

AD_____

Award Number: DAMD17-03-1-0704

TITLE: Cell Cycle Inhibitors as Breast Cancer Prevention Agents

PRINCIPAL INVESTIGATOR: Rafael Herrera, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, TX 77030

REPORT DATE: September 2004

TYPE OF REPORT: Revised Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				<i>Form Approved</i> OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE September 2004		2. REPORT TYPE Revised Final		3. DATES COVERED 1 September 2003- 31 August 2004	
4. TITLE AND SUBTITLE Cell Cycle Inhibitors as Breast Cancer Prevention Agents				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER DAMD17-03-1-0704	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Rafael Herrera, Ph.D. Ashby Morrison, Ph.D. E-Mail: trice@bcm.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Baylor College of Medicine Houston, TX 77030				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT There is evidence from our own work and suggesting that histone H1 phosphorylation by cyclin E/CDK2 relaxes condensed heterochromatin, increasing vulnerability to DNA-damaging agents. We provide further evidence that inhibitors of cyclin E/CDK2 can reverse this vulnerability, and that H1 phosphorylation acts by disrupting H1's association with another component of condensed heterochromatin, Heterochromatin Protein 1. This association may thus provide a relatively specific target for intervention to reduce DNA damage probability and the accompanying likelihood of cancer formation, which deserves further exploration.					
15. SUBJECT TERMS- histone H1 phosphorylation; DNA damage susceptibility; chromatin organization; breast cancer prevention targets.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	5
Reportable Outcomes.....	5
Conclusion.....	6
References.....	6
Appendices.....	7

Introduction

The retinoblastoma gene, RB, is one of the most often inactivated genes in human cancer. As such it is a prototypical tumor suppressor gene. One of the functions of the protein product of the gene, pRb, is to repress the cyclin E promoter through its interaction with the transcription factor E2F. Upon pRb inactivation through phosphorylation, mutation, or interaction with viral oncoproteins, its repression of the promoter is relieved and levels of cyclin E protein are increased. This leads to increased cyclin E-associated kinase activity after the protein binds its catalytic partner, the cyclin dependent kinase CDK2. The active cyclin E/CDK2 complex then phosphorylates its substrates. This cyclin E/CDK2 activity is required for transition through the late stages of G1 and into S phase.

One cyclin E/CDK2 substrate is the linker histone H1 (Herrera et al., 1996). Histone H1 binds to the linker region of chromatin between nucleosomes and helps to compact chromatin into higher order structures. Phosphorylation of H1 leads to its decreased affinity for DNA and therefore a more relaxed chromatin structure (Roth and Allis, 1992).

While RB is lost in only 10-20% of breast carcinomas, cyclin E is often overexpressed in breast cancer (Nielsen et al., 1998). Indeed, overexpression of cyclin E in mice leads to induction of mammary gland hyperplasia and carcinomas (Bortner and Rosenberg, 1997). Both RB inactivation and cyclin E overexpression would lead to an increase in H1 phosphorylation. Indeed, one is a subset of the other in that RB loss should almost always lead to cyclin E overexpression. In addition, it has been shown that almost any lesion that pushes cells towards transformation leads to an increase in H1 phosphorylation (Chadee et al., 1995).

The consequences of RB loss and cyclin E overexpression seem to be obvious. Cells go through G1 more quickly than their wild-type counterparts and seem to have a higher proliferative rate (Herrera et al., 1996; Bortner and Rosenberg, 1997). However, this does not necessary explain why RB is such a potent tumor suppressor and is so often mutated in human cancer. A clue to further consequences of loss of RB or cyclin E overexpression comes from experiments that show an increase in susceptibility to nuclease digestion in cells that lack pRB. Indeed this is seen in all the cells mentioned above that display an increase in levels of phosphorylated H1. These studies show that the chromatin of these cells is more open to attack.

Body

Chromatin Structure and Damage Susceptibility

Our DOD-funded studies demonstrated that loss of RB and subsequent overexpression of cyclin E results in an increased sensitivity to DNA-damaging agents. These include those more likely to be encountered by a mutant cell *in vivo* such as UV and gamma irradiation. These studies suggest that many of the most common lesions seen in breast cancer possess a least two properties that lead to tumor progression. One is their effect on cell cycle progression while the other is potentially their most damning – their rendering the genome more vulnerable. Furthermore, and very importantly, we found that inhibitors (UCN-01, 7-hydroxystaurosporine) of cyclin E/CDK2 activity reversed the increased DNA damage susceptibility. This demonstrates the possible efficacy of cell cycle inhibitors as cancer prevention agents.

In our studies we exposed primary mouse embryo fibroblasts from RB homozygous knockout mice and their wild-type littermates to varying doses of oxidative stress and UV radiation. Immediately thereafter, their DNA was analyzed for single- and double- strand breaks as well as the damage-induced adducts, cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts [(6-4) PDs]. DNA breaks were analyzed by electrophoresis and the levels of CPDs and (6-4) PDs through the use of radio-immunoassays (Mitchell, 1999). It should be noted that DNA damage was analyzed before the cells were able to respond through induction of repair mechanisms or apoptotic pathways. This eliminated the potential confounding factors supplied by the cell's response to the damage. In this way we directly investigated only the damage.

While these studies did not monitor breast cancer directly or investigate the effects of mutagens that may be likely to play a role in breast cancer progression, they could lead to elucidation of a very important concept in RB and cyclin E function. This concept could lead to new strategies to combat tumor progression by protecting the genome from damage through reduction of cyclin E/CDK2 activity or by altering the levels of histone H1 phosphorylation through other means.

Heterochromatin Protein 1 and Histone H1 Interaction

Two key components of mammalian heterochromatin that play a structural role in higher order chromatin organization are the Heterochromatin Protein 1 α (HP1 α) and the linker histone H1. Our DOD-funded studies show that these proteins interact *in vivo* and *in vitro* through their hinge and C-terminal domains respectively. The phosphorylation of H1 by CDK2, which is required for efficient cell cycle progression, disrupts this interaction. We propose that phosphorylation of H1 provides a signal for the disassembly of higher order chromatin structures during interphase, independent of histone H3-lysine9 (H3-K9) methylation, by reducing the affinity of HP1 α for heterochromatin. This work has now been published (Hale, 2006).

