synchronized G1 and G1–S HeLa cells, revealing ~77% of binding sites at G1 localizing on promoters, as did 86% of PHF8 binding sites shared in both G1 and G1–S border. For all those shared PHF8 binding sites, 85% of which showed increased PHF8 tags when cells reached G1–S border (Fig. 3a and Supplementary Fig. 9), at which point H4K20me1 reaches its lowest levels⁶¹ (Fig. 2g). Moreover, ~13,000 new PHF8 binding sites were detected, mostly at intra- or extragenic loci (>70%), which likely mitigated the inappropriate appearance of H4K20me1 (Fig. 3a). ChIP analysis confirmed that PHF8 binding increased on promoter regions of selected G1–S transition-regulated cell cycle genes (Fig. 3b). In concert with association between PHF8 and HCF-1, HCF-1 was similarly recruited (Supplementary Fig. 10a). There was also a decrease in H4K20me1 levels (Fig. 3c) and L3MBTL1 binding (Fig. 3d), but an increase in H3K4me3 (Supplementary Fig. 10b) during G1–S transition. Both PHF8 and HCF-1 siRNAs blocked induction of selected G1–S transition-regulated genes (Supplementary Fig. 11). PHF8 and HCF-1 co-immunoprecipitated (Fig. 3e) and the interacting region was mapped to the amino terminus of HCF-1 (Fig. 3f), which has been suggested to be essential for its function as G1–S transition regulator⁶⁷, further supporting their functional interactions. Furthermore, PHF8 knockdown impaired HCF-1 and

SET1A recruitment (Fig. 3g, h), whereas HCF-1 knockdown did not impair PHF8 recruitment (Supplementary Fig. 12).

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CHAPTER 3

Regulation of M phase by PHF8

We examined chromatin fractions isolated from different phases in cell cycle, confirming PHF8 disappearance from chromatin when cells enter mitosis, which correlates with an increase in H4K20me1 and Pr-Set7 (also known as SETD8; Fig. 2g and Fig. 4a), whereas minimal or no increase in other marks was observed (Supplementary Fig. 13a). Immunostaining of PHF8 without pre-extraction showed no significant signal intensity change between interphase and prophase cells (Supplementary Fig. 13b), whereas with pre-extraction it revealed its dissociation from chromatin in prophase and re-association in telophase (Fig. 4b), as similarly found for green fluorescent protein (GFP)-tagged PHF8 (Supplementary Fig. 13c). In contrast, H4K20me1 levels increased markedly in prophase (Supplementary Fig. 13c, d). PHF8 dissociation became more evident as chromosomes condensed (Supplementary Fig. 13e). These results indicate that PHF8 dissociation in prophase might be involved in H4K20me1 increase at this stage, as similarly proposed due to the increase of methyltransferase, Pr-Set7 (ref. 61). Interestingly, the five-subunit condensin II complex begins loading on chromosomes starting in prophase⁶⁸⁻⁷¹. Cellular fractionation showed that PHF8 dissociation from chromatin paralleled chromosomal loading of condensin II during M-phase (Supplementary Fig. 13f). We therefore wanted

to test whether PHF8 dissociation from chromatin permits accumulation of H4K20me1 and, potentially, condensin II loading.

To test these hypotheses, we first investigated the mechanisms underlying PHF8 dissociation from chromatin. CDK1/cyclin B1 (also known as CCNB1) activity is known to fluctuate during cell cycle⁷², with its activity being inverse to PHF8 association with chromatin. In vitro kinase assays showed CDK1 phosphorylates PHF8 (Fig. 4c). Two putative phosphorylation sites at serine 33 and 84 were identified by mass spectrometry (Supplementary Fig. 14a, b), and mutation at either site proved to be a poor substrate, with double mutation further diminishing the phosphorylation (Supplementary Fig. 14c). Inhibition of CDK activity impaired PHF8 dissociation from chromatin in mitosis (Supplementary Fig. 14d). Similarly, mutation of the phosphorylation sites disrupted PHF8 dissociation from chromatin and increase in H4K20me1 levels that occurred in colcemid-treated cells (Fig. 4d). Thus, PHF8 phosphorylation at both serine 33 and 84 seems to be required for triggering its dissociation from chromatin and accumulation of H4K20me1 levels in prophase. In accord with the possibility that PHF8 dissociation from chromatin could potentially participate in condensin II loading, M-phase-synchronized cells transfected with an empty vector or wild-type PHF8, but not mutant PHF8, exhibited significant increase of chromatin-associated condensin II components compared with asynchronized cells (Fig. 4e).

The condensin II complex from HeLa mitotic extracts was selectively pulled down by H4K20me1 and weakly by H4K20me2, but not by other

histone tails tested (Fig. 4f and Supplementary Fig. 15a). No interaction was detected between H4K20me1 and the non-SMC subunits in the condensin I complex or one component in the cohesin complex, Rad21 (also known as RAD21), as similarly found for condensin II from asynchronized cells (Supplementary Fig. 15b). Furthermore, purified condensin II bound preferentially to mononucleosomes mono-methylated at H4K20 (Supplementary Fig. 15c). We then asked which subunit in condensin II could mediate these interactions. As shown in Fig. 4g, N-CAPD3 strongly and N-CAPG2, to a lesser extent, interacted with H4K20me1 histone tails, but not the other three subunits. Little, if any, interaction was detected with H4K20me3 (Supplementary Fig. 16a). Since none of the subunits in condensin II contains known motifs associating with H4K20me1, we hypothesized that a distinct motif harboured in N-CAPD3 and N-CAPG2, the HEAT repeat^{14,73-75}, might be responsible for these events (Supplementary Fig. 16b). Indeed, the three predicted HEAT repeat clusters in N-CAPD3 and N-CAPG2 had interactions with H4K20me1 histone tails, with the ones from N-CAPD3 showing stronger affinity than those from N-CAPG2. In contrast, minimal, if any, interaction with H4K20me3 was detected (Fig. 4h, lanes 2, 3 and 4). As control, H4K20me1 histone tails failed to pull down N-CAPD3 carboxy terminus (Fig. 4h, lane 1). Furthermore, ChIP-Seq analysis found SMC4 and H4K20me1 co-localized predominately at intra- and extragenic regions (Fig. 4i), with ~55% of SMC4-bound regions occupied by H4K20me1 (Fig. 4j). The specificity of a number of SMC4 binding sites was validated by ChIP-qPCR (Supplementary Fig. 17b, c).

Figure 4: Phosphorylation-dependent PHF8 dissociation from chromatin in prophase links H4K20me1 with condensin II. **a**, Chromatin-bound fractions from HeLa cells synchronized to different cell cycle phases were analysed by immunoblotting. **b**, Asynchronously growing HeLa cells were pre-extracted with 0.1% Triton X-100, fixed and stained with PHF8 and Hoechst dye (DNA). Representative images for different cell cycle phases were shown as indicated. Scale bar, 5 μ m. **c**, His-tagged PHF8 phosphorylation (indicated by p-) by CDK1/cyclin B1 in vitro. **d**, HeLa cells transfected with GFP-tagged wild-type or phosphorylation mutant PHF8 were treated with or without colcemid and chromatin bound fractions were analysed by immunoblotting. **e**, HeLa cells transfected with GFP empty vector, wild-type or phosphorylation mutant PHF8 in the presence or absence of colcemid were sorted. Chromatin-free (S) and -bound (P) fractions were analysed by immunoblotting. **f**, Peptide pull-down assays performed by mixing HeLa mitotic extracts with biotinylated histone tails. Pull-downs were analysed by immunoblotting. **g**, Peptide pull-down assays performed mixing bacterially expressed full length proteins with biotinylated histone tail as indicated. Input (upper panel) and pull-downs (bottom panel) were analysed by immunoblotting. **h**, Schematic representation of N-CAPD3 and N-CAPG2 proteins²⁷. Peptide pull-down assays were performed by mixing His-tagged C terminus of N-CAPD3 (lane 1) or HEAT repeat clusters from N-CAPD3 or N-CAPG2 (lanes 2–4) with biotinylated histone tails as indicated. Input (upper panel) and pull-downs (bottom two panels) were analysed by immunoblotting. **i**, The genomic distribution of SMC4 and H4K20me1 ChIP-Seq peaks in M phase HeLa cells (n = 8,263 and 40,162, respectively). **j**, Bar graph displaying the association of SMC4 and H4K20me1 peaks in M phase HeLa cells.

