

2009

Regulation of the Heterochromatin Protein 1 by Phosphorylation of Histone H3 and the HP1 Hinge Domain

Holger Dormann

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**REGULATION OF HETEROCHROMATIN PROTEIN 1
BY PHOSPHORYLATION
OF HISTONE H3
AND THE HP1 HINGE DOMAIN**

A Thesis Presented to the Faculty of
The Rockefeller University
in Partial Fulfillment of the Requirements for
the degree of Doctor of Philosophy

by
Holger Dormann
June 2009

REGULATION OF HETEROCHROMATIN PROTEIN 1 BY PHOSPHORYLATION OF HISTONE H3 AND THE HP1 HINGE DOMAIN

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The Rockefeller University 2009

Chromatin, a polymer formed from DNA, histones, and associated proteins, is the physiological form of genetic information in all eukaryotic cells. Posttranslational modification of histones, such as acetylation, methylation, and phosphorylation, regulates various DNA-dependent processes, ranging from transcription to replication, DNA repair, and apoptosis. A key mechanism by which histone modifications exert these effects is by recruitment of specific binding partners (effector proteins), that in turn direct downstream functions. Insight into the underlying mechanisms are of great importance for a full understanding of chromatin structure and function.

One of these effector proteins, Heterochromatin Protein 1 (HP1), plays important roles in heterochromatin formation. It is recruited to chromatin by interaction with methylated lysine 9 of histone H3 (H3K9me). However, it has remained enigmatic how HP1 reversibly dissociates from chromatin during mitosis, while the histone mark that recruits the protein, H3K9me, persists.

In the first part of my thesis, my collaborators and I show through a combination of *in vitro* and *in vivo* experiments that this release depends on a novel mechanism, “methyl-phos switching”, in which two nearby histone marks collaborate to accomplish the dynamic regulation of effector protein binding. Phosphorylation of histone H3 at serine 10, immediately adjacent to HP1’s binding site at H3K9me, at the onset of mitosis interferes with HP1 binding to H3K9me, resulting in the release of the effector protein.

In the second part of my thesis, I investigate to what extent posttranslational modification of HP1 itself is involved in the regulation of the effector protein. I identify ten novel phosphorylation sites for the three human HP1 isoforms (α , β , γ), most of which map to the HP1 “hinge region” and are specifically phosphorylated in mitosis. For one highly conserved site, HP1 α serine 92 phosphorylation, I identify Aurora B as the responsible kinase *in vivo*. *In vitro* data suggest that mitotic phosphorylation of the HP1 α hinge may play a role in the regulation of HP1 association with RNA.

My thesis work indicates that HP1’s behavior and interactions in mitosis are regulated by posttranslational modifications on two levels: phosphorylation of histone H3 as well as phosphorylation of HP1 itself.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my mentor, Dr. David Allis, for his support and guidance. His creativity and enthusiasm for science have been a source of inspiration throughout my thesis, with his constructive criticism, he encouraged me to think independently as a scientist, and when I felt discouraged, he cheered me up - I want to thank him for all that.

I thank my faculty advisory committee, Dr. Günter Blobel, Dr. Hironori Funabiki, and Dr. Wolfgang Fischle, for their time, their interest in my work, and all their helpful suggestions. I also thank Dr. Ming-Ming Zhou for agreeing to serve on my thesis committee as my external member.

I am grateful to Dr. Blobel also for the training and advice he gave me during my stay in his lab as an undergraduate and diploma student. This time has been extremely valuable for me, and I want to thank him for giving me the opportunity to work in his lab at a very early stage of my studies.

I would like to thank Dr. Funabiki for serving as the chair of my committee, for the fruitful collaboration on the "methyl-phos switching" project and for many helpful discussions also outside of my committee meetings.

I owe Dr. Fischle thanks for many different things: He not only got me started in the Allis lab and trained me at the bench during the first years of my Ph.D., but he later on also served on my thesis committee, gave me the chance to work in his lab in Göttingen as a guest scientist and finally has made the long way from Germany for my defense. I want to thank him for all this, as well as for his friendship.

I thank our collaborators Hillary Montgomery, Dr. Beatrix Ueberheide, Dr. Jeffrey Shabanowitz and Dr. Donald Hunt for their excellent mass spectrometry work as well as the pleasant interaction during our collaboration.

Boo Shan Tseng was our collaborator on the "methyl-phos switching" project and I thank her for all her work and contributions to this project. I also would like to acknowledge her for generously providing important reagents for some of my experiments.

Dr. Gwen Lomberk and Dr. Raul Urrutia deserve many thanks for generously sharing their antibody against phosphorylated HP1 α with me.

Dr. Alex Ruthenburg has helped me tremendously at various stages on my way to this thesis manuscript. From advice at the bench, comments on the manuscript, rendering of X-ray figures in Pymol ("mystic crystal revelations") to many late-night hours during the lay-out of this thesis, his help has been unwavering. I deeply appreciate this support and his friendship.

I thank Aaron Goldberg, Dr. Monika Lachner, Dr. Tom Milne, and Dr. Greg Wang for critically reading parts of my thesis and for their helpful comments on my thesis talk. Amrita Basu helped me with computational analysis, and Nicolai Siegel with proof-reading and the final production process of this thesis.

I would like to thank Dr. Emily Bernstein for help with RNA experiments, and her and Owen Wu for our ongoing collaboration on the RNA interaction of Heterochromatin Protein 1.

I appreciate the collaboration with Dr. David Shechter and Dr. Sandra Hake for our joint publication on techniques of histone purification and analysis, and with Dr. Greg Wang on leukemogenic PHD finger fusions.

I am very grateful to all past and present members of the Allis lab for their willingness to always share reagents and advice. There have been far too many occasions to list, so I can only acknowledge them collectively for all their help over the years.

I also want to thank them for being excellent colleagues and for keeping up a good lab spirit. Many of them have become good friends, and I thank

them for numerous late-night hours shared at the bench, entertaining lunches, and for teaching me English words that I can't share here, because otherwise this page would have to be censored.

I thank the lab manager of the Allis lab, Jamie Winshell, for running the daily lab business and helping me out at countless little problems and disasters, and the laboratory administrator of the Allis lab, Chandra Egger, for help with many organizational things.

The members of the Fischle lab in Göttingen were very generous in sharing their time, bench space, protocols and reagents with me, and I greatly appreciate their welcoming me during my stay in their lab.

I thank Dr. Elias Coutavas and other members of the Blobel lab for advice, reagents, and the permission to use their cell disruptor.

Upstate Biotechnology has helped me generously with reagents, and I want to thank the colleagues there for the many friendly interactions.

I appreciate the friendly and professional assistance of the staff at the Rockefeller Bioimaging Resource Center, Proteomics Resource Center, Flow Cytometry Resource Center. I also thank the mass spectrometry facility in Göttingen for their excellent work.

I am indebted to the Dean's office of the David Rockefeller Graduate Program for their help at countless occasions.

Financial support was provided by the Boehringer Ingelheim Fonds and the David Rockefeller Graduate Program, and I want to thank them for it.

I am grateful to my friends at Rockefeller and in New York for making my time in New York such a pleasant one.

I thank my family, in particular my parents Elmar and Adelheid Dormann, for their support and constant encouragement.

Lastly, I want to thank my wife Dorothee for her love and support and for being there for me at every step of the way.

TABLE OF CONTENTS

| | |
|--|---------|
| ACKNOWLEDGMENTS | iii |
| TABLE OF CONTENTS | vi |
| LIST OF FIGURES | xii |
| LIST OF TABLES | xvii |
| ABBREVIATIONS | xviii |
| CHAPTER 1: GENERAL INTRODUCTION | page 1 |
| Chromatin | page 2 |
| Nucleosomes and histones..... | page 3 |
| Regulatory role of chromatin..... | page 5 |
| Histone modifications – histone marks..... | page 8 |
| Correlations of histone modifications with biological effects..... | page 10 |
| "Histone Code"..... | page 10 |
| Enzymes that establish histone marks..... | page 12 |
| Molecular mechanisms of histone modifications..... | page 13 |
| Histone modifications and disease..... | page 17 |
| Heterochromatin Protein 1 | page 19 |
| Identification of HP1 and its gene..... | page 19 |
| Conservation of HP1 in different organisms..... | page 21 |
| HP1 domains and molecular interactions..... | page 26 |
| <i>Chromo domain</i> | page 26 |
| <i>Chromoshadow domain</i> | page 31 |
| <i>Hinge region</i> | page 35 |
| Cellular localization and functions of HP1..... | page 38 |
| <i>HP1 in heterochromatin</i> | page 38 |
| <i>HP1 in euchromatin</i> | page 39 |
| Changes in HP1 localization during the cell cycle... | page 42 |

| | |
|--|---------|
| Molecular mechanism of HP1 recruitment..... | page 44 |
| <i>Essential role of CD-H3K9me interaction</i> | page 44 |
| <i>Contribution of other mechanisms</i> | page 44 |
| <i>H3K9me-independent chromatin association of HP1</i> | page 46 |
| Dynamics of HP1 binding..... | page 47 |
| HP1, heterochromatin nucleation and RNAi..... | page 48 |
| HP1 phosphorylation..... | page 49 |
| Open questions..... | page 51 |

CHAPTER 2: MATERIALS AND METHODS..... page 55

| | |
|---|---------|
| Reagents | page 55 |
| Chemicals, media and recombinant proteins..... | page 55 |
| Peptides and peptide modification..... | page 55 |
| Bacterial media..... | page 56 |
| Cell lines and tissue culture media..... | page 56 |
| Antibodies..... | page 56 |
| Molecular Biology | page 58 |
| Plasmids..... | page 58 |
| General DNA manipulation..... | page 59 |
| PCR and mutation of plasmids..... | page 60 |
| Ligation-independent cloning..... | page 61 |
| Cell-based methods | page 61 |
| Mitotic arrest..... | page 61 |
| Transfection of tissue culture cells and selection for stable cell lines..... | page 62 |
| siRNA-mediated gene silencing..... | page 62 |
| Cell synchronization by double thymidine block..... | page 63 |
| Harvesting tissue culture cells..... | page 63 |
| Immunofluorescence staining and confocal microscopy..... | page 64 |
| Cell cycle analysis and flow cytometry..... | page 65 |
| Biochemical methods | page 65 |
| Gel electrophoresis..... | page 65 |
| Transfer of proteins to membrane and western blotting..... | page 66 |
| Peptide competitions..... | page 67 |

| | |
|---|---------|
| Expression of recombinant proteins in <i>E.coli</i> | page 67 |
| Ni-NTA purification of His ₆ -tagged proteins..... | page 67 |
| Column chromatography..... | page 68 |
| GST purification of Aurora B..... | page 69 |
| Acid extraction..... | page 69 |
| Preparation of Dignam extracts..... | page 70 |
| Alternative biochemical fractionation of cells..... | page 71 |
| Peptide pull-down..... | page 72 |
| Anti-FLAG immunoprecipitation of tagged HP1 constructs / Immunoprecipitation of endogenous HP1.. | page 72 |
| RP-HPLC..... | page 73 |
| <i>In vitro</i> kinase reactions and analysis..... | page 74 |
| "Bioinformatics" methods | page 74 |
| Other methods | page 75 |
| Fluorescence anisotropy binding measurements..... | page 75 |
| Mass spectrometry..... | page 75 |
| Phage display screen..... | page 75 |
| Electrophoretic mobility-shift assay (EMSA)..... | page 77 |
| CHAPTER 3: REGULATION OF THE CHROMATIN ASSOCIATION OF HP1 THROUGH PHOSPHORYLATION OF HISTONE H3: "METHYL-PHOS SWITCHING" | page 78 |
| Introduction | page 78 |
| Results | page 80 |
| HP1 is released during mitosis, even though the H3K9me3 mark persists..... | page 80 |
| The Binary Switching Hypothesis..... | page 82 |
| Reduced binding affinity of HP1 to H3K9me3S10ph peptides <i>in vitro</i> | page 85 |
| Reconstitution of the release mechanism in the test tube..... | page 87 |
| Identification of kinases capable of phosphorylating H3S10 in the presence of H3K9 methylation..... | page 88 |
| Reconstitution of the "methyl-phos switching" mechanism in the test tube..... | page 93 |

| | |
|---|----------|
| "Methyl-phos switching" in the test tube with full-length HP1..... | page 97 |
| "Methyl-phos switching" in the test tube with MSK1..... | page 99 |
| Reversibility of "methyl-phos switching" in the test tube..... | page 100 |
| H3S10 phosphorylation coincides with the release of HP1 <i>in vivo</i> | page 103 |
| Inhibition of Aurora B kinase prevents the release of HP1 <i>in vivo</i> | page 104 |
| HP1 binds to metaphase chromosomes lacking H3 serine 10 phosphorylation in <i>Xenopus</i> egg extracts..... | page 106 |
| Discussion | page 107 |
| A novel mechanism how histone modifications control the recruitment of effector proteins..... | page 107 |
| Identity of the phosphatase responsible for the removal of H3S10ph <i>in vivo</i> | page 110 |
| Potential contribution of other mechanisms to the mitotic release of HP1 <i>in vivo</i> | page 110 |
| "Methyl-phos switching" of HP1 at H3 lysine 9/serine 10 outside of mitosis..... | page 113 |
| "Methyl-phos switching" of HP1 at other sites..... | page 115 |
| Binary switching as a general mechanism in the cell..... | page 117 |
| CHAPTER 4: REGULATION OF HP1 THROUGH EFFECTOR PHOSPHORYLATION: PHOSPHORYLATION OF THE HP1 HINGE REGION | page 120 |
| Introduction | page 120 |
| Results | page 125 |
| Mapping of HP1 <i>in vitro</i> phosphorylation by the CPC to a conserved cassette in the hinge region..... | page 125 |
| Specific antibody for HP1 α S92ph..... | page 128 |
| HP1 α S92 phosphorylation is a mitotic mark <i>in vivo</i> | page 129 |
| Detailed characterization of HP1 α S92ph by immunofluorescence microscopy..... | page 133 |
| Presumed centrosomal staining..... | page 133 |
| HP1 α S92ph labels a pool of HP1 α that is still excluded from chromatin at cytokinesis..... | page 136 |
| Aurora B is the kinase responsible for HP1 α S92 phosphorylation <i>in vivo</i> | page 138 |
| Aurora B inhibition with hesperadin..... | page 139 |
| Aurora B knock-down by RNA interference..... | page 141 |

| | |
|---|----------|
| Hypothesis: Does HP1 α serine 92 phosphorylation play a role in regulating HP1 α chromatin association?..... | page 143 |
| S92 phosphorylation correlates with increased extractability of HP1 α | page 146 |
| A pool of HP1 α associated with metaphase chromosomes is not recognized by the Phos-HP1 α antibody in IF experiments..... | page 146 |
| Stable cell lines expressing HP1 α with serine 92 point mutations..... | page 148 |
| No obvious effect of serine 92 mutation on HP1 α localization..... | page 150 |
| Hypothesis: HP1 α serine 92 phosphorylation regulates the HP1 α 's association with a specific binding partner... | page 151 |
| <i>Peptide pull-downs to identify protein interactions controlled by HP1α serine 92 phosphorylation.....</i> | page 152 |
| <i>Phage display screen for interacting proteins regulated by HP1α serine 92 phosphorylation.....</i> | page 155 |
| Redundancy / Requirement for cooperation..... | page 157 |
| Purification of HP1..... | page 159 |
| <i>Acid extraction and HPLC enrichment of endogenous HP1 followed by analysis from SDS-PAGE gel.....</i> | page 160 |
| <i>Cell fractionation and immunoprecipitation of endogenous HP1 followed by analysis on the beads.....</i> | page 163 |
| <i>Cell fractionation, immunoprecipitation and on-beads analysis of exogenous tagged HP1.....</i> | page 164 |
| Most phosphorylations cluster within the hinge region of HP1..... | page 169 |
| Conservation of phosphorylation sites..... | page 171 |
| Higher levels of phosphorylation in mitosis..... | page 172 |
| Hypothesis: Multiple HP1 α hinge phosphorylations collaborate to control the mitotic release of HP1 α from chromatin..... | page 174 |
| Analysis of HP1 α aStA localization in cells devoid of endogenous HP1 α , HP1 β and HP1 γ | page 176 |
| Hypothesis: HP1 α hinge phosphorylation regulates reassociation of HP1 α with chromatin in metaphase..... | page 180 |
| Cell cycle FACS..... | page 183 |
| Hypothesis: HP1 α hinge phosphorylation regulates molecular interactions of the hinge region..... | page 185 |
| Experiments to test if HP1 α hinge phosphorylation controls HP1 α 's interaction with histone H1..... | page 186 |

| | |
|---|----------|
| Experiments to test if HP1 α hinge phosphorylation controls HP1 α 's interaction with RNA..... | page 187 |
| Discussion | page 191 |
| Novel phosphorylations of HP1..... | page 191 |
| Intriguing new experimental directions..... | page 194 |
| Hinge phosphorylation may regulate RNA binding to HP1 α | page 195 |
| CHAPTER 5: GENERAL DISCUSSION | page 196 |
| "Methyl-phos switching" | page 196 |
| Release of HP1 from chromatin by phosphorylation of histone H3: "Methyl-phos switching"..... | page 196 |
| Why does the cell use a "methyl-phos switching" mechanism for the mitotic release of HP1?..... | page 199 |
| Potential contribution of other mechanisms to the mitotic release of HP1 <i>in vivo</i> | page 201 |
| Why does HP1 have to be removed from chromatin during mitosis?..... | page 204 |
| <i>A challenging question to address experimentally....</i> | page 204 |
| <i>Hypotheses on the biological function of HP1 release in mitosis</i> | page 207 |
| HP1 phosphorylation | page 211 |
| HP1 isoforms are subject to phosphorylation..... | page 211 |
| No effect of HP1 phosphorylation on localization dynamics in mitosis..... | page 213 |
| Possible existence of other HP1 binders regulated by HP1 hinge phosphorylation..... | page 214 |
| Reasons for exclusion of HP1 α S92ph signal from chromatin at telophase..... | page 215 |
| HP1 and RNA binding / Nature of the bound RNA(s)..... | page 216 |
| Next experimental steps..... | page 220 |
| Hypotheses on the biological function of the HP1-RNA interaction and of HP1 α hinge phosphorylation..... | page 223 |
| Conclusion | page 231 |
| APPENDIX | page 233 |
| REFERENCES | page 237 |

LIST OF FIGURES

| | | |
|------------------------|---|--------|
| Figure 1.1: | Chromatin and the organization of DNA within the cell. | 2 |
| Figure 1.2: | Composition of the nucleosome. | 4 |
| Figure 1.3: | Posttranslational modifications of histone molecules..... | 9 |
| Figure 1.4: | Molecular mechanisms of histone modifications. | 14 |
| Figure 1.5: | Position effect variegation in <i>Drosophila melanogaster</i> | 20 |
| Figure 1.6: | Domain structure of HP1..... | 22 |
| Figure 1.7: | Alignment of HP1 proteins of different organisms.. | 24 |
| Figure 1.8: | The chromo domain of HP1 binds methylated lysine 9 of histone H3..... | 27 |
| Figure 1.9: | Models for HP1-mediated chromatin condensation and the spreading of heterochromatic domains..... | 34 |
| Figure 1.10: | Cellular localization of HP1 in mammalian cells..... | 43 |
| Figure 3.1: | HP1 dissociates from chromatin during mitosis, even though the H3K9me3 mark persists.. | 81 |
| Figure 3.2: | The Binary Switching Hypothesis. | 83 |
| Figure 3.3: | Binding of the HP1 chromo domain to H3K9me3 is likely to be impaired by the presence of H3S10ph..... | 84 |
| Figure 3.4: | The binding affinity of the HP1 β chromo domain to methylated H3 peptides is significantly reduced by the presence of serine 10 phosphorylation. | 86 |
| Figure 3.5: | Phosphorylation activity of different H3 serine 10 kinases on methylated H3 peptides (<i>In vitro</i> kinase assays)..... | 89 |
| Figure 3.6: | <i>In vitro</i> phosphorylation of methylated H3 peptides. | 90 |

| | | |
|---------------------|--|-----|
| Figure 3.7: | <i>In vitro</i> reconstitution of release mechanism by methyl-phos switching": The HP1 chromo domain is released from H3K9me3 upon serine 10 phosphorylation by the CPC <i>in vitro</i> | 94 |
| Figure 3.8: | Additional specificity controls for <i>in vitro</i> reconstitution of release mechanism by "methyl-phos switching" (Controls for HP1 β CD shown). | 96 |
| Figure 3.9: | Isoform-specific phosphorylation of HP1 by the CPC <i>in vitro</i> | 98 |
| Figure 3.10: | Full-length HP1 β is released from H3K9me3 upon serine 10 phosphorylation by the CPC <i>in vitro</i> | 99 |
| Figure 3.11: | Dissociation of the HP1 β CD from H3K9me3 upon serine 10 phosphorylation by MSK1 <i>in vitro</i> | 101 |
| Figure 3.12: | Reassociation of the HP1 β CD upon phosphatase treatment of H3K9me3S10ph peptides. | 102 |
| Figure 3.13: | The mitotic release of HP1 coincides with the appearance of H3K9me3S10ph <i>in vivo</i> | 103 |
| Figure 3.14: | Retention of HP1 on chromatin upon inhibition of mitotic serine 10 phosphorylation <i>in vivo</i> | 105 |
| Figure 3.15: | "Methyl-phos switching" controls HP1 dissociation during mitosis. | 109 |
| Figure 3.16: | Examples of HP1 "methyl-phos switching" at sites other than H3K9me3S10ph. | 116 |
| Figure 3.17: | Regulation of the chromatin association of the chromatin remodeller CHD by a "methyl-phos" and a "methyl-methyl" switch. | 118 |
| Figure 3.18: | High density and clustering of posttranslational modifications in the histone H3 tail. | 119 |
| Figure 4.1: | <i>In vitro</i> phosphorylation of HP1 by the Chromosomal Passenger Complex. | 123 |
| Figure 4.2: | Indications for <i>in vivo</i> phosphorylation of HP1 in the literature | 124 |
| Figure 4.3: | Mapping of the site of HP1 α and HP1 γ <i>in vitro</i> phosphorylation by the Chromosomal Passenger Complex. | 126 |
| Figure 4.4: | Sequence alignment of the HP1 hinge region. | 127 |

| | | |
|---------------------|---|-----|
| Figure 4.5: | Characterization of the HP1 α S92ph antibody in immunoblotting..... | 130 |
| Figure 4.6: | HP1 α S92 phosphorylation is a mitotic mark. | 131 |
| Figure 4.7: | Detailed characterization of the HP1 α S92ph signal by immunofluorescence microscopy. | 134 |
| Figure 4.8: | Effect of HP1 α RNAi on centrosomal staining of the Phos-HP1 α antibody..... | 135 |
| Figure 4.9: | Differences between HP1 α S92ph and general HP1 α stainings in telophase. | 137 |
| Figure 4.10: | Aurora B inhibition with the small molecule inhibitor hesperadin decreases HP1 α S92 phosphorylation. | 140 |
| Figure 4.11: | Aurora B RNAi decreases HP1 α S92 phosphorylation. | 142 |
| Figure 4.12: | Hypothesis: HP1 α serine 92 phosphorylation is a necessary step for the dissociation of HP1 α from chromatin <i>in vivo</i> | 145 |
| Figure 4.13: | Biochemical and IF experiments suggest that HP1 α serine 92 phosphorylation may indeed regulate HP1 α chromatin association. | 147 |
| Figure 4.14: | Characterization of HeLa cell lines stably expressing FLAG-HA-tagged HP1 α | 149 |
| Figure 4.15: | No obvious effect of HP1 α serine 92 mutations on HP1 α localization..... | 150 |
| Figure 4.16: | Peptide pull-down to identify phosphorylation-dependent binders of HP1 α | 154 |
| Figure 4.17: | Principle of phage display screening. | 156 |
| Figure 4.18: | Phage display screen to identify phosphorylation-dependent binders of HP1 α | 157 |
| Figure 4.19: | Purification of HP1 for analysis of modification profile by mass spectrometry..... | 161 |
| Figure 4.20: | Tandem mass spectrum of a singly phosphorylated HP1 α peptide (S92ph). | 165 |
| Figure 4.21: | Tandem mass spectrum of a doubly phosphorylated HP1 α peptide (S85phS92ph). | 166 |
| Figure 4.22: | Tandem mass spectrum of an HP1 γ peptide defining the HP1 γ N-terminus..... | 167 |

| | | |
|---------------------|---|-----|
| Figure 4.23: | Cartoon depiction of HP1 phosphorylation sites identified by mass spectrometry. | 170 |
| Figure 4.24: | Alignment of the hinge regions of HP1 proteins from different species indicating the position of the newly identified phosphorylation sites. | 172 |
| Figure 4.25: | Localization of HP1 α hinge mutations after knock-down of endogenous HP1 isoforms by RNAi. | 177 |
| Figure 4.26: | Hypothesis: HP1 α hinge phosphorylation regulates reassociation of HP1 α with chromosomes in metaphase. | 181 |
| Figure 4.27: | Mutation of phosphorylation sites in the HP1 α hinge has no obvious effect on HP1 α reassociation with metaphase chromosomes..... | 182 |
| Figure 4.28: | Effect of HP1 α hinge mutations on cell cycle progression. | 184 |
| Figure 4.29: | Hypothesis: HP1 α hinge phosphorylation controls a molecular interaction that has no connection to HP1 chromatin association. | 186 |
| Figure 4.30: | HP1 α hinge phosphorylation may regulate the interaction of HP1 α with RNA. | 189 |
| Figure 5.1: | Regulation of HP1 α by phosphorylation on two different levels..... | 197 |
| Figure 5.2: | HP1 dissociation might be required for full mitotic chromatin condensation (Possible biological function of mitotic HP1 dissociation from chromatin). | 207 |
| Figure 5.3: | Dissociation of HP1 may be required for the release of condensin from chromosome arms (Possible biological function of mitotic HP1 dissociation from chromatin). | 209 |
| Figure 5.4: | HP1 release may be necessary for proper resolution/segregation of chromatids (Possible biological function of mitotic HP1 dissociation from chromatin). | 211 |
| Figure 5.5: | RNA as "molecular glue" to stabilize and compact HP1-dependent heterochromatic structures (Hypothetical model for the biological role of the HP1 α -RNA interaction). | 225 |

| | | |
|--------------------|---|-----|
| Figure 5.6: | RNA binding of HP1 recruits HP1 to euchromatic loci (Hypothetical model for the biological role of the HP1 α -RNA interaction)..... | 227 |
| Figure 5.7: | HP1 tethers nascent heterochromatic RNA transcripts to the heterochromatic locus (Hypothetical model for the biological role of the HP1 α -RNA interaction). | 230 |

LIST OF TABLES

| | | |
|-------------------|--|-----|
| Table 1.1: | Molecular characteristics of euchromatin and heterochromatin | 8 |
| Table 1.2: | Examples of histone marks, functions they correlate with and the responsible enzymes | 11 |
| Table 1.3: | Examples of binding modules and effector proteins for histone marks | 16 |
| Table 1.4: | Examples of HP1 interactions | 30 |
| Table 2.1: | Primary antibodies..... | 57 |
| Table 2.2: | Secondary antibodies..... | 58 |
| Table 2.3: | Bacterial strains | 60 |
| Table 2.4: | siRNA oligos..... | 63 |
| Table 3.1: | Dissociation constants (K_d) for the binding of human HP1 chromo domains to different H3 peptides..... | 87 |
| Table 4.1: | Known interaction partners of HP1 found in the HP1 α IP | 168 |
| Table 4.2: | Proteins found in HP1 α IP that are components of complexes known to interact with HP1 | 168 |
| Table 4.3: | Potential novel interaction partners of HP1 α | 170 |
| Table 4.4: | Relative phosphorylation levels obtained by MS | 173 |

LIST OF ABBREVIATIONS

| | |
|--------|--|
| aa | amino acid |
| Ab | antibody |
| ADP | adenosinediphosphate |
| aStA | "all serines to alanine", i.e. an HP1 α construct with all serines in the hinge region mutated to alanine |
| aStE | "all serines to glutamate", i.e. an HP1 α construct with all serines found phosphorylated in the hinge region |
| ATP | adenosinetriphosphate |
| bp | base pairs |
| BSA | bovine serum albumin |
| C | celsius |
| CD | chromo domain |
| cDNA | complementary DNA |
| ChIP | chromatin immunoprecipitation |
| chromo | chromatin organization modifier |
| CPC | chromosomal passenger complex |
| cpm | count per minute |
| CSD | chromoshadow domain |
| 2D | two dimensional |
| DAPI | 4',6-diamidino-2-phenylindole |
| dCTP | deoxy cytidine triphosphate |
| dGTP | deoxy guanosine triphosphate |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DNA | deoxyribonucleic acid |
| DNase | deoxyribonuclease |
| dNTP | deoxy nucleotide triphosphate |
| dsDNA | double-stranded DNA |
| dsRNA | double-stranded RNA |
| DTT | DL-1,4-dithiothreitol |
| EDTA | ethylenediaminetetraacetic acid |
| EMSA | electrophoretic mobility-shift assay |
| EST | expressed sequence tag |

| | |
|--------------------|---|
| f.c. | final concentration |
| FL | full-length |
| FPLC | fast protein liquid chromatography |
| FRET | fluorescence resonance energy transfer |
| g | grams |
| gen. | general |
| GFP | green fluorescent protein |
| GST | glutathione-S-transferase |
| GTP | guanosine triphosphate |
| h | hour |
| H3 | histone H3 |
| H3S10ph | H3 phosphorylated at serine 10 |
| H3K9me | H3 methylated at lysine 9 |
| H3K9me3 | H3 tri-methylated at lysine 9 |
| H3K9meS10A | H3 methylated at lysine 9 and and serine 10 mutated to alanine |
| HA | hemagglutinin |
| HAT | histone acetyltransferase |
| HDAC | histone deacetylase |
| HeLa | Henrietta Lacks |
| HEPES | 2-[4-(2-hydroxyethyl)-1-piperazinyl]- ethanesulfonic acid |
| His | histidine |
| HMTase | histone methyltransferase |
| HP1 | Heterochromatin Protein 1 |
| HP1 α (N) | FLAG-HA-FLAG tagged HP1 α (wild-type sequence), tag on N-terminus |
| HP1 α S92ph | phosphorylation of HP1 α at serine 92 |
| HPLC | high performance liquid chromatography |
| HRP | horse-radish peroxidase |
| IEF | isoelectric focusing |
| IF | immunofluorescence |
| Ig | immunoglobuline |
| IMAC | immobilized metal affinity chromatography |
| INCENP | inner centromere protein |

| | |
|-------------------|--|
| IP | immunoprecipitation |
| IPTG | isopropylthio-b-D-galactoside |
| K9 | lysine 9 |
| kb | kilo bases |
| Kd | dissociation constant |
| kDa | kilo dalton |
| l | liter |
| LB | Luria-Bertani (broth) |
| m | meter |
| M | molar |
| MAB | monoclonal antibody |
| MALDI-TOF | matrix-assisted laser desorption ionisation time-of-flight |
| MDa | mega dalton (10^6 dalton) |
| min | minute |
| mRNA | messenger RNA |
| MS | mass spectrometry |
| MSK1 | Mitogen- and stress-activated kinase 1 |
| MW | molecular weight |
| NGS | normal goat serum |
| Ni-NTA | nickel nitrilo-triacetic acid |
| nt | nucleotide |
| OD600 | optical density at 600 nm |
| P | phosphorylation |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PEV | position effect variegation |
| ph | phosphorylation |
| Phos-HP1 α | antibody recognizing phosphorylated serine 92 of HP1 α |
| pI | isoelectric point |
| PMSF | phenylmethanesulfonyl fluoride |
| PP1 | protein phosphatase 1 |
| rb | rabbit |
| PV | pellet volume |
| RC | reverse complement |

| | |
|----------|--|
| rec. | recombinant |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| RNAi | RNA interference |
| RP-HPLC | reversed-phase high-performance liquid chromatography |
| PCR | polymerase chain reaction |
| PVDF | polyvinylidene fluoride |
| RNAi | RNA interference |
| rpm | rotations per minute |
| RT | room temperature |
| RT-PCR | reverse transcriptase PCR |
| s | second |
| S10 | serine 10 |
| s. d. | standard deviation |
| SDS | sodium dodecyl sulfate |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| siRNA | short interfering RNA |
| ssDNA | single-stranded DNA |
| S to A | serine to alanine mutation |
| Su(var) | suppressor of variegation |
| T | thymine |
| TAE | tris-acetate-EDTA |
| Taq | thermus aquaticus |
| TBS | tris-buffered saline |
| TCA | trichloro acetic acid |
| TEA | tri-ethanol amine |
| TPR | translocated promoter region |
| Tris | tris(hydroxymethyl)aminomethane |
| tRNA | transfer RNA |
| U | unit |
| UTR | untranslated region |
| UV | ultra violet |
| wt | wild-type, original sequence without mutation |

CHAPTER 1

GENERAL INTRODUCTION

Posttranslational modifications of histone proteins control chromatin structure and function, thereby playing a regulatory role in many fundamental cellular processes. An important mechanism how histone modifications accomplish their biological function is by recruiting specific binding proteins (effectors) to chromatin, that then in turn induce downstream functions.

Insight into the mechanisms for how these effectors are recruited and how their downstream functions are controlled lies at the heart of a deeper understanding of chromatin function. In my Ph.D. project, I studied how an important chromatin effector protein, Heterochromatin Protein 1 (HP1) is regulated by posttranslational modifications, both of the histone and of the effector protein itself.

In this introductory chapter, I will review relevant literature to provide a framework for describing my research course. The first part of the chapter will give a general overview of what is known about chromatin, its molecular components and the mechanisms that guide its functions. In the second part, I summarize the current knowledge about Heterochromatin Protein 1, highlight important findings already reported, point out open questions, and put the research of my Ph.D. into context.

Chromatin

The genetic information of eukaryotic cells is stored in the form of DNA in the cell nucleus (Avery et al., 1944). In order to fit 2 meters worth of DNA (in human cells) into a cell nucleus only a few micrometers in diameter, the DNA ‘fibers’ must be significantly condensed. This enormous compaction is achieved by association of the DNA with a set of nuclear proteins, resulting in the formation of *chromatin* (Figure 1.1), which constitutes the physiological form of all genetic and inheritable information (Felsenfeld and Groudine, 2003).

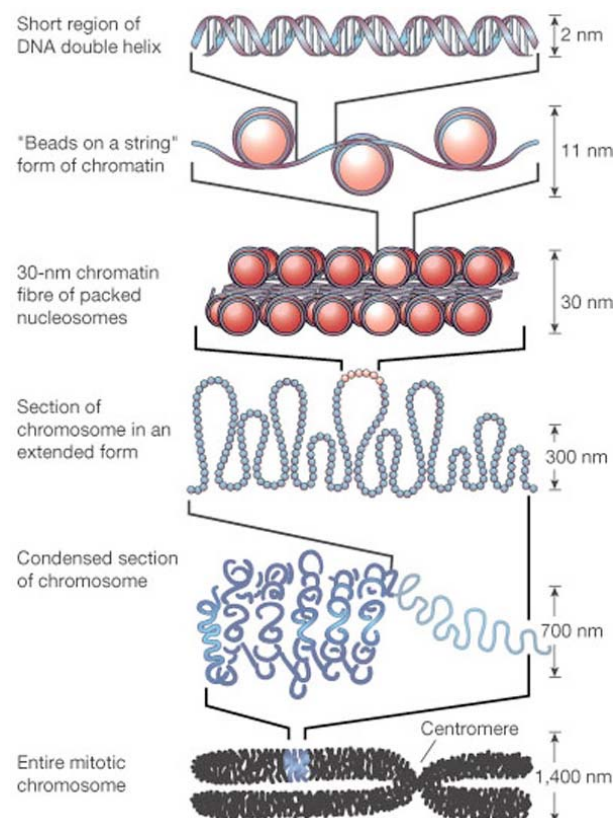


Figure 1.1: Chromatin and the organization of DNA within the cell.

At regular intervals, the double helix of DNA is wrapped around complexes of histone molecules, forming nucleosomes that are arranged like beads on a string. By mechanisms that are currently not well understood, the nucleosome units are then folded into a fiber 30 nm in diameter and further into higher-order structures. The overall compaction of DNA by this organization is more than 10,000-fold (adapted from Felsenfeld and Groudine, 2003).

Early cytological studies established the existence of two different forms of chromatin (Heitz, 1928): *euchromatin* has an open, accessible conformation, replicates early in S-phase, and contains the majority of active genes; *heterochromatin*, in contrast, is tightly compacted, replicates late in the cell cycle, and contains very few active genes. Some regions of the genome, such as centromeres, pericentric regions and telomeres, are heterochromatic in all types of cells and at all times, and have therefore been called *constitutive heterochromatin*. Other heterochromatic regions of the genome can change their status during development or differentiation and are therefore referred to as *facultative heterochromatin*.

Nucleosomes and histones

The fundamental repeating unit of chromatin is the *nucleosome* (Kornberg, 1974; Olins and Olins, 1974), which is formed by wrapping a stretch of 147 bp of DNA around a protein core of eight *histone* proteins – two copies each of the histones H2A, H2B, H3 and H4 (Figure 1.2) (Luger et al., 1997). An additional histone, linker histone H1 (and, in some organisms, H5), interacts with the nucleosomal core as well as with the adjoining linker DNA, forming higher levels of chromatin organization and architecture (Thomas, 1999).

Histones are a family of small basic proteins with remarkable conservation among distantly related species. Structurally, they consist of two parts (Arents et al., 1991): The C-terminal domain is mainly α -helical; by forming histone-histone and histone-DNA interactions these histone-fold domains are critical for the formation of the nucleosome core. The N-terminal tails of histones, on the other hand, are far less structured. Because of their accessibility to proteases, histone tails are thought to

protrude outwards from the core nucleosome (Luger and Richmond, 1998). This exposed localization makes them available for contacts with adjacent nucleosomes and with other chromatin-associated proteins (Ausio et al., 1989; Hansen et al., 1998; Shogren-Knaak et al., 2006; Whitlock and Simpson, 1977).

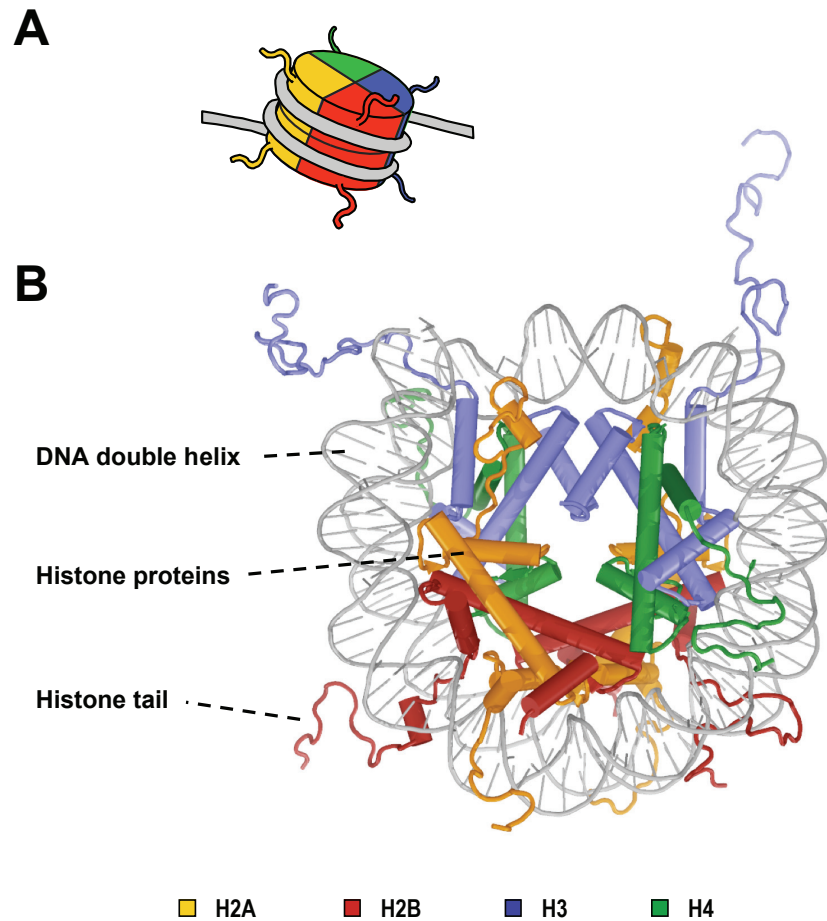


Figure 1.2: Composition of the nucleosome.

(A) Cartoon depiction and **(B)** crystal structure of the nucleosome (solved with *Xenopus laevis* histones (Davey et al., 2002); view down the DNA superhelical axis shown). The nucleosome core is formed by an octamer of histone proteins, two copies each of the four core histones H2A, H2B, H3 and H4. Around this protein core of mostly α -helical structure (shown as cylinders), the DNA is wound in roughly two superhelical turns (147 bp). Binding of the linker histone H1 to this assembly (not shown) confers additional stability and compaction. The N-terminal histone tails protrude outwards from the nucleosome core.

Regulatory role of chromatin

For a long time it was believed that the only function of chromatin was to serve as a protective and static scaffold for storage of the genetic information encoded in the DNA sequence. However, in the last decade it has become clear that dynamic changes in chromatin actively regulate numerous genomic processes. Every nuclear process that requires access to DNA has to function in the context of chromatin, and thus all template-dependent processes, such as transcription, replication, mitotic chromosome condensation, and recombination as well as apoptosis and DNA repair are impacted and controlled by structural changes in chromatin.

Chromatin function is regulated by two general and overlapping mechanisms: (A) through changes in chromatin structure ("cis" mechanisms) and (B) through the recruitment of specific effector proteins ("trans" mechanisms).

A. Changes in chromatin structure

Structural changes of chromatin result in the adoption of more open or condensed conformations of chromatin. Such structural changes significantly affect the accessibility of DNA and thus can control various processes that require access to the DNA template (Hansen, 2002). On a molecular level, changes in chromatin structure are achieved in three different ways: 1) through chromatin remodelling complexes, 2) through the incorporation of histone variants, and 3) through the addition or subtraction of posttranslational modifications of histone proteins (Allis et al., 2007). Each will be briefly discussed below as follows:

1. *Chromatin remodeling complexes* are ATP-hydrolyzing machines that modify DNA-histone contacts, facilitate nucleosome sliding, relocate nucleosomes, or even actively remove core histone dimers,

thus modulating the exposure of DNA within chromatin (Cairns, 2007; Johnson et al., 2005). Based on their composition and activities, several families (such as the Swi/Snf, ISWI or NuRD families) with various members have been delineated. Their mechanisms of action are not yet completely understood (Cairns, 2007), but it is clear that they mediate chromatin rearrangements to facilitate processes such as transcription, DNA repair, replication, chromatin assembly, and homologous recombination (Becker and Horz, 2002; Georgel et al., 1997; Lee et al., 2001).

2. Chromatin structure and function can be altered through the incorporation of *histone variants* (Kamakaka and Biggins, 2005). These variants differ from the corresponding major histones in their primary sequence, in some cases by very subtle differences of a few amino acids, in others dramatically by the presence of specialized domains (Bernstein and Hake, 2006). In several cases, histone variants are localized to specific regions of the genome: macroH2A, e.g., is found at the inactive X chromosome of female mammals (Costanzi and Pehrson, 1998), CENP-A at centromeric chromatin (Black and Bassett, 2008), or H3.3 at actively transcribed genes (Ahmad and Henikoff, 2002). For the deposition of some of these histone variants specialized chromatin remodeling complexes have been identified (Mizuguchi et al., 2004; Tagami et al., 2004). It is believed that the sequence differences amongst histone variants gives them distinct biophysical characteristics that alter the properties of the nucleosome. In addition, the sequence variation allows them to acquire specific patterns of histone modifications (such as the phosphorylation of H2A.X on a serine unique to this

variant at DNA repair sites; Fernandez-Capetillo et al., 2004; Rogakou et al., 1998) and to undergo specific and distinct molecular interactions.

3. Changes in chromatin structure are also brought about by *covalent posttranslational modifications of histone proteins*, especially of the N-terminal tails of the core histones. Many histone modifications change the electrostatic charge of the histone tails, thereby strengthening or weakening histone-DNA and histone-histone contacts ("cis" mechanism). This, in turn, is thought to modulate higher-order chromatin structure (Kouzarides, 2007; Shogren-Knaak et al., 2006; Workman and Kingston, 1998; see below Section "Mechanism of histone modifications").

B. Recruitment of effector proteins

Besides direct changes in chromatin structure, the function of chromatin can also be modulated in an indirect fashion: Distinct patterns of covalent modifications within histones act as "signaling platforms", recruiting specific nuclear factors that in turn mediate downstream functions (Seet et al., 2006; Strahl and Allis, 2000; Taverna et al., 2007; see below, Section "Mechanism of histone modifications").

These mechanisms to regulate chromatin structure and function are also evident in the molecular characteristics of euchromatin and heterochromatin. The two forms of chromatin show differences in the patterns of their histone modifications as well as in their non-histone protein constituents (Dillon and Festenstein, 2002; see Table 1.1).

Since my thesis work focuses on a specific effector protein, Heterochromatin Protein 1, which is recruited to chromatin by a histone modification

("trans" mechanism), I will in the following section provide a more detailed background on histone modifications, the enzymes that establish them, and the mechanisms underlying their functions.

Table 1.1: Molecular characteristics of euchromatin and heterochromatin

| | Euchromatin | Heterochromatin |
|---------------------------------|--------------------|--------------------------|
| Conformation during interphase | decondensed | condensed |
| Gene density | high | low |
| Replication | mainly early | late |
| Levels of H3 and H4 acetylation | high | low |
| Other specific modifications | H3K4me | H3K9me, H3K27me, H4K16Ac |
| Effector proteins | e.g. BPTF, WDR5 | e.g. HP1, Pc |
| Histone variants | H3.3, H2A.Z | |
| Levels of DNA methylation | low | high |

Histone modifications – histone marks

As early as the 1960s Vincent Allfrey and colleagues discovered that histones can be extensively post-translationally modified (Allfrey, 1966). In fact, some types of post-translational protein modifications (like acetylation and phosphorylation) were actually first discovered on histone proteins.

Over the last decades, an enormous number of distinct post-translational modification types and sites have been identified on histones (so-called "marks") have been unveiled, especially since the introduction of mass spectrometric approaches to histone biology a few years ago. In particular the histone tails have been found to be subject to a great variety and high density of posttranslational modifications (Figure 1.3). Lysine

residues in histones can be modified by acetylation, (mono-, di-, or tri-) methylation, ubiquitylation and sumoylation; arginine residues can be (mono-, and symmetrically/asymmetrically di-) methylated, serine and threonine residues can be phosphorylated, and glutamate residues can be ADP-ribosylated (Kouzarides, 2007; Peterson and Laniel, 2004). All in all, close to a hundred individual histone marks have been identified to date, about half of which have been confirmed by independent experimental methods (i.e. detected by mass spectrometry and immunological/enzymatic methods).

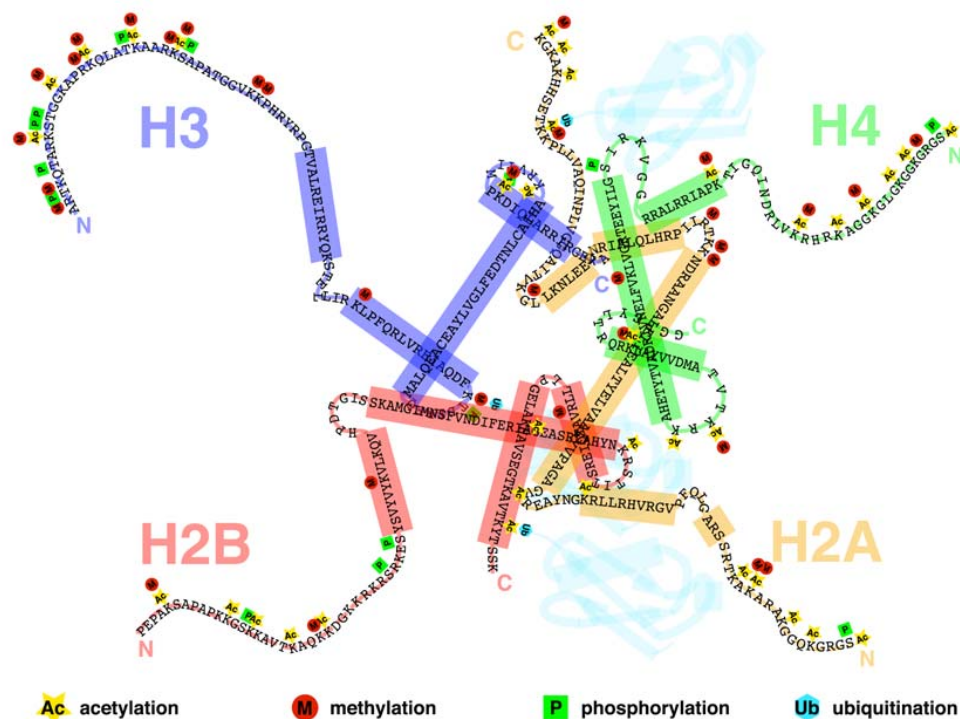


Figure 1.3: Posttranslational modifications of histone molecules.

Histone proteins, in particular their N-terminal tails, are subject to a great diversity and extreme density of posttranslational modifications. Depiction of a half nucleosome (one copy of each of the core histones), DNA shown in turquoise. Ac: acetylation. M: methylation. P: phosphorylation. Ub: ubiquitylation. Note that lysine residues can be mono-, di-, or tri-methylated and arginine residues can be mono- and di-methylated. In addition, other classes of modifications have been found, such as sumoylation or ADP-ribosylation. Figure courtesy of Dr. Wolfgang Fischle.

Correlation of histone modifications with biological effects

The idea that histone modifications may play important regulatory roles within the cell was raised by Allfrey and colleagues more than thirty years ago, when they described a positive correlation between the levels of histone acetylation and gene expression (Allfrey, 1966; Allfrey et al., 1964; Pogo et al., 1968). Since then, it has been possible to directly correlate many histone marks with defined cellular events. For example, besides H3 and H4 acetylation (reviewed in Kurdistani and Grunstein, 2003), gene activation also correlates with methylation of H3 at K4 (Honda et al., 1975; Ruthenburg et al., 2007a). Gene repression correlates with hypoacetylation of H3 and H4 (Allfrey et al., 1964; Green, 2005; Peterson, 2002), with methylation of H3 at K9 (Grewal and Jia, 2007; Peters et al., 2001) and with methylation of H3 at K27 (Schuettengruber et al., 2007). And mitotic chromosome condensation correlates with the phosphorylation of H3 at S10 and S28 (Gurley et al., 1973; Hendzel et al., 1997; Nowak and Corces, 2004) (for more examples see Table 1.2).

"Histone Code"

Understanding how an ever-increasing number of post-translational histone modifications can work together to bring about specific biological outcomes has been a challenge for the field. As solution to this problem, the existence of a "histone code" has been proposed. According to this concept, different combinations of post-translational histone modifications are established and maintained in particular regions of chromatin, and function in a sequential or combinatorial fashion to specify unique downstream biological functions (Jenuwein and Allis, 2001; Strahl and Allis, 2000; Turner, 2000).

Table 1.2: Examples of histone marks, functions they correlate with and the responsible enzymes (*Homo sapiens*, *Mus musculus*)

| | Mark | Function | Enzyme(s) |
|------------|---------|--|---|
| H2A | K119ub1 | Polycomb silencing, UV damage response | RING1B |
| H2B | S14ph | Apoptosis, Somatic hypermutation and class switch recombination | Mst1/krs2 |
| | K120ub | Cell cycle progression, meiosis | |
| H3 | R2me | Gene Expression | CARM1 (me2a) |
| | K4me | Transcriptional activation Trithorax activation | SET7/SET9 (me1) MLL1/MLL2/MLL3 (me3) |
| | K9ac | Nuclear receptor coactivator | SRC1 |
| | K9me | Transcriptional repression, Im- printing Pericentric heterochromatin, Rb-mediated silencing | G9a (me1, me2) SUV39H1/2 (me3) |
| | S10ph | Transcriptional activation of im- mediate early genes Mitotic chromosome condensation | MSK1/2, RSK2 Aurora B |
| | K14ac | Transcriptional activation | Gcn5, P300, PCAF, TAFII230, TAFII250 |
| | K18ac | Transcriptional activation | P300, CBP |
| | K23Ac | Transcriptional activation | CBP |
| | K27me | Polycomb repression Early B cell development X chromosome inactivation | EZH2 (me3) |
| | S28ph | UVB induced phosphorylation Mitotic chromosome condensation | MSK1 Aurora B |
| | K79me | Telomeric silencing, pachytene checkpoint | DOT1L (me2) |
| H4 | R3me | Transcriptional activation | PRMT1 |
| | K5ac | Histone deposition Transcriptional activation | Hat1 p300 |
| | K8ac | Transcriptional activation | PCAF/p300 |
| | K12ac | Excluded from Xi, Histone deposition | Hat1 |
| | K16ac | Sequence-specific TF | ATF2 |
| | K20me | Transcriptional silencing, mitotic condensation | Pr-SET7/Set8 (me1) |

Adapted from Allis et al., 2007. For references and a more complete list, see there.

Enzymes that establish histone marks

In 1996, it was discovered that certain transcriptional coactivators and corepressors had histone acetyltransferase (HAT) and deacetylase (HDAC) activity, respectively (Brownell et al., 1996; Taunton et al., 1996). These findings provided the first real functional link between histone acetylation and transcription, and therefore precipitated a significant wave of interest in the role of histone modifications in the regulation of DNA-dependent processes. As a consequence, over the last decade a remarkable number of enzymes that establish histone marks ("writers") has been identified. Among these are histone acetyltransferases (HATs; Sterner and Berger, 2000), histone kinases (Nowak and Corces, 2004), histone methyltransferases (HMTases; Qian and Zhou, 2006; Zhang and Reinberg, 2001), and enzymes that mediate histone ubiquitylation (Weake and Workman, 2008), sumoylation (Nathan et al., 2006), and ADP-ribosylation (Hassa et al., 2006) (see Table 1.2 for specific examples).

Many histone marks have turned out to be reversible. Indeed, several classes of enzymes have been identified that can remove specific histone modifications ("erasers"), such as histone deacetylases (HDACs, Holbert and Marmorstein, 2005) and phosphatases that work on histones (Nowak and Corces, 2004). Histone methylation was for a long time considered to be more stable than the other modifications, based on early studies looking at the turnover of methyl groups in bulk histones (Byvoet, 1972; Duerre and Lee, 1974). However, recently several classes of enzymes have been identified that can reverse specific methyl marks on histones (Lan et al., 2008; Shi et al., 2004; Tsukada et al., 2006; Wang et al., 2004; reviewed in Lan et al., 2008).

In addition to the removal of individual histone modifications by specific enzymes, proteolytic clipping of histone tails (Allis et al., 1980; Duncan et al., 2008) and histone replacement (Ahmad and Henikoff, 2002) also result in the removal of histone marks.

Molecular mechanisms of histone modifications

As described above, correlations with distinct biological events have been found for many histone modifications. However, the exact molecular mechanisms how these histone marks exert their physiological functions is in many cases not fully understood. As outlined before, histone modifications use two general (albeit not mutually exclusive) mechanisms: They either modulate chromatin structure in *cis* by directly affecting internucleosomal contacts and histone-DNA interactions, or they act in *trans* by recruiting binding partners that then induce and direct downstream functions (Figure 1.4; Allis et al., 2007).

Several mutational studies have led to observations that can best be explained by direct or *cis* effects of histone marks. For example, deletions of large regions of the histone tails of H3 and H4 in yeast have very little effect (Ling et al., 1996; Smith and Stirling, 1988), pointing to redundancy among lysine acetylation marks. Similarly, the effects of several lysine residues within the H3 tail that can be acetylated have been explained in the sense of charged patches: Only the number of charged residues/lysines was important for transcriptional activation in *S. cerevisiae*, whereas their exact position was irrelevant (Kristjuhan et al., 2002).

Published reports have repeatedly confirmed the importance of histone tails for the regulation of higher-order chromatin structure (reviewed in Hansen, 2002). The extensive involvement of the histone

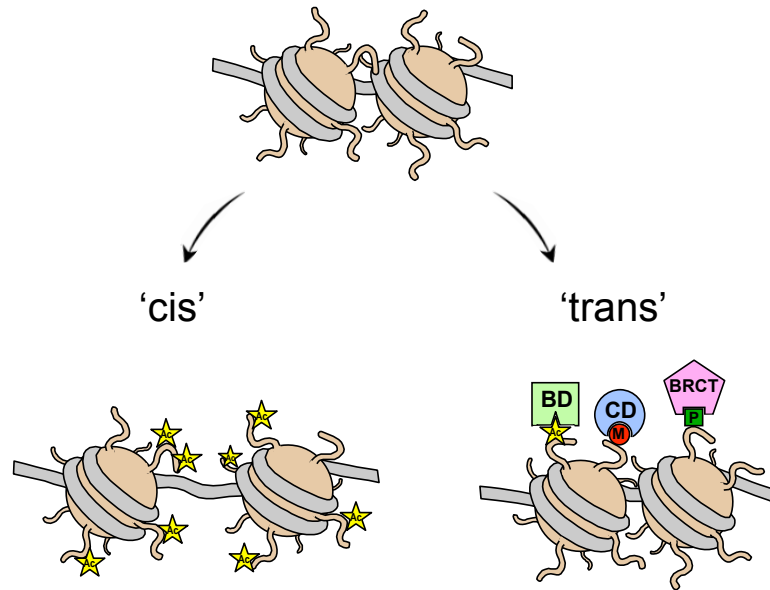


Figure 1.4: Molecular mechanisms of histone modifications.

Histone modifications fulfill their function by two general mechanisms. They either change the net charge of the histone tail, which affects inter/intranucleosomal contacts and results in structural changes of the chromatin fiber ("cis" mechanisms, left). Or they recruit proteins with specific binding modules, such as bromodomains (acetyl-lysine binders), chromo domains (methyl-lysine binders), or BRCT domains (phospho-serine/threonine binders). Upon recruitment, these effector proteins mediate downstream functions ("trans" mechanisms, right).

Ac: acetylation. M: methylation. P: phosphorylation. BD: bromodomain. CD: chromodomain. BRCT: Breast-cancer-susceptibility protein-1 C-terminal domain.

tails in folding of the chromatin fiber suggests that at least some histone marks may have an effect on chromatin fiber dynamics, and in specific cases there is already experimental evidence that strongly supports such a conclusion (e.g. for H4K16ac; Shogren-Knaak et al., 2006). However, at this point biophysical and structural studies of mononucleosomes and nucleosomal arrays containing modified histones have not yet succeeded in demonstrating a significant difference to their corresponding less or unmodified counterparts.

The *trans* mechanism has attracted even more interest in recent years, and several histone modification-specific effector modules (some of which had been observed in other context before, Seet et al., 2006) and a number of specific binding proteins ("readers") have been identified over the last years: *Bromodomains*, e.g., are binding modules that recognize acetylated lysines (Dhalluin et al., 1999). They are found in several transcription factors (Gcn5, TAF_{II}250, PCAF) and chromatin remodeling complexes (Swi/Snf, RSC) and recruit these proteins to sites of active transcription (Zeng and Zhou, 2002). In fact, successive recruitment of multiple bromodomain containing proteins to promoter regions during the course of transcriptional activation has been demonstrated in several model systems (Agalioti et al., 2002; Agalioti et al., 2000; Cosma et al., 1999).

Chromo domains, in contrast, act as binding modules for methylated lysines. The list of chromo domain-containing proteins includes HP1 (Heterochromatin Protein 1), which recognizes histone H3 methylated at lysine 9 and mediates gene silencing and heterochromatinization (Jacobs et al., 2001; Lachner et al., 2001), and Polycomb, a repressive protein complex in *Drosophila* with important functions in development (Fischle et al., 2003c). In addition, several other binding modules have been identified, including domains recognizing methyl-arginine and phospho-serine/threonine residues (Seet et al., 2006; Taverna et al., 2007; see Table 1.3 for more examples).