Rafael Herrera (PI), Ashby Morrison (graduate student), and Tracy Hale (postdoctoral Associate) were all partially supported on this study.

Key Research Accomplishments

- Lesions commonly found in breast cancer frequently increase susceptibility to DNA damage, and inhibitors of cyclin E/CDK2 activity can protect against this susceptibility.
- Phosphorylation of linker histone H1, e.g. by cyclin E/CDK2, relaxes higher order heterochromatin structure, thus increasing vulnerability to DNA damage.
- Phosphorylation of histone H1 by CDK2 disrupts the association of H1 with Heterochromatin Protein 1 α , indicating a mechanism for the relaxation of heterochromatin and suggesting a more specific target for inhibiting DNA damage susceptibility.

Reportable Outcomes

Hale TK, Contreras A, Morrison AJ, Herrera RE (2006) Phosphorylation of the linker histone H1 by CDK regulates its binding to HP1 α . *Molecular Cell*; 22:693-699 [see Appendix]

Conclusions

The results of these studies give firm support to our hypothesis that histone H1 phosphorylation by cyclin E/CDK2 relaxes highly condensed heterochromatin, which is necessary for replication but also increases vulnerability to DNA damage. Cyclin E/CDK2 inhibitors like UCN-01 would therefore be predicted to reduce DNA damage, and this was confirmed. But before proceeding to mouse tumor experiments with such broadly active inhibitors which might be expected to damage the cell cycle and thus cause more genetic damage, we explored the interactions of histone H1 with other chromatin stabilizers and indeed found a specific interaction with HP1 α which is disrupted by H1 phosphorylation, and thus might provide a more specific target for intervention. We feel that this hypothesis and this specific target deserve further examination, in hopes of discovering safe ways to reduce DNA damage which can lead to tumor formation.

References

Bortner DM, Rosenberg MP (1997) Induction of mammary gland hyperplasia and carcinomas in transgenic mice expressing human cyclin E. *Mol Cell Biol*;17(1):453-9

Chadee DN, Taylor WR, Hurta RA, Allis CD, Wright JA, Davie JR (1995) Increased phosphorylation of histone H1 in mouse fibroblasts transformed with oncogenes or constitutively active mitogen-activated protein kinase kinase. *J Biol Chem*; 270(34):20098-105

Herrera RE, Chen F, Weinberg RA (1996) Increased histone H1 phosphorylation and relaxed chromatin structure in Rb-deficient fibroblasts. *Proc Natl Acad Sci U S A*; 93(21):11510-5

Mitchell DL (1999) Quantification of photoproducts in mammalian cell DNA using radioimmunoassay. *Methods Mol Biol*;113:165-73.

Roth SY, Allis CD (1992) Chromatin condensation: does histone H1 dephosphorylation play a role? *Trends Biochem Sci*;17(3):93-8

Appendix

Hale TK, Contreras A, Morrison AJ, Herrera RE (2006) Phosphorylation of the linker histone H1 by CDK regulates its binding to HP1 α . *Molecular Cell*; 22:693-699

Phosphorylation of the Linker Histone H1 by CDK Regulates Its Binding to HP1 α

Short Article

Tracy K. Hale,^{1,2} Alejandro Contreras,¹
Ashby J. Morrison,^{1,3} and Rafael E. Herrera^{1,3,*}

¹The Breast Center
Department of Molecular and Cellular Biology
Baylor College of Medicine
One Baylor Plaza
Houston, Texas 77030

Summary

Two key components of mammalian heterochromatin that play a structural role in higher order chromatin organization are the heterochromatin protein 1 α (HP1 α) and the linker histone H1. Here, we show that these proteins interact *in vivo* and *in vitro* through their hinge and C-terminal domains, respectively. The phosphorylation of H1 by CDK2, which is required for efficient cell cycle progression, disrupts this interaction. We propose that phosphorylation of H1 provides a signal for the disassembly of higher order chromatin structures during interphase, independent of histone H3-lysine 9 (H3-K9) methylation, by reducing the affinity of HP1 α for heterochromatin.

Introduction

Heterochromatin, the cytologically dense regions of the genome found near centromeric and telomeric regions, contains highly condensed chromatin whose maintenance is required for genomic stability and control of gene expression (Maison and Almouzni, 2004). Although heterochromatin domains are structurally stable entities, their components need to be dynamic to cope with the disrupting events that occur during the cell cycle, such as DNA replication (Maison and Almouzni, 2004). Both the linker histone H1 and HP1 families are important modulators of chromatin function. Mammalian cells contain three HP1 isoforms (α , β , and γ) that, although structurally similar, differ in their nuclear location (Minc et al., 1999). HP1 α is associated mainly with centromeric heterochromatin, whereas HP1 β additionally resides in euchromatic regions and HP1 γ is mainly localized to euchromatic sites. The isoforms consist of a conserved amino (N)-terminal chromodomain (CD) and a structurally related carboxy (C)-terminal chromoshadow domain (CSD), connected by a poorly conserved hinge region (Eissenberg and Elgin, 2000). It has been proposed that HP1 primarily controls the formation and propagation of heterochromatin through the interaction of the CD with methylated H3-K9 (Bannister et al., 2001; Jenuwein and Allis, 2001). However, in

