Heterochromatin und epigenetische Kontrolle der Centromere in *Dictyostelium discoideum*

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Zusammenfassung

Heterochromatin Protein 1 (HP1) ist ein evolutionär konserviertes Protein, dass an der Ausbildung höhergeordneter Chromatinstrukturen und an epigenetischem Gen-Silencing beteiligt ist.

Das Ziel der vorliegenden Arbeit war es, HP1-ähnliche Proteine aus *Dictyostelium discoideum* funktionell zu charakterisieren und ihre Funktion bei der Heterochromatinbildung und transkriptionellem Gen-Silencing zu untersuchen.

Im Genom von *Dictyostelium* liegen drei für HP1-ähnliche Proteine codierende Gene (*hcpA, hcpB und hcpC*) vor. Für *hcpA* und *hcpB*, jedoch nicht für *hcpC*, konnte Genexpression während des vegetativen Wachstums und des Entwicklungszyklus gezeigt werden. Obwohl *hcpC* kein offensichtliches Pseudogen darstellt, wurde es nicht in die Analyse mit einbezogen.

Sowohl HcpA als auch HcpB zeigten die charakteristische, konservierte Domänenstruktur von HP1-Proteinen, die aus einer N-terminalen chromo-Domäne und einer C-terminalen chromo shadow-Domäne, die durch eine sog. „hinge“-Region getrennt werden, besteht.

Beide Proteine zeigten alle für HP1-Proteine charakteristischen biochemischen Eigenschaften, u.a. Homo- und Heterodimerisierung *in vivo* und *in vitro*, sowie DNA-Bindung. HcpA zeigte ausserdem *in vitro* Bindung an K9-methyliertes Histon H3. Die Proteine scheinen daher sowohl strukturell als auch funktionell konserviert in *Dictyostelium* vorzuliegen.

Beide Proteine wiesen eine grösstenteils identische subnukleäre Verteilung in mehreren kleineren Punkten und Konzentration in einem Hauptbereich an der Kernperipherie auf. Die Lokalisation dieses Hauptbereichs in direkter Nachbarschaft zum Kern-assoziierten Centrosom und sein Verhalten während der Mitose deuteten stark darauf hin, dass er pericentromerisches Heterochromatin darstellt. Beide HP1-Proteine kolokalisierten ausserdem mit der Histon H3K9-dimethylierung, einem weiteren Kennzeichen für Heterochromatin in *Dictyostelium.*

Aus diesem Grund bestand ein Hauptaspekt der vorliegenden Arbeit darin, die bisher grösstenteils unbekannte strukturelle Organisation von pericentromerischem Heterochromatin zu charakterisieren.

Das *Dictyostelium*-Homolog des inneren Centromer-Proteins INCENP, DdINCENP, kolokalisierte sowohl mit Histon H3K9-dimethylierung als auch mit HcpA während der

mitotischen Metaphase, was ein weiteres Indiz dafür lieferte, dass es sich bei der durch H3K9me2 und HcpA/B charakterisiertem Kernregion um pericentromerisches Heterochromatin handelt.

Zwei Typen von hochrepetetiven Retrotransposons, *DIRS*-1 und s*kipper,* liegen in grossen unregelmässigen Anhäufungen an den Chromosomenden vor, von denen vermutet wird, dass sie die Centromere enthalten. Mittels Chromatin-Immunpräzipitation (ChIP) konnte gezeigt werden, dass diese beiden Retrotransposon-Typen, jedoch nicht die euchromatische Actin-Genfamilie, präferentiell mit H3K9me2 assoziiert sind.

Weder Überexpression von HcpA oder HcpB, noch der Verlust einer der beiden Proteine führte zu einer Änderung der Retrotransposon-Transkriptmengen. Überexpression eines C-terminal trunkierten HcpA-Proteins, von dem vermutet wurde, dass es einen dominant negativen Effekt ausübt, führte hingegen zu einem Anstieg der *skipper*-Transkriptmengen. Ausserdem verursachte die Überexpression dieses Proteins schwere Wachstumsdefekte in axenischer Suspensionskultur und eine verringerte Lebensfähigkeit der Zellen.

Um die Funktion beider Proteine bei der Ausbildung pericentromerischen Heterochromatins zu charakterisiern, wurden Funktionsverlustmutanten für *hcpA* und *hcpB* erzeugt. Beide Gene konnten gezielt über homologe Rekombination ausgeschaltet werden. Überraschenderweise, jedoch nicht gänzlich unerwartet, war die funktionell Redundanz beider Isoformen sehr hoch. Beide Einzelmutanten zeigten keine offensichtlichen Phänotypen unter Standardlaborbedingungen, und nur der Verlust von *hcpA*, jedoch nicht von *hcpB*, führte zu schwachen Wachstumsdefekten bei niedrigen Temperaturen.

Alle Versuche, eine Doppelmutante zu erzeugen, schlugen fehl. Allerdings konnten beide endogenen Gene dann ausgeschaltet werden, wenn die Zellen zuvor mit einem „Rettungskonstrukt“, das eine der beiden Isoformen entweder als 6xHis- oder GFP-Fusionsprotein ektopisch exprimierte, transformiert worden waren. Die Daten implizieren, dass das Vorhandensein von mindestens einer der beiden Isoformen in *Dictyostelium* essentiell ist. Die Lethalität der *hcpA*/*hcpB*-Doppelmutanten erschwerte die funktionelle Analyse der beiden Proteine enorm. Allerdings lieferte dieses Experiment auch den genetischen Beweis, dass das GFP-Fusionsprotein von HcpA durch seine Fähigkeit, den Verlust von endogenem HcpA-Protein zu kompensieren, funktional ist.

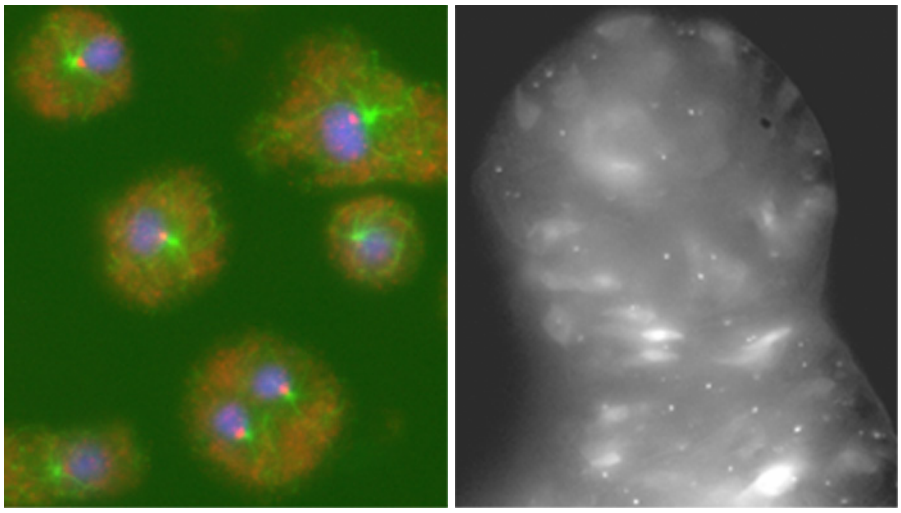
Sowohl HcpA als auch HcpB zeigten quantitative Unterschiede im Dimerisierungsverhalten, die durch die unterschiedlichen C-Termini der Proteine verursacht wurden. Dimerisierungspräferenzen in aufsteigender Reihenfolge sind HcpA-HcpA << HcpA-HcpB << HcpB-Hcp. Überexpression von GFP-HcpA oder einem chimerischen Protein, dass den C-terminus von HcpA enthielt (GFP-Hcp$B_N A_C$), jedoch nicht von GFP-HcpB oder GFP-Hcp$A_N B_C$ führte zu erhöhten Frequenzen von Anaphasebrücken in späten mitotischen Zellen, von denen angenommen wird, dass sie durch Telomer-Telomer-Fusionen verursacht wurden.

Die Zielsteuerung beider Proteine zum Chromatin wird durch mindestens zwei unterschiedliche Mechanismen bewerkstelligt. Die N-terminale chromo-Domäne und die hinge-Region werden für die Zielsteuerung zum pericentromerischen Heterochromatin benötigt, während die C-terminale chromo shadow-Domäne für die Zielsteuerung an andere Bereiche des Chromatins benötigt wird, die an der Kernperipherie liegen und durch Histon H3K9-dimethylierung gekennzeichnet sind. An der Zielsteuerung zum pericentromerischen Heterochromatin ist wahrscheinlich direkte DNA-Bindung beteiligt.

Das Genom von *Dictyostelium* enthält die Gene für alle sechs Untereinheiten des origin recognition complex (ORC), einer Proteinkomponente, die möglicherweise an der Zielsteuerung von HP1 zum Chromatin beteiligtsein könnte. Die Überexpression eines GFP-Fusionsproteins von OrcB, dem Orc2-Homolog in *Dictyostelium*, zeigte eine distinkte subnukleäre Verteilung des Proteins, die partiell mit der von HcpA überlappte. Ausserdem zeigte GFP-OrcB eine Lokalisation am Centrosom während des gesamten Zellzyklus, die auf eine Beteiligung von OrcB an der Centrosomenfunktion hindeutet.

DnmA als einzige DNA-Methyltransferase ist für die gesamte DNA(Cytosin-)-Methylierung in *Dictyostelium* verantwortlich. Um eine *in vivo*-Aktivität des Protein zu detektieren, wurden verschieden Zelllinien etabliert, die DnmA als GFP- oder myc-Fusionsprotein überexprimierten. Es wurde vermutet, dass eine Überexpression des Proteins zu erhöhten 5-Methylcytosin-Mengen in der genomischen DNA aufgrund von genomischer Hypermethylierung führen würde. Obwohl DnmA-GFP präferentielle Lokalisation im Zellkern zeigte, konnten jedoch keine erhöhten 5-Methylcytosin-Mengen in der genomischen DNA über Kapillarelektrophorese festgestellt werden.

Heterochromatin and Epigenetic Control of Centromeres in *Dictyostelium discoideum*



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Publications

Parts of the present thesis are published or are going to be published in the following papers.

Kaller, M., B. Földesi, and W. Nellen. Mitotic Analysis of Protein Factors involved in chromosome segregation in Dictyostelium. (manuscript in preparation)

Kaller, M., U. Euteneuer, and W. Nellen. 2006. Differential Effects of Heterochromatin Protein 1 Isoforms on Mitotic Chromosome Distribution and Growth in Dictyostelium discoideum. *Eukaryot Cell*. 5:530-43.

Chubb, J.R., G. Bloomfield, Q. Xu, M. Kaller, A. Ivens, J. Skelton, B.M. Turner, W. Nellen, G. Shaulsky, R.R. Kay, W.A. Bickmore, and R.H. Singer. 2006 Feb 7; [Epub ahead of print]. Developmental timing in Dictyostelium is regulated by the Set1 histone methyltransferase. *Dev Biol*.

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Summary

Heterochromatin Protein 1 (HP1) is an evolutionarily conserved protein required for formation of a higher-order chromatin structures and epigenetic gene silencing.

The objective of the present work was to functionally characterise HP1-like proteins in *Dictyostelium discoideum*, and to investigate their function in heterochromatin formation and transcriptional gene silencing.

The *Dictyostelium* genome encodes three HP1-like proteins (*hcpA, hcpB, hcpC*), from which only two, *hcpA* and *hcpB*, but not *hcpC* were found to be expressed during vegetative growth and under developmental conditions. Therefore, *hcpC*, albeit no obvious pseudogene, was excluded from this study.

Both HcpA and HcpB show the characteristic conserved domain structure of HP1 proteins, consisting of an N-terminal chromo domain and a C-terminal chromo shadow domain, which are separated by a hinge.

Both proteins show all biochemical activities characteristic for HP1 proteins, such as homo- and heterodimerisation *in vitro* and *in vivo*, and DNA binding activtity. HcpA furthermore seems to bind to K9-methylated histone H3 *in vitro*. The proteins thus appear to be structurally and functionally conserved in *Dictyostelium*.

The proteins display largely identical subnuclear distribution in several minor foci and concentration in one major cluster at the nuclear periphery. The localisation of this cluster adjacent to the nucleus-associated centrosome and its mitotic behaviour strongly suggest that it represents centromeric heterochromatin. Furthermore, it is characterised by histone H3 lysine-9 dimethylation (H3K9me2), which is another hallmark of *Dictyostelium* heterochromatin. Therefore, one important aspect of the work was to characterise the so-far largely unknown structural organisation of centromeric heterochromatin.

The *Dictyostelium* homologue of inner centromere protein INCENP (DdINCENP), co-localized with both HcpA and H3K9me2 during metaphase, providing further evidence that H3K9me2 and HcpA/B localisation represent centromeric heterochromatin.

Chromatin immunoprecipitation (ChIP) showed that two types of high-copy number retrotransposons (*DIRS*-1 and *skipper*), which form large irregular arrays at the chromosome ends, which are thought to contain the *Dictyostelium* centromeres, are characterised by H3K9me2.

Neither overexpression of full-length HcpA or HcpB, nor deletion of single Hcp isoforms resulted in changes in retrotransposon transcript levels. However, overexpression of a C-terminally truncated HcpA protein, assumed to display a dominant negative effect, lead to an increase in *skipper* retrotransposon transcript levels. Furthermore, overexpression of this protein lead to severe growth defects in axenic suspension culture and reduced cell viability.

In order to elucidate the proteins functions in centromeric heterochromatin formation, gene knock-outs for both *hcpA* and *hcpB* were generated. Both genes could be successfully targeted and disrupted by homologous recombination. Surprisingly, the degree of functional redundancy of the two isoforms was, although not unexpected, very high. Both single knock-out mutants did not show any obvious phenotypes under standard laboratory conditions and only deletion of *hcpA* resulted in subtle growth phenotypes when grown at low temperature.

All attempts to generate a double null mutant failed. However, both endogenous genes could be disrupted in cells in which a rescue construct that ectopically expressed one of the isoforms either with N-terminal 6xHis- or GFP-tag had been introduced. The data imply that the presence of at least one Hcp isoform is essential in *Dictyostelium*. The lethality of the *hcpA/hcpB* double mutant thus greatly hampered functional analysis of the two genes.

However, the experiment provided genetic evidence that the GFP-HcpA fusion protein, because of its ability to compensate the loss of the endogenous HcpA protein, was a functional protein.

The proteins displayed quantitative differences in dimerisation behaviour, which are conferred by the slightly different hinge and chromo shadow domains at the C-termini.

Dimerisation preferences in increasing order were HcpA-HcpA << HcpA-HcpB << HcpB-HcpB.

Overexpression of GFP-HcpA or a chimeric protein containing the HcpA C-terminus (GFP-Hcp$B_N A_C$), but not overexpression of GFP-HcpB or GFP-Hcp$A_N B_C$, lead to increased frequencies of anaphase bridges in late mitotic cells, which are thought to be caused by telomere-telomere fusions.

Chromatin targeting of the two proteins is achieved by at least two distinct mechanisms. The N-terminal chromo domain and hinge of the proteins are required for targeting to centromeric heterochromatin, while the C-terminal portion encoding the CSD is required for targeting to several other chromatin regions at the nuclear

periphery that are characterised by H3K9me2. Targeting to centromeric heterochromatin likely involves direct binding to DNA.

The *Dictyostelium* genome encodes for all subunits of the origin recognition complex (ORC), which is a possible upstream component of HP1 targeting to chromatin. Overexpression of GFP-tagged OrcB, the *Dictyostelium* Orc2 homologue, showed a distinct nuclear localisation that partially overlapped with the HcpA distribution. Furthermore, GFP-OrcB localized to the centrosome during the entire cell cycle, indicating an involvement in centrosome function.

DnmA is the sole DNA methyltransferase in *Dictyostelium* required for all DNA(cytosine-)methylation. To test for its *in vivo* activity, two different cell lines were established that ectopically expressed DnmA-myc or DnmA-GFP. It was assumed that overexpression of these proteins might cause an increase in the 5-methyl-cytosine(5-mC)-levels in the genomic DNA due to genomic hypermethylation. Although DnmA-GFP showed preferential localisation in the nucleus, no changes in the 5-mC-levels in the genomic DNA could be detected by capillary electrophoresis.

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1 Introduction

1.1 Chromatin

In eukaryotes, the genetic information of the DNA is organised in the form of chromatin. Chromatin is a nucleoprotein complex, which is organised into special higher order structures that facilitate packaging of the DNA in the nucleus. The requirement to package DNA is best illustrated by the fact that 2 m of human DNA need to be placed into each cell´s nucleus that is only 6 µm in diameter.
Apart from a mere packaging function, the organization of eukaryotic DNA into chromatin fundamentally influences the accessibilty of the DNA to protein factors, and thus affects most DNA-based processes, such as replication, transcription, recombination and repair.

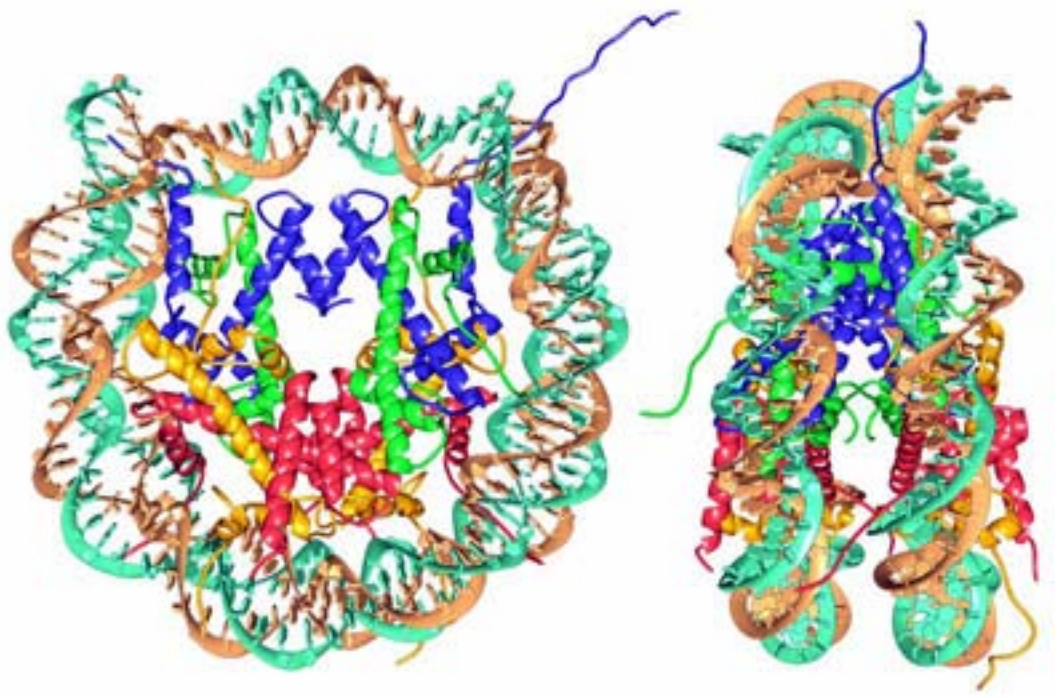

Fig. 1.1 : **Crystal structure of the nucleosome core particle** (From Luger et al., 1997). The DNA is shown in brown and turquoise (blue: histone H3; green: histone H4; yellow: histone H2A; red: histone H2B).

The basic building block of chromatin is the nucleosome. It is composed of two molecules each of the histones H2A, H2B, H3 and H4, which form an octameric, disc-shaped histone core around which 146bp of DNA are wound in 1.7 left-handed turns

(Fig. 1.1). According to their fundamental role in DNA packaging, the core histones represent some of the most highly conserved eukaryotic proteins. Another, less well-conserved histone, histone H1, stabilizes the nucleosome core structure by interactions with both the DNA and the nucleosome core and facilitates higher order chromatin structuring (see below). In the X-ray structure, the N-terminal extensions of the core histones are largely unstructured, suggesting that their conformation is flexible (Luger et al., 1997).

Individual nucleosome core particles are separated by up to 80bp of linker DNA, thereby forming a 10nm wide so-called “beads-on-a-string” array. The 10nm-fiber has the intrinsic property to condense into higher order structures *in vitro*. Upon addition of low concentrations of divalent cations, the nucleosomal arrays condense and fold into the so-called 30nm-fiber (Horn and Peterson, 2002). This fiber is likely the most prominent occuring form of chromatin *in vivo*, since it is the form of chromatin observed after cell lysis with physiological salt concentrations, whereas the extended 10nm-fiber is only observed in the absence of divalent cations (Hansen, 2002). Formation of specific higher-order chromatin structures is facilitated by histone H1, but also by transcriptional repressor proteins such as Polycomb group proteins (Francis et al., 2004; Fig. 1.2) or Heterochromatin Protein 1 (HP1, see below).

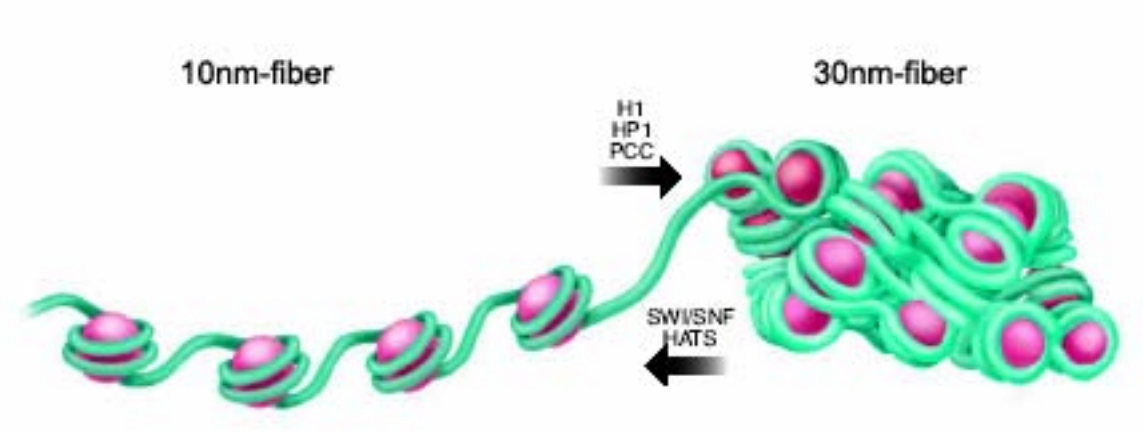

Fig. 1.2: **Schematic representation of chromatin-higher order structuring** (Adapted from: Mohd-Sarip and Verrijzer, 2004). Nucleosome core histones are in purple, DNA is in green. Histone H1, HP1 and Polycomb complex PCC facilitate formation of the transcriptionally repressive 30nm-fiber, while opposing factors such as the SWI/SNF-family of ATP-dependent chromatin remodelling complexes or histone acetylases (HATs) act in decondensation and opening of the chromatin structure.

In the 30nm-fiber, the N-terminal histone tails, rather than simply protruding from the nucleosome core as implied by the nucleosome crystal structure (Luger et al., 1997),

aid in the stabilization of higher order structuring by interacting with both the DNA and the histone core from adjacent nucleosomes (Dorigo et al., 2003; Dorigo et al., 2004; Schalch et al., 2005).

1.2 Regulation of chromatin structure by post-translational histone modifications

Posttranslational modifications of the N-terminal histone tails dramatically alter protein-protein or protein-DNA interactions and thus chromatin folding. For example, acetylation of lysine residues on the histone N-termini catalyzed by histone acetyl transferases (HATs) removes the positive charge from the lysine, thereby weakening the histone tail-DNA interactions in the 30nm-fiber and opening the chromatin structure. Conversely, histone deacetylation by histone deacetylases (HDACs) mediates compaction of chromatin and transcriptional repression (Roth et al., 2001).
In the past few years, a vast body of evidence has accumulated showing that histones, apart from playing a crucial role in DNA packaging, are subjected to a large number of post-translational modifications such as acetylation, methylation, phosphorylation, ADP-ribosylation and ubiquitylation, especially in their N-terminal extensions. These posttranslational modifications, either alone or in combination with others, ultimately lead to changes in the chromatin structure and thus regulate all DNA-based processes. The concept of combinatorial histone modifications that “define” specific chromatin states, has been introduced as the “histone code” theory (Strahl and Allis, 2000; Jenuwein and Allis, 2001).