Frequently, effector proteins carry multiple binding modules (Seet et al., 2006), for example the chromatin remodeler BPTF (two PHD fingers) or the methyltransferase MLL1 (bromo domain, several PHD fingers). Furthermore, often several proteins with binding affinity for histone marks are united in a

multisubunit complex, for example in the chromatin remodeling complexes RSC (yeast RSC contains eight bromodomains in different subunits of the complex) or NURF (which contains, in addition to BPTF, several complex members with multiple WD40 repeat domains) (Ruthenburg et al., 2007b). The observation that binding modules are often linked is mirrored by the finding that different histone marks often seem to collaborate to achieve

Table 1.3: Examples of binding modules and effector proteins for histone marks

| Binding module | Mark | Examples |
|-----------------------|---------------------------------|---|
| Bromo domain | acetylated lysine | PCAF (H4K16ac), TAF _{II} 250 (H3K9ac, H3K14ac) |
| Chromo domain | di/tri-methylated lysine | HP1 (H3K9me), Polycomb (H3K27me) |
| PHD finger | (methylated) lysine | BPTF (H3K4me _{2/3}), BHC80 (H3K4me ₀) |
| MBT domain | mono/di-methylated lysine | L3MBT, pocket 2 (a. o. H4K20me _{1/2}) |
| Tudor domain | methylated arginine | JMJD2A (H4K20me _{1/2}) 53BP1 (H4K20me _{1/2}) |
| WD40 repeats | di-methylated arginine | WDR5 (H3K4me ₂) |
| 14-3-3 | phosphorylated serine/threonine | 14-3-3 ζ (H3S28ph) |
| BRCT domain | phosphorylated serine/threonine | MDC1 (H2A.XS139ph) |

PCAF: p300/CBP-associated factor. TAF_{II}250: TATA-binding protein-associated factor II 250. Chromo: Chromatin organization modifier. HP1: Heterochromatin protein 1. PHD: Plant homeodomain. BPTF: bromodomain and PHD finger transcription factor. BHC80: BRAF-HDAC complex 80. MBT: malignant brain tumor. L3MBTL1: lethal-(3) malignant brain tumor repeat-like protein-1. JMJD2A: Jumonji domain-containing protein-2A. 53BP1: p53-binding protein 1. WDR5: WD repeat domain 5. BRCT: Breast-cancer-susceptibility protein-1 C-terminal. MDC1: Mediator of DNA damage checkpoint 1. See Ruthenburg et al., 2007; Seet et al., 2006; Taverna et al., 2007 for more details.

specific biological effects, as illustrated by the coexistence of H3S10ph and H3K14ac at sites of immediate early gene activation (Cheung et al., 2000), H3K4me2/3 and H4K16ac at transcriptionally active homeotic genes (Dou et al., 2005), or H3K27me3 and H2AK119ub1 at silent homeotic genes (Bernstein et al., 2005; Schuettengruber et al., 2007). Linking together multiple binding modules may lead, through thermodynamic and kinetic effects, to dramatic increases in affinity and specificity, while keeping the effector recruitment dynamic and susceptible to competition (Ruthenburg et al., 2007b).

While histone modifications can this way work together to recruit an effector protein/complex with multiple binding modules, they can also influence each other directly in various ways ("cross-talk" of histone modifications; Fischle et al., 2003b). For example, it has been found that the presence of certain marks enhances or decreases others. In the simplest case, this is just by directly blocking the site (e.g. H3K9 acetylation blocks H3K9 methylation). But there are also effects of enzyme recruitment and substrate recognition/turnover (e.g. the activity of the H3K79 methyltransferase Dot1 is activated by the presence of ubiquitylated histone H2B in the same nucleosome; McGinty et al., 2008). Such "cross-talk" between modifications (for recent reviews, see Fischle, 2009; Suganuma and Workman, 2008) represents, together with the finding that multivalency is important in effector recruitment, additional support for the existence of a histone code.

Histone modifications and disease

Mutations in various factors involved in chromatin formation and regulation have been linked to the development of disease, in particular cancer (Wang

et al., 2007). Through diverse mechanisms, these mutations lead either to repression of normally active genes (e.g. tumour suppressor genes) or result in the activation of genes that are normally silent (such as oncogenes).

Connections have been found for various modifications and at all levels of chromatin formation/regulation: for "writers" such as the H3K27 methyltransferase EZH2 (overexpressed in metastatic prostate cancer, Varambally et al., 2002), "readers" like the H3K9me-binder HP1 (downregulated in invasive metastatic breast cancer, Norwood et al., 2006), for "erasers" such as HDAC1 (aberrant recruitment to promoters due to fusion with DNA-binding proteins, Lin et al., 1998), and for chromatin remodelers such as the NuRD complex (deletion of its ATPase subunit in certain neuroblastomas, Bagchi et al., 2007).

The multitude and variety of these connections foreshadows the enormous potential that intervention with these processes may have for the treatment of cancer in the clinic. In fact, first steps to exploit the insights of chromatin biology in the interest of the patient have already been taken: Small molecule inhibitors of histone deacetylases have been tested with promising results for the treatment of tumors, and many such compounds have already entered into clinical trials (Bolden et al., 2006).

It is clear at this point, however, that a much deeper understanding of the principle mechanisms regulating chromatin structure and function and the specific mechanisms of chromatin-dependent tumorigenesis is required, and much basic research will still be necessary, to achieve the full potential of chromatin-based therapeutics. The close connections between chromatin and cancer make such research imperative.

Having outlined the basic principles of chromatin biology, I will in the following give a more detailed view of one specific non-histone component of chromatin: Heterochromatin Protein 1, the chromatin effector protein that was at the center of my Ph.D. research.

Heterochromatin Protein 1

Identification of HP1 and its gene

As early as 1930, it had been observed in experiments with flies that genes translocated by X-rays from euchromatic regions into the vicinity of pericentric heterochromatin acquire a variegated pattern of expression (Muller and Altenburg, 1930). The effect, which is caused by the repressive properties of heterochromatin, was called Position Effect Variegation (PEV, Figure 1.5), and it was exploited starting in the 1980s to systematically screen for factors that positively or negatively regulate heterochromatin formation.

One of the genes identified in such screens was the gene of Heterochromatin protein 1 (HP1). HP1 had previously been described as a protein that localizes to heterochromatin in immunofluorescence stainings (James and Elgin, 1986). The HP1 mutation *Su(var)2-5* turned out to be a strong suppressor of PEV, suggesting that HP1 plays a key role in the formation of heterochromatic structures (Eissenberg and Hartnett, 1993; Eissenberg et al., 1990; Eissenberg et al., 1992; Wustmann et al., 1989). Complete loss of *Drosophila* HP1 in homozygous *Su(var)2-5* null mutants is lethal, underlining the importance of HP1 in normal development. Flies die at the late third instar larval stage, at the time when the maternal supply of HP1 becomes exhausted (Eissenberg and Hartnett, 1993; Lu et al., 2000), probably due to the failure of HP1 null cells to segregate their

chromosomes faithfully (Fanti et al., 1998). Deletion of the HP1 homolog Swi6 in *S. pombe* results in impaired silencing at the centromeres and a significant increase in chromosome loss (Ekwall et al., 1995).

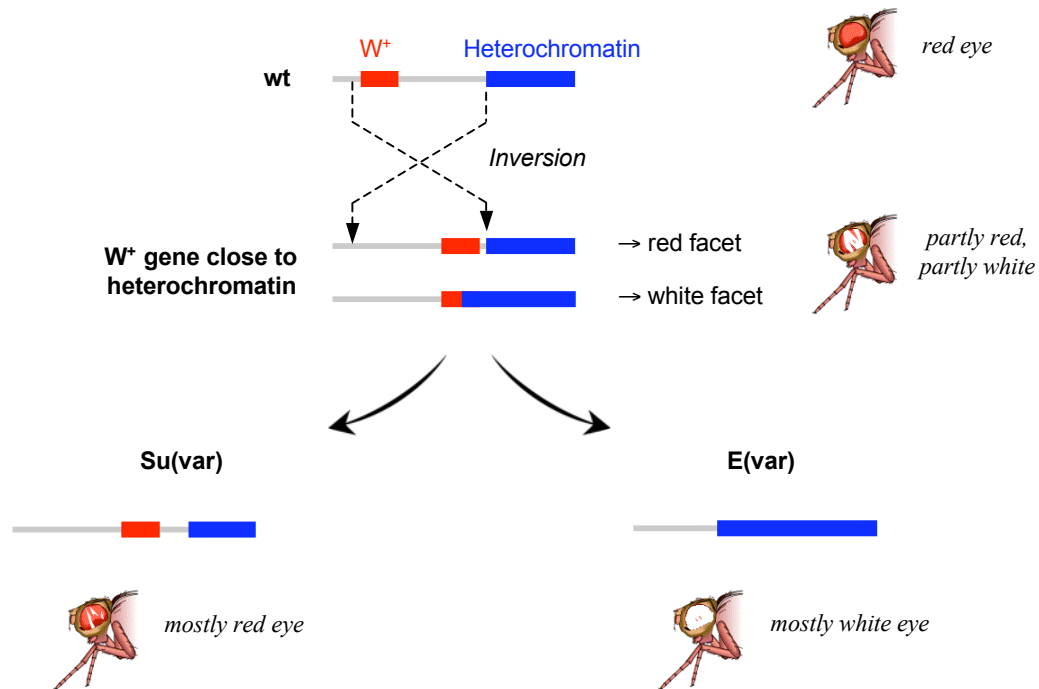


Figure 1.5: Position effect variegation in *Drosophila melanogaster*.

The white gene (W^+) is essential for normal red pigmentation of the fly eye. In wild-type flies, its gene locus (depicted in red) is located within distal euchromatin (grey). Through X-ray-induced inversion, W^+ can end up in the proximity of pericentric heterochromatin (blue), resulting in a variegating phenotype: In some cells, the white gene is still regularly expressed at this new location, leading to red eye facets; in others, heterochromatic proteins spread over the break points, condensing the relocated W^+ locus and silencing the gene, leading to white eye facets. Based on this position effect variegation, selection for second-site mutations allows the identification of proteins that affect heterochromatic structure: Suppressors of variegation (mutation leads to reduced silencing and thus more red eye facets) encode proteins that contribute to heterochromatic silencing. Enhancers of variegation (mutation results in further spreading of heterochromatin and thus more white eye facets) encode proteins that contribute to the active state. Similar PEV screens have been carried out in fission yeast. (Fly depiction adapted from Grewal and Elgin, 2002.)

Conservation of HP1 in different organisms

Heterochromatin protein 1 is a rather small, phylogenetically conserved protein of about 200 amino acids (Singh et al., 1991). Characteristic of all HP1 proteins in different organisms is their specific domain structure: a highly conserved N-terminal chromo (chromatin-organization modifier) domain is connected by a less conserved "hinge region" to a well conserved C-terminal chromoshadow domain (see Figure 1.6A for schematic).

HP1 is found in virtually all higher eukaryotes, ranging from yeast (*S. pombe*) and plants to flies, frogs, and mammals (for an overview see Kwon and Workman, 2008). A notable exception is the budding yeast *S. cerevisiae*, in which silent information regulatory (SIR) proteins likely fulfill a similar role. In many organisms there are several different HP1 isoforms (see Figure 1.6B for examples). While there is one HP1 protein found in *Tetrahymena thermophila* (Hhp1p) and *S. pombe* (Swi6; recently it has been suggested that another chromatin protein in fission yeast, Chp2, may also be part of the HP1 family; Sadaie et al., 2008), there are two HP1 isoforms in *C. elegans* (HPL-1 and HPL-2) and three in *Drosophila* (HP1a, HP1b, HP1c; HP1a was the first HP1 protein identified, and the literature refers to it often as "*Drosophila* HP1") and *Xenopus laevis* (HP1 α , HP1 β , HP1 γ). Mammals also have three HP1 isoforms (HP1 α , HP1 β , HP1 γ), which share an overall sequence identity of about 50% with *Drosophila* HP1; my thesis will focus on the mammalian HP1 isoforms.

The different isoforms have similarities in their amino acid sequence (see Figure 1.7 for an alignment; ; see Appendix for human HP1 sequences with numbered residues). To what extent there is redundancy among isoforms is currently not completely clear. In *Drosophila*, deletion of one isoform (dHP1a) is lethal (Eissenberg and Hartnett, 1993), causing cell

Figure 1.6: Domain structure of HP1.

A: HP1 proteins comprise three domains, an N-terminal chromo domain, a hinge region, and a C-terminal chromoshadow domain. For details on the molecular interactions of the three domains, see section "HP1 domains and molecular interactions".

B: Left: Schematic representation of HP1 isoforms in human, mouse, *Xenopus laevis*, *Drosophila melanogaster* and *Schizosaccharomyces pombe*. Even though there is some variation in the length and amino acid sequence of the domains, all HP1 proteins have a chromo domain, a hinge region and a chromoshadow domain. Especially dHP1b, dHP1c and Swi6 have extensive additional sequence stretches. **Right:** Table showing the conservation (% identity) of the different domains, compared to the most intensely studied HP1 protein, *Drosophila* HP1a. The hinge region is less conserved than the CD and CSD. Table adapted from Li et al., 2002.

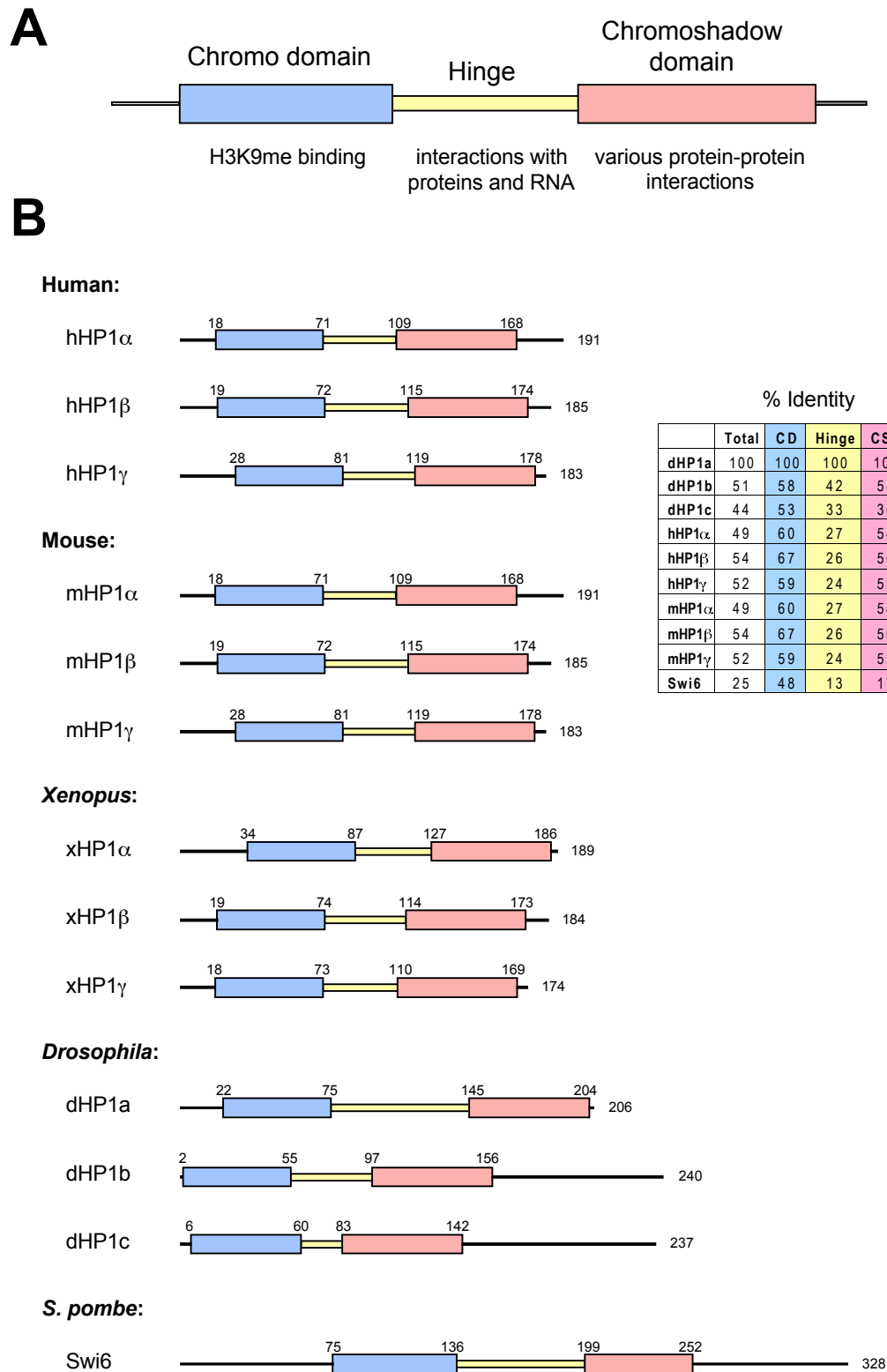


Figure 1.6

Figure 1.7: Alignment of HP1 proteins of different organisms.

Amino acid alignment of HP1 proteins from different organisms. The chromo domain and chromoshadow domain are highly conserved, while there is less conservation of the hinge region (for exact % identity see Table in Figure 1.6B, right).

hHP1: human HP1. mHP1: mouse HP1. xHP1: *Xenopus laevis* HP1. dHP1: *Drosophila melanogaster* HP1. Identical residues in red (identity threshold 0.75), similar residues in yellow. The CD is marked with a red line, the hinge region with yellow and the chromoshadow domain with blue. Green asterisks indicate amino acids of the aromatic cage. Residue numbers for human HP1 α are given above the sequence.

Alignment generated with ClustalW. The *S. pombe* homolog Swi6 was not included in the alignment, because its significantly larger length and lower sequence conservation made an inclusion in this multiple-species alignment difficult. For dHP1b and dHP1c, not the full sequence is shown, but the last 58 aa resp. 69 aa of their long C-terminal tails are omitted (compare to Figure 1.6B, left).

```

hHP1alpha  -----1-----*-----29
hHP1beta   -----MGKK--TKRT---ADSSSSSEEEYVVEKVLDRR
hHP1gamma  -----MASNKTTLQKMGKKQNGK---SKKVEEAPEEFVVEKVLDRR
mHP1alpha  -----MGKK--TKRT---ADSSSSSEEEYVVEKVLDRR
mHP1beta   -----MGKKQNKKK---VEEVLEEEEEEYVVEKVLDRR
mHP1gamma  -----MASNKTTLQKMGKKQNGK---SKKVEEAPEEFVVEKVLDRR
xHP1alpha  MDASDTSTGPRPNRESTVGKK--SKRA---SDASSSE-EEYVVEKVLDRR
xHP1beta   -----MGKKQNKKK---MEEVADEEEEYVVEKVLDRR
xHP1gamma  -----MGKKQNGK---SKKVEEAPEEFVVEKVLDRR
dHP1a      -----MGKKIDNPSSAKVSDAEEEEEYAVEKIIDRR
dHP1b      -----MAEFSSVERVEDKR
dHP1c      -----MVKNEPNFVVERIMDKR

30-----*-----78
hHP1alpha  VV-KGQVEYLLKWKGFSEEHNTWEPEKNLDCPELISEFMKKYKKMKEGEN
hHP1beta   VV-KGQVEYLLKWKGFSDENNTWEPEENLDCPDIAEFLOSQKTAH----
hHP1gamma  VV-NGKVEYFLKWKGFTDADNTWEPEENLDCPELIEAFINSQKAG----
mHP1alpha  MV-KGQVEYLLKWKGFSEEHNTWEPEKNLDCPELISEFMKKYKKMKEGEN
mHP1beta   VV-KGQVEYLLKWKGFSDENNTWEPEENLDCPDIAEFLOSQKTAH----
mHP1gamma  VV-NGKVEYFLKWKGFTDADNTWEPEENLDCPELIEDFLNSQKAG----
xHP1alpha  VV-KGQVEYLLKWKGFSEEHNTWEPEKNLDCPELISEFMKKYKKAKEAE-
xHP1beta   VV-KGQVEYLLKWKGFSDENNTWEPEENLDCPDIAEFLOSQKSAY----
xHP1gamma  VV-NGKVEYLLKWKGFTSDNTWEPEENLDCPELIEAFINSQKAG----
dHP1a      VR-KGQVEYLLKWKGYPETENTWEPENLDCQDLIQQYEASRKDEEKSA
dHP1b      TV-NGRTEYLLKWKGYPRSENTWEPVENLDCPDLIANFEESLKN--
dHP1c      ITSEGGKVEYLIKWRGYTSADNTWEPEECDCPNLIQKEESRAKSK----

79-----109
hHP1alpha  NK-----PREKSESNKRKSNFSN--SADDIKSKKKREQ
hHP1beta   -----ETDKSEGGKRKADSDSEDKGEESKPKKKKE-
hHP1gamma  -----KEK-DGTKRKSLSDS--ESDDSKSKKKRD-
mHP1alpha  NK-----PREKSEGNKRKSSFSN--SADDIKSKKKREQ
mHP1beta   -----ETDKSEGGKRKADSDSEDKGEESKPKKKKE-
mHP1gamma  -----KEK-DGTKRKSLSDS--ESDDSKSKKKRD-
xHP1alpha  -----PKAKTESTKRKA-----GSDDIKAKRRRE-
xHP1beta   -----ESDKSEGGKRKADSDT-EVGEENKPKKKKD-
xHP1gamma  -----KEKPDNKRKSVSDS--ESDDSKSKKKRE-
dHP1a      SKKDRPSSSAKAKETQGRASSSTSTASKRSEPT--APSGNKRRTDA
dHP1b      -----KETKKRLSTST--PESIRSKRKSFL
dHP1c      -----KRGEKKPKCE

110-----154
hHP1alpha  SNDIAR----GFERGLEPEKIIIGATDSCGDLMLFMKWKDTDEADLVLA
hHP1beta   ESEKPR----GFARGLEPERIIIGATDSSGELMFLMKWKNSDEADLVPA
hHP1gamma  AADKPR----GFARGLDPERIIIGATDSSGELMFLMKWKDSDEADLVLA
mHP1alpha  SNDIAR----GFERGLEPEKIIIGATDSCGDLMLFMKWKDTDEADLVLA
mHP1beta   ESEKPR----GFARGLEPERIIIGATDSSGELMFLMKWKNSDEADLVPA
mHP1gamma  AADKPR----GFARGLDPERIIIGATDSSGELMFLMKWKDSDEADLVLA
xHP1alpha  SNDIAR----GFERGLEPEKIIIGATDSCGELMFLMKWKDSDEADLVLA
xHP1beta   EAEKPR----GFVRGLEPERIIIGATDSSGELMFLMKWKTSDEADLVPA
xHP1gamma  SVDKPR----GFARALDPERIIIGATDSSGELMFLMKWKDSDEADLVPA
dHP1a      EQDTIPVSGSTGFDRGLEAKKILGASDNNGRLTFLIQFKGVDAQEMVPS
dHP1b      EDDEEQKKLI GFERGLEASKILGATDSSGHLMLFMKWKGSDEADLVPA
dHP1c      EIQKLR----GYERGLELAETVGATDVTDIKYLVRNQFCDEFDLVPSA

155-----191
hHP1alpha  EANVKCPQIVIAFYEEERLTWHAYPEDAENKEKETAKS-----
hHP1beta   EANVKCPQVVISFYEEERLTWHSYPSEDDDKKDDKN-----
hHP1gamma  EANMKCPQIVIAFYEEERLTWHSYPS-EDEAQ-----
mHP1alpha  EANVKCPQIVIAFYEEERLTWHAYPEDAENKEKESAKS-----
mHP1beta   EANVKCPQVVISFYEEERLTWHSYPSEDDDKKDDKN-----
mHP1gamma  EANMKCPQIVIAFYEEERLTWHSYPS-EDEAQ-----
xHP1alpha  EANLKCQIVIAFYEEERLTWHAYPEESESEKEAVKS-----
xHP1beta   EANVKCPQVVISFYEEERLTWHSYPS-EDDDKKDEKN-----
xHP1gamma  EANVKCPQVVISFYEEERLTWHSYPS-EDEAQ-----
dHP1a      VANEKIPRMVIHFYEEERLSWYSDNED-----
dHP1b      LANTRCPQVVIQFYEEERLTWHTGSGNGNGNTNSVNLGSSGGLGSGVGGSGA ...
dHP1c      QIVEKDPQMLIDYFQKMAPYSRHIAMRMKGVPPEELRLAASRTSYPHISSA ...

```

Figure 1.7

death through chromosome fusions (Fanti et al., 1998). In mammals, knock-down of HP1 α by RNA interference does not lead to directly observable effects, while HP1 α /HP1 γ double RNAi results in the formation of micronuclei, indicative of aberrant mitotic chromosome segregation (Obuse et al., 2004). This indicates differences between organisms in how the multiple functions of HP1 are distributed among the isoforms, as well as organism-specific differences in the exact functions fulfilled by HP1. In line with such an interpretation, various differences in the molecular interactions and cellular localization of the different isoforms have been observed (see Sections "HP1 domains and molecular interactions" and "Cellular localization and functions of HP1").

HP1 domains and molecular interactions

In the following, I will give a brief summary of what is known about the three domains of HP1 and the molecular interactions they undergo.

A. Chromo domain

The chromo domain (CD) was originally described as an evolutionarily highly conserved domain of about 40 amino acid with an obscure biological role that was present in various proteins involved in chromatin organization and gene regulation (Koonin et al., 1995). The CD of HP1 was actually the first chromo domain for which finally a molecular function could be identified: A series of landmark papers published in 2001 demonstrated that the HP1 chromo domain is a binding module for a methyl mark on the histone H3 tail, methylated lysine 9 (H3K9me, see Figure 1.8A; Bannister et al., 2001; Jacobs et al., 2001; Lachner et al., 2001). Subsequently, it was shown that chromo domains in general are methyl-lysine binding domains. The chromo domain of HP1

in particular has since developed into a highly studied paradigm for methyl-lysine binding and the recruitment of effector proteins.

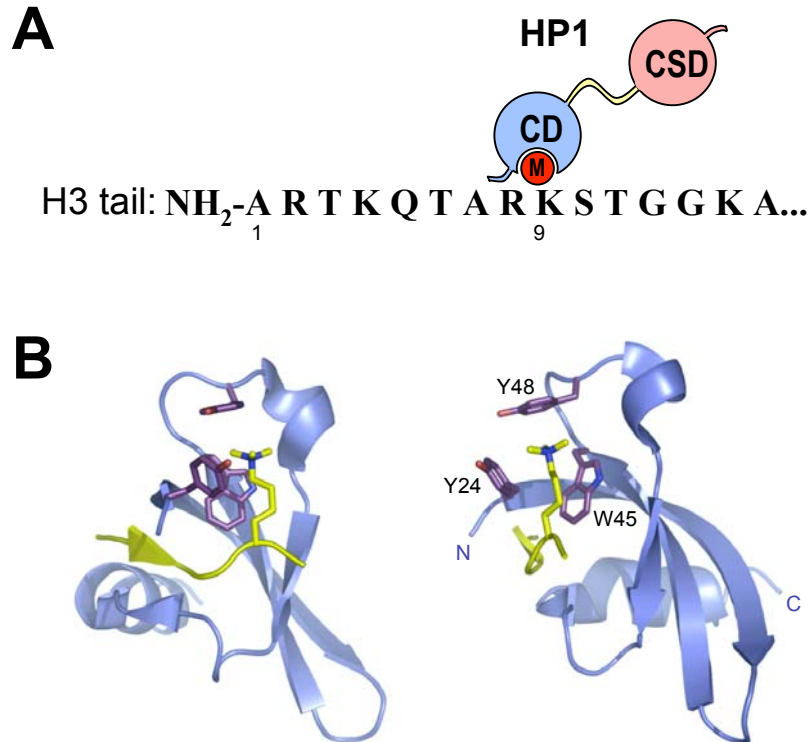


Figure 1.8: The chromo domain of HP1 binds methylated lysine 9 of histone H3.

A: The HP1 CD binds methylated lysine 9, a histone mark that is part of a sequence motif "ARKS" within the N-terminal tail of histone H3.

B: Crystal structure of the CD of *Drosophila* HP1a (23-76) bound to a trimethyl-K9 H3 tail peptide (1-15) (Jacobs and Khorasanizadeh, 2002). Two different views of the same structure are shown, the view on the right is rotated by 90° to the left around the vertical axis. The peptide (yellow, trimethyl-K9 shown) is bound by the CD (in blue) in a weak (K_d in the lower μ M), but specific interaction. No density was seen for H3 residues 1 to 4 and 11 to 15, suggesting that they are not directly involved in binding. **Left:** The peptide inserts into the chromo domain in an extended conformation, forming a β -strand that completes the β sandwich architecture of the CD. **Right:** The methylammonium group of lysine 9 is coordinated by three aromatic residues (Y24, W45, Y48; shown in purple), the "aromatic cage". Mutation of any of these completely abolishes binding.

Di- and trimethylation of H3 lysine 9 can be found at pericentric heterochromatin in virtually all higher eukaryotes and is considered a hallmark of silenced chromatin (Grewal and Jia, 2007). The enzyme mainly responsible for this methylation is the histone methyltransferase Su(var)3-9, as first shown with the *S. pombe* homolog Clr4 (Rea et al., 2000), and then confirmed with the mammalian homolog SUV39H (Peters et al., 2001) and the *Drosophila* homolog Su(var)3-9 (Schotta et al., 2002). Like HP1, Su(var)3-9 is a suppressor of PEV, confirming its importance in the formation of pericentric heterochromatin. By methylating H3 at lysine 9 through its C-terminal SET domain (Rea et al., 2000), Su(var)3-9 "writes" the histone mark that the HP1 CD then reads.

Interestingly, the amino acid context of H3K9 ("ARKS", Figure 1.8A) is found in identical or similar form at multiple other sites in histones and other proteins. In several of these instances the corresponding lysine has been found to be a methylation site as well. Moreover, even recruitment of interaction partners with chromo domains has been shown (e.g. H3K27: ARKmeS binds Polycomb, Fischle et al., 2003c; H1K26: ARKmeS binds HP1, Daujat et al., 2005; G9aK165: ARKmeT binds HP1, Sampath et al., 2007), and additional modifications such as lysine acetylation and serine phosphorylation are known to happen in this sequence stretch. Thus, ARKS and related sequences are typical examples of "modification cassettes" that are found in multiple proteins and confer the ability for the recruitment of specific binding partners (Fischle et al., 2003a).

The crystal structure of the HP1 chromo domain bound to a methylated H3 peptide shows that the main structural features

of the chromo domain are an antiparallel three-stranded β -sheet packed against an α -helix (Figure 1.8B). Of critical importance for the interaction of the CD with the methylated lysine are three aromatic amino acids within the chromo domain (the "aromatic cage", marked by green asterisks in the alignment of Figure 1.7 and shown in purple in the crystal structure of Figure 1.8B; Jacobs and Khorasanizadeh, 2002; Nielsen et al., 2002). Mutation of any of these residues results in loss of methyl-K9 binding *in vitro* and functional defects *in vivo* (Platero et al., 1995).

Biophysical measurements by fluorescence anisotropy have determined dissociation constants for the binding of the CD of *Drosophila* and human HP1s to methylated H3 tail peptides (Fischle et al., 2005; Fischle et al., 2003c). The K_d values are in the low micromolar range and little difference is observed between the chromo domains of the three isoforms. However, there is some discrimination between the level of K9 methylation: the affinity of the HP1 CD for H3K9me3 and H3K9me2 is about five- to tenfold higher than for H3K9me1.

Binding of the chromo domain to the modified H3 tail is essential for recruitment of HP1 to chromatin, and disruption of this interaction by mutations in the CD or removal of the H3K9me mark results in mislocalization of HP1 in flies, *S. pombe* and mammals (Fischle et al., 2003c; Platero et al., 1995; Stewart et al., 2005; Thiru et al., 2004).

Besides its interaction with H3K9me, the HP1 CD has been reported to bind components involved in nuclear architecture and to Psc3, a subunit of the mitotic cohesin complex in *S. pombe* (see Table 1.4 for references).

Table 1.4: Examples of HP1 interactions

(Adapted from Kwon and Workman, 2008; Li et al., 2002; Lomberk et al., 2006.)

Chromo domain:

| Interaction partner | Shown with | References |
|---------------------|--|--|
| H3K9me | Swi6, DmHP1a, mHP1 α , mHP1 β , mHP1 γ | Bannister et al., 2001 Lachner et al., 2001 Jacobs et al., 2001 Nakayama et al., 2001 |
| Lamin B, LAP2beta | mHP1 β | Kourmouli et al., 2000 |
| Psc3 | Swi6 | Nonaka et al., 2002 |

Chromoshadow domain:

| | | |
|-----------------------|--|--|
| HP1 | Swi6, mHP1 α , mHP1 β , mHP1 γ | Le Douarin et al., 1996 Brasher et al., 2000 Cowieson et al., 2000 Thiru et al., 2004 |
| Su(var)3-9 | DmHP1a, mHP1 α , mHP1 β , hHP1 β | Aagaard et al., 1999 Yamamoto and Sonoda, 2003 Schotta et al., 2002 |
| TIF1alpha | mHP1 α | Nielsen et al., 1999 Le Douarin et al., 1996 |
| KAP-1/TIF1beta | mHP1 α , mHP1 β , mHP1 γ , hHP1 α , hHP1 γ | Nielsen et al., 1999 Le Douarin et al., 1996 Lechner et al., 2000 |
| Rb | mHP1 γ , hHP1 γ | Nielsen et al., 2001b |
| Su(var)3-7 | DmHP1a | Cleard et al., 1997 Delattre et al., 2000 |
| CAF-1 (p150) | mHP1 α , mHP1 β , hHP1 α | Murzina et al., 1999 Brasher et al., 2000 |
| TAF _{II} 130 | hHP1 α , hHP1 γ | Vassallo and Tanese, 2002 |
| AF10 | DmHP1a | Linder et al., 2001 |
| ORC1-6 (CD+CSD) | DmHP1a | Pak et al., 1997 |
| Ku70 | hHP1 α | Song et al., 2001 |
| ATRX | mHP1 α , mHP1 β | McDowell et al., 1999 |
| Su(z)12 | hHP1 α , hHP1 γ | Yamamoto et al., 2004 |
| Ki67 | mHP1 α , mHP1 β , mHP1 γ | Scholzen et al., 2002 |
| PIM1 | hHP1 γ | Koike et al., 2000 |
| SP100B | hHP1 α , hHP1 β , hHP1 γ | Lehming et al., 1998 Seeler et al., 1998 |
| Lamin B receptor | hHP1 α , hHP1 β , hHP1 γ | Polioudaki et al., 2001 Ye et al., 1997 |

(continued on next page)

Hinge:

| | | |
|--------|-------------------------------|------------------------|
| RNA | mHP1 α , mHP1 γ | Muchardt et al., 2002 |
| INCENP | hHP1 α , hHP1 γ | Ainsztein et al., 1998 |
| H1b | hHP1 α | Hale et al., 2006 |

Interacting HP1 domain not determined:

| | | |
|-----------------|--|--|
| Dnmt3a/b | mHP1 α , mHP1 β , hHP1 α , hHP1 β , | Lehnertz et al., 2003 Bachman et al., 2001 Fuks et al., 2003 |
| Histone H3 core | hHP1 α , hHP1 β , hHP1 γ | Nielsen et al., 2001a |
| Su(var)4-20 | mHP1 α , mHP1 β , mHP1 γ | Schotta et al., 2004 |

B. Chromoshadow domain

Distantly related to the chromo domain in primary amino acid sequence (Aasland and Stewart, 1995), the chromoshadow domain (CSD) is also highly conserved between HP1 proteins of different organisms. The structure of the CSD bears some resemblance to the chromo domain structure (a three-stranded β sheet packed against two α helices; Cowieson et al., 2000). However, it does not engage a histone modification, but rather mediates a wide array of protein interactions. More than three dozen different molecular interactions of the HP1 chromoshadow domain have been identified to date (see Table 1.4 for examples).

Of particular interest among the interactions of the chromoshadow domain is the interaction with HP1 itself: as shown for *Drosophila* and mammalian HP1, the HP1 CSD mediates homodimerization with the same HP1 isoform as well as heterodimerization between different HP1 isoforms. The finding has been observed by various independent methods, such as two hybrid interaction assay (Gaudin et al., 2001; Le Douarin et al., 1996), *in vitro* pull-downs (Cowieson et al., 2000; Ye et al., 1997), gel filtration, equilibrium sedimentation analysis, and

structural studies (Brasher et al., 2000; Cowieson et al., 2000; Thiru et al., 2004). The dimer interaction is quite tight ($K_d < 150$ nM). Even so, it can be disrupted by single point mutations (e.g. I161E, Y164E in HP1 β ; Brasher et al., 2000).

Dimerization of HP1 through the CSD is an intriguing observation, because it suggests a mechanism how HP1 could achieve condensation and silencing of specific chromosomal domains. As depicted in Figure 1.9A, HP1 may crosslink H3K9-methylated nucleosomes, thus condensing regions of chromatin through a "handcuffing" mechanism (Jenuwein and Allis, 2001). Direct testing of this hypothesis by point mutations has been difficult, though, because deletions of the CSD or mutations abolishing HP1 dimerization also affect HP1 chromatin association in general (Thiru et al., 2004; Vogel et al., 2006).

A phage display screen carried out with the HP1 CSD identified a pentapeptide motif PXVXL/I (X = any amino acid) that interacts specifically with the chromo shadow domain (Smothers and Henikoff, 2000). The peptide motif was subsequently shown to be present in many of the molecules reported to bind the HP1 CSD, for example in KAP-1 (Brasher et al., 2000), Su(var)3-7 (Delattre et al., 2000), CAF-1 p150 (Brasher et al., 2000), the TAF_{II}130 component of TFIID (Vassallo and Tanese, 2002), and AF10 (Linder et al., 2001). Binding of proteins with the PXVXL/I motif to HP1 requires dimerization of the CSD, which generates a hydrophobic groove that the peptide motif associates with (Thiru et al., 2004).

As described above, Su(var)3-9 functionally interacts with the chromo domain of HP1: It methylates H3K9 and thus generates a binding site for the HP1 CD that is essential for HP1 recruitment to

chromatin. However, it has been found that Su(var)3-9 also interacts physically with HP1. In co-immunoprecipitation experiments, it was shown that mouse HP1 β binds the mouse homolog of Su(var)3-9, SUV39H1 (Aagaard et al., 1999). The interaction was mapped to the CSD of HP1 and the extreme N-terminus of SUV39H1 (residues 1 – 42, outside of the chromo domain of SUV39H1 as well as its SET domain; Melcher et al., 2000).

The interaction between SUV39H1 and HP1 has potentially far-reaching biological implications, because it suggests a mechanism for the spreading and maintenance of heterochromatic structures and epigenetic gene silencing. According to this model, SUV39H1, the histone H3 tail and HP1 collaborate to form a self-sustaining loop: methylated H3K9me recruits HP1, which in turn directs more SUV39H1 histone methyltransferase to chromatin, enabling further methylation (Figure 1.9B).

Besides dimerization and the interaction with Su(var)3-9, several transcriptional regulators have been shown to bind to the HP1 CSD, such as the transcriptional repressors TIF1 α , KAP-1/TIF1 β , and Rb (Le Douarin et al., 1996; Nielsen et al., 1999; Nielsen et al., 2001b). This could provide means for initial recruitment of HP1 to specific regions of chromatin. Rb, for example, has been reported in human cells to recruit HP1 β to the cyclin E promoter and induce tri-methylation of H3K9 by SUV39H1 (Nielsen et al., 2001b). The reported interaction of the HP1 chromoshadow domain with class II HDACs (Zhang et al., 2002; reports of HP1 interaction with HDACs also in *S. pombe*, Yamada et al., 2005) may contribute to gene repression and the initiation of stable silencing through hypoacetylation of histones H3 and H4 and deacetylation of H3K9, which allows for K9 methylation.

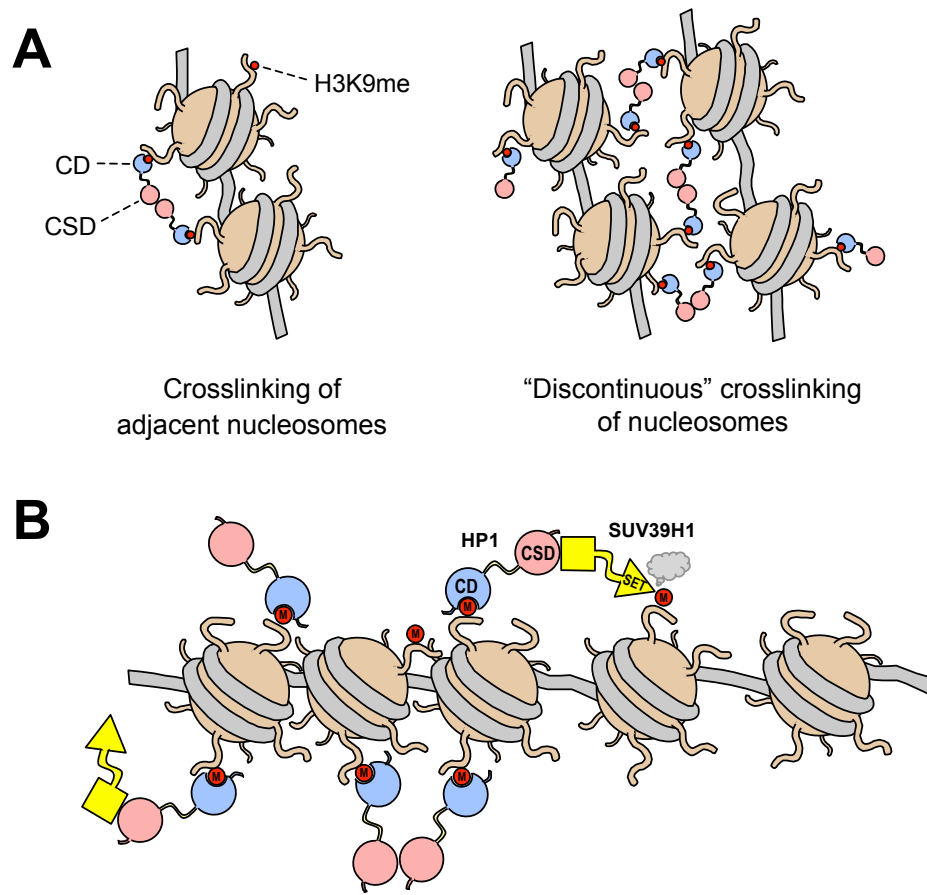


Figure 1.9: Models for HP1-mediated chromatin condensation and the spreading of heterochromatic domains.

A: Model for chromatin condensation through nucleosome crosslinking via HP1 dimerization. According to this model, HP1 binds to the H3K9me mark (small red dot) through its chromo domain (blue). Dimerization of HP1 molecules through the CSD (in pale red) can bridge individual adjacent nucleosomes, thus preventing a more open chromatin conformation of loci carrying H3K9me (left). "Discontinuous" bridging between different chromatin fibers could even cause condensation of larger heterochromatic domains (right). The structures may then be further stabilized by interactions with additional heterochromatic factors.

B: Model for the spreading of heterochromatin by interplay of SUV39H1, histone H3, and HP1. The histone mark H3K9me (small red circle "M") recruits the effector protein HP1 via its chromo domain (CD) to chromatin, possibly with the contribution of other stabilizing interactions. HP1 crosslinks nucleosomes and forms condensed, heterochromatic structures. In addition, however, HP1 also recruits the HMTase SUV39H1 (in yellow). Upon recruitment to chromatin, SUV39H1 methylates through its SET domain adjacent unmethylated H3 tails at lysine 9, forming new H3K9me binding sites for HP1. Thus, this three-component system could explain spreading and maintenance of heterochromatic gene silencing.

In addition to these, various other molecular interactions of HP1 have been described, ranging from histone chaperones over Su(var)3-7, a PEV modifier with an unknown molecular function, to proteins involved in replication, DNA repair and nuclear architecture. For more examples and references see Table 1.4.

C. Hinge region

Compared to the chromo and chromoshadow domains, the hinge region that connects them has received much less attention. The hinge is somewhat variable in length between the different isoforms (ranging for *Drosophila*, e.g., from 60 aa in HP1a to 23 aa in HP1c, Figure 1.6B). As can be seen from the Table in 1.6B, its sequence conservation is significantly lower than that of the CD or the CSD, with the notable exception of a few clusters of mostly basic amino acids (see alignment in Figure 1.7).

The sequence variation between the hinge regions of the different HP1 isoforms has led to the suggestion that the hinge may be the key to explain the differences that have been observed in localization and behaviour between the isoforms. Indeed, most of the molecular interactions that have been described for the hinge region so far have only been observed for specific isoforms (Ainsztein et al., 1998; Hale et al., 2006).

Despite its low level of sequence conservation, the hinge regions of all three isoforms have a common characteristic found in various organisms: a striking density of charged amino acids (K, E, D) and serines. The presence of multiple serine residues has led to speculation that this region of HP1 may be the site of phosphorylation (see below), but no phosphorylation sites in the hinge have been unambiguously identified so far.

The hinge region is assumed to have no defined secondary structure, but instead is probably unstructured, in keeping with a very high accessibility by proteases (Ball et al., 1997). Even though much less is known about the molecular interactions of the hinge region than those of the CD and CSD, nevertheless several molecular interactions of this domain of HP1 have been discovered. Since much of my Ph.D. research focuses on the HP1 hinge, I will describe the interactions of the hinge in a little more detail.

The HP1 hinge binds to INCENP (inner centromere protein), a member of the chromosomal passenger complex (Ainsztein et al., 1998). The chromosomal passenger complex (CPC) is a mitotic kinase complex that shows very distinct localization changes during mitosis and plays an important regulatory role in multiple steps of chromosome segregation and cell division. After identification of human HP1 α and HP1 γ from a two hybrid screen with INCENP, the interaction was reproduced for HP1 α in pull-down experiments with recombinant/*in vitro* translated components and has since been confirmed by co-IP from *Xenopus* egg extracts (Tseng, 2008). The interaction is not required for targeting of INCENP to centromeres, and the exact biological function of this interaction is currently still elusive.

An interaction of the HP1 hinge region with RNA was demonstrated in mouse cells by Muchardt and colleagues (Muchardt et al., 2002). Through immunofluorescence-overlay assays, it was shown that besides the chromo domain the hinge region and an RNA component was required for the proper localization of HP1 to regions of pericentric heterochromatin, in keeping with observations by Maison et al. (Maison et al., 2002). The hinge of HP1 α and HP1 γ directly interacts with RNA by

EMSA (electrophoretic mobility shift assay). Competition experiments showed that a mixture of nuclear RNA bound best, while control sequences (AU- and GC-rich RNA oligomers, tRNA, ssDNA, dsDNA) did not. This suggests that there may be a specific secondary structure or sequence motif that is present in some of the nuclear RNAs and that is specifically bound by the HP1 hinge, but to date nothing more is known about the nature and sequence of any RNAs HP1 may bind to.

Recently, it has been reported that the hinge region of human HP1 also interacts with the linker histone H1b, as shown by both *in vitro* binding and *in vivo* FRET experiments (Hale et al., 2006). The interaction, which was mapped to the C-terminal domain of H1b, is specific for the isoform HP1 α (the HP1 isoform that also has been specifically linked to breast cancer metastasis, see below; Kirschmann et al., 2000). Interestingly, binding of H1b to HP1 α is controlled by posttranslational modification of H1b. Phosphorylation of the histone by CDK2/cyclin E at late G1/S abolishes the interaction, a step that is required for efficient progression of the cell cycle, possibly because it leads to a more relaxed structure that facilitates DNA replication.

Despite these published interactions, the HP1 hinge remains a region about which very little is known compared to the other domains of HP1. The identification of additional interaction partners, understanding the contribution of the HP1 hinge to the differences observed between isoforms, and potential phosphorylation of hinge serines remain wide open questions in the understanding of HP1.

Cellular localization and functions of HP1

HP1 in heterochromatin

Thorough localization studies of HP1 in *Drosophila* and mammals have shown that HP1 proteins are localized primarily to regions of constitutive heterochromatin around the centromeres and at the telomeres, which are rich in repetitive DNA sequences. For example in polytene chromosomes of *Drosophila*, mainly the chromocenter (i.e. regions of pericentric chromatin) and the telomeres are stained with HP1 antibodies (Fanti et al., 1998; James and Elgin, 1986; Li et al., 2002). The *S. pombe* homolog Swi6 is found at silent mating-type loci, centromeres and telomeres (Ekwall et al., 1995), and in mouse cells, colocalization of HP1 with DAPI-dense regions of constitutive heterochromatin is observed during interphase (Figure 1.9; Lachner et al., 2001).

As is evident from its function as a dominant suppressor of variegation, HP1 plays an essential role in the the formation of silenced domains of constitutive heterochromatin at pericentric regions, which in turn is crucial for the faithful segregation of chromosomes during mitosis. HP1 mutation also leads to defective telomere protection in *Drosophila* (it is required for telomere capping, silencing and telomere length control; Perrini et al., 2004) and loss of silencing at mating type loci in *S. pombe*.

But besides this role in gene silencing, HP1 also has a function in *promoting* gene expression within heterochromatin. Some genes located within pericentric heterochromatin require a heterochromatic environment for their normal expression, as first described for the "light" and "rolled" genes (Lu et al., 2000). These genes depend on HP1 to generate the heterochromatic structures needed for their proper expression, and similar

observations have by now been made for many other genes (de Wit et al., 2007). Thus HP1 plays different, quite opposing roles within constitutive heterochromatin, which are not understood in mechanistic terms.

HP1 in euchromatin

However, it is well documented that HP1 does not only localize to regions of constitutive heterochromatin. A small fraction of HP1 is also found at sites within euchromatic regions of the chromosomes. In polytene chromosomes of *Drosophila*, e.g., HP1a is present at about 200 sites within the chromosome arms (Fanti and Pimpinelli, 2008; Li et al., 2002). This suggests that HP1 may also play a role in the repression of individual genes within euchromatin, an interpretation supported by reports demonstrating recruitment of HP1 by several transcriptional repressors (Le Douarin et al., 1996; Nielsen et al., 1999; Nielsen et al., 2001b) and the observation that some HP1-bound genes in euchromatin are upregulated upon mutation of HP1a in *Drosophila* (Hwang et al., 2001).

Several experimental observations argue that HP1 by itself can initiate heterochromatic structures and may indeed directly induce gene silencing within euchromatin. When HP1 is tethered to sites within euchromatic regions of *Drosophila* chromosomes through an ectopic binding domain, this is almost always sufficient to nucleate the formation of heterochromatin and silencing of nearby reporter genes (Li et al., 2003). In mammalian cells, targeting of HP1 α through a GAL/lacR system to euchromatic regions leads to local condensation of the higher order chromatin structure (Verschure et al., 2005). These experiments suggest that HP1 could indeed play a role in gene repression within euchromatic regions of chromosomes.

However, the involvement of HP1 in the regulation of euchromatic regions is even more complex and goes beyond its well established role in gene silencing. Quite surprisingly, at some euchromatic loci HP1 association clearly correlates with *increased* gene expression, such as in some developmental (ecdysone-) and heat-shock induced chromosome puffs in *Drosophila* (chromosome puffs are morphological features caused by strong decondensation of chromatin due to high levels of gene expression). Upon induction of gene expression at these sites, *Drosophila* HP1a is specifically recruited to these decondensed regions of extremely active transcription (Piacentini et al., 2003), suggesting that HP1 can indeed promote gene expression without inducing heterochromatic structures. Support for this interpretation comes from recent high-resolution mapping of HP1 binding sites in *Drosophila* that finds HP1 at transcriptionally active loci (de Wit et al., 2007; Johansson et al., 2007), from the observation that HP1 deletion reduces mRNA levels of some euchromatic genes (Cryderman et al., 2005), and from ChIP (Chromatin immunoprecipitation) experiments in mouse cells that find HP1 γ at actively transcribed regions (Vakoc et al., 2005). Thus, despite its name, HP1 seems to have functions in euchromatin that go beyond its role as an initiator of gene silencing and heterochromatin formation, at least at a subset of loci.

In addition to constitutive heterochromatin and sites of repressed and activated euchromatic genes, HP1 has also been detected at a few other cellular structures in mammalian cells, such as in PML nuclear bodies (discrete subnuclear structures of unknown function that are disrupted in acute promyelocytic leukemia; Everett et al., 1999; Seeler et al., 1998) or at the periphery of nucleoli (Minc et al., 1999).

To further complicate the localization of HP1, in many instances subtle differences between the three HP1 isoforms are observed. It has repeatedly been noted, for example, that euchromatic localization and function is more pronounced in the case of HP1 γ /HP1c than in the case of the other two isoforms (Hwang and Worman, 2002; Minc et al., 2000; Smothers and Henikoff, 2001; Vakoc et al., 2005). High-resolution mapping in *Drosophila* Kc cells confirms these findings: As opposed to HP1a, little enrichment of HP1c at pericentric regions is observed, but rather a focal distribution to a few "hotspots" near highly active genes, where HP1c colocalized with many transcription factors and other regulatory proteins (de Wit et al., 2007; Moorman et al., 2006). From a molecular point of view, differences between the isoforms are perhaps not completely surprising, because there is clear sequence variation between the three isoforms, in particular in the hinge region. Differences in the molecular interactions of the three isoforms (e.g. HP1 α interacts with histone H1b, while HP1 β and HP1 γ do not), and in their involvement in human disease (e.g. HP1 β , but not HP1 α , is downregulated in the progression of human melanoma; Nishimura et al., 2006) have been known for a long time.

In summary, HP1 is clearly involved in multiple, sometimes seemingly opposing cellular events. It is a central player in the formation/maintenance of constitutive heterochromatin at pericentric regions and telomeres, which is crucial for the faithful segregation of chromosomes during mitosis, as well as for the normal expression of certain genes located within pericentric heterochromatin. Besides its function in heterochromatin, HP1 clearly also plays a part in euchromatin, where it represses transcription at some loci, while promoting gene expression at others. On a molecular level, little is known how HP1 fulfills these multiple functions.

The paradigm that the presence of HP1 always accompanies and promotes constitutive heterochromatin is clearly not completely true, since ample evidence exists for both euchromatic localization and positive involvement in gene expression. However, by far the largest fraction of cellular HP1 is indeed localized to regions of constitutive heterochromatin by H3K9me-dependent mechanisms, where it is involved in heterochromatin formation and gene silencing.

Changes in HP1 localization during the cell cycle

Detailed studies of HP1 in cultured cells have revealed dramatic changes in HP1 localization during mitosis in *Drosophila* (Kellum et al., 1995) as well as human cells (Hayakawa et al., 2003; Minc et al., 1999; Murzina et al., 1999). When chromosome condensation is initiated during prophase, all three HP1 isoforms are released from their interphase binding sites on chromatin and become dispersed throughout the cytoplasm (see Figure 1.10). No earlier than telophase/early G1 does the bulk of HP1 isoforms reassociate with chromatin.

Quite surprisingly, the binding site of HP1 at constitutive heterochromatin, methylated lysine 9 (K9) of H3, is not removed during this dissociation (Bannister et al., 2002). This is surprising, because in most other cases where release of a recruited effector protein is observed, this is achieved by removal of the histone mark. Before our work, the mechanisms of the reversible ejection of HP1 from chromatin during mitosis were thus still enigmatic.

Several studies have reported another subtlety of HP1 dynamics during mitosis observed in human cells: A small fraction of the HP1 α isoform (and, according to some reports also of HP1 β and HP1 γ) reassociates

at metaphase with centromeric regions of chromosomes (Hayakawa et al., 2003; Minc et al., 1999; Sugimoto et al., 2001). Hayakawa and colleagues demonstrated that this reassociation is chromo domain-independent and rather requires the CSD and part of the hinge region (HP1 α 101-180). Recently, it has been reported that HP1 interacts with shugoshin, a factor that is required for protection of centromeric cohesins during mitosis/meiosis (Yamagishi et al., 2008). The interaction is conserved in both *S. pombe* and humans, and shugoshin is mislocalized in the absence of HP1, suggesting that shugoshin recruitment may be the biological function of the reassociation of HP1 α with centromeric regions of chromosomes during metaphase.

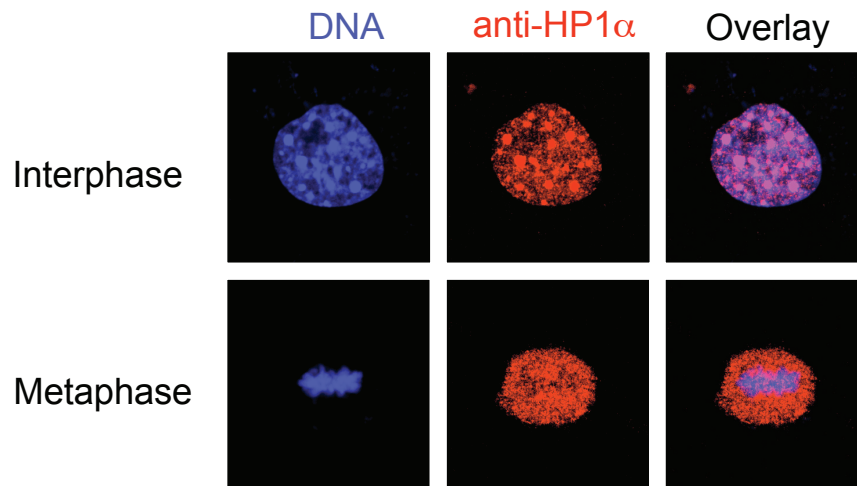


Figure 1.10: Cellular localization of HP1 in mammalian cells.

Localization of HP1 in interphase and metaphase. Immunofluorescence staining of 3T3 mouse fibroblast cells. In interphase the HP1 α signal colocalizes with densely staining regions of DNA, indicating that HP1 α is concentrated at regions of pericentric heterochromatin. (Note: These striking dots of condensed heterochromatin are specifically observed in mouse cells and not seen as clearly in other, e.g. human, cells.) In metaphase, HP1 α is dispersed throughout the cytoplasm and for the most part excluded from the condensed DNA, which is arranged in the metaphase plate. Similar observations are made with the other HP1 isoforms. DNA stained with DAPI.

The mechanism of the reversible HP1 release has not been uncovered (or had not been uncovered at the time when I started with my thesis), and the biological function of the striking changes in HP1 localization during mitosis are completely unknown.

Molecular mechanism of HP1 recruitment

Essential role of CD-H3K9me interaction

Many studies have shown that the CD interaction with H3K9me is absolutely essential for the recruitment of HP1 to chromatin. If this interaction is abolished by point mutations or deletions of the chromo domain (e.g. Cheutin et al., 2003; Thiru et al., 2004) or by removal of the K9-methyl mark (Ekwall et al., 1996; Lachner et al., 2001), HP1 is not localized to pericentric heterochromatin anymore in several *in vivo* systems. Point mutations that abolish CD binding to H3K9me in flies exhibit the same phenotypic effects as HP1 deletions (suppression of PEV, homozygous lethality) and such constructs are incapable of rescuing the deletions (Eissenberg et al., 1990; Eissenberg et al., 1992; Platero et al., 1995).

Contribution of other mechanisms

However, several *in vivo* observations suggest that CD binding to H3K9me may not be sufficient for stable chromatin association of HP1 in the cell. The chromo domain alone is not targeted properly to heterochromatin *in vivo* and shows much lower resistance to extraction with detergents (Cheutin et al., 2003; Smothers and Henikoff, 2001; Thiru et al., 2004). Even certain point mutations in the chromoshadow domain are sufficient to reduce the stability of HP1's association with heterochromatin significantly (Fanti et al., 1998; Thiru et al., 2004). Moreover, the three HP1 isoforms

have somewhat different localization patterns, even though their chromo domains bind H3K9me equally well *in vitro*. This suggests that additional molecular interactions contribute to HP1 chromatin association *in vivo*.

Several *in vitro* observations support the understanding that stable chromatin binding of HP1 involves additional interactions besides CD-H3K9me binding, especially in the context of the nucleosome (in contrast to experiments with histone peptides). In pull-down experiments with recombinant GST-HP1, the CD alone could not pull down native soluble oligonucleosomes from chicken erythrocyte extracts (Meehan et al., 2003). Binding of bacterially expressed HP1 to mononucleosomes required the full-length protein (Zhao et al., 2000). And, as Eskeland and colleagues reported, the association of recombinant *Drosophila* HP1 with highly methylated reconstituted chromatin was much weaker than with methylated peptides and presumably not sufficient to maintain a heterochromatic structure *in vivo* (Eskeland et al., 2007).