the context of chromatin, it is clear that multiple interactions determine the targeting of HP1 α and the establishment of silenced domains (Maison and Almouzni, 2004). Studies have shown that the presence of methylated H3-K9 within chromatin is insufficient for HP1 specificity (Cowell et al., 2002) and recruitment (Stewart et al., 2005), whereas the targeting of the three homologs to different chromosomal locations is not solely determined by the CD of HP1 (Platero et al., 1995; Powers and Eissenberg, 1993). In addition, the ability of HP1 α to bind RNA is thought to play a structural role in heterochromatin formation (Maison et al., 2002; Muchardt et al., 2002). Importantly, the CD lacks affinity for native chromatin, because the binding of *Xenopus* HP1 α to native chromatin is shown to be dependent on the presence of linker H1 histones and not methylated H3-K9 (Meehan et al., 2003). The H1 histones are a family of basic proteins that are essential dynamic components of the 30 nm chromatin fiber (Fan et al., 2003) shown to direct and stabilize higher order folding of the fiber (Carruthers et al., 1998; Contreras et al., 2003; Th'ng et al., 2005). Like the core histones, the tail domains of H1 are posttranslationally modified (Garcia et al., 2004; Jenuwein and Allis, 2001). A prominent modification of H1 is its cell cycle-dependent phosphorylation (Talasiz et al., 1996). It has been proposed that this phosphorylation of H1, by cyclin-dependent kinases (CDKs), controls the level of chromatin condensation as cells traverse the cell cycle (Roth and Allis, 1992). Recent data suggest that H1 phosphorylation destabilizes H1-chromatin interactions (Contreras et al., 2003), leading to a relaxed chromatin structure (Herrera et al., 1996). Exactly how it fulfills this role in chromatin condensation and the consequences of its phosphorylation during the cell cycle remain unclear (van Holde, 1989), as very few studies have addressed the interaction of H1 with other components of the chromatin machinery involved in higher-order folding such as the HP1 isoforms (Hansen, 2002; Nielsen et al., 2001; Vaquero et al., 2004). In this study, we investigate the interaction between HP1 α and the linker histone H1b, demonstrating that phosphorylation of H1b by CDK2 can regulate this interaction. We propose that one mechanism by which H1 phosphorylation leads to the decondensation of chromatin during interphase is by disrupting the association of HP1 α with the chromatin fiber.

Results and Discussion

Compared to euchromatin, the heterochromatic regions of eukaryotic nuclei have an enrichment of H1 that is more statically bound (Contreras et al., 2003). Of the five somatic variants of histone H1, the predominant variant found in heterochromatin is H1b (Th'ng et al., 2005). To explore the interaction of unmodified human H1b and HP1, *in vitro* binding assays were performed. Bacterially expressed H1b only associates with HP1 α , the HP1 isoform that resides in heterochromatin, but not with HP1 β and HP1 γ (Figure 1A). This finding agrees with a previous study showing that only the HP1 α isoform bound to

*Correspondence: rherrera@mdanderson.org

²Present address: Children's Cancer Research Group, Christchurch School of Medicine, 2 Riccarton Ave, Christchurch 8001, New Zealand.

³Present address: University of Texas M.D. Anderson Cancer Center, Science Park Research Division, 1808 Park Road 1-C, Smithville, Texas 78957.

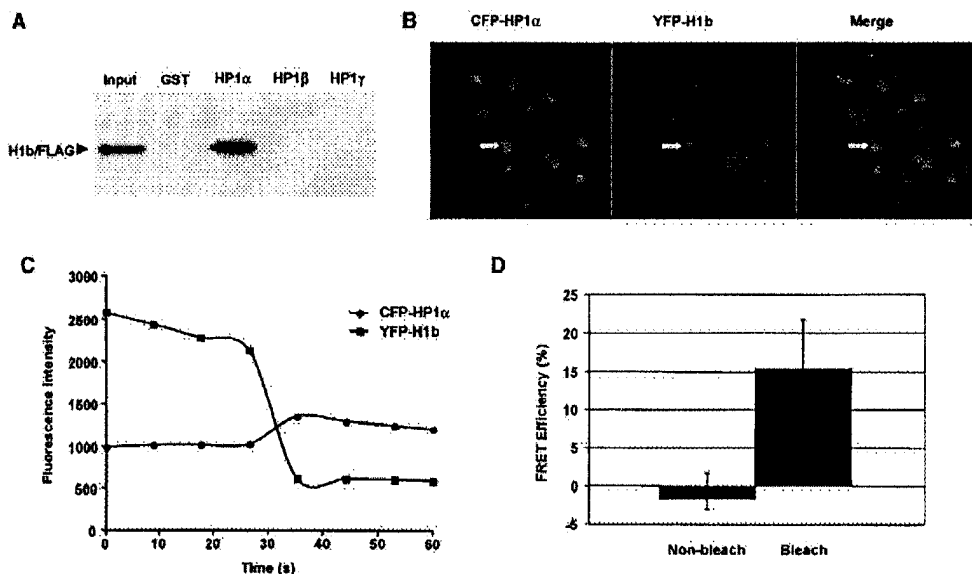


Figure 1. HP1 α Interacts with H1b In Vitro and In Vivo

(A) Unmodified H1b/FLAG incubated in a batch assay with either GST or the GST fusion proteins HP1 α , β , or γ . The GST fusion proteins were immobilized and the bound H1b/FLAG detected by immunoblotting using anti-FLAG. "Input" represents 10% of prebound H1b/FLAG. (B) Mouse NIH 3T3 fibroblasts were cotransfected with plasmids expressing CFP-HP1 α (green) and YFP-H1b (red) and imaged with the Zeiss LSM 510 confocal microscope. Arrow indicates an example where H1b is present in heterochromatin that also contains HP1 α . Areas of colocalization result in the yellow pseudo color observed when panels one and two are overlaid (Merged). (C and D) Detection of an in vivo interaction between CFP-HP1 α and YFP-H1b using FRET (Karpova et al., 2003). (C) Quantification of the fluorescent intensity of CFP-HP1 α and YFP-H1b in a representative region of a NIH 3T3 cell expressing CFP-HP1 α and YFP-H1b imaged in (B). The fluorescent intensity of the donor CFP-HP1 α increased after photobleaching of the acceptor YFP-H1b at 27 s. (D) Average change in CFP-HP1 α intensity in nonbleached regions (internal negative control) versus bleached regions. Error bars represent standard deviations derived as described in the Experimental Procedures.

a mixture of linker histones isolated from calf thymus (Nielsen et al., 2001).