Given that SMC4 is present in both condensin I and II complexes, which have different distributions along chromosomes²⁷, the 55% overlap of SMC4 and H4K20me1 loci supports the specific association between H4K20me1 and condensin II.

To assess the function of PHF8 re-association with chromatin in late mitosis, fluorescence microscopy imaging was used to monitor living HeLa cells. We started imaging the GFP-Histone H2B-expressing cells 48 h after control or PHF8 siRNA transfection. We found that, as expected, the majority of cells arrested at G1 phase and were of smaller size compared to control cells. However, of the 15 cells that entered mitosis during the filming period (5 h) in this experiment, none exited from mitosis and 6 of them eventually died. In contrast, all the control siRNAs transfected cells exited mitosis normally (Fig. 5a and Movie S1). To allow more cells to escape through the G1/S border, we started to film cells 24 h after siRNA transfection, when PHF8 break down was less effective. A significant increase in the metaphase to anaphase interval was observed in the PHF8 siRNA-transfected cells, but only a slight increase in the nuclear envelope breakdown to metaphase interval (Fig. 5b, c and Movie S2). To further characterize the anaphase onset defect,

Figure 5: PHF8 is required for proper anaphase onset and subsequent chromosome segregation. a-b, Selected frames from a live-imaging sequence of HeLa cells expressing Histone H2b:GFP treated with control or PHF8 siRNA for 48 h (a) or 24 h (b). Time points are in minutes relative to nuclear envelope breakdown (NEBD). Scale bar, 5 μm. In panel b, two examples for both control and PHF8 siRNA transfected cells were shown. c, Bar graph displaying the average time from nuclear envelope breakdown to metaphase or metaphase to anaphase for control or PHF8 siRNA transfected HeLa cells from (b) ($\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

we performed a double thymidine block following the modified protocol shown in Fig. S18a, which would allow cells to escape the G1-S transition delay as mentioned above. We found that knock-down of PHF8 resulted in a failure of the decrease in Cyclin B1 and Securin protein levels (Fig. S18b) and of Condensin II complex release from chromosomes (Fig. S18c), which are characteristics of telophase. The anaphase onset delay described here could also be the consequence of an earlier defect and whether this delay in PHF8-

depleted cells can be linked to the proposed role of H4K20me1 in Condensin II recruitment remains unclear.

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CHAPTER 4

PHF8 in Zebrafish

To determine PHF8 function in the context of a developing organism, we took advantage of the zebrafish model system. We injected Morpholino oligo (MO) directed against the zebrafish PHF8 to knock down its translation (Fig. S19 a, b), which caused morphological defects at day 2 (Fig. 6a) and death by the second week. On day 1, TUNEL staining revealed that the *PHF8* MO-injected embryos exhibited apoptosis throughout their body and most dramatically in the brain (Fig. 6b). Because PHF8 family members, including PHF2 and KIAA1718 or other cell-type specific H4K20 demethylases yet to be identified, might have redundant functions, they may be protective against phenotypes even more severe than the mental retardation reported in patients with PHF8 gene truncation or mutations^{76,77}. The apoptosis observed was detectable in 17-18 h old embryos and peaked around 24-28 h (data not shown). Alcian blue staining revealed cranial cartilage defects, including absence of branchial arches and ceratohyal in PHF8 MO injected embryos on day 4 (Fig. 6c). Whole mount *in situ* staining showed that cranial neural crest marker *sox9a* was lost in the branchial arches in 2-day-old embryos (Fig. 6d). However, 16 h old embryos showed no difference in early neural crest cells, suggesting that initial differentiation of neural crest was not affected in *PHF8* morphants, but following proliferation and cell migration could be affected. In

Figure 6: PHF8 functions *in vivo*. **a**, a live view of control or PHF8 Morpholino injected zebrafish embryos (2 days old). **b**, TUNEL staining of control or PHF8 morpholino injected zebrafish embryos (18 h old). Red dots denote TUNEL positive cells. **c**, Alcian blue staining of zebrafish embryos cartilage (4 days old). Gills were absent in PHF8 morphants. **d**, *sox9a in situ* labeled control and PHF8 embryos (2 days old). Staining of gills is greatly reduced in PHF8 morphants. **e**, *islet1 in situ* labeled eyes of control and PHF8 embryos (2 days old). **f**, Cells in telophase from Zebrafish embryos injected with control (upper panels) or PHF8 (bottom panels) Morpholino were stained with Tubulin (green) and Phospho-Histone H3 serine 10 (red).

addition, the retinal marker *islet1* was greatly reduced in the eyes of PHF8 morphants compared with controls (Fig. 6e). To analyze the mitotic phenotype in zebrafish embryos, we stained cells spread on microscope slides prepared from control or PHF8 morphants with antibodies against β -Tubulin and

phospho-Histone H3 serine 10. The cells in telophase from control samples always had two intact nuclei connected with spindles. In contrast, abnormal spindle structures were observed in telophase cells in PHF8 MO injected samples, such as one cell with two spindles attached and either a dying cell or no cell at the other end of the spindles (5 defects/600 cells) (Fig. 6f). We also found there were around 2 times more mitotic cells in PHF8 morphants than controls at 17 hours post fertilization (hpf). These in vivo results were in agreement with the analysis of PHF8 in human culture cells.

Figure S1: PHF8 mediated histone H4 lysine 20 demethylation events involved in cell cycle progression. a, Model of regulation of cell cycle genes in G1-S transition by PHF8 based on its H4K20me1 demethylase activity. b, Model of regulation of mitosis by PHF8, with phospho-PHF8 dissociation from chromatin in early mitosis linking accumulation of H4K20me1 with Condensin II complex recruitment.

Figure S2: PHF8 demethylase activity. a, PHF8 demethylase activity assessed in both dose-dependent (left panels) and time-course experiments (right panels). Left panels: His-tagged full length wild-type (with increasing amount) or mutant PHF8 proteins were incubated with mononucleosomes for 4 hrs, analyzed by immunoblotting as indicated (-, +, ++ and +++ represent 0, 2.5, 5 and 10 μ g protein, respectively). Right panels: 5 μ g of His-tagged full-length wild-type or mutant PHF8 proteins were incubated with mononucleosomes for various time periods, analyzed by immunoblotting as indicated. b, Demethylase activity of Flag-tagged full-length wild-type and mutant (H247A) PHF8 immunoprecipitated from HEK293T cell lysates was assessed using core histones as substrates. Asterisk denotes potential substrate. c, ChIP-Seq analysis and peak (island) finding for histone marks as indicated and percentage of these peaks associated with PHF8 promoter peaks. d, Peptide pull-down assay mixing purified bacterially-expressed His-tagged delta PHD PHF8 (aa 55-1024) with various biotinylated histone tails as indicated. Pull-downs were analyzed by immunoblotting. e, HeLa cells were transfected with control or PHF8 siRNA and empty vector, wild-type or mutant (H247A) PHF8 with non-sense mutations at siRNA targeting sequence, and analyzed by immunoblotting as indicated. (S.R.=siRNA resistant) f-n, Immunohistochemical analysis of HeLa cells transfected with Flag-tagged wild-type or mutant (H247A) PHF8. Cells were stained with anti-flag (red) and anti-H4K20me1 (f), anti-H4K20me2 (g), anti-H4K20me3 (h), anti-H3K9me1 (i), anti-H3K9me2 (j), anti-H3K9me3 (k), anti-H3K27me1 (l), anti-H3K27me2 (m) or anti-H3K27me3 (n) (green). Nuclei were counterstained with DAPI (blue). White arrows indicate cells transfected with PHF8.