1.3 Heterochromatin and Euchromatin

The term “heterochromatin” was introduced in 1928 by Emil Heitz (Heitz, 1928) to describe a form of chromatin that stays in a condensed form throughout the cell cycle, in contrast to the rest of the chromatin (“euchromatin”) that undergoes extensive condensation / decondensation cycles from interphase to metaphase.
Heterochromatin probably represents a more compact structure than the 30nm-fiber. Many additional non-histone proteins are thought to facilitate an additional level of higher-order structuring (Alberts et al., 2002; Horn and Peterson, 2002); however, heterochromatin is structurally not well understood.

Nevertheless, heterochromatin has “defining” properties that distinguish it from euchromatin. Heterochromatin represents genomic regions that contain few genes, but large blocks of repetetive DNA. These regions are (largely) transcriptionally silent, inaccessible to DNA-modifying agents, poorly digested by nucleases and do replicate late in S-phase. Typically, this condensed form of chromatin is found at centromeres and telomeres (Grewal and Moazed, 2003; Richards and Elgin, 2002). Since the heterochromatic DNA sequences mostly consist of tandem repeats and transposable elements, the packaging into higher order chromatin structures is believed to prevent recombination events between homologous DNA sequences, thus ensuring genomic integrity and stability.

On the molecular level, heterochromatin differs from euchromatin in its histone modification pattern. Characteristic heterochromatic histone modifications are histone H3 methylation at the lysine 9 (K9) and lysine 27 (K27) residues, histone H4 methylation at the lysine 20 (K20) residue and histone hypoacetylation. In contrast, histones in euchromatic regions are mostly hyperacetylated and display a different methylation pattern (Noma et al., 2001; see below). Furthermore, heterochromatic repetetive DNA itself is subjected to DNA methylation in many organisms, which functions to reinforce the silent state of these chromosomal regions (Richards and Elgin, 2002).

1.4 Centromeres

The centromere is a unique chromosomal locus that ensures that the sister chromatids properly segregate and that each daughter cell receives a chromosome copy during cell division. Centromeric chromatin serves as an assembly site for the kinetochore, the protein complex that makes contacts with the spindle microtubules during mitosis (Chan et al., 2005).

The centromere is a striking example of an epigenetically determined chromosomal locus. Except for *S. cerevisiae*, no specific sequences that would determine the position of the centromere have been defined in any organism (Cleveland et al., 2003). Despite this lack of underlying sequence conservation, centromere core domains in most organisms are defined by chromatin in which a divergent histone H3 variant, CENP-A, is incorporated into the nucleosomes (Henikoff and Dalal, 2005). This centromere core is embedded within arrays of repetetive elements. The

chromatin in these regions is characterised by specific histone modifications, such as histone H3K9 methylation, and is bound by additional proteins such as HP1 (see below). These chromosomal regions, usually referred to as pericentromeric heterochromatin, are crucial for proper centromere function during mitosis (see 1.11).
Although the underlying centromeric DNA sequences do not display any obvious sequence conservation, centromeres are embedded within pericentromeric heterochromatin in many different organisms (Fig. 1.3). Although centromeric chromatin is characterised by a distinct histone modification pattern that is distinct from both euchromatin and heterochromatin (Sullivan and Karpen, 2004), heterochromatin with its specific molecular marks surrounding the core domain can be regarded as a defining feature of centromeres.

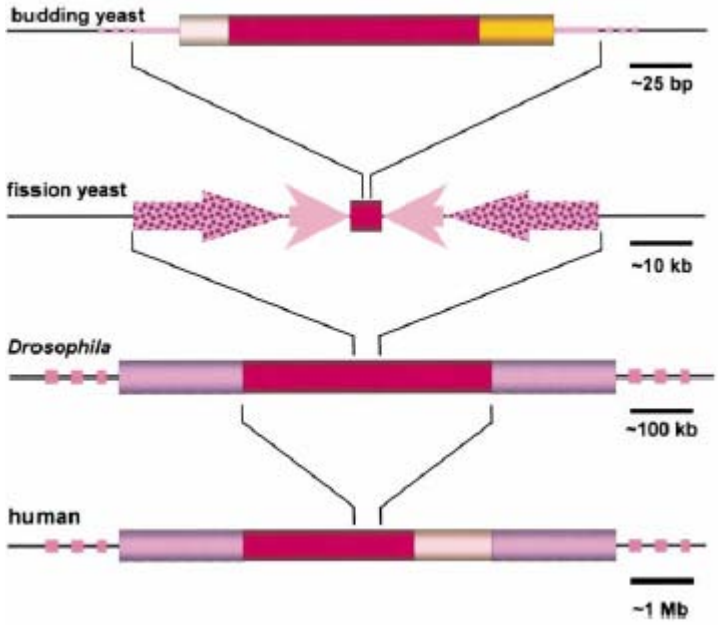

Fig. 1.3: **Structural organisation of centromeres from different organisms.** (From: Cleveland et al., 2003). The 125bp centromeres of budding yeast are defined by three conserved sequence elements pink, red, yellow). Fission yeast centromeres are comprised of a nonconserved central core (red), flanking inner repeats (pink arrows) at which the CENP-A-containing nucleosomes assemble, and conserved heterochromatic outer repeats (stippled purple). The *Drosophila* centromere core spans ~400 kb (red) and is embedded in constitutive heterochromatin (purple). Human centromeres are comprised of different types of repetetive DNA (pink and red), which are flanked by heterochromatin (purple).

1.5 Histone lysine methylation and regulation of chromatin function

Methylation of histones primarily occurs on the N-terminal extensions protruding from the nucleosome core, and to a lesser extent in the core domain. Methylation occurs on lysine, but also on arginine residues of histones H3 and H4.

In most cases histone lysine methylation is catalyzed by histone methyltransferases (HMTases) that contain a conserved SET-domain (named after the proteins Su(var)3-9, Enhancer of zeste and Trithorax, Jenuwein et al., 1998; Rea, et al., 2000) but also by non-SET-domain-containing proteins (Dot1 and its homologues), whereas arginine methylation is catalyzed by the PRMT1 family (Martin and Zhang, 2005). The enzymatic machinery required to establish the “chromosomal imprint” by introducing specific histone methylation marks is evolutionarily conserved, but the downstream effects of a given modification can be remarkably different between different organisms, indicating that the information carried by specific histone modifications can be used to trigger different effector pathways (see below).

There are six residues on the histones H3 and H4 known to be methylated: K4, K9, K27, K36 and K79 of histone H3, and K20 of histone H4 (Fig. 1.4). While histone acetylation generally correlates with transcriptional activation, histone methylation can either mark transcriptionally active or inactive chromatin, depending on the lysine residue which is methylated. Furthermore, in contrast to acetylation, lysine residues can either be mono-, di- or trimethylated, which adds further complexity to the methyl

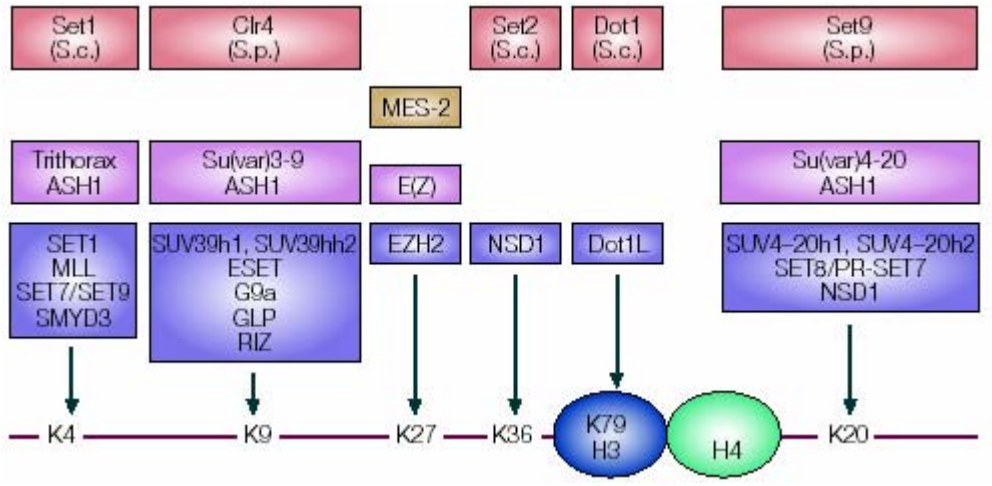

Fig. 1.4: **Known histone methyltransferases and their target lysine residues on histone H3 and H4.** Red: yeast, purple: *Drosophila*, brown: *C. elegans*, blue: mammalian orthologous proteins. (From: Martin and Zhang, 2005)

mark, since different methylation states of a given lysine residue have been shown to have different functional implications. For example, trimethylation of histone H3K9 is particularly enriched and thus marks pericentromeric regions in mammals, while the dimethylated form of H3K9 is mainly found in silenced euchromatic regions (Rice et al., 2003; Peters et al., 2003)

Histone H3K4, H3K36 and H3K79 methylation sites have been correlated with transcriptional activity. In contrast, histone H3K9, H3K27 and histone H4K20 methylation are markers for transcriptional repressive chromatin. One remarkable exception from this rule is *S. cerevisiae*, where H3K9 methylation is absent and which uses H3K4 and H3K79 methylation to index transcriptionally silent regions such as telomeres or portions of the ribosomal DNA (Bryk et al., 2002; van Leeuwen et al., 2002). Histone H3K79 methylation is catalyzed by the Dot1 family of HMTases that lack a SET domain. The function of H3K79 methylation in mammals, despite its association with transcribed genomic regions, is currently unclear. On possible role seems to lie in DNA damage signalling, since 53BP1 (p53-binding protein1) binds to K79-methylated histone H3 at sites of DNA damage (Huyen et al., 2004). A possible involvement in gene transcription stems from the observation that H3K79-methylation of the *hoxA9* gene by the human Dot1 homologue hDOT1L results in HOXA9 upregulation in leukaemic transformation (Okada et al., 2005).

The enzymatic machinery responsible for H3K4 and H3K36 methylation is directly coupled with the transcription process by physical association of the histone methyltransferases with RNA polymerase II, resulting in histone methylation within the coding regions. It is hypothesized that these histone methylation marks serve as a long-term signature to stably inherit the active state through cell division cycles (Martin and Zhang, 2005).

Histone H3K9 and H4K20 methylation are mainly found in constitutive pericentromeric chromatin (Fischle et al., 2003). Among the first HMTases identified were the products of the su(var)3-9 gene in *Drosophila*, and its homologues clr4 in *S. pombe* and suv39h1 and –h2 in humans (Martin and Zhang, 2005). The proteins, which had previously been shown to be required for proper heterochromatin formation, are responsible for the methylation of histone H3 at the lysine 9 residue. Methylation of the lysine 9 residues creates a binding site for HP1 proteins (Bannister et al., 2001; Lachner et al., 2001). This is thought to lead to transcriptional repression by formation of compacted higher order chromatin structures in a functionally conserved pathway.

By now, additional mammalian H3K9-specific HMTases have been identified, such as G9a and ESET/SETDB1, which are mainly required for gene-specific silencing of single-copy loci in euchromatic regions (Tachibana et al., 2002; Tachibana et al., 2005; Dodge et al., 2004;).

Methylation of H4K20 is another marker for mammalian heterochromatin, which is catalyzed by the Suv4-20h1/2 enzymes. In *Drosophila*, mutations of the Su(var)4-20 homologue impair heterochromatin formation as shown by suppression of position effect variegation (PEV, see below) (Schotta et al., 2004). Remarkably, H4K20 methylation in fission yeast is not involved in heterochromatin formation, but rather is required for proper DNA-damage signalling, most likely through recruitment of Crb2, a homologue of mammalian 53BP1, a Tudor-domain (see below) containing protein (Sanders et al., 2004).

H3K27 methylation, which is missing from pericentromeric heterochromatin in *Drosophila* and mammals, indexes transcriptionally repressed euchromatic loci (Fischle et al., 2003). H3K27 methylation is catalyzed by the E(z) (enhancer of zeste) HMTase, which is a component of one of the Polycomb group (PcG) complexes (Müller et al., 2002; Kuzmichev et al., 2003). The PcG group of proteins constitute a conserved gene regulatory system that is required to maintain repressed transcriptional states of many loci in the genome, among them several developmental master regulatory genes. Thereby, Polycomb group proteins establish a “cellular memory” that stabilizes cellular differentiation into different lineages. Targets for Polycomb-mediated silencing are for example the *hox* genes, a highly conserved class of transcription factors defining the positions of structures and appendages along the animal anterior-posterior axis (Ringrose and Paro, 2004, Orlando, 2003). The H3K27 methylation mark is selectively bound by the chromo domain-containing Polycomb (Pc) protein (see below), which then leads to stable repression of the targeted loci.

1.6 *Dictyostelium* histone lysine methyltransferases

The *Dictyostelium* genome encodes for five SET-domain containing proteins (Fig. 1.5). One of them, (DDB0188336; setA; www.dictybase.org) has been characterised as a histone H3 lysine 4-specific methyltransferase (Chubb et al., 2006), the function of which on heterochromatin structure has been partly analyzed in this study. A second putative histone methyltransferase (DDB0190352, suvA) displays high homology (E-value $5e^{-41}$) to the histone H3 K9 methyltransferase Su(var)3-9 from *Drosophila* and its

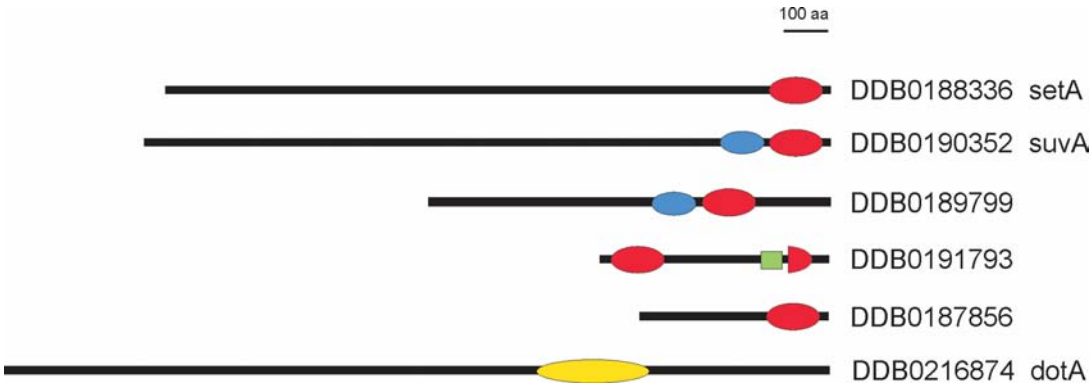

Fig. 1.5: **Schematic representation of known and putative *Dictyostelium* histone methyltransferases**. For explanations, see text. SET domains are marked as red ellipsoids, pre-SET domains as blue ellipsoids; histidine-rich region is marked as green box. DOT1-homologous region is marked in yellow. Bar represents 100 amino acids (aa).

mammalian homologues Suv39h1/2 (hence its name suvA). However, the functional analysis of the gene and the protein encoded by it is still lacking. This is partially due to the inability to obtain a gene knock-out by homologous recombination, suggesting that the gene is essential (M. Essid, Diploma thesis, 2003). A third putative histone methyltransferase is DDB0189799. It also contains a cysteine-rich pre-SET domain, which is very often (and only) found in histone methyltransferases and specifies these proteins as histone methyltransferases; BLAST searches (Altschul et al., 1997) reveal high homologies to histone H3K36-specific methyltransferases such as SDG8 (E value $2e^{-53}$) from *Arabidopsis* (Zhao et al., 2005) or human NSD1 (E value $2e^{-47}$) (Rayasam et al., 2003).

DDB0191793 displays a rather unusual domain architecture, since it contains a weakly conserved N-terminal SET domain, and an additional truncated C-terminal SET domain. The protein lacks a pre-SET domain, but contains a histidine-rich region N-terminal to the C-terminal SET domain, which displays weak homologies (E-value >0,01) similar to histidine rich regions in some zinc-binding proteins, and may be involved in DNA binding. BLAST searches reveal a homology (E-value $2e^{-10}$) to histone H3-lysine 4 (H3K4)-specific SMYD-like histone methyltransferases, which intriguingly contain MYND-type zinc fingers (Hamamoto et al., 2004).

DDB0187856 also lacks a pre-SET domain, but contains a C-terminal SET-domain and displays weak homologies (E-value 0,001) to known histone methyltransferases such as murine Suv39h2, however, precise indications for substrate specificity could not be deduced from the BLAST search results.

A further putative *Dictyostelium* HMTase is DDB0216874, which lacks a SET domain and has homologies (E-value: $3e^{-13}$) to DOT1-like histone methyltransferases specific for histone H3K79 methylation. It has been named dotA, accordingly.

1.7 Deciphering the histone code: Methyl-lysine-binding proteins

Unlike acetylation, histone methylation does not lead to charge changes in the modified histones, it thus very likely does not alter chromatin structure directly. In order to be converted into a distinct functional chromatin state, the methylation signal on the histone has to be “read” by specific proteins that recognize and bind to the modified lysine residues. To date, at least three protein motifs that are capable to specifically interact with methylated lysine residues have been identified: the chromo domain, the Tudor domain, and WD40-repeat domain (Fig. 1.6).

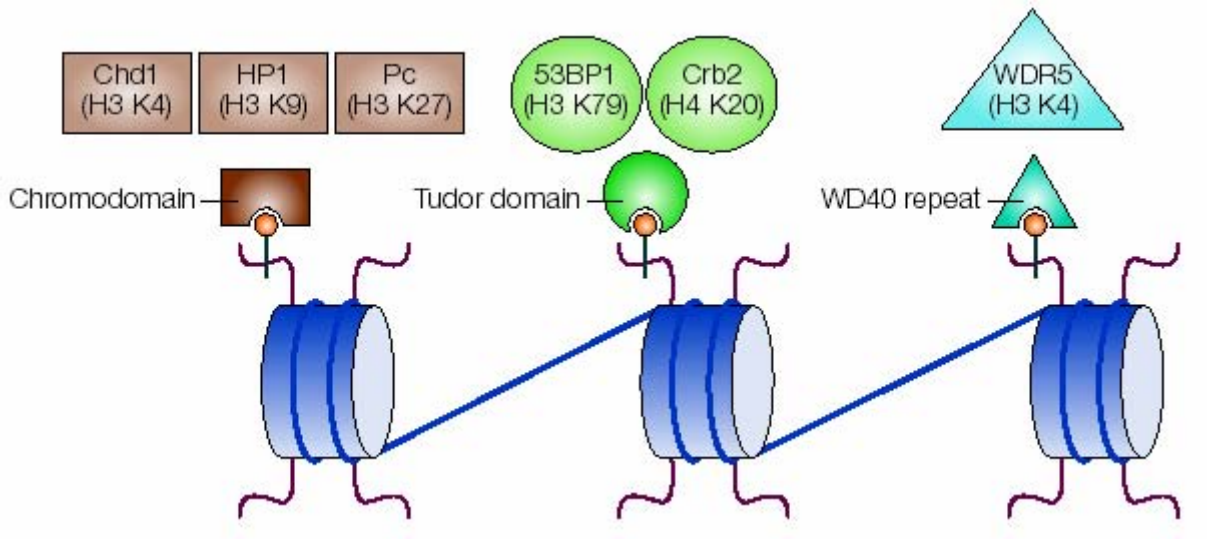

Fig. 1.6: **Protein domains and examples of proteins required for recognition of specific histone methylation marks.** For explanation, see text. (From: Martin and Zhang; 2005)

The chromo domain (for Chromatin organisation modifier; Paro and Hogness, 1991) is a conserved protein module that is found in many proteins involved in chromatin organisation, such as Heterochromatin Protein 1 (HP1), Polycomb group proteins, or Chd1-chromo helicases (see Fig. 1.4). Furthermore, it is present in some homologues of H3K9 specific-histone methyltransferases, such as Clr4 from *S. pombe* or Su(var)3-9 from *Drosophila*, or the RITS component Chp1 from *S. pombe* (see below). In these cases, the interaction of the chromo domain with the H3K9 methyl mark is thought to

stabilize chromatin binding of these proteins and to reinforce heterochromatin establishment (Martin and Zhang, 2004).

Several subclasses of the chromo domain exist, which differ in their binding specificity for methylated histone lysine residues. Others serve as protein-RNA-interaction modules, as exemplified by the chromo domain of MOF1, a protein involved in dosage-compensation in *Drosophila* (Akhtar et al., 1998).

The chromo domain of HP1 binds specifically to methylated lysine 9 of histone H3 (Bannister et al., 2001; Lachner et al., 2001), while the Polycomb (Pc) chromo domain is specific for lysine 27 of histone H3. The difference in substrate specificity is conferred by the discriminative binding of amino acids in the n-4, n-5 and n-7 positions (where n corresponds to the methylated lysine residue) of the histone tail (Fischle et al., 2003).

Human Chd1 binds to histone H3 methylated at K4 via its chromo domain, but methyl lysine binding involves only two aromatic residues, in contrast to the three-residue aromatic cage used by chromodomains of HP1 and Polycomb proteins (Flanagan et al., 2005).

The Tudor domain binds methyl-lysine or methyl-arginine residues present in a wide range of proteins, of which many are involved in RNA transport. The Tudor domain of 53BP1 has been shown to bind to methylated H3K79 (Huyen et al., 2004). Tudor domain proteins are involved in a wide range of RNA-based regulatory processes such as RNAi, mRNA splicing or mRNA transport, however, the exact function of the Tudor domain in these processes is still unclear (Bernstein and Allis, 2005).

The WD40 repeat protein WDR5 has only recently been shown to selectively bind to methylated histone H3K4 (Wysocka et al., 2005). Intruigingly, the WD40 repeat is found in a large number of proteins involved in chromatin remodelling and chromatin assembly, indicating a broader function of this protein motif in the regulation of chromatin structure (Hennig et al., 2005).

1.8 HP1 structure

Heterochromatin Protein 1 (abbreviated HP1) was the first non-histone heterochromatin component to be discovered in *Drosophila* 20 years ago in polytene chromosomes (James and Elgin, 1986). Since then, a large number of genetic and biochemical experimental approaches have elucidated its function in heterochromatin

and made it one of the best characterised chromatin proteins to date (Hiragami and Festenstein, 2005).

The HP1 domain structure is evolutionarily conserved from *S. pombe* to humans. HP1-like proteins are characterised by an N-terminal chromo domain (CD) and a C-terminal chromo shadow domain (CSD), which are separated by less-well conserved linker region, the hinge (Fig. 1.7). While the chromo domain is found in a number of other proteins involved in chromatin regulation (see above), the chromo shadow domain is only found in HP1 proteins and thus serves as a defining feature of the HP1 family.

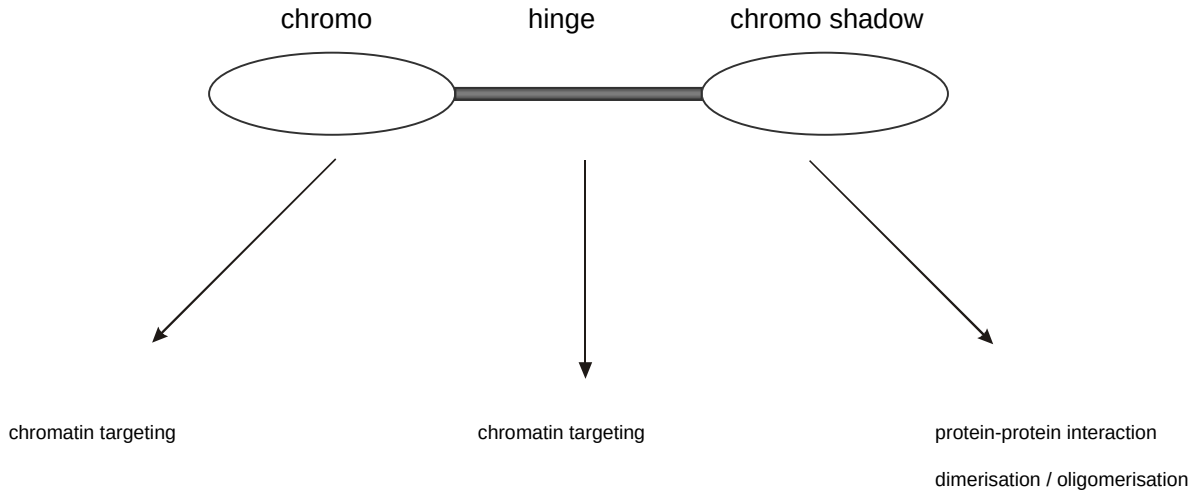

Fig. 1.7: **Schematic representation of the relationships between HP1 domains and their function.** For explanations, see text.