Using HP1 α and H1b fluorescently tagged with CFP and YFP, respectively, H1b is shown to strongly colocalize with HP1 α in mouse NIH 3T3 fibroblast cells (Figure 1B), demonstrating that both H1b and HP1 α are enriched in heterochromatin. In an attempt to detect a direct H1b-HP1 α interaction in situ, we employed fluorescence resonance energy transfer (FRET) by the acceptor photobleaching method. This technique reveals protein-protein interaction in living cells by taking advantage of the energy transfer that occurs between two fluorescent tags with overlapping emission/absorption spectra attached to the proteins of interest. Proteins must be no more distant than 10–80 Å for energy transfer to occur (Truong and Ikura, 2001), which will result in an increase of donor fluorophore (CFP) intensity upon acceptor fluorophore (YFP) photobleaching (Karpova et al., 2003). Photobleaching of YFP-H1b, in regions that colocalize with CFP-HP1 α (Figures 1B and 1C), results in a 15.4% $\pm$ 6.2% increase in fluorescence of CFP-HP1 α (Figure 1D). In nonbleached portions of the cell, the percent change of CFP-HP1 α intensity was $-1.73\% \pm 3.3\%$, indicating an average decrease in fluorescence due, most likely, to CFP photobleaching by the image capturing process (Figure 1D). The FRET efficiency of 15% is very comparable to that observed between other interacting proteins. For example, Karpova et al. [2003] observed efficiencies of 8.48% and 23.1%

between TRAF2 (TNF-receptor-associated factors) homodimers and TRAF1-TRAF2 heterodimers, respectively. TRAF factors had previously been shown to interact strongly by various methods, including laser light scattering, analytical ultracentrifugation, and crystallography studies (Karpova et al., 2003). In addition, no increase in CFP intensity after photobleaching of YFP was observed in cells expressing CFP-HP1 α only (data not shown). To control for chance dimerization of CFP and YFP due to high concentration, no FRET was observed in cells coexpressing CFP-HP1 α and a fusion protein where a nuclear localization sequence was tagged to YFP (data not shown). Thus, HP1 α directly interacts with histone H1b in vitro and in vivo (Figure 1).

To characterize the domains of HP1 α and H1b (Figure 2A) required for this interaction, further in vitro binding assays were performed. Only the hinge domain of HP1 α interacts with H1b (Figure 2B). Because the HP1 α hinge domain can interact with RNA (Muchardt et al., 2002), RNase A was also added to the in vitro binding assay to demonstrate that the interaction of HP1 α with H1b is not mediated by RNA from the bacterial preparation (Figure 2C). Thus, consistent with the observation that the hinge domain of HP1 differs in length and composition between the isoforms and has been shown to functionally discriminate these proteins in vivo (Platero et al., 1995; Powers and Eisenberg, 1993), HP1 α specificity for H1 (Figure 1A) is mediated through its hinge region. Just as the hinge region determines the

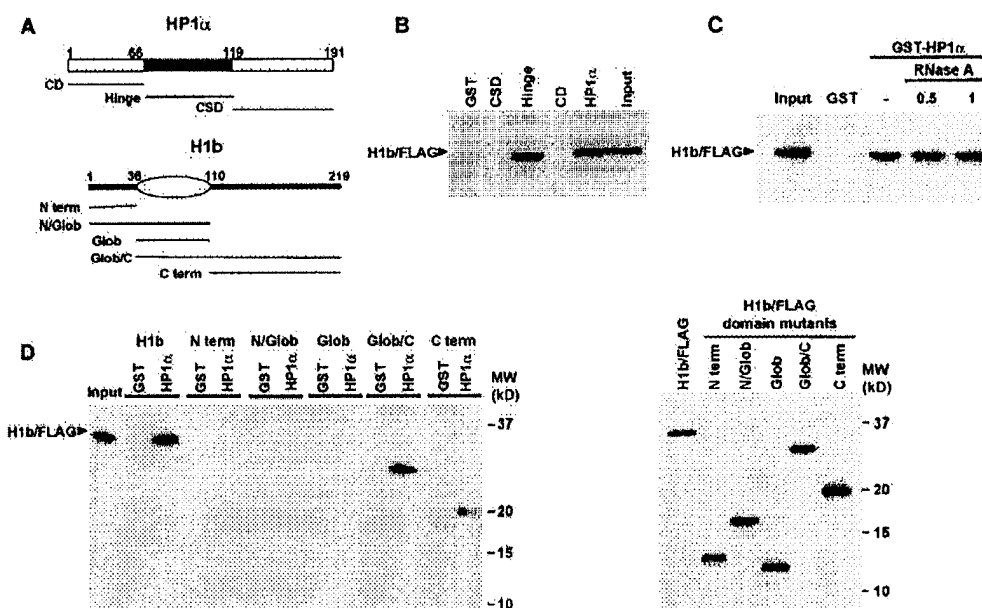


Figure 2. The Hinge Domain of HP1 α Interacts with the C-Terminal Tail of H1b In Vitro

(A) The various GST-HP1 α and H1b/FLAG domain mutants used in the in vitro binding assays below.
(B-D) In vitro binding assays where the GST fusion proteins were immobilized and the bound H1b/FLAG detected by immunoblotting using anti-FLAG.
(B) H1b/FLAG incubated in a batch assay with either GST or the GST-HP1 α domain mutants (CD, Hinge, or CSD). Input represents 10% of pre-bound H1b/FLAG.
(C) Unmodified H1b/FLAG incubated with either GST or GST-HP1 α and RNase A to a final concentration of 0.5 ng/ml or 1 ng/ml. Input represents 10% of pre-bound H1b/FLAG.
(D) The domain mutants of H1b/FLAG incubated in a batch assay with either GST or GST-HP1 α (top). The right panel shows 10% of the input H1b/FLAG and H1b/FLAG domain mutants used in the binding assay of the left panel to assure equal protein amounts, as detected by immunoblotting using anti-FLAG.

interaction of *Xenopus* HP1 α with native chromatin (Meehan et al., 2003), HP1 α -H1b binding in vivo may be required for stabilization of condensed chromatin. This observation allows for the simultaneous binding of HP1 α to both methylated H3-K9 and H1 via the CD and hinge domain, respectively.