Figure S3: PHF8 demethylase activity. a, Immunohistochemical analysis of HeLa cells transfected with Flag-tagged PHF8 (1-447) fused with a nuclear localization signal. Cells were stained with anti-flag (red) and anti-H4K20me1, anti-H3K9me1, anti-H3K9me2, anti-H3K9me3, anti-H3K27me1, anti-H3K27me2 or anti-H3K27me3 (green), as indicated. Nuclei were counterstained with DAPI (blue). White arrows indicate PHF8 transfected cells. b, Immunohistochemical analysis of U2OS cells transfected with Flag-tagged PHF8. Cells were stained with anti-flag (red) and anti-H4K20me1, anti-H3K9me2, anti-H3K9me1 or anti-H3K27me2 (green), as indicated. Nuclei were counterstained with DAPI (blue). White arrows indicate cells transfected with PHF8. c-d, H3K9me2 (c) or H3K27me2 (d) ChIP-Seq tags distribution over PHF8 promoter regions in control and PHF8 siRNA transfected HeLa cells. e-f, Graphs displaying the relationship between levels of PHF8 ChIP-Seq tags and the change in H4K20me1 (e) or H3K9me1 (f) levels on individual promoter for all promoters in the genome. Promoters were binned by the levels of PHF8 ChIP-Seq tags (within +/- 500 bp) from the TSS. Difference in H4K20me1 (e) or H3K9me1 (f) levels between PHF8 and control siRNA were reported as an average for all promoters in the same bin. As shown, the difference in H4K20me1 (e) or H3K9me1 (f) levels between PHF8 siRNA transfected cells and control cells correlated with PHF8 ChIP-Seq tags on all promoters in the genome (e.g., the less PHF8 tags a promoter has, the less change of H4K20me1 or H3K9me1 could be observed when depleting PHF8, and vice versa).

Figure S4: Characterization of PHF8 protein. a, **Validation of PHF8 binding by ChIP-qPCR.** HeLa cells were subjected to standard ChIP assay. ChIP signals for PHF8 were presented as fold enrichment over that of IgG ($\pm$ s.e.m.). All the selected PHF8 binding sites identified by ChIP-Seq showed enrichment over IgG and a predicted negative control (GAPDH promoter region). b, Gene ontology analysis of PHF8 bound promoters (n=10,495) revealed by ChIP-Seq in HeLa cells. c, Gene ontology analysis of genes positively regulated by PHF8 revealed by RNA profiling using samples from HeLa cells transfected with control or PHF8 siRNA. d, Validation of PHF8 positively regulated genes revealed by RNA profiling through RT-qPCR. RNA samples extracted from HeLa cells transfected with either control or PHF8 siRNAs were subjected to RT-qPCR analysis by using primers specific for the genes as indicated. Data shown is the relative fold change comparing PHF8 siRNA to control siRNA transfected samples for each individual gene after normalized to actin ($\pm$ s.e.m.). e, HeLa nuclear extracts were analyzed by size exclusion chromatography (Superose-6). Collected fractions were then subjected to western blotting analysis as indicated. From fractions 15 to 21, a peak of PHF8/E2F1/HCF-1/SET1A is seen at fraction 18, as indicated by red arrow. Molecular mass markers are shown on top.

Figure S5: E2F1 ChIP-Seq and its association with PHF8. a, Genomic distribution and top enriched motifs of E2F1 ChIP-Seq peaks (n=16,093) in HeLa cells. b, Venn diagram showing overlap between PHF8 and E2F1-bound promoters (n=10,495 and 8,968, respectively). c, Validation of E2F1 binding by ChIP-qPCR as described in Fig. S4a. All the selected E2F1 binding sites identified by ChIP-Seq showed enrichment over IgG and a predicted negative control (GAPDH promoter region) ($\pm$ s.e.m.).

Figure S6: PHF8 knock-down leads to G1-S transition delay in HeLa cells. a, Cell viability analysis of HeLa cells transfected with control, PHF8, HCF-1 or SET1A siRNA at various time points as indicated. b, HeLa cells were transfected with control or PHF8 siRNAs, immunostained with anti-PHF8 (top panel) or DAPI (bottom panel). Scale bar, 5 μ m. c, Kinetics of BrdU incorporation in HeLa cells transfected with either control or PHF8 siRNAs and released from a mitotic arrest. S phase entry was measured by determining BrdU incorporation at 6, 10 and 12 h, presumably, cells are in late G1, early S and mid S phases, respectively. Cells were stained with BrdU (green) and DAPI (Blue) as indicated. Arrowheads denote mitotic cells with condensed chromosomes. d, Bar graph displaying percentage of BrdU positive stained cells in HeLa cell transfected with control or PHF8 siRNAs and released from a mitotic arrest as described in (c) (n=500). e, Modified synchronization protocol followed to collect cells in S, G2-M or G1-S with control or PHF8 siRNA transfection, which allowed cells to escape the G1-S border since PHF8 was still not significantly depleted after 23 h siRNA transfection, but 8 h or 10.5 h after releasing from the second thymidine treatment, cells presumably entered into late S or M phase, respectively, with a significant depletion of PHF8 protein, as shown by immunoblotting. f, Ratios of H4K20me1 between PHF8 and control siRNA samples in G1-S, S and M phases as described in Fig. 2g. Densitometry was carried out in Photoshop. g, Chromatin-bound fractions from HeLa cells transfected with control or PHF8 siRNAs and synchronized to different cell cycle phases as indicated were analyzed by immunoblotting. h, HeLa cells were transfected with control or PHF8 siRNAs, immunostained with H4K20me1 (top panel) and DAPI (bottom panel). Interphase and mitotic cells are indicated by arrowheads and arrows, respectively. A significant increase of H4K20me1 was observed in interphase cells, but not in mitotic cells.

Figure S7: Changes in PHF8, H4K20me3, H3K9me1/2/3, H3K27me1/2/3 and H3K4me3 occupancy on RBL1(p107), CDC25A, CCNE1, E2F1 and GAPDH promoter regions upon PHF8 knock-down. ChIP analysis of PHF8 (a) or histone marks, including H4K20me3 (b), H3K9me1 (c), H3K9me2 (d), H3K9me3 (e), H3K27me1 (f), H3K27me2 (g), H3K27me3 (h) and H3K4me3 (i) on selected cell cycle-regulated gene as well as GAPDH promoter region in HeLa cells transfected with control or PHF8 siRNAs as indicated. ChIP signals were presented as percentage of input ($\pm$ s.e.m., *p<0.05, **p<0.01). ChIP for H4K20me3 on centromeric region was shown as a positive control.