Considering the enormous increase in affinity that can be achieved through multivalency (Ruthenburg et al., 2007b), it seems possible that additional interactions besides CD binding to H3K9me could be the key to understand HP1 chromatin association *in vivo*. Several studies have proposed specific factors that may stabilize HP1 binding to chromatin. Eskeland and colleagues, e.g., found that the addition of the chromatin remodeler ACF1 (ATP-utilizing chromatin assembly and remodeling factor 1) or the HMTase Su(var)3-9 increased binding of recombinant HP1 to methylated reconstituted chromatin arrays. Both factors are suppressors of variegation known to interact with the CSD of HP1, and the effect was independent of Su(var)3-9 HMTase activity, suggesting an increase in affinity through bimodal binding of HP1 to methylated chromatin (Eskeland et al., 2007).

RNA is another molecule that has been implicated in HP1 chromatin association, based on the findings that RNase treatment of nuclei results in the release of HP1 binding in mammalian tissue culture cells (Maison et al., 2002), that HP1 recruitment to transcriptionally active heat-shock puffs depends on RNA (Fanti and Pimpinelli, 2008) and that HP1 interacts directly with RNA (Muchardt et al., 2002).

In addition, several other interactions of HP1 have been suggested to play a role in HP1's association with chromatin *in vivo*, such as the interactions with H1 and the histone H3 core (Meehan et al., 2003; Nielsen et al., 2001a) or with HOAP (Heterochromatin protein 1/origin recognition complex-associated protein; Badugu et al., 2003). However, the exact nature of these interactions is still quite enigmatic, and it seems there may be multiple factors among the huge number of HP1's molecular interactions capable of contributing to HP1's chromatin association.

H3K9me-independent chromatin association of HP1

As discussed above, the stabilization of HP1 on chromatin is a complex phenomenon and involves the CD-H3K9me interaction and additional interactions mediated by factors associated with HP1. However, the reality is actually even more complicated, because abundant observations show that there are also mechanisms of HP1 chromatin association that are completely independent of H3K9 methylation.

It has been noted, for example, that in *Drosophila* polytene chromosome stainings there is no full overlap of HP1 and H3K9me staining, indicating that particularly on euchromatic arms of chromosomes there are sites of HP1 binding that do not depend on the interaction with methylated lysine (Fanti and Pimpinelli, 2008; Li et al., 2002). In keeping with this, in chromo

domain point mutants that abolish K9me3 binding, association with sites on chromosome arms is still observed (Fanti et al., 1998). HP1 association with *Drosophila* telomeres is mediated by two mechanisms: One population of HP1 binds in a chromo domain-dependent fashion and is essential for silencing of telomeric sequences and telomere length control. The other fraction binds independently of the chromo domain and is required for telomere stability (Perrini et al., 2004).

While there are some hints about the mechanisms that could be involved in such K9me3-independent recruitment to chromatin (for example binding of the HP1 CD to H1K26me, Daujat et al., 2005); or HP1-binding proteins with DNA-binding affinity, Nielsen et al., 1999), it seems that the enigmatic multiple functions of HP1 may be related to the many means by which the protein is targeted to its site of action. Clearly much more careful research will be required to tease apart all of these different mechanisms of HP1 recruitment and chromatin binding.

Dynamics of HP1 binding

Even though the overall structure of heterochromatic domains is quite stable over time, HP1 binding to chromatin is actually highly dynamic. As demonstrated by FRAP (fluorescence recovery after photobleaching) experiments in mammalian cells, HP1 association with chromatin is characterized by rapid on-off kinetics (Cheutin et al., 2003; Festenstein et al., 2003; Schmiedeberg et al., 2004). These experiments also showed that there are at least three different subpools of HP1 with different mobilities in heterochromatin.

As described in more detail later in this thesis, I observed in my own experiments with mammalian cells some differences in the fractionation/

extraction behaviour of exogenous GFP-HP1 compared to endogenous HP1 (see Chapter 4). Thus, these results on HP1 dynamics have to be interpreted with caution.

HP1, heterochromatin nucleation and RNAi

According to current understanding, heterochromatin nucleation is distinct from the subsequent steps of heterochromatin spreading and maintenance. Two alternative mechanisms seem to be involved in the nucleation process. One way is the recruitment of HP1 by factors with DNA-binding affinity, such as Rb or KAP-1 (see Section "HP1 domains and molecular interactions: Chromoshadow domain"). HP1 in turn recruits methyltransferase activity and mediates spreading/maintenance of heterochromatic structures. This appears to be the predominate mechanism for the repressive function of HP1 in euchromatic regions.

Formation of constitutive heterochromatin, however, seems to be mediated by other mechanisms. Besides DNA-binding proteins (Jia et al., 2004; Kim et al., 2004; Yamada et al., 2005), it can be mediated by mechanisms that require the presence of repetitive DNA elements and involves the RNAi machinery, as suggested by observations made in genetic experiments with *S. pombe*. Loss of/mutations in components of the RNA interference pathway (Argonaute 1, Dicer, RNA-dependent RNA polymerase) prevent heterochromatin-specific chromatin modifications (such as H3 K9 methylation), binding of Swi6 and formation of heterochromatin at centromeres and mating type regions (Hall et al., 2002; Volpe et al., 2002). The result has been confirmed by similar findings in *Tetrahymena* (Mochizuki et al., 2002), *Drosophila* (Pal-Bhadra et al., 2004) and mouse (Kanellopoulou et al., 2005).

Through a series of other observations, a pathway is emerging that involves transcription of repetitive elements within centric regions by RNA Polymerase II into double-stranded RNAs, processing of these dsRNAs into short interfering RNAs by the RNase Dicer, and the RNA-induced transcriptional gene silencing complex (RITS). It is clear at this point that the repetitive elements are sufficient to nucleate Swi6-dependent heterochromatin at ectopic sites. However, the exact molecular details of many steps of the pathway, and how siRNAs generated during this process localize histone-modifying activities, in particular the Ctr4 HMTase, is currently not understood (see Grewal and Jia, 2007 and Kwon and Workman, 2008 for more details and references).

HP1 phosphorylation

In several organisms, it has been reported that HP1 is multiply phosphorylated. These studies detected HP1 phosphorylation upon metabolic ^{32}P -phosphate labelling of cells, and confirmed the presence of several differently charged isoforms by immunoblotting after 2D or acid urea gel electrophoresis, leading to the observation of seven to eight differently charged isoforms in *Drosophila* (Eissenberg et al., 1994), four in *Tetrahymena* (Huang et al., 1999), and three (HP1 α) and five (HP1 γ) in human tissue culture cells (HP1 β was found not to be phosphorylated in this study; Minc et al., 1999).

Changes in the phosphorylation state of HP1 correlate with specific biological events. The number of differently charged HP1 isoforms in *Drosophila*, e.g., increases at hour 1.5 – 2 of early embryonic development, around the time when cytologically visible heterochromatin appears and HP1 concentrates in heterochromatin (Eissenberg et al., 1994). In *Tetrahymena*,

HP1 hyperphosphorylation correlates with chromatin changes induced by starvation (Huang et al., 1999). And in human HeLa cells, the number of charged isoforms is specifically increased during mitosis (Minc et al., 1999).

Despite these interesting correlations, an in depth analysis of HP1 phosphorylation and its role has turned out to be difficult. Due to the lability of the modifications and the challenges in purifying a protein that is strongly associated with heterochromatic structures, none of the phosphorylation sites have really been mapped *in vivo* to date.

A few sites have been proposed based on *in vitro* phosphorylation: Pim-1 kinase can phosphorylate HP1 γ in the hinge region (the exact site was not mapped) in *in vitro* kinase assays (Koike et al., 2000). Three sites (one in the N-terminus at S15, two at the very C-terminus at S199 and S202; additional phosphorylation suggested in the hinge) were found phosphorylated in recombinant HP1a after incubation with nuclear extracts from *Drosophila* embryos (Zhao and Eissenberg, 1999). While these may indeed represent sites of *in vivo* phosphorylation of *Drosophila* HP1a, due to the known promiscuity of kinases in the test tube these results have to be interpreted with caution.

Naturally, the lack of knowledge about the exact sites of phosphorylation has hindered an understanding of the biological function of HP1 phosphorylation beyond the described correlative data. Where results were obtained, they have often not been fully conclusive, despite some curious observations. For example, while Eissenberg and colleagues had found that HP1 becomes hyperphosphorylated at the stage of development when HP1 seems to localize to heterochromatin in *Drosophila* embryos (Eissenberg et al., 1994), another study reported that hyperphosphorylated

HP1a is extracted more easily with salt from chromatin of *Drosophila* embryos (Huang et al., 1998). When Zhao and Eissenberg tested mutants of phosphorylation sites for their effect on HP1 silencing, they found that both S to A and S to E mutation showed the same effect (reduced silencing; Zhao et al., 2001). However, another study mutated nine serine residues in the hinge region of *Drosophila* HP1a (most of these residues not conserved in other organisms), including the same sites as Zhao et al. In this case *enhanced* silencing activity for both S to G and S to E mutants was observed (Badugu et al., 2005). In addition, Badugu et al. reported that HP1 dimerization and binding to H3K9me were enhanced by the mutations, while interactions with components of the *Drosophila* Origin of Replication Complex were reduced. No other interactions besides these were tested.

In summary, the sites of HP1 phosphorylation have not been successfully mapped in any organism at this point, nor have the biological function(s) of HP1 phosphorylation been fully understood. This is particularly true for the human HP1 isoforms, where not a single site has been mapped so far. Similarly, the role of HP1 phosphorylation in mitosis has not been studied at all, despite the curious observation that HP1 becomes hyperphosphorylated at this stage of the cell cycle. Some of these questions, I have tackled in my thesis project (see below).

Open questions

In recent years, enormous progress has been made in our understanding of the general principles how chromatin is organized and functions within the cell. HP1 is today one of the best-studied factors among all chromatin components. Since its discovery in the 1980s, much has been learned

about its multiple important roles in the cell, the molecules it binds to and the pathways it interacts with.

However, there are still many fundamental questions in HP1 biology that have not been answered. For example, a full understanding of the different ways how HP1-dependent heterochromatin is nucleated, of the different roles of the three HP1 isoforms, of the regulation of HP1's diverse molecular interactions, or of the exact molecular mechanism(s) of HP1-mediated gene silencing will still require plenty of research. HP1 may have additional, still undiscovered roles in the cell, as suggested by its binding partners (e.g., structural components of the nucleus, implicating HP1 in nuclear organization). When I began my Ph.D., little was known about HP1's posttranslational modifications beyond the fact that they exist. Furthermore, the molecular mechanisms underlying the relocalization of HP1 at the onset of mitosis and the biological reason for these striking localization changes remained exciting, but still unaddressed questions.

In the past few years, several studies have implicated genetic alteration of HP1 in the development of several types of human cancer. In highly invasive/metastatic breast cancer cells, HP1 α (not HP1 β or HP1 γ) is down-regulated compared to poorly invasive/nonmetastatic breast cancer cells (Kirschmann et al., 2000). Similarly, HP1 β downregulation correlates with the invasiveness of several melanoma cell lines (Nishimura et al., 2006). The involvement of HP1 in human disease underlines the importance of learning more about this important chromatin protein. A better understanding of the multiple roles that HP1 plays in the cell and the molecular mechanisms underlying its functions will not only contribute hugely to our understanding of chromatin and its functions in the cell, but may also lead to insights that could one day be used for the benefit of human patients in the clinic.

The research of my Ph.D. thesis focused on understanding the remarkable changes that HP1 undergoes during mitosis, both with respect to its localization and to its posttranslational modification profile.

Fascinated by the question of how reversible dissociation of HP1 could be achieved without any changes to the histone mark that recruits the effector, in the first part of my thesis I sought to understand the molecular mechanisms of the mitotic chromatin dissociation of HP1. Through the combination of *in vitro* and *in vivo* experiments presented in this thesis, I show that the transient release of HP1 during mitosis is achieved by a novel mechanism, methyl-phos switching, in which two histone marks cooperate to bring about dynamic release of an effector protein: Phosphorylation of H3 at serine 10 at the onset of mitosis interferes with HP1 binding to methylated lysine 9 and thus ejects the effector protein from its binding site at chromatin. *In vivo* inhibition experiments show that methyl-phos switching is a necessary step for the mitotic release of HP1.

Prompted by observations made during the study of methyl-phos switching, in the second part of my thesis I set out to learn more about the dramatic changes in HP1's posttranslational modification profile during mitosis. This led to purification/mass spectrometry identification of almost a dozen phosphorylations on the three human HP1 isoforms, most of which map to the HP1 hinge region and are upregulated in mitosis. For one particularly conserved site, I was able to identify Aurora B as the responsible kinase. In various attempts, no substantial evidence for a connection between mitotic HP1 phosphorylation and the mitotic release of HP1 or the reassociation of a subpool of HP1 to centromeres could be established. I conclude with *in vitro* data that suggests that mitotic phosphorylation of the HP1 α hinge may play a role in the regulation of HP1 association with RNA.

In sum, my thesis has revealed that HP1's behavior and interactions in mitosis are regulated by posttranslational modifications on two levels: phosphorylation of histone H3 as well as phosphorylation of HP1 itself. Some of the mechanistic findings presented in this thesis may have implications even beyond the realm of chromatin biology.

CHAPTER 2

MATERIALS AND METHODS

Reagents

Chemicals, media and recombinant proteins

Nocodazole, MG132, Microcystin LR, and Thymidine were purchased from Sigma, β -glycerolphosphate from Calbiochem, and propidium iodide from Molecular Probes. Calyculin A was from LC Laboratories. Hesperadin was received as a gift from Boehringer Ingelheim, Austria. Radiolabelled nucleotides were obtained from Amersham/GE Healthcare, and IPTG was from Acros Organics. Normal goat serum was from Jackson Immunoresearch. All tissue culture media and G-418 were purchased from Gibco. Recombinant MSK1, RSK2, and IKK α were from Upstate Biotechnology. Immunoprecipitated *Xenopus laevis* Chromosomal Passenger Complex was a kind gift of Boo Shan Tseng (Funabiki lab, Rockefeller University). All other reagents that were not generated by ourselves were from Fisher Scientific.

Peptides and peptide modification

Unmodified and posttranslationally modified peptides of the histone H3-tail (residues 1-15 and 1-20) and of the HP1 α hinge region (residues 83-101) were synthesized either at the Baylor College of Medicine Protein Chemistry Core Laboratory or the Rockefeller University Proteomics Resource Center. For H3 peptides, a non-native tyrosine residue was added at the C-terminus (for concentration determination by UV absorption measurements). For pull-down experiments, an artificial biotin group was added to the C-terminus (H3 peptides) or N-terminus (HP1 α peptides)

during synthesis. Peptides were purified by RP-HPLC (reversed-phase high-performance liquid chromatography) and characterized by MALDI-MS (Matrix-assisted laser desorption/ionization mass spectrometry) by the Proteomics Resource Center.

For fluorescence anisotropy measurements, peptides were labelled using fluorescein-5-EX succinimidyl ester (Molecular Probes) according to manufacturer's instructions and then purified by gel filtration (column: 0.5 ml G10 Sephadex, Pharmacia; buffer: 100 mM KPO₄ (pH 7.5)) and RP-HPLC.

Bacterial media

Bacteria were grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl), usually at 37° C. For selection, the following antibiotics were included in the media: ampicillin (100 µg/ml), kanamycin (50 µg/ml), or chloramphenicol (25 µg/ml).

Cell lines and tissue culture media

Human HeLa, HeLa S3, HEK293, HeP2, mouse NIH3T3, and 10T1/2 cell lines were all from ATCC. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, with 4.5 g/l D-Glucose, L-Glutamine and 110 mg/l Sodium pyruvate; Gibco) supplemented with 10% fetal bovine serum (FBS, PAA Laboratories) and penicillin–streptomycin (10 000 U/ml and 10 000 µg/ml, respectively; Invitrogen), at 37 °C in a humidified atmosphere with 5% CO₂. Cell monolayers were detached by one wash with PBS (phosphate buffered saline) followed by incubation in 0.05% trypsin/0.5 mM EDTA (Gibco).

Antibodies

For primary and secondary antibodies used, including dilutions, see Table 2.1 and Table 2.2, respectively.

Table 2.1: Primary antibodies

| Antibody | Source | Western | IF |
|---|--|----------------|-----------|
| HP1 α S92ph ¹ ("Phos-HP1 α ") | gift of R. Urrutia (Mayo Clinic, Rochester, MN) | 1:2 000 | 1:200 |
| Anti-HP1 α | MAB3446 (Chemicon) | 1:7 500 | --- |
| Anti-HP1 α | #05-689 (Upstate) (cl15.19s2) | 1:2 000 | --- |
| Anti-HP1 α | MAB3584 (Chemicon) | --- | 1:5 000 |
| Anti-HP1 β | MAB3448 (Chemicon) | 1:10 000 | 1:7 500 |
| Anti-HP1 γ | MAB3450 (Chemicon) | 1:40 000 | 1:15 000 |
| Anti-H3K9me3 | #07-442 (Upstate) | 1:1 000 | 1:1 000 |
| Anti-H3S10ph | #05-598 (Upstate) | 0.5 μ g/ml | --- |
| Anti-H3S10ph | #06-570 (Upstate) | 1:5 000 | --- |
| Anti-H3S10ph | #05-806 (Upstate) | --- | 1:4 000 |
| Anti-H3K9me3S10ph | #05-809 (Upstate) | 1:1 000 | 1:1 000 |
| Anti-H3 | Ab1791 (Abcam) | 0.5 μ g/ml | --- |
| Anti-H3 | #06-755(Upstate) | 1:1 000 | --- |
| Anti-GFP | #11814460001 (Roche) | 1:10 000 | --- |
| Anti-Aurora B | gift of S. Taylor (Ditchfield et al., 2003) | --- | 1:750 |
| Anti-Aurora B | #611082 (BD Biosciences) | 1:250 | --- |
| Anti-Aurora A | #603301(Biolegend) | 1:500 | --- |
| Anti-Flag M2 | F1804 (Sigma) | 1:2 000 | 1:1 500 |
| Anti-HA (HA.11) | MMS-101P (Covance) | 1:1 000 | 1:1 500 |
| Anti-Cyclin B1 | #05-373 (Upstate) | 1:1 000 | --- |

¹ purified rabbit IgG against HP1 α peptide 85-98 (SESNKRKSpHNFNSA)

Table 2.2: Secondary antibodies

| Antibody | Source | Western | IF |
|--------------------------------------|---------------------------|----------------|-----------|
| Anti-rabbit(IgG)-HRP | Amersham/GE Healthcare | 1:2 500 | --- |
| Anti-rabbit(IgG)-HRP | #PO399, Dakocytomation | 1:2 500 | --- |
| Anti-mouse(IgG)-HRP | Amersham/GE Healthcare | 1:2 000 | --- |
| Goat-anti-rabbit AlexaFluor488 | Molecular Probes | --- | 1:2 000 |
| Donkey-anti-rabbit AlexaFluor488 | Invitrogen | --- | 1:2 500 |
| Sheep-anti-mouse- Rhodamine Red X | Jackson ImmunoResearch | --- | 1:1 500 |

Molecular Biology

Plasmids

Constructs were generated by standard techniques of DNA cloning (Sambrook and Russel, 2001 ; for a brief description see next section), using the commercially available vectors pET11a (Novagen) for His-tagged constructs, pEGFP-N and pEGFP-C (Clontech) for GFP-tagged constructs, and pGEX (Amersham/GE Life Science) for GST-tagged constructs.

To generate His₆-tagged full-length or chromo domain constructs of the three HP1 isoforms, the full sequence of human HP1 α (GenBank BC006821; residues 1-191), HP1 β (GenBank BC002609; residues 1-185) and HP1 γ (GenBank BC000954; residues 1-183), or regions encompassing the chromodomains (HP1 α : 15-71, HP1 β : 15-72, HP1 γ : 24-81) were fused to His₆-tags by PCR with EST (expressed sequence tag) clones as templates

(HP1 α I.M.A.G.E. Consortium Clone ID 3448801; HP1 β : I.M.A.G.E. Consortium Clone ID 5013102; HP1 γ : I.M.A.G.E. Consortium Clone ID 3450099). PCR products were then cloned into the pET11a vector via the Nde I and BamH I restriction sites.

The vector pHD-N (N-terminal Flag-HA-Flag tag for expression in mammalian cells) for ligation-independent cloning (see below) was constructed by inserting an oligonucleotide cassette into the vector pIRESneo (Clontech) via the Nhe I and BamH I sites, pHD-C (C-terminal Flag-HA-Flag tag for expression in mammalian cells) was generated by inserting a cassette into pIRESneo (Clontech) via the EcoR V and BamH I sites.

The GST-Aurora B plasmid (in pGEX, Amersham/GE Healthcare) was a gift of Ciaran Morrisio (National University of Ireland, Galway). The His6-H1.4 plasmid (in pET16, Novagen) was a gift from Szabolcs Sörös (MPI for Biophysical Chemistry, Göttingen)

General DNA manipulation

For cloning and plasmid amplification, recombinase-deficient TOP10 and XL1-Blue *E. coli* strains were used (see Table 2.3). Plasmid DNA was purified with the Quagen Mini and Midi prep kits (Qiagen). DNA digestions were carried out with restriction enzymes from NEB according to manufacturer's instructions. DNA fragments were isolated with the QIAquick Gel extraction kit (Qiagen). Cut vectors were treated with calf intestine phosphatase (NEB) and then purified using the QIAquick PCR purification kit (Qiagen). Ligation of DNA was carried out with T4 DNA ligase (NEB) for 3 h at RT. Bacterial transformations were carried out by 42° C heat shock according to standard protocols (Sambrook and Russell,

2001). All plasmid DNA constructs were fully sequenced, and sequencing was carried out by Genewiz Inc. Synthesized DNA and oligonucleotides for cloning and introducing mutations were purchased from Operon, Sigma Genosys, and GenScript.

Table 2.3: Bacterial strains

| Strain | Genotype | Source |
|--------------------|--|------------|
| TOP10 | <i>F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG</i> | Invitrogen |
| XL1-Blue | <i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA lac[F['] proAB lacI^qΔM15 Tn10 (Tet^r)]</i> | Stratagene |
| BL21 | <i>F⁻ ompT hsdSB(r_B⁻ m_B⁻) gal dcm</i> | Stratagene |
| RosettaBlue | <i>endA1 hsdR17(r_{K12}⁻m_{K12}⁺) supE44 thi-1 recA1 gyrA96 relA1 lac pRARE (Cm^R) [F['] proA⁺B⁺ lacI^qΔM15 ::Tn10(Tc^R)]</i> | Stratagene |
| Rosetta(DE3) pLacI | <i>endA1 hsdR17(r_{K12}⁻m_{K12}⁺) supE44 thi-1 recA1 gyrA96 relA1 lac pLacIRARE (Cm^R) [F['] proA⁺B⁺ lacI^qΔM15 ::Tn10(Tc^R)] (DE3)</i> | Stratagene |

PCR and mutation of plasmids

PCR to amplify DNA was carried out with Pfu polymerase (dPromega) according to manufacturer's instructions. Typically, 50 µl reactions were set up with 10 ng template, 400 nM of each primer, 0.2 mM of each dNTP, 5 µl 10X Pfu buffer, and 1 U of Pfu polymerase. A typical cycling program was 95°C/3:00min-[92/0:45-48/0:30-72/1:00]_{6x} - [95/0:45-56/0:30-72/1:00]_{26x}-72/2:00-4/pause. PCR products were purified with the QIAquick PCR purification kit (Qiagen).

Mutations were introduced into plasmids with a protocol based on the "QuickChange Site-Directed Mutagenesis" procedure (Stratagene). Two primers of complementary sequence with the mutated bases in the middle

and about annealing 15-20 bases up- and downstream were designed with the QuikChange Primer Design software (at www.stratagene.com). The PCR product was purified with the QIAquick PCR purification kit (Qiagen), digested for 1 h at 37° C with Dpn I (NEB) and an aliquot used to transform bacterial cells.

Ligation-independent cloning

Vectors were linearized with the blunt-end cutter BsaB I (NEB) and PCR purified (QIAquick PCR purification kit, Qiagen). Inserts were generated by PCR. Single-stranded ends for annealing ("sticky ends") are generated in this protocol through a T4 polymerase reaction in the presence of only one dNTP, which by its 3'→5' exonuclease activity removes the second strand to the first position where this nucleotide is in the sequence. The vector and the insert are constructed in a way that their resulting annealing sequences match and can be annealed. Because the annealing sequence is much longer than in standard ligations, the annealed sequences are so stable that no ligation enzyme is required. Vector "sticky ends" were generated by carrying out a T4 polymerase reaction (NEB) in the presence of dGTP, insert "sticky ends" in the presence of dCTP. The resulting vector and insert products were mixed (molar ratio or about 1:3), after 5 min of incubation at room temperature EDTA was added to a final concentration of 10 mM. The annealing reaction was incubated for another 10 min at RT and then transformed into competent *E. coli* cells.

Cell-based methods

Mitotic arrest

To arrest HeLa cells in mitosis, 200 ng/ml nocodazole (Sigma) was added to culture media at 40-60% cell confluence. Cells were processed after

a 12 to 16 h incubation. Typical enrichment rates were about 70-80% mitotic cells.

Transfection of tissue culture cells and selection for stable cell lines

Transfection of DNA into HeLa cells was performed using Fugene 6 reagent (Roche) in accordance with the instructions of the manufacturer, typically using 3 μ l Fugene reagent and 1 μ g of DNA per 6-well. HEK293 cells were transfected by CaPO_4 precipitation following established protocols (Sambrook and Russel, 2001).

Selection was started 5 days after transfection. HeLa cells were selected for four weeks with 0.5 μ g/ml geneticin (G-418 sulfate; Gibco), HEK293 cells with 1 μ g/ml. Untransfected cells were treated at the side as a control to verify that the treatment was working. No clonal selection was carried out and no clonal cells were used in this study (because this might have been a bias towards cells expressing the transgene at higher/lower levels). For storage of cell lines, cells were frozen in fetal calf serum containing 10% DMSO (Sigma) and stored in a liquid nitrogen tank.

siRNA-mediated gene silencing

For a list of the siRNAs used, see Table 2.4. siRNA duplexes (20 μ M or 100 μ M) were stored at -80°C . For transfection with siRNAs, HeLa cells were grown for one day in DMEM/FBS without antibiotics/antimycotics. Transfection was carried out at 30-50% confluency in 24-well plates with Oligofectamine (Invitrogen), using 60 pmol siRNA duplexes and 3 μ l Oligofectamine reagent per well. To optimize transfection efficiencies, BLOCK-iT fluorescent oligos (Invitrogen) were used and transfected cells analyzed by flow cytometry. Knock-down was analyzed by SDS-PAGE of

whole-cell extracts and subsequent immunoblotting. Pilot experiments were carried out to determine which time span of incubation after transfection resulted in maximal knock-down (for HP1 protein: 3.5 d).

Table 2.4: siRNA oligos

| Name | Sequence | Source |
|-----------------------|---------------------------------|---|
| Aurora B | 5'-AACGCGGCACUUCACAAUUGA-3' | Dharmacon (Lampson and Kapoor, 2005) |
| HP1 α (5'-UTR) | 5'-GGGACCUGGUGGCCUUAGUCUUUCA-3' | Dr. W. Fischle (MPI Göttingen, Germany) |
| HP1 β | 5'-UGACACCAUAGAGGUGGCUUGAGAA-3' | Dr. W. Fischle |
| HP1 γ | 5'-GGUUACUUUGAACAAAUAA-3' | Qiagen |

Cell synchronization by double thymidine block

To obtain a highly synchronized culture of HeLa cells, thymidine (2 mM final concentration) was added to the medium of cultures grown to 20-30% confluency (first block). After 15 h, cells were washed with PBS, fresh thymidine-free medium added and the cells grown for 9 h. Thymidin was re-added (2 mM f.c.) for another 15 h (second block). To release the second block, cells were washed in PBS and fresh thymidine-free medium was added. Cell synchrony was checked by light microscopy, by western blotting with mitotic markers, and by cell cycle flow cytometry.

Harvesting tissue culture cells

Cells were harvested commonly by scraping them off the plate with a cell scraper. For highly enriched mitotic cells, mitotic shake-off was carried out (i.e. rounded-up and detached mitotic cells were selectively pipetted off the plate). If cells were to be analyzed for phosphorylation, phosphatase

inhibitors (10 mM NaF, 2 mM Orthovanadate, 10 nM Calyculin A) were added, then cells were collected by centrifugation (500 g/5 min). Cells were washed once with PBS (including phosphatase inhibitors) and then flash frozen in liquid nitrogen and stored at -80°C until further processing.

Whole cell extracts were generated by resuspending the cells in protein sample buffer, heating the mixture for 5 min to 95°C , and subsequent shearing the the DNA by sonication in a BioRuptor (Diagenode) for 5 min at setting High.

Immunofluorescence staining and confocal microscopy

For analysis by immunofluorescence microscopy, tissue culture cells were grown on cover slips in 24-well plates. Where indicated, cells were treated with hesperadin (200 nM, in DMSO) for 1 h prior to fixation. Cells were fixed with 3.7 % paraformaldehyde in PBS for 15 min and permeabilized with 0.2% Triton X-100/0.2% NP-40 in PBS. Where mentioned, the order was reversed, and the cells were first extracted with 0.2% NP-40 for 5 min and then fixed. Cells were blocked for 30 min in Blocking solution (PBS with 2.5% bovine serum albumine, 10% normal goat serum, and 0.1% Tween-20). Primary and fluorescently labelled secondary antibodies were applied in Blocking solution for 1 h at RT/over night at 4°C (primary antibodies) or for 30 min at RT (secondary antibodies), followed by three washing steps with PBS containing 0.1% Triton X-100. DNA was stained with DAPI (1 $\mu\text{g}/\text{ml}$ in PBS; Sigma) for 30 seconds. The cover slips were mounted onto microscope slides with Prolong Gold Antifade (Molecular Probes). All steps were carried out at RT unless noted otherwise.

Stainings were analyzed on an inverted LSM 510 laser scanning confocal microscope (Zeiss Axiovert 200) with a 63x/1.4 N.A. oil immersion lens

using a pinhole diameter of 1 Airy Unit. Image capture and processing was performed with the LSM 510 confocal software (Zeiss).

Cell cycle analysis and flow cytometry

Staining for cell cycle analysis was carried out following standard protocols (Darzynkiewicz et al., 1999). Cells were detached by trypsinization, collected by centrifugation (200 x g, 5 min), washed in PBS and resuspended to about 10^6 cells per 0.5 ml PBS. Ice-cold 70% ethanol was added under vortexing (to achieve a single cell suspension) and the cells were kept in fixative for at least 2 h on ice. Cells were centrifuged (500 x g, 5 min), the ethanol removed, and the cells washed in PBS. For staining of DNA, cells were resuspended in Propidium iodide staining solution, consisting of 0.1% Triton X-100 in PBS with 0.2 mg/ml DNase free RNase A (prepared by boiling RNase A for 5 min; Sigma) and 20 µg/ml of propidium iodide (Molecular Probes). After 30 min incubation at RT, the cells were analyzed on a FACSCalibur Flow Cytometry System (Becton-Dickinson). Cell cycle modelling was carried out with the software package FloJo (Tree Star) using the Watson Pragmatic Model.

Biochemical methods

Gel electrophoresis

SDS-PAGE was carried out according to the protocol by Laemmli (Laemmli, 1970) with adaptations. Usually 12% or 15% Tris-glycine gels were used, for peptides 15% Tris-tricine gels (Schagger and von Jagow, 1987; Strom et al., 1993). Gel pouring and running was carried out with the Bio-Rad minigel system. For samples intended for mass spectrometry, the Novex precast system (Novagen) was used.

Samples were boiled for 5 min in protein sample buffer (60 mM Tris (pH 6.8) 10% glycerol, 2% SDS, 140 mM β -mercaptoethanol, 0.03% bromophenol blue). Gels were run typically for 40 min at 200 V in SDS-PAGE running buffer (25 mM tris base, 200 mM glycine, 0.1% SDS). Novex gels were run according to manufacturer's instructions in the provided MOPS or MES buffer systems. Gels were stained with Coomassie (10% acetic acid, 50% methanol, 0.05% Coomassie R-250) and destained with 40% methanol/10% acetic acid.

Transfer of proteins to membrane and western blotting

Proteins separated by SDS-PAGE were transferred to PVDF membranes (Immobilon-P Millipore) in a semi-dry system (Hoefer) using Towbin buffer (192 mM glycine, 25 mM Tris-Cl, 0.1% SDS, 20% methanol, pH 8.0). Alternatively, proteins were transferred to PVDF or nitrocellulose membranes by slot blotting, using the Bio-Dot SF microfiltration apparatus (Bio-Rad) following the manufacturer's instructions.

Transferred proteins were visualized by Amido black staining (0.1 % (w/v) amido Black, 40% methanol, 10% acetic acid). Membranes were blocked for 1 h at room temperature TBS-milk, which is 5% non-fat dry milk in TBS (20 mM Tris (pH7.6), 137 mM NaCl, 0.0015% phenol red). After incubation with primary antibody (dilutions see Table 2.1) for 1 h at RT, the membrane was washed three times for 5 min with TBS. The membrane was incubated with the HRP-coupled secondary antibody (see Table 2.2) in TBS-milk for 45 min at RT, and afterwards washed three times with TBS. Blot were developed with the ECL plus chemiluminescence detection system (Amersham Biosciences) and exposures were carried out with BioMax film (Kodak).

Peptide competitions

For peptide competition experiments in western blotting, antibodies were preincubated in TBS-milk (20 mM Tris (pH7.6), 137 mM NaCl, 0.0015% phenol red, 5% non-fat dry milk) with 2 µg/ml peptide, tumbling for 1 h at room temperature. For peptide competitions in immunofluorescence stainings, antibodies were preincubated with 2 µg/ml peptide in Blocking solution (PBS with 2.5% bovine serum albumine, 10% normal goat serum, and 0.1% Tween-20) for 1 h rotating at RT. Afterwards, the preincubated antibodies were used for western blotting or immunofluorescence stainings, respectively, following the usual procedure.

Expression of recombinant proteins in *E.coli*

For expression of recombinant proteins, the recombination-deficient (and codon-optimized) *E. coli* strains BL21, RosettaBlue, and RosettaBlue(DE3)pLacI were used (see Table 2.3). A single colony from a plate was used to inoculate a 50 ml culture of the respective bacterial strain in LB including the respective antibiotic for selection, and the culture was grown over night in a shaker-incubator (37° C, 250 rpm). The next morning, the overnight culture was used to inoculate a 2 l culture of LB (37° C, 250 rpm). At an OD₆₀₀ of about 0.6, the cells were induced with 1 mM IPTG final concentration. After induction, cells were usually grown for another 3 to 5 h at 37° C. After harvesting the cells (6 000 rpm/ 10 min/4° C in SLA-1500), the pellet was immediately frozen at -80° C.

Ni-NTA purification of His₆-tagged proteins

The bacterial cell pellet was resuspended in 30 ml Lysis buffer (20 mM Tris (pH 8.0), 300 mM NaCl, 0.1 mM MgCl₂, 0.1 % Triton X-100, 2 mM imidazol,

5 mM β -Mercaptoethanol, Complete EDTA-free protease inhibitor (Roche), 1 mM PMSF), and the cells were lysed on ice with an Emulsiflex-C5 cell disrupter (10 000 psi, three passages). Insoluble cell debris was pelleted (15 500 rpm/15 min/ 4° C in SS34), meanwhile Ni-NTA beads (nickel nitrilotriacetic acid, Qiagen; 1 ml per 5 mg of expressed recombinant protein) were equilibrated with 50 ml Lysis buffer. The soluble supernatant of the disrupted cells was applied to the Ni-NTA beads and incubated rotating for 20 min at 4° C. After three washes "in batch" with Lysis buffer, the beads were transferred to a plastic chromatography column, washed with 4 column volumes of Column Wash buffer (20 mM Tris (pH 8.0), 1 M NaCl, 0.1 % Triton X-100, 0.1 mM MgCl_2 , 30 mM Imidazol, 5 mM β -Mercaptoethanol). The recombinant protein was eluted with 6 column volumes of Elution buffer (20 mM Tris (pH 8.0), 300 mM NaCl, 0.1 mM MgCl_2 , 150 mM Imidazol, 5 mM β -mercaptoethanol), dialyzed against Dialysis buffer (25 mM Tris (pH 8.0), 150 mM NaCl, 1 mM DTT) and concentrated with Centricon centrifugal filter units (Millipore). Protein concentrations were determined by absorbance spectroscopy using predicted extinction coefficients. Proteins were stored at 4° C or shock frozen and stored at -80° C.

Column chromatography

All protein purification chromatography with the exception of Ni-NTA purification was carried out on ÄKTA Purifier fast protein liquid chromatography (FPLC) instruments (Amersham/GE Healthcare). Gel filtration as a second purification step for GST-Aurora B was performed with a Superdex 75 (Pharmacia; 20 mM imidazole (pH 8.0), 25 mM NaCl, 2 mM DTT). Separation of RNA from the recombinant HP1 hinge was carried

out on a MonoS column (Amersham/GE Healthcare) with a linear gradient from 50 mM KCl to 1 M KCl. Purity was routinely confirmed by SDS-PAGE and Coomassie staining.

GST purification of Aurora B

GST-tagged recombinant Aurora B was prepared by GST purification according to the protocol from Dr. Ciaran Morrison, National University of Ireland, Galway. All steps of the procedure were carried out at 4° C. Briefly, bacterial cells were lysed in Lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 50 mM EDTA, 1 mM DTT, 10% glycerol, 1 mM PMSF) with an Avestin EmulsiFlex C5 cell disruptor (three passages). Insoluble cellular debris was removed by centrifugation (15 500 rpm, 15 min, SS34 rotor). The supernatant was applied to Glutathione Sepharose (Fast flow 4B, Amersham/GE) and incubated on a rotator for 1.5 h. Unbound proteins were washed off (four washes with Lysis buffer), transferred into a plastic column (Bio-Rad) and eluted with Elution buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 10 mM EDTA, 1 mM DTT, 10% glycerol, 25 mM GSH (reduced glutathione), 1 mM PMSF). The eluate was eluted over night against 50% glycerol/50% XBE2 (10 mM K-HEPES (pH 7.7), 50 mM sucrose, 100 mM KCl, 2 mM MgCl₂, 0.1 mM CaCl₂, 2 mM K-EGTA, pH adjusted to 7.9). After protein concentration with a Centricon centrifugal filter units (Millipore), aliquots were flash frozen in liquid nitrogen and stored at -80° C.

Acid extraction

Cells were lysed by resuspending them directly in 0.2 M H₂SO₄ and agitated on a rotator for 1 h at 4° C. Insoluble matter was pelleted by centrifugation and the soluble proteins were precipitated by adding trichloroacetic acid

(TCA) to a final concentration of 25%. After 1 h incubation on ice, proteins were collected by centrifugation, washed once with acetone containing 0.1% HCl, a second time with pure acetone, and dissolved in a buffer for further processing or dried and stored at -80°C .

Preparation of Dignam extracts

Nuclear extracts for peptide pull-downs with the HP1 α (83-101) S92ph peptide were prepared following the procedure by Dignam and colleagues (Dignam et al., 1983). All steps were carried out at 4°C and protease inhibitors (Complete protease inhibitor cocktail, Roche) and phosphatase inhibitors (10 mM NaF, 5 mM Orthovanadate, Calbiochem Phosphatase Inhibitor Cocktail Set I [final concentrations 25 μM Bromotetramisole oxalate, 5 μM Cantharidin, 5 nM Microcystin-LF]) were used in all buffers.

The cell pellet was resuspended in 5 PV (pellet volumes) of buffer A (10 mM HEPES, 1.5 mM MgCl_2 , 10 mM KCl, 0.5 mM DTT, pH 7.9) and incubated on ice for 5 min to swell the cells. Then the cells were collected by centrifugation (750 rpm, 10 min), the supernatant was removed and the cells resuspended in 2 PV buffer A. Cell lysis was carried out with a Dounce homogenisator (pestle B) with 15 to 20 strokes. Nuclei were collected by centrifugation (1500 rpm, 7 min), and the supernatant flash frozen in liquid nitrogen (cytosolic fraction). The nuclei were resuspended in 4 ml buffer B (20 mM HEPES, 25 % (v/v) glycerol, 45 mM NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, 0.5 mM DTT, pH 7.9) and broken by another round of douncing in this buffer. The suspension was centrifuged at $25000 \times g$ for 30 min. The supernatant was dialyzed against buffer C (50 mM Tris-Cl, 120 mM NaCl, 0.5 mM EDTA, 0.1% NP-40, 0.5 mM DTT, 1 mM PMSF,

pH 7.5) for 5 h. Aliquots were flash frozen in liquid nitrogen and stored at -80°C until further use.

Alternative biochemical fractionation of cells

As an alternative to the Dignam protocol, the procedure of Mendez and Stillman was used to fractionate cells (Mendez and Stillman, 2000). All steps were carried out on ice and phosphatase inhibitors were included in all buffers (10 nM Calyculin A, 10 mM NaF, 5 mM Orthovanadate, Calbiochem Phosphatase Inhibitor Cocktail Set I [final concentrations 25 μM Bromotetramisole oxalate, 5 μM Cantharidin, 5 nM Microcystin-LF]). HeLa cells ($5 \cdot 10^8$ cells) were resuspended in 25 ml of Buffer A (10 mM HEPES (7.9), 10 mM KCl, 1.5 mM MgCl_2 , 0.34 M sucrose, 10% glycerol) including 0.2% NP-40 and incubated for 7 min on ice to lyse the cells. Cell nuclei were pelleted with a low-speed spin (1500 rpm, 5 min, in Eppendorf 5810R table-top centrifuge). While the supernatant (Cytosolic fraction) was transferred to a new tube and the salt concentration was adjusted to 250 mM NaCl, the nuclei were washed once with Buffer A (without NP-40) and then disrupted by resuspending them in 25 ml NoSalt buffer (3 mM EDTA, 0.2 mM EGTA). The nuclei were incubated for 30 min on ice, interrupted by occasional vortexing. By centrifugation (3000 rpm, 5 min, in Eppendorf 5810R), chromatin was collected as the pellet. The supernatant (soluble nuclear fraction and proteins stripped off the chromatin with NoSalt buffer) was adjusted to 250 mM NaCl. To solubilize most chromatin-bound proteins, the chromatin pellet was extracted with 5 ml HighSalt solubilization buffer (50 mM Tris (8.0), 2.5 M NaCl), vortexed, and chromatin sheared with a BioRuptor sonicator (Diagenode) for 10 min at setting High. Unsolubilized material was pelleted (4000 rpm, 5 min) and the supernatant (solubilized chromatin proteins) diluted down to 250 mM NaCl.

Peptide pull-down

Peptide pull-downs were carried out as previously described (Wysocka, 2006; Wysocka et al., 2005). Typically, 100 µg of biotinylated peptide in PBS were prebound to 400 µl of avidin-coated beads (Pierce) for 5 h at 4° C with rotation. Unbound peptide was removed by three washes with PBS/0.1% Triton X-100. Immobilized peptides were incubated with cellular extracts for 3 h at 4° C. Beads were washed (six washes with Washing buffer (20 mM HEPES (pH 7.9), 150 mM KCl, 20% v/v glycerol, 0.2 mM EDTA, 0.1% Triton X-100). For more or less stringent washing, 3 washes with 300 mM or 50 mM KCl were carried out, respectively. In case phosphorylated peptides were used, phosphatase inhibitors (10 mM NaF, 5 mM Orthovanadate, Calbiochem Phosphatase Inhibitor Cocktail Set I [final concentrations 25 µM Bromotetramisole oxalate, 5 µM Cantharidin, 5 nM Microcystin-LF]) were included at all steps,. Protease inhibitors (Roche Protease inhibitor cocktail and 1 mM PMSF) were included from the point of adding the cellular extract. Peptides and bound proteins were eluted by boiling the beads in protein sample buffer. Samples were run on SDS-PAGE gels and stained with Coomassie or by silver staining (SilverSnap Staining Kit, Pierce).

Anti-FLAG immunoprecipitation of tagged HP1 constructs /

Immuno-precipitation of endogenous HP1

Immunoprecipitation of exogenous, Flag-tagged HP1 proteins expressed in HeLa cells was carried out with anti-Flag M2 beads (Sigma). Phosphatase inhibitors were included in all buffers (10 mM NaF, 2 mM Orthovanadate, 10 nM Calyculin A). Beads were washed twice in Wash buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.05% NP-40), then the beads were incubated

with the extracts (Cytosolic, NoSalt-soluble and HighSalt-soluble) for 45 min at 4° C. Beads were washed three times with 1M-Wash buffer (50 mM Tris-Cl, pH 8.0, 1 M NaCl, 0.1% NP-40), brought down in Wash buffer to a volume of 1 ml, spun down again and resuspended in Storage buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl). Aliquots were taken during the whole procedure and the different steps subsequently analyzed by western blotting. Samples were shock frozen in liquid nitrogen, stored at -80° C, shipped to the Hunt lab on dry ice and analyzed there by mass spectrometry.

Immunoprecipitation of endogenous HP1 isoforms was carried out essentially by the same procedure, with a few minor differences: monoclonal HP1 antibodies (HP1 α : MAB3584, HP1 β : MAB3448, HP1 γ : MAB3450 (Chemicon)) were immobilized on magnetic sheep-anti-mouse beads (M280 Sheep-anti-mouse IgG, Dynal Biotech). During washes, beads were collected not by centrifugation, but with a magnet.

RP-HPLC

Separation of acid extracted proteins was carried out by RP-HPLC (reversed-phase HPLC) on a C8 column (220 x 4.6 mm Aquapore RP-300 (PerkinElmer) or Vydac 208TP510). A linear gradient of 35-60% solvent B (solvent A: 5% acetonitrile, 0.1% trifluoroacetic acid; solvent B: 90% acetonitrile, 0.1% trifluoroacetic acid) was applied over 75 min at 1.0 ml/min flow-rate on a Beckman Coulter System Gold 126 Pump Module and 166/168 Detector. Peptides were purified by a similar procedure on a C18 column. Fractions were subsequently lyophilized and stored at -80° C.

***In vitro* kinase reactions and analysis**

Kinase reaction with H3 peptides (1-5 μ M) or recombinant HP1 proteins (500 ng) were typically carried out in a 50 μ l volume of Kinase Buffer (15 mM MOPS (pH 7.2), 30 mM MgCl_2 , 25 mM NaCl, 10 mM β -glycerolphosphate, 2 mM EGTA, 1 mM DTT) with 150 μ M ATP and 2-3 μ Ci [γ - ^{32}P]-ATP. 5 μ l of immunoprecipitated chromosomal passenger complex (CPC) (kindly provided by Boo Shan Tseng, Funabiki Laboratory, Rockefeller University), 100 ng of commercial recombinant kinases (MSK1, RSK2, IKK α ; UBI/Millipore), or 2 μ g of recombinant Aurora B kinase (own preparation) were used. Where indicated, hesperadin was added at a final concentration of 4 μ M. Reactions were stopped after 60-90 min by adding acetic acid to 30% (v/v) and then either run on 15% Tris-Glycine or 20% Tris-Tricine gels for analysis by autoradiography or analyzed by filter binding assay.

For filter binding assays, the reaction mixture was spotted onto P81 filter paper (Whatman). The filter paper was washed three times with 0.75% phosphoric acid, once with acetone, dried and then analyzed by scintillation counting on the ^3H -channel [sic] in a Beckman LS6000IC scintillation counter.

For non-radioactive kinase reactions with fluorescinated peptides, 100 nM peptide, 1 mM ATP and 10 μ l CPC were used. The reactions were stopped with a final concentration of 0.5% (v/v) trifluoro acetic acid and peptides were analysed by MALDI-TOF mass spectrometry.

"Bioinformatics" methods

Alignments were generated with ClustalW (at www.ebi.ac.uk/Tools/clustalw2), graphic depictions of alignments with Boxshade (at <http://mobyli.pasteur.fr>).

Kinase predictions were carried out with the Group Based Prediction System (Xue et al., 2008). Depiction of crystal structures were generated with PyMol (DeLano, 2008). Extinction coefficients for recombinant proteins were calculated from their amino acid sequence using the ProtParam tool (ExPASy, <http://ca.expasy.org>).

Other methods

Fluorescence anisotropy binding measurements

Fluorescence anisotropy binding assays were typically performed in a 100 μ l volume of 20 mM imidazole (pH 8.0), 25 mM NaCl, 2 mM DTT, and 100 nM fluorescein-labelled peptide (Jacobs et al., 2004). Measurements were made on a Plate Chameleon multilabel counter (Hidex), and fluorescence polarization (P) values were converted to anisotropy (A) values by the equation $A = 2P / (3-P)$. For the analysis of binding curves, non-linear least-squares fitting of the data was carried out with the software KaleidaGraph (Synergy Software) using the equation $A = [A_f - (A_b - A_f)] \cdot [\text{protein}] / (K_d + [\text{protein}])$ where A_f and A_b are the anisotropy values of the free and bound peptides, respectively.

Mass spectrometry

Mass spectrometry analysis of HP1 proteins was carried out by Hillary Montgomery (Hunt lab, University of Virginia). Analysis of peptides was carried out by MALDI-TOF mass spectrometry on a Voyager RP STR instrument (ABI) in linear mode using hydroxy cinnamic acid as matrix.

Phage display screen

The phage display screen for proteins binding to HP1 α in a manner dependent on HP1 α S92 phosphorylation was carried out with the T7Select

system (Novagen). This screen was carried out by myself in the laboratory of Dr. Wolfgang Fischle (Goettingen, Germany).

Two commercially available cDNA phage libraries (T7Select Human Liver cDNA Library and T7Select Human Brain cDNA Library, Novagen) were used. Upon amplification of the libraries, an aliquot of the liver library was supplemented with a T7 phage with the HP1 α gene cloned into it (kindly provided by Henriette Franz, Fischle lab) in a 1:10⁶ dilution, to serve as a positive control in the screen. For amplification of T7 phages, BLT5403 *E. coli* cells grown in TB (12 g bacto tryptone, 24 g yeast extract, 4 ml glycerol, 2.31 g KH₂PO₄ and 12.54g K₂HPO₄ per 1000 ml) were used. Phage concentration was determined by plaque assay (a diluted aliquot of the phage-containing solution was mixed with uninfected *E. coli* bacteria, plated, and incubated over night at 37° C; plaques of killed bacteria were counted the next day and phage titers calculated).

Biotinylated HP1 α peptides (83-101, unmodified and phosphorylated at serine 92) and histone H3 peptides (1-20, unmodified and tri-methylated at lysine 9) were immobilized on avidin beads (Pierce). For the biopanning selection procedure, the phage mixture was incubated with the beads for 2 h rotating at 4° C. Unbound phage was washed off (three washes with PBS including 0.05% Tween-20). During all these steps, buffers included 25 mM NaF and 25 mM β -glycerolphosphate as phosphatase inhibitors. Phages selected in the pull-down were amplified by adding the beads with bound phages to a 50 ml culture of BLT5403 bacteria and growing the culture over night at 37° C. Phages obtained from this amplification were used for the next round of biopanning selection. Five rounds of biopanning were carried out.

For a qualitative analysis of the selection procedure, a PCR reaction was carried out with (template: aliquots from the phage solution taken at each round of selection; primers" specifically designed to anneal at the 5'- and 3'-ends of the phage insert).

Electrophoretic mobility-shift assay (EMSA)

Electrophoretic mobility-shift assays (EMSA) were carried out as described (Akhtar et al., 2000). Briefly, single-stranded probe RNA was *in vitro* transcribed with the MEGAscript T7 Kit (Ambion) using a 500 nucleotide fragment of the human cyclin E gene as template. The RNA was radioactively labeled by 5'-end-labeling with T4 Polynucleotide kinase (NEB) and [γ -³²P]ATP and purified with the RNeasy kit (Qiagen). Binding reactions were carried out in 20 μ l of Binding Buffer (20 mM HEPES (pH 7.6), 100 mM KCl, 2 mM EDTA, 0.01% NP-40, 1 mM DTT) including 20 U RNasin Ribonuclease inhibitor (Promega), 10,000 cpm labelled RNA (about 1 pmol). Reactions were incubated for 30 min on ice, and then run on a 4% native 0.5x Tris-borate-EDTA gel (prerun for 30 min at 100V at RT) for 3 h at 250 V at 4° C. Data was imaged with a FLA-5000 phosphorimager (Fujifilm).

CHAPTER 3

REGULATION OF THE CHROMATIN ASSOCIATION OF HP1 THROUGH PHOSPHORYLATION OF HISTONE H3: "METHYL-PHOS SWITCHING"

Introduction

In recent years, it has become increasingly clear that dynamic changes in chromatin structure actively regulate all kinds of DNA-dependent processes, ranging from transcription and mitotic chromosome condensation to DNA-repair and apoptosis (Felsenfeld and Groudine, 2003). Functions of chromatin are controlled, among other ways, by posttranslational modifications of N-terminal histone tails (Kouzarides, 2007; Peterson and Laniel, 2004). These modifications can serve as binding sites for the recruitment of modification-specific binding proteins (effectors), which then direct downstream functions (de la Cruz et al., 2005; Strahl and Allis, 2000).

Recruitment of binding partners to chromatin is often dynamic, and the ability to release effector proteins from their binding site enables the cell to react to changes in environmental cues (Felsenfeld and Groudine, 2003). In many cases, reversibility is achieved efficiently by the enzymatic removal of relatively short-lived posttranslational modifications. For example, removal of histone acetylation can be achieved by histone deacetylases (Keogh et al., 2006; Sterner and Berger, 2000). Alternatively, histone tails can be clipped proteolytically (Allis et al., 1980; Duncan et al., 2008) or even the complete histone can be replaced (Ahmad and Henikoff, 2002). What all these mechanisms have in common is that they result in elimination of the posttranslational modification that recruits the effector.

However, there are also situations where a histone modification appears to be more stable, yet dynamic behaviour of an effector protein is nevertheless observed. This suggests the existence of other, so far unknown mechanisms to control the release of effector proteins from their binding sites at chromatin.

One such case where an effector protein dissociates from its binding site at chromatin, while the histone mark persists, is the mitotic release of Heterochromatin protein 1 (HP1).

HP1, an effector protein with important functions in heterochromatin formation and gene silencing, is recruited to chromatin by histone H3 methylation at lysine 9 (H3K9me) (Bannister et al., 2001; Lachner et al., 2001). In interphase cells, HP1 is tightly associated with chromatin. At the onset of mitosis, however, the protein is released from its binding site at H3K9me (see Introduction, Figure 1.10; Hayakawa et al., 2003; Minc et al., 1999; Schmiedeberg et al., 2004), even though the methyl-mark it binds to is not reduced. Although this phenomenon has been observed for years (Bannister et al., 2002), the mechanism of this dissociation was completely unknown.

In this chapter, I describe experiments that I carried out in collaboration with a postdoctoral fellow in the Allis laboratory, Dr. Wolfgang Fischle, to elucidate the mechanism of HP1 release from chromatin during mitosis. Other key experiments, using the *Xenopus* cell-free system, were carried out in collaboration with the laboratory of Dr. Hironori Funabiki (data not presented in this thesis). In a combination of biophysical *in vitro* and *in vivo* immunofluorescence experiments, we show that the mitotic release of HP1 from chromatin is mediated by histone H3 phosphorylation, thus implicating histone H3 phosphorylation in the regulation of HP1

chromatin association. Our findings define a novel mechanism to regulate binding of an effector protein, "methyl-phos switching", in which two modifications work together to bring about dynamic control of binding to a histone mark, with far-reaching implications, inside as well as outside of the realm of chromatin.

Results

HP1 is released during mitosis, even though the H3K9me3 mark persists

We began our investigation into HP1's chromatin association by reproducing the observation that HP1 is released from chromatin, while the H3K9me3 mark persists (Bannister et al., 2002; Kellum et al., 1995; Minc et al., 1999). Immunofluorescence microscopy (IF) of mouse fibroblast cells (Figure 3.1A) clearly shows that HP1 dissociates from chromatin in mitosis, while the H3K9me3 signal is still observed at the DNA.

To analyze the levels of the H3K9me3 mark throughout the cell-cycle in a more quantitative way, we carried out western blotting experiments. Comparison of asynchronously growing HeLa cells with cells arrested in mitosis by nocodazole treatment clearly shows that the overall level of H3K9me3 in the cell does not change at the different stages of the cell cycle (Figure 3.1B).

Taken together, the IF and immunoblotting experiments confirm that HP1 is released during mitosis from chromatin, even though the mark that is largely responsible for its recruitment, H3K9me3, remains unchanged. Naturally, this finding raises the puzzling question of how an effector protein like HP1 can be released, when the histone mark mainly responsible for its recruitment is stable.

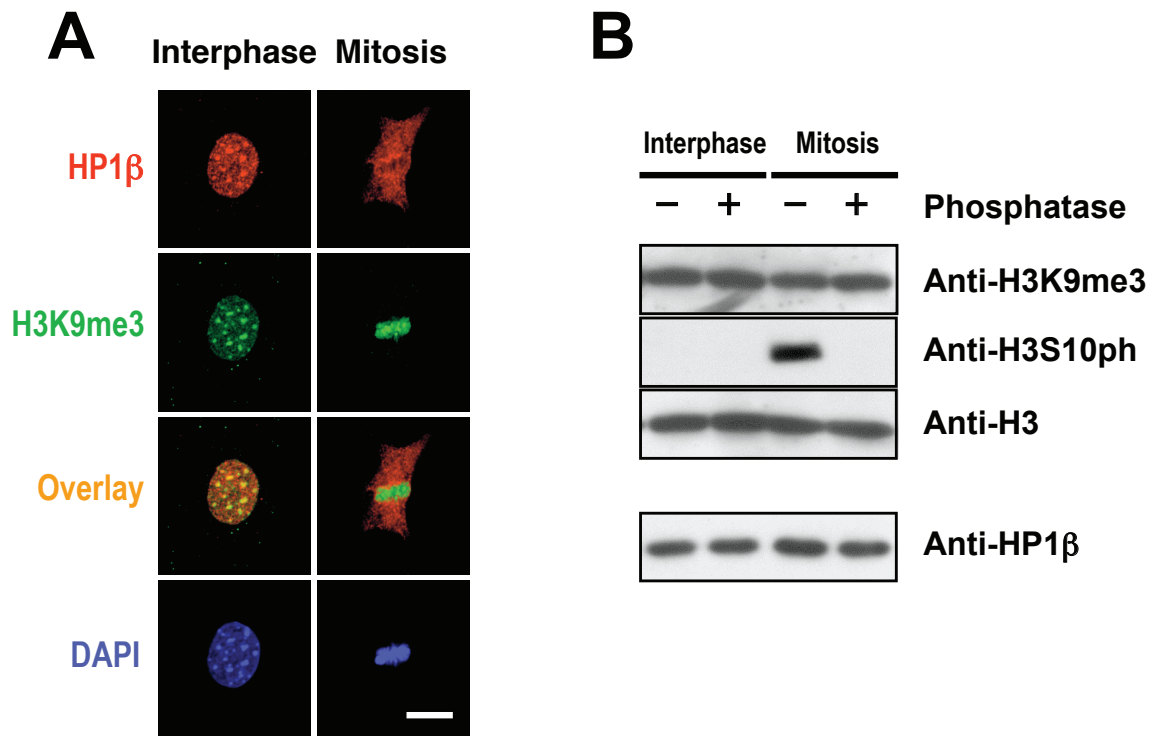


Figure 3.1: HP1 dissociates from chromatin during mitosis, even though the H3K9me3 mark persists (in collaboration with Dr. Wolfgang Fischle).

A: Immunofluorescence microscopy of mouse 10T1/2 fibroblast cells. In interphase, HP1 β localizes to dot-like structures throughout the cell nucleus that coincide with H3K9me3 and DAPI-dense regions, indicating HP1's association with pericentric heterochromatin Maison and Almouzni, 2004. In mitosis, HP1 β is dispersed throughout the cytoplasm, while the H3K9me3 signal still overlaps with the DNA aligned in the metaphase plate. Similar observations were made for HP1 α and HP1 γ . Overlay generated from HP1 β and H3K9me3 channels. Scale bar, 10 μ m.