H1 histones have a tripartite structure consisting of a central globular domain flanked by two lysine rich tails (Figure 2A). H1b binds HP1 α predominantly through the C-terminal tail domain (Figure 2D). It is this domain that plays a prominent role in determining the binding properties of the individual H1 variants in vivo, with H1b having one of the highest binding affinities for chromatin (Th'ng et al., 2005). Interestingly, it is also the domain that has four of the five CDK phosphorylation sites that are present in this variant (Figure 3A). Given that phosphorylation of H1 decreases the binding affinity of H1 for chromatin in vivo (Contreras et al., 2003) and high levels of H1 phosphorylation, due to increased CDK2 activity, are associated with a more relaxed chromatin structure (Herrera et al., 1996), we explored the consequence of H1b phosphorylation on the interaction with HP1 α . Wild-type (wt) H1b or a mutant H1b (M1-5), in which the five consensus CDK phosphorylation sites (S/TPXK/R) in the N- and C-terminal tails were mutated to alanines (Figure 3A), were phosphorylated in vitro with baculovirus-produced human CDK2/cyclin E. We have previously shown that M1-5 cannot be phosphorylated

by CDK/cyclin complexes in vitro or in vivo (Contreras et al., 2003). Figure 3B displays that phosphorylated H1b can no longer interact with HP1 α . Binding of M1-5 is not affected by treatment with the baculovirus-produced CDK2/cyclin E, ruling out any confounding effects from the baculovirus lysate. Thus, H1 phosphorylation by CDK2/cyclin E abolishes its ability to interact with HP1 α in vitro and suggests a mechanism where H1 phosphorylation modulates chromatin folding by disrupting the interaction of HP1 α with the condensed chromatin fiber.

Although the interaction of H1b and HP1 α occurs in vivo (Figure 1), the phosphorylation status of H1 in these experiments is unknown. Immunofluorescent studies using antibodies against HP1 α and phosphorylated H1 were performed to see if phosphorylated H1 colocalizes with HP1 α (Figure 3D). As can be seen, HP1 α localizes predominantly to the dense regions of chromatin, which stain strongly with DAPI (Minc et al., 1999; Nielsen et al., 2001). In stark contrast, phosphorylated H1 resides outside of heterochromatic regions, in agreement with previous studies showing H1b is underphosphorylated in heterochromatin (Contreras et al., 2003). These studies show that phosphorylated H1 and HP1 α do not reside in similar regions of the cell, suggesting that, in vivo, HP1 α binds to underphosphorylated H1 in heterochromatin.

Because cell cycle progression requires the reorganization of higher order chromatin structure, it is proposed

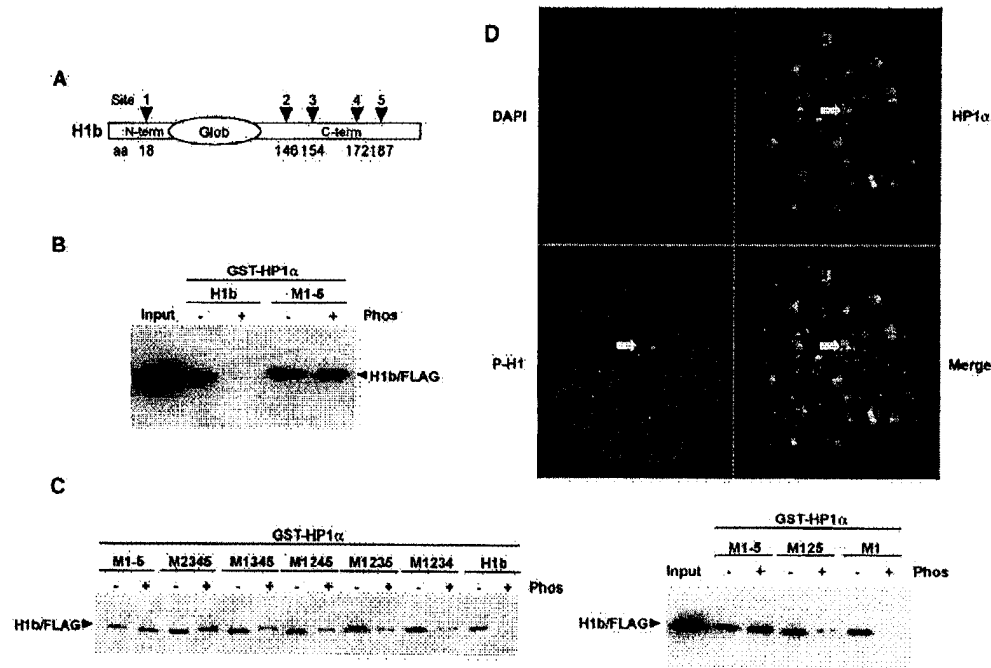


Figure 3. Phosphorylation of H1b Prevents Its Interaction with HP1 α

(A) A representation of the tripartite structure of the human linker histone H1b consisting of a globular domain flanked by N- and C-terminal tails. The numbered CDK consensus sites are shown above, whereas the location of the five phosphate acceptor residues that were mutated to alanine in the phosphorylation mutant M1-5 are shown below.

(B and C) H1b/FLAG proteins from in vitro kinase reactions with either inactive CDK2 (– phos) or the active kinase complex CDK2/cyclin E (+ phos) were incubated with GST-HP1 α in batch assays. Bound H1b/FLAG was detected by immunoblotting using anti-FLAG. Input represents 10% prebound H1b/FLAG. (B) H1b/FLAG or mutant M1-5/FLAG. (C) H1b/FLAG, M1-5/FLAG, and phosphorylation mutants of H1b/FLAG that can only be phosphorylated on one CDK site each (M2345, M1345, M1245, M1235, and M1234, where the number refers to the phosphorylation site mutated within the protein to prevent phosphorylation). Compare lanes for M1-5 and M2345 to the other phosphorylation mutants (left). The right panel shows the M1-5, M125, and M1 phosphorylation mutants of H1b/FLAG.