Three dimensional structures of the CD and the CSD have been obtained by NMR spectroscopy and X-ray cristallography (Ball et al., 1997; Brasher et al., 2000; Cowieson et al., 2000). Both the CD and the CSD are globular domains, while the hinge is largely unstructured. The CD and the CSD consist of an anti-parallel, three-stranded beta-sheet, against one or two a-helices are packed, respectively. Although the domain structures of the CD and the CSD are very similar, the two domains have strikingly different functions.

1.9 Functions of the chromo domain (CD)

The CD of HP1 is required for chromatin binding, and consequently, mutations in the CD largely abolish binding to centromeric heterochromatin. A breakthrough finding in

the elucidation of HP1 function was the discovery that the HP1 CD specifically binds to the histone H3 tail when it is methylated at K9 (Bannister et al., 2001; Lachner et al., 2001). This finding provided a direct explanation for the heterochromatic localisation of HP1 proteins, and their co-localisation with H3K9 methylation, which was known to be highly abundant in (pericentromeric) heterochromatin. Accordingly, inhibition of histone methylation, either by mutations of histone methyltransferases such as Suv39h1/2 or Clr4 or by treatment with the HDAC inhibitor trichostatin A (TSA), interferes with HP1 binding to heterochromatin. Interaction with the methylated K9 residue is mediated by a “hydrophobic box” of three conserved aromatic amino acid side chains: Y-21 (which can also be F), W-42 and F-45. Other residues in the HP1 CD, V-23, L-40, W-42, L-58 and C-60 (counting is for mouse HP1β) are required for recognition specificity by binding the A-7 residue of the histone H3 tail (Nielsen et al., 2002).

The affinity of HP1 increases with the methylation level of H3K9 residues (Fischle et al., 2003). At least in mammalian cells, the trimethylated form of the K9 residue of histone H3 is predominantly found in pericentromeric heterochromatin, whereas the mono- and dimethylated forms are found in euchromatic regions. Thus, the stability of HP1 binding to specific chromatin regions may be determined by the methylation status of histone H3.

The interaction of the HP1 CD with methylated H3K9 is rather weak (Bannister et al., 2001; Lachner et al., 2001; Jacobs and Khorasanizadeh, 2002; Fischle et al., 2003), although HP1 displays high affinity to chromatin *in vivo*. This implies that additional interactions of HP1 are required for chromatin binding (Maison and Almouzni, 2004).

1.10 Functions of the chromo shadow domain (CSD)

While the CD stays monomeric, CSDs readily dimerise in solution (Brasher et al., 2000). HP1 proteins therefore can interact with each other and form homo- and heterodimers among or between different HP1 isoforms. Furthermore, bacterially expressed and purified HP1 proteins form complexes ranging in size from 158 to 443 kDa, which is at least more than four times the size of individual HP1 proteins (~ 20-35 kDa), suggesting the formation of oligomeric complexes (Wang et al., 2000; Festenstein et al., 2003).

In addition, dimerisation of HP1 proteins via their CSDs is required for interaction with other protein partners (Brasher et al., 2000). The CSD is an interaction module responsible for binding a wide range of other proteins that, in most cases, contain a loosely conserved PxVxL motif (where P = proline; V = valine; L = leucine and x is any amino acid; Smothers and Henikoff, 2000; Thiru et al., 2004). The pentapeptide motif is present in the CSD itself and allows for the aforementioned homo- and heterodimerisation. The pentapeptide motif binds as a monomer into a hydrophobic pocket that is formed by the dimerized CSDs. Until now, a broad variety of proteins containing the consensus motif has been shown to interact with HP1 proteins via its CSD (Table 1.1). However, there are also other proteins which have been shown to bind HP1 that lack the PxVxL consensus sequence. Moreover, in some instances, proteins interact with the CD or the hinge of HP1.

On the one hand, the CSD as a protein interaction module is required for HP1 targeting to chromatin, for example through interactions with gene-specific co-repressor proteins (see 1.11). On the other hand, it stabilizes HP1 binding to chromatin by mediating incorporation into larger protein assemblies at heterochromatin regions. This is particularly important due to the rapid on/off kinetics of HP1 proteins in these areas, which has been revealed by FRAP (fluorescence recovery after photobleaching) studies for various HP1 homologues (Cheutin et al., 2003; Festenstein et al. 2003; Schmiedeberg et al.,2004; Cheutin et al., 2004).

1.11 Functions of the hinge region

The CD and CSD are separated by a less conserved linker region, the “hinge”, that contributes to heterochromatin targeting by its ability to bind DNA and RNA (Muchardt et al., 2002; Zhao et al., 2000).

The hinge region does not contain a known DNA binding motif, and the nucleic-acid binding activities are sequence-unspecific *in vitro*. Responsible for nucleic-acid interactions are lysine- and arginine-rich basic patches within the hinge (Muchardt et al., 2002). In addition, mutations in the conserved C-59 within the chromo domain of HP1 abolish DNA- and RNA-binding (Sugimoto et al., 1996; Schmiedeberg et al., 2004). It has been suggested that HP1 can directly bind to DNA in certain unusual chromatin structures specific for heterochromatin. This actually might be a major force of heterochromatic targeting, since under physiological conditions, the methylated

Table 1.1: Overview of known HP1 interaction partners, the HP1 variant involved in the interaction and the HP1 interacting domains (From: Hiragami and Festenstein, 2005).

Protein	Species	HP1 variant	Interacting domain
Chromatin components/ modifiers and transcription regulators			
Histone H1	*Drosophila*	HP1	?
Histone H3	*Drosophila*; mouse	HP1a, HP1α, HP1β, HP1γ	CD (for mouse HP1s)
Methyl K9 histone H3	*S. pombe*; *Drosophila*;	Swi6, HP1a, HP1α, HP1β, HP1γ.	CD
mouse; human	$HP1^{Hs}α$, $HP1^{Hs}β$, $HP1^{Hs}γ$	[23, 24]	
Histone H4	*Drosophila*; mouse	HP1a, HP1β	CSD (for HP1a)
Members of	*Drosophila*; mouse;	HP1a, HP1α, HP1β, $HP1^{Hs}β$	CSD (for HP1a, HP1α)
SUVAR3-9 HMTase	human		
Suvar3-7	*Drosophila*	HP1a	CSD
Suvar4-20 HMTase	mouse	HP1α, HP1β, HP1γ	?
Su(z)12	human	$HP1^{Hs}α$, $HP1^{Hs}γ$	CSD
Dnmt3a	mouse	HP1α	?
Dnmt3b	mouse, human	HP1α, HP1β, $HP1^{Hs}α$, $HP1^{Hs}β$	?
Kap-1/TIF1β	mouse, human	HP1α, HP1β, HP1γ; $HP1^{Hs}α$, $HP1^{Hs}γ$	CSD
TIF1α	mouse	HP1α	CSD
SNF2β/BRG1	mouse	HP1α	CSD
ATRX	mouse	HP1α, HP1β	CSD
$TAF_{II}130$	human	$HP1^{Hs}α$, $HP1^{Hs}γ$	CSD
RNA	mouse	HP1α, HP1γ	Hinge (for HP1α)
Chromatin	*Xenopus*	xHP1α	Hinge
Proteins involved in DNA replication/chromosomal segregation/cell cycle			
CAF-1 (p150 subunit)	mouse, human	HP1α, HP1β, $HP1^{Hs}α$	CSD
Ku70	human	$HP1^{Hs}α$	CSD
ORC1-6	*Drosophila*	HP1a	CD, CSD
HOAP	*Drosophila*	HP1a	?
Psc3	*S. pombe*	Swi6	CD
INCENP	human	$HP1^{Hs}α$, $HP1^{Hs}γ$	Hinge (for $HP1^{Hs}α$)
Hsk1/CDC7	*S. pombe*	Swi6	?
Proteins involved in nuclear organisation			
Nuclear envelope	mouse	HP1α, HP1β, HP1γ	CD
Lamin B receptor	human	$HP1^{Hs}α$, $HP1^{Hs}β$, $HP1^{Hs}γ$	CSD
Lamin B	mouse	HP1β	CD
LAP2β	mouse	HP1β	CD

histone tails (which are bound by HP1 *in vitro*) themselves are engaged in interactions with the DNA and thus might be inaccessible (Meehan et al., 2003).

The hinge region of HP1 proteins contains multiple putative phosphorylation sites. Phosphorylation of HP1 proteins has been shown to alter interactions with its binding partners, such as subunits of the origin recognition complex (ORC) complex or heterochromatin protein 1 / ORC associated protein (HOAP) in *Drosophila* (Badugu et al., 2005). Furthermore, mutations in several phosphorylation sites impair the gene silencing activity of HP1 (Zhao et al., 1999; Zhao et al., 2001).

It is conceivable that phosphorylation within the hinge may neutralize the positive charges of the basic amino acid residues and thus alter the nucleic acid binding activity of HP1 proteins.

1.12 An Overview of HP1 functions *in vivo*

Despite its name and most prominent heterochromatic localisation, HP1 proteins are versatile protein factors involved in a broad variety of chromatin regulating actvities, which are seemingly contradictory.

In several organisms, HP1 proteins have been shown to be crucial for heterochromatin function of constitutive heterochromatin, e.g. at centromeres or telomeres (Hiragami and Festenstein, 2005). Formation of functional heterochromatin at these regions is crucial for cellular survival for various reasons. Pericentromeric heterochromatin in *S.pombe* is required for recruitment of cohesin complexes that regulate sister chromatid cohesion and thus mitotic chromosome segregation. Consequently, a loss of the H3K9-specific HMTase Clr4, which disrupts heterochromatin binding of the HP1-homologue Swi6, or the loss of the *S. pombe* HP1 homologue Swi6 itself leads to elevated rates of lagging anaphase chromatids and chromosome loss (Ekwall et al, 1995; Ekwall et al., 1996; Bernard et al., 2001; Nonaka et al., 2002). Similarly, the loss of mammalian histone methyltransferases Suv39h1 and Suv39h2 leads to altered pericentromeric histone methylation patterns and chromosomal instabilities (Peters et al., 2001).

In addition to the centromeric function, HP1 proteins are also involved in telomere capping by binding directly to telomeric DNA, which serves to protect the ends of linear chromosomes that may otherwise be recognized as damaged DNA by the repair machinery (Perrini et al, 2004). Loss of HP1 at telomeres causes end-to-end chromosome fusions in *Drosophila* (Fanti et al., 1998). Association of HP1 proteins with telomeres was also observed in mammalian cells using chromatin immunoprecipitation (Garcia-Cao et al., 2004). Moreover, derepression of subtelomeric reporter genes correlates with delocalisation of HP1 proteins (Koering et al., 2002).

At the silent-mating-type locus in fission yeast, heterochromatin formation by the HP1 homologue Swi6 is required for proper recombination between the mating-type loci and mating-type switching (Nakayama et al., 2000).

Heterochromatin is often found at the nuclear periphery. Furthermore, gene silencing, for example of immunoglobulin genes, frequently involves sequestration of the silenced locus to the nuclear periphery (Skok et al., 2001; Kosak et al., 2002). Since HP1 binds to components of the nuclear envelope such as the lamin B receptor, lamin B or lamina associated polypeptide (LAP) 2β (Kourmouli et al., 2000; Ye et al., 1997), HP1 may tether heterochromatin and silenced genes to the nuclear periphery, thus creating transcriptionally repressive microenvironments within the nucleus (Ayyanathan et al., 2003).

HP1 proteins also interact with the p150 subunit of chromatin assembly factor CAF-1. CAF-1 contains the consensus PxVxL motif that is recognized by the HP1 CSD. At sites of ongoing replication, HP1 is localised to pericentromeric heterochromatin independent of histone H3K9 methylation via this interaction (Quivy et al., 2004). One possible function could be the stable maintenance of heterochromatic structures after DNA replication. Regulated deposition of HP1 onto newly replicated DNA may reinforce the silenced state, while methylated histones are depleted from the newly synthesized DNA. A possible involvement of HP1 in the epigenetic inheritance of silenced states during DNA replication also stems from the observation that HP1 interacts with the origin recognition complex (ORC), a highly conserved multiprotein complex composed of six proteins (Orc1-6) that is required for initiation of DNA replication in all eukaryotes tested (Bell, 2002).

HP1 proteins also play a role in repression of individual euchromatic genes. Strikingly, the *Arabidopsis* HP1 homologue LHP1 exclusively localizes to euchromatic regions and is involved in regulation of individual genes (Gaudin et al., 2001; Kotake et al., 2003; Libault et al., 2005; Nakahigashi et al., 2005).

The molecular machinery required for epigenetic silencing of euchromatic single-copy genes is remarkably similar to that active in heterochromatin formation (see below). The generation of the silenced state depends on interactions of HP1 with sequence-specific corepressor proteins such as Rb or members of the TIF family, which additionally recruit histone-modifying enzymes such as Suv39, SETDB1 and histone deacetylases, for example to cyclin E genes (Ryan et al., 1999; Nielsen et al., 1999; Lechner et al., 2000; Nielsen et al., 2001a). Several other endogenous genes have been shown to be silenced by HP1 on a similar basis, for example myc-responsive genes such as E2F-1, c-myc and cdc25A. Targeting of HP1γ to the promoters of these

genes by interaction with the E2F-6 complex containing G9a and EuHMTase, two H3K9-specific HMTases, has also been shown (Ogawa et al., 2002).

One important difference compared to constitutive heterochromatin is that the small patch of heterochromatin generated at the promoters of individual euchromatic genes does not spread into adjacent regions, allowing for gene-specific transcriptional repression. For example, Rb-mediated repression of the cyclin E gene is realised by histone deacetylation of a single nucleosome at the transriptional start position (Morrison et al, 2002).

The exact function of HP1 in these silencing events is unclear and difficult to elucidate, since it cannot be analysed by simply knocking-out HP1. However, the presence of HP1 in these repressive complexes is crucial, since for example a mutation in the HP1-interacting box of TIFβ, which specifically abolishes HP1 binding, causes defects in cellular differentiation of primitive endoderm-like (PrE) cells into parietal endoderm-like (PE) cells by de-regulation of genes essential for differentiation (Cammas et al., 2004).

In contrast to its well-accepted role as a transcriptional repressor, HP1 in some cases seems to serve as a transcriptional activator. For example, transcription of several heterochromatic genes is reduced in an HP1-null background in *Drosophila* (Lu et al., 2000). Moreover, several pericentromeric genes expressed at medium or high levels are bound by HP1 or Su(var)3-9 (Greil et al., 2003). Association of HP1 with the coding regions of activated genes in heat-shock induced puffs on *Drosophila* polytene chromosomes depends on the presence of RNA transcripts, which might indicate that HP1 either stabilizes the mRNA or somehow controls transcription rates (Piacentini et al., 2003). Furthermore, the mammalian HP1γ isoform, which exclusively localizes to euchromatin, is present in the transcribed region of active genes, where it is associated with transcription elongation (Vakoc et al, 2005; Lomberk et al., 2006). In this context, it should be stressed that HP1 has been shown to bind to RNA *in vitro* by its hinge region (see above). HP1-associating transcriptional regulators such as TIF1s and BRG1/SNF2b can act as both transcriptional co-repressors and co-activators (Nielsen et al., 1999; Rooney and Calame, 2001). When these proteins bind to HP1 in a co-activator form, it is possible for HP1 to act also as a co-activator.

Due to its multitude of different functions, HP1 proteins are essential for viability in several organisms. Loss-of function mutations in *Drosophila* and mammalian cells, but

not in *S. pombe*, are lethal (Eissenberg et al., 1992; Ekwall et al., 1995; Filesi et al., 2002).

In *Arabidopsis*, *C. elegans* or *Tetrahymena*, the respective HP1 proteins, although structurally conserved, are not involved in formation of constitutive heterochromatin, but serve rather specialized functions in silencing of individual single-copy genes. Knock-out of HP1 proteins therefore results in phenotypically distinct and less drastic effects (Gaudin et al., 2001; Huang et al., 1999; Couteau et al., 2002).

1.13 The role of HP1 in heterochromatin formation and transcriptional gene silencing

Originally, HP1 was identified as the protein product of the su(var)-2-5 gene in *Drosophila* (Eissenberg et al., 1990, Eissenberg et al., 1992), which is a strong suppressor of position-effect variegation (PEV).

Position-effect variegation is a gene-silencing phenomenon that occurs when a normally euchromatic gene is juxtaposed adjacent to heterochromatin due to chromosomal rearrangements. Since heterochromatin tends to spread into adjacent regions of the chromatin fiber, this gene is heterochromatinized, e.g. transcriptionally silenced, in a stochastic manner. In the absence of HP1, transgenes stay active, which can be explained by the inability of heterochromatin to spread into the region of the transgene. In addition to that, loss of HP1 influences the targeting of H3K9 methylation and the subnuclear distribution of the latter. In the absence of HP1, histone H3K9 dimethylation is no longer restricted to heterochromatin, but found dispersed across the chromosome arms, indicating that heterochromatin association of Su(var)3–9 and HP1 are interdependent.

Largely similar observations are made when transgenes are integrated into silent mating-type or pericentromeric heterochromatin in *S. pombe*. In the absence of the HP1 homologue Swi6, the frequency of transgene silencing is reduced, while additional doses of Swi6 (upon overexpression) facilitate transgene silencing (Allshire et al., 1994; Nakayama et al., 2000). Similarly, in mammalian PEV, alleviated HP1 dosage also increases gene silencing frequencies (Festenstein et al., 1998). In contrast to that, only slight effects were observed by overexpression of the *S. pombe* H3K9-HMTase Clr4 (Nakayama et al., 2000). Nevertheless, Clr4 acts upstream of Swi6 in *S. pombe*, since *clr4*-deletion strains loose Swi6 localisation to heterochromatin (Ekwall et al., 1996).

The precise function of HP1 proteins in heterochromatin formation is still not clear; however, given the biochemical *in vitro* activities of the individual domains of the HP1 proteins, it can be imagined that HP1 proteins function as adaptor proteins that cross-connect different protein complexes and thus lead to formation of higher-order chromatin structure (see Fig. 1.2).

A possible series of events is schematically depicted in Fig. 1.8. Methylation of the H3K9 residue by an HMTase leads to HP1 targeting to chromatin (possibly in combination with direct recruitment by protein-protein interactions).

HP1 dimerisation on chromatin leads to further recruitment of HDACs and HMTases to chromatin and deacetylation/methylation of adjacent nucleosomes, which are in turn bound by HP1 and so on. As a consequence, H3K9-methylated and HP1-bound chromatin spreads, and is furthermore compacted into higher-order nucleosomal arrays, possibly by HP1 oligomers that cross-link adjacent nucleosomes. Chromatin condensation into the 30nm-fiber by Polycomb group proteins has been shown (Francis et al., 2004); but experimental (structural) evidence for a similar function of HP1 proteins is still lacking. However, the HP1-containing complexes have been shown to recruit DNA methyltransferases Dnmt1 and and Dnmt3a/b and histone

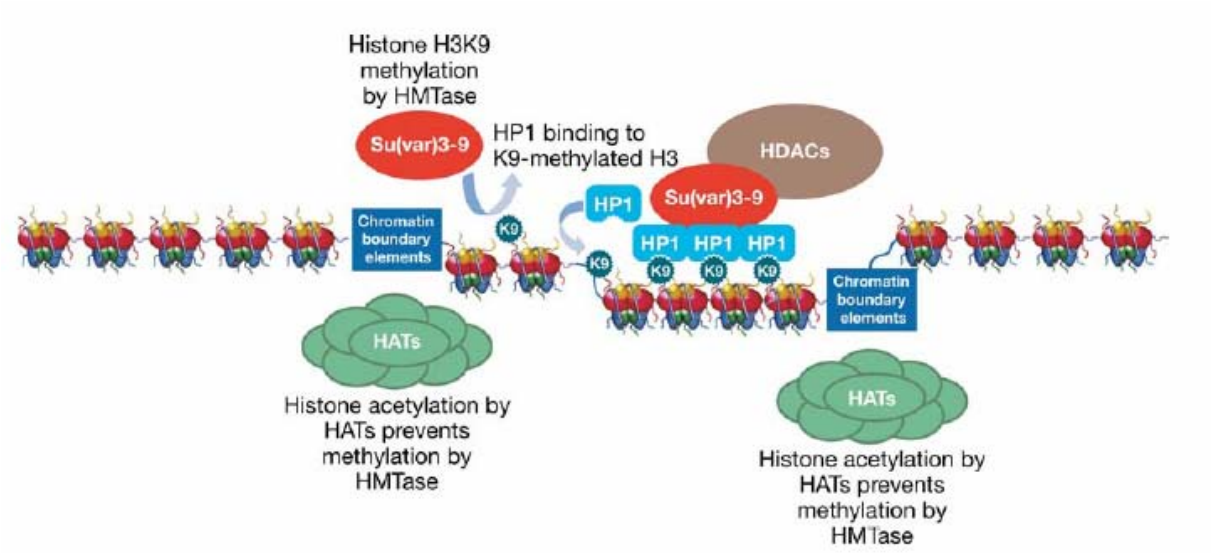

Fig. 1.8: **Proposed model of heterochromatin formation by H3K9methylation and HP1 binding**. Histone deacetylation by an HDAC-containing complex allows histone H3K9 methylation at targeted regions. HP1 recognizes the H3K9 methyl mark, dimerizes and recruits further HMTase- and HDAC-containing complexes, which then act on the neighbouring nucleosomes. Heterochromatin spreading is stopped by boundary elements, which are considered to be genomic targets of histone actelytransferase (HAT) activities that prevents further histone methylation. (From: Shilatifard, 2006)

deacetylases by physical interaction (Bachman et al., 2001; Fuks et al., 2003), resulting in the establishment of further repressive epigenetic marks.

Taken together, HP1-containing complexes are thought to create repressive chromatin environments, in which several different activities such as DNA methylation, histone deacetylation and histone methylation are highly abundant, and which lead to a self-reinforcing and self-propagating silenced chromatin state.

A detailed analysis of heterochromatin spreading has been performed on the silent-mating-type locus in *S. pombe*. High-resolution mapping by chromatin immunoprecipitation showed that in the absence of Swi6, histone methylation is concentrated around the entry site of the *cenH* repeat, which is targeted by the RNAi machinery (see below), but does not spread over the entire mating-type locus, indicating that Swi6 is indeed required for heterochromatin spreading (Hall et al., 2002).

Although HP1 functions in formation of higher-order chromatin assemblies, heterochromatin is not a static structure of cross-linked nucleosomes. HP1 proteins themselves are highly mobile within heterochromatin (Cheutin et al., 2003; Festenstein et al., 2003). Therefore, HP1 binding to chromatin is essentially a stochastic event, that is likely to be facilitated by the presence of HP1 binding sites such as methylated H3K9, and to be stabilized by a multitude of binding events to other heterochromatic proteins present at the same loci.

Heterochromatin integrity and heterochromatin-induced gene silencing are maintained in a dynamic competition between protein factors required for opening chromatin structure and transcriptional activation, and for protein factors involved in chromatin condensation and gene repression. For example, modulated expression of an transcriptional activator protein can overcome transcriptional silencing in heterochromatin by shifting the dynamic equilibirum between activating and repressive protein factors towards chromatin opening (Ahmad and Henikoff, 2001). This competition can counterbalance the tendency of heterochromatin to spread into adjacent chromatin regions.

1.14 HP1 and DNA methylation

In addition to H3K9 methylation and HP1 deposition, heterochromatin is characterised by extensive DNA methylation. This additional epigenetic modification involved is known to be involved in various gene silencing processes such as genomic imprinting, X-chromosome inactivation or transcriptional silencing of transposons and retrotransposons (Robertson, 2005). Genetic studies in different organisms have shown that H3K9methylation acts upstream from DNA methylation. Consequently, loss of the H3K9-HMTases DIM5 in *N. crassa*, the KRYPTONITE methyltransferase in *Arabidopsis* or murine Suv39h1/2 causes loss of DNA methylation (Tamaru and Selker, 2001; Jackson et al., 2002; Lehnertz et al., 2003). Furthermore, HP1 is essential for DNA methylation in *N. crassa* (Freitag et al., 2003). In numerous cases of transcriptional inactivation, it has been shown that establishment of repressive histone modification patterns chronologically precedes the establishment of DNA methylation, which is therefore regarded as a “final” lock to switch off transcription (Heard et al., 2001; Bachman et al., 2003; Feldman et al., 2006).