Figure S8: H4K20me1 and H3K9me1 occupancy on selected PHF8-bound promoter regions. ChIP analysis of histone marks, including H4K20me1 and H3K9me1 on selected PHF8-bound promoter regions in HeLa cells transfected with control or PHF8 siRNAs as indicated. ChIP signals were presented as percentage of input ($\pm$ s.e.m.). Representative promoters with only H4K20me1 (a) or H3K9me1 (c) change, or both H4K20me1 and H3K9me1 changes (b) were shown.

Figure S9: PHF8 ChIP-Seq in G1 phase and G1-S border. a, Tags distribution over PHF8 binding sites common in G1 phase and G1-S border revealed by ChIP-Seq. Both promoter and non-promoter regions were shown as indicated. b, PHF8 ChIP-Seq tags in G1 phase or G1-S border on promoter region of RBL1(p107) gene locus in HeLa cells.

Figure S10: HCF-1 binding and H3K4me3 occupancy during G1-S transition in cell cycle. ChIP analysis of HCF-1 (a) or H3K4me3 (b) on selected cell cycle-regulated gene promoter regions in HeLa cells synchronized to G1 phase or G1-S border phases as indicated. ($\pm$ s.e.m., ** $p < 0.01$, *** $p < 0.001$).

Figure S11: PHF8 and HCF-1 knock-down blocked induction of G1-S transition-regulated genes bound by both PHF8 and E2F1. a, mRNA levels of selected genes in HeLa cells transfected with control, PHF8 or HCF-1 siRNAs alone or with or without wild-type or mutant (H247A) PHF8 measured by RT-qPCR. (S.R.= siRNA resistance). b. mRNA levels of PHF8 (left panel) and HCF-1 (right panel) in HeLa cells transfected with either control, PHF8 or HCF-1 siRNAs, as described in (a), detected by RT- qPCR. Data shown is the relative fold change compared to control siRNA transfected samples after normalized to actin ($\pm$ s.e.m., * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure S12: HCF-1 knock-down has no effect on PHF8 binding. ChIP of PHF8 on selected cell cycle gene promoter regions in HeLa cells transfected with control, PHF8 or HCF-1 siRNAs as indicated. ChIP signals for PHF8 were presented as fold enrichment over IgG ChIP signals ($\pm$ s.e.m.).

Figure S13: PHF8 dissociates from chromatin in prophase. a, Chromatin-bound fractions from HeLa cells synchronized to different cell cycle phases were analyzed by immunoblotting as indicated. b, Asynchronously growing HeLa cells were directly fixed and stained with PHF8. Nuclei (DNA) were stained with Hoechst dye. Representative images for different cell cycle phases were shown as indicated. Scale bar, 5 μ m. c, HeLa cells transfected with GFP-tagged PHF8 were pre-extracted with 0.1% Triton X-100, fixed and stained with anti-H4K20me¹ (red). Nuclei were stained with DAPI. Arrow and arrowhead denote interphase and prophase cells, respectively. d, Asynchronously growing HeLa cells were pre-extracted with 0.1% Triton X-100, fixed and stained with H4K20me¹ as indicated. Nuclei (DNA) were stained with Hoechst dye. Representative images for different cell cycle phases were shown as indicated. Scale bar, 5 μ m. e, PHF8 gradually dissociates from chromatin as cell condenses its chromosomes in prophase. Asynchronously growing HeLa cells were pre-extracted with 0.1% Triton X-100, fixed and stained with PHF8. Nuclei (DNA) were stained with Hoechst dye. Representative images for interphase and prophase cells were shown as indicated. Scale bar, 5 μ m. f, Chromatin-free (S, soluble) and -bound (P, pellet) fractions were prepared from HeLa cells synchronized to different cell cycle phases and analyzed by immunoblotting as indicated.

Figure S14: Tandem MS spectrum revealed two putative phosphorylation sites on PHF8 by CDK1/cyclinB1 at Serine 33 and 84. a-b, In vitro kinase assay was performed by mixing CDK1/cyclinB1 with purified bacterially-expressed PHF8 protein. The reaction mixture were then digested with Trypsin singles proteomic grade and subjected to Capillary-LC analysis. InsPecT was applied to search for phosphorylated peptides on a serine, threonine or tyrosine with a mass shift of 80 Da. The parent ion mass tolerance was set to be 2 Da and b, y ion mass tolerance was set as 0.5 Da. Tandem MS spectrum revealed two putative phosphorylation sites on PHF8 by CDK1/cyclinB1 at Serine33 (a) and Serine 84 (b). P represents H3PO4. c, In vitro phosphorylation of His-tagged wild-type and phosphorylation mutant PHF8 by CDK1/cyclinB1. Protein expression was visualized by commassie blue staining (bottom panel). d, HeLa cells were pre-treated with or without colcemid (1 μ m) for 20 h before adding DMSO vehicle or Roscovitine (10 μ m) and chromatin bound fractions were analyzed by immunoblotting as indicated.

Figure S15: Specific interaction between Condensin II complex and H4K20me¹ histone tails. a, Peptide pull-down assays were performed mixing HeLa mitotic extracts with biotinylated histone tails. Pull-downs were analyzed by immunoblotting as indicated. b, Peptide pull-down assay by mixing HeLa mitotic (left panels) or asynchronized (right panels) cell extracts with biotinylated H4K20me¹ histone tails. Pull-downs were analyzed by immunoblotting with antibodies against individual subunits in both Condensin I and II complexes or Rad21, a component of the cohesin complex as indicated. c, Immunoprecipitates from a negative IgG control or a specific antibody against N-CAPD3 were mixed with mononucleosomes purified from HeLa cells. Pull-downs were analyzed by immunoblotting as indicated.

Figure S16: Heat repeat clusters in N-CAPD3 and N-CAPG2. a, Peptide pull-down assays were performed by mixing purified bacterially-expressed full length N-CAPD3, N-CAPG2, N-CAPH2, SMC2 or SMC4 with biotinylated H4K20me₃ histone tails. Pull-downs were analyzed by immunoblotting as indicated. b, HEAT repeats in N-CAPD3 and N-CAPG2. HEAT repeat cluster (a) in N-CAPD3 contains 4 continuous Heat repeats from aa 443-655; Heat repeat cluster (b) in N-CAPD3 contains 4 continuous HEAT repeats from aa 902-1052; HEAT repeat cluster (c) in N-CAPG2 contains 5 continuous HEAT repeats from aa 329-537. Consensus sequence in HEAT repeat was highlighted in red. Please also see reference (Ono, et al).

Figure S17: SMC4 and H4K20me1 ChIP-Seq in M phase HeLa cells. a, Specificity of the H4K20me1/2/3 antibodies shown by immunoblotting with histone H4 tails mono-, di- or tri-methylated at K20. b, HeLa cells transfected with either control or SMC4 siRNAs were lysed 72 h after transfection and subjected to immunoblotting analysis as indicated. c, Validation of SMC4 binding sites in M phase HeLa cells revealed by ChIP-Seq. Cells transfected with siRNAs as described in (b) were subjected to standard ChIP-qPCR analysis. ChIP signals were presented as % of Inputs ($\pm$ s.e.m.). ChIP signals for all 36 selected putative SMC4 binding sites showed enrichment over a negative control region and decreased after knock-down of SMC4.

Figure S18: PHF8 knock-down leads to anaphase onset delay. a, HeLa cells were synchronized and transfected with siRNAs following the protocol shown on the top. PHF8 protein level was detected by immunoblotting. b, As in (a), cells transfected with siRNAs for indicated time period were analyzed by immunoblotting as indicated. c As in (a), Chromatin free (S, soluble) and bound (P, pellet) fractions were prepared from cells transfected with siRNAs for indicated time period and analyzed by immunoblotting as indicated.