(D) Deconvolution microscopy of asynchronously growing mouse NIH 3T3 fibroblasts fixed and costained with antibodies against phosphorylated histone H1 (P-H1) and HP1 α . Arrow indicates a region containing HP1 α (green) but that is devoid of phosphorylated H1 (red). Note the lack of yellow pseudo color in the Merged panel, in contrast to Figure 1B.

that phosphorylation of H1 destabilizes the chromatin structure, allowing for access to the chromatin template of factors required for gene expression and DNA replication (Roth and Allis, 1992). CDK2 activity, with cyclin E in late G₁ and cyclin A during S phase, is essential for progression into and through S phase (Ohtsubo et al., 1995). To demonstrate the importance of H1 phosphorylation by CDK2 and, hence, its ability to disrupt HP1 α binding, we investigated the ability of cells that tran-

siently overexpress M1-5 to enter S phase. This H1b mutant cannot be phosphorylated but is incorporated into chromatin (Contreras et al., 2003). Cell cycle profiles of Saos-2 human osteosarcoma cells overexpressing wt H1b and M1-5 along with a CD20 cell surface marker demonstrate that expression of either H1b wt or M1-5 leads to an increase in the G₁ fraction of CD20-positive (and therefore transfected) cells (Figure 4). Addition of cyclin E reduced the G₁ fraction of cells transfected

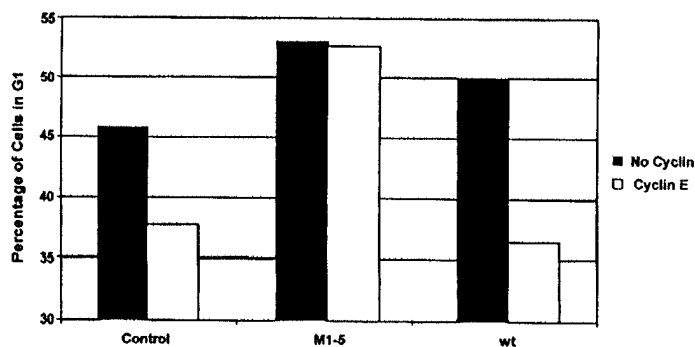


Figure 4. Phosphorylation of H1b Is Required for Efficient Cell Cycle Progression

Human Saos-2 cells were transiently cotransfected with a vector expressing CD20 and vectors expressing M1-5, H1b (wt), or no histone (Control), in combination with a vector expressing cyclin E or the vector alone (No Cyclin). Cells were harvested for flow cytometry analysis to determine the cell cycle profiles of cells that coexpressed the CD20 cell surface marker. The percentage of FITC-CD20-positive cells in G₁, determined by DNA content, is shown.

with control and wt constructs. However, the increase in G₁ fraction persisted in cells transfected with the M1-5 mutant. Therefore, overexpression of the mutant M1-5 causes cells to stall in G₁, which cannot be overcome by increased CDK2 activity. This observation furthermore suggests that when unphosphorylated H1 (that can bind HP1 α) is bound to chromatin, cells cannot efficiently progress through late G₁ into S phase. Nevertheless, this observation is correlative and does not rule out the possibility that the effect is due to activities of H1 in addition to its ability to bind HP1. However, that H1 phosphorylation (and a concomitant relaxation of chromatin structure) is required for efficient cell cycle progression is supported by the recent finding that recruitment of CDK2/cyclin A to replication foci during S phase results in phosphorylation of H1 and chromatin decondensation (Alexandrow and Hamlin, 2005).

When CDK2 is active in late G₁/S phase, phosphorylation of H1b is limited to no more than three phosphates per molecule (Talaszi et al., 1996). To identify the level of phosphorylation required to disrupt the interaction with HP1 α , mutants with a varying number of CDK sites in the C-terminal tail of H1b were created (Figure 3A). Phosphorylation of only one site within the C terminus of H1b extensively affects HP1 α binding (Figure 3C), and significantly, there was no preference for which particular CDK site in the C-terminal tail was phosphorylated (Figure 3C). This demonstrates that the interaction of HP1 α and the C terminus of H1 is charge dependent and disruption of the interaction is not dependent on the phosphorylation of a particular CDK phosphorylation site but rather involves a cumulative effect of the four phosphorylation sites within the H1 tail (Figure 3C and data not shown). Therefore, the level of phosphorylation seen on H1b during interphase can severely disrupt the HP1 α -H1b interaction.

These findings show a direct interaction in vitro and in vivo between HP1 α and H1b that is regulated by the posttranslational phosphorylation on the tail domain of H1. Interestingly, a recent study (Daujat et al., 2005) has shown that in vitro, the CD of each HP1 isoform can bind to H1b when it is methylated on lysine 26 (H1-K26). Preliminary studies in our laboratory have shown that the phosphorylation of methylated H1b by CDK2 can also disrupt the interaction of either HP1 α or β with methylated H1 (data not shown). This suggests the interaction between H1b and the HP1 isoforms is dependent on the type and combination of posttranslational modifications present on the linker histone. Therefore, the interaction of an HP1 isoform with a particular chromatin domain is likely to be regulated by the modification status not only of the core histones but also of histone H1. If a "histone code" does exist for histone H1 (Jenuwein and Allis, 2001), our findings are consistent with several recent studies that support the idea of a simple redundant histone code (Dion et al., 2005; Henikoff, 2005; Liu et al., 2005).

From our findings, the C-terminal tail of H1 is likely to be involved in directing the HP1 α isoform to heterochromatic regions that are enriched in unphosphorylated H1b, suggesting a more active mechanism by which H1 directs chromatin compaction beyond simply binding to linker DNA. The potential for HP1 α to self-associate through the CSD (Nielsen et al., 2001), while interact-

ing with methylated H3-K9 through the CD and with H1 and RNA through the hinge domain, could provide the framework to create and stabilize the condensed domains required of heterochromatin. Conversely, the phosphorylation of H1 by CDK2 at specific loci during late G₁/S could disrupt the interaction of HP1 α with H1 and the chromatin fiber, resulting in a relaxation of the chromatin structure required for DNA replication to proceed (Alexandrow and Hamlin, 2005). This suggests that at least part of the reason H1 phosphorylation influences chromatin structure is because it leads to a decreased affinity of HP1 α for chromatin. This model provides a mechanism by which HP1 α could dissociate from heterochromatin during the cell cycle without a concomitant change in the methylation status of H3-K9 and H1-K26. Therefore, H1 phosphorylation could be a primary signal for the disassembly of specific higher-order chromatin structures, namely heterochromatin, during interphase.

Experimental Procedures

Expression Constructs and Recombinant Proteins

As described previously (Contreras et al., 2003), pET3d constructs bacterially express the C-terminal FLAG epitope-tagged fusion proteins of human H1b. The human pGEX2T-HP1 α , β , and γ constructs have been described (Bannister et al., 2001). The domain mutants of GST-HP1 α and H1b/FLAG, as well as the phosphorylation mutants of H1b/FLAG, were created by standard methods.