1.15 HP1 isoforms

Many organisms, for example *Drosophila*, *C. elegans* or vertebrates, have two or more different HP1 isoforms. Though functionally overlapping, these are largely non-redundant, as shown by genetic analysis or biochemical characterization (Couteau et al., 2002; Meehan et al., 2003; Smothers and Henikoff, 2001). Single HP1 isoforms in organisms such as *Drosophila* or mammals are rather essential for viability, or have at least profoundly distinct functions that cannot be fully compensated by the other isoforms. Furthermore, different isoforms often display a distinct subnuclear distribution. In mammals, HP1α and HP1β are predominantly found in heterochromatin, whereas HP1γ localises to euchromatin in interphase (Minc et al., 1999). Similarly, *Drosophila* HP1a is predominantly found in the (heterochromatic) chromocenter, whereas the two other isoforms HP1b and HP1c localize to both heterochromatin and euchromatin, or exclusively to euchromatin (Smothers and Henikoff, 2001). Single HP1 isoforms interact isoform-specifically with other proteins (see Table 1.1). The two mammalian isoforms HP1α and HP1γ are phosphorylated throughout the cell cycle (with hyperphosphorylation during mitosis), while HP1β is

largely unphosphorylated (Minc et al., 1999). Although largely identical, subtle differences in primary amino acid sequence lead to distinct phosphorylation states of the different isoforms, which may help to explain some of the isoform-specific effects observed *in vivo*.

1.16 The RNAi-heterochromatin-connection

Although heterochromatin formation and spreading are rather well understood, little is known about the mechanisms that target histone modifications, HP1 and/or DNA methylation to genomic regions such as centromeric repeats or telomeres. One possible pathway for targeted changes in chromatin structure has been revealed by studying dosage compensation in *Drosophila* and mammals. Dosage compensation describes the phenomenon that RNA- and protein levels from genes expressed from the X chromosomes are equalized, either by upregulation of transcription from the single male X chromosome, or inactivation of one of the two female X chromosomes (Lucchesi et al., 2005).

The first of these mechanisms is realised in *Drosophila*. The dosage compensation complex (DCC) contains two non-coding RNAs, rOX1 and rOX2, that target the DCC to ~ 35 entry sites on the male X chromosome (among them are the rOX genes themselves), from which histone acetylation of H4K16 spreads along the chromosome. The H4K16 hyperacetylation, which is in part responsible for the upregulation of transcription, is catalyzed by the chromo domain-containing MOF1 histone acetyltransferase, which is a key component of the DCC (Gilfillan et al., 2004).

The second mechanism of dosage compensation is realised in mammals, where the second female X chromosome is inactivated by heterochromatinisation. During random X chromosome inactivation, which takes place in later development (and is different from an imprinted inactivation mechanism in extraembryonic tissues), transcription of the non-coding 17kb-long *Xist* RNA initiates a series of events that ultimately lead to an entirely silenced chromosome that is covered with histone H3 methylated at K9 and K27, histone H4 methylated at K20, and DNA methylation (Heard, 2004).

Involvement of RNAs in heterochromatin formation is further demonstrated by the functional interplay between the RNA interference (abbreviated RNAi) machinery and

heterochromatin silencing pathways (Bernstein and Allis, 2005; Lippman and Martienssen, 2004).

The term RNAi was introduced in 1998 to describe a double-stranded RNA-triggered mechanism that degrades homologous mRNAs (Fire et al., 1998). Since then, RNAi has been identified as an evolutionarily conserved pathway that is used by many organisms to shut down unwanted gene expression of retrotransposons, but also to regulate endogenous genes (for review see Meister and Tuschl, 2004 and references therein).

Although the set of proteins and effector complexes of the RNAi pathway differ from organism to organism, the canonical effector molecules of the RNAi machinery are small interfering RNAs (siRNAs). These siRNAs are generated from dsRNA precursors (derived from different origins and varying in length) by the action of Dicer, a member of the RNaseIII family of ribonucleases, which processes the dsRNA into short RNA duplexes fo 21 to 28 nucleotides in length. The siRNAs are loaded into different effector complexes, such as RISC (RNA-induced silencing complex), or serve as primers for an RNA-dependent RNA polymerase (RdRP)-dependent mechanism, which synthesizes new dsRNA from the targeted complementary RNA-template. The RISC complex is guided to homologous mRNAs, which are cleaved through the endonucleolytic activity of Argonaute-2, a RISC component.

RNAi-like mechanisms have previously been shown to control transposon activity or transcription of tandem transgene arrays in various organisms (Matzke et al. 2000; Sijen and Plasterk 2003; Cogoni and Macino 1999; Mourrain et al. 2000; Svoboda et al. 2004a). On the other hand, heterochromatin very often is composed of arrays of repetetive DNA elements, e.g. retrotransposons, which suggested that the RNAi machinery might be involved in the regulation of these genomic loci.

Early evidence for an RNAi-like mechanism targeting transcriptional silencing came from studies of RNA-mediated DNA methylation (RDM) in viroid-infected plants (Wassenegger et al., 1994). The involvement of RNAi in transcriptional silencing and heterochromatin formation has been further elaborated by a series of elegant studies in *S. pombe*. One of the breakthrough findings of these studies was that the traditional view of heterochromatin as to be inaccessible for transcription factors and thus, transcriptionally silent, was essentially wrong. Paradoxically, maintenance of the silent state seems to require low-level transcription of the “silent” locus. In *S. pombe*, subunits of the RNA Pol II complex are required for centromeric transcription and

RNAi-mediated heterochromatin formation (Kato et al, 2005; Djupedal et al., 2005). Long non-coding RNAs homologous to the centromeric repeats were found to accumulate in strains mutated for the RNAi components *dcr1*, *ago1* and *rdp1*, the fission yeast homologues of Dicer, an Argonaute protein and RNA-dependent RNA polymerase, but not in wild-type cells. Maintenance of the heterochromatic state and "transcriptional silencing" at centromeres thus is reinforced by an auto-regulatory feedback loop that post-transcriptionally degrades these centromeric transcripts via the RNAi pathway (Volpe et al., 2002). Similarly, a specialized DNA-directed RNA polymerase (DDRP), known as RNA Pol IV or *RPD*, is required for maintenance of

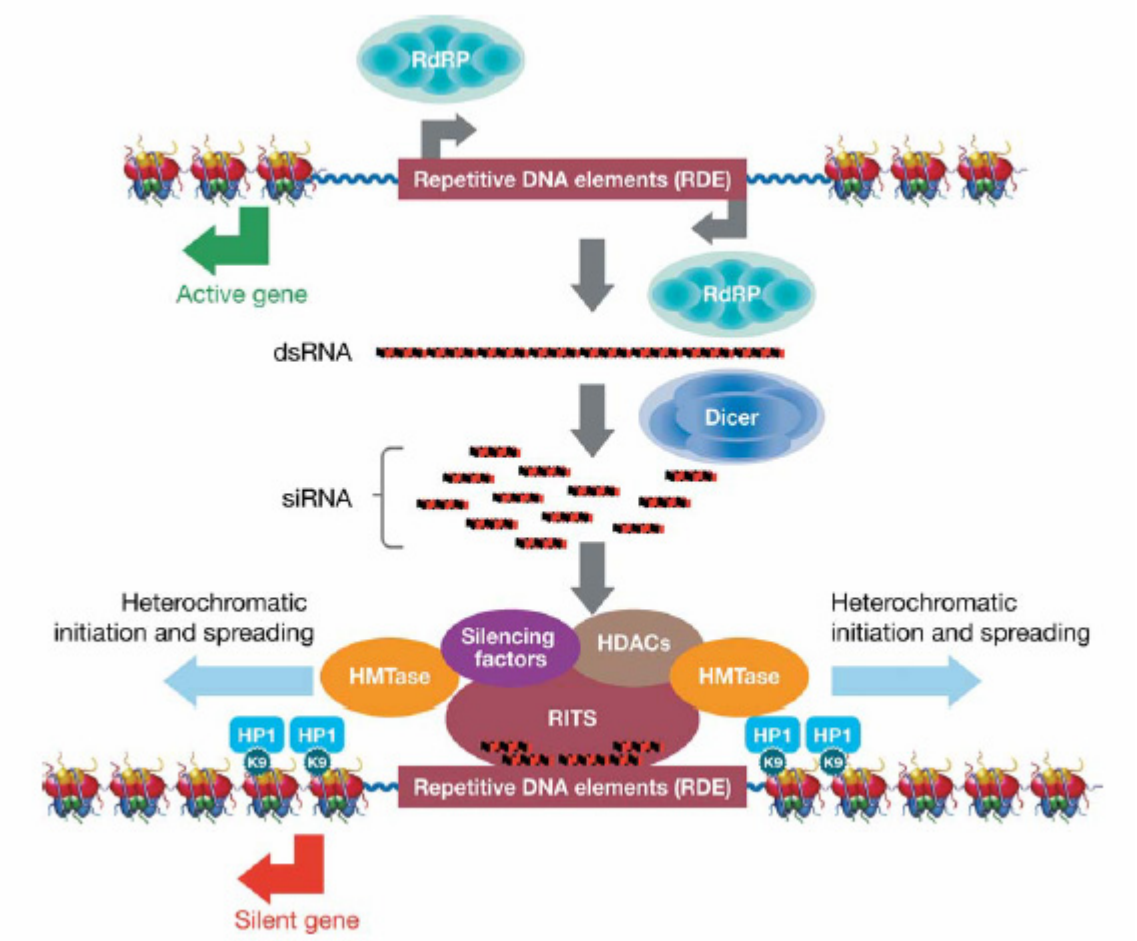

Fig. 1.9: **Proposed model for RNAi-mediated heterochromatin formation**
Transcripts derived from repetetive DNA elements are converted into dsRNA by the action of an RdRP. The dsRNA is cleaved into siRNAs by the Dicer, which are incorporated into the RITS complex and target HMTases, HDACs and other silencing factors, through interaction with RITS, to homologous sequences. At these loci, the process of heterochromatin formation and spreading described in Fig. 1.6 then is initiated. (From: Shilatifard, 2006)

transcriptional silencing of various loci in *Arabidopsis* (Herr et al., 2005; Onodera et al., 2005). Transcripts derived from repetetive DNA elements are converted into dsRNA by the action of an RNA-dependent RNA polymerase (RdRP). The dsRNA is cleaved into siRNAs by Dicer (Fig. 1.9). In fission yeast, the resulting siRNAs recruit protein factors that mediate heterochromatin formation at these loci (Sugiyama et al., 2005; Verdel et al., 2004). siRNAs are either incorporated into the RITS complex (RNA induced transcriptional silencing), the nuclear counterpart of RISC. This complex is composed of the chromodomain protein Chp1, Tas3 (a protein of unknown function) and Ago1, the sole argonaute protein in *S. pombe*. RITS binds to all known heterochromatic loci in *S. pombe*, e.g. centromeres, telomeres and the silent mating type locus (Noma et al., 2004), indicating a general function in heterochromatin formation. Guiding of siRNA-loaded RITS to chromatin is thought to involve DNA-RNA pairing of homologous sequences, or - in analogy to cytoplasmic RISC - base pairing with nascent transcripts derived from centromeric regions. A second protein complex, named RDRC (RNA-dependent RNA polymerase complex), contains the RNA-dependent RNA polymerase Rdp1, a novel RNA helicase and a member of the poly A polymerase family and interacts with RITS through Ago1 in an siRNA dependent manner. Mutants in any of the RDRC components fail to localize RITS to heterochromatin (Motamedi et al. 2004).

Since (peri-)centromeric heterochromatin is crucial for centromere and kinetochore function during mitosis, the RNAi machinery contributes to the maintenance of genomic integrity and, consequently to mitotic chromosome segregation. In addition, RNAi mutants in *S. pombe* are defective in telomeric clustering and meiotic chromosome segregation; however, in this case the siRNAs derived from the RNAi pathway seem to act as a molecular scaffold that holds telomeric chromatin from different chromosomes together (Provost et al., 2003; Hall et al., 2003; Volpe et al., 2003).

Although most of the experimental data described above are derived from studies in fission yeast, the basic principles and mechanisms seem to be conserved in other organisms. In *Drosophila*, loss of the *piwi*, *aubergine* (two argonaute family members) and *homeless* genes suppresses PEV, correlating with a reduction in H3K9 methylation and delocalization of HP1, most dramatically observed in the *hls* mutants (Pal-Bhadra et al., 2004). In *Arabidopsis*, the Argonaute-family member Ago4 controls locus-specific siRNA accumulation and histone as well as DNA methylation at these

loci (Zilberman et al., 2003). In mammals, Dicer-null ES cells have centromeric silencing defects (Kanellopoulou et al., 2005). RNA components also seem to be required for the structural integrity of pericentromeric heterochromatin in mammalian cells (Maison et al., 2002), although it is not clear if these RNAs are derived from the RNAi pathway.

Although RNAi-based pathways provide a integrative explanation of how heterochromatin is targeted, there are several other pathways required for heterochromatin formation that can act in parallel.

Fission yeast CenpB homologues directly bind to centromeric α-satellite DNA and induce heterochromatin formation through recruitment of HP1/Swi6 (Nakagawa et al., 2002). Furthermore, it has been demonstrated that Swi6 can be directly recruited to heterochromatin during DNA replication through interaction with DNA polymerase α (Nakayama et al., 2001a). At the silent mating-type locus, Atf/Pcr1 transcription factors provide a second entry site for HP1/Swi6 that is targeted independently from the cenH repeat, which serves as an entry site for RNAi-mediated heterochromatin formation (Jia et al., 2004). Other possibilities of RNAi-independent heterochromatin targeting involve the interaction of HP1 with the origin recognition complex (see below).

1.17 The Role of the origin recognition complex in HP1 targeting and heterochromatin formation

The origin recognition complex (ORC) is a highly conserved multiprotein complex composed of six proteins (Orc1-6) and is required for initiation of DNA replication in all eukaryotes tested. Upon binding to DNA, it facilitates the assembly of the so-called pre-replicative complex (pre-RC) by recruitment of other DNA replication factors such as Cdc6, Cdt1 and the MCM (minichromosome maintenance) complex. Moreover, formation of pre-RCs ensures that DNA is replicated only once per cell cycle, thus ensuring genomic integrity (Bell, 2002). In addition to its role in formation of pre-replication complexes (pre-RCs) at origins of replication, ORC is required for transcriptional gene silencing at the silent mating type loci in *S. cerevisiae* (Shore, 2001) and for position-effect variegation (PEV) in *Drosophila* (Pak et al., 1997). In higher eukaryotes such as *Drosophila*, *Xenopus* and mammals, ORC interacts with HP1 and is associated with heterochromatin. Furthermore, disruption of ORC interferes with HP1 localisation to heterochromatin (Pak et al., 1997; Shareef et al.,

2001). Especially, mutants of the Orc2 subunit display impaired HP1 localisation into heterochromatin in *Drosophila* and human cells, indicating that Orc2 functions in HP1 targeting (Huang et al., 1998b; Prasanth et al., 2004).

1.18 Crawling into focus : Epigenetics in *Dictyostelium discoideum*

Chromatin remodelling has become an important area of research and is closely linked to epigenetic gene regulation. In recent years, non-mendelian inheritance and information not encoded in the DNA has attracted substantial attention and provided new insight into developmental processes that, when impaired, may cause diseases in humans that were difficult to trace by standard genetics (Bickmore and van der Maarel, 2003). Efforts are now focusing on understanding the mechanisms by which large sections of the genome may be reversibly inactivated by modification of DNA and/or chromatin. “Model systems” range from yeasts to highly complex mammals, but there is a gap in the complexity scale that could be conveniently filled by *Dictyostelium.* For example, several components of the protein machinery required for epigenetic gene silencing in higher eukaryotes were also identified in *Dictyostelium*, but not in the yeast *S. cerevisiae* and some not in *S. pombe,* that has more simple chromatin systems.

Histone deacetylases (HDACs) and histone acetyl transferases (HATs), ATP-dependent chromatin-remodeling enzymes, chromodomain proteins (including three HP1 isoforms), bromodomain proteins, histone methyltransferases, including a su(var)3-9 homolog (see 1.6), a DNA methyltransferase, and a putative histone demethylase have been identified in the *Dictyostelium* genome using standard BLAST approaches. In addition, a set of known proteins required for RNA interference has been identified and the function of individual proteins in RNAi was established (Martens et al., 2002; Kuhlmann et al., 2005; Popova et al., 2006).

Several of these proteins occur in large families, which may cause experimental problems due to functional redundancy of the different family members. For example, there are nine members in the family of putative histone deacetylases but it should be noted that the substrates of these proteins may not exclusively be histones. Other domains that are typical of chromatin proteins have been identified and include, for example, PHD zinc fingers, jumonji domains and Tudor domains.

Homologs of all the core nucleosomal histones can be identified and variant histones (eg. H3.3 and H2AZ) and a linker histone are also present. It seems that the three major histone H3 genes are all H3.3-like (Ahmad and Henikoff, 2002), based on the amino acid substitutions in their histone fold and N-terminal domains.

The majority of amino acid residues that are known to be post-translationally modified in histones (lysine, arginine and serine/threonine) are present in the *Dictyostelium* histone homologs. The five conserved lysine residues of histone H3 known to be subjected to methylation are fully conserved in the major *Dictyostelium* histone H3 isoforms (Fig. 1.10). Methylation of at least some of at these residues can be detected in *Dictyostelium* cells using commercially available antisera. Strong nuclear-wide staining is observed with antibodies (Abcam, Upstate and others) against (tri-) methylated H3K4. Staining is lost in a *setA* knock-out mutant confirming the identity of the gene product as a H3K4-methyltransferase (Chubb et al., 2006). Antisera against di-methylated H3K9, reveal a distinct nuclear distribution and different antibodies against acetylated lysines give rise to speckled nuclear staining patterns (M. Kaller, Diploma thesis, 2002).

Dictyostelium discoideum is a biochemically and genetically highly accessible organism, which has been established as a “model system” to study various cell biological aspects such as signal transduction and cellular differentiation. Upon starvation, unicellular amoebae enter a developmental program that leads to formation of a multicellular aggregate, which ultimately differentiates into two distinct cell types: spore and stalk cells. A comparable cellular differentiation program is lacking from the yeasts, thus making *Dictyostelium* an ideal system to study epigenetically regulated developmental processes. For example, SH2-domain mediated signalling is present in *Dictyostelium* (Kawata et al., 1997), but absent from the yeasts. Furthermore, an orthologue of the mammalian Rb protein has recently been identified and described to be required for lineage commitment during *Dictyostelium* development. The presence of an Rb-like protein in *Dictyostelium* is particularly intruiging, since Rb proteins have been shown to mediate epigenetic modifications at the targeted gene promoters in mammals (Macwilliams et al., 2006).

Little is known about the signal transduction pathways that lead to epigenetic silencing of individual euchromatic genes, but understanding of the mechanisms that lead to

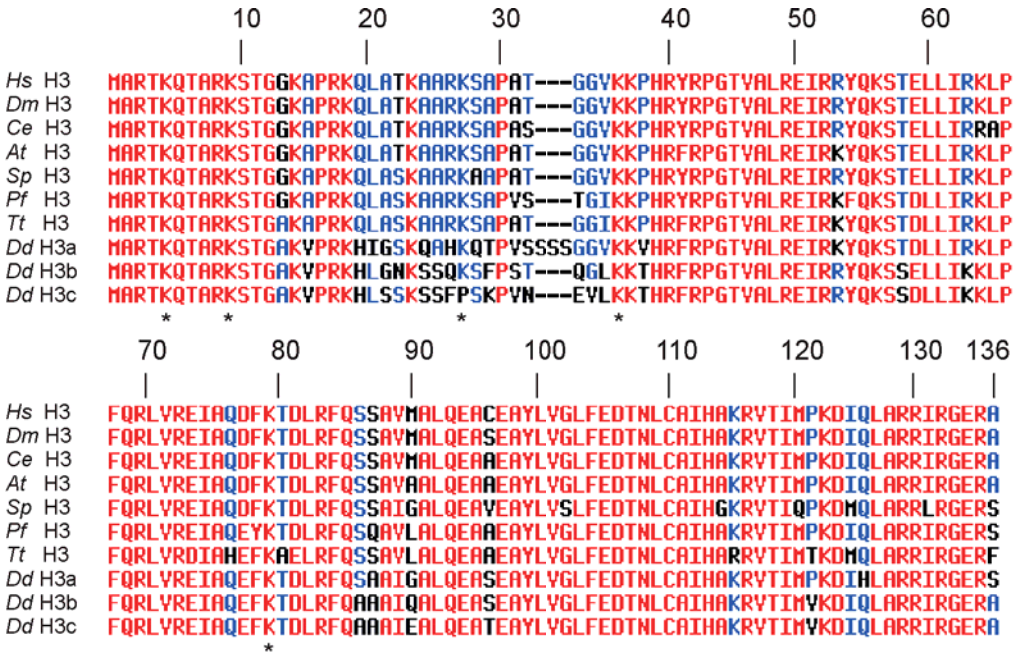

Fig. 1.10: **Amino acid alignments of histone H3 isoforms from various evolutionary lineages.** Conserved lysine residues subjected to methylation (K4, K9, K27, K36 and K79) are indicated by asterisks. Amino acid numbering is according to all histones except *Dictyostelium* histone H3a. Amino acid residues identical in all H3 proteins are highlighted in red; amino acid residues identical in at least 4 out of ten histone H3 proteins are highlighted in blue. Abbreviations: *Hs*: *Homo sapiens, Dm*: *Drosophila melanogaster*, *Ce*: *Caenorhabditis elegans, At*: *Arabidopsis thaliana, Sp*: *Schizosaccharomyces pombe, Pf*: *Plasmodium falciparum, Tt*: *tetrahymena thermophila, Dd*: *Dictyostelium discoideum.*

silencing, e.g. of tumor suppressor genes, is of great importance, since alterations in these pathways can lead to pathological cellular states such as cancer cell transformation. *Dictyostelium* combines highly elaborated cell-cell signalling and signal transduction pathways with high biochemical and, due to its haploid genome, genetic accessibility. It thus provides a covenient system to dissect signalling cascades leading to epigenetic changes in gene expression.

1.19 Aims of this work

HP1 proteins have been chosen as candidate proteins to explore heterochromatin structure and function in *Dictyostelium*, since they are structurally and functionally highly conserved and homologues from various organisms had already been characterised in detail. The present work, by characterising homologues of these well-known proteins, should provide the tools to further analyze chromatin-mediated mechanisms of gene regulation, which are likely to be conserved in *Dictyostelium*.

In contrast to other organisms, very little is known about heterochromatin structure and function in *Dictyostelium.* Although the *Dictyostelium* genome is almost completely sequenced (Eichinger et al., 2005), the information on centromeric and telomeric structures is limited. It is assumed that centromeres reside within clusters of repetetive DNA (e.g. transposons and retrotransposons) that are preferentially located at chromosome ends. The lack of a conserved underlying DNA sequence strongly argues that epigenetic factors probably play a role in centromere positioning, maintenance and function in *Dictyostelium*. Similarly, telomeres in *Dictyostelium* have not been defined by sequence. They appear not to be composed of specific hexanucleotide repeats, but are rather composed of complex arrays of repetetive DNA. Intruigingly, rDNA sequences from the extrachromosomal rDNA copies can be found at the ends of *Dictyostelium* chromosomes (Eichinger et al., 2005). Thus, HP1 proteins were chosen to provide molecular tools to study centromere and telomere organisation in *Dictyostelium*.

Furthermore, they were used to evaluate the subnuclear localisation of other proteins, which are assumed to display a centromeric localisation. The localisation of these proteins, such as DdINCENP (Chen et al., 2005), could not be analyzed before due to a lack of proper centromeric markers.

It was recently shown that chromatin at various gene loci is differentially methylated during *Dictyostelium* development and that a disruption of the histone H3K4 methyltransferase SetA results in a global developmental defect (Chubb et al., 2006). Therefore, one task of this work was to analyze if HP1 proteins are required for cellular differentiation and development in *Dictyostelium*.

2 Materials and Methods

All standard materials and methods have been described elsewhere (M. Kaller, Diploma thesis, 2002).