Figure S19: PHF8 protein and mRNA levels in control and PHF8 morpholino injected zebra fish embryos. a, Immunoblotting analysis of lysates prepared from control or PHF8 morpholino injected zebra fish embryos as indicated. b, RNA extracted from control or PHF8 morpholino injected zebra fish embryos were analyzed by RT-qPCR. Data shown is the relative fold change compared to control morpholino oligos injected embryos after normalized to the expression of a housekeeping gene, actin ($\pm$ SEM).

Sample	Total Reads	Aligned Reads (uniquely aligned reads & the reads that align to repetitive regions)	Uniquely Aligned Reads
PHF8 (unsynchronized)	37,439,557	12,429,834	9,768,104
PHF8 (G1 phase)	20,037,406	11,782,825	9,584,043
PHF8 (S phase)	31,939,089	14,944,104	11,845,100
H3K4me2 (unsynchronized)	2,029,718	1,566,645	1,240,010
E2F1 (unsynchronized)	16,367,661	7,785,017	7,339,848
H4K20me1 (M phase)	16,361,110	12,923,394	9,263,695
SMC4 (M phase)	13,437,787	7,224,333	5,261,263

Figure S20: Summary of ChIP-Seq reads for PHF8, H3K4me2, E2F1, H4K20me1 and SMC4.

CHAPTER 5

Conclusions

Together these studies have revealed that, while PHF8 has multiple substrates (H4K20me1, H3K9me1/2 and H3K27me2) *in vitro*, in the intact cells it could act as a cell cycle regulator partially based on its H4K20me1 demethylase activity. However, functional gene-specific effects of H3K9me1/2 or H3K27me2 demethylation can not be excluded, as the presence of many other demethylases for these residues could account for the lack of broad alterations of these marks following PHF8 knock-down. PHF8, the first identified H4K20me1 demethylase, acts as a cell cycle regulator: its absence leads to a delay at G1/S transition and its dissociation from chromatin in early mitosis, in conjunction with the increase expression of Pr-Set7, leads to the surge of H4K20me1, which recruits Condensin II complex through interactions with HEAT repeat clusters harbored in two non-SMC subunits, N-CAPD3 and N-CAPG2. These data reveal a new type of histone tail “reader”, with HEAT repeats recognizing the H4K20me1 mark, and link a specific histone mark associated with cell cycle progression to the specific machinery required to complete cell division.

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assistance with the ChIP-Seq data analysis; C. Nelson for cell culture assistance; J. Hightower and D. Benson for assistance with figure and manuscript preparation and the UCSD BIOGEM laboratory for RNA profiling. M.G.R. is an Investigator with the Howard Hughes Medical Institute. This work was supported by grants from NIH and NCI to A.K.A., P.C.D., C.K.G, A.D. and M.G.R. and from DOD and PCF to M.G.R. Chapter 1 to 5 has been published in Nature, 2010 Jul 22; 466(7305):508-12. Wen Liu; Bogdan Tanasa; Oksana V. Tyurina; Tian Yuan Zhou; Reto Gassmann; Wei Ting Liu; Kenneth A. Ohgi; Chris Benner; Ivan Garcia-Bassets; Aneel K. Aggarwal; Arshad Desai; Pieter C. Dorrestein; Christopher K. Glass; Michael G. Rosenfeld. "PHF8 Mediates Histone H4 Lysine 20 Demethylation Events Involved in Cell Cycle Progression". The dissertation author was the primary investigator and author of this paper.

CHAPTER 6

Methods

PHF8 protein purification and demethylation assay. Flag-PHF8 wild type and enzymatically-impaired mutant were expressed in HEK293T cells and cells were lysed in lysis buffer containing 50mM Tris HCl, pH 7.4, 450mM NaCl, 1mM EDTA, 1% TRITON X-100 followed by sonication. High salt concentration and sonication were applied here due to PHF8 is mainly and tightly associated with chromatin. Flag-PHF8 proteins were then affinity purified by using Anti-flag M2-agarose as described in the technical bulletin (Sigma A2220) and washed extensively. Before elution with 3X Flag peptides (Sigma), the affinity resins were washed with demethylation buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM $[\text{NH}_4]_2\text{Fe}[\text{SO}_4]_2$, 1 mM α -ketoglutarate, and 2 mM ascorbic acid) twice. His-tagged PHF8 wild type, enzymatically-impaired mutant or deletions were expressed in BL21 (DE3) bacterial cells (Stratagene) and purified by using Ni-NTA Agarose (Qiagen). For demethylation reactions, PHF8 proteins were incubated with 5 μg of bulk histones (Sigma H9250) or mononucleosomes prepared from HeLa cells in demethylation buffer at 37 °C, as described⁷⁸.

Histone peptide pull-down assay. Biotinylated histone peptides (Millipore and Abgent), either modified or unmodified, were pre-incubated with Dynalbeads M-280 streptavidin blocked with BSA (Invitrogen) at room temperature for 1 h and then washed with washing buffer (0.01% Tween-20 in

PBS) twice before mixing with HeLa Nuclear extracts, total cell lysates or bacterial expressed His-tagged proteins. After incubation in the incubation buffer (20mM Hepes pH 8.0, 1.5mM MgCl₂, 0.42 M NaCl, 25% Glycerol, 1mM DTT, 0.2mM EDTA, 0.01% Tween-20) overnight at 4 °C, pull downs were washed with washing buffer (same as incubation buffer) for 5 times before resolving by 4-12% Bis-Tris gel (Invitrogen) and analyzing by immunoblotting. HeLa nuclear extracts were prepared as described⁷⁹. For preparing mitotic HeLa cell extracts, cells were treated with Colcemid (10⁻⁶M) for 20 h to enrich the mitotic cell population. To express full length individual subunits in the Condensin II complex and HEAT repeat cluster domains in bacterial cells, cells were induced with low concentrations of IPTG and at room temperature.

Cell cycle synchronization. HeLa cells were synchronized by two sequential treatments with 2mM thymidine (Sigma) as described previously^{64,80}. Briefly, HeLa cells were grown in the presence of 2mM thymidine for 12 h and then the thymidine containing medium was replaced by normal growth medium for 11 h before the second treatment with 2mM thymidine for another 12h. The cells were then harvested (G1/S border) or released into normal growth medium and harvested at 4, 8, 10.5, 15 hr after release, which represent Mid S, S/G2, G2/M, Mid G1 phases, respectively. In some cases, M phase population was enriched by treating cells with Colcemid (10⁻⁶M) (Sigma) for 20 h. To collect cells in S and M phases with *PHF8* siRNA transfection, a modified synchronization protocol was applied (see Fig. S6e).

Morpholino injection. Morpholino injections were done as described previously⁸¹. Essentially, PHF8 splicing blocking Morpholino oligo (MO) (5'-AATTAGAGAACAACCTAACCCGGAGT) and Fluor standard control MO (5'-CCTCTTACCTCAGTTACAATTTATA) (Gene Tools, LLC, Corvallis, OR) were injected into two to four-cells stage embryos about 2.5 ng per embryo. The PHF8 protein level was examined by immunoblotting and PHF8 transcript level was checked by RT-qPCR using primers: Forward 5'-AACAGCCCAAACAGAGATCG and reverse 5'-AGGTTCTCCACCCATGACAG.

Cloning procedures. PHF8 full length or truncations was PCR amplified from cDNA samples from HeLa cells by using KOD Hot Start Master mix (Novagen) and cloned into p3XFLAG-CMV™-10 (Sigma), pET-28a(+) (Novagen) or pEGFP-C3 (Clontech) vectors. Full length subunits in the Condensin II complex, including N-CAPD3, N-CAPG2, N-CAPH2, SMC2 and SMC4, and HEAT repeat clusters, were PCR amplified from cDNA templates (Open biosystems or OriGene) by using KOD extreme Hot Start DNA polymerase (Novagen). PHF8 siRNA-resistant (S.R.) non-sense mutants, enzymatic deficient or phosphorylation mutants were generated by using QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene).