Purification of the H1b/FLAG fusion proteins has been described (Contreras et al., 2003), whereas GST and GST/HP1 fusion proteins were expressed in *Escherichia coli* BL21 (Stratagene) and cleared cell lysates prepared by sonication in coupling buffer (CB: 1 $\times$ PBS, 100 mM EDTA [pH 8], and 1 $\times$ complete protease inhibitor cocktail [Roche]) with 1.5% Triton X-100.

The eukaryotic expression vectors pECFP-HP1 α and pEYFP-H1b express the fusion proteins CFP-HP1 α and YFP-H1b under the control of the human cytomegalovirus promoter. The eukaryotic expression vectors pcDNA3.1/neoH1b, pcDNA3.1/neoH1b, M1-5, and pRc/cycE (Hinds et al., 1992) were used in the cell cycle analysis.

In Vitro Binding Assay

GST fusion proteins were purified from the cleared lysates by coupling to glutathione Sepharose 4B beads (Amersham Bioscience) in CB according to the manufacturer's instructions, followed by three washes and resuspension in binding buffer (BB: 25 mM Tris-HCl [pH 8], 287 mM NaCl, 1 mM EDTA [pH 8], 10% glycerol, 0.22% NP-40, and 1 $\times$ complete protease inhibitor cocktail [Roche]).

H1b/FLAG proteins were phosphorylated by kinase complexes coimmunoprecipitated from Sf9 cell lysates containing baculovirus-expressed CDK2/cyclin E or CDK2 as described (Herrera et al., 1996). The kinase reaction or purified H1b/FLAG fusion proteins was then added to glutathione Sepharose 4B-coupled GST-HP1 fusion proteins. Incubation was carried out overnight at 4°C with gentle agitation and reactions washed exhaustively. The bound proteins were eluted, separated on a 12% or 15% SDS-PAGE, and visualized by immunoblotting with anti-FLAG M2 antibody (Sigma).

Indirect Immunofluorescence Analyses

Asynchronously growing mouse NIH 3T3 fibroblasts were fixed (2% paraformaldehyde/PBS) and permeabilized (0.2% Triton X-100/PBS) before incubation with antibodies against phosphorylated histone H1 (Upstate) and HP1 α (Chemicon). Cells were then incubated with the appropriate secondary antibody (Molecular Probes) before deconvolution microscopy with a Zeiss AxioVert S100 TV microscope and the DeltaVision Restoration Microscopy System (Applied Precision, Inc.). For deconvolved images, captured raw images were deconvolved with the DeltaVision constrained iterative algorithm. All images were digitally processed for presentation with Adobe Photoshop.

FRET

Mouse NIH 3T3 fibroblasts were cotransfected for 48 hr with constructs expressing N-terminal CFP-tagged HP1 α and C-terminal YFP-tagged H1b. Cells were fixed in 4% paraformaldehyde and analyzed for FRET with the Zeiss LSM 510 confocal microscope. FRET was demonstrated by acceptor bleaching experiments as described by Karpova et al. (2003) where the donor fluorophore (CFP) increases upon acceptor fluorophore (YFP) photobleaching. For each region analyzed for FRET, four images were captured before photobleaching of YFP, followed by the capture of four images after photobleaching. A total of 14 different regions were photobleached from four different cells. As a negative internal control, 12 unbleached regions from four different cells were analyzed for changes in CFP intensity. FRET efficiency was calculated as previously described (Karpova et al., 2003).

Cell Cycle Analysis

Human osteosarcoma Saos-2 cells were processed for flow cytometry on a Becton-Dickinson FACScan machine 48 hr posttransfection, as described (Zhu et al., 1993). The DNA content of FITC-CD20-positive cells was determined by the intensity of propidium iodide staining. Because the cell cycle distributions of the control samples varied between experiments, but the distributions within each experiment were consistent, the data presented are representative of multiple experiments.

Acknowledgments

This work was supported by a Research Project Grant from the American Cancer Society (R.E.H.) and the New Zealand Child Cancer Foundation (T.K.H.). A.J.M. is currently supported by the Odyssey Program and the Theodore N. Law Award for Scientific Achievement at The University of Texas M.D. Anderson Cancer Center. We thank Rachel Purcell and Eric Wooten for critically reading the manuscript.

Received: October 17, 2005

Revised: March 8, 2006

Accepted: April 14, 2006

Published: June 8, 2006

References

- Alexandrow, M.G., and Hamlin, J.L. (2005). Chromatin decondensation in S-phase involves recruitment of Cdk2 by Cdc45 and histone H1 phosphorylation. *J. Cell Biol.* 168, 875–886.
- Bannister, A.J., Zegerman, P., Partridge, J.F., Miska, E.A., Thomas, J.O., Allshire, R.C., and Kouzarides, T. (2001). Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* 410, 120–124.
- Carruthers, L.M., Bednar, J., Woodcock, C.L., and Hansen, J.C. (1998). Linker histones stabilize the intrinsic salt-dependent folding of nucleosomal arrays: mechanistic ramifications for higher-order chromatin folding. *Biochemistry* 37, 14776–14787.
- Contreras, A., Hale, T.K., Stenoien, D.L., Rosen, J.M., Mancini, M.A., and Herrera, R.E. (2003). The dynamic mobility of histone H1 is regulated by cyclin/CDK phosphorylation. *Mol. Cell. Biol.* 23, 8626–8636.
- Cowell, I.G., Aucott, R., Mahadevaiah, S.K., Burgoyne, P.S., Huskisson, N., Bongiorno, S., Prantera, G., Fanti, L., Pimpinelli, S., Wu, R., et al. (2002). Heterochromatin, HP1 and methylation at lysine 9 of histone H3 in animals. *Chromosoma* 111, 22–36.
- Daujat, S., Zeissler, U., Waldmann, T., Happel, N., and Schneider, R. (2005). HP1 binds specifically to K26 methylated histone H1.4, whereas simultaneous S27 phosphorylation blocks HP1 binding. *J. Biol. Chem.* 280, 38090–38095.
- Dion, M.F., Altschuler, S.J., Wu, L.F., and Rando, O.J. (2005). Genomic characterization reveals a simple histone H4 acetylation code. *Proc. Natl. Acad. Sci. USA* 102, 5501–5506.
- Eissenberg, J.C., and Elgin, S.C. (2000). The HP1 protein family: getting a grip on chromatin. *Curr. Opin. Genet. Dev.* 10, 204–210.