2.1 Primers

A list of all primers used in this study is given below. All primer sequences are in 5´ to 3´ orientation. Given in brackets is the original lab nomenclature.

hcpA1 (HP5)	GAATTCAAAATGGGAAAAAGAGATAAGAG
hcpA2 (HP10)	GTCGACATGGGAAAAAGAGATAAGAGAATAATAG
hcpA3 (HP3)	CATATGGGAAAAAGAGATAAGAGAATAATAG
hcpA4 (HP4)	GGATCCTTTTAACTTTGTTGACCCTTATAACC
hcpA5 (HP6)	GGATCCACTTTGTTGACCCTTATAACC
hcpA6 (HP1)	CTCGAGTCGACGATAAAAGGGTTCAGCATGG
hcpA7 (HP2)	GGTACCGAATTCGTTTAAGGATTTCATTTAATACCC
hcpB1 (hcrb1)	GCTAAAAGAATTCAAAATGGGAAAAAGAG
hcpB2 (hcrB5)	CAGCTAAAATTGTCGACATGGGAAAAAGAG
hcpB3 (hcrB3)	GCTAAAATTGTAACATATGGGAAAAAGAG
hcpB4 (hcrB2)	GGATCCACTTGGCTGACCACTATAACC
hcpB5 (hcrB4)	ATTGGATCCTTAACTTGGCTGACCACTATAACC
hcpA (HP1) KO LA 5´	ATAGGATCCGAAGACCCTTTTAAGAAATGTTGTC
hcpA (HP1) KO LA 3´	TTGAAGCTTTTTTCTTGTTCTCTTGAACTTTC
hcpA (HP1) KO RA 5´	ATTGCATGCCAAATCATTTAATTGATGATG
hcpA (HP1) KO RA 3´	ATGGGCCCGAAGACGAATTATTTAGTAATTCTTTAAAATATG
hcpB (hcrB) KO LA 5´	ATTGGGCCCGAAGACAAAATTCACCATATAAGGGG
hcpB (hcrB) KO LA 3´	ATTGCATGCCCATGTATCTTAATCTGATG
hcpB (hcrB) KO RA 5´	ATTAAGCTTCATCAACAACAGCAGCAGCACC
hcpB (hcrB) KO RA 3´	ATTGGATCCGAAGACTTACCCCATCCAAACAATGAG
hcpA 5´ outer	TGGTGATATGTCATAAACCTGC
hcpA (HP1) 3´ outer	CAGTTACTTGTTTCATTATGGC
hcpB (HcrB) 5´ outer	AAAAGATATAGAATCTACAACTATC
hcpB 3´ outer	GGAAATTAACGAACATTTTGAC
DnmA1 (MT14)	ATTGAATTCAAAATGGAACAATTGAGAGTATTAG
DnmA2 (MT15)	AGGAAGCTTATTGGATCCTTTTTTTCCTTCTTTTTCCTTTTG
c-Myc 5´ *Bam*HI	GATCCGAACAAAAATTAATTTCAGAAGAAGATTTAAATTA
c-Myc 5´ *Hind*III	AGCTTAATTTAAATCTTCTTCTGAAATTAATTTTTGTTCG
orcB 1 (HPint 5´)	GTCGACATGGAAGAATATACAGATTCAGG
orcB 2 (HPint 3´)	CTCGAGTTATGATATTGAATTTTCTAATTGTG

His-tag for (hcpab_uni)	ATTGAATTCAAAATGGGCAGCAGCCATCATCATCATCATCAC
hcpABΔCSD	ATTGGATCCACCTTCAATATAAGGTACACC
hcpC1 (hcrC1)	ATTAGATCTAAAATGGGTAAAACAAATAAAAAAAAAATTG
hcpC2 (hcrC2)	ATTGGATCCATTTTCTTCACCTTTATAACC
trx for	GAACGAGCTCCATGGCCAATAGAGTAATTCATG
trx rev	CGCGGATCCTTATTTGTTTGCTTCTAGAGTACTTC
DIRS 2for	GTATGCCCTGTTCGCCACCTTGC
DIRS 1rev	CGTAGAAGGTATCTACAGTATC
skipper GAG for	TGAAGCTAAAACCATTGACGC
skipper GAG rev	CTAATTGAACTTCAGCAGTACC
skipper RT for	CTGTTACCTTAGTGAAGATGGG
skipper RT rev	GGGCATCTATTGTCTTATGACATGG
act for	GATAACGGTTCTGGTATGTG
act rev	CCTGAATCCATAACGATACC
BS^R G1 5´	CGCTACTTCTACTAATTCTAGA
GFP KI 5´	CTCTGCAGGAATTCATGAGTAAAGGAGAAGAAC
qact6 for	AACGGTTCTGGTATGTGTAAAGC
qact6 rev	CAATTGATGGGAAAACAGCAC
qskip for	CTGCCACTGCTGTCATCTTC
qskip rev	TTGGTTCAACACCATTCTGG
qdirs for	TCAATGGAAGCAATGTCTGG
qdirs rev	TTCTCCAGGATAGAAGGCAAAA

2.2 Plasmids

2.2.1 pET15b-HcpA / pet15b-HcpB: Recombinant His-tagged HcpA and HcpB was produced by cloning their coding sequences into the NdeI and BamH1 sites of pET15b (Invitrogen). Primers used for amplification of the respective cDNAs were hcpA3 and hcpA4 for *hcpA*, and hcpB3 and hcpB5 for *hcpB*. PCR products were cloned into pGEM-T-EASY (Promega) and excised by restriction digestion with NdeI and BamHI.

2.2.2 pdneoGFP-HcpA / HcpB: For N-terminal GFP-fusions, *hcpA* was amplified from oligo-dT-primed cDNA using primers hcpA2 and hcpA4, and *hcpB* with primers hcpB2 and hcpB5. PCR products were cloned into pGEM-T-EASY (Promega), excised by restriction digestion with SalI and BamHI, and cloned into the SalI and BamHI sites of pdneo2-GFP (Rauchenberger et al., 1997).

2.2.3 pdneo*hcpA*i: The central portion of the *hcpA* coding region, covering the entire hinge region, a 3´portion of the chromo and a 5´ portion of the chromo shadow domain were amplified from (intronless) genomic DNA with primers hcpA6 and hcpA7 and cloned into pGEM-T-Easy (Promega). HcpA fragments were excised with EcoRI and SalI and cloned into pdneo2 disc as (Martens et al, 2002), from which the 5´ portion of the discoidin antisense fragment had been excised, to obtain an *hcpA* insert in sense orientation of the actin6-promoter (pdneo *hcpa* sense). The *hcpA* antisense strand was created by excision of the central *hcpA* sequence from pGEM-T-EASY with XhoI and Acc65I and ligated into pdneo *hcpa* sense to create pdneo*hcpA*i. The mRNA transcribed from the upstream actin6-promoter should fold back into a hairpin structure with ~ 500bp dsRNA and a ~300nt loop composed of remaining discoidin sequences. This plasmid was used to knock-down *hcpA* expression by RNAi-mediated cleavage of the *hcpA*-dsRNA and subsequent *hcpA* mRNA degradation.

2.2.4 pDd-HcpA-GFP / pDd-HcpB-GFP: To generate C-terminally GFP-tagged HcpA and HcpB, the respective cDNA was amplified using primers hcpA1 and hcpA5 for *hcpA*, and hcpB1 and hcpB4 for *hcpB*. PCR products were cloned into pGEM-T-EASY (Promega), excised by restriction digestion with EcoRI and BamHI and cloned into the EcoRI and BamHI sites of pDd-GFP (Gerisch et al., 1995).

2.2.5 pDd-HcpA-RFP: For the HcpA-RFP fusion, the GFP coding sequence of pDd-HcpA-GFP was replaced by mutant RFP (RedStar) from p415Gal1-Redstar (Knop et al., 2002) by digestion with BglII / XhoI and ligation into BamHI / XhoI-digested pDd-HcpA-GFP.

2.2.6 pdneoGFP-OrcB: The *orcB* coding sequence was amplified with primers orcB1 and orcB2 from oligo-dT-primed cDNA, and cloned into pGEM-T-Easy (Promega). The *orcB* sequence was excised with SalI and BamHI and cloned into pdneoGFP (Rauchenberger et al., 1997).

2.2.7 pdneo-myc-HcpA / HcpB: To create N-terminal myc-fusions of the Hcp proteins, the respective coding sequence was excised from pdneoGFP-HcpA or –HcpB, respectively, with SalI and SacI, and cloned into pdneo-myc-Cofilin (Drengk et

al., 2003), from which the cofilin coding sequence had been completely deleted by a SalI / SacI digestion.

2.2.8 pdneoGFP-HcpA$_N$B$_C$ / pdneoGFP-HcpB$_N$A$_C$:
Chimeric proteins of HcpA and HcpB were derived from the previously described plasmids pdneoGFP-HcpA and pdneoGFP-HcpB. Both plasmids were digested with *Pst*I and *Bam*HI, thereby excising the sequences encoding for most of the hinge and the entire chromo shadow domain of HcpA and HcpB. The excised fragments were exchanged between the two plasmids, resulting in pdneoGFP-HcpA$_N$B$_C$ (HcpA N-terminus and HcpB C-terminus) and pdneoGFP-HcpB$_N$A$_C$ (HcpB N-terminus and HcpA C-terminus). To verify the chimeric constructs, the respective GFP-fusion genes were amplified from plasmid DNA by PCR with primers GFP KI 5´ and hcpA5 for GFP-HcpA and GFP-HcpB$_N$A$_C$, and primers GFP KI 5´ and hcpB5 for GFP-HcpB and GFP-HcpA$_N$B$_C$. The PCR products were digested with BcuI or Acc65I. These restriction sites were present in the 5´ region (chromo domain) or the 3´ region (chromo shadow domain) of the *hcpB* coding sequence, respectively, but absent from the *hcpA* sequence, and thus allowed discrimination between the two genes and the respective chimeric fusion constructs.

2.2.9 pDd-HcpAΔC-GFP / pDd-HcpBΔC-GFP The chromo shadow domain deletions were designed with primers hcpA1 and hcpABΔCSD for *hcpA*, hcpB1 and hcpABΔCSD for *hcpB*, and cloned into the EcoRI and BamHI sites of pDd-GFP. For these constructs, identical reverse primers for both isoforms were used, since the primer binding sites were identical in both coding sequences.

2.2.10 pDEX-His-HcpA / pDEX-His-HcpB: For expression of 6xHis-tagged fusion proteins in *Dictyostelium*, the coding sequences were amplified from the respective pET-15b-derived plasmids using primers His-tag for and hcpA4 for *hcpA*, and His-tag for and hcpB5 for *hcpB*, and cloned into the EcoRI and BamHI sites of pDEX-DnmA, from which the DnmA coding seqence had been excised.

2.2.11 pDEX-DnmA-myc: the *dnmA* cDNA was amplified using primers and cloned into pDEX_RH (Faix and Dittrich, 1996), thus creating pDEX-DnmA. pDEX-DnmA was digested with BamHI and HindIII, and two complementary oligos (c-Myc 5´ BamHI and

c-Myc 5´ HindIII) encoding the myc-tag and with complementary BamHI/HindIII overhangs were introduced, thus creating pDEX-DnmA-myc .

2.2.12 pGEM7z- hcpA KO / pGEM7z-hcpB KO

Knock-out constructs were generated using a Blasticidin resistance (BS^R) cassette (Sutoh, 1993) with flanking arms of 625 bp and 501 bp for *hcpA*, or 703 bp and 821 bp for *hcpB*. Primers to create recombinogenic arms were hcpA KO LA 5´ and hcpA KO LA 3´ (left arm), hcpA KO RA5´ and hcpA KO RA 3´ (right arm) for *hcpA*; hcpB KO LA 5´ and hcpB KO LA 3´ (left arm), hcpB KO RA5´ and hcpB KO RA 3´ for *hcpB*. All PCR products were ligated into pGEM-T-Easy (Promega), excised with either Bsp120I and SphI (left arm *hcpB*, right arm *hcpA*) or BamHI and HindIII (left arm *hcpA*, right arm *hcpB*) and cloned into pGEM7z-BSr, which had been linearized with the corresponding restriction enzymes.

Knock-out constructs were designed to disrupt the respective open reading frames between the N-terminal chromo domain and the nuclear localisation signal (NLS) within the hinge region. The truncated genes should thus not generate any functional, correctly localized proteins.

Prior to electroporation, both constructs were linearized by digestion with BpiI. DNA was precipitated with ethanol, dried and dissolved in water.

2.2.13 pGEM-7z-hcpA^{flox} KO / pGEM-7z-hcpB^{flox} KO

For disruption of both endogenous *hcp* genes, the BS^R cassette of both targeting constructs pGEM7z-hcpA KO or pGEM7z-hcpB KO (see above) was excised with XbaI and HindIII, and replaced by a BS^R cassette with flanking *loxP* sites excised from pLPBLP (Faix et al., 2004) with BcuI and HindIII. Following this strategy, the orientation of the blasticidin resistance cassette was reversed compared with the original knock-out vectors pGEM7z-hcpA KO and pGEM7z-hcpB KO (see above).

A graphical overview of all knock-out constructs is given in Fig. 2.1.

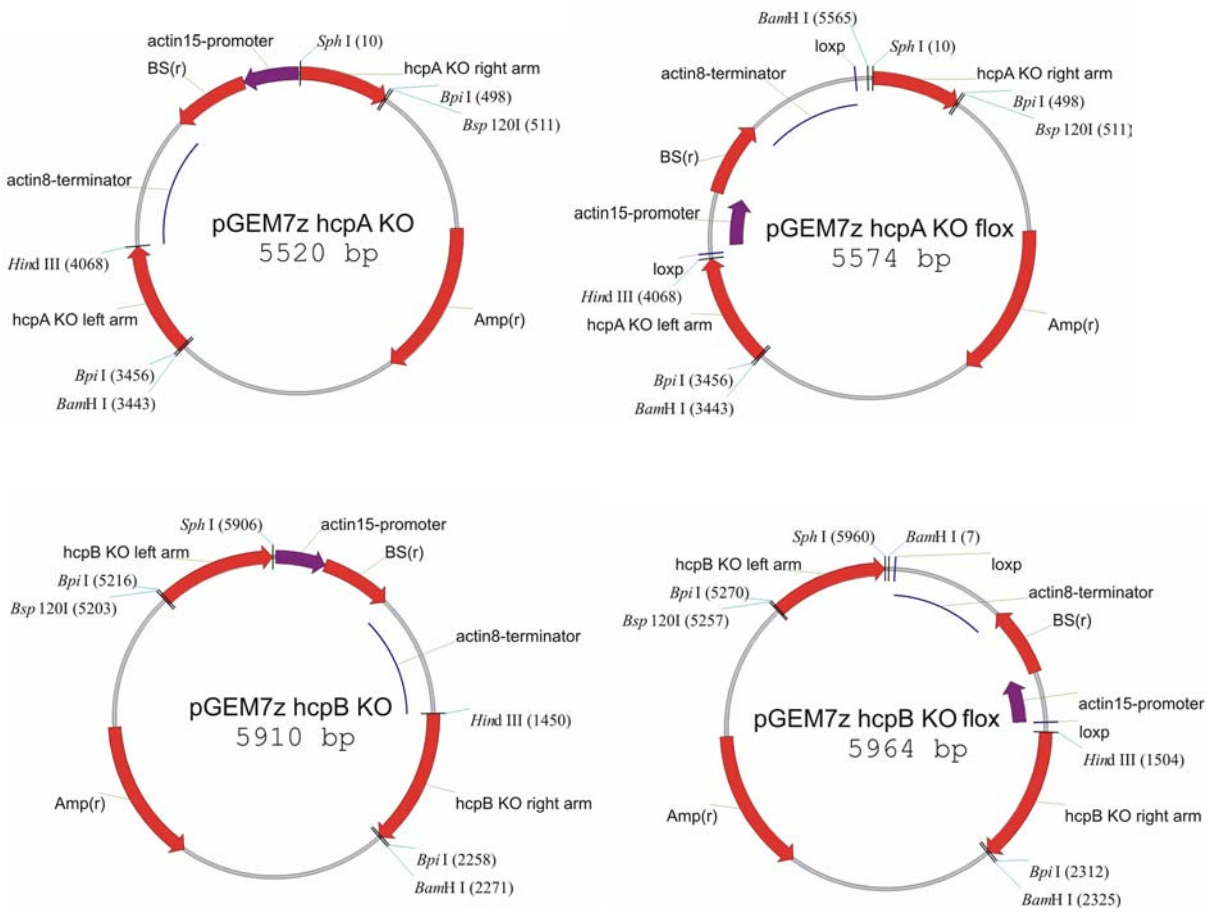

Fig. 2.1: **Plasmid maps of pGEM7z-derivatives used as gene targeting constructs for *hcpA* and *hcpB*** (Bs(r): blasticidin resistance cassette).

2.3 Strains

All standard manipulations were performed using standard methods and have been described elsewhere (M. Kaller, Diploma thesis, Kassel University, 2002). All transformations and co-transformations were carried out as described previously (Nellen et al., 1987; Nellen and Firtel, 1985). A detailed list of all *Dictyostelium* strains generated and used in this study is given in Table 2.1.

2.4 Generation of knock-out and double knock-out cell lines

Gene disruptions and subcloning were done by homologous recombination (Witke et al., 1987). Clones were analyzed by PCR as described (Martens et al., 2002).

For *hcpA*, primers hcpA 3´outer and BS^R G1 5´ were used to verify site-specific integration of the BS^R cassette. For screening of the disrupted wild-type allele, primers

TABLE 2.1: List of strains used in this study

strain	transformed vector
Ax2	pDd-HcpA-GFP
Ax2	pDd-HcpB-GFP
Ax2	pdneoGFP-HcpA
Ax2	pdneoGFP-HcpB
Ax2	pDd-HcpA-RFP / pDd-HcpB-GFP
Ax2	pdneo-myc-HcpA
Ax2	pdneo-myc-HcpB
Ax2	pDEX-His-HcpA
Ax2	pDEX-His-HcpB
Ax2	pDEX-His-HcpA/pdneoGFP-HcpA
Ax2	pDEX-His-HcpA/pdenoGFP-HcpB
Ax2	pGEM-7z-hcpA KO
Ax2	pGEM-7z-hcpB KO
Ax2	pGEM-7z-hcpA^{flox} KO
Ax2	pGEM-7z-hcpB^{flox} KO
hcpA^{lp}	pGEM-7z-hcpB KO
hcpA^{lp}	pGEM-7z-hcpB^{flox} KO
hcpA^{lp}	pdneoGFP-HcpA
hcpA^{lp}	pDEX-His-HcpA
hcpA^{lp} / pDEX-His-HcpA	pGEM-7z-hcpB^{flox} KO
hcpA^{lp} / pdneoGFP-HcpA	pGEM-7z-hcpB^{flox} KO
Ax2	pDEX-DnmA-myc
Ax2	pDd-DnmA-GFP
Ax2	pDd-HcpA-RFP / pTX-INCENP-GFP
Ax2	pTX-INCENP-GFP
Ax2	pdneoGFP-OrcB
Ax2	pdneoGFP-OrcB / pDd-HcpA-RFP
Ax2	pdneoGFP-HcpA$_N$B$_C$
Ax2	pdneoGFP-HcpB$_N$A$_C$
Ax2	pDd-HcpAΔC-GFP
Ax2	pDd-HcpBΔC-GFP
setA$^-$	pDd-HcpA-GFP
rrpA$^-$	pDd-HcpA-GFP
rrpB$^-$	pDd-HcpA-GFP
rrpC$^-$	pDd-HcpA-GFP

hcpA 3´outer and hcpA1 were used. In knock-out clones with a disrupted *hcpA* gene, theses primers should not generate a PCR product under the chosen PCR conditions. For *hcpB*, primers hcpB 5´outer and BS^R G1 5´ were used to verify site-specific integration of the BS^R cassette. For screening of the disrupted wild-type allele, primers hcpB 5´outer and hcpB4 were used.

For the generation of *hcpA/hcpB* double knock-out strains, the Ax2 strain was first transformed with the *hcpA* targeting construct containing a BSr cassette with flanking *lox*P sites. Isolation of *hcpA*$^-$ cells was carried out as described above, but with primers hcpA 5´outer and BS^R G15´ due to a different orientation of the BS^R cassette in the targeting construct. Transient expression of Cre-recombinase was carried out as described (Faix et al., 2004), except that after 5 days of selection, selective medium

was exchanged by non-selective HL5 medium. Further culturing, serial dilution and plating on bacterial plates then was performed until growing cells were microscopically detectable in the culturing dishes. Screening for blasticidin and G418 sensitivity were carried out as described (Faix et al., 2004).

The generated *hcpA*^{lp} allele was verified by PCR amplification with primers hcpA3 and hcpA4, followed by restriction digestion with BamHI. The BamHI site is flanking the remaining *lox*P site after Cre-mediated recombination, but is absent from the wild-type *hcpA* gene, thus allowing discrimination between the *hcpA*^{lp} and wild-type *hcpA* alleles.

The generated *hcpA*^{lp} strain was then transformed with the *hcpB* targeting construct, or transformed with pDEX-His-HcpA or pdneoGFP-HcpA prior to *hcpB* targeting. *HcpB* gene disruptions were detected with primers hcpB 3´outer and BSr G1 5´. Screening for the presence of the wild type *hcpB* alleles was performed as described for the single knock-out. The presence of the *hcpA*^{lp} allele was verified by PCR amplification of the endogenous genomic *hcpA* locus with primers hcpA5´outer and hcpA5, followed by restriction digestion with NcoI. The chosen primers only amplified the endogenous *hcpA* locus with flanking upstream genomic regions, but not the transgenic *hcpA* sequences introduced by transformation with *hcpA* expression vectors, which only contained the *hcpA* coding sequence.

2.5 Cell culture

Growth curves of Ax2, *hcpA*$^{-}$ and *hcpB*$^{-}$ were done in axenic suspension culture in HL5 medium on a rotary shaker at 150rpm. For the overexpression strains, HL5 medium supplemented with 20 µg/ml geniticin was used. The starting cell density was 1-3x10^5/ml for growth at 22°C and at 15°C. Cell densities were measured with a Coulter Counter®. All growth curves were measured in duplicate.

In order to determine cell viability, the plating efficiency of cell suspensions that had been cultured for 48 hours on rotary shakers was determined. Cell densities were measured before serial dilution in phosphate buffer. Cells were plated together with *Klebsiella aerogenes* suspension on non-selective Sm plates and allowed to grow for 72 hours. Colonies were counted, and the plating efficiency was calculated :

$$\text{plating efficiency} = \frac{\text{colony number x dilution factor}}{\text{suspension volume (ml) x cell number / ml}}$$

2.6 Immunodetection

Dictyostelium cells were grown on coverslips in petri dishes containing the appropriate selective medium for 20-24 hours. Cells were then fixed in –20°C methanol for 20 min, washed three times in 1x PBS and blocked with PBG buffer (1x PBS with 3% BSA and 0.045% cold water fish gelatin; Sigma) for 1 hour at 37°C. Primary antibodies were applied in appropriate dilutions and incubated overnight at 4°C.

For detection of histone H3K9 dimethylation, a polyclonal rabbit antibody (UpstateBiotechnology, CA, USA) diluted 1:100 in PBG buffer was used. For staining of centrosomes, the anti-DdCP224 antibody 2/165 (Graf et al., 2000) was used in a 1:100 dilution. After six washes in PBG, Cy3-conjugated secondary antibodies (Dianova, Hamburg) were applied in a 1:1000 dilution in PBG for 1 hour at 37°C. DNA was stained with DAPI (1 mg/ml) diluted 1:15,000 in 1x PBS for 15 min. After 3x 5 min washing with 1xPBS, coverslips were once rinsed with destilled water, briefly dried on tissue paper and embedded in gelvatol.

2.7 Mitotic analysis

For mitotic assays, cells precultured in petri dishes at the indicated temperatures were diluted to $3x10^5$/ml at 22°C and $7x10^5$/ml at 15°C, respectively. 500 µl of cell suspension were placed on 18x18 mm^2 coverslips and allowed to attach to the glass surface for 30 min. Cells were then grown on coverslips in petri dishes covered with 5 ml of HL5 medium for 20-24 hours and fixed as described. Mitotic cells were detected by anti-DdCP224 or by anti-α-tubulin staining. The mouse monoclonal anti-DdCP224 antibody 2/165 (Graf et al., 1999; Graf et al., 2000), was applied in a 1:100 dilution, the rabbit polyclonal anti-α-tubulin antibody (Kilmartin et al., 1982) in a 1:50 dilution.