B: Immunoblotting of extracts obtained from asynchronously growing (Interphase) or nocodazole-arrested (Mitosis) HeLa cells. Acid-extracted histones (H3K9me3, H3S10ph and H3 blots) or total cell extracts (HP1 β blot) were treated with or without alkaline phosphatase and then analyzed by western blotting. The H3S10ph signal confirms the enrichment for mitotic cells upon nocodazole arrest. No significant changes in the global H3K9me3 levels or in the expression of HP1 β through the cell cycle are observed (similar results obtained for HP1 α and HP1 γ).

The Binary Switching Hypothesis

To explain this enigma of effector protein release from a stable histone mark, Drs. Fischle and Allis proposed a novel mechanism for how cells might control binding of effector proteins to posttranslational modifications. According to their "Binary Switching" hypothesis (Fischle et al., 2003a), dynamic regulation of the read-out of a stable histone mark can be achieved through an adjacent reversible modification. Addition of the reversible modification would interfere with the binding of the effector protein and thus releases the effector from its binding site. Removal of the reversible modification, on the other hand, would allow the effector protein to reassociate (Figure 3.2).

How could such a binary switch be constructed in the specific case of H3K9me3 and HP1? H3K9me3 might form a "methyl-phos switch" by collaborating with the adjacent phosphorylation of H3 at serine 10 (H3S10ph), a mitotically robust histone mark that has been known for years (Hendzel et al., 1997).

H3S10 phosphorylation is a very prominent mark in all eukaryotic cells during mitosis, when it is observed globally and on all chromosomes (Hsu et al., 2000; Kaszas and Cande, 2000; Van Hooser et al., 1998; Wei et al., 1998; Wei et al., 1999). In fact, due to its abundance serine 10 phosphorylation has been used widely as a general marker for mitosis. However, although this mark and the enzymes responsible for its steady-state balance have been characterized quite well, the biological role of this modification is still elusive (Hsu et al., 2000; Prigent and Dimitrov, 2003; Wei et al., 1999). According to the binary switching theory, at least one of the biological functions of serine 10 phosphorylation in mitosis might be to regulate binding of the effector protein HP1 to H3K9me, a process that we refer to as "methyl-phos switching".

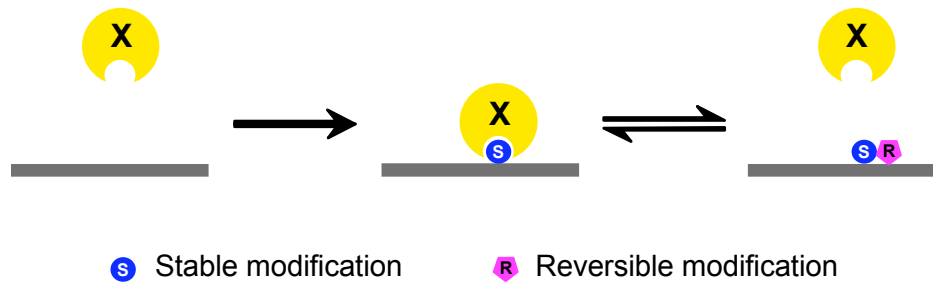


Figure 3.2: The Binary Switching Hypothesis.

According to the Binary Switching Hypothesis Fischle et al., 2003, dynamic read-out of a stable histone mark can be achieved through an adjacent reversible modification. A stable modification S (blue circle) serves as a binding site to recruit the effector protein X. Addition of the reversible modification R (pink pentagon) immediately adjacent to the stable modification sterically interferes with the binding of X to the stable modification and therefore causes the release of the effector protein. Upon removal of the reversible modification, the effector X can reassociate with its binding site. Together the two modifications work like a switch for the recruitment and release of a specific binding protein. Such switches are possible for various combinations of modifications, such as "methyl-phos", "acetyl-phos", or "methyl-acetyl" switches.

Several observations suggested to us that this might indeed be the case. Firstly, we had evidence for the existence of the dual modification mark H3K9meS10ph *in vivo* through mass spectrometry. In collaboration with the laboratory of Dr. Donald Hunt (University of Virginia, Charlottesville), Dr. Fischle had examined the modification profile of histones obtained from mitotic HeLa cells. This had led to the identification of a novel dual modification mark, where lysine 9 methylation and serine 10 phosphorylation are present at the same time on one and the same H3 tail (H3K9me1S10ph, H3K9me2S10ph, and H3K9me3S10ph).

Secondly, Dr. Fischle had validated the identification of this novel dual histone mark through an independent method. After raising an antiserum

that specifically recognizes the dually-modified H3K9me3S10ph epitope, western blotting confirmed that the dual-mark epitope is specifically detected on histones of mitotic cells, while it is not found in histones purified from asynchronously growing cells.

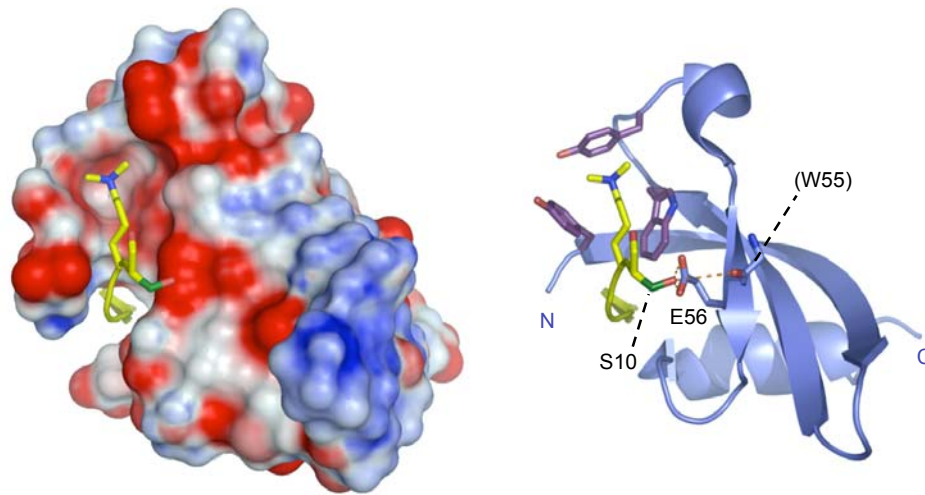


Figure 3.3: Binding of the HP1 chromo domain to H3K9me3 is likely to be impaired by the presence of H3S10ph.

Crystal structure of the *Drosophila melanogaster* HP1 chromo domain (23-76) interacting with the H3 tail tri-methylated at K9 (Jacobs and Khorasanizadeh, 2002). Peptide in yellow, K9me3 and S10 shown in detail, C β of serine 10 in green.

Left: Electrostatic surface charge rendering of the HP1 CD (negative charge in red, positive charge in blue) interacting with an H3K9me3 tail peptide. H3 serine 10 is close to a negative patch of the CD. From this depiction it is evident that phosphorylation of serine 10 will lead to steric interference with the CD, as well as repulsion between the negative charge of the phosphate and the negative patch of the CD. **Right:** Ribbon depiction of the HP1 CD interacting with an H3K9me3 tail peptide. The three caging residues of HP1 that coordinate trimethyl-K9 of H3 are shown in purple. H3 serine 10 undergoes multiple hydrogen bonds with glutamate 56 and a carbonyl group of the HP1 backbone (W55). Phosphorylation of S10 would prevent formation of this network of hydrogen bonds. The sum of these effects (steric hindrance, electrostatic repulsion, and loss of hydrogen bonds) is likely to abolish the interaction between the H3 tail and the HP1 chromo domain upon phosphorylation of H3 serine 10.

Thirdly, a close examination of the co-crystal structure of the HP1 chromodomain bound to a methylated H3 tail peptide (Fischle et al., 2003b; Jacobs and Khorasanizadeh, 2002; Nielsen et al., 2002) revealed that serine 10 phosphorylation was likely to interfere with chromodomain binding by effects of steric hindrance, electrostatic repulsion, and loss of hydrogen bonds (see Figure 3.3).

Taken together, these observations all pointed in the direction that dissociation of HP1 from chromatin during mitosis might indeed be achieved by a binary switching mechanism involving the H3K9me3S10ph dual mark.

Reduced binding affinity of HP1 to H3K9me3S10ph peptides *in vitro*

As a first test of this hypothesis, we attempted to verify the prediction from the co-crystal structure that HP1 binding to H3K9me3 would be affected by the presence of adjacent serine 10 phosphorylation. In *in vitro* binding experiments by fluorescence anisotropy, we compared HP1 binding to a singly modified H3K9me3 peptide with binding to a peptide carrying the dual modification (H3K9me3S10ph). These experiments revealed that binding of HP1 β to a dually modified H3K9me3S10ph peptide is significantly weaker than binding to a H3K9me3 peptide (Figure 3.4). Similar measurements were also made for the chromo domains of the other human HP1 isoforms and for full-length HP1 proteins (Table 3.1), therefore confirming that binding of HP1 to methylated lysine 9 is indeed impaired by phosphorylated serine 10.

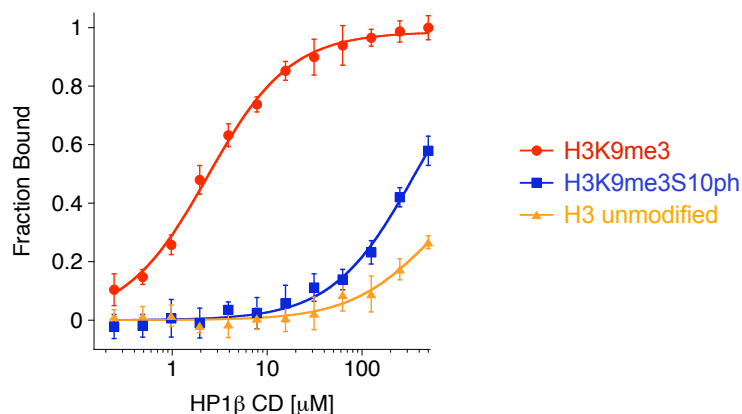


Figure 3.4: The binding affinity of the HP1 β chromo domain to methylated H3 peptides is significantly reduced by the presence of serine 10 phosphorylation.

Binding curves determined by fluorescence anisotropy for interaction of the HP1 β chromo domain with the indicated peptides. The HP1 β chromo domain binds to a H3 (1-20) K9me3 peptide with the expected binding affinity in the low micromolar range (red plot). The interaction with a dually modified peptide H3 (1-20) K9me3S10ph was significantly weaker ($> 500 \mu\text{M}$; blue plot). (The K_d can be estimated from this graph as the concentration of HP1 β CD for which the Fraction bound is 0.5.)

Table 3.1: Dissociation constants (K_d) for the binding of human HP1 chromo domains to different H3 peptides (data generated by Dr. Wolfgang Fischle)

| | H3 unmodified | H3K9me3 | H3K9me3S10ph |
|----------------------------------|----------------------|----------------|---------------------|
| HP1α CD | > 500 | 13 \pm 3 | > 500 |
| HP1β CD | > 500 | 3 \pm 1 | 360 \pm 60 |
| HP1γ CD | > 500 | 7 \pm 2 | 500 \pm 100 |
| FL-HP1α | > 500 | 18 \pm 4 | > 500 |
| FL-HP1β | > 500 | 6 \pm 1 | 450 \pm 80 |
| FL-HP1γ | > 500 | 7 \pm 2 | > 500 |

(values in $\mu\text{M} \pm \text{s.d.}$)

Even though K_d values for the interaction of HP1 proteins with H3K9me3S10ph are not quite as low as for the unmodified H3 peptide, they are two orders of magnitude lower than for H3K9me3. Note that dissociation constants greater than 500 μM cannot be determined by this assay. H3 peptides: residues 1-20. CD: chromo domain. FL: full-length.

Reconstitution of the release mechanism in the test tube

While these results were encouraging, simple measurement of binding affinities does of course not at all reproduce the dynamic environment within the cell. Here, serine 10 would have to be phosphorylated by a kinase, while the neighboring H3K9me3 is engaged by HP1, and this phosphorylation would subsequently result in the release of the effector protein.

We realized that it would be almost impossible to tease apart the molecular mechanism of mitotic HP1 release directly by *in vivo* experiments. Redundancy among H3 genes (about a dozen in human cells, Marzluff

et al., 2002) and the enormous complexity of the cellular events during mitosis makes it very difficult to obtain more than correlative data in this question from the tissue culture system. Therefore, we decided to pursue an extreme reductionist approach, trying to reconstitute the complete release mechanism of HP1 with purified components in the test tube.

Identification of kinases capable of phosphorylating H3S10 in the presence of H3K9 methylation

As a first step towards this reconstitution, I sought to identify conditions for efficient and specific *in vitro* phosphorylation of H3 peptides. The mammalian kinase responsible for mitotic phosphorylation of histone H3 at serine 10 is Aurora B kinase, a component of the Chromosomal Passenger Complex (CPC) (Hsu et al., 2000; Vagnarelli and Earnshaw, 2004). However, it had been reported that Ipl1, the homolog of Aurora B in *S. cerevisiae*, is not capable of phosphorylating H3 *in vitro*, if lysine 9 is methylated (Rea et al., 2000). I therefore tested not only recombinant Aurora B kinase for its ability to phosphorylate H3 serine 10 *in vitro*, but also several other H3 kinases, such as IKK α (I κ B kinase α), MSK1 (Mitogen- and stress-activated kinase 1), and RSK2 (Ribosomal S6 kinase 2), that have been reported to phosphorylate serine 10 at specific genomic loci upon immediate early gene activation (Anest et al., 2003; Sassone-Corsi et al., 1999; Strelkov and Davie, 2002).

To my surprise, all these kinases were capable of phosphorylating H3 peptides methylated at lysine 9 in radioactive *in vitro* kinase assays (Figure 3.5). Phosphorylation by recombinant Aurora B (even though with a rather low specific activity) was rather unexpected in light of the published findings on Ipl1 (Rea et al., 2000). We therefore compared the

activity of Aurora B on unmodified and methylated H3 peptides directly. Both peptides were phosphorylated by recombinant Aurora B with almost identical efficiency (Figure 3.6A).

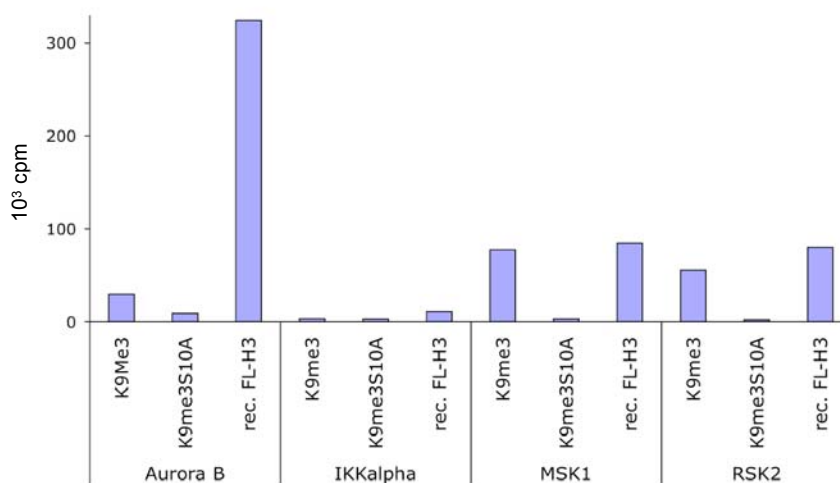


Figure 3.5: Phosphorylation activity of different H3 serine 10 kinases on methylated H3 peptides (*In vitro* kinase assays).

Radioactive *in vitro* kinase assay with different recombinant kinases. Phosphorylation of H3 peptides methylated at K9 (residues 1-15, 3 μ M) was determined in filter binding assays by scintillation counting.

All four kinases tested were able to phosphorylate the positive control, recombinant H3 (2 μ g; the high levels of phosphorylation observed for Aurora B are probably caused by Aurora B's ability to phosphorylate full-length H3 on multiple sites). The specific activity of recombinant Aurora B kinase is much lower than that of MSK1 and RSK2 (amounts of enzyme used in this experiment: 3 μ g of Aurora B, 100 ng of IKK α , MSK1, RSK2). Aurora B, MSK1 and RSK2 also show significant activity on H3K9me3 peptides (in other experiments, a weak activity of IKK α on H3K9me3 peptides was detected as well). The H3K9me3S10A control proves that the phosphorylation of the peptides is specific for serine 10. Recombinant IKK α , MSK1, RSK2 from UBI, recombinant Aurora B from own purification.

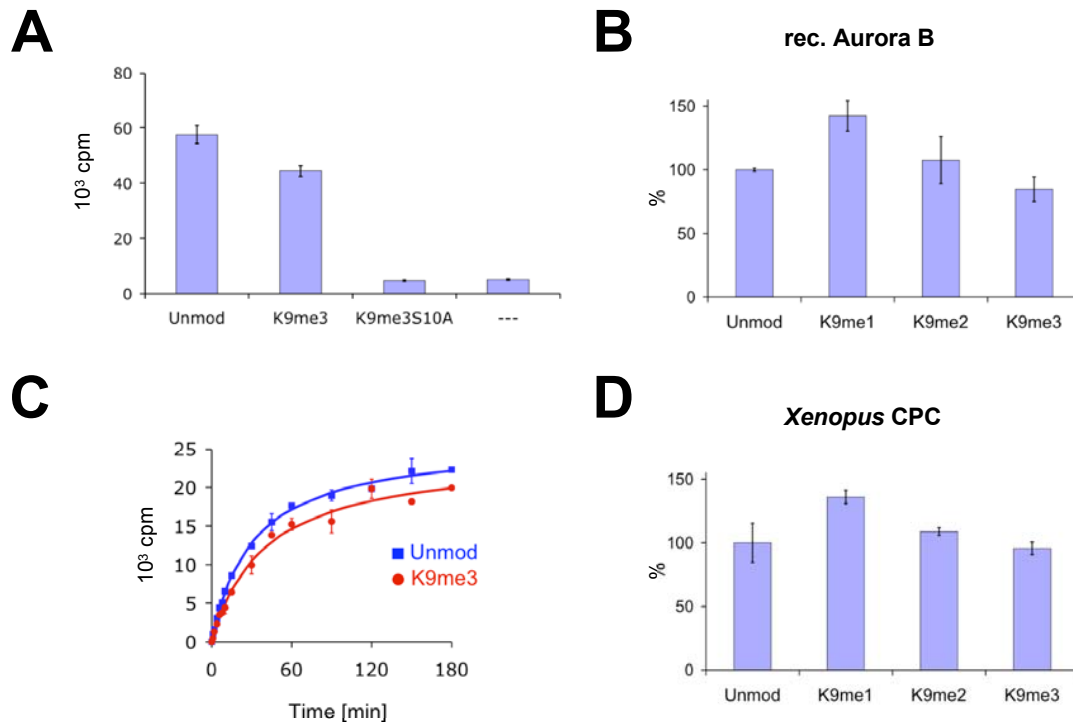


Figure 3.6: *In vitro* phosphorylation of methylated H3 peptides.

A: Radioactive *in vitro* kinase assay to determine the activity of recombinant Aurora B on unmodified and K9me3 H3 peptides (1-15). The methylated H3 peptide is phosphorylated almost as well as the unmodified peptide. Comparison of the K9me3S10A reaction with the No peptide control (---) reveals exquisite specificity of the peptide phosphorylation for serine 10; the low levels of background phosphorylation are mostly caused by autophosphorylation of the kinase.

B: Quantitative comparison of *in vitro* phosphorylation of different H3 peptides (1-15) by recombinant Aurora B (n=3; error bars indicate s.d.). Reactions were evaluated by filter binding assay and scintillation counting. Counts were corrected for background and exact peptide concentration (quantitative mass spectrometry), and normalized to the unmodified H3 peptide (100%). Unmodified H3 peptides and peptides mono-, di-, and tri-methylated at K9 are all phosphorylated by Aurora B with similar efficiencies *in vitro*. Phosphorylation of H3 serine 10 by the CPC is thus not inhibited by K9 methylation.

C: Reaction kinetics (radioactive filter binding assay) of the phosphorylation of unmodified and K9-methylated H3 peptides (1-15) by Aurora B. Values corrected for autophosphorylation of the kinase and background peptide phosphorylation. The unmodified (blue) and H3K9me3 (red) peptide are phosphorylated with very similar kinetics (n=3, error bars show s.d.).

D: Similar experiment as in panel B carried out with the Chromosomal Passenger Complex immunoprecipitated from *Xenopus* egg extracts (provided by Boo Shan Tseng, Funabiki laboratory, Rockefeller University). Again, unmodified and mono/di/tri-methylated H3 peptides are phosphorylated with very similar efficiencies.

Rea and colleagues had used dimethylated H3 peptides (Rea et al., 2000), while I used trimethylated peptides. Although an effect of this difference would be unexpected, I directly compared the phosphorylation of H3 peptides with different stages of K9 methylation. To make sure my measurements of peptide concentration were not affected by degraded peptide (photometric measurement through a tyrosine added to the C-terminal end of the peptides), the exact peptide concentrations were determined in these experiments by quantitative mass spectrometry. Once again, my results showed that peptides of all methylation stages are phosphorylated by Aurora B about equally well (Figure 3.6B).

The discrepancy between my findings with mammalian Aurora B and the published observations with the yeast homolog could be explained by an effect of reaction kinetics: If phosphorylation of unmodified and methylated H3 peptides proceeds at a very different rate, then samples taken at an early time-point will show quite different phosphorylation levels; the end point of the reaction, however, would in both cases be a fully phosphorylated peptide. I therefore carried out an experiment that followed the kinetics of the peptide phosphorylation by Aurora B kinase (Figure 3.6C). Unmodified H3 peptide and H3K9me3 peptide were found to be phosphorylated with almost identical kinetics.

Aurora B phosphorylates peptides of all methylation stages equally well, and no differences were observed in the phosphorylation kinetics of unmodified and K9me3 H3 peptides. Thus, it seems that the observed differences between mammalian Aurora B and its yeast homolog Ipl1 in their ability to phosphorylate serine 10 in the presence of K9-methylation are probably a real distinction between these enzymes rather than an artifact of the experimental conditions. It should be noted that *S. cerevisiae*

chromatin does not have H3K9 methylation (Briggs et al., 2001). Thus, it is possible that the mammalian kinase or the complex that it resides in (see below) has acquired the ability to phosphorylate serine 10 in the presence of K9 methylation, while its yeast homolog has not.

While recombinant Aurora B kinase was capable of phosphorylating H3 peptides, the specific activity was extremely low. In particular recombinant MSK1 showed similar site specificity and phosphorylated all methylation stages equally well, but with a much (about 100-fold) higher specific activity, making MSK1 the most efficient serine 10 kinase for H3K9me3 peptides among all enzymes tested. Since an *in vitro* test of the "methyl-phos switching" mechanism of course represents an artificial system anyway and the higher activity of MSK1 might be quite helpful for a principal test of the feasibility of binary switching, we decided to also proceed with this kinase.

We subsequently found that the Chromosomal Passenger Complex immunoprecipitated from *Xenopus* egg extracts (kindly provided by Boo Shan Tseng, Funabiki lab, Rockefeller University) had a several-fold higher specific activity on H3 peptides than recombinant Aurora B. It is unclear whether this increased activity is due to the presence of other complex members or reduced activity of bacterially expressed Aurora B. Like recombinant Aurora B, *Xenopus* CPC phosphorylated H3 peptides irrespective of their methylation status (Figure 3.6D). Moreover, the complex could be easily removed after kinase reactions, due to its immobilization on beads from the immunoprecipitation. We therefore decided to use the immunoprecipitated *Xenopus* CPC rather than recombinant Aurora B for our attempt to reconstitute the release mechanism of HP1 in the test tube.

Reconstitution of the "methyl-phos switching" mechanism in the test tube

In contrast to the situation in these *in vitro* kinase experiments on methylated peptides, operation of a "methyl-phos switching" mechanism inside of the cell involves an additional level of complexity: The phosphorylation site at serine 10 may often not be freely accessible to the CPC, since methyl-K9 recruits HP1 which in turn might prevent access to serine 10. Thus, to complete the *in vitro* reconstitution of "methyl-phos switching", HP1 was included in the reaction.

The activity of the CPC on H3K9me3 peptides in the presence of the effector protein was again measured by radioactive kinase assay. In these experiments, the kinase complex was able to efficiently phosphorylate H3K9me3 peptides. The efficiency declined at higher HP1 concentrations (probably due to competitive inhibition by HP1 at concentrations resulting in stoichiometric binding), but even in the presence of a significant excess of the chromo domain, there was still considerable phosphorylation of the peptide observed, as observed for all three HP1 isoforms (Figure 3.7A, C, E).

In parallel, we examined with fluorescein-labelled H3K9me3 peptides whether phosphorylation of H3S10 would eject the HP1 chromo domain from its binding site. Upon addition of CPC, the effect of serine 10 phosphorylation on HP1 CD association was monitored over time by fluorescence anisotropy measurements. In keeping with the binary switching hypothesis, phosphorylation of a H3K9me3 peptide by the CPC in the test tube results in dynamic loss of HP1 CD binding, an observation made with the CD of all three isoforms α , β , and γ (Figure 3.7B, D, F).

Figure 3.7: *In vitro* reconstitution of release mechanism by "methyl-phos switching": The HP1 chromo domain is released from H3K9me3 upon serine 10 phosphorylation by the CPC *in vitro*.

A, C, E: Phosphorylation of H3K9me3 peptides in the presence of HP1 α chromo domain (A), HP1 β chromo domain (C) and HP1 γ chromo domain (E). H3 peptides (1-15, 1 μ M) were incubated in *in vitro* radioactive kinase assays with [γ - 32 P]ATP and CPC (immunoprecipitated from *Xenopus* egg extracts) in the presence of increasing concentrations of recombinant HP1 chromo domain (1 to 67.5 μ M). Reactions were run on SDS-PAGE, stained with Coomassie (top) and analyzed by autoradiography (bottom). Brackets and arrow heads indicate where the HP1 CD and the peptide runs on the gel, respectively.

Reactions with a pre-phosphorylated peptide (H3K9me3S10ph) or in the presence of the Aurora B-inhibitor hesperadin confirm the specificity of the reaction. Even in the presence of a large molar excess of the HP1 CD, significant phosphorylation is observed.

B, D, F: Fluorescence polarization measurement of HP1 release upon serine 10 phosphorylation. Different concentrations of recombinant HP1 α chromo domain (B), HP1 β chromo domain (D), HP1 γ chromo domain (F) were mixed with the indicated fluorescein-labelled H3K9me3 peptides (100 nM). CPC immunoprecipitated from *Xenopus* egg extracts was added in the presence or absence of the Aurora B inhibitor hesperadin (4 μ M). By fluorescence polarization, the dissociation of the HP1 CD from the peptide over time was recorded (end point after over night reaction shown; n= 3, error bars indicate s.d.).

In the presence of hesperadin (no serine 10 phosphorylation) the peptide remains associated with the HP1 β chromo domain (yellow plot). Without hesperadin (serine 10 becomes phosphorylated), the chromo domain dissociates from the peptide (blue plot), approaching the curve observed for the H3K9me3S10ph peptide (red plot).

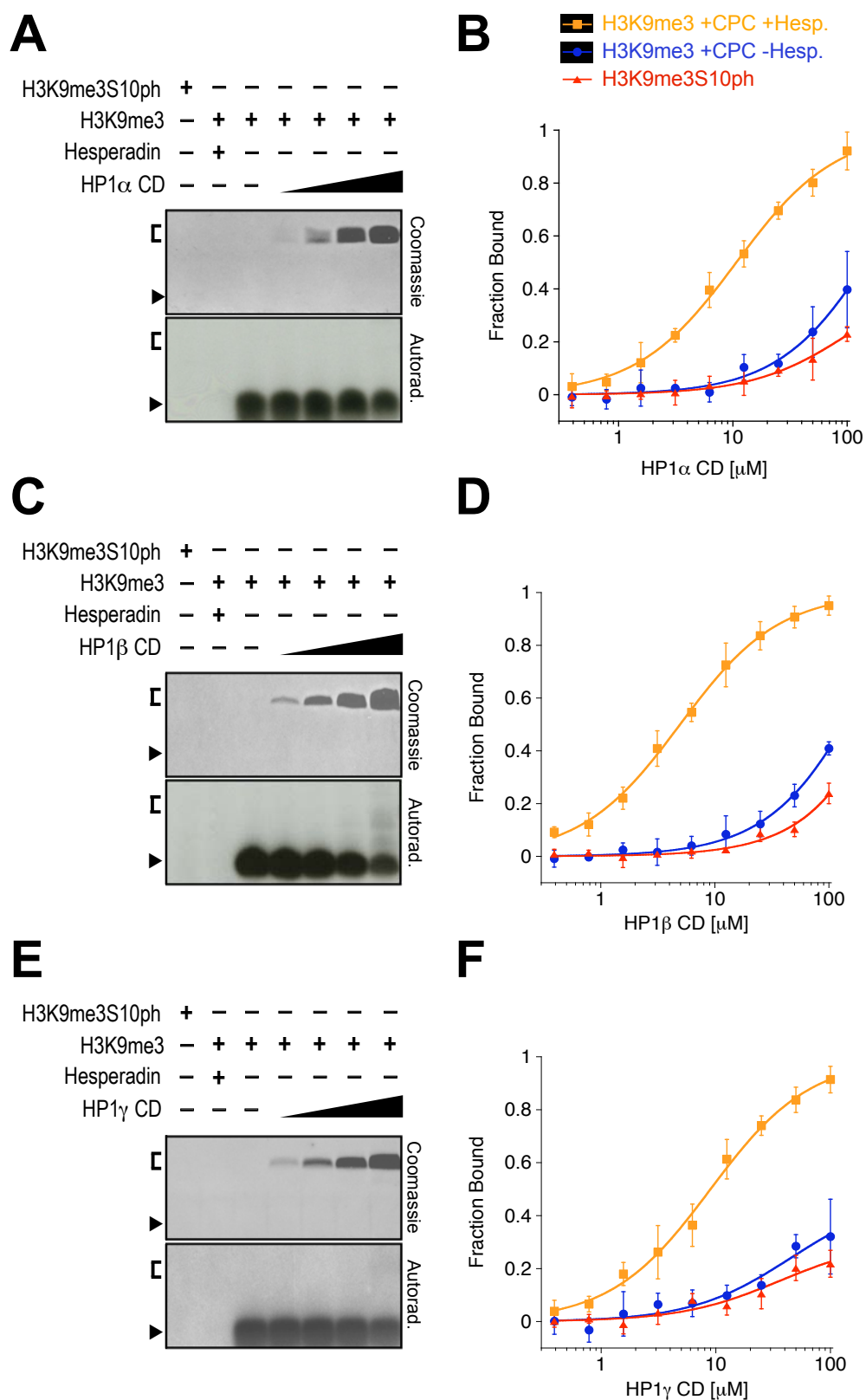


Figure 3.7

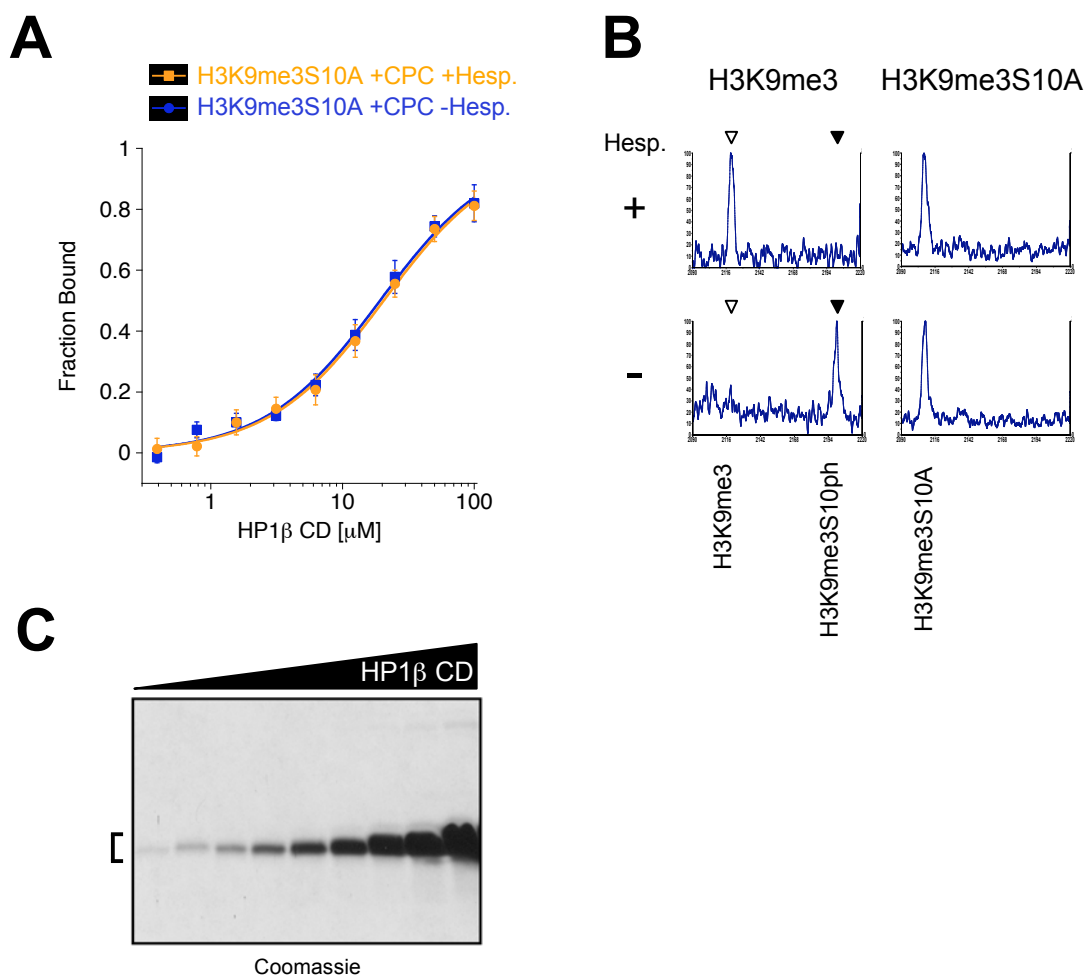


Figure 3.8: Additional specificity controls for *in vitro* reconstitution of release mechanism by "methyl-phos switching" (Controls for HP1 β CD shown).

A: Control reaction carried out with H3K9me3S10A peptides, which cannot phosphorylated by the CPC. Addition of the CPC has no effect on the binding of the HP1 β chromo domain. (H3K9me3S10A peptides generally show about fivefold reduced affinity for the HP1 β chromo domain compared to H3K9me3 peptides.)

B: Mass spectrometric analysis (MALDI-TOF) of CPC reactions from Figure 3.7D containing 50 μ M of HP1 β chromo domain. In the absence of the Aurora B inhibitor hesperadin, the H3K9me3 peptide (*) becomes completely phosphorylated to H3K9me3S10ph (**). As expected, there is no effect on the H3K9me3S10A control peptide.

C: Samples of the reactions analyzed in Figure 3.7D were run out on SDS-PAGE and stained with Coomassie, proving that the HP1 β chromo domain is not degraded in the course of the experiment. Bracket indicates position of HP1 β chromo domain. Note: Identical controls as presented here for the HP1 β CD were also carried out for the reactions with all other isoforms, with very similar results.

The release of the HP1 chromo domain from the H3K9me peptide is dependent on the kinase activity of the CPC, since no such changes are observed if Aurora B activity is inhibited with the small molecule inhibitor hesperadine (Hauf et al., 2003; Figure 3.7B, D, F) or if an unphosphorylatable H3K9me3S10A peptide is used (Figure 3.8A). Mass spectrometry confirmed that the peptide was indeed phosphorylated specifically at serine 10 (Figure 3.8B). Aliquots of the reactions were subsequently run out on SDS-PAGE gels, to exclude the trivial explanation that the HP1 CD had simply been degraded during the incubation (Figure 3.8C).

"Methyl-phos switching" in the test tube with full-length HP1

Taking these observations made with the chromo domains of HP1 a step further, we attempted to repeat the analysis with full-length HP1 proteins. However, in pilot experiments we observed that full-length HP1 itself was subject to *in vitro* phosphorylation by the CPC. While it is not clear whether this curious observation actually reflects a real *in vivo* event, it of course represents a problem for the analysis of the "methyl-phos switching" mechanism in the test tube, because it makes it difficult, if not impossible to specifically attribute dissociation of the effector protein to phosphorylation of the H3 tail. Closer analysis of the *in vitro* phosphorylation of full-length HP1 by the CPC revealed, however, that the phosphorylation was isoform-specific (Figure 3.9). Only full-length HP1 α and HP1 γ were phosphorylated, while full-length HP1 β was not. Thus, we could avoid the problem of HP1 *in vitro* phosphorylation by focusing our analysis to the β isoform of HP1.

Our experiments with full-length HP1 β showed ejection by serine 10 phosphorylation very similar to the observations with the CDs (Figure 3.10). This proves that the release of HP1 from H3K9me3 in our reductionist *in*

vitro system is not just limited to the isolated chromo domain, but can also happen in the context of the full-length protein.

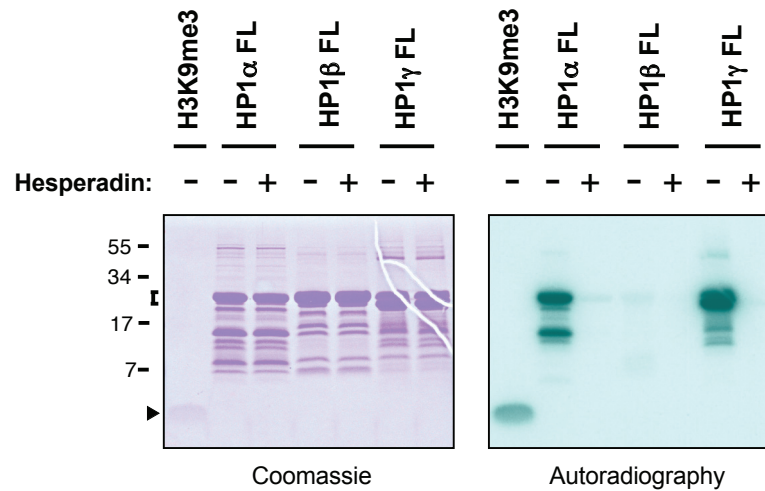


Figure 3.9: Isoform-specific phosphorylation of HP1 by the CPC *in vitro*.

Isoform-specific *in vitro* phosphorylation of HP1 by the CPC (radioactive *in vitro* kinase reaction followed by autoradiography). Recombinant full-length HP1α and HP1γ are phosphorylated by the CPC. No phosphorylation is observed for full-length HP1β. Bracket indicates position of full-length HP1 isoforms (additional bands are mostly degradation products), arrow head indicates position of the control peptide.

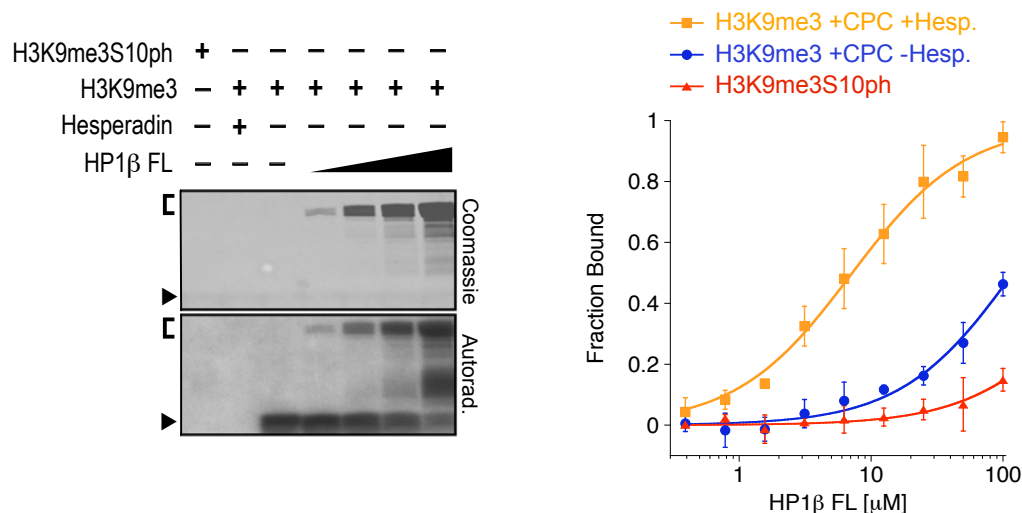


Figure 3.10: Full-length HP1 β is released from H3K9me3 upon serine 10 phosphorylation by the CPC *in vitro*.

An experiment analogous to the one described in Figure 3.7 was carried out with full-length HP1 β (see Figure 3.7 for experimental details). **Left:** The radioactive kinase assay shows that H3K9me3 peptides are phosphorylated even in the presence of high concentrations of full-length HP1 β . **Right:** Fluorescence anisotropy measurements indicate that, as the HP1 CDs, full-length HP1 β dissociates from H3K9me3 peptides upon serine 10 phosphorylation by the CPC.

All the controls depicted in Figure 3.8 were carried out for this experiment as well, showing no effect of CPC treatment on the association of full-length HP1 β with H3K9me3S10A peptides (fluorescence anisotropy), specific phosphorylation of serine 10 (mass spectrometry), and little degradation of the effector protein in the course of the reaction (SDS-PAGE).

"Methyl-phos switching" in the test tube with MSK1

While our controls clearly show that Aurora B-dependent phosphorylation of serine 10 is required for the release of HP1 in this reconstituted system, they cannot exclude that there are additional effects caused by the presence of other components in the test tube. In particular, it is possible that the other complex members of the CPC, which are always present in the reactions as well, also play a part.

Unfortunately, the very low specific activity of recombinant Aurora B made it difficult to carry out similar experiments with the isolated kinase. However, taking advantage of our identification of MSK1 as another kinase capable of phosphorylating H3K9me3 peptides *in vitro*, we were able to reproduce the CPC results with this kinase: MSK1 was capable of efficiently phosphorylating H3K9me3 peptides in the presence of the HP1 chromo domain (Figure 3.11A), and the phosphorylation resulted in the ejection of the chromo domain from its binding site on the methylated H3 tail (Figure 3.11B). This proves that the release of the HP1-H3K9me3 interaction by serine 10 phosphorylation does not require any of the other complex members of the CPC, but that serine 10 phosphorylation is sufficient for the release of CD binding in our reductionist *in vitro* system.

Reversibility of "methyl-phos switching" in the test tube

In the cell, the release of HP1 from its binding site is reversible, and the protein reassociates with chromatin at the end of mitosis. To test the assumption that this reversibility could be achieved by "methyl-phos switching", we examined the reverse reaction and sought to find out if this observation could be reproduced in our *in vitro* system. In these experiments, removal of serine 10 phosphorylation from H3K9me3S10ph peptides by PP1, a phosphatase known to dephosphorylate H3S10ph (Prigent and Dimitrov, 2003), resulted in reassociation of the HP1 β chromo domain to the peptide (Figure 3.12). The effect can be abolished by inclusion of the phosphatase inhibitor microcystin LR, showing that it indeed depends on the dephosphorylation.

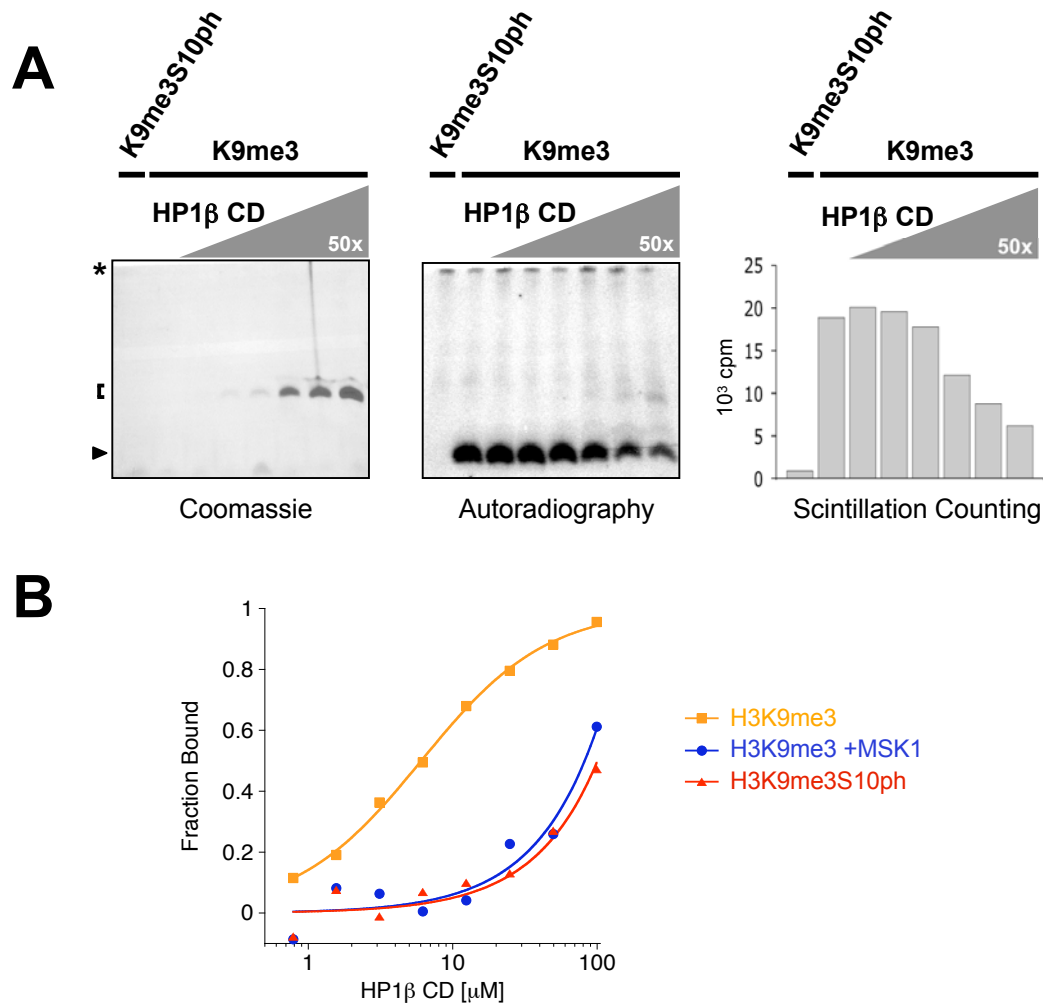


Figure 3.11: Dissociation of the HP1 β CD from H3K9me3 upon serine 10 phosphorylation by MSK1 *in vitro*.

A: H3K9me3 peptides are phosphorylated by MSK1 even in the presence of a large excess of HP1 β chromo domain (radioactive kinase experiment). In addition to the analysis by SDS-PAGE and autoradiography, peptide phosphorylation was quantified by filter binding assay and scintillation counting. While high concentrations of the HP1 β chromo domain slow down the peptide phosphorylation, there is still significant phosphorylation activity observed. Bracket, arrow head and asterisk indicate positions of HP1 β CD, peptide and MSK1, respectively.

B: Release of the HP1 β CD from H3K9me3 peptides upon treatment with the serine 10 kinase MSK1 (fluorescence anisotropy). Phosphorylation of H3K9me3 peptides by MSK1 shifts the binding curve to the right, close to the curve observed with a H3K9me3S10ph control peptide. This indicates dissociation of the chromo domain from the peptide.

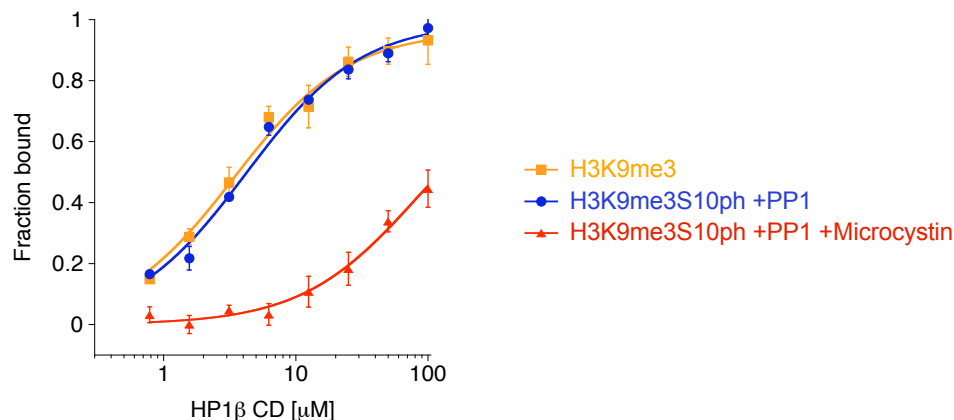


Figure 3.12: Reassociation of the HP1β CD upon phosphatase treatment of H3K9me3S10ph peptides.

Dephosphorylation of a H3K9me3S10ph peptide results in restoration of the interaction with the HP1β chromo domain. Binding of the HP1β chromo domain to a fluorescein-labelled H3K9me3S10ph peptide was measured by fluorescence anisotropy. In the absence of the phosphatase inhibitor microcystin LR, the phosphatase (recombinant PP1, NEB) removes serine 10 phosphorylation (as determined by mass spectrometry, data not shown) and binding of the HP1β chromo domain to the peptide is restored (blue plot). In the presence of the microcystin, serine 10 phosphorylation is unaffected by phosphatase treatment and no reassociation is observed (red plot).

Taken together, the presented data demonstrate that in our reductionist system enzymatic phosphorylation of serine 10 results in the release of HP1 from methylated H3 peptides, while removal of S10ph causes reassociation. Thus, these experiments unambiguously demonstrate the operation of a "methyl-phos switching" mechanism for the dynamic and reversible regulation of HP1 binding to the stable K9-methyl mark in the test tube.

H3S10 phosphorylation coincides with the release of HP1 *in vivo*

The described *in vitro* findings suggest that the "methyl-phos switching" mechanism could account for the observations made *in vivo* and that transient, reversible phosphorylation of serine 10 during mitosis might also be the mechanism how the dynamic interaction between HP1 and H3K9me3 over the cell cycle is achieved within the cell. To test this hypothesis, we turned to a combination of inhibition experiments and microscopy.

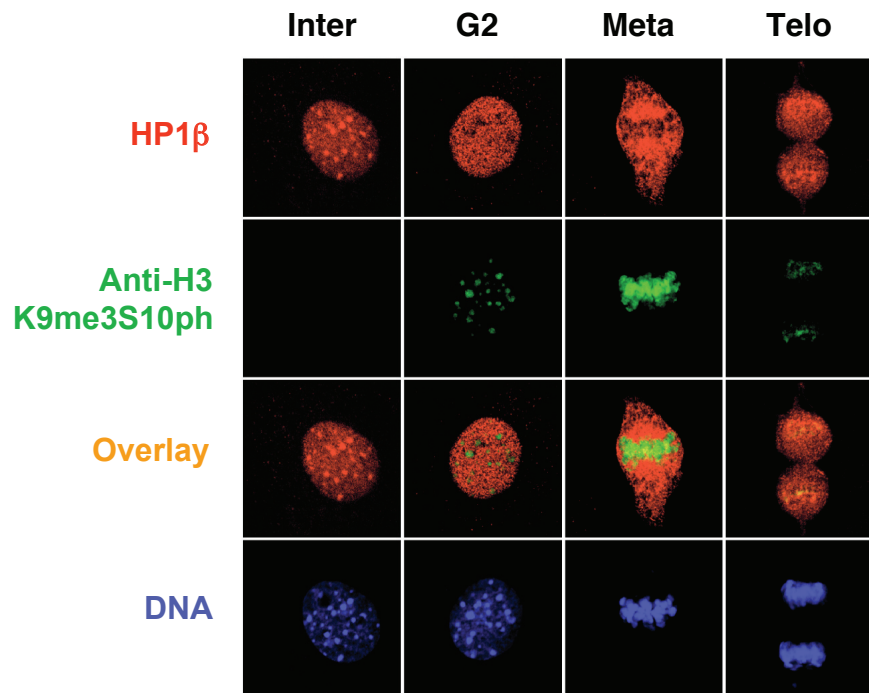


Figure 3.13: The mitotic release of HP1 coincides with the appearance of H3K9me3S10ph *in vivo*.

Immunofluorescence analysis of 10T1/2 mouse fibroblasts. The dual H3K9me3S10ph epitope occurs at late G2, at the same time when the typical dot-like staining pattern of HP1 β starts to become diffuse and HP1 β is released from chromatin. Only at telophase, when the dual epitope is removed again, does HP1 start to reassociate with chromatin. Similar observations were made in HeLa cells and with the HP1 isoforms α and γ . DNA stained with DAPI. Figure courtesy of Dr. Wolfgang Fischle.

Taking advantage of the dual-epitope(H3K9me3S10ph)-specific antiserum that he had generated, Dr. Fischle carried out a careful IF analysis in cultured mammalian cells, comparing the time of dual epitope appearance with the release of HP1. This analysis revealed a close temporal correlation between the occurrence of the H3K9me3S10ph mark and the dissociation of HP1 from chromatin at late G2 (Figure 3.13). Significant H3K9me3S10ph staining is only observed in mitotic cells that have lost the characteristic chromatin-associated HP1 localization pattern and instead show diffuse HP1 staining throughout the cytoplasm. Thus, there is a close link between the temporal and local occurrence of the dual epitope in mitosis and the dissociation of HP1 from chromatin.

Inhibition of Aurora B kinase prevents the release of HP1 *in vivo*

The observed close correlation between S10 phosphorylation and HP1 dissociation prompted us to carry out a direct *in vivo* test to see if there was a causal link between mitotic serine 10 phosphorylation and the release of HP1 (experiments carried out by Dr. Fischle).

To this end, asynchronously growing HeLa cells were treated with hesperadin and the effect of this inhibition on the mitotic dissociation of HP1 was studied by immunofluorescence microscopy. We found that upon inhibition of Aurora B, all three HP1 isoforms were retained on mitotic chromosomes (Figure 3.14A).

Since these observations were dependent on stainings with HP1 antibodies, we reproduced the experiment with cells expressing GFP-tagged HP1 isoforms. Similar to endogenous HP1, GFP-HP1 was retained at chromatin upon hesperadin treatment (data not shown). Thus, our experiments with GFP-HP1 confirm the findings with endogenous HP1 and exclude the possibility that our *in vivo* observations are simply a peculiar artifact of epitope masking.

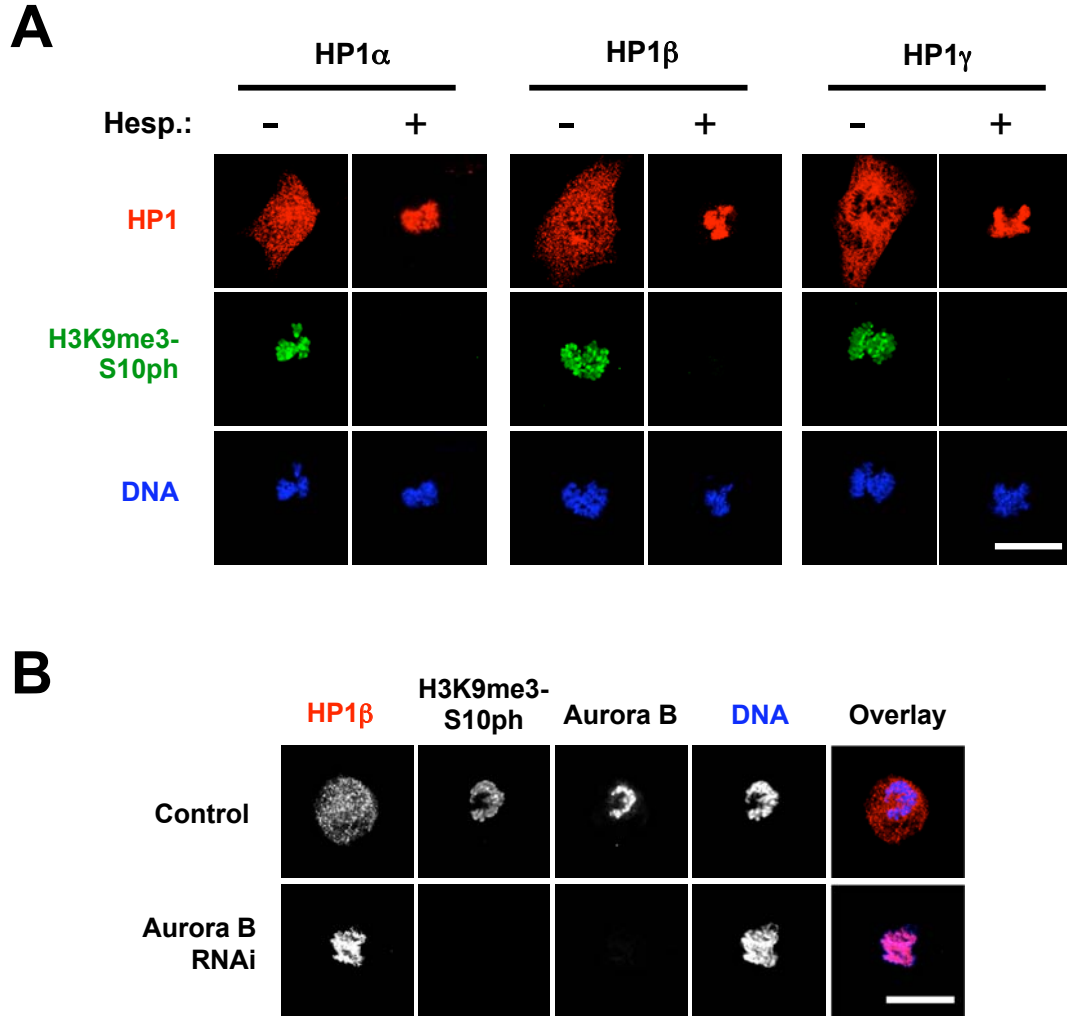


Figure 3.14: Retention of HP1 on chromatin upon inhibition of mitotic serine 10 phosphorylation *in vivo* (Figure courtesy of Dr. Wolfgang Fischle).

A: Upon inhibition of Aurora B kinase activity, endogenous HP1 isoforms are retained on mitotic chromatin. 10T1/2 cells were either mock treated or treated with hesperadin (200 nM), and then analyzed by immunofluorescence microscopy with the indicated antibodies. In cells treated with hesperadin, no H3K9me3S10ph signal is observed and HP1 does not dissociate from chromatin at metaphase. DNA stained with DAPI. Bar, 10 μ m.

B: Retention of HP1 on mitotic chromatin upon knock-down of Aurora B kinase by RNA interference. HeLa cells either mock treated (Control) or treated with Aurora B siRNAs (Aurora B RNAi) were analyzed by IF microscopy. Aurora B RNAi resulted in loss of the dual epitope and failure of HP1 β to dissociate from mitotic chromatin. Staining for HP1 α and HP1 γ gave very similar results. DNA stained with DAPI. Overlay generated from HP1 β and DNA channels. Bar, 10 μ m.

Retention of HP1 on chromatin *in vivo* upon Aurora B inhibition was also confirmed by biochemical fractionation experiments with HeLa and 10T1/2 cells arrested in mitosis with nocodazole. Upon Aurora B inhibition, we observed increased association of all three HP1 isoforms with chromatin. At the same time, there was no change in the levels of HP1, H3K9me3 or the acetylation of H3 at lysine 14 (data not shown).

Since all these observations depended on the use of hesperadin, it was possible that our observations were just the result of unspecific side-effects of this small molecule inhibitor. To exclude this possibility, we examined the effect of Aurora B depletion by RNA interference on HP1 dynamics in mitosis. In keeping with the results obtained from the inhibition experiments, knock-down of Aurora B prevented the dissociation of HP1 from chromatin during mitosis (Figure 3.14B), while not affecting the localization of HP1 in interphase cells.

In sum, these *in vivo* experiments strongly suggest that serine 10 phosphorylation of H3 is a necessary step for the release of HP1 from chromatin in mitosis.

HP1 binds to metaphase chromosomes lacking H3 serine 10 phosphorylation in *Xenopus* egg extracts

We realized that the tissue culture system offers only very limited possibilities to manipulate experimental parameters in order to investigate the molecular mechanism of mitotic HP1 release. An experimental system that allows for detailed examination and highly controlled experimental manipulation of cell cycle events are *Xenopus* egg extracts. Therefore, we collaborated with Boo S. Tseng and Dr. Hironori Funabiki, experts in the use of this cell-free system (who had actually already independently made observations related to "methyl-phos switching" in *Xenopus* egg extracts).

In keeping with our observations in tissue culture cells, Boo Tseng and Dr. Funabiki demonstrated simultaneous occurrence of the dual mark K9me3S10ph and release of GFP-tagged *Xenopus* HP1 from chromosomes in egg extracts entering mitosis (data not shown).