Antibodies. Antibodies recognize different histone modifications, including H4K20me1 (ab9051), H4K20me2 (ab9052), H4K20me3 (ab9053), H3K9me1 (ab8896), H3K9me2 (ab1220), H3K9me3 (ab8898), H3K27me2 (ab24684), H3K36me1 (ab9048), H3K36me3 (ab9050), H3K79me2 (ab3594),

H3K4me2 (ab7766) were purchased from Abcam; H3K9me2 (07-441), H3K27me1 (07-448), H3K27me3 (07-449), H3K36me2 (07-569), H3K4me3 (07-473) and phospho-Histone H3 (Ser10) (05-806) were purchased from upstate; H4K20me1 (39175), H4K20me2 (39173) and H4K20me3 (39180) were purchased from Active Motif. Information on other antibodies is indicated as below. Anti-H2B (07-371) is from Upstate. Anti-Histone H4 (39269) and anti-Histone H3 (39163) are from Active Motif; PHF8 (ab35471, used for ChIP and IF), anti-Pr-Set7 (ab3744), anti -L3MBTL1 (ab51880), anti-SMC4 (ab17958, used for ChIP and IB), anti-SMC2 (ab10412, used for IB), anti-N-CAPD3 (ab70349, used for IB), anti-N-CAPG2 (ab70350, used for IB) and anti-securin (ab79546) are from Abcam; anti-PHF8 (A301-772A, IHC-00343, used for IB), anti-Set1A (A300-290A, used for IB), anti-HCF-1(A301-399A, used for IB), anti-SMC4(A300-064A, used for ChIP), anti-SMC2 (A300-058A, used for IB), anti-N-CAPH2 (A302-275A, used for IB), anti-N-CAPD2 (A300-601A, used for IB), anti-N-CAPG (A300-602A, used for IB) and anti-N-CAPH (A300-603A, used for IB) are from Bethyl Laboratories, Inc.; anti-His (SC-803), anti-E2F1(SC-193 & SC-22820), anti-E2F2(SC-632), anti-E2F3(SC-879), anti-E2F4(SC-866), anti-E2F5(SC-1083), anti-GAPDH (SC-25778) and anti-CyclinB1(SC-752) are from Santa Cruz Biotechnology, Inc.; anti-Flag (F1804) is from Sigma. Anti-HA affinity (3F10) (11867423001) is from Roche. anti-GFP antibody was from Dr. Arshad Desai's laboratory. Anti-HCF-1 and Set1A used for ChIP were gifts from Dr. Winship Herr. ChIP: Chromatin-Immunoprecipitation; IB: immunoblotting; IF: immunofluorescence.

Chromatin Immunoprecipitation. Chromatin immunoprecipitation (ChIP) was performed as described⁸². Briefly, Cells were cross-linked with 1% formaldehyde (Sigma) and Chromatin DNA was sheared to 300-500bp average in size through sonication. Resultant was immunoprecipitated with control IgG or specific antibodies overnight at 4°C and followed by incubation with protein A-sepharose beads (Sigma) for an additional 2 h. After washing and elution, the protein-DNA complex was reversed by heating at 65°C overnight. Immunoprecipitated DNA was purified by using QIAquick spin columns (Qiagen) and analyzed by qPCR using Stratagene Mx3000 machine. Primers used are specific for regions tested and their sequences are available upon request. All ChIP and qPCRs were repeated at least three times and representative results were shown.

ChIP-Seq sample preparation and Computational Analysis of Illumina GA I/II data. Asynchronized HeLa cells or cells synchronized to G1 or G1/S phases by double thymidine block or M phase by Colcemid treatment were subjected to standard ChIP, as described above. ChIP DNA samples were then subjected to preparation for ChIP-Seq.

Library construction. The libraries were constructed following Illumina's Chip-Seq. Sample prep kit. Briefly, Chip DNA was end-blunted and added with an "A" base so the adaptors from Illumina with a "T" can ligate on the ends. Then 200-400 bp fragments are gel isolated and then purified. 18 cycles of PCR was done to amplify the library.

Primary analysis of ChIP-seq datasets. The image analysis and base calling were performed by using Illumina's Genome Analysis pipeline. The sequencing reads were aligned to the human genome UCSC build hg18 by using BOWTIE⁸³ alignment programs in 2 ways: only uniquely aligned reads were kept or both uniquely aligned reads and the sequencing reads that align to repetitive regions were kept for downstream analysis (if a read aligns to multiple genome locations, only one location is arbitrarily chosen). The multiplicate reads were collapsed in order to reduce the PCR biases. The aligned reads were used for peak/island finding with MACS⁸⁴ (PHF8, E2F1 and H3K4me2 ChIP-Seq. datasets) or SICER⁸⁵ (SMC4 and H4K20me1 ChIP-Seq. Datasets), respectively. Both MACS and SICER peak/island predictions were adjusted for genome instabilities (amplifications, deletions) either by a considering a local background area (MACS) or a random background model (SICER) that was used as a reference for the subsequent calculation of the enrichment scores.

Annotating and comparing the ChIP-seq peaks. The ChIP-Seq. peaks were mapped on the UCSC hg18 genes and RepeatMasker repeating elements. A peak was considered to be associated with a particular genome feature (promoter, intron, exon, repetitive element) if the peak summit (MACS peaks) or the middle of the peak (SICER peak) was located within 1kb distance of the transcription start site (the promoter region) or within an exon, intron, or a repetitive element (SINE elements, LINE elements, LTR retroposons, endogenous retroviruses, simple repeats, low complexity

repeats, satellite repeats, tRNA, rRNA, snRNA, scRNA, srpRNA). If a peak intersected with multiple genome features, all the corresponding genome features were considered when computing the genome distributions. PHF8 peaks in G1 and G1/S phases were considered intersecting or common if the predicted peaks intersected over at least 1bp. SMC4 and H4K20me1 peaks in M phase were considered intersecting if they overlap at least half of their average peak length.

Gene ontology analysis. the gene ontology analysis was carried out by using DAVID/ EASE⁸⁶ or BINGO⁸⁷ and the sequence motif enrichment analysis was performed by using HOMER. The sequence reads for each ChIP-Seq. were provided in Fig. S20. All the ChIP-Seq. experiments were performed using samples collected from HeLa cells.

Protein immunoprecipitation and immunoblotting. Cells were lysed in lysis buffer (50mM Tris HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 1% TRITON X-100) and pre-cleared with protein A beads (Sigma) for 1 h before incubation with antibodies overnight at 4 °C, followed by adding protein A beads for another 2 h before washing for 5 times with washing buffer (same as lysis buffer). Immunoprecipitates, pull downs, cell lysates or reaction mixtures from in vitro demethylase assay or in vitro kinase assay were boiled in SDS sample buffer and resolved by 4-12% Bis-Tris NuPAGE gel (Invitrogen), transferred onto Nitrocellulose membrane (Bio-Rad) and western blotting was performed following standard protocols.

siRNA transfection, RNA isolation and RT-qPCR. Two independent sources of siRNAs against PHF8 were validated in this study, PHF8 siRNA from Qiagen (CCG GAG ACA GTG CGA ACC GTA) and PHF8/ siGENOME SMARTpool from Dharmacon (M-004291-00-0005). Pr-set7/SETD8 siGENOME SMARTpool (M-031917-00-0020) was from Dharmacon. HCF-1(CAG CGA AAT CTC AGC CTT TAA), Set1A (CAG CGT ATT ATG AAA GCT GGA), SMC2 (CAG GTG GTT ATT GGT GGT AGA), SMC4 (AAG GGA CTT TGT TGA ACT TTA), N-CAPD3 (TCC GTC GTC AGC CAA AGA GAA) and N-CAPG2 (CCC TGA TGA AAT GGA GAA GAA) siRNAs were purchased from Qiagen. siRNA transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. siRNA transfection efficiency was determined by immunoblotting and/or RT-qPCR. Total RNA was isolated from HeLa cells using RNeasy Mini Kit (Qiagen) following manufacturer's protocol. First-strand cDNA synthesis from total RNA was carried out using SupreScript III (Invitrogen). Resulting cDNA was then analyzed by Quatitative PCR (qPCR) using Stratagene Mx3000 machine. Primers are specific for genes tested and their sequences are available upon request. All RT-qPCRs were repeated at least three times and representative results were shown.