For determination of chromosome missegregation frequencies, only late mitotic cells with spindles $\geq$ 5 µm were counted, and the number of mitoses with DNA bridges was determined.

2.8 Fluorescence microscopy

For microscopic analysis, a Leica DM IRB inverted fluorescence microscope and for image acquisition a Leica DC 350F digital camera and IM50 software was used. Images were processed in AdobePhotoshop.

2.9 Thiabendazole-Sensitivity-Assay

To analyse sensitivity towards the microtubule-destabilizing drug thiabendazole, different *Dictyostelium* strains were cultured in HL5 medium with 10 µM thiabendazole. Cultures that had been precultured on rotary shakers were adjusted to a cell density of 1-2x10^5/ml. Thiabendazole (from a 2 mg/ml stock in DMSO) was diluted 1:1000 in the medium. Cell densities were measured in regular intervals using a CoulterCounter®.
In parallel, cells grown on coverslips were incubated in medium supplemented with 10 µM thiabendazole for 3-4 hours, fixed with methanol and immunostained with anti-DdCP224-antibodies as described to detect mitotic cells.

2.10 Analysis of *Dictyostelium* Development

To analyse possible developmental phenotypes of mutant strains, cells were spotted on non-nutrient phosphate agar plates with cell densities of either 2x10^5/cm^2 or 8x10^5/cm^2. 10ml of axenic suspension cultures with a density of 1x10^6/ml were centrifuged, washed with phosphate buffer and adjusted to cell densities of either 2x10^5/ 50 µl or 8x10^5/ 50 µl. As a rule of thumb, 50 µl of cell suspension were required to cover a 1 cm^2 area. Cells were spotted in triplicate on phosphate agar plates. Excess buffer was allowed to evaporate for 20-30 min under a sterile hood. Developmental stages were analysed at regular intervals.

2.11 Spore Germination Assay

10 ml of exponentially growing axenic suspension cultures (1x10^6/ml) were centrifuged, washed once with phosphate buffer and adjusted to a cell density of 1x10^6/ 50 µl with phosphate buffer. 500 µl of the suspension were placed on an 10cm^2-area on non-nutrient phosphate agar plate. Excess buffer was allowed to

evaporate for 20-30 min under a sterile hood. Spores were allowed to mature for 3-4 days. Spores were then resuspended in phosphate buffer. Spore density was determined using a Neubauer counting chamber. Spores were serially diluted and 10-20 µl of the diluted spore suspension plated with 200 µl of *Klebsiella aerogenes* suspension on Sm agar plates and incubated at room temperature. The number of forming colonies was determined, and plating efficieny as an indicator of spore viability was calculated :

$$\text{plating efficiency} = \frac{\text{colony number x dilution factor}}{\text{suspension volume (ml) x spore number / ml}}$$

2.12 Preparation of *Dictyostelium* whole cell extracts

Cells precultured in 24 well-plates or petri dishes were resuspended and 1 ml was transferred into 1,5 ml tubes. The cells were spun down for 5 min at 2.300 rpm and washed once with phosphate buffer. The cell pellet was suspended in 95µl lysis buffer (20 mM Tris pH 7,5, 300 mM NaCl and 0,5 % NP40) by brief vortexing. 15 µl 6xLaemmli buffer were added, vortexed again and boiled for 10 min at 95°C.

2.13 Preparation of nuclear extracts

5ml of cell cultures with a density of $3x10^6$ /ml were centrifuged at 1.200 rpm for three minutes. The cell pellet was washed once with 5-10 ml of phosphate buffer. The cells then were resuspended in cell lysis buffer (20 mM HEPES pH 7.5, 40 mM $MgCl_2$, 20 mM KCl, 5% sucrose (w/v)) Afterwards, 500 µl of 10% NP40 solution and 5 µl β-mercaptoethanol were added. Cells were lysed for 15 min with gentle rotation at 4°C. The nuclear pellet was centrifuged for 15 min at 4.000 rpm. The pellet was resuspended in 2x Laemmli buffer and boiled for 10 min at 95°C. For an equivalent of $1x\ 10^6$ cells, 1 µl 2x Laemmli buffer was added.

2.14 Bacterial expression and purification of His-HcpA, His-HcpB and His-HP1α

Vectors for bacterial expression were transformed into *E. coli* BL21 (DE3) pLysS (Invitrogen) using standard procedures. Single clones were then first tested for protein expression. A 5 ml-preculture was inoculated overnight at 37°C. 2-3 ml of this

preculture were then used to inoculate 50 ml-cultures. Cultures were grown at 30°C until an OD of 0,4 – 0,5 was reached. Protein expression was induced with 1 mM IPTG for 3 h at 30°C. Induced cell cultures were centrifuged for 5 min at 4.000 rpm. The cell pellet was either stored at -20°C or used directly. The pellet of induced *E. coli* cells was lysed in 10 ml lysis buffer (20 mM Tris pH 7,5, 10 mM imidazole, 300 mM NaCl, 0,5% NP40, 0,5 mM PMSF), and sonified on ice 3 times 30 sec with 30 sec intervals. PMSF was taken from a 200 mM stock solution in ethanol. For sonification, a UP 200 S sonifier (Dr. Hielscher GmbH, Stansdorf, Germany) with an S3 microtip was used. The settings of the sonifier were 100% intensity and duty cycle 0,5. The cell lysate was centrifuged at 10.000 rpm for 10 min at 4°C. The supernatant was collected, and one aliquot was stored as input fraction.
Ni Sepharose^{TM} 6 Fast Flow (Amersham Biosciences) in Poly-Prep® Chromatography Columns (Bio Rad) with a column volume of 300-500 µl were equilibrated with 5 ml of with lysis buffer. The supernatant then was loaded on the Ni-sepharose bed. An aliquot of the flowthrough was stored as the flowthrough fraction. The columns were washed with at least 20 column volumes of washing buffer (20 mM Tris-HCl pH 7.5, 50 mM imidazole, 300 mM NaCl). The His-tagged proteins then were eluted with elution buffer (20 mM Tris-HCl pH 7.5, 250 mM imidazole, 300 mM NaCl) stepwise with 500 µl for each step. The second fraction contained the majority of the His-tagged proteins. Aliquots of all fractions were boiled with 6x Laemmli buffer for 10 min at 95°C. Aliquots of the input, flowthrough, wash and elution fractions were then analyzed by SDS-PAGE and Western blot.

2.15 Pull-Down-Assays

His-tagged HcpA and HcpB were purified on Ni-Sepharose beads (Amersham) as described above. Instead of eluting the immobilzed proteins with imidazole, the beads were directly challenged with *Dictyostelium* cell extracts. Cell lysates from a *Dictyostelium* strain expressing GFP-tagged fusion proteins were prepared by sonifying 1 x 10^8 cells from axenic suspension culture for three times 15 sec in 10 ml lysis buffer (20 mM Tris pH7.5, 300 mM NaCl, 10 mM imidazole, 0.5% NP40). Cell lysates were mixed with pre-coated Ni-sepharose beads and rotated for 1-2 hours on a spinning wheel. Beads were spun down by brief centrifugation at 1.000 rpm for 1 min. The supernatant was stored, and the beads were washed with at least 20 column

volumes of binding buffer. The respective Hcp and bound proteins were then eluted with 250 mM imidazole. Eluted fractions were analyzed by 12% SDS-PAGE. Proteins were stained with Coomassie and detected on Western blots by mouse monoclonal anti-GFP- or anti-His-antibodies (all Dept. of Cell Biology, Kassel University).

For competition experiments, His-tagged HcpA or HcpB was bound to Ni-sepharose as described above. Lysates from *Dictyostelium* cells expressing GFP-HcpA and GFP-HcpB or the respective chimeric proteins were mixed and incubated with pre-coated beads as described above. After washing, bound proteins were eluted with 250 mM imidazole. Detection of GFP-tagged proteins was done by Western blotting with an α-GFP-antibody.

The same protocol was applied for determining *in vivo* interactions, except that extracts from His-HcpA/GFP-HcpB and His-HcpA/GFP-HcpA cotransformed cell lines were directly incubated with the Ni-sepharose beads.

2.16 Immunoprecipitation of myc-tagged HcpA/B proteins

2.16.1 Preparation of *Dictyostelium* nuclei

2 x 10^8 cells of myc-HcpA or myc-HcpB expressing *Dictyostelium* strains were centrifuged for 3 min at 1.200 rpm at 4°C. The cell pellet was washed once with 30 ml cold phosphate buffer. The cells were resuspended in 27 ml of cell lysis buffer (see 2.13), then 3 ml of 10% NP40 were added. The suspension was carefully inverted until it became clear, which indicated lysis of the cells. Under these conditions, the nuclei remained intact. The nuclei were centrifuged at 4.000 rpm for 15 min at 4°C. A swinging bucket rotor was used, which allowed the nuclear pellet to be collected at the bottom of the tube. The supernatant was discarded. The nuclei were resuspended in 1.5 ml of RIPA buffer (50mM Tris-HCl, pH 7.4, 1% NP40, 0,25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF), and the suspension was transferred into a 15 ml tube. For complete lysis of the nuclei, the suspension was sonified twice for 5 sec (90% duty cycle, 40% intensity). The suspension was centrifuged at 10.000 rpm for 10 min at 4°C. The supernatant then was transferred into a fresh 1,5 ml tube. An aliquot was stored as the input fraction.

2.16.2 Immunoprecipitation

To the nuclear lysate, 50 µl of a 50% ProteinA-sepharose CL4B (Amersham

Biosciences) slurry in 1xPBS were added, and the mixture was rotated for 30 min to 1 h at 4°C. The beads were briefly spun down for 5 sec at 14.000 rpm. The supernatant was transferred into a fresh tube. This pre-clearing step was required to minimize unspecific binding of proteins to the beads.

The supernatant was divided into three parts. Either a) an anti myc-antibody against the protein of interest, b) an unspecific anti-GFP-antibody or c) no antibody was added. For the anti-myc and anti-GFP antibodies (all Dept. of Cell Biology, Kassel University), 20 µl of the hybridoma supernatant (9E-10 or 264-449-1, respectively) were used. The mixture was incubated on a rotating wheel overnight at 4°C. To enhance binding of the protein-antibody complexes to the beads, 3µl of rabbit anti mouse bridging antibody were added and incubated for 1-2 h. After that, 50 µl of ProteinA-sepharose slurry were added, and incubated for further 1-2 h. The beads were spun down at 14.000 rpm for 5 sec, and the supernatant carefully discarded. An aliquot of the supernatant was stored. The beads were washed with 800µl cold RIPA buffer. This washing step was repeated three times. To the beads, 60 µl of 2x Lämmli buffer were added. The beads were boiled for 5 min at 95°C. This boiling step released the protein-antibody complex from the beads. The beads were briefly spun down at 14.000 rpm and supernatant, which is the immunoprecipitated fraction, was transferred into a fresh tube. Input and immunoprecipitated fractions were analyzed by SDS-PAGE and Western blotting.

2.17 Chromatin immunoprecipitation (ChIP)

2.17.1 Fixation

100ml of *Dictyostelium* cell cultures with a density of $3x10^6$ cells / ml were spun down and washed once with phosphate buffer. The cell pellet was resuspended in a total volume of 3,1 ml of phosphate buffer and transferred into 15 ml tubes. 400 µl of 10x PBS and 500 µl of a freshly prepared 8% paraformaldehyde solution were added. The 8% paraformaldehyde solution was generated by mixing 0.08 g paraformaldehyde (Merck, no. 4005) with 900 µl destilled water and incubation on a heating block at 40°C. 100 µl of 1 M NaOH solution were added and further incubated at 40°C with rotation, until the paraformaldehyde was dissolved. Cells were fixed for 30 min at room temperature with gentle rotation. The reaction was quenched by addition of 250 µl of a

2 M glycine solution and further incubation with rotation for 5 min at room temperature. The cells were spun down for 3 min at 1.2000 rpm. The cell pellet was washed 4 times with 4 ml of 1xTBS (see below) and once with 1xPBS. At this step, the pellet could be stored at –80°C.

2.17.2 Sonification

The pellet was resuspended in 1,5 ml of 1xTBS. 1,5 ml of 2xTTS/1xTBS (see below) and 15 µl 200 mM PMSF were added and incubated for 15 min on ice. For sonification, a UP 200 S sonifier (Dr. Hielscher GmbH, Stansdorf, Germany) with an S3 microtip is used. The settings of the sonifier were 40% intensity and duty cycle 1. The sonification was done 5x 10 sec with 30 sec intervals. After that, each sample was again sonified twice for 10 sec with a 30 sec interval. The lysate was centrifuged for 10 min at 4.000 rpm at 4°C. The supernatant was transferred into a fresh tube and centrifuged again.

2.17.3 Control of sonification conditions

150 µl of the supernatant were diluted with 750µl E buffer (see below) and incubated with 3 µl RNaseA (1 mg/ml) for 1 hour at 37°C. 36 µl 5 M NaCl solution were added and incubated overnight at 65°C for cross-link-reversal. Then, 6,5 µl Proteinase K (25 mg/ml) und 3 µl RNaseA (1 mg/ml) were added and incubated for 1h at 45°C. Out of 500 µl, proteins were extracted with phenol-chloroform. The DNA was precipitated with ethanol. The pellet was air-dried and dissolved in 50 µl water. 10-20 µl of the DNA were analyzed on a 1 % agarose gel.

2.17.4 Immunoprecipitation

500 µl of the supernatant were diluted with 1 ml 1xTTS (see below), and incubated with 40 µl ProteinA-sepharose CL4B (Amersham) (1:3 slurry equilibrated in 1xPBS) for 2 h at 4°C on a spinning wheel. This step was used to pre-clear the cross-linked material to avoid unspecific protein interactions with the ProteinA-sepharose. The beads were briefly spun down at 14.000 rpm. 20 µl of the supernatant were kept as the input fraction. 120 µl of the supernatant were diluted with 680 µl 1xTTS, 5 µl 200 mM PMSF and 32 µl anti-myc-antibody (9E-10) or 3 µl of anti-(histone H3) dimethyl-K9-antibody (UpstateBiotechnology, #02-712). As a control, samples without addition

of antibody were prepared in parallel. The protein amounts used for immuno-precipitation were thus equivalent to ~ $5x10^6$ cells. The mixture was incubated overnight on a spinning wheel at 4°C. After that, 3 µl of a rabbit-anti-mouse bridging antibody were added (not to the minus-antibody controls, and not for the polyclonal rabbit anti-(histone H3) dimethyl-K9-antibody) and further incubated for 1,5 h. 70 µl of ProteinA-sepharose were added and incubated for 1-2 h. The beads were spun down for 1min at 1.000 rpm, the supernatant was carefully removed and the beads were washed 4 times with 1 ml 1xTTS and twice with 1 ml LiDN buffer (see below). Washing of the beads was performed for 3 min on a rocking platform. The supernatant was removed completely. The antibody-protein-DNA–complex was eluted with 100 µl E buffer for 15 min at 37°C on a rocking incubator. Beads were spun down briefly at 14.000 rpm, and the elution step was repeated. Both eluted fractions were pooled, and 8 µl 5 M NaCl were added. In parallel, 20 µl of the input fractions were diluted with 180 µl E buffer, and 8 µl 5 M NaCl were added. Cross-link reversal was done by overnight incubation at 65°C. 1 µl RNaseA (1 mg/ml) were added and incubated for 1 h at 37°C. After that, 1,5 µl Proteinase K (25 mg/ml) were added and incubated for 1 h at 45°C. Proteins were removed by phenol-chloroform extraction. The DNA was precipitated with ethanol. The pellet was air-dried and dissolved in 150 µl TE buffer. Samples were stored at –20°C until further use.

2.17.5 Quantification of immunoprecipitated DNA

For quantification of the DNA precipitated with the α-myc-antibody, the input fractions, the precipitated fractions, and minus-antibody-controls were analyzed by PCR. The input fractions were diluted 1:10 in TE buffer. PCR cycle numbers had to be adjusted empirically to stay under non-saturated PCR conditions. The number of PCR cycles varied, dependent on the copy number of the chosen target genes in the genome. For amplification of *DIRS*-1, 24 cycles were run, and for actin and *skipper* 25 cycles were run. Primer pairs used were DIRS1for/DIRS2rev (*DIRS*-1), skipperRT for/rev (*skipper*), act for/rev (actin). The multi-copy actin gene family served as a euchromatic copy number control, which should not be targeted by HP1 proteins or histone H3K9 methylation. For quantification of the DNA precipitated with the α-H3K9me2-antibody, the input fractions were diluted 1:20 in TE buffer. Primers used for PCR are specified above. PCR cycles were 26 (*DIRS*-1), 27 (*skipper*) and 29 (actin).

To adjust PCR conditions for each gene, additional primers for each target were

designed with the Universal probe finder 2.0 software (www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp). PCR conditions for these optimized primers were 95°C 5min, 95°C 15sec, 60°C 1min.

1xTBS:	50 mM Tris pH 8.0, 150 mM NaCl
1xTTS:	50 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X100, 2 mM EDTA, 0.05% SDS
2xTTS/1xTBS:	50 mM Tris pH 8.0, 150 mM NaCl, 2% Triton X100, 4 mM EDTA, 0.1% SDS
LiDN buffer:	10 mM Tris pH 8.0, 250 mM LiCl, 1% deoxycholate, 1% NP40, 1 mM EDTA
E buffer:	50 mM Tris pH 7.5, 1% SDS, 1 mM EDTA

2.18 Far Western blot

Basically, Far Western blots were performed as described (Cavailles et al., 1994). Nuclear extracts from *Dictyostelium* cells were prepared as described (see 2.13), separated by SDS-PAGE and blotted on a nitrocellulose membrane. The blotted proteins were denatured / renatured by a guanidinium hydrochloride concentration gradient. For this purpose, the membrane was incubated for 20 min each in HB-buffer (25 mM HEPES pH7.7, 25 mM NaCl, 5 mM $MgCl_2$,1 mM DTT) containing 6 M, 4 M, 3 M, 2 M, 1,5 M, 1 M, 0,75 M, 0,5 M, 0,25 M und 0,125 M guanidinium hydrochloride. Afterwards, the membrane was blocked for 15 min in HB-buffer containing 5% milk powder and for 15 min in HB-buffer containing 5% milk powder and 0,05% NP40. His-tagged HcpA was bacterially expressed as described. Cell pellets of induced *E. coli* cultures were resuspended in 10 ml H buffer (20 mM HEPES pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.05% NP-40, 1%milk powder,1 mM DTT) and sonified as described. The lysate then was centrifuged for 10 min at 10.000 rpm. 5 ml of the supernatant were diluted with 5 ml H buffer and incubated with the membrane at 4°C with agitation overnight. As a control, a second membrane was incubated with cell lysates from induced *E. coli* cultures transformed with empty pET15b. Membranes were washed 3x 5 min with H buffer. The primary anti-His-antibody was applied in a 1:5 dilution in H buffer with 2% milk powder overnight at 4°C. Membranes were then washed once with H buffer and twice with 1xPBS/0,05% Tween20. Incubation with

secondary antibodies and development of the blot was similar to the Western blot procedure described.

2.19 Gel Retardation Assay

Bacterial expression and purification of His-tagged HcpA, HcpB and HcpBΔCSD were carried out as described. After elution, the purified proteins were dialyzed overnight against 2 l of storage buffer (10 mM Tris-HCl pH 8,0, 300 mM NaCl, 0.1 mM EDTA) and kept on ice at 4°C. A 93 bp PCR product was gel-purified and radioactivelly end-labelled using [γ-^{32}P-ATP] and T4 Polynucleotide Kinase (MBI) :

PCR product	15 µl (1-20pmol of 5´-termini)
10x forward reaction buffer (MBI)	3 µl
[γ-^{32}P-ATP]	8 µl (~20pmol)
water	to 28 µl
T4 Polynucleotide Kinase (10u)	2µl

The reaction mixture was incubated at 37°C for 45 min. After that, the enzyme was inactivated for 10 min at 60°C. The endlabelled DNA was purified and separated from free nucleotides on a sepharose-G50-column by centrifugation at 1.500 rpm for 10 min. For further purification, the DNA was precipitated with 100% ethanol. After precipitation, the DNA was air-dried and resuspended in 10 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$. Increasing amounts of protein (1-5 µl, which corresponded to 0,2 -1µg protein) were mixed with 3 µl of end-labelled DNA in the presence or absence of cold competitor DNA. As competitor DNA, 1 µl of column-purified plasmid DNA (~ 0,5 µg) was used. The mixture was filled up with 1x assay buffer to a total reaction volume of 20 µl. The reaction mixtures were kept on ice for 1 h or for 30 min at room temperature. Glycerol was added to a final concentration of 10%. The samples were then run on a 5% polyacrylamide gel in 1xTBE. The gel had been pre-run for at least 30-60 min at 120 V. The gel was run at 120 V for 3-5 hours and exposed on radiosensitive screens to visualize the radiolabelled bands. Image acquisition was performed using a Fuji X BAS 1500 Phosphorimager. Image analysis was done using the TINA 2.0 software.

3 Results

3.1 HP1-like proteins in *Dictyostelium discoideum*

A database BLAST search using the human HP1α sequence and a tBLASTN algorithm (Altschul et al., 1997) revealed the presence of three genes encoding putative HP1-like proteins in the *Dictyostelium* genome. All three displayed the characteristic hallmarks of HP1 proteins: an N-terminal CD and a C-terminal CSD separated by a less conserved hinge region. The genes were denominated *hcpA*, *hcpB* and *hcpC* (Fig. 3.1). HcpA displays 70% identity (77% similarity) with HcpB, but only 62% identity (75% similarity) with HcpC. Sequence analysis further showed that amino acid residues within the CD that are required for function, such as methyl-lysine binding, were highly conserved in the *Dictyostelium* proteins, whereas functionally important amino acid residues within the CSD were less conserved (Fig. 3.1). The HcpA CD displayed highest similarity to that of human HP1γ (79%), and slightly less to the α- and β-isoforms (73%, and 77%, respectively). For comparison, the CSD of HcpA displayed 52%, 48% and 46% similarity to the human α-, β- and γ-isoforms.
RT-PCR analysis showed that *hcpA* and *hcpB* were expressed in axenically grown cells (Fig. 3.2) and throughout development (data not shown). *hcpC* transcripts were not detected under these conditions while the gene specific primers readily showed a PCR product when using genomic DNA. To see if *hcpC* was induced as a potential compensatory mechanism, we investigated its transcription in *hcpA*$^-$ and *hcpB*$^-$ knock-out strains but could not detect any RT-PCR signal (Fig. 3.2). Apparently, *hcpC* is not expressed at all or has a very restricted expression pattern. It was therefore excluded from further studies.

3.2 HcpA and HcpB display largely identical subnuclear localisation

To study the subnuclear organisation of *Dictyostelium* heterochromatin, we used C-terminally GFP-tagged HcpA and HcpB (termed HcpA-GFP, HcpB-GFP), respectively. Electron microscopy revealed that the HcpA-GFP fusion protein colocalized with electron dense structures at the nuclear periphery close to the centrosome (Fig. 3.3). These structures contain the kinetochores/centromeres of *Dictyostelium* chromosomes (Moens, 1976).