Upon removal of Aurora B through immunodepletion of the CPC, as expected H3 serine 10 phosphorylation was clearly diminished, while the levels of H3K9me3 were unaffected. Most notably, however, binding of HP1 α to chromosomes was significantly increased in extracts depleted of the CPC compared to control extracts. This retention of HP1 on chromatin was dependent on the chromodomain-H3K9me3 interaction, since the interaction could be competed with an H3K9me3 peptide (no effect of unmodified H3 peptide) and since mutation of one of the caging amino acids (W57A) drastically reduced the amount of bound GFP-xHP1 α in Δ CPC extracts. Thus, these data show clearly that HP1 cannot bind to metaphase chromosomes when H3 is phosphorylated at serine 10, in the absence of serine 10 phosphorylation, however, it associates through mechanisms that depend on chromo domain-binding to H3K9me3.

Discussion

A novel mechanism how histone modifications control the recruitment of effector proteins

With the described experiments, I have, together with my collaborators, provided answers for two longstanding questions. Firstly, we have identified a biological role for mitotic H3 serine 10 phosphorylation (H3S10ph), a prominent mitotic histone mark with so far elusive biological function. We have demonstrated that H3S10 phosphorylation plays a crucial role in the regulation of the chromatin association of Heterochromatin protein

1 (HP1), an effector protein with important functions in the formation of heterochromatic structures that dissociates from chromatin during mitosis.

Secondly, the experiments presented here led to the discovery of the mechanism underlying the mitotic release of HP1 from chromatin. They show that the reversible dissociation of HP1 from its binding site at histone H3 during mitosis is achieved through histone phosphorylation.

Reconstitution of the release mechanism with purified components in the test tube and subsequent inhibition studies *in vivo* clearly demonstrated that Aurora B phosphorylation of H3 at serine 10 at the onset of mitosis is crucial for the ejection of HP1 from mitotic chromatin. H3 serine 10 phosphorylation sterically and electrostatically interferes with binding of HP1 to its binding site at methylated lysine 9 of histone H3, resulting in the release of the effector protein. Thus, in this "methylation-phosphorylation switching" mechanism, two histone modifications collaborate to control association of an effector protein with chromatin (Figure 3.15). While the actual binding site (the methylation mark) is stable, the dynamic histone phosphorylation mark serves to regulate dynamic binding and release of the effector protein.

"Methyl-phos switching" is a novel mechanism how two histone modifications collaborate to bring about dynamic regulation of effector binding, that defines a new class of cross-talk between histone modifications in general (Fischle et al., 2003c; Fischle et al., 2003d). Our findings have been confirmed by other investigators (see below), and it is becoming increasingly clear that there are broader implications for this mechanism within the field of chromatin and beyond.

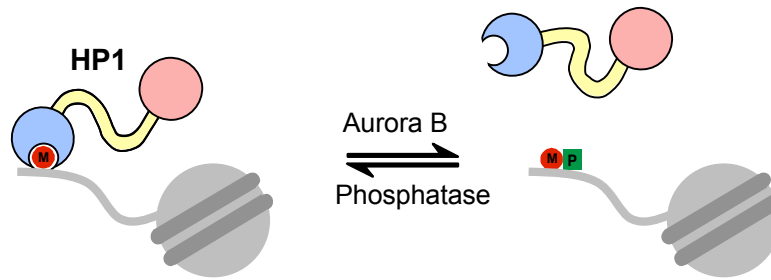


Figure 3.15: "Methyl-phos switching" controls HP1 dissociation during mitosis.

A "methyl-phos switching" mechanism controls the dynamic release of HP1 during mitosis. Model summarizing the mechanism of mitotic HP1 dissociation by "methyl-phos switching", as it is established by the experiments described in chapter 3 of my thesis.

HP1 is recruited to chromatin by methylated lysine 9 on the histone H3 tail. During mitosis, H3 serine 10 is phosphorylated by the Chromosomal Passenger Complex (CPC) with its kinase Aurora B. This sterically interferes with binding of HP1's chromo domain to H3K9me, resulting in HP1 dissociation. At the end of mitosis, serine 10 phosphorylation is removed by phosphatases and HP1 binding to H3K9me is restored. Thus, serine 10 phosphorylation allows for a reversible release of HP1 from chromatin, even though its binding site at H3K9me3 is stable.

In the following, I will briefly discuss different aspects of mitotic "methyl-phos switching" of HP1, "methyl-phos switching" in other cellular contexts and binary switching in general in light of the literature. In the first part of this discussion, I will briefly address what the responsible phosphatase might be and if there may be other supportive mechanisms besides "methyl-phos switching" that contribute to the mitotic release of HP1. In the second part of the discussion, I will go into the general implications of our findings.

Identity of the phosphatase responsible for the removal of H3S10ph *in vivo*

Observations *in vivo* indicate that the mitotic dissociation of HP1 is a fully reversible process. In our *in vitro* reconstitution system, the methylation-phosphorylation switch could be readily reversed by phosphatase treatment, and removal of serine 10 phosphorylation by phosphatase PP1 resulted in full reassociation of the HP1 chromo domain with H3K9me3 (Figure 3.12). The responsible phosphatase *in vivo*, however, is still unknown. Even so, there are some interesting observations and worthwhile speculation.

One of the genes identified in PEV screens, Su(var)3-6, has been shown to encode a catalytic subunit for type 1 serine-threonine protein phosphatases (PP1; Baksa et al., 1993). Interestingly, mutation of this gene leads not only to strong PEV suppression, but also to abnormal mitosis, mitotic defects, and hyperploidy. Considering the role that HP1 "methyl-phos switching" plays in the mitotic dissociation of HP1 and (possibly) in the definition of boundaries of heterochromatin (see below), this phenotype comes very close to what would be expected for a phosphatase that is involved in "methyl-phos switching" of HP1.

Potential contribution of other mechanisms to the mitotic release of HP1 *in vivo*

The existence of the "methyl-phos switching" mechanism and its importance for the mitotic dissociation of HP1 has been confirmed by multiple groups, *in vitro* and *in vivo*, and in both mammalian cells and *S. pombe* (Chen et al., 2008; Hirota et al., 2005; Kloc et al., 2008; Sabbattini et al., 2007; Terada, 2006).

In extreme reductionist systems (only H3 peptides and the HP1-CD) "methyl-phos switching" is always sufficient for HP1 release (Fischle et al.,

2005; Terada, 2006), allowing to study the mechanism without overlapping other effects and thus establishing binary switching as a mechanism for the regulation of effector protein association with histone marks in general. However, in more complex systems there seem to be other effects that also affect experimental observations. Already when full-length HP1 proteins are used instead of the isolated chromo domain, "methyl-phos switching" is not always sufficient. We have shown that full-length HP1 β is released from H3 peptides upon serine 10 phosphorylation, while Terada reports that full-length HP1 α does not dissociate in very similar experiments (in parallel control experiments, the HP1 α CD was ejected; Terada, 2006). Thus, there are differences between isoforms, and additional binding mechanisms seem to prevent the release of full-length HP1 α after "methyl-phos switching". This raises the question whether "methyl-phos switching", while clearly a *necessary* step, is also *sufficient* for the release of HP1, particularly in an *in vivo* context where HP1 undergoes many different interactions.

By Aurora B inhibition and depletion in various experimental systems, *in vivo* experiments have clearly demonstrated the importance of "methyl-phos switching" for the release of HP1, for example in mammalian cells (Fischle et al., 2005; Sabbattini et al., 2007; Terada, 2006), in *Xenopus* egg extracts (Fischle et al., 2005) and in *S. pombe* (Kloc et al., 2008). Further support came from a different approach, the overexpression of an exogenous H3 protein with a S10A mutation in mammalian cells, which also led to increased retention of HP1 at chromatin (Terada, 2006). However, these experiments can only prove that "methyl-phos switching" is *necessary* for the release of HP1, while they do not address whether it is *sufficient*.

When HP1 binding to full-length H3 or chromatin was tested in the presence of cellular extracts, binding of HP1 in spite of serine 10

phosphorylation of H3 has been reported. In pull-downs with recombinant full-length HP1 γ from acidic-soluble cellular extracts, for example, Fass and colleagues found that serine 10 phosphorylation does not prevent H3 binding (Fass et al., 2002), suggesting that the reversal of the CD-H3K9me interaction is not sufficient for the release of HP1 from chromatin *in vivo*. Such a conclusion is also supported by an experimental observation made by our collaborators Boo Shan Tseng and Dr. Hironori Funabiki. In *Xenopus* egg extracts, exogenous HP1 with a mutation in the chromo domain still showed partial retention on chromosomes (in extracts depleted of CPC), even though mutation of the chromo domain abolishes H3K9me-dependent binding of HP1, therefore mimicking the effect of complete "methyl-phos switching" (Fischle et al., 2005).

HP1 is known to undergo various molecular interactions and bind to chromatin by multiple different mechanisms *in vivo*. Considering the clear evidence for a role of other interactions in stable chromatin association of HP1 (see Introduction), it is very likely that there may be other interactions besides the CD-H3K9me interaction that have to be reversed for the mitotic release of HP1. It is therefore expected that other mechanisms besides "methyl-phos switching" do play a part in the mitotic dissociation of HP1 from chromatin *in vivo*. Interestingly, there are indications in mammalian cells that HP1 becomes multiply phosphorylated during mitosis (Minc et al., 1999). While the sites of these phosphorylations had not been mapped and little, if anything, was known about the function of these mitotic phosphorylations, it is tempting to speculate that mitotic HP1 phosphorylation could provide a mechanism for the reversal of molecular interactions of HP1 and thus contribute to the release of the protein during mitosis.

"Methyl-phos switching" of HP1 at H3 lysine 9/serine 10 outside of mitosis

As shown in this chapter of my thesis, in the regulation of mitotic HP1 dissociation "methyl-phos switching" plays a central role. However, there are other instances where there are indications that "methyl-phos switching" may regulate HP1 behaviour.

In a recent publication by Sabbattini and colleagues (Sabbattini et al., 2007), it was reported that H3 S10 phosphorylation by Aurora B in postmitotic cells leads to delocalization of HP1 β . While HP1 β is localized to regions of facultative heterochromatin in activated B cells, it becomes displaced from facultative heterochromatin in an Aurora B-dependent fashion during the differentiation process to terminally differentiated postmitotic plasma cells. The displacement of HP1 may allow binding of other chromatin proteins involved in transcriptional silencing to these regions of the genome. The study thus establishes a non-mitotic role for "methyl-phos switching" in differentiation.

Another instance, where "methyl-phos switching" of HP1 may play a role, is meiosis. H3 serine 10 phosphorylation has been reported in meiosis as well, and it is possible that HP1 might have to be released for accurate progression of meiotic divisions (Kaszas and Cande, 2000; Wei et al., 1998; Wei et al., 1999).

"Methyl-phos switching" of HP1 could also be involved in transcriptional activation. It is well established that HP1 has, besides its function in heterochromatin, also a role in the repression of euchromatic genes (see Introduction, Section "Cellular Localization and Functions of HP1"). This is reflected by the multiple interactions HP1 undergoes with transcriptional repressors, such as TIF1 α (Le Douarin et al., 1996), TIF1 β /

KAP1 (Nielsen et al., 1999), Rb (Nielsen et al., 2001), or E2F (Ogawa et al., 2002). In keeping with this interpretation, it has been reported that transcriptional activation of a silenced transgene array in human cells involved the dissociation of associated HP1 proteins (Janicki et al., 2004). Clearly, HP1-mediated gene repression is reversible, and mechanisms must have evolved to overcome this form of gene silencing. Interestingly, H3 serine 10 phosphorylation has also been observed in connection with gene activation. During a process called immediate early gene response which involves the rapid induction of gene expression and cell growth upon treatment of quiescent mammalian cells with mitogens (Mahadevan et al., 1991), a burst of H3 serine 10 phosphorylation is observed at specific loci that are activated during this process (e.g. Chadee et al., 1999; DeManno et al., 1999; Yamamoto et al., 2003). Several kinases involved in the phosphorylation of serine 10 during this process have been identified (MSK1, RSK2, IKK α ; Anest et al., 2003; Sassone-Corsi et al., 1999; Strelkov and Davie, 2002).

Interestingly, in my experiments on HP1 "methyl-phos switching" *in vitro*, the immediate early kinase MSK1 was capable of ejecting the HP1 chromo domain from its binding site at H3K9me3 (see Section "Methyl-phos switching" in the test tube with MSK1"). Thus, while it is not clear at this point if HP1 really participates in the regulation of immediate early genes, it remains an interesting hypothesis to test if "methyl-phos switching" of HP1 by serine 10 phosphorylation is indeed a step in the activation of these genes.

"Methyl-phos switching" of HP1 may also be the key to explain another curious observation in *Drosophila* cells. Reduced levels of the H3S10 kinase JIL-1 lead to spreading of HP1 and H3K9 methylation to ectopic

locations along the arms of chromosomes (Zhang et al., 2006). A possible explanation for this finding is that in a wild-type situation JIL-1 controls HP1 association by "methyl-phos switching", preventing HP1 association at specific target sites, blocking the spreading of heterochromatin, and thus defining heterochromatic domains through "methyl-phos switching".

All in all, there are multiple pieces of evidence suggesting an involvement of HP1 "methyl-phos switching" in biological processes outside of mitosis.

"Methyl-phos switching" of HP1 at other sites

HP1 chromatin association is mediated by the interaction of the chromo domain with H3K9me. However, lysine 9 and serine 10 of H3 are part a sequence motif "ARKS", which is a typical "modification cassette" (Fischle et al., 2003a), a sequence motif that is multiply posttranslationally modified. A brief review of the predicted human proteome shows that there are almost 70 proteins with an "ARKS" motif in the human genome (A. Basu, personal communication), thus conferring to these proteins the ability to undergo regulated molecular interactions with binding partners. At several instances, it has been reported already that the "ARKS" motif or related motifs are methylated at the lysine and/or phosphorylated at the serine or threonine. Examples have been found in the H3 tail at K9 and K27, in H1.4 at K26, in G9a at K165, and in the hinge of macroH2A at K157. Intriguingly, HP1 itself contains a very similar "KRKS" motif (see Chapter 4 of this thesis). In several cases HP1 binding to these sites has been reported (Daujat et al., 2005; Sampath et al., 2007).

The linker histone H1.4 contains an "ARKS" cassette (Figure 3.16, top). Lysine 26 within this motif is methylated by the HMTase Ezh2 *in*

in vivo, and in reporter assays methylation of this site is necessary for Ezh2-mediated transcriptional repression (Kuzmichev et al., 2004). In a recent publication, Daujat et al. report that HP1 binds through its chromo domain to H1K26me *in vitro* and in pull-downs from cellular extracts (Daujat et al., 2005). It is tempting to speculate that H1K26 methylation may be one of the suspected ways of H3K9me-independent recruitment of HP1 to chromatin (see Introduction). However, it has also been found that the neighbouring S27 is subject to phosphorylation (Garcia et al., 2004), and the interaction of HP1 with H1.4K26me is abolished by simultaneous phosphorylation of the neighbouring serine 27, as shown in various pull-down and competition experiments (Daujat et al., 2005). The observations raise the intriguing possibility that release of HP1 from H1.4K26me by "methyl-phos switching" may be the explanation for observed decondensation effects of S27 phosphorylation (Roth and Allis, 1992).

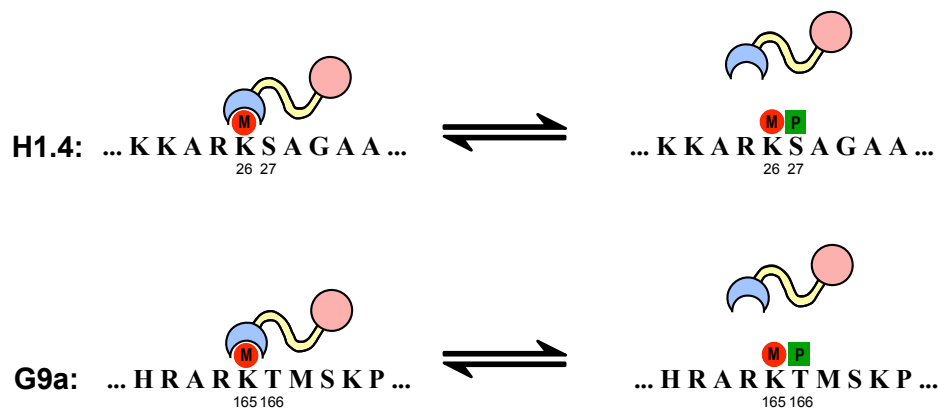


Figure 3.16: Examples of HP1 "methyl-phos switching" at sites other than H3K9me3S10ph.

Top: Histone H1.4 contains a modification cassette "ARKS" that is methylated at K26 and binds HP1. Phosphorylation of the neighbouring S27 abolishes HP1 association (Daujat et al., 2005). **Bottom:** The histone methyltransferase G9a contains an "ARKT" motif that is methylated at K165 and recruits HP1. A phosphorylation of T166 right next to the methylated lysine prevents HP1 binding.

Another case of possible HP1 "methyl-phos switching" is G9a, a histone methyltransferase that methylates H3 at lysine 9 (Tachibana et al., 2001). G9a contains an "ARKT" motif (Figure 3.16, bottom), and autocatalytic methylation of lysine 165 within the G9a sequence generates a binding site that HP1 γ associates with *in vivo* (Sampath et al., 2007). In *in vitro* binding assays, this interaction is reversed by phosphorylation of the adjacent threonine 166. While the biological function of G9aK165 methylation and the existence of T166ph *in vivo* still have to be established, this may turn out to be another case of HP1 "methyl-phos switching" in the cell.

Thus, H3K9meS10ph is not the only site where "methyl-phos switching" of HP1 is observed. Several other proteins, both in histones and in non-histone proteins, have modification cassettes that give them the ability to recruit HP1 in a manner that is regulated by "methyl-phos switching".

Binary switching as a general mechanism in the cell

From these examples, it is clear that "methyl-phos switching" is a versatile mechanism that is used by the cell in many different contexts, for the regulation of HP1 binding in mitosis, during differentiation, or during for the definition of boundaries of heterochromatin, and for the association with different histones and non-histone proteins alike.

However, the demonstration of binary switching has even broader implications. Since various different histone marks serve as binding sites to recruit effector proteins, it is obvious that the mechanism will not be limited to HP1 and "methyl-phos" switching, but that various other proteins binding to different types of modifications may be regulated by this mechanism and variations of the mechanism with other combinations of marks will occur, such as "acetyl-phos", "methyl-acetyl", or even "methyl-methyl" switching.

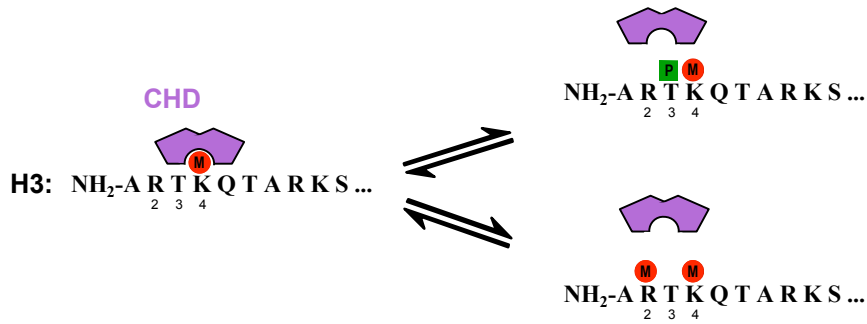


Figure 3.17: Regulation of the chromatin association of the chromatin remodeller CHD by a "methyl-phos" and a "methyl-methyl" switch.

Example of a "methyl-phos" and "methyl-methyl" switch involving the chromatin remodeller CHD. CHD binds to methylated lysine 4 in the H3 tail. Both phosphorylation of threonine 3 (top, "methyl-phos switch") and methylation of arginine 2 (bottom, "methyl-methyl switch") abolish association of CHD with H3K4me.

This is illustrated by the example of CHD (chromo-ATPase/helicase-DNA-binding), a nucleosome remodeling factor with two chromo domains (Figure 3.17). Cooperating with each other in a tandem arrangement, these two CDs bind to H3K4me, a hallmark modification of active chromatin (Flanagan et al., 2005). The two neighbouring residues of H3, threonine 3 and arginine 2, have both been found to be modified *in vivo* as well. H3 T3 is phosphorylated during mitosis by the kinase haspin, a modification that is required for proper chromosome alignment in metaphase (Polioudaki et al., 2004). H3 R2 is the site of methylation by the methyltransferase CARM1 and plays a role in transcriptional activation (Chen et al., 1999). In fluorescence anisotropy experiments, it was found that the presence of either additional modification (i.e. a dual epitope H3R2meK4me or H3T3phK4me) significantly reduces binding of CHD to H3K4me (Flanagan et al., 2005). Thus, binding of the chromatin remodeller CHD to H3K4me is another case of binary switching, which in this case involves both a "methyl-phos" and a "methyl-methyl switch" and may control recruitment of the chromatin remodeller.

Within the histone tails, there are many regions with a very high density of clustered histone marks (see Figure 1.3 for an overview). This is exemplified by the histone H3 tail (Figure 3.18). Within the first 30 amino acids of the H3 sequence, there are multiple regions of very densely clustered histone marks found. For many of these sites, binding proteins have been found, and thus it seems very likely that other cases will be discovered in the future where chromatin effector proteins are regulated by this mechanisms (Fischle et al., 2003a).



Figure 3.18: High density and clustering of posttranslational modifications in the histone H3 tail.

The histone H3 tail shows a very high density of posttranslational modification sites, many of which cluster together. This suggests the existence of other binary switches within the H3 tail. Red circle: (mono/di/tri-) methylation. Green square: phosphorylation. Yellow star: acetylation.

While the recruitment of effector proteins by histone marks has received a lot of attention in recent years, the regulation of protein interactions by posttranslational modifications is a general principle that has been studied intensely for years (Pawson, 1995; Seet et al., 2006). Many of the principles observed with histones have also been found in non-histone proteins. It is therefore likely that the mechanism of binary switching will be found in many interactions between non-histone proteins as well. Future research will tell how far beyond the field of chromatin biology the consequences of this novel mechanistic principle will reach.

CHAPTER 4

REGULATION OF HP1 THROUGH EFFECTOR PHOSPHORYLATION: PHOSPHORYLATION OF THE HP1 HINGE REGION

Introduction

The recruitment of specific effector proteins by posttranslational histone modifications represents a key mechanism for controlling chromatin structure and function. The elusive mechanisms for how these effector proteins are directed to chromatin and released, and how they fulfill their functions in the dynamic environment of the nucleus, remain major challenges for the field of chromatin biology.

Arguably one of the more famous of these effector proteins, Heterochromatin Protein 1 (HP1) has over the last few years been the focus of intense research. This has led to a number of key findings, and has not only provided insights into the functions and mechanisms of action of this important chromatin factor, but has also shaped our understanding of chromatin in general. For example, as described in previous chapters, the discovery that chromo domains are modules for methyl-lysine binding provided a paradigm for how proteins can be recruited to modified histones (Bannister et al., 2001; Jacobs et al., 2001; Lachner et al., 2001).

However, despite the progress made, a number of important questions in HP1 biology remain unresolved, such as a full understanding of the interplay of the different mechanisms involved in HP1 recruitment to chromatin, the different roles of the three HP1 isoforms, the exact sites and function of HP1 posttranslational modifications (especially in mammalian

cells), or the mechanisms that regulate HP1's multiple molecular interactions. Addressing these questions will be of vital importance for a full comprehension of the biological role of HP1 as well as for a deeper understanding of the mechanisms that control chromatin structure and function.

In Chapter 3, I presented data that finally provides the mechanism of reversible HP1 dissociation from chromatin at the onset of mitosis. HP1 is released by a mechanism called methyl-phos switching, through which the interaction of the HP1 CD with methyl-K9 H3 is reversibly abolished by transient phosphorylation of the histone at serine 10. An essential step in the ejection of HP1 from mitotic chromatin, this release mechanism illustrates how HP1 and its dynamics during mitosis are controlled by histone phosphorylation.

But histone phosphorylation may not be the only way how posttranslational modifications control HP1 behaviour. Indeed, it has been observed in various organisms that HP1 itself is subject to phosphorylation. Highly intriguing changes in HP1 phosphorylation at specific stages of the cell cycle suggest that phosphorylation may be a critical mechanism to control the protein's function, making HP1 phosphorylation an important frontier that urgently needs to be addressed.

Studying the posttranslational modifications of HP1 may also be of more general interest. Much has been learned in recent years about the mechanisms of how histone modifications recruit effector proteins to chromatin to bring about specific effects. However, far less attention has been given to the possibility that the functions of effector proteins may also be controlled by posttranslational modifications of the effector proteins themselves. It is tempting to speculate that such a regulation on side of the

effector proteins may exist as well, since it would allow the cell to modulate the read-out of the histone code. Indeed, it has been observed that the "modification cassettes" that are found in histone proteins are also present in non-histone proteins (sequences closely related to the "ARKS" motif in H3 are present, e.g., in the histone methyltransferase G9a or even in HP1; see Chapter 3, Discussion, section "Methyl-phos switching at other sites"). Modulation of the read-out of histone marks by modification of effector proteins would add yet another layer of complexity to the regulation of chromatin structure and function. Phosphorylation of HP1 may be a good model to address this fundamental question.

While carrying out *in vitro* kinase experiments for the reconstitution of methylation-phosphorylation switching in the test tube (Chapter 3), I made the observation that the Chromosomal Passenger Complex (CPC, a mitotic kinase complex with important regulatory roles in mitotic chromosome segregation and cell division) phosphorylates not only histone H3, but also full-length HP1 *in vitro*. This phosphorylation is isoform-specific, since only HP1 α and HP1 γ are phosphorylated, while there is no phosphorylation observed for HP1 β (Figure 4.1). The finding appeared immediately intriguing, because it is in keeping with an observation in the published literature that had suggested mitotic phosphorylation of the HP1 isoforms HP1 α and HP1 γ before.

In a paper published in 1999, E. Minc and colleagues for the first time presented evidence that HP1 is phosphorylated in human cells (Minc et al., 1999). When they immunoprecipitated HP1 from HeLa cells grown in the presence of radioactive phosphate, they found that HP1 α and HP1 γ were radioactively labelled in IPs from both interphase and mitotic extracts, while no labelling was observed for HP1 β . Following up on this finding,

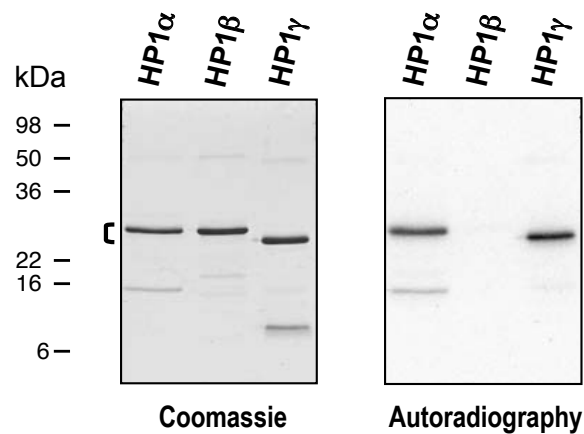


Figure 4.1: *In vitro* phosphorylation of HP1 by the Chromosomal Passenger Complex.

Isoform-specific phosphorylation of HP1 by the Chromosomal Passenger Complex *in vitro*. Recombinant HP1 α , HP1 β and HP1 γ were incubated with *X. laevis* Chromosomal Passenger Complex (a gift from Boo Tseng, Funabiki lab) in radioactive *in vitro* kinase assays. Autoradiography shows that the isoforms HP1 α and HP1 γ are phosphorylated in this reaction, while HP1 β is not. Bracket indicates position of full-length HP1 isoforms on the gel. Faster migrating bands are probably degradation products.

they carried out 2D gel electrophoresis to analyze the number of charged isoforms of HP1 (Figure 4.2). In mitosis, additional species with more negative charge (either due to gain of negative charge or neutralization of positive charge through posttranslational modification) appeared for HP1 α and HP1 γ , suggesting that the proteins acquired additional posttranslational modifications such as additional phosphorylations. It was never confirmed whether these sites are indeed phosphorylations, where the sites of modification lie within the HP1 sequence, or what the biological function of such curious hyperphosphorylation of HP1 during mitosis might be.

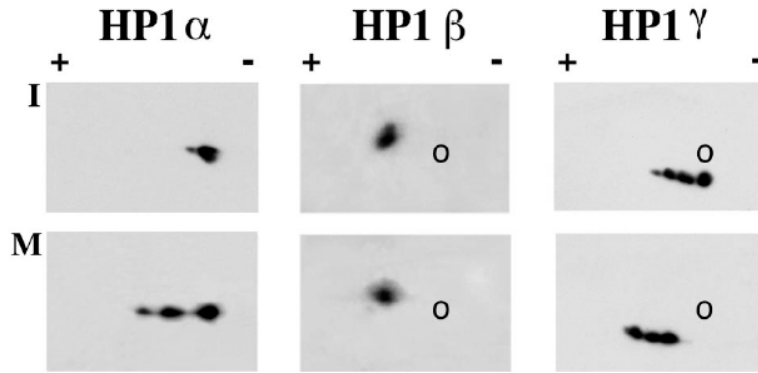


Figure 4.2: Indications for *in vivo* phosphorylation of HP1 in the literature (Figure from Minc et al., 1999).

HP1 α and HP1 γ may be hyperphosphorylated in mitosis *in vivo*. Total cell extracts from interphase (I) and mitotic (M) HeLa cells were separated by 2D gel electrophoresis, followed by immunoblotting for the different HP1 isoforms. Plus/minus signs indicate the orientation of the electrodes during the isoelectric focusing step. The black circle marks the place on the membrane where the interphase signal for HP1 α was detected. The appearance of additional dots in the mitotic samples suggests that HP1 α and HP1 γ are modified by a posttranslational modification that adds negative charge or reduces positive charge. Additional phosphorylations in mitosis could explain this observation.

In light of the findings reported by Minc and colleagues, several lines of evidence suggested that my *in vitro* observations might reflect a real *in vivo* event: (1) The observed *in vitro* phosphorylation showed the same isoform-specificity as the metabolic labelling described by Minc et al. (2) In the cell, the Chromosomal Passenger Complex is active specifically in mitosis and could therefore indeed be responsible for the additional charged HP1 species observed by Minc et al. in mitotic samples. And (3) an interaction of HP1 α and HP1 γ with INCENP, a component of the CPC, has been described in the published literature before (Ainsztein et al., 1998). Therefore, I decided to follow up on my observation of HP1 *in vitro* phosphorylation by the CPC.

Focusing mostly on HP1 α , in this chapter I summarize the mapping of this phosphorylation to a conserved site in the hinge region, confirmation and characterization of the mark *in vivo*, the identification of the responsible kinase, and experiments to elucidate the biological function of the mark. I also report the identification of a series of additional, novel phosphorylations in the hinge regions of all three HP1 isoforms by mass spectrometry, describe the tests I performed to ask if these modifications contribute to the regulation of HP1 α dynamics during mitosis, and end with observations that suggest that the HP1 α hinge phosphorylations may play a role in the regulation of RNA binding to HP1.

Results

Mapping of HP1 *in vitro* phosphorylation by the CPC to a conserved cassette in the hinge region

Through a combination of *in vitro* kinase assays, deletion/mutation analysis and computer-based predictions, I succeeded in identifying the sites of the observed *in vitro* phosphorylation of HP1 α and HP1 γ by the CPC (Figure 4.3). The sites of modification are serine 92 in HP1 α and serine 93 in HP1 γ ¹. These serine residues fall within the hinge region of HP1 that connects the chromo and chromoshadow domains. Recombinant Aurora B kinase (without the other CPC components) showed slightly reduced site specificity compared to the complex, but otherwise reproduced the results obtained with the CPC (data not shown).

¹ For a long time it has been unclear, where exactly translation of the mammalian HP1 γ open reading frame starts. By mass spectrometry, my collaborator Hillary Montgomery (Hunt lab, University of Virginia) and I could confirm the exact identity of the HP1 γ N-terminus. In some other studies, a translation start ten amino acids downstream had been assumed. Thus, what I refer to as HP1 γ S93 by some other studies would refer to as HP1 γ S83.

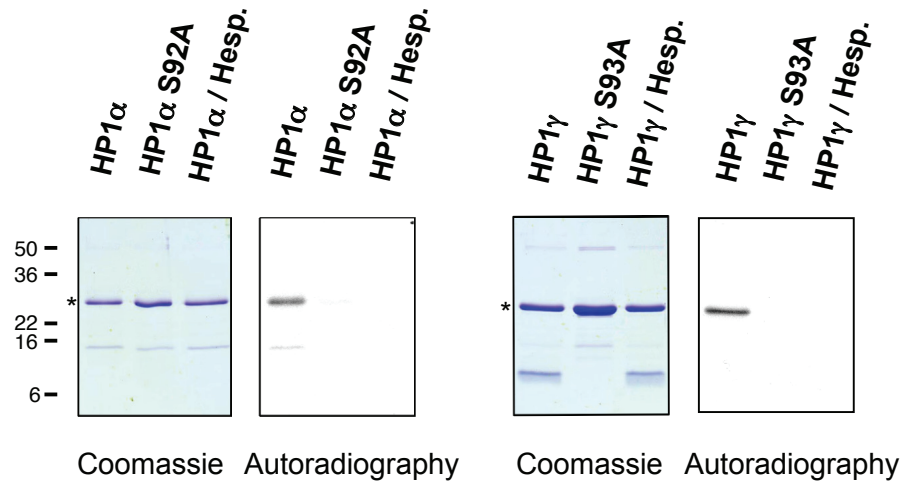


Figure 4.3: Mapping of the site of HP1 α and HP1 γ *in vitro* phosphorylation by the Chromosomal Passenger Complex.

Radioactive *in vitro* kinase assays. Phosphorylation of HP1 α and HP1 γ is completely abolished by mutation of serine 92 and serine 93, respectively. Inclusion of hesperadin, a small molecule inhibitor of Aurora B kinase activity (Hauf et al., 2003), completely prevents phosphorylation, indicating that phosphorylation is indeed caused by Aurora B activity and not by another component that copurified with the CPC during immunoprecipitation from *X. laevis* egg extracts. Asterisk indicates full-length HP1 α / γ .

In order to assess the sequence context and level of conservation of the serines identified as phosphorylation sites, I generated a sequence alignment of the human HP1 isoforms with the HP1 proteins of other organisms. As shown in Figure 4.4, the overall sequence conservation within the hinge region is rather low. HP1 α S92ph/HP1 γ S93ph is part of a highly conserved region right in the middle of the hinge sequence. This sequence motif (KRKS) is found in HP1 proteins from mammals down to flies. Similar, though not identical, sequence motifs are found in *S. pombe* (SKRK, KSRK) and *T. thermophila* (RKS, KRS) (not shown in the alignment).

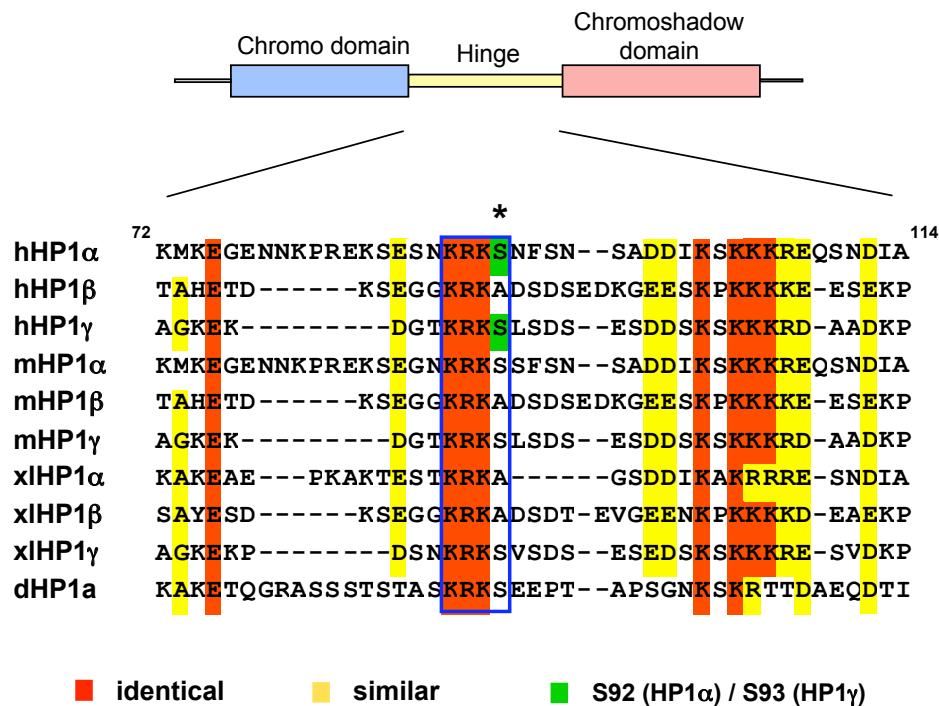


Figure 4.4: Sequence alignment of the HP1 hinge region.

Sequence alignment of the hinge regions of HP1 isoforms from different organisms (equivalent to human HP1α amino acids 72-114). Overall, the conservation of the hinge region is not very high, with the exception of two conserved stretches of basic amino acids in the middle and in the C-terminal part of the hinge. HP1α S92 and HP1γ S93 (asterisk) are homologous residues and are part of a conserved sequence motive KRK(S) (blue box). hHP1: human, mHP1: mouse, xHP1: *X. laevis*, dHP1: *Drosophila melanogaster*. *Drosophila* HP1b was not included, because the ClustalW algorithm used did not align its KRKS motive to the cassette in the other HP1s (compare to Figure 1.7).

In the human HP1β isoform, the serine is replaced with an alanine (KRKA), which explains the isoform-specificity of the observed phosphorylation. That the alanine is not always found in exactly the same isoforms (in *X. laevis*, HP1α has an alanine, while HP1β and HP1γ have serines; in flies KRKS is found in HP1a and HP1b, but not in HP1c) may indicate that in distinct organisms biological functions may be distributed differently between the isoforms.

The KRKS motif resembles the Aurora B target site on histone H3 (ARKS around lysine 9 and lysine 27). The ARKS sequence has the characteristics of a "modification cassette" (Fischle et al., 2003a) that includes sites of methylation, effector protein binding (HP1 and Polycomb, respectively; see Chapter 1), phosphorylation, and methyl-phos switching (see Chapter 3). Even in non-histone proteins, a closely related modification cassette has been found to be a site of methylation, and simultaneous phosphorylation of the neighbouring site leads to "methyl-phos switching" (the ARKT motif in the methyltransferase G9a seems to function as a "histone mimic", Sampath et al., 2007; see also Chapter 3, Discussion, section "Methyl-phos switching at other sites"). This suggested to me that the very similar, conserved KRKS motif might play an interesting role in the biology of the HP1 hinge.

Specific antibody for HP1 α S92ph

To examine the phosphorylation sites *in vivo*, I decided to rely on modification-specific antibodies against the sites. An HP1 α S92ph serum was made available to me through the generosity of Dr. Raul Urrutia (Mayo Clinic, Rochester, MN). This group had just carried out computer-based phospho-site predictions of HP1 for various cellular kinases. On this basis, they had generated several sera for predicted phospho-serines in the HP1 sequence, and two of these sera were against HP1 α S92ph and HP1 γ S93ph. To avoid direct competition, we decided to collaborate with a division of labor such that I would focus on the HP1 α phosphorylation, while they would work on HP1 γ .

As a first step, I characterized the HP1 α S92ph serum and tested its specificity in western blotting experiments with unmodified and *in vitro* phosphorylated HP1 isoforms. As shown in Figure 4.5A, the serum ("Phos-

HP1 α antibody") turned out to be quite specific for HP1 α S92ph, with little cross-reactivity to the other HP1 isoforms or unmodified HP1 α .

The HP1 α S92ph antibody was also tested in peptide competition experiments with extracts of nocodazole-arrested HeLa cells (which carry the HP1 α S92 phosphorylation; see following section). Again, in these experiments the antibody proved highly specific for its epitope (Figure 4.5B).

HP1 α S92 phosphorylation is a mitotic mark *in vivo*

Taking advantage of the HP1 α S92ph antibody, I set out to test whether the S92 phosphorylation mark indeed also existed *in vivo*. Since I had observed *in vitro* phosphorylation of serine 92 with a mitotic kinase complex (the CPC), I decided to look for the signal in cells arrested in mitosis. These western blotting experiments from cellular extracts confirmed the existence of HP1 α S92 phosphorylation *in vivo* and showed that HP1 α S92ph is specifically enriched in cells arrested in mitosis (Figure 4.6A).

Similar observations were made in immunofluorescence experiments carried out with HeLa cells. In these experiments mitotic cells were stained strongly, while for interphase cells only a very weak background staining was observed (Figure 4.6B). That the observed IF signal was indeed specific for the HP1 α S92ph epitope was confirmed by IF peptide competition experiments (Figure 4.6C).

To study the kinetics of the HP1 α S92ph mark in more detail, I carried out a time-course experiment with cells synchronized by double-thymidine block (Figure 4.6D). Consistent with my findings obtained with nocodazole-arrested samples, the HP1 α S92ph signal was specifically observed in mitosis, showing similar kinetics as the mitotic marker H3 serine 10 phosphorylation.

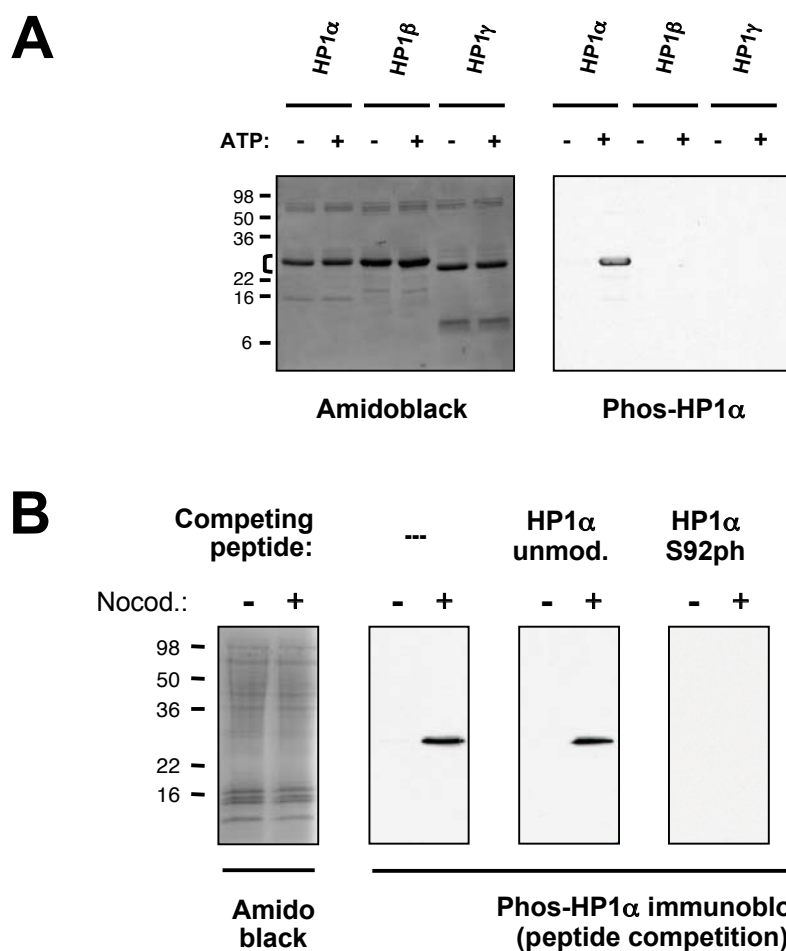


Figure 4.5: Characterization of the HP1 α S92ph antibody in immunoblotting.

A: Recombinant HP1 isoforms were incubated with recombinant Aurora B kinase in the presence or absence of ATP. Immunoblotting of these samples shows that the HP1 α S92ph antibody ("Phos-HP1 α " antibody) distinguishes with exquisite specificity between the isoforms as well as between the phosphorylated and unphosphorylated form. Bracket indicates area where full-length recombinant HP1 proteins run.

B: Peptide competition experiment with Phos-HP1 α antibody. The antibody (1:2000) was preincubated with different peptides (2 μ g/ml) and then used for immunoblotting of HeLa total cell extracts (asynchronous cells or cells arrested in mitosis with nocodazole). Only the phosphorylated HP1 α peptide can compete the signal. HP1 α peptides: amino acids 83-101. H3 peptide: amino acids 1-20.

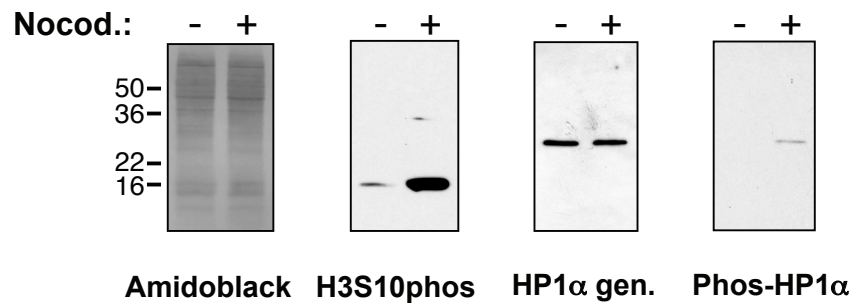
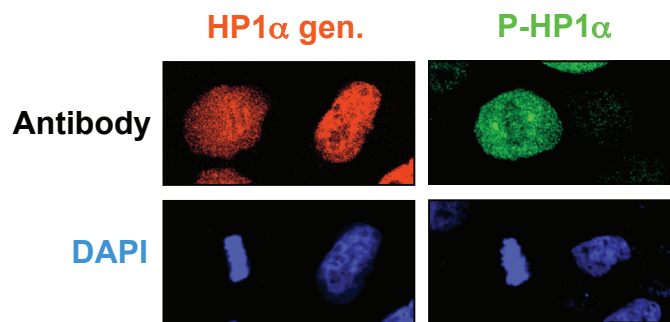
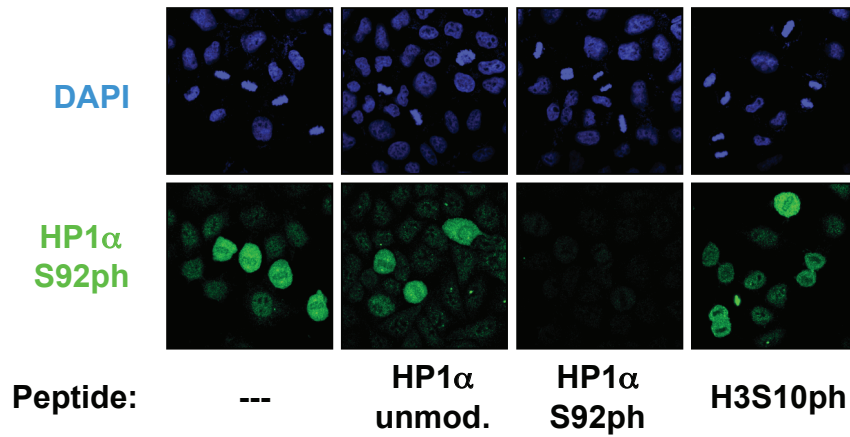
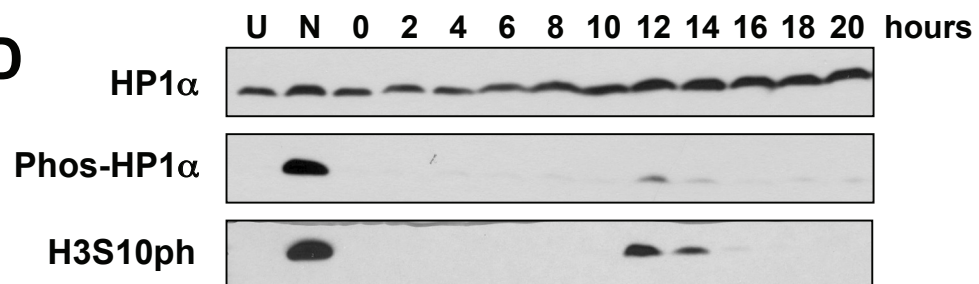
Figure 4.6: HP1 α S92 phosphorylation is a mitotic mark.

A: Whole cell extracts of HeLa cells (asynchronous or arrested in mitosis with nocodazole) were analyzed by western blotting. The mitotic marker H3S10ph confirms enrichment for mitotic cells upon nocodazole treatment. While HP1 α levels do not change, HP1 α serine 92 phosphorylation is specifically observed in mitotic cells.

B: Immunofluorescence microscopy of HeLa cells. The mitotic cell shows intense HP1 α S92ph staining, while the interphase cell next to it is not stained at all. General HP1 α staining confirms that this observation is not simply explained by changes in the levels of HP1 α or differences in antibody accessibility.

C: Peptide competition. The HP1 α S92ph antibody (1:200) was preincubated with different peptides (2 μ g/ml) and then used for immunofluorescence staining of HeLa cells. Competition with a phosphorylated HP1 α peptide completely abolishes the observed signal, while control peptides (unmodified HP1 α peptide, H3S10ph peptide with the very similar ARKS sequence) have no effect. HP1 α peptides: amino acids 83-101. H3 peptide: amino acids 1-20.

D: HeLa cells were synchronized by double-thymidine block. After removal of the second S-phase block, samples were taken at regular intervals and whole cell extracts analyzed by immunoblotting. The Phos- HP1 α signal comes up in mitosis (around 12 h), at the same time as the mitotic marker H3 serine 10 phosphorylation. U: Unsynchronized cells; N: Nocodazole-arrested cells.

A**B****C****D****Figure 4.6**

Overall, these western blotting and IF experiments confirm that HP1 α serine 92 is indeed a site of phosphorylation *in vivo*, and that this site is specifically phosphorylated in mitosis.

Detailed characterization of HP1 α S92ph by immunofluorescence microscopy

In order to take a closer look at the temporal correlation between HP1 α S92ph and the well characterized mitotic marker H3S10ph, I carried out an IF costaining of HP1 α S92ph and H3S10ph by (Figure 4.7A). This analysis confirmed that there is a close temporal correlation in the appearance of the two marks (both at late G2/early prophase). The staining of both antibodies disappears slowly around the stage of cytokinesis (Figure 4.7A). At first sight, the localization of the HP1 α S92ph signal with its exclusion from chromatin during mitosis looks very similar to stainings with general HP1 α antibodies (data not shown).

Closer inspection of immunofluorescence stainings, however, yielded two intriguing observations (Figure 4.7B): The first is that the HP1 α S92ph antibody stains two dot-like structures that look in their cellular position and their migration behaviour like spindle poles/centrosomes. The second, that at telophase there are differences between the stainings of the HP1 α S92ph antibody and a general HP1 α antibody (Millipore MAB 3584) (see below).

Presumed centrosomal staining

Dot-like structures suggestive of centrosomal staining had on occasion also been observed in prophase (Figure 4.7A) or even interphase (Figure 4.6C), even though these stainings were not very reproducible. To investigate

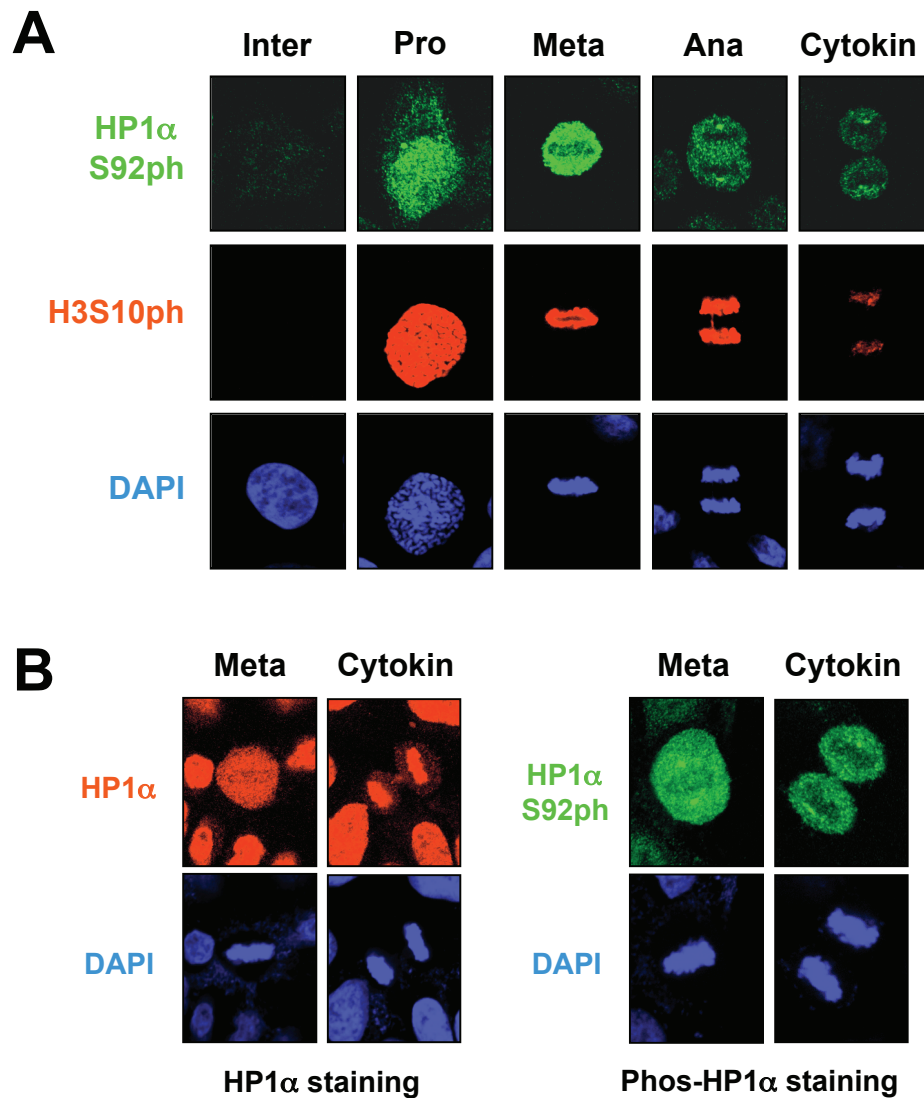


Figure 4.7: Detailed characterization of the HP1 α S92ph signal by immunofluorescence microscopy.

A: Immunofluorescence microscopy of HeLa cells after costaining for HP1 α S92ph and H3S10ph. The signal of the two antibodies appears with a very similar timing in early prophase. HP1 α S92ph then follows the movement of HP1 α as it dissociates from chromatin. The signal becomes weaker as mitosis proceeds, but is still visible until cytokinesis.

B: Immunofluorescence microscopy of HeLa cells after staining with the indicated antibodies. The HP1 α S92ph antibody stains dot-like structures, most likely centrosomes, in metaphase as well as cytokinesis. In cytokinesis, the general HP1 α staining shows that most of HP1 α has already reassociated with chromatin. However, HP1 α S92ph staining is at this point still excluded from chromatin. Note that the general HP1 α and Phos-HP1 α stainings depicted in this figure are *not* co-stainings, but rather individual stainings.

whether the spindle pole signal was the result of a small amount of highly phosphorylated HP1 α at the centrosomes or just a crossreactivity of the HP1 α S92ph antibody, I studied the effect of HP1 α knock-down by RNAi on the staining of HeLa cells (Figure 4.8).

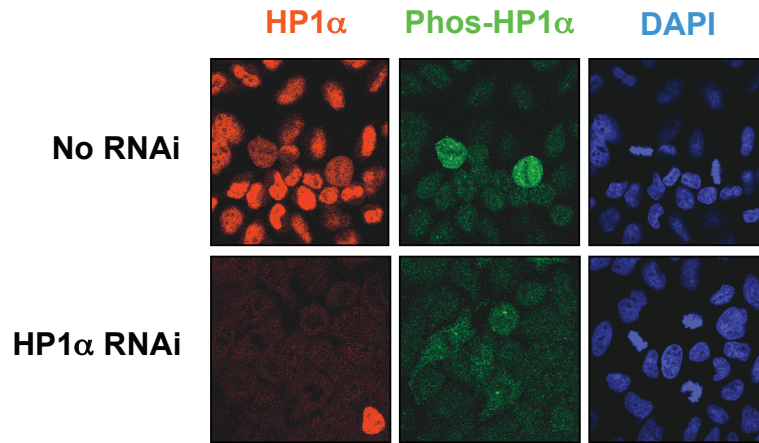


Figure 4.8: Effect of HP1 α RNAi on centrosomal staining of the Phos-HP1 α antibody.

Immunofluorescence microscopy with HP1 α S92ph and HP1 α antibodies after transfection with HP1 α or mock siRNAs. HP1 α RNA interference clearly reduced the levels of HP1 α , as well as the Phos-HP1 α signal in the cytoplasm/nucleus. The dot-like structures seem less affected.

It is difficult to judge from these pictures to what extent the signal of the dot-like structures is affected by the RNAi, because partial removal of strong dot-like staining may be hard to detect and quantify, and the effects were somewhat variable. My overall conclusion is, however, that there is little if any effect of HP1 α RNA interference on the dot-like staining. Because, in addition to this finding, I had never observed myself nor seen in the literature any reports of spindle pole/centrosome staining with any general HP1 α antibody, I concluded that the centrosomal staining may be a crossreactivity and decided not to follow up on this observation any further.

HP1 α S92ph labels a pool of HP1 α that is still excluded from chromatin at cytokinesis

The second curious observation made from the IF stainings of Figure 4.7B are the differences between HP1 α S92ph and general HP1 α stainings at the end of mitosis. In late telophase/cytokinesis, the majority of HP1 α is already back at its binding site at chromatin, as can be seen from the staining with the general HP1 α antibody. At the same time, however, the (fading) Phos-HP1 α signal is still seen largely in the cytoplasm, indicating that HP1 α molecules carrying the S92 phosphorylation mark have not yet reassociated with chromatin. Thus, it seems that the Phos-HP1 α antibody marks a specific subpool of HP1 α that has not yet re-engaged into a molecular interaction with chromatin.

The observation was confirmed by costainings in several human (HeLa, HeP2 cells; data not shown) as well as mouse cell lines (NIH3T3, 10T1/2; see Figure 4.9A). In addition to reproducing the exclusion of the HP1 α S92ph signal from chromatin at telophase/cytokinesis (see Figure 4.9B for blow-up), these experiments also confirmed that HP1 α S92 phosphorylation exists in mouse cells and behaves exactly as in human cells. (In peptide competitions, the general HP1 α signal (Monoclonal Antibody #3584 from Chemicon/Millipore) was unaffected by both unmodified and phosphorylated HP1 α peptides in immunofluorescence and western blotting experiments (data not shown).)