- Fan, Y., Nikitina, T., Morin-Kensicki, E.M., Zhao, J., Magnuson, T.R., Woodcock, C.L., and Skoultschi, A.I. (2003). H1 linker histones are essential for mouse development and affect nucleosome spacing in vivo. *Mol. Cell. Biol.* 23, 4559–4572.
- Garcia, B.A., Busby, S.A., Barber, C.M., Shabanowitz, J., Allis, C.D., and Hunt, D.F. (2004). Characterization of phosphorylation sites on histone H1 isoforms by tandem mass spectrometry. *J. Proteome Res.* 3, 1219–1227.
- Hansen, J.C. (2002). Conformational dynamics of the chromatin fiber in solution: determinants, mechanisms, and functions. *Annu. Rev. Biophys. Biomol. Struct.* 31, 361–392.
- Henikoff, S. (2005). Histone modifications: combinatorial complexity or cumulative simplicity? *Proc. Natl. Acad. Sci. USA* 102, 5308–5309.
- Herrera, R.E., Chen, F., and Weinberg, R.A. (1996). Increased histone H1 phosphorylation and relaxed chromatin structure in Rb-deficient fibroblasts. *Proc. Natl. Acad. Sci. USA* 93, 11510–11515.
- Hinds, P.W., Mitnacht, S., Dulic, V., Arnold, A., Reed, S.I., and Weinberg, R.A. (1992). Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell* 70, 993–1006.
- Jenuwein, T., and Allis, C.D. (2001). Translating the histone code. *Science* 293, 1074–1080.
- Karpova, T.S., Baumann, C.T., He, L., Wu, X., Grammer, A., Lipsky, P., Hager, G.L., and McNally, J.G. (2003). Fluorescence resonance energy transfer from cyan to yellow fluorescent protein detected by acceptor photobleaching using confocal microscopy and a single laser. *J. Microsc.* 209, 56–70.
- Liu, C.L., Kaplan, T., Kim, M., Buratowski, S., Schreiber, S.L., Friedman, N., and Rando, O.J. (2005). Single-nucleosome mapping of histone modifications in *S. cerevisiae*. *PLoS Biol.* 3, e328. 10.1371/journal.pbio.0030328.
- Maison, C., and Almouzni, G. (2004). HP1 and the dynamics of heterochromatin maintenance. *Nat. Rev. Mol. Cell Biol.* 5, 296–304.
- Maison, C., Bailly, D., Peters, A.H., Quivy, J.P., Roche, D., Taddei, A., Lachner, M., Jenuwein, T., and Almouzni, G. (2002). Higher-order structure in pericentric heterochromatin involves a distinct pattern of histone modification and an RNA component. *Nat. Genet.* 30, 329–334.
- Meehan, R.R., Kao, C.F., and Pennings, S. (2003). HP1 binding to native chromatin in vitro is determined by the hinge region and not by the chromodomain. *EMBO J.* 22, 3164–3174.
- Minc, E., Allory, Y., Worman, H.J., Courvalin, J.C., and Buendia, B. (1999). Localization and phosphorylation of HP1 proteins during the cell cycle in mammalian cells. *Chromosoma* 108, 220–234.
- Muchardt, C., Guilleme, M., Seeler, J.S., Trouche, D., Dejean, A., and Yaniv, M. (2002). Coordinated methyl and RNA binding is required for heterochromatin localization of mammalian HP1 α . *EMBO Rep.* 3, 975–981.
- Nielsen, A.L., Oulad-Abdelghani, M., Ortiz, J.A., Remboutsika, E., Chambon, P., and Losson, R. (2001). Heterochromatin formation in mammalian cells: interaction between histones and HP1 proteins. *Mol. Cell* 7, 729–739.
- Ohtsubo, M., Theodoras, A.M., Schumacher, J., Roberts, J.M., and Pagano, M. (1995). Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. *Mol. Cell. Biol.* 15, 2612–2624.
- Platero, J.S., Hartnett, T., and Eissenberg, J.C. (1995). Functional analysis of the chromo domain of HP1. *EMBO J.* 14, 3977–3986.
- Powers, J.A., and Eissenberg, J.C. (1993). Overlapping domains of the heterochromatin-associated protein HP1 mediate nuclear localization and heterochromatin binding. *J. Cell Biol.* 120, 291–299.
- Roth, S.Y., and Allis, C.D. (1992). Chromatin condensation: does histone H1 dephosphorylation play a role? *Trends Biochem. Sci.* 17, 93–98.
- Stewart, M.D., Li, J., and Wong, J. (2005). Relationship between histone H3 lysine 9 methylation, transcription repression, and heterochromatin protein 1 recruitment. *Mol. Cell. Biol.* 25, 2525–2538.
- Talasz, H., Helliger, W., Puschendorf, B., and Lindner, H. (1996). In vivo phosphorylation of histone H1 variants during the cell cycle. *Biochemistry* 35, 1761–1767.

Th'ng, J.P., Sung, R., Ye, M., and Hendzel, M.J. (2005). H1 family histones in the nucleus. Control of binding and localization by the C-terminal domain. *J. Biol. Chem.* **280**, 27809–27814.

Truong, K., and Ikura, M. (2001). The use of FRET imaging microscopy to detect protein-protein interactions and protein conformational changes in vivo. *Curr. Opin. Struct. Biol.* **11**, 573–578.

van Holde, K. (1989). *Chromatin* (Heidelberg, Germany: Springer-Verlag).

Vaquero, A., Scher, M., Lee, D., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2004). Human SirT1 interacts with histone H1 and promotes formation of facultative heterochromatin. *Mol. Cell* **16**, 93–105.

Zhu, L., van den Heuvel, S., Helin, K., Fattaey, A., Ewen, M., Livingston, D., Dyson, N., and Harlow, E. (1993). Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. *Genes Dev.* **7**, 1111–1125.