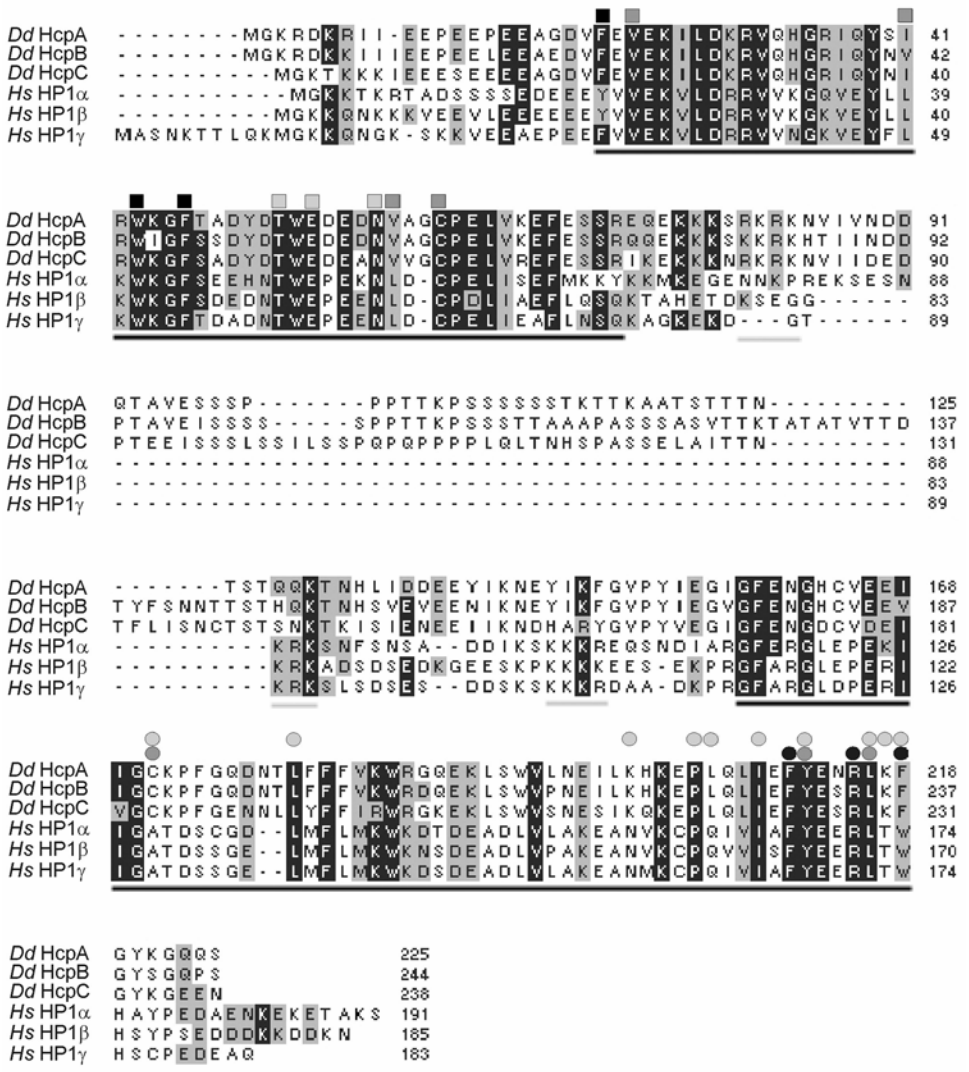

Fig. 3.1: **Alignment of HP1-like proteins.** HcpA, HcpB and HcpC were aligned with human HP1 orthologues HP1α, HP1β and HP1γ. The highly conserved CD and CSD are underlined in black. Functionally important amino acid residues in the chromo domain are indicated as squares. Black: aromatic residues that form a pocket required for methyl-lysine binding; light grey: additional residues required for methyl-lysine recognition; dark grey: residues required for recognition of Ala 7 in the histone H3 N-terminus (Nielsen et al., 2002). Functionally important amino acid residues in the chromo shadow domain are indicated as circles. Light grey: residues that form the CSD dimer interface (Brasher et al., 2000); Residues required for recognition of the PxVxL motif in HP1 interacting proteins are shown in dark grey (central valine) and black (proline and leucine) (Thiru et al., 2004).The nuclear localisation signals within in the hinge region are underlined in grey. Note that the previously described bipartite NLS within the hinge region of the human HP1 proteins (Smothers and Henikoff, 2001) is missing in the *Dictyostelium* proteins, but lies adjacent to the chromo domain. Abbreviations: *Dd Dictyostelium discoideum*, *Hs Homo sapiens*.

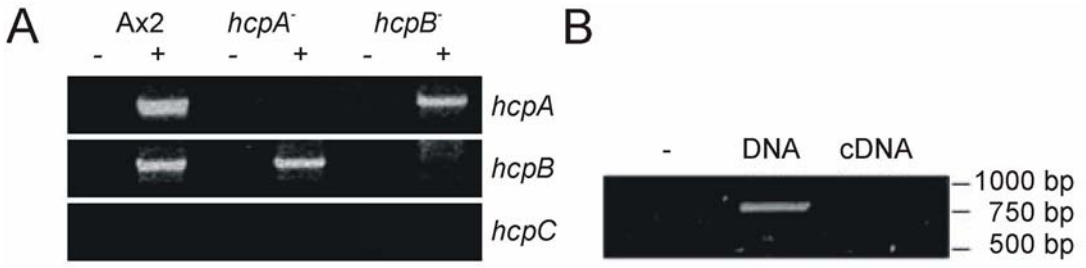

Fig. 3.2: **RT-PCR analysis of *hcpA*, *hcpB* and *hcpC* transcript levels in wild-type and mutant strains.** A) Whereas *hcpA* and *hcpB* are transcribed in wild-type Ax2 cells, but not in the respective knock-out mutants, *hcpC* is neither transcribed in wild-type cells nor in the *hcpA*$^-$ and *hcpB*$^-$ knock-out mutants (- minus reverse transcription; + plus reverse transcription). B) Primers used for amplification of the *hcpC* gene generate a PCR product on genomic DNA, but not on cDNA (- negative control). Note that the genomic *hcpC* sequence contains 106bp intronic sequence and that the PCR product is thus slightly larger than the expected PCR product on cDNA.

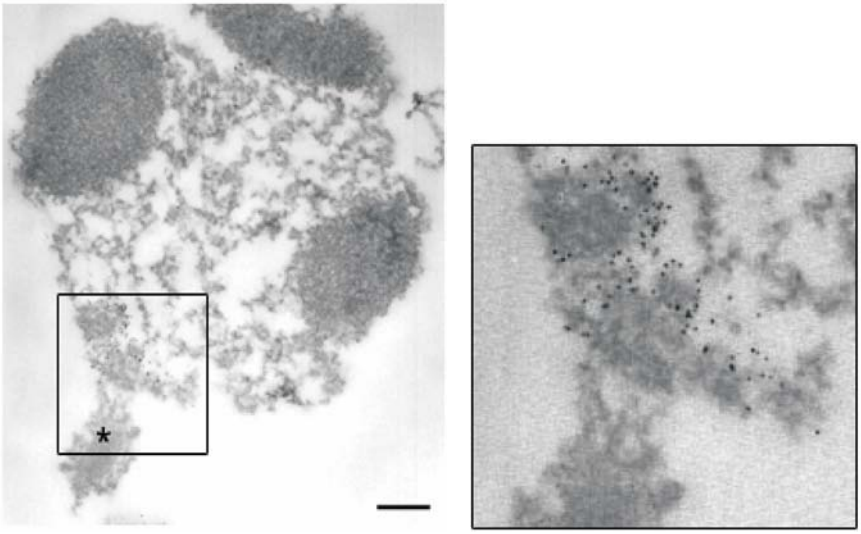

Fig. 3.3: **Ultrastructural analysis of subnuclear localisation of HcpA-GFP**. An isolated nucleus/centrosome complex from cells expressing HcpA-GFP is shown. The centrosome is marked with an asterisk. The three dark areas represent nuclear caps (nucleoli). The magnification on the right shows that 5nm gold labelled HcpA-GFP (arrows) is predominantly found in the vicinity of the centrosome in a strictly confined nuclear area. The nuclear envelope has been extracted by Triton X 100 during the isolation procedure. Bar: 200 nm. (Image by U. Euteneuer, LMU Munich; from: Kaller et al., 2006).

Fluorescence microscopy revealed that both HcpA-GFP and HcpB-GFP localized to one major cluster at the nuclear periphery, and several minor foci (Fig. 3.4A). Due to higher expression levels of the HcpB-GFP fusion, a higher nucleoplasmic staining background was observed compared to the HcpA-GFP-fusion. Although both constructs were driven by an actin15-promoter, this difference could also consistently

be detected in co-transformants expressing HcpA-RFP and HcpB-GFP fusion proteins. However, coexpression of HcpA-RFP and HcpB-GFP showed that HcpA and HcpB strictly colocalize (Fig. 3.4B), indicating that the subnuclear distribution of both isoforms is very similar. The remarkably different expression levels of HcpA-GFP and HcpB-GFP could also be demonstrated by SDS-PAGE of nuclear extracts from several independent clones of HcpA-GFP and HcpB-GFP expressing strains and Western blotting with an α-GFP-antibody (Fig. 3.4C).

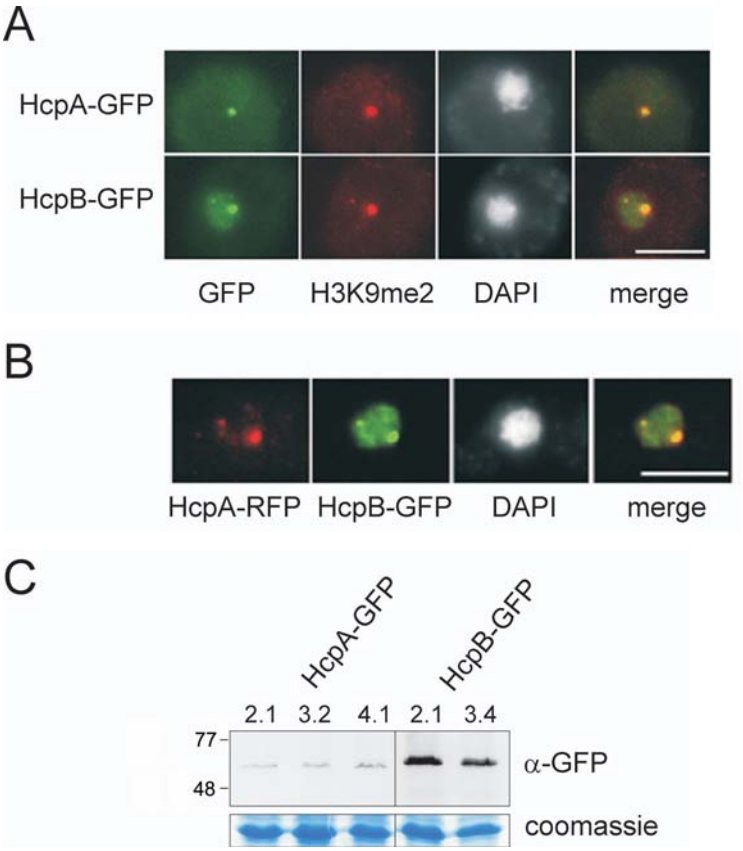

Fig. 3.4: **Analysis of subnuclear localisation of HcpA-GFP and HcpB-GFP**. A) HcpA-GFP and HcpB-GFP localize to one major and several minor foci at the nuclear periphery. Since the plain of the major spot is shown, only one or two minor foci are seen. Although driven by identical promoters, the fusion proteins display significant differences in expression levels. Note the increased nucleoplasmic staining of HcpB-GFP. Both HcpA-GFP and HcpB-GFP co-localize with endogenous histone H3K9 dimethylation (H3K9me2). Bar: 5 µm. B) Co-transformed HcpA-RFP- and HcpB-GFP-constructs demonstrate co-localization of HP1 isoforms. In this example, both proteins are seen in one major and one minor focus at the nuclear periphery. C) Western blot analysis of nuclear extracts from HcpA-GFP and HcpB-GFP expressing *Dictyostelium* cell lines shows highly dissimilar expression levels of the fusion proteins. Several independent clones of each transformation are shown.

Histone H3 dimethyl-K9 (H3K9me2) is a further hallmark for heterochromatin and serves as a binding site for HP1 proteins (Bannister et al., 2001; Lachner et al., 2001). To test for this association in *Dictyostelium*, double labeling experiments were performed and showed that both HcpA-GFP and HcpB-GFP co-localized with H3K9me2 (Fig. 3.4A). The same was true for the respective N-terminal GFP-fusion proteins (termed GFP-HcpA, GFP-HcpB; data not shown). The specificity of the H3K9me2 antibody, originally raised against methylated human histone H3, was verified by Western blotting. It recognized a single 17 kDa-protein which corresponds to the size of the *Dictyostelium* histone H3 (Fig. 3.5B).

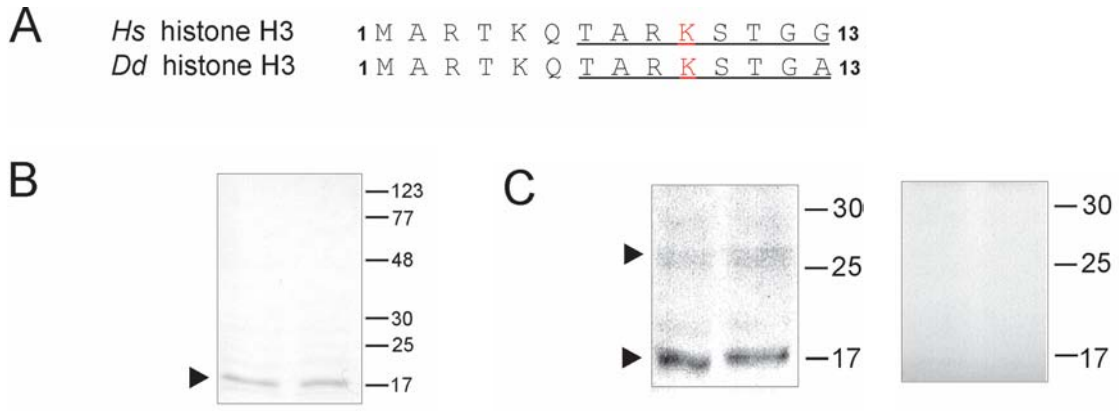

Fig. 3.5: **Validation of the α-H3K9me2 antibody.** A) Amino acid alignment of the histone H3 N-termini of human and *Dictyostelium* histone H3. The peptide used for rabbit immunization and antibody generation is underlined. The lysine-9 residue of histone H3 is marked in red. B) Nuclear extracts from two different *Dictyostelium* cell lines were separated by SDS-PAGE and analysed with the α-H3K9me2-antibody by Western blot. The antibody recognized a ~ 17kDa protein, which fits with the *in silico* calculated size of the three predicted *Dictyostelium* histone H3 variants (~15,7 kDa). C) His-HcpA binds to a protein with similar size as histone H3 in Far Western blots. Nuclear extracts from two different *Dictyostelium* cell lines were separated by SDS-PAGE, blotted onto a nitrocellulose membrane and incubated with *E.coli* lysates from cells expressing His-HcpA (left) or with *E.coli* lysates from cells transformed with an empty pET15b vector (right). Arrows indicate proteins that are bound by His-HcpA.

In contrast to that, attempts to detect histone H3K9-trimethylation (H3K9me3) with H3K9me3-specific antibodies (a kind gift from T. Jenuwein, Vienna), either by Western blot or immunocytochemistry, failed (data not shown). Although the presence of monomethyl-K9 (H3K9me1) has not been tested, it is likely that H3K9me2 is the main heterochromatic histone modification state of the lysine 9 residue in *Dictyostelium*.

Apart from the observed co-localisation of HcpA/HcpB with H3K9me2 *in vivo*, His-tagged HcpA also bound to a protein of the size of histone H3 in Far Western blots

(Fig. 3.5C). It cannot be ruled out that the protein bound by His-HcpA is different from histone H3, because no competetion with histone H3-derived peptides, which would allow to evaluate binding specificity, was performed on the Far Western blots. However, the data indicate that binding of HcpA (and likely HcpB) to H3K9me2 is likely to be conserved in *Dictyostelium*.

3.3 HcpA and HcpB form homo- and heterodimers *in vitro* and *in vivo*

A conserved feature of HP1 proteins is their ability to form multimeric complexes by homo- and heterodimerisation through their CSDs (Brasher et al., 2000; Nielsen et al., 2001). Since we found largely identical subnuclear distribution of both Hcp isoforms, we determined if HcpA and HcpB are capable of dimerisation *in vitro*. Bacterially expressed His-tagged HcpA, HcpB and murine HP1α were immobilized and purified on Ni-sepharose columns (Fig. 3.6).

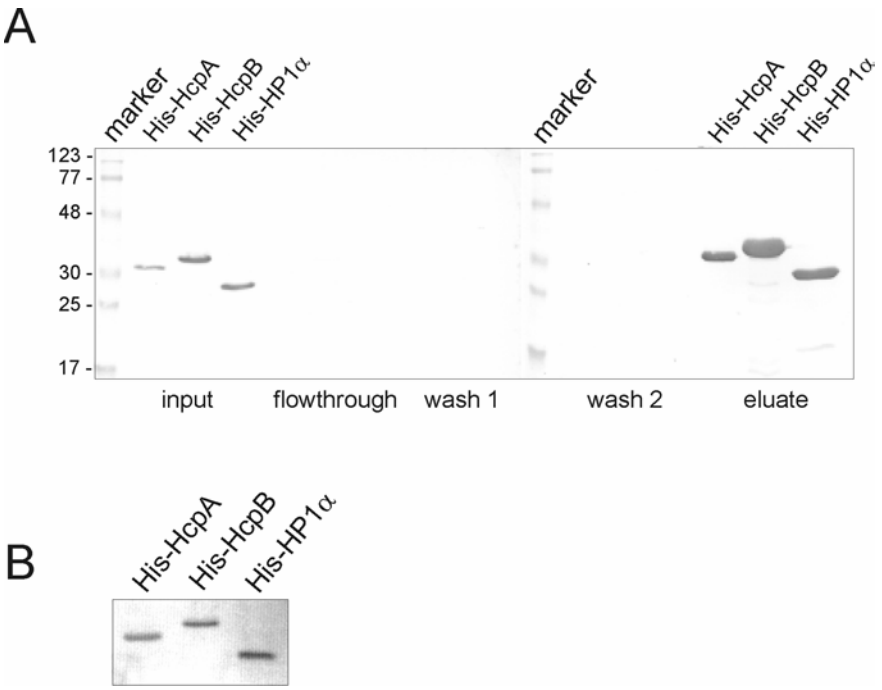

Fig. 3.6: **Purification of His-HcpA, His-HcpB and His-HP1α by affinity chromatgrophy with Ni-sepharose.** A) Western blot with an α-6xHis-antibody on the input, flowthrough and the two wash fractions of each protein purification. B) Purified His-HcpA, His-HcpB and murine His-HP1α from the eluates was separated by SDS-PAGE and stained with Coomassie. Note the almost identical protein amounts in the eluates.

Extracts from *Dictyostelium* cells expressing GFP-HcpA were passed over the matrix and bound proteins were eluted with imidazole (Fig. 3.7A). Both His-HcpA and His-HcpB were able to retain GFP-HcpA and GFP-HcpB on the column (Fig. 3.7B). This showed that the *Dictyostelium* proteins could form both homo- and heterodimers.

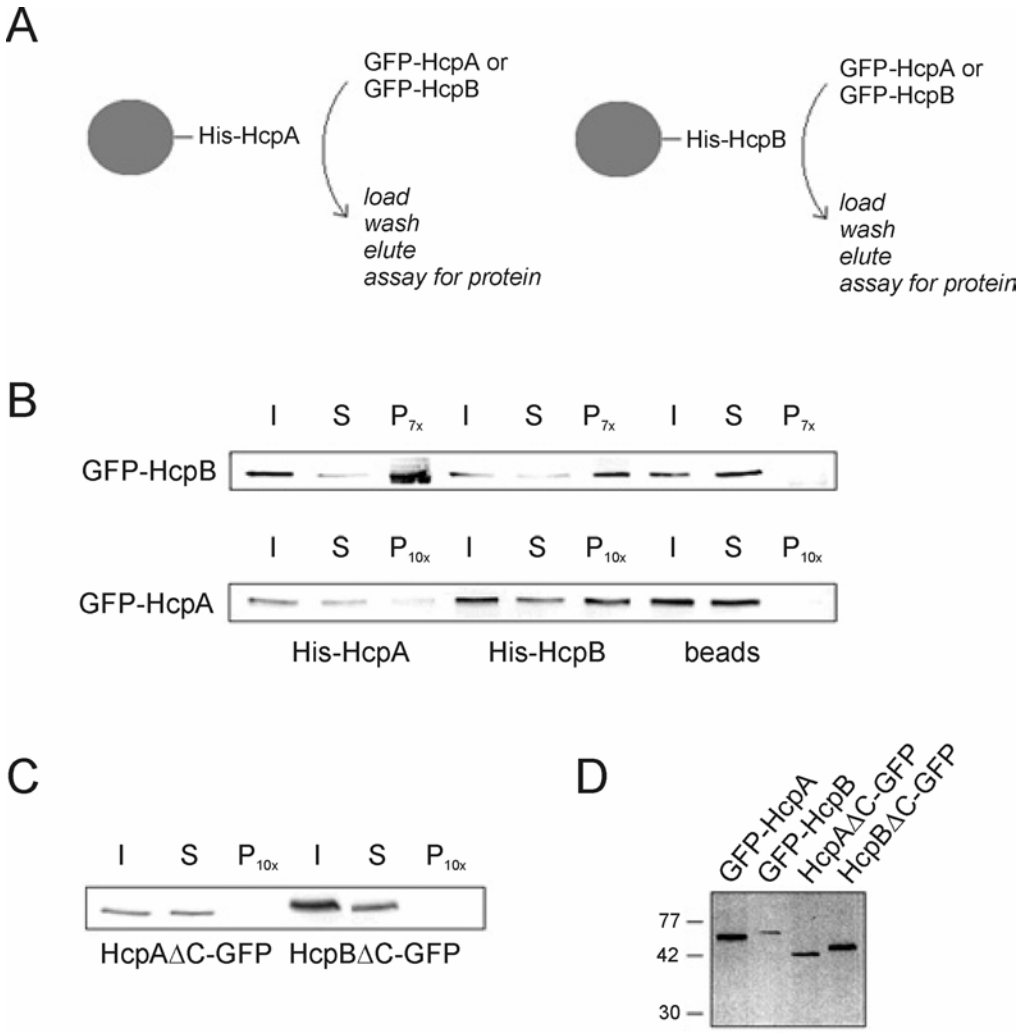

Fig. 3.7: **Homo- and heterodimer formation of HcpA and HcpB proteins *in vitro.***

A) Scheme for pull-down analysis. Ni-sepharose beads pre-loaded with either His-HcpA or His-HcpB were incubated with *Dictyostelium* cell lysates containing GFP-HcpA and GFP-HcpB were treated as shown. B) GFP-HcpA and GFP-HcpB bind to both His-HcpA or His-HcpB, but not to empty beads.

C) Neither HcpAΔC-GFP nor HcpBΔC-GFP can bind to His-HcpA. His-HcpA was immobilized on Ni-sepharose beads and incubated with cell lysates containing either HcpAΔC-GFP or HcpBΔC-GFP. E) Western blots showing proteins used in this study. Left: bacterially expressed His-HcpA and His-HcpB. Right: Cell lysates from indicated *Dictyostelium* overexpression strains were probed with α–GFP antibody. Abbreviations: I:Input; S:supernatant; P:pellet. Pellet fractions were either 7- or 10-fold concentrated (as indicated).

Although both proteins in general were capable of homo- and heterodimer formation, it appeared that the affinities for homo- and heterodimerisation were significantly different between HcpA and HcpB. This was indicated by a significantly weaker amount of GFP-HcpA than GFP-HcpB that was reproducibly retained on the columns.
HcpA-homodimer formation appeared to be less favourable than HcpA-HcpB-heterodimer or HcpB-homodimer formation (HcpB-HcpB >> HcpA-HcpB >> HcpA-HcpA). Although the functional significance of this is currently unclear, it may indicate that the two isoforms differentially contribute to heterochromatin formation by differential oligomerisation dynamics. Alternatively, differential posttranslational modifications, such as phosphorylation (Badugu et al., 2005), in the GFP-tagged proteins obtained from *Dictyostelium* extracts had an influence on binding.
GFP fusions of HcpA or HcpB lacking the CSD (HcpAΔC-GFP, HcpBΔC-GFP) could not form any dimers with immobilized His-HcpA (Fig. 3.7C), indicating that the CSD is required for dimerisation. This is consistent with findings for HP1 homologues from other organisms.
Interestingly, mouse HP1α, although structurally closely related, was not able to bind GFP-HcpA (Fig. 3.8). This is reminiscent of domain swap experiments between *S. pombe* Swi6 and mouse HP1β (M31), where neither M31 nor a chimeric Swi6 protein with an M31 CSD was able to complement the *swi6*$^-$ phenotype in *S. pombe* (Wang et al., 2000). Both results indicate that, although being structurally and functionally conserved among species, slight alterations in the primary amino acid sequence of the CSD determine the species-specificity of protein-protein-interactions.