That the S92 phosphorylation staining is excluded from chromatin is of course just a correlation and does not prove any causal link between this phosphorylation and the regulation of HP1 α chromatin association. An alternative explanation would be for example the existence of a chromatin-associated phosphatase that removes the phospho-mark as soon as HP1 α

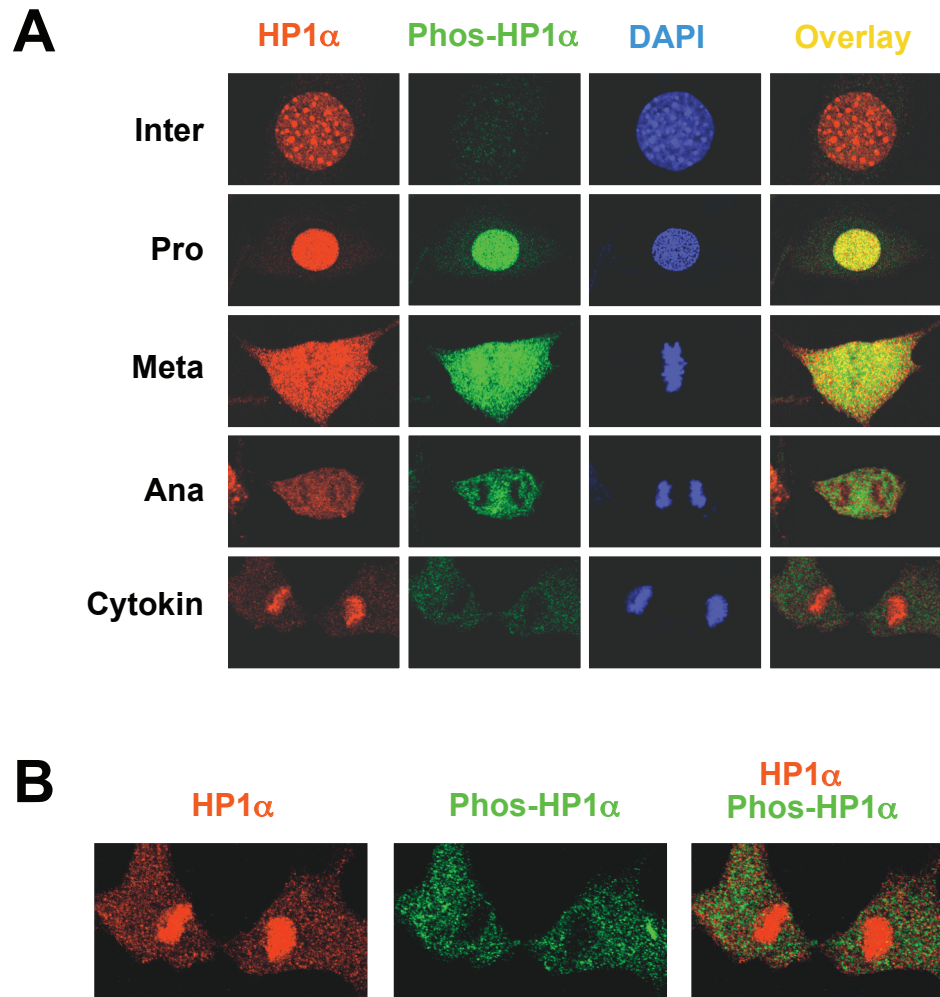


Figure 4.9: Differences between HP1 α S92ph and general HP1 α stainings in telophase.

A: Detailed analysis of HP1 α S92ph in mouse 10T1/2 fibroblast cells (IF). Kinetics and localization of the HP1 α S92ph staining are very similar to the stainings observed in human cells. (Note: The pronounced punctate staining of HP1 α in interphase cells is a known characteristic of mouse cells.)

B: Blow-up from panel A with increased settings for sensitivity and contrast. As in human cells, Phos-HP1 α labels in mouse cells specifically the pool of HP1 α that has not yet reassociated with chromatin at telophase/cytokinesis.

comes close. However, it is tempting to speculate that the exclusion of phosphorylated HP1 α from chromatin may be indicative of a causal relationship, that S92ph controls HP1 α chromatin association and that the phosphorylation has to be removed for stable reassociation of HP1 α at the end of mitosis.

Experiments that I carried out to follow up on this observation are described below (Section "A role for HP1 α serine 92 phosphorylation in regulating HP1 α chromatin association?"). However, before I go into detail about these experiments, I will first present data in which I confirm that Aurora B is indeed the kinase responsible for S92 phosphorylation *in vivo*.

Aurora B is the kinase responsible for HP1 α S92 phosphorylation *in vivo*

As shown in Figure 4.7A, HP1 α S92 phosphorylation and H3 S10 phosphorylation take place at about the same time in late G2/early prophase, suggesting that Aurora B may be responsible not only for H3 S10, but also for HP1 α serine S92 phosphorylation. In addition, the sequence context of HP1 α serine 92 (KRKS) resembles known Aurora B target sites (e.g. in H3S10: ARKS), and Aurora B efficiently phosphorylates HP1 *in vitro*. Furthermore, HP1 α (as well as HP1 γ) has been reported to interact with another component of the Chromosomal Passenger Complex, INCENP (Ainsztein et al., 1998). Thus, I decided to directly test the hypothesis that Aurora B is responsible for HP1 α S92 phosphorylation *in vivo*.

To begin, I turned to immunofluorescence costaining to show that Aurora B and HP1 α indeed colocalized at the time of HP1 α phosphorylation. As expected for a chromosomal passenger protein, in prophase at the time

when HP1 α S92 phosphorylation occurs, Aurora B was found all over the chromosomes, therefore significantly overlapping with the localization of HP1 α serine 92 phosphorylation (data not shown). Encouraged by this correlative observation, I went ahead to test involvement of Aurora B in mitotic HP1 α phosphorylation in a more direct way.

Aurora B inhibition with hesperadin

To test directly whether Aurora B is indeed the kinase responsible for mitotic HP1 α phosphorylation *in vivo*, I carried out Aurora B inhibition experiments with the small molecule inhibitor hesperadin (Hauf et al., 2003). However, Aurora B activity is required for multiple important steps in mitosis, and without it the spindle check-point does not function, preventing mitotic arrest by chemicals commonly used for this purpose. Since detection of HP1 α S92ph in western blots requires enrichment for mitotic cells, I was forced to adapt a more indirect approach (Hauf et al., 2003).

HeLa cells were arrested in mitosis by treatment with nocodazole for 12 h. Then the arrested cells were treated with hesperadin. Simultaneously with hesperadin, the proteasome inhibitor MG132 was added to the cells, which precludes the degradation of anaphase inhibitor and thus prevents cells from leaving metaphase. After a brief incubation of 15 min with hesperadin/MG132, cells were harvested and tested by western blotting.

Generally, phosphatases are globally present and constitutively active, while kinases are highly regulated in their localization and activity. The phosphorylation of a specific site is determined by the balance between these counteracting forces. Consequently, if Aurora B is the kinase responsible for serine 92 phosphorylation, inhibition of this kinase should

shift the phosphorylation/dephosphorylation balance in the arrested cells towards the unphosphorylated form, resulting in a reduced HP1 α S92ph signal.

With control immunoblots I verified that my basic experimental set-up was working (Figure 4.10). When I subsequently checked the effect of the hesperadin/MG132 treatment on HP1 α S92ph, I found that the phosphorylation signal accumulated upon nocodazole arrest was significantly reduced and brought down to levels observed in unsynchronized cells. This strongly supports the hypothesis that Aurora B is indeed the kinase responsible for HP1 α S92 phosphorylation *in vivo*.

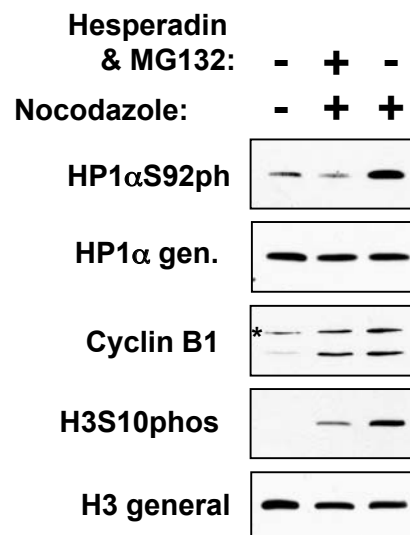


Figure 4.10: Aurora B inhibition with the small molecule inhibitor hesperadin decreases HP1 α S92 phosphorylation.

HeLa cells were arrested in mitosis with nocodazole. Then they were either treated with hesperadin/MG132 or vehicle. Asynchronously growing cells (no nocodazole treatment) serve as control.

As expected, nocodazole arrest results in accumulation of the mitotic cyclin B1 (asterisk indicates a crossreacting band) as well as increased levels of H3S10ph and HP1 α S92ph. Blots with antibodies against general H3 and HP1 α confirm equal loading. Upon treatment with hesperadin/MG132, the cyclin B1 signal persists, proving that cells did not leave mitosis. Levels of H3S10ph and HP1 α S92ph, on the other hand, are reduced upon hesperadin/MG132-treatment.

However, it should be noted that the described inhibition experiment is based on a chemical compound, which might have unspecific effects on other kinases. Indeed, off-target effects of hesperadin have already been reported (e.g. on Aurora A, Hauf et al., 2003). I therefore decided to complement the small molecule inhibition experiment with a second, independent method.

Aurora B knock-down by RNA interference

To verify the observations made in the inhibition experiment through a second approach that was independent of a chemical compound, I carried out knock-down experiments of Aurora B by RNA interference.

Because Aurora B knock-down prevents the straightforward use of nocodazole to arrest cells in mitosis (see above), I generated a synchronized population of HeLa cells by double-thymidine block. Between the first and second block, the cells were transfected with siRNAs targeted at the Aurora B sequence. After the release of the second block and transition through S phase, the synchronized cell population was harvested upon entry into mitosis (as judged from cells deattaching and rounding up). Cell synchrony and enrichment for mitotic cells in samples transfected with Aurora B and mock siRNAs was confirmed by flow cytometry (Figure 4.11, left).

Western blotting shows that Aurora B RNAi resulted in almost complete removal of the kinase, while Aurora A protein levels were unaffected (Figure 4.11, right). When I examined how this specific removal of Aurora B affected mitotic HP1 α S92 phosphorylation, I found a significant decrease in the levels of this mark. This strongly supports the hypothesis that Aurora B is indeed the kinase responsible for phosphorylation of HP1 α serine 92 during mitosis *in vivo*.

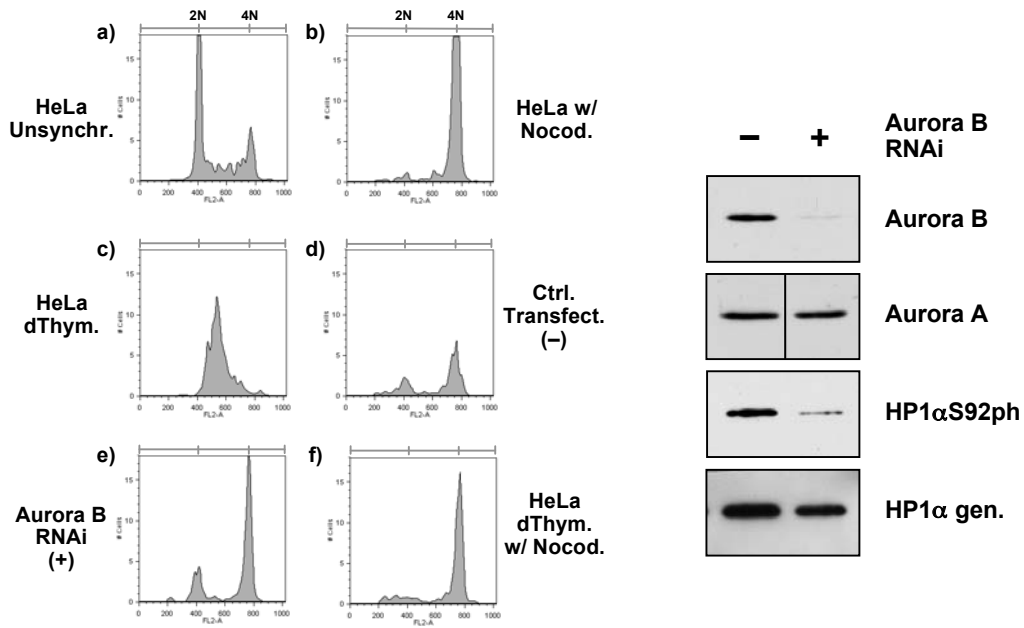


Figure 4.11: Aurora B RNAi decreases HP1αS92 phosphorylation.

Left: Analysis of DNA content of cells synchronized by double-thymidine block. Cells were stained with propidium iodide, analyzed by flow cytometry and the data plotted as histograms. 2N: DNA content of diploid cell (before replication). 4N: doubled DNA content after replication.

a: In a control population of unsynchronized HeLa cells, most cells are in G1 phase (2N DNA content) and only few cells in S (between 2N and 4N) or G2 (at 4N). **b:** Positive control: HeLa cells in mitosis (arrested in prophase with nocodazole) uniformly have 4N DNA content. **c:** HeLa cells 2h after double-thymidine block proceed through S phase as a homogeneous population. **d, e:** Cells synchronized by double-thymidine block, transfected with mock (d) or Aurora B siRNAs (e), and harvested at mitosis. Most cells have 4N DNA content, indicating reasonably good cell synchrony. d) and e) are the samples used for analysis by western blotting, see on the right. **f:** Synchronized HeLa cells prevented from proceeding through mitosis by nocodazole block. Comparison of d and e with f shows that a small number of cells in d and e had already progressed through mitosis and reentered G1 at the time of harvesting. However, there is still significant enrichment for mitotic cells observed in d) and e).

Right: Western blots of synchronized HeLa cells treated with either Aurora B or mock siRNAs, and harvested when the majority of cells were in mitosis (see left, panels d and e). Aurora B RNAi efficiently reduces Aurora B protein levels, while not affecting Aurora A. Even though HP1α protein levels are similar in both samples, HP1α serine 92 phosphorylation is significantly decreased in the sample treated with Aurora B siRNAs.

In sum, I have presented multiple experiments that all support the interpretation that Aurora B is the kinase responsible for mitotic phosphorylation of HP1 α at serine 92. Colocalization of Aurora B and HP1 α at the time of phosphorylation, chemical inhibition, knock-down of Aurora B by RNA interference, site-specific *in vitro* phosphorylation as well as the published observation reporting an interaction of HP1 α with another member of the Chromosomal Passenger Complex, INCENP (Ainsztein et al., 1998), all point in the same direction. While it cannot be ruled out that there are other kinases that contribute to this phosphorylation at the onset of mitosis, the simplest interpretation of my data is that Aurora B is the main kinase responsible for HP1 α serine 92 phosphorylation *in vivo*.

Hypothesis: Does HP1 α serine 92 phosphorylation play a role in regulating HP1 α chromatin association?

The observation that HP1 α S92ph specifically stains a subpopulation of HP1 α which has not yet reassociated with chromatin is highly intriguing, because it raises the possibility that serine 92 phosphorylation may play a role in the regulation of HP1 α chromatin association.

As conclusively shown by the findings described in Chapter 3 of my thesis, H3 serine 10 phosphorylation is essential for the mitotic release of HP1 from chromatin, and other groups have confirmed this observation (Hirota et al., 2005; Terada, 2006b). However, these different reports do not fully agree as to whether H3 serine 10 phosphorylation is actually also *sufficient* for HP1 α dissociation. While in peptide binding assays reduced binding of the chromo domains of all three HP1 isoforms and of full-length HP1 β to H3K9me3S10ph peptides was found (Fischle et al., 2005a; Hirota et al., 2005), H3S10 phosphorylation seems not sufficient to prevent binding

of full-length HP1 α and HP1 γ (Fass et al., 2002; Terada, 2006a). Differences in the experimental systems cannot fully account for these conflicting observations. One intriguing explanation to this conundrum is, however, a possible contribution of other HP1 domains besides the chromo domain to the association of full-length HP1 α and HP1 γ with chromatin. If in addition to the chromo domain-H3K9me interaction other molecular interactions contribute to stable association of HP1 α and HP1 γ with chromatin *in vivo*, then reversal of chromo domain-binding by methyl-phos switching may not be sufficient for the release of the full-length proteins.

This interpretation is supported by observations that our collaborators Boo Tseng and Dr. Hironori Funabiki made with *Xenopus* egg extracts during the study of methyl-phos switching. While exogenous full-length xHP1 α was released from chromatin in the presence of CPC and retained upon depletion of the CPC, a point mutation that abolished the CD-H3K9me interaction displayed an intermediate effect and clearly some binding in CPC-depleted extracts (Fischle et al., 2005b).

Contributions of additional interactions besides the CD-H3K9me to HP1 chromatin interaction have been suggested repeatedly in the literature (Dialynas et al., 2006; Eskeland et al., 2007; Muchardt et al., 2002; see Chapter 1, Section "Molecular Mechanisms of HP1 Recruitment"). The molecular mechanism for the reversal of such interaction(s) may well be phosphorylation on the side of the effector protein HP1.

Given the phosphorylation of HP1 α and HP1 γ , it might seem surprising that HP1 β is not phosphorylated. However, considering the differences in the interactions of the different isoforms, it is possible that HP1 β does not require Aurora B phosphorylation, because it simply does not undergo the specific interaction that is reversed by HP1 α/γ phosphorylation. Of

interest in this context, in none of the publications reporting interaction of full-length HP1 with H3 carrying serine 10 phosphorylations was retention of HP1 β reported. In our own experiments full-length HP1 β was readily released from H3K9me peptides upon S10 phosphorylation (Figure 3.7B).

Based on the observation that HP1 α S92ph closely correlates with HP1 α release and specifically stains a subpopulation of HP1 α which is largely not associated with chromatin in immunofluorescence, and the possible importance to release other HP1 interactions besides the CD-H3K9me interaction as described above, I formulated the hypothesis that HP1 α S92 phosphorylation might play a role in the mitotic release of HP1 α from chromatin *in vivo* (Figure 4.12).

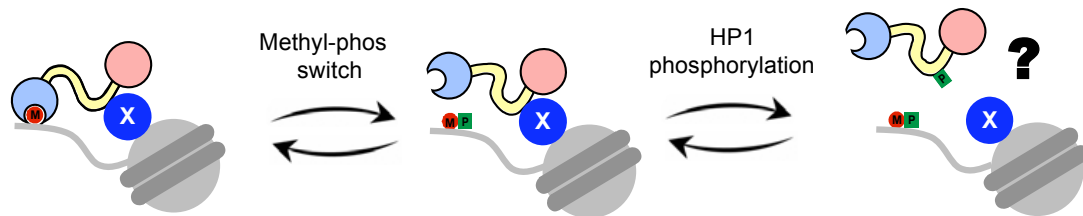


Figure 4.12: Hypothesis: HP1 α serine 92 phosphorylation is a necessary step for the dissociation of HP1 α from chromatin *in vivo*.

This hypothesis assumes that stable HP1 α binding to chromatin involves, in addition to chromo domain binding to H3K9me, a second interaction mediated by the hinge region (to an unknown chromatin component X). Efficient release of HP1 α from chromatin during mitosis is therefore a two-step process and involves not only methyl-phos switching, but also HP1 α serine 92 phosphorylation to reverse the hinge interaction. Stable reassociation of HP1 α to chromatin at the end of mitosis, on the other hand, requires the removal of HP1 α S92ph.

S92 phosphorylation correlates with increased extractability of HP1 α

Support for the hypothesis came from observations I made in biochemical experiments. Following a protocol commonly used in the Allis lab to prepare chromatin on a small scale, I extracted chromatin prepared from mitotic HeLa cells with a low-salt buffer (Mendez and Stillman, 2000). When I then compared the S92 phosphorylation of extracted and retained HP1 α by western blotting, I found that the level of HP1 α S92 phosphorylation correlates with the extractability of HP1 α (Figure 4.13A).

A pool of HP1 α associated with metaphase chromosomes is not recognized by the Phos-HP1 α antibody in IF experiments

A second observation in support of the hypothesis that serine 92 phosphorylation may play a role in HP1 α chromatin association came from immunofluorescence microscopy. In metaphase cells, I observed on occasion a pool of HP1 α that specifically localized to chromatin. However, this pool of HP1 α was only detected by general HP1 α staining and not with the Phos-HP1 α antibody (Figure 4.13B).

One possible explanation for this finding is that some HP1 α protein is not phosphorylated and therefore remains chromatin-associated throughout metaphase. This would be in keeping with the hypothesis that HP1 α serine 92 phosphorylation plays a role in the release of HP1 α from chromatin (for alternative explanations see below).

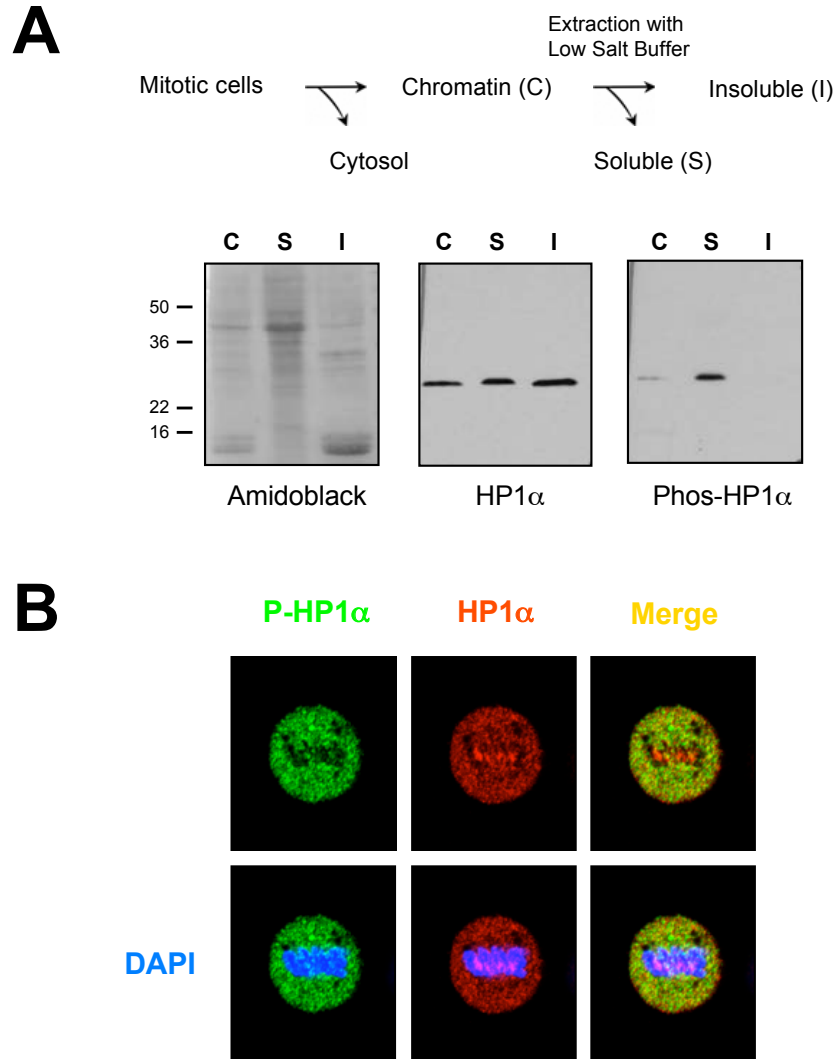


Figure 4.13: Biochemical and IF experiments suggest that HP1 α serine 92 phosphorylation may indeed regulate HP1 α chromatin association.

A: Mitotic HeLa cells were lysed with 0.2% NP40 and purified chromatin was extracted with a buffer of low ionic strength ("low-salt buffer": 2 mM KCl/3 mM EDTA/0.2 M EGTA). Serine 92 phosphorylation of extracted (S, soluble) and unextracted (I, insoluble) HP1 α was compared by western blotting. Levels of phosphorylation are much higher in the extracted fraction. C: chromatin fraction before extraction; S: soluble in low-salt buffer; I: insoluble in low-salt buffer.

B: Immunofluorescence microscopy of HeLa cells. Staining with a general HP1 α antibody labels a pool of HP1 α that is associated with the mitotic chromosomes of metaphase cells. This pool of HP1 α is not recognized by the Phos-HP1 α antibody.

Stable cell lines expressing HP1 α with serine 92 point mutations

Encouraged by these findings, I decided to test the hypothesis that HP1 α phosphorylation at serine 92 might be involved in the control of HP1 α dissociation from chromatin directly. However, I did not have specific knowledge of which interaction(s) might be stabilizing HP1 α 's chromatin binding. In fact, the details of HP1 chromatin binding *in vivo* are exceedingly complex, possibly requiring several steps of binding and stabilization and multiple interactions (see Chapter 1). While it is clear that the CD-H3K9me3 interaction is a particularly important interaction for binding of HP1 to chromatin, HP1 chromatin association *in vivo* is not completely understood. This made it impossible for me to set up an *in vitro* system of which I could be sure that it fully reflected the *in vivo* situation. Thus, I reasoned that the best way to test my hypothesis was the expression of HP1 α constructs with S92 mutations in cultured mammalian cells. If the hypothesis in Figure 4.12 was correct, then an unphosphorylatable HP1 α should be retained at chromatin (at least in the most extreme scenario). Similarly, an HP1 α construct that mimics constitutive phosphorylation should not reassociate properly with chromatin at the end of mitosis. (Since the interaction controlled by S92 phosphorylation might be only of a stabilizing nature, it was possible that the phenotype would be a little less pronounced.)

To this end, I generated HeLa cell lines that stably expressed tagged HP1 α with either wt (wild-type) sequence or with a point mutation at serine 92 (mutated to alanine to prevent phosphorylation; mutated to glutamate to mimic constitutive phosphorylation). Originally, I developed stable cell lines expressing HP1 α fused with Green Fluorescent Protein (GFP). However, with these cells I observed significant differences in the

fractionation behaviour of wt GFP-HP1 α and endogenous HP1 α (data not shown). Thus, I generated a second set of cell lines with a much smaller FLAG-HA-FLAG tag. As a control for effects caused by the tag, I generated two sets of cell lines with the constructs tagged on the N-terminal and the C-terminal side of the protein, respectively.

In western blotting experiments, the cell lines for both N-terminally (Figure 4.14) and C-terminally tagged (data not shown) HP1 α were characterized, proving expression of a protein of the expected size (Figure 4.14, left). Tagged HP1 α is phosphorylated with the same efficiency as endogenous HP1 α (Figure 4.14, right), and in fractionation experiments as well as peptide pull-down experiments with H3 peptides tagged HP1 α behaved like the endogenous protein (data not shown).

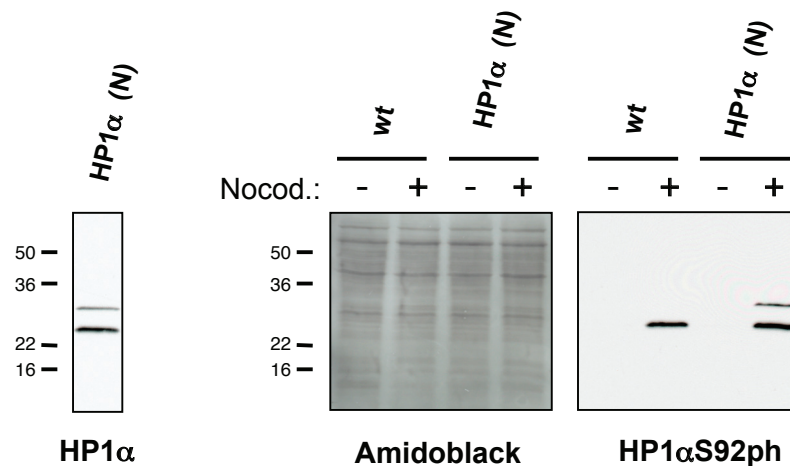


Figure 4.14: Characterization of HeLa cell lines stably expressing FLAG-HA-tagged HP1 α .

Cell line expressing N-terminally FLAG-HA-tagged HP1 α shown. Whole cell extracts of cell lines were analyzed by immunoblotting. **Left:** An additional band of the expected size is detected in the HP1 α western blot, showing the expression level of the tagged HP1 α . **Right:** Upon nocodazole arrest, exogenous tagged HP1 α is phosphorylated to the same extent as endogenous HP1 α (compare with ratio of bands in HP1 α blot). HP1 α (N): HeLa cells stably expressing N-terminally FLAG-HA-tagged HP1 α . wt: Unaltered HeLa control cells. Amidoblack shows equal loading of the lanes. Similar results were obtained with the C-terminally tagged constructs.

No obvious effect of serine 92 mutation on HP1 α localization

The effect of serine 92 mutation on the localization of HP1 α was analyzed by immunofluorescence microscopy after staining with an HA antibody. Clearly, the tag did not alter the cellular localization of HP1 α in these experiments, because tagged wt HP1 α (no mutation) showed the same localization as observed before for endogenous wt HP1 α (Figure 4.15, top row).

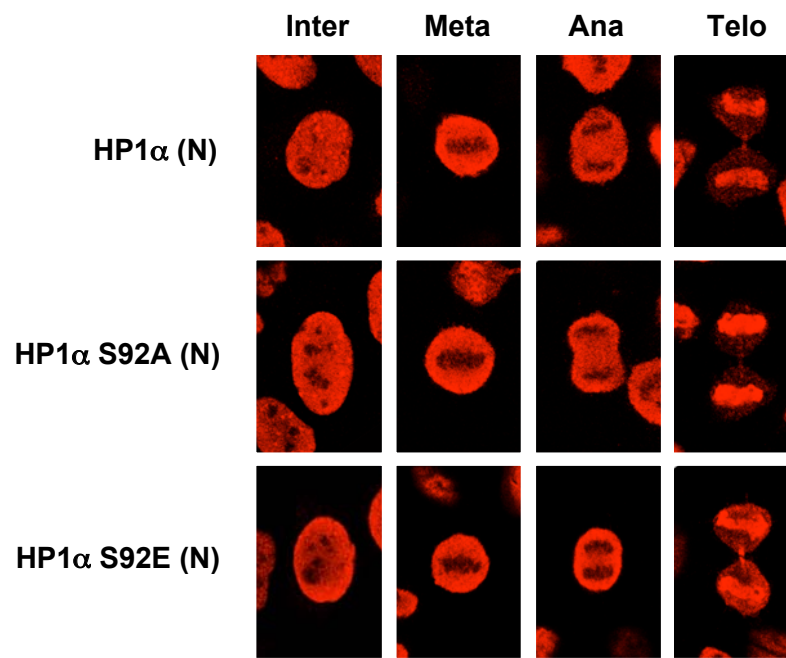


Figure 4.15: No obvious effect of HP1 α serine 92 mutations on HP1 α localization.

Immunofluorescence microscopy (anti-HA) of HeLa cell lines stably expressing FLAG-HA-tagged HP1 α constructs with and without serine 92 point mutations. Typical stainings are shown. Point mutation of serine 92 does not have any clear effect on HP1 α localization or dynamics at the different stages of mitosis.

Stages of mitosis are indicated above the panels. (Since only mouse interphase cells show clear heterochromatic dots, no such dots are observed in these HeLa cell stainings.) HP1 α (N): HP1 α with wt sequence, N-terminally tagged; HP1 α S92A (N): HP1 α with serine 92 mutated to alanine, N-terminally tagged; HP1 α S92E (N): HP1 α with serine 92 mutated to glutamate, N-terminally tagged. Comparable observations were made with the C-terminally tagged constructs.

If serine 92 phosphorylation was indeed essential for the regulation of HP1 α dissociation during mitosis, I reasoned that mutating this serine should affect the localization of HP1 α during mitosis. Mutation of serine 92 to alanine precludes phosphorylation and may therefore prevent the release of HP1 α from chromatin; mutation of serine 92 to glutamate, on the other hand, might mimic constitutive phosphorylation (phosphomimic) and may thus delay or even prevent stable reassociation of HP1 α with chromatin at the end of mitosis. However, my analyses failed to show any obvious effects of the serine 92 point mutations on HP1 α localization or dynamics (Figure 4.15).

There are several possible explanations why these point mutations of serine 92 do not have any clear effect on HP1 α localization. S92ph could be redundant with other phosphorylations or other covalent marks, or may need to cooperate with additional modifications in order to bring about an effect on HP1 α 's chromatin association (see below section "Redundancy / Requirement for cooperation"). But it is of course also possible that serine 92 phosphorylation does indeed fulfill its function on its own, but that this function is simply not the regulation of HP1 chromatin binding. Serine 92 phosphorylation could for example control a different molecular interaction of HP1 α that has to be released/established during mitosis, but that does not affect the association of HP1 with chromatin.

Hypothesis: HP1 α serine 92 phosphorylation regulates the HP1 α 's association with a specific binding partner

If such an interaction exists, I reasoned that a yet unidentified binding partner would display differential binding to unmodified versus S92-phosphorylated HP1 α . Based on this assumption, I set out to identify

molecules that bound to the serine 92 region of HP1 α if it was unmodified, but were abrogated, if S92 was phosphorylated, or vice versa.

Peptide pull-downs to identify protein interactions controlled by HP1 α serine 92 phosphorylation

My first approach to identify such phosphorylation-dependent binders was by peptide pull-down (Wysocka, 2006). Protein modules binding in dependence of a single modifications often recognize only a short sequence stretch around the modification site (Seet et al., 2006); thus, a peptide was generated that included the site of modification and about ten amino acids upstream and downstream (HP1 α residues 83-101). Phosphorylated/unmodified HP1 α peptides were immobilized on avidin-coated agarose beads and incubated with cellular extracts. After washing steps, bound proteins were eluted and run out on SDS-PAGE gels, to identify specific bands after silver-staining. Since a binding protein might be a factor that is only expressed at a certain time during the cell cycle, extracts from both asynchronously growing and nocodazole-arrested HeLa cells were used. To minimize the risk of peptide dephosphorylation by phosphatases present in the extract, high concentrations of phosphatase inhibitors (25 mM NaF, 25 mM β -glycerolphosphate, 6 mM Orthovanadate, CalBiochem Phosphatase Inhibitor Set (contains Cypermethrin, Dephosphatin, Okadaic acid, and NIPP-1); these are levels known to protect endogenous HP1 α from dephosphorylation) were present at all times during the pull-down. However, no phospho-specific bands were detected reproducibly in these first pull-downs.

Several technical aspects of the experimental procedure could account for this. Therefore, I repeated the experiment under various conditions,

such as different methods of extract preparation, using HeLa S3 suspension cells (larger cell number results in higher extract concentration, in case the binding partner was only a low-abundance protein) and lowering the number and stringency of washing steps (to reduce the risk of washing off a low-affinity interactor). To make sure even weak specific binders were not missed in this complex protein mixture, whole gel lanes were analyzed by mass spectrometry (carried out by the MS facility of the Max-Planck-Institute for Biophysical Chemistry in Göttingen, Germany). Subsequently, the complete lane was compared in its protein content and abundance with control pull-downs.

To validate the approach, I also included a positive control by using K9me3 and unmodified H3 peptides (Figure 4.16). As expected, in the H3K9me3 pull-down all three HP1 isoforms HP1 α , HP1 β and HP1 γ were found. In contrast, no HP1 peptides were detected in the pull-down with unmodified H3 peptide. Thus, the control pull-downs specifically enriched for the expected modification-dependent interactors and verified the approach.

Comparison of the proteins in pull-downs with HP1 α S92ph and unmodified HP1 α peptides (see Figure 4.16 for example), however, did not lead to the identification of any convincing and reproducible candidates. Minor differences in the binding to the two peptides seemed either caused by handling (different keratins), unspecific binding of abundant and sticky cellular proteins (e.g. chaperonin, ribosomal proteins, actin, tubulin) or could be excluded due to their cellular localization (e.g. mitochondrial proteins).

Besides the possibility that S92 phosphorylation simply does not regulate a protein interaction at all, there are several other possible reasons why pull-down experiments may not have led to the identification of an

interactor. It could be that the binding partner was not present in sufficient abundance in the extracts used or that the affinity of the interaction is very low. Indeed, interactions depending on a single posttranslational modification have often been found to be rather weak (Fischle et al., 2003b; Pawson, 1995; Ruthenburg et al., 2007). The low affinity may even be a prerequisite for dynamic regulation, and in the context of the full-length protein such interactions could be stabilized by additional contacts with other partners. In this case a classical biochemical pull-down with a peptide may simply not be sensitive enough for the identification of the interaction partner.

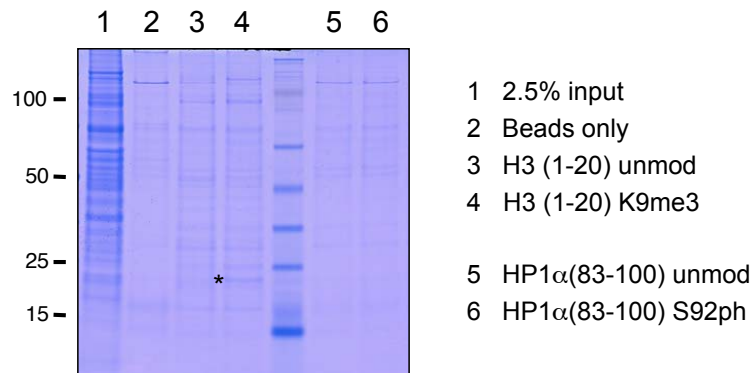


Figure 4.16: Peptide pull-down to identify phosphorylation-dependent binders of HP1α.

Peptide pull-down carried out with a nuclear extract of unsynchronized HeLa S3 cells. (Similar experiments were completed with extracts from nocodazole-arrested cells.) Extracts were incubated with biotinylated peptides immobilized on avidin-coated agarose beads. After washes the beads were boiled in protein sample buffer, and the mixture was run out on an SDS-gel and stained with Coomassie. Lanes were subsequently cut into small pieces and subjected to analysis by mass spectrometry. Lanes 3 and 4 show the control pull-down with unmodified and K9-methylated H3 peptides. Some differences are so clear that they can even be seen in the coomassie staining. All three HP1 isoforms were subsequently identified specifically in the H3 K9me3 lane (asterisk marks a band that is most likely HP1γ). Lanes 5 and 6 show an example of an HP1α (83-101) peptide pull-down (unmodified vs. S92ph). Only little protein binds to the HP1α peptides (compare to lane 2), and banding patterns for the two pull-downs are virtually identical.

Phage display screen for interacting proteins regulated by HP1 α serine 92 phosphorylation

To exclude that an interaction partner was missed due to these limitations of the biochemical pull-down, I applied a complementary approach by phage display screening, which I carried out (as some other experiments described in this thesis) in the laboratory of Dr. Wolfgang Fischle at the Max-Planck-Institute for Biophysical Chemistry in Göttingen (Germany). Its multiple rounds of clonal enrichment (see Figure 4.17) make this approach particularly suited for the identification of weak interactions with rare binding partners.

To verify that the screen was working as expected, control peptides (unmodified H3 peptide and H3K9me3 peptide) were probed with a library containing minute amounts of an HP1-expressing phage (diluted 1:10⁶). Within a few rounds of selection, the H3K9me3 peptide significantly enriched for the HP1-expressing phage (Figure 4.18, left), proving that this phage display screening protocol can indeed be used for efficient and highly selective enrichment for specific binders of posttranslational modifications.

The actual screen for binding proteins controlled by HP1 α S92 phosphorylation was carried out with two different phage libraries (from liver and brain tissue; Novagen), to increase the chances that a binding protein was found in the library. For every peptide I worked in duplicates, to estimate the reproducibility of the results. To my disappointment, the screening with the HP1 α peptides did not result in the enrichment of any specific phages, as judged by a preliminary analysis by PCR (Figure 4.18, right). Any follow-up experiments would have involved a significant investment in time and effort (screening of clones from last round of selection; sequencing of fused mammalian cDNA sequence; verification of

identified interactions). The preliminary analysis by PCR suggested that it was unlikely that a binding partner would be identified in the end (even though the positive control showed that the procedure was working in general). I therefore decided to abandon the phage display screen.

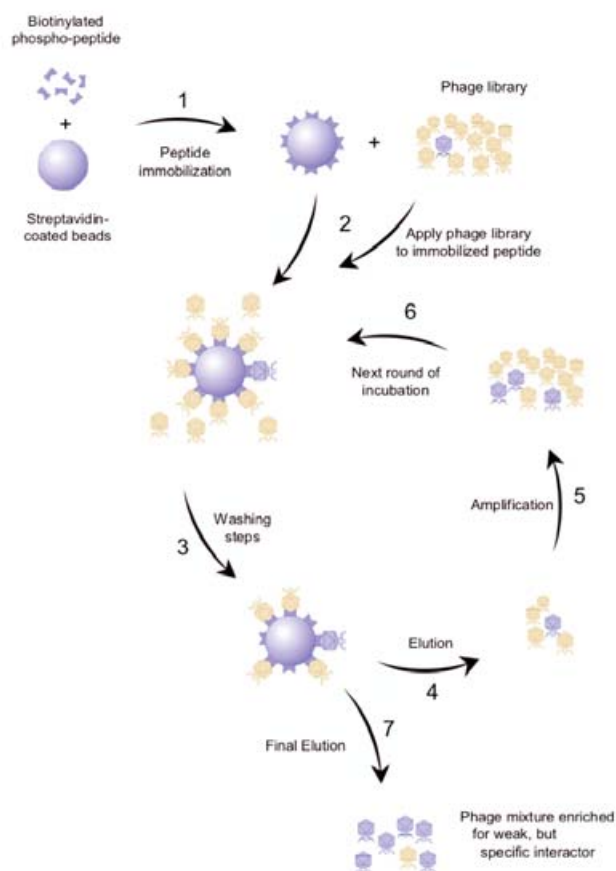


Figure 4.17: Principle of phage display screening.

Principle of the phage display screen for factors specifically interacting with HP1 α S92ph. An immobilized HP1 α S92ph peptide (1) is incubated with a library of phages expressing different cDNAs on their surface (2). After washing steps (3), phages binding to the peptide are released (4) and the eluted phages amplified in bacteria (5). The resulting mixture of phages is used for a second round of peptide binding (6). By successive rounds of binding and amplification phages expressing specific binding proteins are enriched, even if the interaction is rather weak and the binding protein present only at very low abundance in the original library (7).

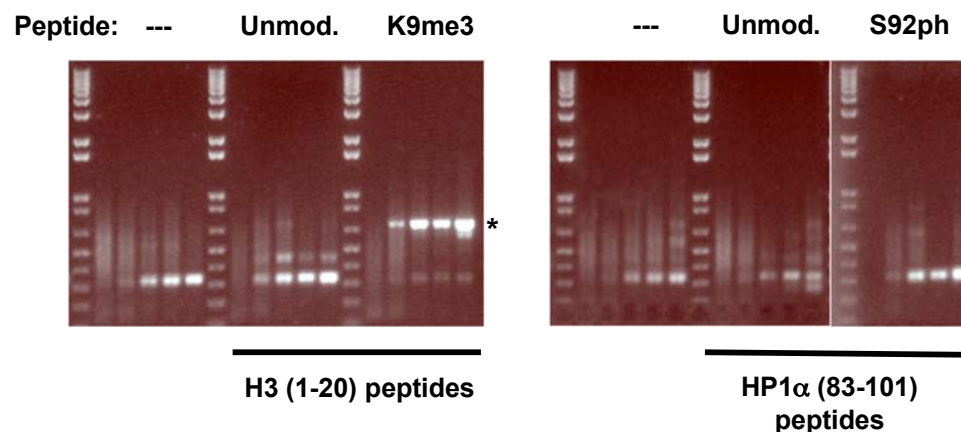


Figure 4.18: Phage display screen to identify phosphorylation-dependent binders of HP1 α .

Phage mixtures eluted after different rounds of selection were analyzed by PCR (with sequencing primers annealing on both sides of the insert within the phage sequence). The appearance of specific bands indicates the enrichment for individual phages. For each peptide, selection rounds 1 to 5 are shown, separated by a lane with DNA molecular weight marker.

Left: Control. For the H3K9me3 peptide, a strong band of the expected size (asterisk) is observed within few rounds of selection, proving fast and strong enrichment for phages expressing HP1. The unmodified H3 peptide shows no significant enrichment besides the band that is also seen in the beads-only control.

Right: Actual screen. Neither with the unmodified HP1 α peptide nor with the HP1 α S92ph peptide specific bands (besides the band seen in the beads-only control) are observed.

Redundancy / Requirement for cooperation

At this point, I pursued several different approaches (and variations of these) in order to identify a phospho-dependent binding partner. Even though I had tried to anticipate and work around limitations of some of these approaches, there were of course still multiple trivial technical explanations possible why the experiments undertaken had not succeeded.

It cannot be excluded, for example, that the length of the HP1 α peptide used in these assays (19 aa) was simply not sufficient for stable binding, even though modules binding in dependence of a single posttranslational modification often recognize only a few amino acids around that mark (Seet et al., 2006). I carried out pilot experiments by passing cellular extracts over a recombinantly expressed HP1 α hinge construct, but obtained protein mixtures too complex to analyze, possibly because the hinge region is not structured and therefore extremely sticky as an isolated domain. As an alternative explanation, it was also possible that a phosphorylation-dependent binding partner existed, but that it was not a protein, but a nucleic acid and therefore not represented in the extracts/libraries I used.

However, besides these possibilities, there was another explanation for the failure to identify S92ph-dependent binding that seemed even more likely: Serine 92 phosphorylation may not fulfill its function on its own, but in concert with other modifications. This could even explain why no phenotype was detected upon S92 mutation *in vivo*.

If there are indeed multiple sites of posttranslational modification in mitosis and modification of some of these is already sufficient for HP1 α dissociation (i.e. there is redundancy among the modifications), this could explain why S92A mutation does not prevent dissociation of HP1 α . The interplay of several sites of posttranslational modification could also be the reason why S92E mutation has no detectable effect: If a threshold number of sites have to be modified simultaneously to cause dissociation of HP1 α from chromatin (i.e. a "requirement for cooperation" of several modifications), mutation of a single site to glutamate will not prevent HP1 α from reassociating at the end of mitosis. Thus, the existence of

other modifications that collaborate with S92ph could explain the lack of a phenotype for point mutations of serine 92 despite all the intriguing correlative observations made.

As far as the type of posttranslational modification that may be collaborating with S92 phosphorylation is concerned, it could in principle be any kind. However, it seems particularly tempting to speculate about a role of phosphorylation. Additional phosphorylation would further increase the negative charge already introduced by S92ph, multiple kinases are known to be active during mitosis, and E. Minc et al. had already made observations suggesting that there may be more than one site of mitotic HP1 α phosphorylation (Minc et al., 1999).

Studying the role of HP1 α S92 phosphorylation in the context of such other mitotic phosphorylations is only possible, if these other phosphorylations (or modifications) are known. Therefore, I decided that it would be a worthwhile undertaking to examine the posttranslational modification profile of HP1 in detail.

Purification of HP1

In order to identify and map the sites of mitotic HP1 phosphorylation/modification, I decided to purify the protein from cultured cells and determine the posttranslational modification profile by mass spectrometry (MS). Besides novel modifications, the MS approach also promised to confirm the existence of mitotic HP1 α S92 phosphorylation *in vivo* through a completely independent method (in addition to the antibody-based methods I had already used), and might possibly even permit quantifying the abundance of the S92ph mark in mitotic and interphase cells.

Three different approaches for the purification of HP1 were

developed, which I briefly summarize below. In each of these cases, the actual mass spectrometric analysis was carried out by my collaborator Hillary Montgomery in the lab of Dr. Don Hunt at the University of Virginia (Charlottesville, VA).

(1) *Acid extraction and HPLC enrichment of endogenous HP1 followed by analysis from SDS-PAGE gel*

I isolated endogenous HP1 by acid extraction from highly enriched mitotic HeLa cells (nocodazole treatment followed by mitotic shake-off, resulting in about 95% enrichment for mitotic cells). Upon further separation by reversed phase HPLC (Figure 4.19A, left), HP1-containing fractions were run out on an SDS-PAGE gel (Figure 4.19A, right), and HP1 bands excised and analyzed by MS.

This approach led to the identification of three phosphorylations (HP1 β S89ph, HP1 γ S93ph and HP1 γ S95ph), but unfortunately no convincing modification was identified for the isoform that I was most interested in, HP1 α . Peptide coverage for all HP1 isoforms was low due to three technical aspects of the procedure: Firstly, acid extraction, even though the method of choice for enrichment of histones, is not very effective for the extraction of HP1, a finding that may be due to differences in isoelectric point (mammalian HP1 has a much lower pI than histones; note that this is in contrast to, e.g., *Tetrahymena* HP1, which has a pI more similar to that of histone proteins). Secondly, the approach requires proteolytic digestion and subsequent recovery of peptides from gel bands, which is often inefficient. And thirdly, in-gel digests are usually done with trypsin, and due to its richness in basic amino acids especially the HP1 hinge is cut by trypsin into pieces too small for most analyses.

Figure 4.19: Purification of HP1 for analysis of modification profile by mass spectrometry.

A: Approach 1: Enrichment for of endogenous HP1 isoforms by acid extraction, RP-HPLC and gel electrophoresis.

Left: Proteins acid extracted from mitotic HeLa cells were separated by RP-HPLC on an acetonitrile gradient. Fractions containing the HP1 isoforms were identified by slot blotting (the three isoforms eluted together, area between red lines). It is clear from the chromatogram that HP1 proteins are, in contrast to e.g. histones, only minor components in the extract. **Right:** HPLC fractions containing HP1 proteins were united and run on an SDS-PAGE gel, to cut bands for MS analysis. Asterisk: band containing (among other proteins) HP1 α and HP1 β . Arrow head: band containing HP1 γ .

B: Approach 2: Purification of endogenous HP1 γ by cell fractionation and immunoprecipitation (HP1 γ shown because HP1 α runs exactly behind antibody band).

Left: HP1 γ western blots after immunoprecipitation from the cytoplasmic fraction of mitotic HeLa cells. Most of the HP1 γ in the fraction is precipitated in the HP1 γ IP, while the control IP shows that this is dependent on the HP1 γ antibody. I: Input. U: Unbound. B: Bound. **Right:** Coomassie-stained gel of the HP1 γ IP and control IPs (25% of total IPs loaded). Compared to the amounts of antibody, the levels of HP1 γ (arrow head) in the IP are rather low. Note, though, that HP1 peptides were not analyzed from the gel, but rather directly from the beads after limited proteolytic digest.

C: Approach 3: Purification of tagged, overexpressed HP1 α from mitotic HeLa cells by cell fractionation and immunoprecipitation from the cytoplasmic fraction. Coomassie-stained gel (10% of total IP loaded). Arrow head: position of tagged HP1 α . Asterisks: antibody bands.

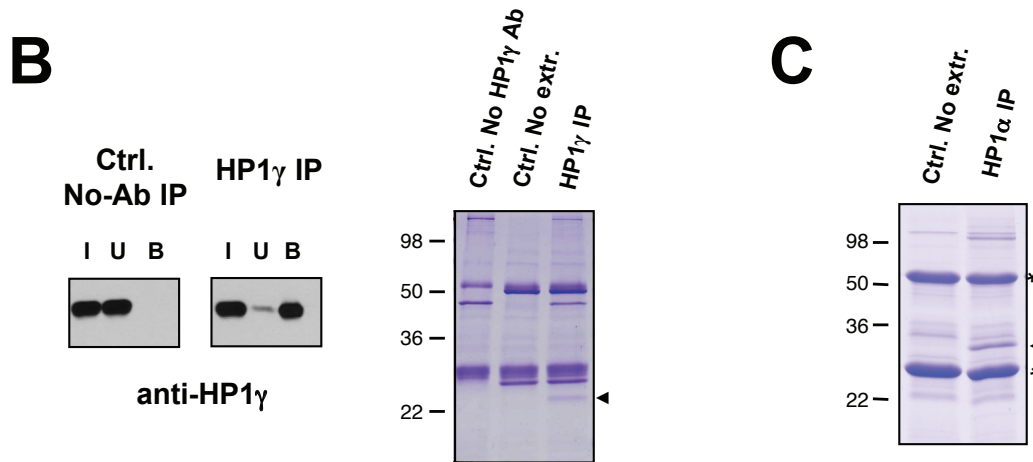
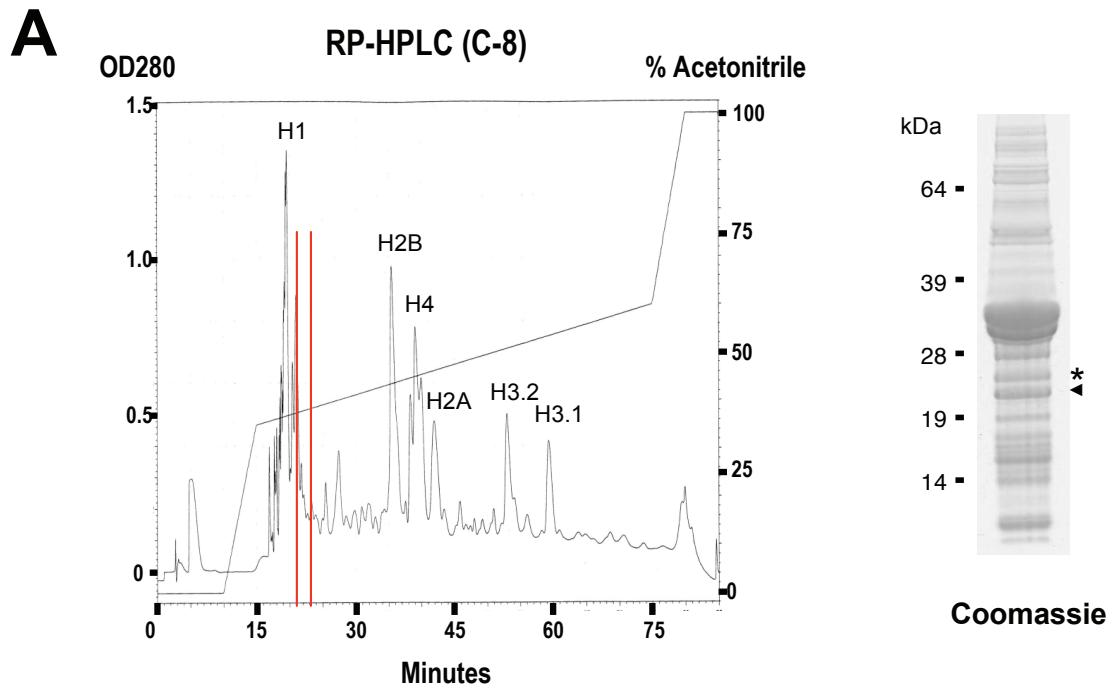


Figure 4.19

(2) *Cell fractionation and immunoprecipitation of endogenous HP1 followed by analysis on the beads*

To overcome these problems, I turned to a second approach for the purification of HP1. For more efficient extraction of HP1, cultured cells were subjected to biochemical fractionation (Wysocka et al., 2001), and all proteins subsequently stripped off of DNA with a high-salt buffer. The HP1 isoforms were further enriched by immunoprecipitation with monoclonal HP1 antibodies (Figure 4.19B, left). HP1 was then digested directly on the beads (not from the gel), and resulting peptides subjected to MS.

Even though only partial digests were carried out and I had verified saturation of the beads with HP1, a large excess of antibody was detected in these MS samples (see Figure 4.19B, right), most likely due to the procedure of antibody immobilization (I used anti-mouse beads to immobilize anti-HP1 monoclonals, and some of the antibody may have been bound in an inactivating fashion). MS analysis of HP1 α against such a background of antibody protein fragments turned out to be next to impossible. Only upon inclusion of an additional enrichment step for phospho-peptides by IMAC (immobilized metal affinity chromatography) could novel HP1 modifications be identified.

The above altered approach resulted in the identification of multiple phosphorylations for all three HP1 isoforms (see below). It also led to the first proof by mass spectrometry for the existence of the S92ph mark *in vivo*. However, inclusion of the IMAC step made any quantification of the levels of phosphorylation impossible.

(3) *Cell fractionation, immunoprecipitation and on-beads analysis of exogenous tagged HP1*

To reduce the amount of antibody in the sample, I took advantage of my cell lines expressing the three HP1 isoforms with a FLAG-HA tag and switched to anti-FLAG agarose beads, while still pursuing an approach of immunoprecipitation/on-beads analysis (Figure 4.19C). The samples obtained by this procedure turned out to be very well suited for the analysis by mass spectrometry and even allowed for quantification of phosphorylation levels.

To investigate whether the phosphorylation levels changed in the course of the cell cycle, I purified and analyzed HP1 from both asynchronously growing and mitotically enriched (nocodazole-treated) cell populations, and diverse fractions (cytoplasmic, nucleoplasmic, chromatin-associated). Overall, the analysis led to the identification of six phosphorylation sites on HP1 α , one on HP1 β and three on HP1 γ (see Figure 4.20 and Figure 4.21 for example mass spectra; see Figure 4.23 for summary of results). I will discuss these phosphorylations in detail in the section below entitled "Most phosphorylations cluster within the hinge region of HP1 α ".

Several other proteins were detected in the IPs that despite rigorous washing steps co-immunoprecipitated with the tagged HP1 α . Especially the HP1 α IPs were analyzed in depth, and we found that many of these coprecipitating proteins were published interaction partners of HP1 α , such as HP1 β , HP1 γ , Su(var) 3-9, KAP-1, INCENP, CAF1 p150, Lamin B1, Lamin B Receptor, Ki-67, BRG1, and NIPBL (see Table 4.1). In addition, a few factors were detected that are members of complexes shown before to interact with HP1, for example Dasra B/Borealin, CAF1 p60, or NSL1

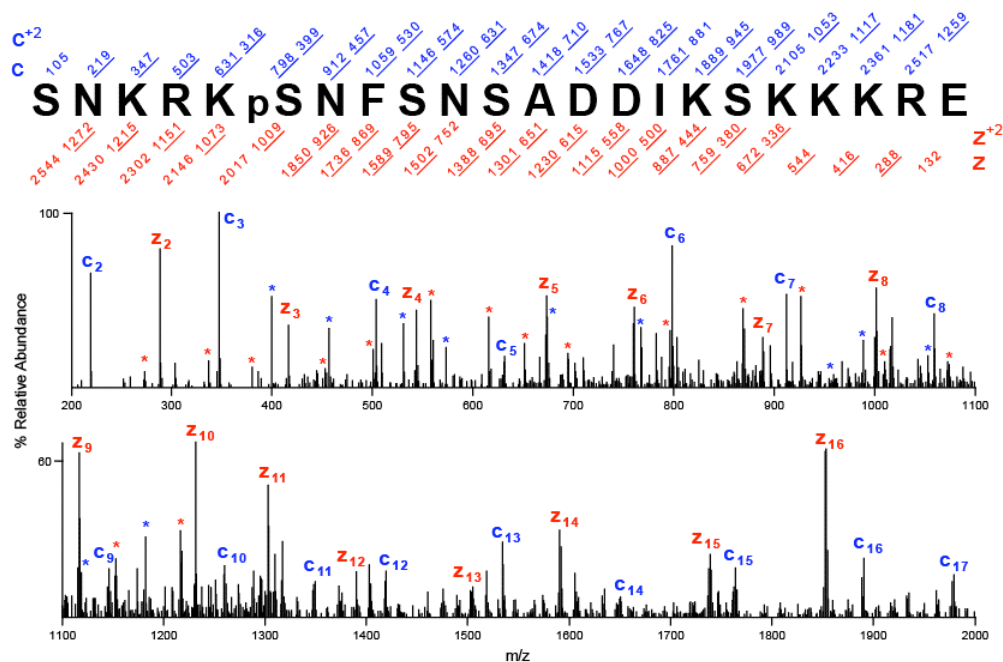


Figure 4.20: Tandem mass spectrum of a singly phosphorylated HP1 α peptide (S92ph) (Figure courtesy of Hillary Montgomery).

Tandem mass spectrum (MS/MS) of a singly phosphorylated HP1 α peptide (S92ph), residues 87-108, fragmented by electron transfer dissociation (ETD). The peptide was generated by an on-beads digest of exogenous HP1 α with endoproteinase Glu-C. A fraction of the resulting peptide mixture was analyzed by on-line nanoflow high performance liquid chromatography micro-electrospray ionization tandem mass spectrometry (nHPLC- μ ESI MS/MS). The instrument cycled through the acquisition of a full-scan mass spectrum (MS) and the top five most abundant masses in this initial MS scan were sequentially chosen for tandem MS (MS/MS) spectra (depicted in this Figure). Full sequence coverage was attained for this +6 charged peptide, 442.40 m/z. The spectrum provides evidence that the HP1 α peptide is phosphorylated at serine 92. c' and z'• fragment ions are denoted in blue and red, respectively. Doubly-charged fragment ions are denoted with asterisks (*).

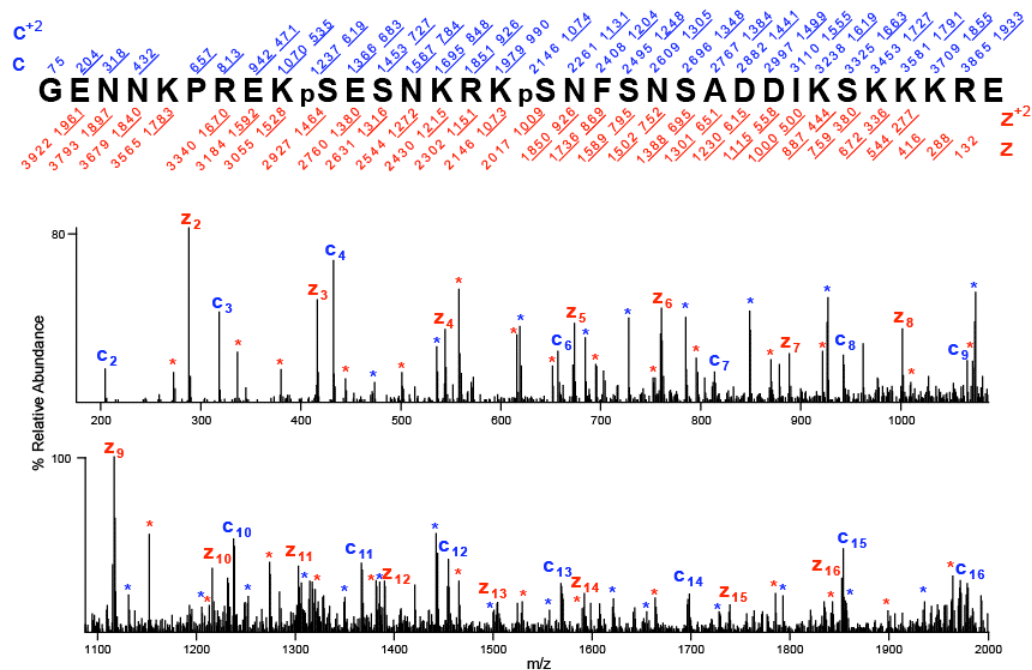


Figure 4.21: Tandem mass spectrum of a doubly phosphorylated HP1 α peptide (S85phS92ph) (Figure courtesy of Hillary Montgomery).