Immunofluorescence. HeLa cells transfected with expression vectors or siRNAs as indicated were pre-extracted with 1% Triton X-100 or directly fixed for 5 min with 4 % formaldehyde in Phem buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl₂, pH 6.9), then permeabilized for 2 min with

0.1 % Triton X-100 in Phem buffer if there was no pre-extraction. After three rinses with Phem buffer, blocking solution (1 % BSA in PBS, pH 7.4) was applied for 30 min and primary antibodies against Flag (Sigma, 1:1000), PHF8 (abcam, 1:1000), H4K20me1 (Actif Motif, 1:1000), H4K20me2 (abcam, 1:250), H4K20me3 (abcam, 1:250), H3K9me1 (Abcam, 1:500), H3K9me2 (Upstate, 1:500), H3K9me3 (Abcam, 1:500), H3K27me1 (Upstate, 1:500), H3K27me2 (Abcam, 1:500) and H3K27me3 (Upstate, 1:500), SMC2 (Bethyl Laboratory, Inc., 1:250) and β -tubulin (1:1000) were added in blocking buffer for 1 h at room temperature. After three 5-min washes with PBS / 0.1 % Triton X-100, cells were incubated with Hoechst dye or DAPI and with secondary antibodies conjugated with fluorescent dyes (Jackson ImmunoResearch) for 1 h, washed again with PBS / 0.1% Triton X-100, and mounted in 20 mM Tris, pH 9 / 0.1 % p-phenylenediamine. Images were recorded on a DeltaVision microscope (Applied Precision) equipped with a CoolSnap charge-coupled device camera (Roper Scientific) at 1 x 1 binning and a 100x NA 1.3 U-planApo objective (Olympus). Z-stacks (0.2 μ m sections) were deconvolved using softWoRx (Applied Precision), projected for maximum intensity and imported into Adobe Photoshop CS4 (Adobe) for further processing. Image intensities for each antibody were scaled identically.

RNA profiling. Control or PHF8 siRNAs were transfected into HeLa cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. siRNA transfection efficiency was determined by immunoblotting and RT-qPCR. Total RNA was isolated from HeLa cells using RNeasy Mini Kit

(Qiagen) following manufacturer's protocol with DNase I in column digestion. The microarray experiments and data normalization were performed at the UCSD BIOGEM laboratory. Briefly, RNA quality was assessed using the Agilent Bioanalyzer. 250ng of total RNA was labeled with biotin using the Ambion Illumina TotalPrep RNA Amplification kit. 1500 ng of cRNA were hybridized to the Sentrix Human-6 v2 expression BeadChIP from Illumina for 18 hours at 58°C and then stained with Steptavidin-Cy3. Slides were scanned using the Illumina BeadArray Reader scanner and raw data extracted with the Illumina BeadStudio software. The data was then processed according to Illumina protocols and normalized using the multi-loess method "mloess" as described previously⁸⁸. Experiments were performed in duplicates. The differential expression analysis was performed by using SAM (significance analysis of microarrays) method⁸⁹ implemented in MeV/TM4⁹⁰ and in the R package "siggenes"⁹¹ and the FDR was sequentially set up at distinct values lower than 0.25. The expression of selected cell cycle genes which are positively regulated by PHF8 was validated by RT-qPCR. The gene ontology analysis was done by DAVID/EASE tools⁸⁶ (<http://david.abcc.ncifcrf.gov/>).

Gel filtration chromatography and purification of proteins associated with PHF8. Gel filtration chromatography was performed using a superpose 6 column (Amersham Biosciences). Briefly, HeLa nuclear extracts were loaded on the equilibrated column and eluted with a buffer containing 20mM Hepes pH 8.0, 1.5mM MgCl₂, 150 mM NaCl, 25% Glycerol, 1mM DTT,

0.2mM EDTA, 1% Triton X-100. Fractions were collected in a volume of 250 μ l and followed by western blotting analysis.

To purify proteins associated with PHF8, nuclear extracts were prepared from HeLa cells stably expressing Flag-PHF8 under inducible system or a control cell line in a buffer containing 20mM Hepes pH 8.0, 1.5mM MgCl₂, 0.42M NaCl, 25% Glycerol, 1mM DTT, 0.2mM EDTA, 1% Triton X-100. PHF8 associated proteins were then affinity purified by using Anti-flag M2-agarose, washed extensively and eluted with 3X Flag peptides. The eluates were concentrated and in solution digested with Trypsin singles proteomic grade (Sigma) following manufacturer's protocols and then subjected to Capillary-LC analysis. Essentially, Nano-capillary columns were prepared by drawing a 360- μ m O.D., 100- μ m I.D. deactivated, fused silica tubing (Agilent) with a Model P-2000 laser puller (Sutter Instruments) (Heat: 330, 325, 320; Vel, 45; Del, 125) and were packed at 600 psi to a length of about 10 cm with C18 reverse-phase resin suspended in methanol. The column was equilibrated with 95% of solvent A (water, 0.1% AcOH) and loaded with 10 μ l (10ng/ μ l) of trypsin digested reaction mix by flowing 95% of solvent A and 5% of solvent B (CH₃CN, 0.1% AcOH) at 200 μ l/min for 15 mins. A gradient for eluting trypsin digested peptides was established with a time-varying solvent mixture [(min, % of solvent A): (15, 95), (50, 60), (70, 30), (75, 5)] and directly electrosprayed into the LTQ-MS inlet (source voltage, 1.8 kV; capillary temperature, 180°C). Two methods setting were used. In both methods, the first scan was a low resolution broadband scan. The subsequent

six scans were data-dependent on the first scan. In each data-dependent scan, the top intensity ions excluded the ones in exclusion list were selected to be fragmented by CID which potentially, generated hundreds of fragmentation spectra collected as individual data events. In method one, the size exclusion list was set to 25 ions and a repeat of 30 s, whereas for the second method the exclusion list option was disabled. The resulting RAW files were converted to mzXML using the program ReAdW (<http://tools.proteomecenter.org>) and analyzed with InsPecT⁹² (version 2007.05.23)(<http://proteomics.ucsd.edu/InspectDocs/index.html>) ($P \leq 0.3$). Proteins pulled down in control samples were subtracted and the resultants were considered to be specifically associated with PHF8. The interaction of many of these proteins with PHF8 was validated by immunoprecipitation and western blotting.

Cell proliferation assay. Cell viability was measured by using CellTiter 96 AQueous one solution cell proliferation assay kit (Promega) following manufacturer's protocol. Briefly, 20 μ l of CellTiter 96 AQueous one solution reagent was added into per 100 μ l of culture medium with cells transfected with siRNAs. The culture plates were incubated for 1 h at 37 °C in a humidified, 5% CO₂ atmosphere. The reaction was stopped by adding 25 μ l of 10% SDS. Data was recorded at wavelength 490nm using CARY 3E UV-visible Spectrophotometer.