Tandem mass spectrum (MS/MS) of a doubly phosphorylated HP1 α peptide (S85phS92ph), residues 76-108, fragmented by electron transfer dissociation (ETD). Full sequence coverage was attained for this +7 charged peptide, 572.10 m/z. The spectrum shows that this HP1 α peptide is doubly phosphorylated, at serine 85 and serine 92. c' and z'• fragment ions are denoted in blue and red, respectively. Doubly-charged fragment ions are denoted with asterisks (*).

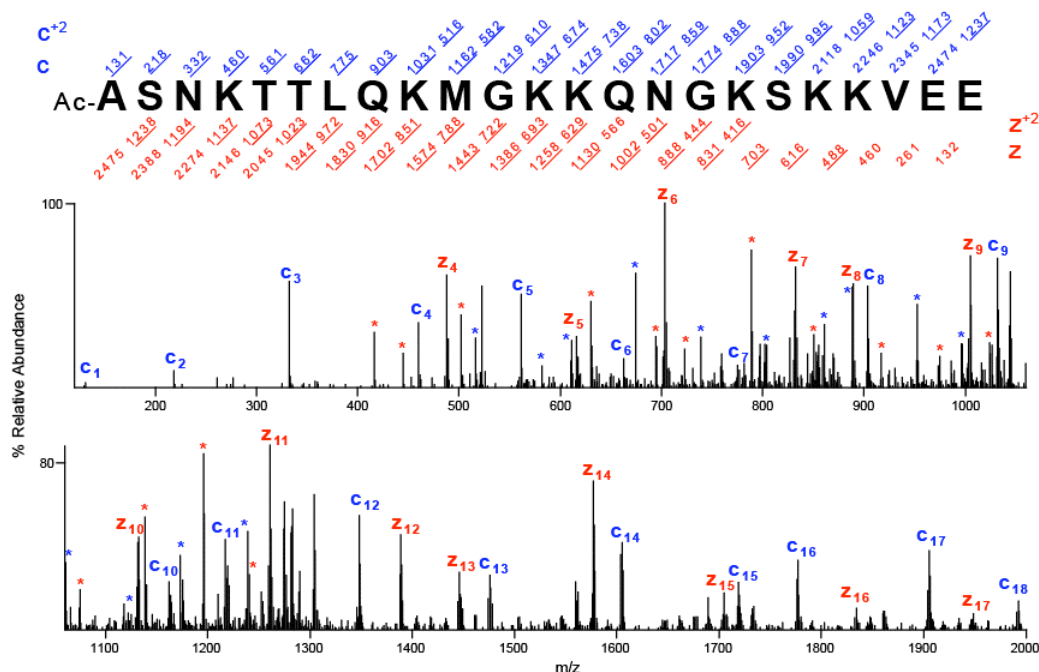


Figure 4.22: Tandem mass spectrum of an HP1 γ peptide defining the HP1 γ N-terminus (Figure courtesy of Hillary Montgomery).

Tandem mass spectrum (MS/MS) of the N-terminal peptide from HP1 γ , residues 2-24, fragmented by electron transfer dissociation (ETD). Full sequence coverage was attained for this +5 charged peptide, 522.10 m/z. The spectrum confirms the existence of an N-acetylated peptide ASNKTTLQKMGKKQN... *in vivo*. This implies that translation of HP1 γ starts ten residues upstream of what is often assumed to be the N-terminus of HP1 γ . c' and z' fragment ions are denoted in blue and red, respectively. Doubly-charged fragment ions are denoted with asterisks (*).

Table 4.1: Known interaction partners of HP1 found in the HP1 α IP

| Identified interaction | Reference |
|------------------------|--|
| HP1beta | Le Douarin et al., 1996 |
| HP1gamma | Le Douarin et al., 1996 |
| SUV39H1 | Aagaard et al., 1999 Czvitkovich et al., 2001 |
| KAP1 | Le Douarin et al., 1996 |
| INCENP | Ainsztein et al., 1998 |
| CAF1 p150 | Murzina et al., 1999 Ryan et al., 1999 |
| BRG1 | Nielsen et al., 2002 |
| Lamin B1 | Kourmouli et al., 2000 |
| Lamin B receptor | Ye et al., 1997 |
| Ki-67 | Scholzen et al., 2002 |
| NIPBL | Lechner et al., 2005 |

SUV39H1: Suppressor of variegation 3-9 homolog 1. INCENP: Inner centromere protein. CAF1: Chromatin assembly factor 1. BRG1: Brahma-related gene 1 (also known as SWI/SNF-related matrix-associated actin-dependent regulator of chromatin). NIPBL: Nipped-B like protein.

Table 4.2: Proteins found in HP1 α IP that are components of complexes known to interact with HP1

| Identified interaction | Complex | Reference |
|------------------------|-------------------------------|--|
| Dasra B/Borealin | Chromosomal Passenger Complex | Sampath et al., 2004 Ainsztein et al., 1998 |
| NSL1 | Mis12 Complex | Euskirchen, 2002 Obuse et al., 2004 |
| CAF1 p60 | CAF1 Complex | Murzina et al., 1999 |

NSL1: NNF1 synthetic lethal. NNF1: Necessary for nuclear function 1. CAF1: Chromatin assembly factor 1.

(Table 4.2). Co-immunoprecipitation of these proteins can be considered as further validation that the tagged HP1 α protein indeed undergoes the same interactions and behaves the same way as endogenous HP1 α .

Of interest, from coimmunoprecipitated HP1 γ , Hillary Montgomery and I could obtain specific information about the HP1 γ N-terminus (Figure 4.22), which settles a long-standing uncertainty about the exact translation start site of the protein. Detection of a peptide ASNKTTLQKMGKKQN... demonstrates that in HeLa cells, translation of HP1 γ starts (also) ten amino acids upstream of what is often assumed as the protein's N-terminus (MGKKQN...). We also found that the terminal methionine of HP1 γ is actually processed, and that the protein is N-acetylated.

In addition to known interaction partners of HP1 α , a number of other proteins were identified from these IPs. These include several transcriptional regulators (BCLAF, ERH, TRAP3), proteins involved in sister chromatid cohesion and cell division (Prohibitin 2, NDR1), and a protein phosphatase (PP1) (see Table 4.3). These proteins co-purified with tagged HP1 α , but unfortunately there was no selection for phospho-dependent binders in this pull-down.

Most phosphorylations cluster within the hinge region of HP1

Most of the identified phosphorylation sites map to the hinge region of HP1 (Figure 4.23). This is intriguing, because it suggests that the phosphorylations may indeed collaborate to bring about a hinge-mediated effect, for example in the regulation of a molecular interaction. Thus, it provides support for the hypothesis that redundancy among the different HP1 phosphorylations may indeed be the reason why mutation of serine 92 to alanine has no observed phenotypic effects.

Table 4.3: Potential novel interaction partners of HP1 α

| Identified interaction | Reference |
|------------------------|----------------------------------|
| BCLAF | Kasof et al., 1999 |
| ERH | Isomura et al., 1996 |
| TRAP3 | Fondell et al., 1996 |
| Prohibitin 2 | Coates et al., 1997 |
| NDR1 | Millward et al., 1995 |
| PP1 | Trinkle-Mulcahy and Lamond, 2006 |

BCLAF1: BCL2-associated transcription factor 1 (also known as Btf, . ERH: Enhancer of rudimentary homolog. TRAP3: Thyroid hormone receptor associated protein 3. NDR1: Nuclear Dbf2-related kinase (also known as Serine-threonine kinase 38). PP1: Protein phosphatase 1.

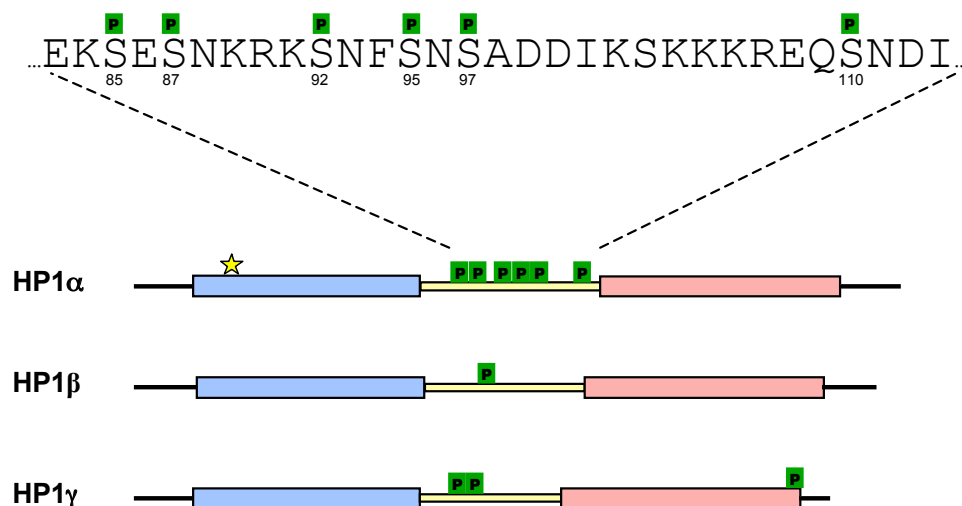


Figure 4.23: Cartoon depiction of HP1 phosphorylation sites identified by mass spectrometry.

The phosphorylation sites identified by mass spectrometry are: S85, S87, S92, S95, S97, and S110 in HP1 α ; S89 in HP1 β ; and S93, S95, and S176 in HP1 γ . Almost all phosphorylation sites fall within the hinge region of the HP1 proteins. Yellow star: acetylation site identified in the HP1 α chromo domain (K24). Mass Spectrometry was carried out by my collaborator Hillary Montgomery (Hunt lab, UVA)

Because of the striking density of serine residues in this region, the HP1 hinge has long been speculated to be phosphorylated (Badugu et al., 2005). However, actual evidence for the existence of such phosphorylations has been lacking so far. The mass spectrometry data generated by Hillary Montgomery and me is the first time that definitive evidence is presented for the existence of such a series of phosphorylations in the hinge region. Compared to the other two domains of HP1, the hinge region has received much less attention. The discovery of these posttranslational modifications of the hinge may therefore contribute to our understanding of this understudied domain of HP1.

Conservation of phosphorylation sites

Sequence conservation of the serines identified as phosphorylation sites was assessed from an alignment of the human HP1 isoforms with the HP1 proteins of other organisms (Figure 4.24). Outside of the conserved blocks of basic amino acids, the hinge regions of most HP1s have a high density of serines, glutamic acid and aspartic acid; however, the exact order of the serines and acidic residues is not conserved between organisms. But despite this low conservation at the level of the exact amino acid sequence, the composition and character of this region is well preserved across the different species.

The conservation of the basic and acidic (i.e. charged) character of these clusters makes it reasonable to assume that a molecular interaction mediated by these patches of positively and/or negatively charged amino acids could be conserved across different organisms. And if the target sequence for the kinase(s) responsible for the observed phosphorylations (besides serine 92) is defined simply by an acidic sequence context, it is

well possible that the phosphorylations as well as the responsible kinase(s) are conserved between different species.

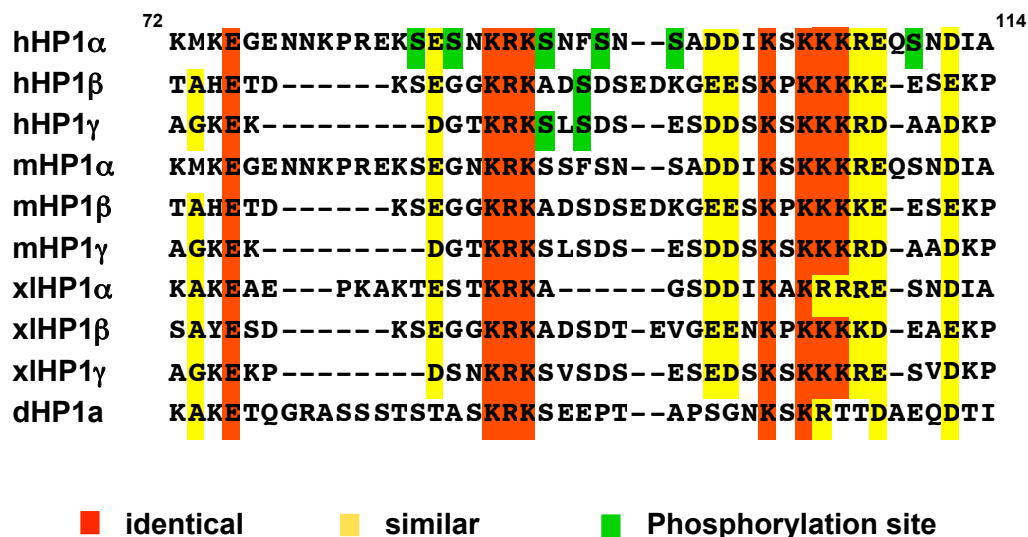


Figure 4.24: Alignment of the hinge regions of HP1 proteins from different species indicating the position of the newly identified phosphorylation sites.

Low sequence conservation between the conserved blocks of basic amino acids makes it difficult to obtain a meaningful alignment. However, the character of these sequence stretches seems very well preserved between species. Identified phosphorylation sites in green. hHP1: human, mHP1: mouse, xHP1: *Xenopus laevis*, dHP1: *Drosophila melanogaster* HP1.

Higher levels of phosphorylation in mitosis

The IPs of tagged exogenously expressed HP1 α allowed me to obtain quantitative data for several of the phosphorylation sites from both asynchronous and mitotic samples. Comparing the phosphorylation levels (see Table 4.4) clearly reveals that there is a significant increase of phosphorylation during mitosis at several of these sites. It seems possible that I have identified the sites for which indications had been found before by the Buendia lab (Minc et al., 1999). While the levels of mitotic

Table 4.4: Relative phosphorylation levels obtained by MS

| Phospho sites | Sequence | Relative phosphorylation (%) | | | | | |
|------------------------|---|------------------------------|----------|-----------|------------------------|----------|-----------|
| | | mitotically arrested | | | asynchronously growing | | |
| | | Cyto | Low Salt | High Salt | Cyto | Low Salt | High Salt |
| pS92 | S ₈₇ NKRK[S]NFSNSADDIKSKKKRE ₁₀₈ | 20 | 8 | 7 | 2 | 1 | ND |
| pS92 & pS95 | S ₈₇ NKRK[S]NFSNSADDIKSKKKRE ₁₀₈ | 0.6 | 0.2 | 0.1 | 0.5 | 0.3 | ND |
| pS92 & pS97 | S ₈₇ NKRK[S]NFSNSADDIKSKKKRE ₁₀₈ | | | | | | |
| pS85 | G ₇₆ ENNKPREK[S]ESNKRKSNFSNSADDIKSKKKRE ₁₀₈ | 29 | 21 | 11 | 3 | 2 | 0.3 |
| pS85 & pS92 | G ₇₆ ENNKPREK[S]ESNKRK[S]NFSNSADDIKSKKKRE ₁₀₈ | 16 | 15 | 8 | ND | ND | ND |

Phosphorylated residues shaded in green. ND: peptide was not detected. Cyto: cytoplasmic fraction. Low Salt: fraction obtained when chromatin was extracted with a low salt buffer (see Materials and Methods). High Salt: Fraction obtained when all proteins were stripped off of chromatin with a high salt buffer. Data generated by Hillary Montgomery.

phosphorylation found in the quantitative MS analysis may not seem high, I assume that the values measured are artificially low due to technical aspects of the experimental procedure (such as incomplete enrichment for mitotic cells or phosphatase activity during extended incubations and handling). This is supported by other observations that I lay out in more detail in the Discussion at the end of this chapter.

In summary, our mass spectrometry analysis of HP1 α posttranslational modifications revealed that the protein is multiply phosphorylated. The sites of phosphorylation specifically cluster in the hinge region in an area with a high density of serines and acidic amino acids between blocks of basic residues, a characteristic that is conserved between species. The levels of phosphorylation at most of these sites are specifically upregulated during mitosis.

Hypothesis: Multiple HP1 α hinge phosphorylations collaborate to control the mitotic release of HP1 α from chromatin

Having identified this series of novel phosphorylations in the HP1 α hinge, I was now in a position to reevaluate the hypothesis that HP1 α phosphorylation may play a role in the release of HP1 α , this time taking the potential collaboration of multiple phosphorylations into consideration. For this purpose I generated stable HeLa cell lines expressing HP1 α constructs in which all the sites of mitotic HP1 α phosphorylation were mutated to alanine (six sites, see Figure 4.23; because I made the cell lines before our MS approach finally succeeded, the construct included an additional seventh mutation, S103A). To control for unwanted effects of the tag, two versions of this HP1 α "aStA" construct (for "all Serines to Alanine") were generated, one with an N- and one with a C-terminal FLAG-HA-tag.

By immunofluorescence microscopy I examined if there was any effect of the mutations on the localization or dynamics of HP1 α in mitosis. If there was indeed a role of the hinge phosphorylations in the mitotic release of HP1 α , then simultaneous mutation of all mapped phosphorylation sites should affect the dissociation of HP1 α from chromatin despite redundancy among the phosphorylation sites. However, even simultaneous mutation of all serines in the HP1 α hinge to alanine did not have any detectable effect on the localization of the protein, neither in interphase nor at any stage of mitosis (data not shown).

At first sight, this seems to reject the hypothesis that HP1 α hinge phosphorylation might play any role in the control of mitotic HP1 dissociation from chromatin. However, upon more careful reflection, it became clear that even after ruling out redundancy of phosphorylations as a potential problem, there was still another major aspect of the experimental system that could significantly complicate the experimental read-out, namely HP1 dimerization.

As described in the Introduction, it has been known that, through its chromoshadow domain, HP1 α can both homodimerize and heterodimerize with HP1 β and HP1 γ (Platero et al., 1995; Smothers and Henikoff, 2000). The interaction is strong enough to be observed in pull-downs with recombinant components (Ye et al., 1997), and it is known that the different HP1 isoforms affect each other in their localization *in vivo* (Dialynas et al., 2007).

In the tissue culture system that I was using for the analysis of the hinge mutations, endogenous unmutated HP1 α , HP1 β and HP1 γ were still present in addition to the exogenous, mutated HP1 α I was testing. Thus, it was possible that interaction with the endogenous, unmutated

HP1 isoforms had a significant impact on HP1 dynamics in mitosis and "masked" the effects of the mutations.

Analysis of HP1 α aStA localization in cells devoid of endogenous HP1 α , HP1 β and HP1 γ

Point mutations abolishing HP1 dimerization have been published by others before (e.g. HP1 α I165E/HP1 β 161E, Brasher et al., 2000). However, these mutations also affect other interactions of the HP1 CSD (some of which actually depend on HP1 dimerization), and have been reported to significantly reduce chromatin association of HP1 and change its localization (Thiru et al., 2004). Thus, I reasoned that my best experimental option was a cell system in which the endogenous HP1 proteins were absent. Genetic knock-outs in mammalian cells have never been reported for any of the HP1 isoforms in the literature. Similarly, it is unlikely that a stable simultaneous knock-down of all three isoforms will be able to survive for extended periods of time, because knock-down of two of the three isoforms by RNA leads already to failure to segregate chromosomes faithfully during cell division (Obuse et al., 2004). Thus, I reasoned that the best way to do this experiment was by triple knock-down through simultaneous transient transfection of siRNAs against all three endogenous HP1 isoforms.

To make sure that only the endogenous HP1 α was knocked down and not my mutated construct, I generated dsRNA oligos directed against the 5' untranslated region of the gene (a gift from Dr. Wolfgang Fischle, Goettingen; see Figure 4.25A). With fluorescent RNA oligos and flow cytometric analysis I optimized the transfection conditions until I reached efficiencies of >95% (data not shown). Even though successful transfection

Figure 4.25: Localization of HP1 α hinge mutations after knock-down of endogenous HP1 isoforms by RNAi.

A: Strategy of specific RNAi against endogenous HP1 α . HP1 α siRNAs were targeted against the 5' untranslated region of the HP1 α gene, which is not present in the mRNA of the exogenous HP1 α construct that carries the phosphorylation site mutations.

B: Immunoblots of cells at time of analysis by IF. Specific and almost complete knock-down of all three endogenous HP1 proteins is observed. Exogenous, tagged HP1 α is unaffected by the RNAi. That there are sometimes traces of signal left after RNAi may be because of the small fraction of cells that were not transfected with the respective siRNA.

Asterisk: Exogenous HP1 α . (Extended periods of culture led to a reduction in the expression levels of N-terminally tagged HP1 α . The specific effects of the RNAi on the HP1 protein levels are therefore clearer in the C-terminally tagged cell lines.)

N: N-terminally tagged HP1 α without mutations. N-aStA: N-terminally tagged HP1 α with all S in hinge mutated to A. C: C-terminally tagged HP1 α without mutations. C-aStA: C-terminally tagged HP1 α with all S in hinge mutated to A.

C: Immunofluorescence microscopy (anti-HA) of HeLa cell lines stably expressing HA-tagged HP1 α constructs, either with wt sequence or with all serines in the hinge mutated to alanine, with or without triple knock-down of endogenous HP1 isoforms (stainings shown are representative of what was commonly observed). No clear differences are seen at any of the cell cycle stages.

HP1 α (N): HP1 α with wt sequence, N-terminally tagged; HP1 α "all S to A" (N): HP1 α with all serines in the hinge region mutated to alanine, N-terminally tagged

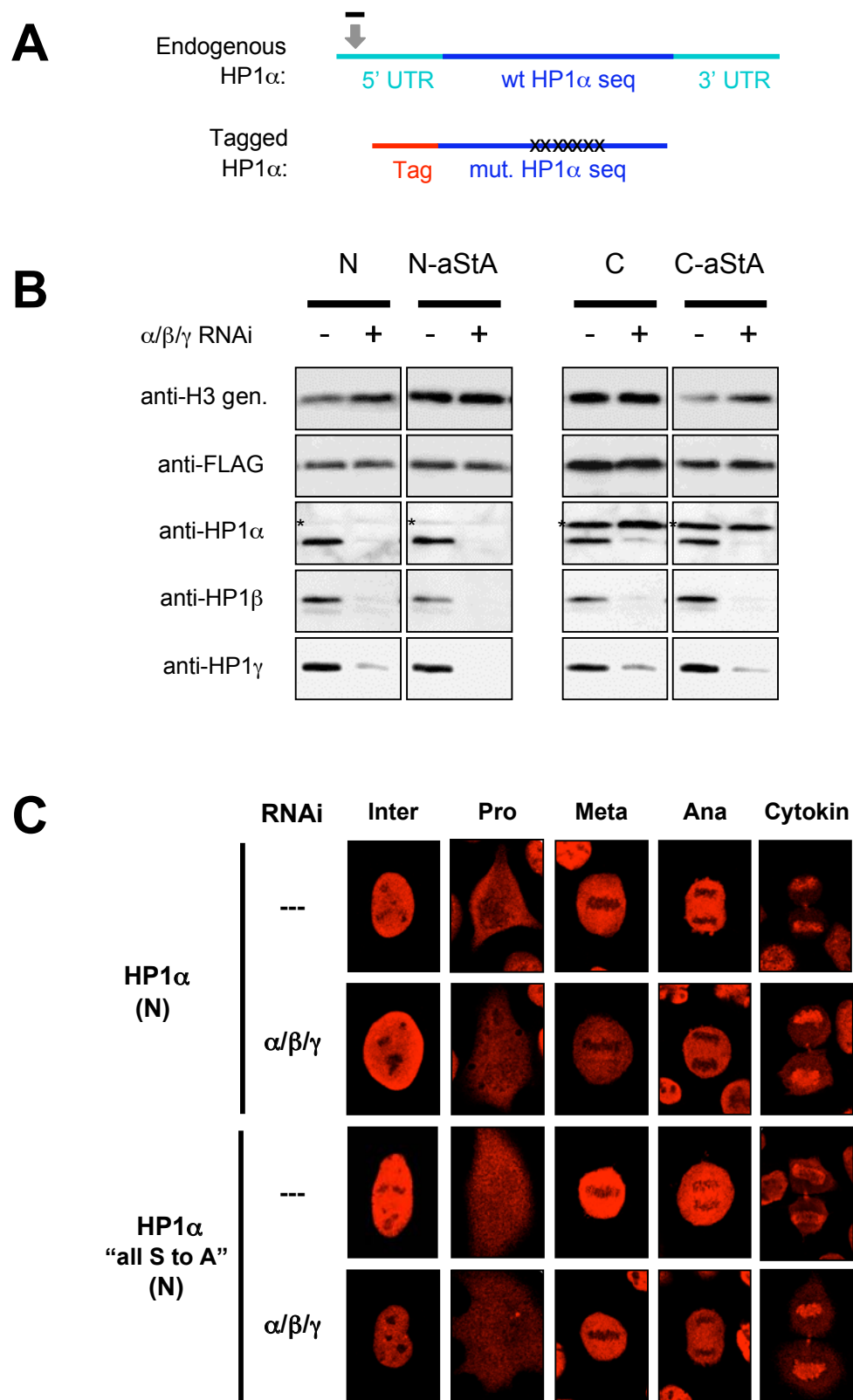


Figure 4.25

of individual cells cannot be tracked during the final experiment, these extremely high transfection efficiencies assure that 80-90% of cells will receive all three oligos.

For the actual experiment, cells were transfected with either HP1 α / β / γ siRNAs or random sequence siRNA. The day after the transfection, cells were split into two fractions and either seeded on cover slips for analysis by IF, or replated for biochemical analysis of the knock-down. Cells were harvested 3.5 d after transfection, according to pilot experiments the time of lowest protein levels (data not shown).

Immunoblotting proved efficient knock-down of all three endogenous HP1 isoforms, while the levels of exogenous HP1 α protein were unaffected (Figure 4.25B). However, even with endogenous HP1 isoforms largely absent, I did not discover any clear differences in the localization of wt and mutated HP1 α in my analysis of immunofluorescence stainings (Figure 4.25C).

Theoretically, it is possible that the efficiency of the knock-down was simply not efficient such that the remaining traces of endogenous HP1 isoforms were still sufficient to prevent an effect of the mutations. However, this scenario seemed not very likely. Despite a number of observations pointing in that direction, despite pursuing diverse experimental approaches, mutating all serine residues in the HP1 α hinge, and even knocking down all endogenous HP1 isoforms simultaneously, I did not find any clear evidence to support the hypothesis that phosphorylation of the HP1 α hinge controls the mitotic release of HP1 α from chromatin. Of course my experiments do not completely rule out such a possibility. However, I decided that at this point that further follow-up experiments on the hypothesis were not justified and that it would be the best for me to reconsider other hypotheses.

Hypothesis: HP1 α hinge phosphorylation regulates reassociation of HP1 α with chromatin in metaphase

Alternatively, HP1 α hinge phosphorylation may be the key to explain a curious experimental observation of HP1 biology that I also pointed out in the Introduction to this thesis: After the global release of HP1 from chromatin at the onset of mitosis, a small fraction of HP1 α (and, possibly, also of HP1 β and HP1 γ) reassociates with the centromeric regions of the chromosomes in metaphase (Hayakawa et al., 2003; Minc et al., 1999). Hayakawa and colleagues mapped the region of HP1 α that is required for the reassociation with centromeres during metaphase. They found that the above reassociation is independent of the chromo domain and rather mediated by a region comprising the C-terminal part of the hinge and the chromoshadow domain (aa 101-180).

Only a fraction of HP1 α reassociates, and it has thus already been suggested that posttranslational modification might be the mechanism how this reassociation of HP1 α is controlled (see Figure 4.26). While most of the identified phosphorylation sites lie just outside of this mapped binding region, due to tertiary structure effects or simply the immediate proximity to the binding region the addition of a series of negative charges to the HP1 α hinge could prevent the interaction that causes this reassociation of HP1 α with the metaphase chromatin. This interpretation is supported by previous observations, where serine 92 phosphorylation seems exclusive with the binding of a pool of HP1 α to metaphase chromosomes (Figure 4.13B). This suggests HP1 α hinge phosphorylation as the molecular mechanisms to keep the majority of HP1 α from reassociating with metaphase chromatin.

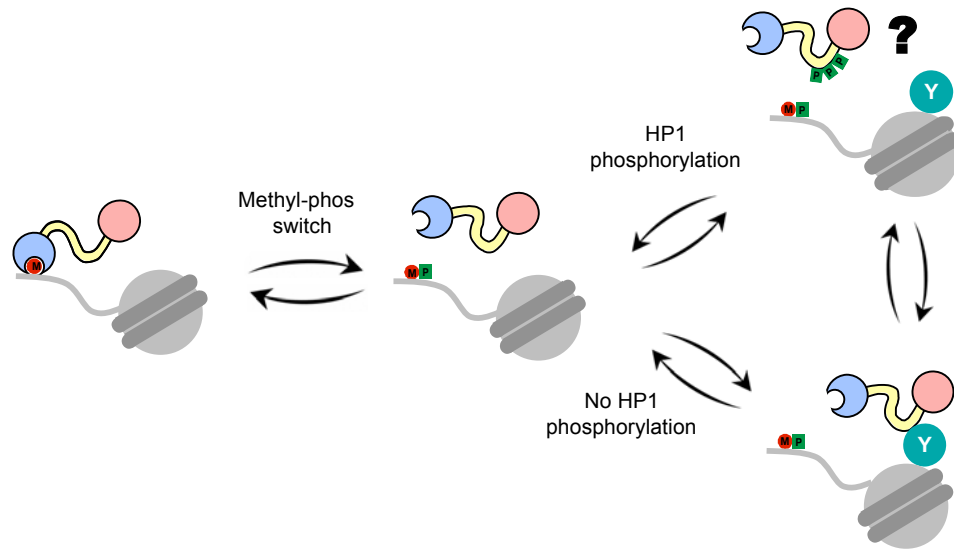


Figure 4.26: Hypothesis: HP1 α hinge phosphorylation regulates reassociation of HP1 α with chromosomes in metaphase.

Schematic overview of the hypothesis: After HP1 α is globally released from chromatin by methyl-phos switching (and possibly other mechanisms), a fraction of HP1 α reassociates with centromeric chromatin through chromo domain-independent mechanisms. This reassociation may be controlled by HP1 α hinge phosphorylation. Only the pool of HP1 α that happens to remain unphosphorylated may be able to rebind, while HP1 α hinge phosphorylation prevents the molecular interactions for reassociation with metaphase chromosomes.

For better visualization of the small pool of chromatin-bound HP1 α against the large background of HP1 α in the cytoplasm at metaphase, an alternative protocol of fixation and extraction for immunofluorescence microscopy was adapted (Figure 4.27A; Dormann et al., 2006). This protocol was then used to compare the reassociated, chromatin-bound pool of two HP1 α constructs, one with all hinge phosphorylation sites mutated to alanine, the other to glutamate.

No differences in the staining intensity were observed (Figure 4.27B) suggesting that HP1 α reassociation with chromatin is not significantly altered by phosphorylation of the identified sites within the HP1 α hinge.

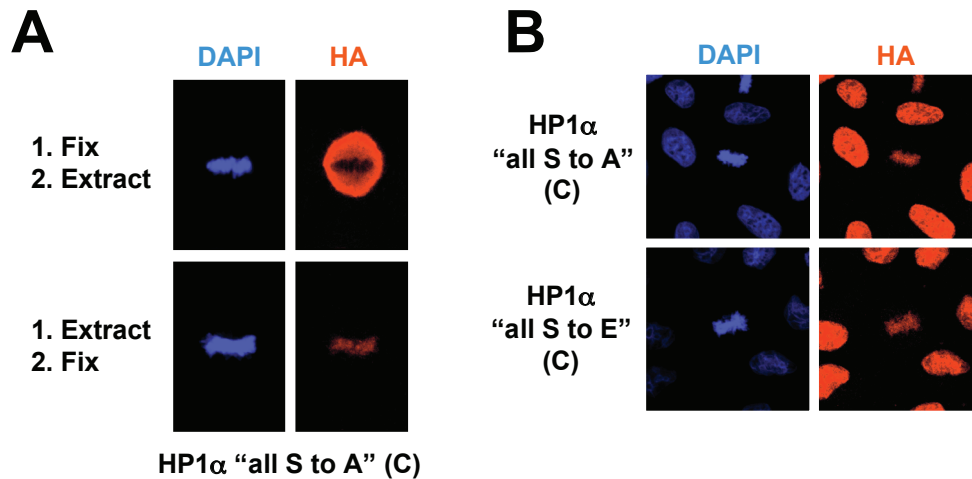


Figure 4.27: Mutation of phosphorylation sites in the HP1 α hinge has no obvious effect on HP1 α reassociation with metaphase chromosomes.

A: IF of HeLa cells stably expressing HA-tagged HP1 α . Comparison of different Fixation-Extraction procedures. If mitotic cells are fixed first and then extracted (top), the excess of HP1 α in the cytoplasm is seen. However, if the order is changed and cells are extracted first and then fixed (bottom), all the unbound HP1 α in the cytoplasm is removed and the small pool of HP1 α bound to metaphase chromatin becomes visible.

B: Immunofluorescence microscopy (anti-HA) of HeLa cells stably expressing tagged HP1 α mutants. Cells were first extracted and then fixed. As commonly observed, no difference in the HP1 α pool associated with metaphase chromatin is observed.

HP1 α "all S to A" (C): HP1 α with all serines in the hinge mutated to alanin and C-terminal FLAG-HA tag. HP1 α "all S to E" (C): HP1 α with all serines in the hinge mutated to glutamate and C-terminal FLAG-HA tag.

Cell cycle FACS

In all the described IF experiments, mutated HP1 α had shown the same cellular localization at the different stages of mitosis as wt HP1 α . In light of these observations, a direct involvement of HP1 α hinge phosphorylation in the regulation of HP1's chromatin dissociation or reassociation seems unlikely. However, cells analyzed in IF experiments are "hand-picked" for being in specific mitotic stages. It is therefore possible that eventually the localization changes are the same for wt and mutant HP1 α , but the *kinetics* of these localization changes are actually different. Cells expressing mutated HP1 α constructs may simply take longer for their progression through mitosis. Interestingly, mutation/deletion of HP1 is connected to lagging chromosomes and delayed mitosis in several organisms (mostly of course because heterochromatin integrity is important for centromere function; Ekwall et al., 1995; Kellum and Alberts, 1995; Obuse et al., 2004).

To investigate whether mutations in the HP1 α hinge region caused such delays in mitosis, I carried out cell cycle FACS analysis of HeLa cells expressing either wt or mutated HP1 α . If the time for transition through one of the steps of mitosis was extended, the fraction of cells in mitosis would increase. However, my analysis of the flow cytometry data led only to the discovery of minor differences between the individual samples (Figure 4.28). Since these differences showed no systematic pattern, they seemed rather caused by random fluctuations or by the fact that these stable cell lines that had been grown independently for a while, leading to minor differences in their growth characteristics.

To exclude that the presence of the endogenous wild-type copy of HP1 α prevented the detection of a clear effect, I repeated the experiment with cells in which the endogenous HP1 α protein had been knocked down

by RNAi. However, once again all the measurements fell within a rather narrow range (data not shown), indicating that even in the absence of endogenous HP1 α there was no significant effect of the mutations on the progression of mitosis.

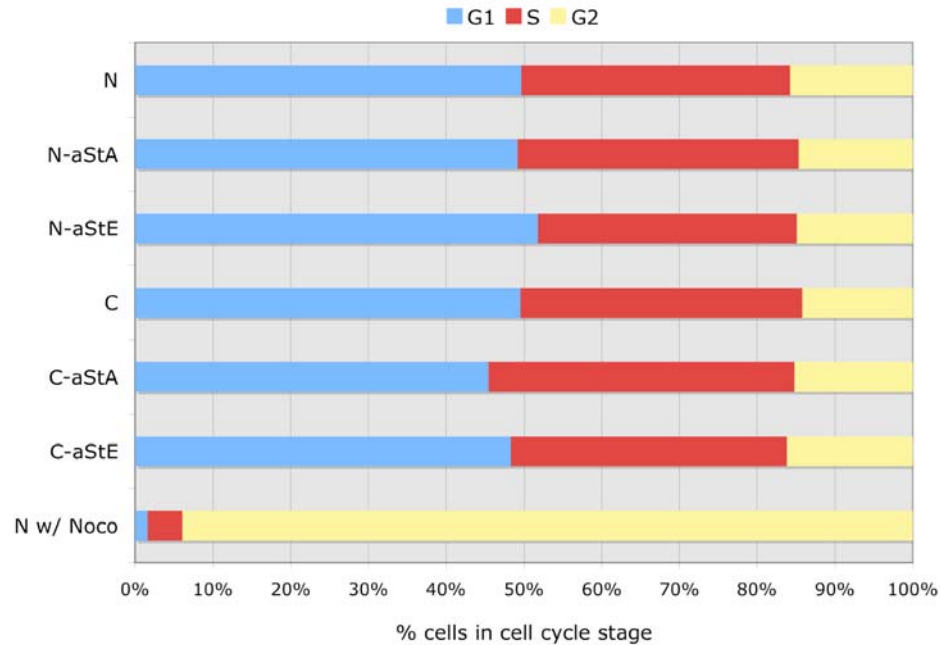


Figure 4.28: Effect of HP1 α hinge mutations on cell cycle progression.

Distribution of cell cycle stages of HeLa cell lines stably expressing various HP1 α constructs. Upon RNase digestion and propidium iodide staining, the DNA content of asynchronously growing cultures was determined by flow cytometry, followed by modelling of the culture's cell cycle distribution (Watson Pragmatic model, FloJo software package).

The cell cycle distribution of the different cell lines is very similar.

N: N-terminally tagged HP1 α without mutations. N-aStA: N-terminally tagged HP1 α with all S in hinge mutated to A. N-aStE: N-terminally tagged HP1 α with all S in hinge mutated to E. C: C-terminally tagged HP1 α without mutations. C-aStA: C-terminally tagged HP1 α with all S in hinge mutated to A. C-aStE: C-terminally tagged HP1 α with all S in hinge mutated to E. N w/ Noco: Control cells (N-terminally tagged HP1 α without mutations) arrested in mitosis with nocodazole.

Hypothesis: HP1 α hinge phosphorylation regulates molecular interactions of the hinge region

My thesis data suggest that hinge phosphorylation controls neither mitotic HP1 α dissociation from chromatin nor reassociation of a pool of HP1 α with chromatin in metaphase. Mutations of the phosphorylation sites also do not affect progression through mitosis. However, the fact that these phosphorylations in the HP1 α hinge specifically occur in mitosis still suggests that they may play a regulatory role at this phase of the cell cycle.

It is unlikely that the phosphorylations fulfill their function by affecting HP1 α structure, because the hinge region is most likely unstructured (Ball et al., 1997). There is also no indication that mitotic HP1 α phosphorylation affects protein stability; neither in the published literature, nor in any of my experiments with synchronized cell populations or nocodazole arrested cultures were changes in HP1 α protein levels ever observed.

The identified modifications all cluster within a narrow region of the HP1 α hinge, adding a significant amount of negative charge to a rather small patch of sequence. This suggests that HP1 α hinge phosphorylation may control a molecular interaction with a binding partner that associates with this region of HP1 α (Figure 4.29).

As outlined in the Introduction of my thesis, several molecules have been described to bind to the hinge region of HP1 α and are therefore candidates: histone H1 (Hale et al., 2006), the chromosomal passenger protein INCENP (Ainsztein et al., 1998), and RNA (Muchardt et al., 2002). Since all these published reports included *in vitro* binding experiments, I decided to test through direct *in vitro* interaction assays, whether any of these interactions was affected by phosphorylation of the HP1 α hinge.

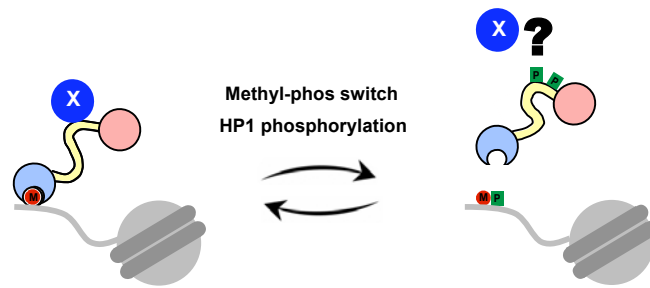


Figure 4.29: Hypothesis: HP1 α hinge phosphorylation controls a molecular interaction that has no connection to HP1 chromatin association.

Hypothesis: At the same time when methyl-phos switching releases HP1 chromatin association at the onset of mitosis, HP1 α hinge phosphorylation reverses (or establishes) a different molecular interaction with an unrelated molecule X.

Experiments to test if HP1 α hinge phosphorylation controls HP1 α 's interaction with histone H1

In 2006, the lab of Rafael Herrera (Baylor College, Houston) reported a specific interaction of HP1 α with H1b, a histone H1 isoform predominantly found in heterochromatin (Hale et al., 2006; Th'ng et al., 2005). The interaction was mapped to the HP1 α hinge, and it was shown that phosphorylation of H1b by CDK2 during interphase interrupts the interaction, possibly to allow disassembly of higher order chromatin structures for better access to the chromatin template (Roth and Allis, 1992). Since already phosphorylation of a single site in the H1b C-terminal domain was sufficient to abolish the interaction, it is tempting to speculate that phosphorylations on the HP1 α hinge may have a similar effect.

In order to address the question in the most direct way, I relied on *in vitro* binding experiments with recombinant proteins. However, although I tried this experiment under various conditions (H1b purified under different

(denaturing and nondenaturing) conditions, various salt concentrations, presence/absence of BSA), I could never reproduce the reported specific *in vitro* binding of H1b to the HP1 α isoform or the specificity of binding to the hinge region (data not shown).

My difficulties to repeat these experiments are mirrored by similar observations made by other labs, who also have only seen unspecific binding of H1b (Fischle, 2008). It cannot be ruled out that some minor difference between our experimental procedures (e.g. different tags) unexpectedly cause these conflicting observations. However, because multiple approaches by multiple labs had not been able to reproduce the reported results, I decided that it was advisable not to spend more time on the interaction of H1b with HP1 α .

Experiments to test if HP1 α hinge phosphorylation controls HP1 α 's interaction with RNA

The second molecular interaction of HP1 α that I decided to examine was the interaction with RNA. Prompted by observations by Maison et al. (Maison et al., 2002), C. Muchardt and colleagues showed in 2002 in a series of experiments (overlay assays and electrophoretic mobility-shift assays) that HP1 α and HP1 γ bind RNA (Muchardt et al., 2002). While their *in vitro* experiments were carried out with a random bacterial RNA, competition demonstrated specificity with respect to the kind of RNA (competition by mammalian nuclear RNA; no competition by AU- or GC-rich oligomers, tRNA, or DNA). However, neither the nature nor the sequence of RNAs bound by HP1 α *in vivo* has been studied so far.

Muchardt and colleagues mapped the RNA binding activity *in vitro* to residues 86 to 108 of HP1 α (Muchardt et al., 2002), corresponding to

the C-terminal half of the hinge, which is rich in basic amino acid (and includes the KRKS sequence motif and most of the phosphorylation sites that I identified within the HP1 α hinge). Mutation of three adjoining lysine residues within this sequence (K103, K104, and K105) to alanine completely abolished RNA binding of HP1 α . If the removal of three positive charges by mutation entirely prevents RNA binding, then it seems quite possible that the addition of multiple negative charges to the same region by phosphorylation may have a similar effect. Thus, I hypothesized that the HP1 α hinge phosphorylations may have a function in the regulation of RNA binding.

Further encouragement came from observations that I made while purifying recombinant HP1 α domains. After affinity purification of HP1 α hinge constructs (wt and with all serines in the hinge mutated to glutamate) from *E. coli* cells, I took a UV-Vis spectrum of the purified protein. To my surprise, the spectrum of the wt hinge had a significant peak at 260 nm (Figure 4.30A, left) that was not present in the spectrum of the mutated hinge construct (Figure 4.30A, right). Since the two samples had been prepared in parallel and undergone exactly the same manipulation, this suggested that a bacterial nucleic acid may be binding to and copurifying with the wt HP1 α hinge, while it did not associate with the mutated hinge.

To verify the presence of the nucleic acid and learn more about its nature, I treated aliquots of the dialyzed protein mixture with DNase and RNase, respectively. Ethidium bromide staining after agarose gel electrophoresis revealed that the 260 nm peak was caused by RNA (Figure 4.30B).

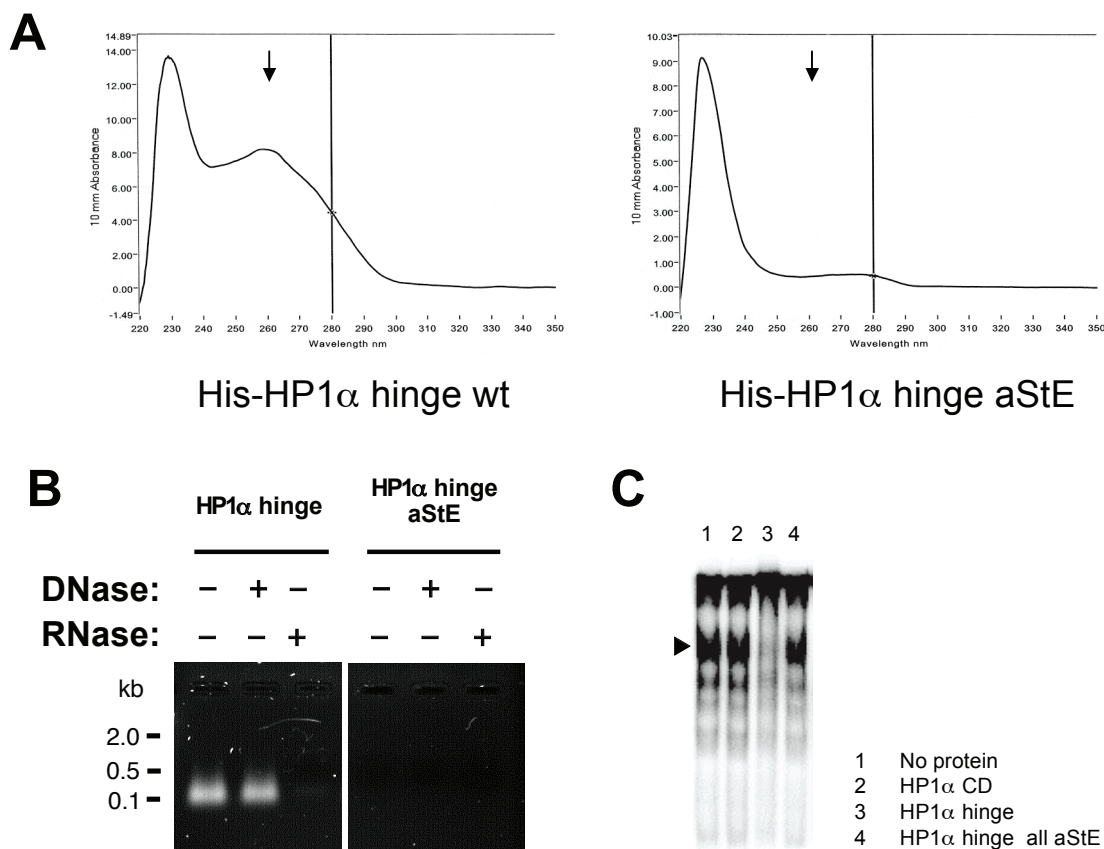


Figure 4.30: HP1 α hinge phosphorylation may regulate the interaction of HP1 α with RNA.

A: UV-Vis spectra of bacterially expressed His-HP1 α hinge (wt or with serines in hinge mutated to glutamate) after elution from Ni-NTA and dialysis. A peak at 260 nm (arrow) indicates that a nucleic acid copurifies with the wt HP1 α hinge. No such peak is observed in the spectrum of the mutated HP1 α hinge.

B: The copurifying nucleic acid is RNA. Upon heat denaturing (5 min at 95° C), aliquots of the elution from panel A were treated with either DNase or RNase A. Only RNase treatment removes the nucleic acid.

C: Electrophoretic mobility shift assay to compare RNA binding of different HP1 α wt and mutant domains (autoradiography). As expected, the control chromo domain construct does not bind the RNA probe and leaves the RNA in the unshifted position (marked by an asterisk). While the wt HP1 α hinge binds to and shifts the RNA, the mutated HP1 α hinge does not. (The signal that is seen in all lanes at the top of the gel indicates that a fraction of the labelled RNA had aggregated.)

This suggests that (possibly due to the absence of its normal target RNAs) the wt HP1 α hinge associates by means of its RNA-binding capacity with bacterial RNA. Mutation of the HP1 α hinge (all serines in the hinge were mutated to glutamate, introducing negative charges and thus mimicking constitutive phosphorylation), on the other hand, seems to abolish the ability of the HP1 α hinge to bind RNA. Obviously, no conclusion can be drawn from this experiment about the size or nature of RNAs bound by HP1 α in mammalian cells, because in this experiment nucleic acids were sheared during cell disruption and the interaction with bacterial RNAs is artificial anyway. Nevertheless, the observation is completely in line with the exciting hypothesis that phosphorylation of the HP1 α hinge region might indeed regulate the binding of RNA to HP1 α .

As discussed above, so far little is known about the connection between HP1 α and RNA *in vivo*. In particular, no RNAs that may bind HP1 α *in vivo* have been identified yet. This makes it very difficult to design experiments that directly test the effect of HP1 α hinge phosphorylation on RNA binding in the cell. Muchardt and colleagues used an *in vitro* method, electrophoretic mobility-shift assays (EMSAs), to learn more about the interaction they had observed in overlay assays (Muchardt et al., 2002). Therefore, I decided to apply the same technique and subject the hypothesis that HP1 α hinge phosphorylation has an effect on HP1 α 's RNA binding to a first test by EMSA.

To obtain recombinant protein for use in the EMSA, I took HP1 α hinge protein affinity purified from bacteria and then added an ion exchange chromatography step to separate the protein component from the bacterial RNA (data not shown). As RNA probe for these assays, a *Drosophila* mRNA sequence (500 nt of the cyclin E gene) was used. In pilot experiments, I had

established that this RNA probe reproduced the domain-specific binding to HP1 α described by Muchardt and colleagues (collaboration with Dr. E. Bernstein, then a postdoc in the Allis lab; data not shown).

When I tested binding of the probe RNA to wt and mutated HP1 α domains, I observed a striking difference. The unmutated wt hinge bound to and shifted the RNA probe. The mutated HP1 α hinge (phospho-sites replaced by glutamate), however, did not shift the probe at all (Figure 4.30C). While these experiments still are preliminary and have to be repeated and extended, they are fully in keeping with the interpretation that phosphorylation of the HP1 α hinge region regulates the binding of RNA to HP1 α .

Discussion

Novel phosphorylations of HP1

In this chapter of my thesis, I have described the identification of a series of phosphorylation sites of HP1. Phosphorylation at these sites is specifically upregulated in mitosis, and most of them cluster in a short sequence stretch of the hinge region, a domain of HP1 that so far has received very little attention compared to the other domains of HP1. For one site, HP1 α S92ph, which is conserved between different species, I carried out a detailed analysis with a modification-specific antibody and identified the Chromosomal Passenger Complex as the responsible kinase *in vivo*.

Intrigued by curious correlations (Figures 3.5, 3.6, 3.9), I tested various hypotheses about the function of these HP1 phosphorylations, ranging from an involvement in the mitotic release of HP1 α and a regulatory role in the reassociation of a subpool of HP1 α with metaphase centromeres to a function in mitotic progression or the regulation of molecular interactions

of HP1 α , but no obvious effects were observed. In the last section, I present experimental observations that suggest HP1 α hinge phosphorylation may regulate the interaction of the HP1 α hinge with RNA.

The findings presented in this chapter of my thesis for the first time identify specific phosphorylation sites of human HP1 (actually, while I was working on this study, two other, non-mitotic phosphorylations of HP1 have been identified by other groups, see Chapter 5, General Discussion).

In 1999, Minc and colleagues had published observations made from radioactive labelling experiments and western blotting after 2D gel electrophoresis that allowed some predictions about the number of human HP1 phosphorylations (Minc et al., 1999; see Figure 4.2). To what extent do my findings match with the number of phosphorylation sites and changes through the cell cycle suggested by the data of Minc and colleagues (Minc et al., 1999)?

The most detailed analysis Hillary Montgomery and I carried out with the HP1 α isoform. For this isoform Minc and colleagues have indications for low phosphorylation in interphase and hyperphosphorylation in mitosis. This is fully in keeping with my findings (from their data, less phosphorylation sites are expected in mitosis, but it is possible that the two additional charged forms observed by Minc et al. are caused by multiple sites, or that some of the phosphorylations in their experiment were lost during handling). While my MS data on HP1 γ is not as detailed, it is also in line with the predictions (several phosphorylations). A major discrepancy seems to exist, however, for HP1 β , since I observed that HP1 β is phosphorylated (at S89), while Minc et al. claim that it was not (Minc et al., 1999). Interestingly, MS data indicates that HP1 β S89 is highly phosphorylated, and to the same extent in both mitotic and unsynchronized

samples (about 90%). Thus, it seems possible that the one spot in the 2D gel of Minc and colleagues is caused by this phosphorylated species. Note that Minc and colleagues claim that HP1 β was not radioactively labelled, but do not show the data, while they show this data for HP1 α and HP1 γ . This may indicate that the HP1 β labelling was ambiguous. Thus, it seems that my mass spectrometry findings are overall in reasonably good agreement with what was expected based on radioactive labelling and 2D gel electrophoresis by Minc and colleagues.

While the MS data generated by Hillary Montgomery and me clearly shows an increase in phosphorylation during mitosis, the levels in mitosis still are not very high, never reaching more than 30% (see Table 4.4). However, for three reasons I suspect that the levels of mitotic phosphorylation within the cell may be significantly higher than suggested by our MS quantification. (1) The enrichment for mitotic cells was not perfect. MS quantification was done from mitotic samples that had not been further enriched by mitotic shake-off (to reduce the time of handling), resulting in only about 75% enrichment for mitotic cells. (2) Despite the use of phosphatase inhibitors there was probably some loss of phosphorylation during the fractionation and immunoprecipitation procedure. (3) In the samples obtained by approach 1 (higher enrichment for mitotic cells, all steps under denaturing conditions), it was possible to obtain quantitative data for one phosphorylation site, HP1 γ S93ph. This serine is the homologous HP1 γ site to HP1 α S92ph, and it was found phosphorylated to more than 70%. Unfortunately, no data for HP1 α S92 could be obtained from this approach. However, these data suggests that *in vivo* the homologous site HP1 α S92 may have high mitotic phosphorylation levels as well. The longer incubation times required for approach 3 (the

approach used to purify HP1 for quantification of phosphorylation by MS, which includes a cell fractionation step; see Section "Purification of HP1") and the lower enrichment for mitotic cells in the starting material may result in significantly underestimated levels of phosphorylation, possibly at all sites.

Intriguing new experimental directions

The findings presented in this chapter open up interesting new experimental directions. I identified Aurora B as the kinase responsible for HP1 α S92 phosphorylation *in vivo*, but it has not been addressed yet which kinases are responsible for the other HP1 phosphorylations. Computational kinase prediction suggests enzymes with a known cell cycle dependence as possible kinases for HP1 α hinge phosphorylation sites. For example, casein kinase 2 (Litchfield, 2003) was predicted for S85, S87, S97, and S110 in HP1 α . And S85 and S97 are predicted targets for the mitotic kinase PLK (Polo-like kinase; Nigg, 1998). Thus, it will be very interesting to follow up on this question in the future.

The identification of serine 89 of HP1 β as a phosphorylation site with high levels of phosphorylation (about 80%) in both interphase and mitosis raises the question what the biological function of this modification may be.

Besides phosphorylation, acetylation was discovered as another type of HP1 posttranslational modification: HP1 α was found to be acetylated at lysine 24 (Figure 4.23). The levels of acetylation were rather low (<5%), and even though this site is close to the aromatic cage, there is no direct indication from the crystal structure that acetylation at this site will affect H3K9me binding of the chromo domain. However, considering that these samples

were prepared without deacetylase inhibitors and that K24 is solvent-exposed, it is possible that K24 acetylation could regulate interactions of the chromo domain other than the CD-H3K9me interaction.

In our mass spectrometry analysis of tagged HP1 α , several copurifying proteins were identified (see Table 4.3). Since the purification conditions were stringent, it is possible that these proteins might be novel interactors of HP1 α .

Together with the reagents that I have generated (tagged HP1 cell lines, point mutants, recombinant HP1 constructs etc.), these data may be promising as a starting point for some graduate student in the future in the quest to expand our knowledge about HP1, the role of its modifications and the factors it interacts with.

Hinge phosphorylation may regulate RNA binding to HP1 α

In vitro observations that I made during the purification of mutated HP1 hinge constructs from bacterial cells and by electrophoretic mobility-shift assay suggest the intriguing possibility that HP1 α hinge phosphorylation may be important for the regulation of the interaction of HP1 with RNA. Clearly, these data on HP1 phosphorylation and RNA binding are still of a preliminary character. They have to be repeated and extended *in vitro*, for example by a further characterization of the *in vitro* interaction by competition experiments. Several observations in the literature suggest an involvement of RNA in HP1-dependent heterochromatin, thus making the observation that HP1 phosphorylation may regulate RNA binding a very interesting alley of research to pursue further. In the next chapter of my thesis (General Discussion) I will suggest experiments to confirm my observation, outline possible follow-up experiments, and speculate on the biological role of this interaction and its regulation by phosphorylation.

CHAPTER 5

GENERAL DISCUSSION

In my thesis studies, I have focused my attention on two different ways how phosphorylation regulates Heterochromatin Protein 1, an important chromatin effector protein and key factor in the formation of heterochromatin in eukaryotic cells (Figure 5.1): on the one hand, I discovered together with my collaborators that phosphorylation of histone H3, the histone that recruits HP1 to chromatin, is an essential step in the reversible ejection of HP1 from chromatin at the onset of mitosis. On the other hand, I identified several novel phosphorylations of HP1 itself, showed that these sites in the HP1 hinge region are hyperphosphorylated specifically in mitosis, and present observations suggesting that these mitotic phosphorylations may control HP1's association with RNA.

In this chapter, I strive to briefly summarize the experimental findings presented in my thesis and then discuss important aspects in light of the literature. In addition, I will outline possible future directions, and suggest models that put my findings in a larger biological context.

"Methyl-phos switching"

Release of HP1 from chromatin by phosphorylation of histone H3:

"Methyl-phos switching"

Heterochromatin Protein 1 (HP1) is recruited to chromatin by binding to methylated lysine 9 of histone H3 (H3K9me) through its chromo domain, probably further stabilized by additional interactions. At the onset of mitosis, the bulk of the protein is released from its binding site to be

dispersed all throughout the cytoplasm (Hayakawa et al., 2003; Kellum et al., 1995; Minc et al., 1999), even though the H3K9me persists, an observation that was a long-standing conundrum in the field prior to this work.

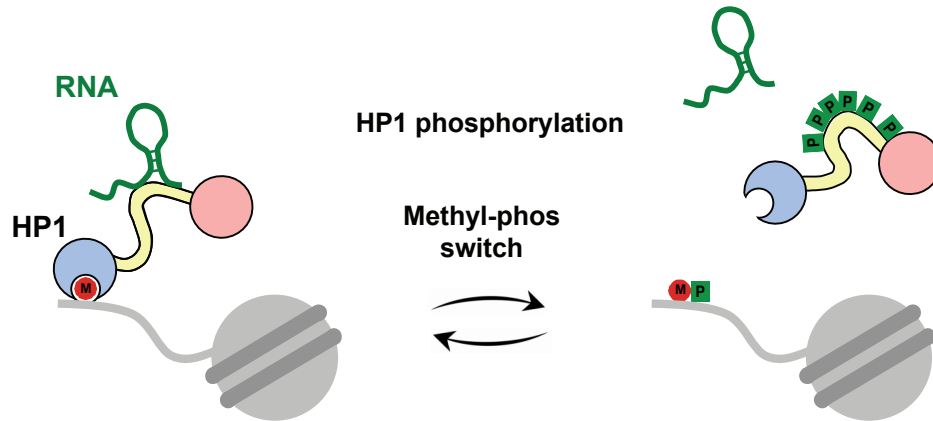


Figure 5.1: Regulation of HP1 α by phosphorylation on two different levels.

During mitosis, HP1 is regulated by phosphorylation on two different levels. Phosphorylation of histone H3 at serine 10 by Aurora B kinase results in the reversible release of the CD-H3K9me interaction by methyl-phos switching and (possibly in combination with other mechanisms) to dissociation of HP1 from chromatin. Simultaneously, phosphorylation of HP1 α itself by Aurora B and other, currently unknown kinases in its hinge domain abolishes the interaction with an RNA component (shown in green) that remains to be further characterized.

In experiments that I carried out in collaboration with Dr. Wolfgang Fischle, then a postdoctoral fellow in the Allis laboratory (now a faculty member at Max-Planck-Institute of Biophysical Chemistry, Göttingen), and with colleagues from the Funabiki lab, Boo Shan Tseng and Dr. Hironori Funabiki, I showed that the release of HP1 depends on phosphorylation of histone H3 at serine 10, a site that is phosphorylated by the Chromosomal Passenger Complex at the onset of mitosis. Serine 10 phosphorylation in immediate proximity to HP1's binding site at methyl-K9 sterically and electrostatically interferes with binding of the chromo domain and therefore

ejects the chromo domain from the histone mark. Removal of serine 10 phosphorylation at the end of mitosis, on the other hand, allows HP1 to reassociate with H3. In addition to providing important insight into the mechanism of mitotic HP1 release, these findings also identify a biological function for mitotic H3 serine 10 phosphorylation, a histone mark with so far elusive biological role (Nowak and Corces, 2004).

The data presented in my thesis is the first evidence for the existence of a novel "binary methylation-phosphorylation switch" mechanism, that controls the recruitment of effector proteins to chromatin through two collaborating histone marks (see Figure 3.12A). Even though the actual binding site of HP1, the methylation mark H3K9me, remains unchanged, addition or removal of the H3S10 phosphorylation mark results in a dynamic regulation of HP1 binding to chromatin.

Our findings close an important gap in the understanding of HP1 behaviour. As outlined in detail in the Discussion section of chapter 3, they also have wide implications: "methyl-phos switching" is not limited to the mitotic release of HP1, but has also been observed for HP1 in other cellular contexts. Furthermore, examples of binary switching have been found in non-histone proteins as well, and with other combinations of modifications, such as "methyl-methyl switches" (see Chapter 3, Section "Discussion"). Thus, binary switching appears to be a mechanism that is employed by the cell in many different contexts. It will be very interesting to see in the next years where else this regulatory principle is used.

However, our findings also immediately open up a whole array of follow-up questions. Why does the cell use this peculiar mechanism to regulate the reversible dissociation of HP1? Is "methyl-phos switching" sufficient for the dissociation of HP1 from chromatin *in vivo*? And why

must HP1 be released from chromatin during mitosis? In the following, I will discuss these questions and speculate in light of the literature.

Why does the cell use a "methyl-phos switching" mechanism for the mitotic release of HP1?

It has been observed on many occasions, that binding of effector proteins to chromatin is often a dynamic and reversible process (Festenstein et al., 2003; McNally et al., 2000; Phair et al., 2004). Generally, effectors are recruited to chromatin by their interaction with specific histone marks (which are established by "writer" enzymes). The release of effector proteins appears to be accomplished by a number of different mechanisms. Besides binary switching, these include removal of the histone mark by enzymes (Lan et al., 2008; Yang and Seto, 2008), clipping of histone tails by proteases (Allis et al., 1980; Duncan et al., 2008, or even replacement of the modified histone as a whole (Ahmad and Henikoff, 2002). This of course raises the question why the mitotic release of HP1 proteins is accomplished by "methyl-phos switching" rather than by any of the other mechanisms. A closer look at "methyl-phos switching" reveals that this mechanism has certain characteristics that set it apart from all the other mechanisms.

In general, kinase function in the cell often controls rapid changes in protein-protein interactions, for example in the recruitment of adaptor proteins in signal transduction pathways (Seet et al., 2006), the control of enzymatic activity via conformational changes (Johnson et al., 1996), or the disassembly of the lamin network underlying the nuclear envelope during mitotic prophase (Moir et al., 1995). Considering the rapid and extensive changes that chromatin has to undergo to enable faithful progression through mitosis, a kinase reaction might be the best way for

the cell to achieve a fast global release of the HP1 effectors in a highly regulated manner. Phosphorylation and dephosphorylation are quite thermodynamically favorable and the kinetics of both enzyme classes are quite rapid relative to other ways to establish or remove posttranslational modifications.

Compared to the other mechanisms, "methyl-phos switching" provides specificity to a mechanism designed to release an effector protein. Since it interferes exclusively with the association of proteins binding in the immediate vicinity of the phosphorylation site, effector proteins associated with other regions of the histone remain completely unaffected. This is in stark contrast to, e.g., tail clipping or histone exchange. While these mechanisms allow to exchange the complete modification profile of a histone in a single step, this comes of course at the price of specificity: many different histone marks and all the interaction partners associated with them are removed at once.

"Methyl-phos switching" is energy-efficient as well. With mechanisms such as tail clipping or histone replacement, re-establishing HP1 association with chromatin comes at a significant energy cost for the cell, because it requires complete synthesis of a new histone and specific methylation of the newly synthesized H3. Reassociation mediated by "methyl-phos switching", in contrast, requires only a single kinase reaction and thus allows dynamic regulation of chromatin read-out at minimal energetic cost for the cell.

"Methyl-phos switching" also fully preserves the epigenetic information encoded in the methyl-marks. Enzymatic removal of modifications through demethylation, in contrast, would erase the "epigenetic memory" of the H3K9 methyl-mark (Trojer and Reinberg, 2006).

In sum, it seems that the different mechanisms to release effector proteins all have their distinct characteristics that make them particularly suited for specific situations. Clipping of histone tails and histone replacement exchange a whole set of modifications at once and thus allow to completely change the character of a histone in a single step. While this may be quite helpful in certain cellular contexts, for example changes of expression profile during differentiation (Duncan et al., 2008), it comes with a significant energy investment and with the loss of the epigenetic information of the histone marks. Enzymatic removal of the histone mark is quick, specific and energy-efficient, but it also erases the epigenetic information deposited in the histone marks. "Methyl-phos switching", on the other hand, is fast, energy-efficient and at the same time preserves the epigenetic information stored in the methylation marks. Thus, it seems perfectly suited for the task of transiently and reversibly releasing the effector protein HP1 during mitosis.

Potential contribution of other mechanisms to the mitotic release of HP1 *in vivo*

It is clear at this point that "methyl-phos switching" is an essential step in the release of HP1 from chromatin. However, as described in more detail in the Discussion section of chapter 3, it is still an open question whether "methyl-phos switching" is actually *sufficient* for HP1 dissociation in mitosis.

In *in vitro* binding experiments with the HP1 chromo domain and H3 peptides, H3 phosphorylation is always sufficient for HP1 release. Experiments with full-length HP1 isoforms, however, have led to different

observations with the individual HP1 isoforms, and even to contradicting results (Fischle et al., 2005; Hirota et al., 2005; Terada, 2006). In binding experiments in the presence of cellular extracts, retention of HP1 upon abolishment of the chromo domain-H3K9me interaction has been observed as well (Fass et al., 2002; Fischle et al., 2005).

In vivo experiments so far could only confirm that "methyl-phos switching" is *necessary* for HP1 release in the cell (Fischle et al., 2005; Kloc et al., 2008; Sabbattini et al., 2007; Terada, 2006), but have not addressed the question whether it is also *sufficient*. Testing this question *in vivo* is not straightforward. Because there are multiple genes for H3 in mammalian cells (Marzluff et al., 2002), it is difficult to do an overexpression experiment with an H3 construct with a S10E mutation: if such an overexpression partially releases HP1, it is hard to tell if the residual chromatin-bound HP1 is all due to HP1 association with simultaneously present wild-type copies of H3, or if some of it is still associated with the mutated H3 through chromo domain-independent mechanisms. Moreover, observations with chromo domain mutants (which do not localize to heterochromatin at all *in vivo*, Platero et al., 1995; Stewart et al., 2005; Thiru et al., 2004) suggest that there may be a sequential order of the different interactions that mediate stable chromatin association of HP1. It seems initial contact is made by the CD and methylated K9. This may increase the local concentration of the protein, and then a subsequent "locking" step by additional interactions stabilizes HP1 binding to heterochromatic sites (Singh and Georgatos, 2002). Thus, constitutive disruption of the CD-H3K9me by mutation of serine 10 may actually prevent CD-independent mechanisms from being established in the first place.

This difficulty is exemplified by a recent publication reporting that phosphorylation in the chromo domain of HP1 β by CK2 (casein kinase 2) is a necessary step in the release of HP1 β from chromatin at sites of UV-induced DNA damage (Ayoub et al., 2008). Mutation of the site of phosphorylation, threonine 51, to glutamate abolished the interaction of HP1 β with chromatin. However, since the T51E mutant does not bind to H3K9me (as shown by *in vitro* binding experiments), it is not clear whether additional mechanisms of binding could be established at all. The system may be used, however for an interesting test: if overexpression of casein kinase 2 leads to the release of HP1, this would be an indication that reversal of the CD-H3K9me3 interaction may indeed be sufficient for HP1 release *in vivo*.

An alternative approach that may at least allow to estimate the severity of the contribution of other mechanisms besides "methyl-phos switching" may be to take advantage of *Xenopus* egg extracts and their options to manipulate experimental parameters in a cellular context that approximates the *in vivo* situation. In this system, the release of HP1 from interphase extracts could be tested with an H3S10 kinase other than Aurora B (judging from my *in vitro* data, MSK1 may be a good starting point; Figure 3.11). While still phosphorylating H3S10 efficiently, MSK1 is not very likely to phosphorylate, besides H3 serine 10, exactly the same targets as the CPC, and it does not initiate and control diverse regulatory events in mitosis. The level of retention of HP1 at chromatin may thus permit to estimate the contribution of mechanisms other than "methyl-phos switching".

Otherwise, directly testing the sufficiency of "methyl-phos switching" for the release of HP1 inside the living cell may require an artificial system

that allows recruitment of a (non-mitotic) H3S10 kinase to a heterochromatic locus bound by HP1. While such a system may be difficult to set up, it should allow to give a final answer to the still open question whether other mechanisms besides "methyl-phos switching" are required for the release of HP1 from chromatin at the onset of mitosis.

Why does HP1 have to be removed from chromatin during mitosis?

In the course of my thesis work, my collaborators and I have studied the mechanisms underlying the localization changes as well as the striking alterations in posttranslational modifications that HP1 undergoes during mitosis. Despite progress made, however, the overarching biological question remains – what is the biological function of HP1 release from chromatin during mitosis?

A challenging question to address experimentally

Experimentally, it is extremely challenging to address this question directly. One obvious way to get insight into this question is to prevent HP1 dissociation by inhibiting/depleting Aurora B and studying the effects on the mitotic cell. As shown by us and others, Aurora B inhibition/depletion causes retention of HP1 (Fischle et al., 2005; Hirota et al., 2005; Sabbattini et al., 2007; Terada, 2006). At the same time, it leads to a number of unusual observations about chromosome structure and behaviour, such as reduced cohesin dissociation from chromosome arms, a less pronounced primary constriction at centromeres, "fuzzy" appearance of chromosomes in hypotonic buffers, and syntelic attachment of the kinetochores of sister chromatids to microtubules of the same spindle pole (Giménez-Abián et

al., 2004; Hauf et al., 2003; Hirota et al., 2005). Eventually, it results in chromosomal defects and polyploidy (Hauf et al., 2003).

However, it is well established that Aurora B is not just a histone H3 kinase, but also has important functions (besides HP1 phosphorylation) at the spindle checkpoint and during cytokinesis with functions in metaphase chromosome alignment, sister chromatid resolution, spindle checkpoint signaling, bipolar spindle assembly, and cytokinesis (Ditchfield et al., 2003; Hauf et al., 2003; Lampson and Kapoor, 2005). Thus, it is impossible to differentiate whether the observed defects are indeed caused by the failure of HP1 to dissociate from chromatin, lack of mitotic H3S10 phosphorylation (which may have other functions in addition to HP1 "methyl-phos switching"), or the inability of Aurora B to phosphorylate other important mitotic targets.

One possible approach to see what the effects of HP1 retention are, while not abolishing Aurora B activity, would be to replace the endogenous H3 with an H3 that cannot be phosphorylated at serine 10, for example by a S to A mutation. However, this is quite difficult to do in mammalian cells, because mammals have a high copy number of H3 genes (twelve; Marzluff et al., 2002). The only established experimental systems that contain an HP1-like protein and have a low complexity of histone genes and thus may allow such a study are *S. pombe* or *Neurospora*. Indeed, in *S. pombe* the phenotype of H3S10 mutation has already been described as having mitotic defects (Mellone et al., 2003).

However, this is hard to interpret due to a second caveat: H3S10A does not only prevent phosphorylation, but also causes by itself already a significant reduction in the affinity of the HP1 chromo domain for H3K9me (by almost a factor of 10; Fischle, 2005; Hirota et al., 2005). This is not

surprising, considering the stabilizing contribution of the hydrogen bonds that serine 10 undergoes with residues of the HP1 CD (see Figure 3.2B). It is therefore questionable whether HP1 would bind to H3S10A at all *in vivo* (presumed that H3S10A is indeed subject to methylation *in vivo*, an assumption that has not been tested yet). These doubts are further fueled by the observation that GFP-Swi6 is mislocalized upon H3S10A mutation in *S. pombe* (Mellone et al., 2003), and if indeed differences were observed, it would be hard to tell if these were not simply due to effects of H3S10A mutation on HP1 recruitment.

A second, alternative approach to get insight into the biological function of mitotic HP1 dissociation would be to design an HP1 protein with mutations in the chromo domain that make it "immune" to phosphorylation of serine 10 (i.e. HP1 mutants that bind to H3K9me3S10ph equally well as to H3K9me3). Due to the multiple effects of serine 10 phosphorylation on the interaction of the CD with the methylated H3 tail (steric hindrance, electrostatic repulsion, and loss of hydrogen bonds; see Figure 3.2B), it is clear that this would be extremely difficult to achieve. Several such mutations have been tested (Fischle et al., 2005). However, neither chromo domain mutants designed to reduce the steric hindrance by the phosphate group (E53A, E53G) nor mutants engineered to relieve the electrostatic repulsion (E53Q, E53K) showed any increase in binding to H3K9meS10ph peptides *in vitro*. Rather, these mutants showed significant loss of affinity for H3K9me3 peptides, as expected owing to the loss of the hydrogen bonds with serine 10. Thus, it will be a real challenge to engineer such a switching-resistant HP1.

If experimental findings identify in the future other mechanisms involved in the mitotic release of HP1, this may open new roads to study

this question: While Aurora B kinase has various different functions in mitosis, these potential other mechanisms might be easier to inhibit specifically without side-effects on many other steps in mitosis.

Hypotheses on the biological function of HP1 release in mitosis

A direct experimental approach to this challenging question may therefore require years of research. However, in the following I will speculate about possible reasons why HP1 has to be released from chromatin during mitosis. Three steps during mitosis come particularly to mind: (1) condensin-mediated chromatin condensation, (2) removal of cohesin from chromosome arms, and (3) proper resolution/segregation of chromatids.

During prophase of the cell cycle, the chromosomes are highly compacted, in preparation of the spatial rearrangements involved in chromosome alignment and segregation. The mechanism of this chromatin condensation at the onset of mitosis seems to be distinct from that of HP1-mediated heterochromatin formation. It is possible that HP1 proteins need to be released from chromatin, in order to permit mitotic chromosome compaction by other factors (Figure 5.2).

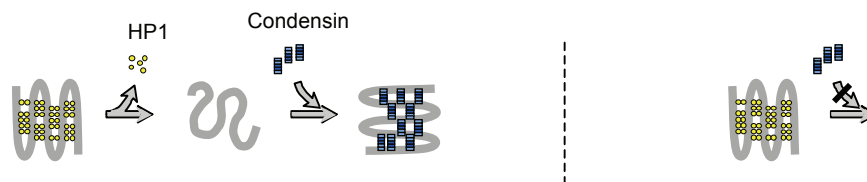


Figure 5.2: HP1 dissociation might be required for full mitotic chromatin condensation (Possible biological function of mitotic HP1 dissociation from chromatin) Figure adapted from Dormann et al., 2006.

Left: In a cell that progresses through M phase, HP1 is released at the onset of mitosis. This process allows condensin to access chromatin, therefore promoting mitotic chromatin condensation. **Right:** If HP1 does not dissociate, condensin cannot get full access to chromatin and mitotic chromatin condensation is inhibited.

One of the components involved in mitotic chromosome compaction is condensin, a multisubunit complex formed by the association of two SMC subunits (Structural Maintenance of Chromosomes, members of a large family of ATPases with key function in higher-order chromosome organization and dynamics (Yokomori, 2003)) with three non-SMC subunits (Kimura and Hirano, 1997). Condensin's exact mechanism of action is not fully understood at this point. However, it is clear that the complex binds to DNA and induces positive supercoiling (Bazett-Jones et al., 2002; Kimura and Hirano, 1997; Kimura et al., 1999; Stray et al., 2005; Stray and Lindsley, 2003), which probably contributes to chromosome compaction. It is tempting to speculate that the presence of HP1 (or the chromatin condensation mediated by HP1) would prevent access of condensin to DNA and thus interfere with mitotic chromosome condensation. In keeping with this model, it has been observed in *S. pombe* that in cells defective in CPC function condensin does not localize to mitotic chromosomes and chromosome condensation is impaired (Morishita et al., 2001). Similarly, in human cells Aurora B depletion results in loss of centromeric localization of condensin (Ono et al., 2004). Thus, from a biological point of view dissociation of HP1 through binary switching may be essential for mitotic chromosome condensation through condensin.

A second hypothesis is that the release of HP1 may be required for the removal of cohesin from chromosome arms (Figure 5.3). Biorientation of chromosomes on the mitotic spindle requires cohesion between sister chromatids (Losada, 2007). This cohesion is accomplished by cohesin, a multisubunit complex related to condensin, that forms a ring-like structure around the sister chromatids (Gruber et al., 2003). Separation of sister chromatids is dependent on the removal of cohesin from chromosomes,

which in vertebrates is a two-step process (Losada, 2007). Originally localized all over the chromosomes, at prophase the bulk of cohesin is removed from chromosome arms. This removal depends on the mitotic kinases Plk1 and Aurora B, and on the phosphorylation of one of the cohesin subunits (Giménez-Abián et al., 2004; Losada et al., 2002; Sumara et al., 2002). Only little cohesin persists on chromosomes, mostly at the centromeric regions (Hoque and Ishikawa, 2001; Waizenegger et al., 2000), where it is protected from removal through the protein Sgo1/MEI-S332 and the phosphatase PP2A (Kitajima et al., 2004; Salic et al., 2004; Sgo1 recruitment depends on centromeric HP1, Yamagishi et al., 2008). This remaining cohesin is then removed at the metaphase-anaphase transition by cleavage through separase, enabling the separation of sister chromatids (Hauf et al., 2001; Waizenegger et al., 2000).

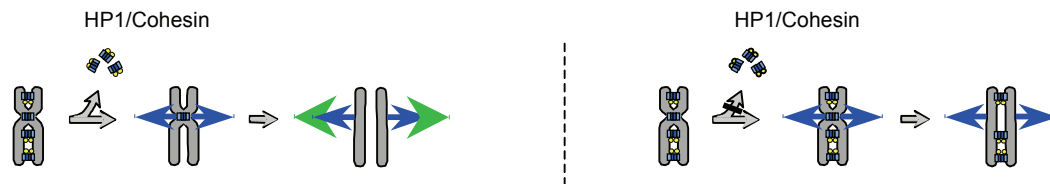


Figure 5.3: Dissociation of HP1 may be required for the release of condensin from chromosome arms (Possible biological function of mitotic HP1 dissociation from chromatin) Figure adapted from Dormann et al., 2006.

Left: HP1 release at the onset of mitosis also removes cohesin, which is associated with HP1, from chromosome arms. This allows for proper segregation of chromatids into the two daughter cells. **Right:** If HP1 is not released, cohesin remains associated with the chromosome arms. The chromatids cannot separate, which may lead to missegregation of chromosomes.

The exact function of Aurora B in the prophase dissociation of cohesin is currently unknown. However, it seems possible that HP1 is involved in this dissociation. In *S. pombe*, cohesin binds to the HP1

homolog Swi6, an interaction that is necessary for cohesin localization to the heterochromatic structures of the pericentric regions and that seems to be conserved in mammalian cells (Nonaka et al., 2002). Moreover, HP1 dissociation from chromatin and the release of cohesin occur with a very similar timing during prophase. Thus, it seems quite possible that cohesin removal from chromosome arms at prophase could be accomplished by Aurora B through the ejection of HP1 by "methyl-phos switching", making the biological function of HP1 release to help resolve the arm regions of sister chromatids from each other.

A third possibility is that HP1 proteins may have to dissociate from mitotic chromosomes to facilitate separation of chromatids (Figure 5.4). HP1 is assumed to mediate chromatin condensation by crosslinking K9-methylated nucleosomes through its ability to dimerize and interact with other chromatin components (Figure 1.9A). If such crosslinking occurs between different chromatids, it may not be possible to segregate sister chromatids to opposing poles of the spindle during anaphase. In this scenario, if HP1 is not removed before anaphase, the consequence might be segregation defects such as lagging chromosomes, chromosome bridges, and aneuploidy. Thus, according to this model, the biological function of mitotic HP1 release of HP1 might be to reverse crosslinks between individual chromatids to allow for the proper resolution and segregation of chromosomes. In keeping with this interpretation, Interestingly, Terada, while overexpressing an H3 construct with a S10A mutation in mammalian cells, observed lagging chromosomes and chromatin bridges (Terada, 2006). It is impossible to differentiate whether these effects are due to the retention of HP1 or reduced levels of K9 methylation and/or HP1 binding to this H3 mutant in the first place (see above). However, the observations

would match the phenotype expected for problems with chromosome separation due to the failure to remove HP1 crosslinking of chromatids.

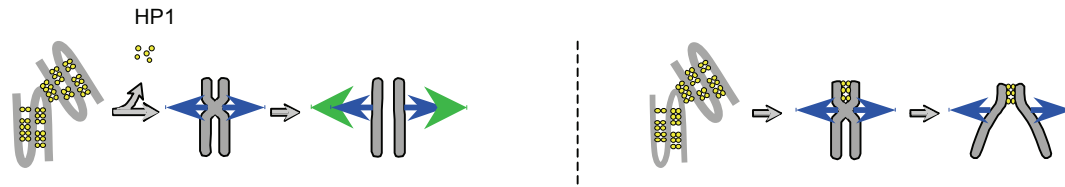


Figure 5.4: HP1 release may be necessary for proper resolution/segregation of chromatids (Possible biological function of mitotic HP1 dissociation from chromatin) Figure adapted from Dormann et al., 2006.

Left: If HP1 is released at the onset of mitosis, the pulling forces exerted by the spindle microtubuli segregate the chromatids into the two daughter cells (as indicated by the green arrow heads). **Right:** If HP1 is not removed, different chromatids (in this case sister chromatids) may remain attached to each other via HP1 cross-linking. This entangling inhibits proper resolution of chromatids and may lead to defective chromosome segregation.

HP1 phosphorylation

HP1 isoforms are subject to phosphorylation

Prompted by experimental observations made during the study of HP1 "methyl-phos switching" in the test tube, in the second part of my thesis I have focused on a potential involvement of posttranslational modifications of HP1 itself during mitosis. Together with my collaborators, I have mapped a set of *in vivo* phosphorylation sites of all three human HP1 isoforms, shown that most of these sites map to the HP1 hinge region (see Figure 4.23), and found evidence that HP1 α and HP1 γ are specifically phosphorylated in mitosis (the levels of the observed HP1 β phosphorylation were equally high in interphase and mitosis, see Chapter 3, Section "Discussion"). One site that is part of a highly conserved sequence motif ("KRKS"), HP1 α S92ph, I characterized in detail and present data that implicates Aurora B as the

responsible kinase *in vivo*. While testing specific candidates for an effect of HP1 α hinge phosphorylation on their association with HP1, I made *in vitro* observations that suggest that HP1 α hinge phosphorylation may be regulating the association of HP1 α with RNA.

The sites of phosphorylation presented in my thesis is among the first sites identified in mammalian HP1 proteins. While this work was in progress, reports on two individual modifications on the two other HP1 isoforms have been published.

The Urrutia lab describes a role for HP1 γ serine 93 phosphorylation (or, assuming a different translation start site, serine 83 phosphorylation) in interphase (Lomberk et al., 2006). According to their interpretation, phosphorylation of this site by PKA leads to exclusively euchromatic localization of HP1 γ , interaction with the DNA repair factor Ku70 (but in the absence of the other complex members that Ku70 usually associates with), and impaired silencing activity. Another study reported phosphorylation of HP1 γ S93 (but not HP1 α and HP1 β) in cells entering senescence, where the modification is required for efficient incorporation of HP1 γ into SAHF (senescence-associated heterochromatin foci), domains of facultative heterochromatin that repress proliferation-promoting genes (Zhang et al., 2007). Ayoub and colleagues have recently implicated phosphorylation of HP1 β at threonine 51 by casein kinase 2 in the mobilization of HP1 β after DNA damage (Ayoub et al., 2008; see above).

HP1 γ S93ph is homologous to HP1 α S92ph, the site of Aurora B phosphorylation that I characterized in more detail in my thesis. However, as opposed to these studies, I do not observe any localization effects upon HP1 α S92 mutation, HP1 α is known to interact with Ku70 already in an unphosphorylated state (Song et al., 2001), and I studied the events

connected to HP1 α S92 phosphorylation specifically in mitosis, which was not investigated in these studies (similar to HP1 α S92, HP1 γ S93, is also upregulated in mitosis, even though that was not part of the study by Lomberk and colleagues; my own observations in western blotting and IF experiments with an antibody kindly provided by the Urrutia lab). Nevertheless, these papers illustrate the potential of HP1 modifications to control HP1 interactions and functions.

No effect of HP1 phosphorylation on localization dynamics in mitosis

In my thesis work, I tested the hypothesis that HP1 α hinge phosphorylation may be involved in the different localization changes observed for HP1 α during mitosis, but did not observe any effect. Is it possible that the HP1 α hinge region nevertheless plays, besides regulating RNA association, a role in HP1 α release *in vivo*, and that this role was missed due to shortcomings of the experimental approaches taken to address those questions?

My experiments designed to test different hypotheses about the function of HP1 α hinge mutations *in vivo* were largely based on mutation of the identified phosphorylation sites. Serine to glutamate mutations were used, for example, for testing a role of HP1 hinge phosphorylation in HP1 α 's reassociation at metaphase; if HP1 α hinge phosphorylation regulated this reassociation of HP1 α with metaphase centromeres, increased/decreased binding of mutated constructs was expected. Glutamate is known to be a good mimic for the negative charge effect of the phosphoryl-group, but it is structurally different (planar instead of tetrahydric). Therefore it may not always function as a mimic for phospho-serine, and problems like this could of course prevent the observation of effects in experimental tests based on such mutations.

However, mechanisms that may besides "methyl-phos switching" be involved in the regulation of HP1 localization changes during mitosis will likely depend on additional interaction partners of HP1. The identity of these interactors is currently unknown, and they may actually not have been identified at all. Thus, there is little alternative for systematic testing of such hypotheses but *in vivo*. While far from perfect, glutamate and alanine are probably still the best mimics for constitutively phosphorylated/not phosphorylatable serine available (aspartate is clearly smaller than phospho-serine).

Considering that HP1 chromatin binding can be guided by different mechanisms, it is possible that only a small subpopulation of HP1 associated with a few loci is affected by HP1 α hinge phosphorylation (see also below, Section "Speculations on the biological function of the HP1-RNA interaction and of HP1 α hinge phosphorylation"). In such a scenario, effects of the mutations at these sites may have been missed in my immunofluorescence analysis. Indeed, this cannot be completely excluded, and it may be worth reconsidering some of my experiments in a more detailed way (different extraction/fixation procedures, chromosome spreads; alternatively even ChIP or examination of different cell types). However, considering the detailed analysis that I have carried out already, it does not seem worthwhile to continue along this path, unless additional evidence for such an involvement of HP1 phosphorylation in HP1 localization should emerge.

Furthermore, the analysis presented in this thesis was carried out with fixed cells and therefore looks only at single time-points in the release/reassociation process. The release and reassociation of HP1, however, is a dynamic process, and it is possible that HP1 phosphorylation affects the dynamics of this process rather than the final outcome. Elegant stud-

ies have already examined the dynamics of HP1 in living cells in other context (during the activation of a silenced locus; Janicki et al., 2004), and it may be worth it to carry out similar experiments to analyze the effect of HP1 phosphorylation mutations on the dynamics of HP1 in living cells.

Possible existence of other HP1 binders regulated by HP1 hinge phosphorylation

In vitro experiments presented in this thesis implicate HP1 phosphorylation in the control of RNA binding to the HP1 α hinge. This exciting finding opens new doors for potentially interesting follow-up experiments and allows for some intriguing hypotheses (see below). However, it should be noted that this observation does not exclude that there may be other, additional interactions besides RNA binding that may be regulated by the same mechanism.

One protein that may be interesting to test in the future for phosphorylation-dependent binding to HP1 may be the CPC component INCENP, because there is literature suggesting that it binds to the HP1 hinge region. Even though it has been demonstrated that the interaction with HP1 α is not required for INCENP localization or localization changes during mitosis (Ainsztein et al., 1998) and vice versa (Terada, 2006), it is possible that this interaction affects for example the enzymatic activity of the CPC on partners associated with HP1 (such as histone H3) by positioning the complex in a favourable position. The ability of HP1 to fulfill such a function might be modulated by HP1 phosphorylation.

It is also possible that so far unknown HP1 binders are regulated through HP1 hinge phosphorylation. My experiments indicate that unbiased

pull-down experiments to identify such factors may not be straightforward with the isolated hinge domain (see Chapter 4, Section "Redundancy / Requirement for Cooperation"), but with careful optimization this may also be a promising approach in the future.

Reasons for exclusion of HP1 α S92ph signal from chromatin at telophase

Originally, my interest in the role of HP1 phosphorylation in the regulation of HP1 α localization during mitosis was raised by IF experiments with the HP1 α S92ph antibody, when I made the observation that the signal of an HP1S92ph antibody is excluded from chromatin, while with a general HP1 α antibody staining is observed (Figures 4.7B, 4.9BC, 4.13B). According my mutation analysis, HP1 α hinge phosphorylation has no effect on HP1 α localization in mitosis. How can the IF observations then be explained?

One possibility is that the S92ph epitope is masked by a molecular interaction that HP1 α undergoes when it reassociates with chromatin. Alternatively, a chromatin-associated phosphatase could remove the phosphorylation as soon as HP1 binds to chromatin. Notably, in a fractionation experiment (Figure 4.13A) I observed by western blotting that HP1 α from a fraction loosely associated with chromatin showed significantly higher levels of S92 phosphorylation than HP1 α that was tightly associated with chromatin. This might point to the latter of the two possible explanations, a chromatin-associated phosphatase.

HP1 and RNA binding / Nature of the bound RNA(s)

In chapter 4 of my thesis, I have presented *in vitro* observations suggesting that HP1 α hinge phosphorylation may control the association of HP1 α

with RNA (Figure 4.30). These observations were made with recombinant HP1 α hinge constructs, that associated with bacterial RNA in the case of the wild-type hinge sequence, but did not associate in the case of a hinge sequence with S to E mutations at the phosphorylation sites. The effect was reproduced in an electrophoretic mobility-shift assay (EMSA) with a *Drosophila* mRNA sequence as probe. The conditions under which these observations were made are certainly quite artificial and raise the question whether this is indeed a real interaction or rather an *in vitro* artifact.

A first evaluation of this question may come from a closer look at what is known in the literature about the interaction of HP1 with RNA. There are reports by several groups that suggest such a connection., and findings demonstrating a direct interaction of HP1 with RNA have been published in mammals (Muchardt et al., 2002) and in *S. pombe* (Motamedi et al., 2008). In addition, observations that could readily be explained by direct RNA binding of HP1 were made in both *Drosophila* (Piacentini et al., 2003) and mammals (Maison et al., 2002).

The first real *in vivo* evidence for a direct interaction of HP1 with RNA has been reported only very recently from RNA immunoprecipitation experiments with Swi6, the HP1 homolog in fission yeast (Motamedi et al., 2008). These experiments demonstrated that Swi6 specifically binds to noncoding *cen*RNAs (transcripts generated from the centromeric regions of the *S. pombe* chromosome). While an indirect involvement of RNA in the formation of Swi6-dependent heterochromatin is well established (Swi6 is essential for an RNAi-dependent silencing loop which requires transcription though noncoding heterochromatin to be sustained, see Introduction), this is the first occasion that direct RNA binding is demonstrated in *S. pombe*. As a model, Motamedi and colleagues suggest that Swi6 association

with *cen* transcripts may tether nascent heterochromatic transcripts to heterochromatin and allow RNAi complexes to assemble on the transcript. It should be noted that detection of the transcripts was carried by RT-PCR and only two transcripts were tested (*cen* and actin mRNA, which does not associate with Swi6). Thus, no conclusion can be drawn from these experiments whether there are other RNAs that may associate with Swi6.

In mammalian cells, there are also observations indicating a direct association of HP1 with RNA, even though these experiments have not been done in genuine *in vivo* systems. In 2002, Maison et al. noted in work with mouse cells that RNase treatment during immunofluorescence experiments destroys the architecture of heterochromatic domains and specifically releases HP1 α , a finding that could be reproduced in overlay assays (Maison et al., 2002). Adding back RNA reversed all observed effect. Maison and colleagues showed that there is some specificity with respect to the species of RNA: only RNase A, and not RNase H (cleaves dsRNA and DNA-RNA duplicates) disrupts the heterochromatic structures and releases HP1, and restoration of heterochromatic architecture and HP1 α foci is observed upon adding back total and especially nuclear RNA, while there is little effect with tRNA and bacterial mRNA.

Prompted by similar observations in overlay assays, Muchardt and colleagues showed that HP1 α and HP1 γ can bind RNA directly *in vitro* and mapped this binding to the C-terminal part of the HP1 α hinge region (Muchardt et al., 2002). By competition experiments with different nucleic acids in EMSAs, they carried out a preliminary characterization of the RNA interaction that allow some conclusions about the specificity of HP1's RNA-binding in this experimental system.

It is unlikely that HP1 recognizes a complex RNA sequence motive, because Muchardt and colleagues were able to carry out their EMSAs with a random bacterial mRNA probe. On the other hand, binding is also not simply dictated by attractive electrostatic forces either, because the interaction is not competed by ssDNA, dsDNA, AU- or GC-rich RNA oligomers or tRNA. Ribosomal RNA and especially total nuclear RNA, in contrast, compete the interaction with the bacterial probe RNA efficiently. This suggests that there is either some promiscuity with regard to the sequence specificity of the HP1 hinge region, or the hinge recognizes a rather short sequence or small structural RNA motif that is found rather often in a complex sequence. It should be noted, though, that these *in vitro* observations do not categorically exclude binding of only one specific RNA by HP1 α *in vivo*. The chromatin proteins MOF and MSL-3, for example, show no or little binding specificity in their RNA binding *in vitro*, although they have a very specific physiological target, roX RNA (Akhtar et al., 2000).

The RNA-binding region of HP1 was mapped by EMSA and overlay assay to residues 86-108 of HP1 α (the C-terminal part of the hinge region). The region has no clear homology to known RNA-binding modules, despite a high density of basic amino acids. However, RNA binding of sequences with similar composition has been demonstrated in several ribosomal proteins and retroviral virulence factors, such as HIV TAT (Brodersen et al., 2002; Weiss and Narayana, 1998). An interacting domain of this size is not capable of contacting more than 6 to 10 nucleotides at once (Ruthenburg, 2009), which would explain the low sequence specificity of HP1 RNA binding. The RNA binding domain includes most of the phosphorylation sites that I have identified in the HP1 α hinge. Interestingly, Muchardt and colleagues demonstrated that simultaneous mutation of only three conserved lysines

within the RNA binding region (K104/105/106A) abolishes RNA binding. Thus, it seems feasible that introduction of a few negative charges by phosphorylation of the RNA interacting domain may have a similar effect.

In addition to the reports in *S. pombe* and in mammals, a connection between HP1 and RNA has been suggested in one other context. In *Drosophila*, HP1a is recruited to the very actively transcribed sites of developmental and heat shock-induced chromosomal puffs (Piacentini et al., 2003). The association of HP1a with these puffs is functionally relevant (reduced expression of HP1a results in decreased levels of transcripts at these sites, overexpression of dHP1a in elevated levels) and RNA-dependent (RNase treatment and induction of puffs without transcription). HP1 binds specifically to coding regions of the actively transcribed hsp70 gene (rather than to promoters). It has not been investigated so far whether there is indeed a direct association of HP1 with RNA at these sites, and if so, what the nature of this RNA may be. Since these findings in *Drosophila* implicate HP1 with euchromatic RNA (as opposed to heterochromatic RNAs as in the reports in *S. pombe* and mammals), HP1's RNA binding may be more complex and possibly directed towards different kinds of RNA (Kellum, 2003).

All in all, my experimental observations are in agreement with published findings on HP1 interaction with RNA, and it seems quite possible that HP1 α hinge phosphorylation may indeed regulate the interaction of HP1 α with RNA *in vivo*. However, my preliminary observations and the very limited data in the literature do not yet allow to draw any definite conclusions to this important question.

Next experimental steps

To expand on my preliminary finding that HP1 phosphorylation regulates RNA binding *in vitro*, the first follow-up step will have to be a rigorous confirmation of this experimental observation will be required. The EMSA could be repeated with new preparations of labeled RNA (hopefully without any aggregation), full-length HP1 α constructs could be tested, and the *in vitro* interaction could be further characterized, e.g. by the use of different salt concentrations that would permit a qualitative evaluation of the interaction's affinity. Competition with different RNA species could be carried out to verify if my HP1 hinge constructs reproduce the results published for RNA binding of full-length HP1 before (Muchardt et al., 2002).

As helpful as further examination of the interaction of HP1 with RNA in the test tube may be, it will not get beyond the basic limitations of the *in vitro* system. It is hard to exclude that observations made in such a system are simply general effects of changes in charge that are amplified by the artificial conditions of the test tube, while no such effect exists under *in vivo* conditions. Thus, for meaningful data it will be necessary to study the HP1 α -RNA interaction *in vivo*.

A quick way to show that HP1 hinge phosphorylation indeed affects RNA binding of HP1 α *in vivo* would be by purification of HP1 from tissue culture cells and autoradiographic analysis of copurifying RNAs after radioactive labelling. From cell lines expressing tagged wt HP1 α or HP1 α with S to A and S to E mutations, HP1 could be purified e.g by immunoprecipitation/pull-down. Coprecipitating RNA could be labelled by polynucleotide kinase end-labelling with ^{32}P -ATP (assuming free 5'-OH), and then analyzed by gel electrophoresis and autoradiography. Differences

in the RNA binding of the constructs should be visible as variations in the banding pattern between the different constructs.

However, it is unclear how interpretable such a pull-down experiment would be. Considering that HP1 undergoes dozens of different interactions and is part of large heterochromatic complexes (see Chapter 1, Introduction), the mixture of copurifying RNAs may simply be too complex to allow any meaningful evaluation from an autoradiograph. Some improvement might be achieved by the use of crosslinking methods such as CLIP (Cross-linking and immunoprecipitation, see below) to allow for more rigorous purification.

By far the most insight into the interaction of HP1 α with RNA, the role of RNA in HP1 biology, and the part that HP1 α hinge phosphorylation plays in this context, will however come from identification of RNAs associated with HP1 α *in vivo*. While the exact experimental approach would depend on the type and the size of the RNA associating with HP1 (e.g. non-coding RNA, mRNA, small RNAs), Various protocols for this different purposes have already been developed by others (e.g. Pfeffer et al., 2005; Rinn et al., 2007). CLIP (Cross-linking and IP), a method specifically developed for the highly specific purification of RNAs binding to protein in intact cells (Ule et al., 2003), could be used. By a UV cross-linking step, specifically interacting RNA is bound covalently to protein and can be purified by harsh purification steps, such as denaturing purification or transfer to nitrocellulose, which discards of free RNA (Ule et al., 2005). After removal of HP1 protein by proteinase K digest, linker RNA can be ligated to the 5' and 3' ends, and the RNAs amplified by RT-PCR with DNA primers complimentary to the RNA linkers. Sequence analysis could be carried out either by microarray analysis or by Solexa sequencing.

Purification and identification of protein-associated RNAs profits immensely from a negative control that allows to distinguish specific interactions from unspecific ones. Since my *in vitro* data indicates that HP1 α hinge phosphorylation abolishes RNA binding to HP1, the hinge phosphorylation may offer a highly useful handle for the identification of HP1-binding RNAs *in vivo* (cell lines expressing HP1 α constructs with S to E mutations in the hinge could be used as negative control and compared to either wt or S to A constructs).

The experimental work and technical expertise required to successfully identify HP1-associated RNAs through this approach is of course significant. Therefore, I have recently entered into a collaboration with Dr. Emily Bernstein, a former colleague in the Allis lab (now a faculty member at Mount Sinai School of Medicine, New York). Dr. Bernstein is an expert in RNA biology, and we are optimistic that our collaboration will soon provide experimental validation whether my *in vitro* observations reflect real *in vivo* behavior of HP1. Successful identification of HP1-associated RNAs in mammalian cells will undoubtedly contribute significantly to our understanding of this highly interesting chromosomal protein and its function in the cell.

Hypotheses on the biological function of the HP1-RNA interaction and of HP1 α hinge phosphorylation

The fundamental biological question underlying these future experiments is of course, what the role of the RNA interaction may be and why the RNA has to be released during mitosis. As pointed out before, a very limited amount of data is available on the connection between HP1 and RNA, making models speculative by necessity. Even so, I want to propose three

hypotheses what the role of RNA may be in HP1 biology and how the regulation by HP1 hinge phosphorylation would fit into these scenarios.

- 1) RNA could play the role of "molecular glue" that stabilizes heterochromatic structures.

The currently favored model of HP1-mediated heterochromatin formation involves recruitment of HP1 by H3K9me and condensation of heterochromatic domains by crosslinking through HP1 dimerization of the chromoshadow domain (Jenuwein and Allis, 2001). Due to the diverse molecular interactions that HP1 undergoes, it has been suggested that HP1-mediated heterochromatin formation may be enhanced by additional factors that, through interactions with HP1 and possibly each other, contribute to the formation of a tightly packed dense heterochromatic structure (Craig, 2005; Dillon and Festenstein, 2002). According to my model, RNA fulfills such a function (Figure 5.5). By interacting with the hinge regions of multiple HP1 molecules, RNA assists HP1 crosslinking in the formation of densely packed heterochromatic regions and further stabilizes the heterochromatic architecture.

The role of HP1 α hinge phosphorylation in this model may be a contribution to the disassembly of large heterochromatic complexes during mitosis, at the time when HP1 is released by "methyl-phos switching" (and, possibly, other mechanisms). HP1 hinge phosphorylation reverses the RNA-HP1 interaction, helping to break down large heterochromatic complexes and to set HP1 molecules free for reassociation with chromatin at the end of mitosis. Removal of HP1 hinge phosphorylation after mitosis (possibly by a chromatin-associated phosphatase) would then reestablish RNA binding.

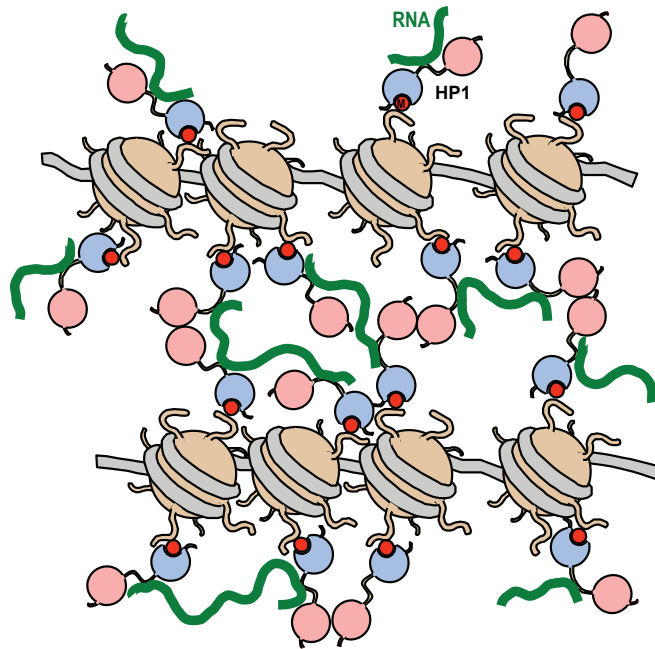


Figure 5.5: RNA as "molecular glue" to stabilize and compact HP1-dependent heterochromatic structures (Hypothetical model for the biological role of the HP1 α -RNA interaction)

According to this model, RNA may have a role in stabilization and compaction of heterochromatic domains. Through the interaction of its chromo domain with H3K9me (and likely, other interactions), HP1 is recruited to chromatin. Dimerization of HP1 molecules through their chromoshadow domain leads to crosslinking of nucleosomes and significant compaction of the domain. Filling in the spaces of this meshwork, RNA may bind to the hinge regions of several HP1 molecules simultaneously. Like a "molecular glue", this further stabilizes and compacts the heterochromatic structure.

This model is in keeping with several observations made by me and others. In 2002 the Almouzni lab showed that an RNA component is required for the maintenance of mouse pericentric heterochromatin organization (Maison et al., 2002). Recently, it has been demonstrated that in *S. Pombe* Swi6/HP1 directly associates with centromeric transcripts (Motamedi et al., 2008), giving further credence that such a model may indeed be possible *in vivo*.

In my own experiments, I observed during a fractionation procedure that (S92-) phosphorylated HP1 α could be easily stripped off of chromatin, while unphosphorylated HP1 α was part of much more stable chromatin structures (Figure 4.13A). That HP1 α hinge phosphorylation had no effect on HP1 localization would be explained in this model by the fact that RNA compacts heterochromatic structures, but is not directly involved in HP1 chromatin binding.

To test this model, one could analyze the complexes that HP1 is part of during mitosis and how they are affected by HP1 hinge phosphorylation or mutation of the phosphorylation sites. Alternatively, a more detailed analysis of the composition and architecture of heterochromatic domains (and, possibly, how these are affected by mutations of the HP1 α hinge) may be a possible approach.

- 2) The interaction of HP1 with RNA could mediate the association of HP1 with euchromatic regions within the genome. Most likely in collaboration with other interactions, interaction with mRNA recruits HP1 to these sites, where the protein plays a role in the promotion of gene expression (Figure 5.6).

This model is in agreement with my experimental findings and several observations in the published HP1 literature. The association of HP1 with euchromatic sites within the genome has been observed in *Drosophila* and mammalian cells by various experimental methods (Cryderman et al., 2005; de Wit et al., 2007; Johansson et al., 2007; Piacentini et al., 2003; Vakoc et al., 2005; see Introduction).

Association of HP1 with such sites seems to be RNA-dependent, as suggested by the observation that HP1 association with heat-shock and developmentally induced puffs (highly expressed regions in *Drosophila*

polytene chromosomes) is only observed in the presence of RNA (Piacentini et al., 2003). High-resolution mapping of HP1a binding sites in *Drosophila* HP1a shows that HP1 localizes specifically to transcribed regions of actively expressed genes, and is excluded from the promoter (de Wit et al., 2007; Johansson et al., 2007). This raises the possibility that HP1 may actually be binding to the transcribed mRNA. However, it is clear that there must be contributions of other mechanisms, because HP1 associates only with few of the many actively transcribed loci in the genome.

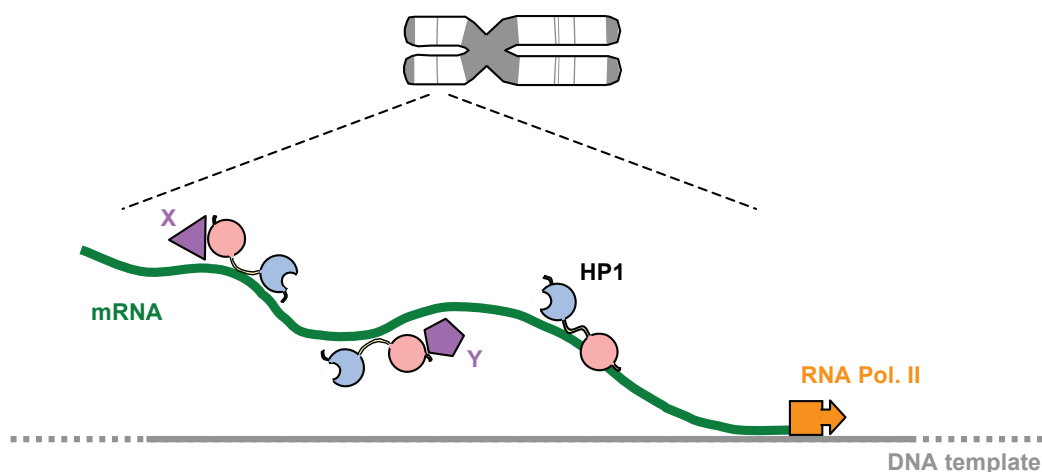


Figure 5.6: RNA binding of HP1 recruits HP1 to euchromatic loci (Hypothetical model for the biological role of the HP1 α -RNA interaction)

RNA binding of HP1 could recruit HP1 to euchromatic loci. Besides abundant association with heterochromatic regions, HP1 is also found at a few euchromatic sites (top). According to this model, the association of HP1 with these euchromatic regions is mediated by HP1 binding to mRNA (in green), most likely in collaboration with other interactions (not shown in this cartoon). At the locus, HP1 promotes gene expression by unknown mechanism (such as a role in mRNA stabilization), and may also recruit other factors (X, Y; shown in purple) to the site.

The function of HP1 at these sites is currently not understood, but the protein seems to positively regulate gene expression (loss of HP1 results in reduced, overexpression in increased gene expression at such sites, Cryderman et al., 2005; correlation of HP1 binding with gene expression, de Wit et al., 2007). On a molecular level, it may be that HP1 promotes transcription at these sites through a role in transcriptional elongation or even in mRNA stabilization (Kellum, 2003).

According to my proposed model, HP1 is recruited to these sites by an interaction with RNA. During mitosis, the protein has to be released from the sites of active transcription, possibly because transcription has to be shut down to allow full condensation of the chromosomes. In the model, the dissociation of HP1 from these sites is accomplished by phosphorylation of HP1 in the hinge region, which abolishes RNA binding.

Upon S to A mutation or S to E mutation of the HP1 phosphorylation sites, HP1 is retained or released, respectively. But since there are only few of such HP1-binding euchromatic loci, this effect is not readily observed in my immunofluorescence assay, because there is a huge excess of heterochromatic HP1 that at the same time dissociates from chromatin by "methyl-phos switching" and this larger population dominates IF observations.

In this model, only the small subpool of HP1 at euchromatic sites depends on HP1 phosphorylation for its release in mitosis. However, my data indicates that there is a significant amount of phosphorylated HP1 present in mitotic cells. This could be explained by the existence of additional functions of HP1 phosphorylation Alternatively, it is possible that the "collateral" phosphorylation of the other pool of HP1 does not hurt the cell and is therefore not selected against.

First hints whether such a model would be feasible at all could come from the identification of the RNA(s) associated with HP1. As a more direct test of the model, ChIP experiments at known euchromatic binding sites of HP1 could be carried out. By comparing the association of HP1 constructs with wt sequence and with mutated phosphorylation sites, it should be possible to verify whether HP1 binding to these sites is regulated by HP1 α hinge phosphorylation.

- 3) HP1 binding to RNA could be important in regions of constitutive heterochromatin for tethering nascent RNA transcripts to heterochromatin.

This hypothesis was recently formulated by Motamedi and colleagues (Motamedi et al., 2008) and is based on their observation of an *in vivo* interaction of Swi6 (the *S. pombe* homolog of HP1) with noncoding *cen* transcripts (transcripts originating from baseline transcription of the silenced centromeric repeat regions) in fission yeast. The model (Figure 5.7) suggests that Swi6/HP1 tethers these nascent heterochromatic RNA transcripts to chromatin. The stable association of the transcripts with the sites of RNA synthesis is required for the subsequent assembly of the RNAi complexes RITS (RNA-induced transcriptional gene silencing complex) and RDRC/Dcr1 (RNA-dependent RNA polymerase complex/Dicer 1) on the RNA (Motamedi et al., 2008), which facilitates efficient processing of the transcripts to double-stranded siRNAs, a step that is required for H3 lysine 9 methylation and heterochromatic silencing (Grewal and Jia, 2007; Volpe et al., 2002). In keeping with the hypothesis, in the absence of Swi6, the levels of centromeric siRNAs are dramatically reduced (Motamedi et al., 2008), and localization of RITS and RDRC to *cen* transcripts (Motamedi et al., 2008), and Rdp1

to *cen* DNA (Sugiyama et al., 2005) is decreased compared to wild-type cells, demonstrating that Swi6 is required for the efficient association of RITS, RDRC and Dicer with their target transcripts at centromeres.

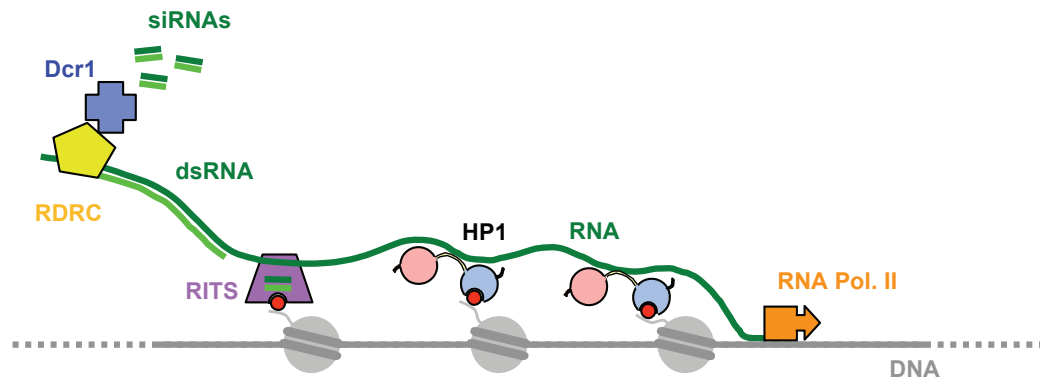


Figure 5.7: HP1 tethers nascent heterochromatic RNA transcripts to the heterochromatic locus (Hypothetical model for the biological role of the HP1 α -RNA interaction)

HP1 could bind nascent heterochromatic RNA transcripts and tether them to the heterochromatic locus. This enables assembly of RNA processing complexes on the RNA that are required for heterochromatin formation/maintenance. According to this model, HP1 associates with heterochromatin through the chromo domain – H3K9me interaction. Through the hinge region, HP1 associates with RNA transcripts (in dark green) that are generated at low levels, and thus stably tethers them to the site. This permits assembly of machinery required for processing of the transcript, such as the RNA-dependent RNA polymerase complex (RDRC, in yellow) that generates double-stranded RNA (dsRNA), Dicer (in blue) that cuts the dsRNA into siRNAs (short interfering RNAs), or RITS (in purple) that contains siRNAs and is required for establishing/maintaining H3K9 methylation at centric regions. Figure adapted from Motamedi et al., 2004.

It is possible that the interaction of Swi6 with *cen* RNA has to be released during mitosis, for example because the heterochromatic complexes associated with RNA processing would impede the efficient

interaction of Swi6 with cohesin (Nonaka et al., 2002). Such an ejection of *cen* RNA from Swi6 binding could conceivably be regulated by Swi6 phosphorylation.

It should be noted, though, that at the moment it is not yet established if Swi6 indeed binds *cen* RNA through its hinge domain, nor has it been investigated in detail to which extent the pathways of heterochromatin nucleation/maintenance are conserved in higher organisms.

All in all, little is known about the connection between HP1 biology and RNA. As outlined above there are some very intriguing possibilities, and my finding that RNA binding to HP1 is regulated by HP1 phosphorylation may open the door for some exciting further discoveries in the future.

Conclusion

In my Ph.D. project I have explored the ways how phosphorylation regulates Heterochromatin Protein 1, an important chromatin effector protein. As I have shown, phosphorylation controls HP1 function on two different levels: On one level, phosphorylation of histone H3 regulates HP1 chromatin release during mitosis through binary switching, and the discovery of this mechanism leads the way to a better understanding not only of the mechanisms that control HP1 chromatin association, but also of many other molecular interactions in chromatin biology and beyond, that are regulated by similar mechanisms. On the other level, mitotic phosphorylation of HP1 itself in its hinge domain appears to control the association of HP1 with RNA. My observations open up new experimental directions that may lead to a better understanding of the role and regulation of RNA in the biology of Heterochromatin Protein 1.

The recruitment of effectors by posttranslational modifications of histone proteins is one of the key mechanisms to control chromatin structure and function. The findings presented in my thesis illustrate, however, that there are additional levels of complexity that involve an intricate interplay between multiple histone modifications, as well as modulation of effector function by posttranslational modification of the effectors themselves. Exploring these layers of regulatory complexity will surely be challenging, but the therapeutic potential of chromatin, especially for the treatment of cancer, indicates that the effort will be well-invested.

APPENDIX

Human HP1 α

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
M G K K T K R T A D S S E D E E E Y V V E K V

26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50
L D R R V V K G Q V E Y L L K W K G F S E E H N T

51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75
W E P E K N L D C P E L I S E F M K K Y K K M K E

76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100
G E N N K P R E K S E S N K R K S N F S N S A D D

101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125
I K S K K K R E Q S N D I A R G F E R G L E P E K

126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150
I G A T D S C G D L M F L M K W K D T D E A D L

151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175
V L A K E A N V K C P Q I V I A F Y E E R L T W H

176 177 178 179 180 181 182 183 184 185 186 187 188 189 190 191
A Y P E D A E N K E K E T A K S

Blue: Chromo domain. Yellow: Hinge region. Red: Chromoshadow domain.
Green: Residues of aromatic cage.

Human HP1β

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
M G K K Q N K K K V E E V L E E E E E Y V V E K
26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50
V L D R R V V K G K V E Y L L K W K G F S D E D N
51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75
T W E P E E N L D C P D L I A E F L Q S Q K T A H
76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100
E T D K S E G G K R K A D S D S E D K G E E S K P
101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125
K K K K E E S E K P R G F A R G L E P E R I I G A
126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150
T D S S G E L M F L M K W K N S D E A D L V P A K
151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175
E A N V K C P Q V V I S F Y E E R L T W H S Y P S
176 177 178 179 180 181 182 183 184 185
E D D K K D D K N

Blue: Chromo domain. Yellow: Hinge region. Red: Chromoshadow domain.
Green: Residues of aromatic cage.

Human HP1 γ

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
MASNKTTLQKMGKKQNGKSKKVEEA

26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50
EPEEFVVEKVLDRRVVNGKVEYFLK

51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75
WKGFTDADNTWEPEFENLDCPELIEA

76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100
FLNSQKAGKEKDGTKRKSLSDSESD

101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125
DSKSKKRDAAADKPRGFARGLDPER

126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150
IGATDSSGELMFLMKWKDSEADL

151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175
VLAKFANMKCPQIVIAFYERLTWH

176 177 178 179 180 181 182 183
SCPEDEAQ

Blue: Chromo domain. Yellow: Hinge region. Red: Chromoshadow domain.
Green: Residues of aromatic cage.

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