Flow cytometry. HeLa cells were trypsinized, washed with PBS and fixed with ethanol at 4 °C overnight. Cells were then washed with PBS and

stained with PI/Triton X-100 staining solution (0.1% (v/v) Triton X-100, 0.2mg/ml DNase-free RNAase A (Sigma), 0.02mg/ml PI (Roche)) at 37 °C for 15 minutes. DNA content was measured using FACSCaliber (Becton-Dickinson). About 105 events were analyzed for each sample and data were analyzed using CellQuest (Becton Dickinson) and ModFit LT (Verity Software House).

BrdU incorporation assay. HeLa cells were transfected with either control or PHF8 siRNAs for 18 h before treated with colcemid for another 12 h. Cells were then cultured in fresh medium containing BrdU (10 µM). S phase entry was measured by determining BrdU incorporation at 6, 10 and 12 h, presumably, cells are in late G1, early S and mid S phases, respectively. BrdU labeling and staining was performed using the BrdU labeling and detection kit I (Roche) following the manufacturer's instruction. Briefly, Cells were fixed with fixative (70% ethonal, 50mM glycine, pH 2.0) for 1 h, washed with PBS and stained with anti-BrdU for 30 min at 37 °C, followed by washing and staining with anit-mouse-IgG-fluorescein for 30 min at 37 °C.

Chromatin free and Chromatin bound fractions isolation. Cellular fractionation was done as described⁹³. Briefly, cells were lysed in a buffer containing 1% NP-40, 5 mM MgCl₂, 10 mM NaCl, and 20 mM Tris (pH 8.0) with protease inhibitors (Roche) for 1 h at 4°C, and the lysates were separated by centrifugation (14, 000 rpm, 10 min) into supernatant (chromatin-free) and pellet (chromatin-bound) fractions. The pellet was then re-suspended in a buffer containing 1% NP-40, 5 mM MgCl₂, 150 mM NaCl, and 20 mM Tris (pH

8.0) with protease inhibitors and incubated for 1 h at 4°C followed by sonication to shear DNA and dissociate chromatin bound proteins. The resultant as well as the supernatant fractions were denatured, fractionated by Bis-Tris gel and analyzed by immunoblotting.

In vitro kinase assay and identification of phosphorylation sites. In vitro kinase reactions were performed by incubating CDK1/cyclinB1 (Upstate) with purified bacterially-expressed PHF8 proteins in a buffer containing 25 mM HEPES, pH 7.9, 25mM glycerophosphate, 25 mM MgCl₂, 2 mM dithiothreitol, and 0.1mM NaVO₃ for 1 h at 30°C. For 30 µl reaction, 1 µl of 10 µCi/ µl ATP, [γ -³²P] was used. The reactions were terminated with SDS sample buffer, boiled and resolved by SDS-PAGE gel (PAGE gel, Inc.). Phosphorylation was detected by autoradiogram. InsPecT was used to search for phosphorylated peptides on a serine, threonine or tyrosine with a mass shift of 80 Da. The database used in this search includes common contaminated proteins such as trypsin and human keratins with PHF8 protein sequence add in and their corresponding shuffled sequences. The parent ion mass tolerance was set to be 2 Da and b, y ion mass tolerance was set as 0.5 Da.

Pull-down assay using mononucleosomes and Condensin II purified from HeLa cells. The Condensin II complex from HeLa cell nuclear extracts was purified as previously described⁷³, Essentially, HeLa cell nuclear extracts were prepared in a buffer containing 20 mM K-Hepes (pH 8.0), 100 mM KCl, 2 mM MgCl₂, 0.2 mM EDTA, 0.5 mM PMSF, 1 mM β -mercaptoethanol, and 10% glycerol. For immunoprecipitations, 25 µl of protein

A-agarose beads (Sigma) were pre-conjugated with 2.5 µg of control IgG or N-CAPD3 antibody (Abcam) before adding to nuclear extracts (0.5mg) at 4 °C for 2 h. Immunoprecipitates (negative control or Condensin II complex) were washed three times with TBS containing 0.2% Triton X-100 and once with TBS.

The pull down assay was then carried out following the previously described protocol³⁵. Briefly, the aforementioned immunoprecipitates were incubated with 200 µg of purified HeLa nucleosomes for 1 h at 40°C, and then washed extensively with phosphate-buffered saline (PBS). The bound material were eluted and denatured by adding SDS-loading buffer, and then fractionated by Bis-Tris gel and analyzed by immunoblotting.

Live-Imaging. Time-lapse sequences of HeLa cells expressing Histone H2b::GFP were acquired at 37 °C on a spinning disc confocal head (McBain Instruments) mounted on an inverted Nikon TE2000e microscope equipped with a 60x 1.4 NA Plan Apochromat lens (Nikon), a krypton-argon 2.5 W water-cooled laser (Spectra-Physics) and a charge-coupled device camera (iXon; Andor Technology). Acquisition parameters, shutters, and focus were controlled by MetaMorph software (MDS Analytical Technologies). Six z-sections 3 µm apart were taken. Z-stacks were projected for maximum intensity and imported into Adobe Photoshop CS4 (Adobe) for further processing.

TUNEL assay. TUNEL assay was performed using the In Situ Cell Death Detection Kit, TMR red (Roche). Embryos were dechorionated and fixed

with 4% paraformaldehyde for 30 min, followed by dehydration in MeOH for 30 min. After re-hydrating by washing 2-3 times in PBS/ 0.1% Triton X-100, embryos were penetrated by incubating in a solution consisting of 1% Sodium Citrate and 1% Triton X-100 on ice for 15 min and washed again with PBS-Triton X-100. Embryos were then covered with labeling mixture and incubated for 1h at 37°C, followed by washing in PBS-Triton X-100 and equilibration with 75% glycerol and mounting on slides. Imaging were done using Olympus FV1000 confocal 2-photon microscope. The resulting images were obtained by compressing multistacks in FV10-ASW1.7 viewer.

Whole mount in situ hybridization. In situ staining was done as previously described⁸¹.

Alcian blue staining. Protocol for Alcian blue staining was described previously⁹⁴. Alcian blue instead of Alcian green was used in this study.

Immunostaining in Zebra fish. For single cell immunostaining, injected embryos were grown for 18 h, dechorionated and fixed in 4% paraformaldehyde for 15 min. After washing in PBS-Triton X-100 buffer two times, embryos were macerated in 50 µl of PBS-Triton X-100, diluted with 250 µl of 0.8% low melting agarose and spread on a microscope slide previously covered with a thin layer of 1% agarose. Prepared slides were dried for 15 min and submerged into blocking solution (10% of Fetal bovine serum, FBS) in PBS-Triton X-100 for 2 h, incubated with anti- β -Tubulin antibody mouse IgG (Sigma, 1:500) overnight or anti-phospho-Histone H3 serine 10 (Upstate, 1:500) for 2 h, washed in PBS-Triton X-100 buffer for 2 h and blocked again

for 15 min. Corresponding secondary antibodies (Alexa Fluor 488 donkey anti-mouse and Alexa Fluor 594 donkey anti-rabbit, Molecular probes) were added for 1h. After washing, slides were mounted in mounting media containing DAPI (Invitrogen, Inc.). Imaging was done using Olympus FV1000 confocal 2-photon microscope. The resulting images were obtained by compressing multistacks in FV10-ASW1.7 viewer.

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