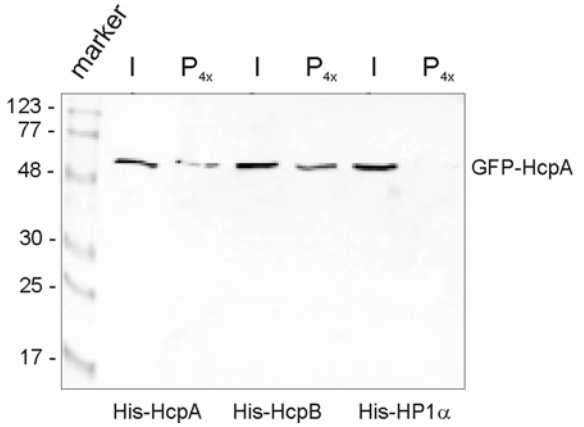

Fig. 3.8: **GFP-HcpA interacts with His-HcpA and His-HcpB, but not with murine His-HP1α.** Pull-downs were basically done as described in Materials and Methods, but with slight alterations. Column volume was 1ml, and proteins were eluted (pellet) in one 2.5ml fraction. A Western blot with anti-GFP-antibody of input (I) and pellet (P) fractions to detect GFP-HcpA is shown. Pellets were 4-fold concentrated.

To confirm that both isoforms are also capable of homo- and heterodimerisation *in vivo*, cell lines that stably expressed both 6xHis-tagged HcpA alone or in combination with either GFP-HcpA or GFP-HcpB were generated. Purification of His-HcpA expressed in *Dictyostelium* by Ni-sepharose beads was highly efficient, since His-HcpA was effectively enriched in the eluate, compared to the almost undetectable amounts in the input fractions due to the very low expression levels of His-HcpA (Fig. 3.9B)

Ni-sepharose beads were then challenged with cell extracts from His-HcpA / GFP-HcpA or His-HcpA / GFP-HcpB cotransformed cell lines (Fig. 3.9A). From extracts of co-transformed cell lines, GFP-HcpA and GFP-HcpB could be co-eluted with His-HcpA, indicating that both isoforms form oligomeric complexes *in vivo* (Fig. 3.9C).

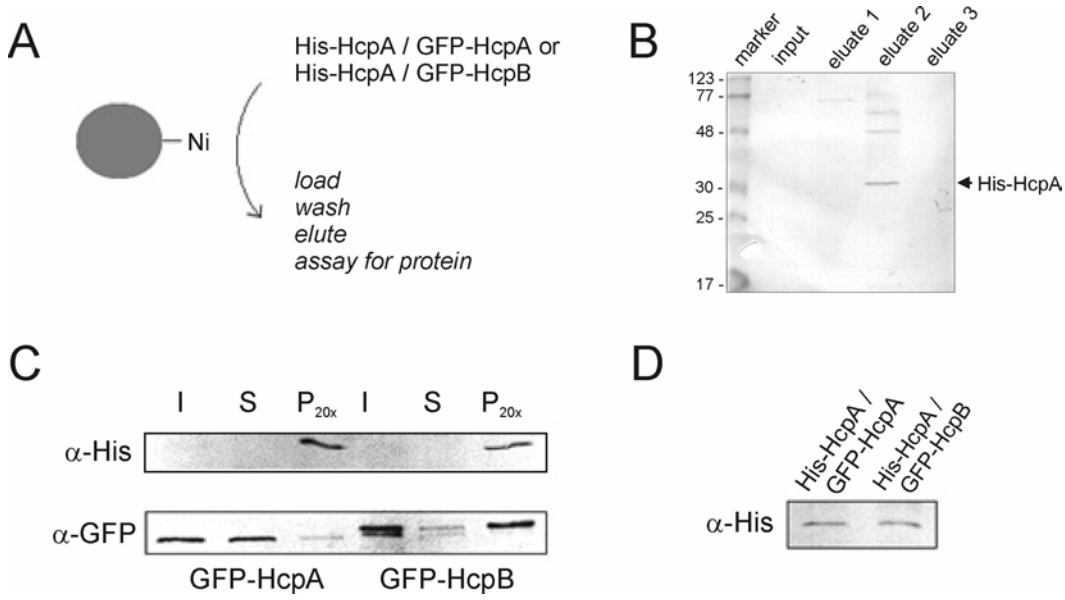

Fig. 3.9: **Homo- and heterodimer formation of HcpA and HcpB proteins *in vivo.***

A) Scheme for pull-down analysis. Ni-sepharose beads were incubated with cell lysates from *Dictyostelium* cell lines cotransformed with His-HcpA and GFP-HcpA or GFP-HcpB. B) Validation of the affinity-purification method of *in vivo* expressed His-HcpA by Ni-sepharose. His-HcpA is under control of an actin15-promoter and expressed, similar to HcpA-GFP (Fig. 3.4A and 3.4C) at very low levels in the cell. It is therefore hardly detectable in the input fraction, but effectively enriched in the eluate. The eluate (fraction) 2 is 20-fold enriched compared to the input. C) Both GFP-HcpA and GFP-HcpB are co-eluted with His-HcpA from Ni-sepharose beads. D) His-HcpA is detectable in nuclear extracts of co-transformants. Nuclear extracts were subjected to SDS-PAGE and Western blot. His-HcpA was detected with an α-His-antibody. Abbreviations: I:Input; S:supernatant; P:pellet. Pellet fractions were 20-fold concentrated.

Remarkably, significantly more GFP-HcpB than GFP-HcpA was co-eluted with His-HcpA, although the expression levels of His-HcpA were very similar in both cell lines (Fig. 3.9D), and the respective GFP-fusion proteins were present in great excess compared to His-HcpA. The data indicate that the stoichiometry of both isoforms within oligomeric complexes was significantly different in the two co-transformed cell lines. Furthermore, the data show that the differential dimerisation preferences of HcpA and HcpB observed *in vitro* are also observed *in vivo*.

3.4 Differential dimerisation dynamics are conferred by the C-termini of HcpA and HcpB

To further validate the differential dimerisation preferences of HcpA and HcpB, competetive pull-downs were performed. For this purpose, extracts of GFP-HcpA and GFP-HcpB expressing *Dictyostelium* cells were mixed and allowed to compete for binding to recombinant His-HcpA or His-HcpB immobilized on Ni-sepharose beads.
In agreement with the previous findings, both the His-HcpA and the His-HcpB matrices retained predominantly the GFP-HcpB protein, indicated by a reversal of isoform ratios from input to the eluted fraction (Fig. 3.10C). Since the C-terminal CSD is required for dimerisation (Cowieson et al., 2000; also see Fig. 3.7C), we tested if exchanging the C-termini of HcpA and HcpB would influence the dimerisation specifity. For this purpose, chimeric Hcp fusion proteins that contained the N-terminus of one isoform and the C-terminus of the other isoform (Fig. 3.10A) were expressed in *Dictyostelium* (Fig. 3.10B). Semiquantitative competitive pull-downs of protein mixtures containing HcpA, HcpB or the respective chimeric proteins showed that those Hcp proteins that contained the C-terminus of HcpB bound more efficiently to matrix-immobilised His-HcpA or His-HcpB than competitor proteins containing the C-terminus of HcpA. This was indicated by a reversal of the respective protein amounts in the eluted fractions compared to the input fractions (Fig. 3.10C). By this experiment, it could be demonstrated that the differential dimerisation preferences of HcpA and HcpB are conferred by the C-termini, including hinge and CSD, of the respective isoform. Although it was shown that the CSD is obligatory for dimerisation (Fig. 3.7C), with these experiments it was not dissected if either the hinge or the CSD, or both in combination are required to confer the specific preferences to the proteins.

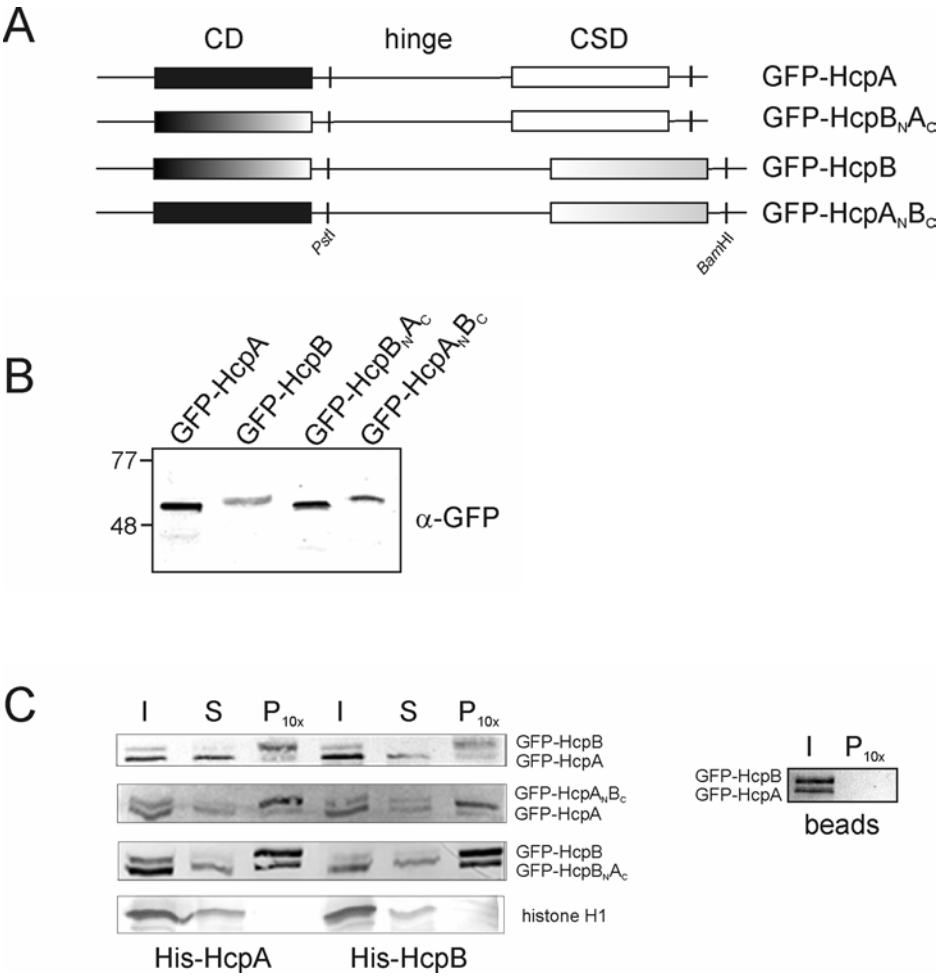

Fig. 3.10: **Exchange of the C-termini of HcpA and HcpB confers distinct dimerisation properties to the chimeric proteins**. A) Scheme for generation of chimeric proteins. The positions of the PstI and BamHI restriction sites used for cloning are indicated. B) Western blot of whole cell extracts form strains expressing GFP-HcpA, GFP-HcpB and the chimeras. Note that the size differences of the different proteins are due to the C-terminal parts of the proteins. C) Competetive pull-downs of protein mixtures indicated. Note that the ratio of the different isoforms in the input fractions in each experiment is almost reversed in the eluted fractions. GFP-tagged proteins can be eluted from beads that were pre-coated with either His-HcpA or His-HcpB, but not from empty beads. For comparison, histone H1 is not bound by immobilised His-HcpA or His-HcpB. For immunodetection of histone H1, a polyclonal α-histone H1 antibody from rabbit (a kind gift from E. Schulze, Göttingen) in a 1:50 dilution was applied.

The pronounced difference in CSD-mediated dimerisation efficiency may also reflect differences in the interaction with other protein partners. Since dimerisation of HP1 proteins is required for recruiting further interacting proteins (Brasher et al., 2000), preferential dimerisation of HP1 isoforms may result in a distinct stoichiometry of different multiprotein complexes.

3.5 Mitotic dynamics of *Dictyostelium* heterochromatin

In interphase cells, the major heterochromatic cluster is closely associated with the centrosome, a nucleus-associated body in *Dictyostelium* (see Fig. 3.3). Since this association was stable in randomly migrating and even in chemotaxing cells (Fig. 3.11), this indicated a functional relationship and was reminiscent of centromeric heterochromatin in *S. pombe*, that is closely connected to the nuclear-membrane-embedded spindle pole body (Funabiki et al., 1993).

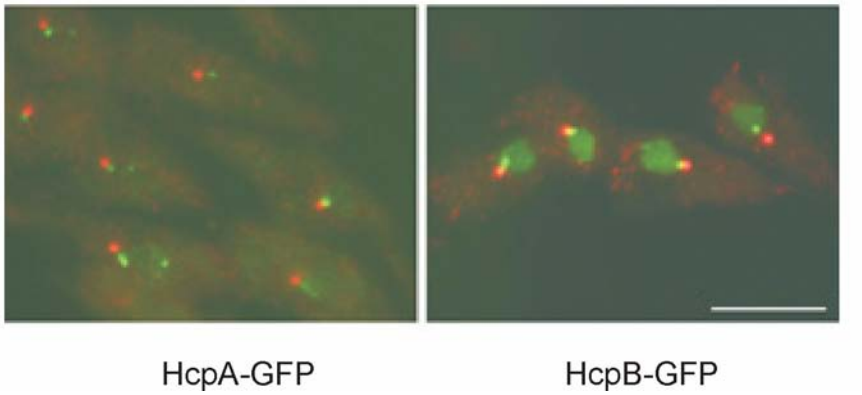

Fig. 3.11: **Centrosome and the major heterochromatin cluster remain associated in chemotaxing cells.** Cells expressing HcpA-GFP (left) or HcpB-GFP (right) were placed on coverslips at a density of 1 x 10^6 cells /ml and starved overnight under phosphate buffer. The emerging streams were fixed and immunostained for the detection of centrosomes with an anti-DdCP224-antibody (red). Bar: 10 µm.

To examine if the major heterochromatic cluster represented *Dictyostelium* centromeres, we analyzed HcpA/HcpB-GFP distribution during mitotic stages in asynchronously growing *Dictyostelium* cells. Mitoses were identified by antibodies directed either against DdCP224, a *Dictyostelium* XMAP215 homologue that stains the centrosome and the mitotic spindle (Graf et al., 2000; Graf et al., 2003), or α-tubulin (Kilmartin et al., 1982).

During interphase, both Hcp-GFP-fusion proteins localized to one prominent cluster at the nuclear periphery directly opposite to the centrosome (Fig. 3.12). In prophase, when *Dictyostelium* centrosomes are duplicated, the cluster split up into several smaller foci. In some preparations up to six spots could be distinguished that may represent the centromeres of the six *Dictyostelium* chromosomes (Fig. 3.12A, prophase inset). This is similar to pericentromeric heterochromatin in *S. pombe* that also splits up during mitotic prophase (Funabiki et al., 1993; Pidoux et al., 2000). In prometaphase, when the duplicated spindle poles separate and penetrate the nuclear envelope, the majority of the nucleoplasmic HcpA/HcpB-GFP enters the cytoplasm,

indicated by a significant increase in cytoplasmic and loss of nucleoplasmic GFP staining. Probably this represents passive diffusion and not an active process since a similar relocalisation has been described for NLS-tagged GFP (Zang et al., 1997). Both observations argue that mitosis may not be completely closed in *Dictyostelium*. In metaphase, heterochromatin localizes between a bilayered structure at the central

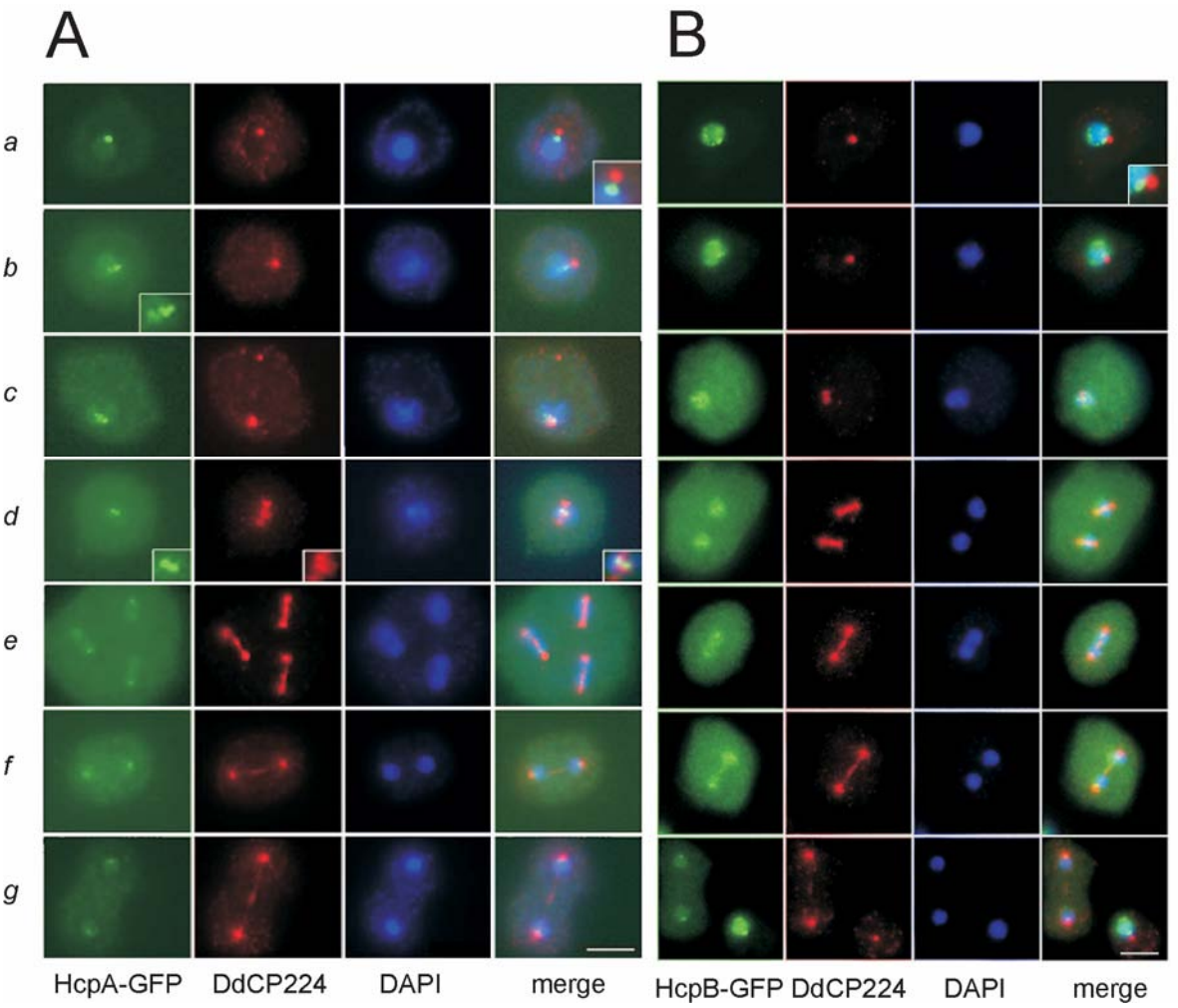

Fig. 3.12: **Mitotic dynamics of *Dictyostelium* heterochromatin.** A) Cells expressing HcpA-GFP. B) cells expressing HcpB-GFP. During interphase, heterochromatin localizes next to the nucleus-associated centrosome (see inset). During prophase, the heterochromatin cluster divides in up to six spots (see inset). During prometaphase, nucleoplasmic protein enters the cytoplasm, indicated by increased cytoplasmic fluorescence. The telophase panel for HcpB-GFP shows a telophase and an interphase cell to illustrate the loss of nuclear fluorescence during mitosis. Centrosomes and mitotic spindle are stained with an anti-DdCP224 antibody. *a*: interphase, *b*: prophase, *c*: prometaphase, *d*: metaphase, *e*: early anaphase, *f*: late anaphase, *g*: telophase. Bar: 5 µm.

spindle that is stained by the anti-DdCP224 antibody (Fig. 3.12) and that was suggested to represent the kinetochore region (Rehberg and Graf, 2002). During anaphase, the heterochromatic clusters divided and moved towards the spindle poles, leading the separated DNA masses. Although GFP-fused Hcp proteins stay associated with heterochromatin throughout the entire cell cycle, we reproducibly detected a significant loss of Hcp proteins at heterochromatin during late mitotic stages compared to interphase (Fig. 3.12, telophase panel). This mitotic loss of Hcp proteins from heterochromatin was observed for both N-terminal and C-terminal GFP-fusions of HcpA and HcpB (data not shown). In contrast, the H3K9me2 signal intensity during late mitotic stages was almost identical to that observed in interphase (Fig. 3.13). The results indicated that heterochromatic localisation of Hcp proteins is dynamically regulated during the cell cycle, whereas H3K9me2 represents a stable heterochromatic imprint that persists during cell division.

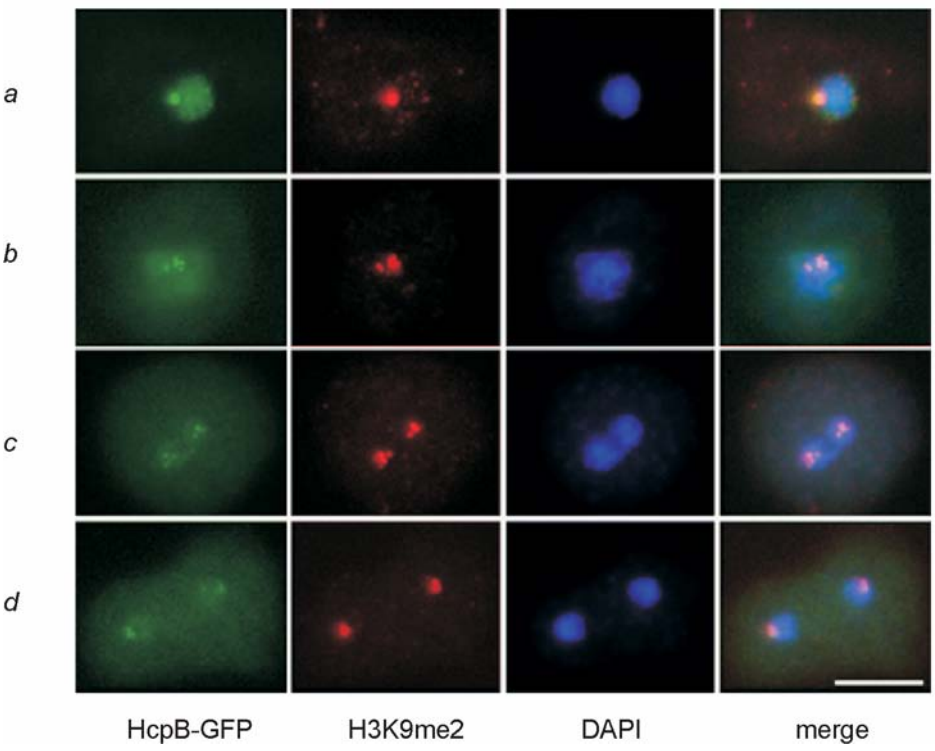

Fig. 3.13: **Mitotic dynamics of HcpB-GFP and histone H3K9me2.** Whereas H3K9me2 is stable during mitosis, the fluorescence intensity of HcpB-GFP at centromeres and in the nucleoplasm significantly decreases. Images in vertical rows were captured with identical exposure times. Similar results were obtained with HcpA-GFP and the respective N-terminal GFP-fusions (data not shown). *a*: interphase; *b*: prophase; *c*: early anaphase; *d*: telophase. Bar: 5 µm.

To further quantify the mitotic delocalisation of Hcp proteins from heterochromatin, we generated a cell line that lacked both endogenous HcpA/HcpB and only expressed GFP-HcpA (*hcp*$^{AB-}$/GFP-HcpA, see 3.7). Thus, this strain allowed us to investigate the localisation of GFP-HcpA to heterochromatin more thoroughly, since GFP-HcpA compensated for both endogenous proteins and should therefore reflect their dynamics more closely than in an overexpression strain, where the recombinant protein competed with the endogenous proteins.

Similarly to overexpression of GFP-HcpA in the Ax2 background (data not shown). GFP-HcpA localisation to heterochromatin during mitosis was largely diminished (Fig. 3.14). This is in agreement with findings from other systems such as mammalian cell lines which report a strong decrease of heterochromatic HP1 binding during mitosis due to Aurora kinase activity, which phosphorylates serine-10 of histone H3. Serine-10 lies adjacent to the methylated lysine-9 residue and its phosphorylation disrupts binding of HP1 (Fischle et al., 2005; Hirota et al., 2005). Furthermore, HP1 proteins themselves have been reported to be differentially phosphorylated during mitosis (Minc et al., 1999), which may contribute to dissociation from heterochromatin.

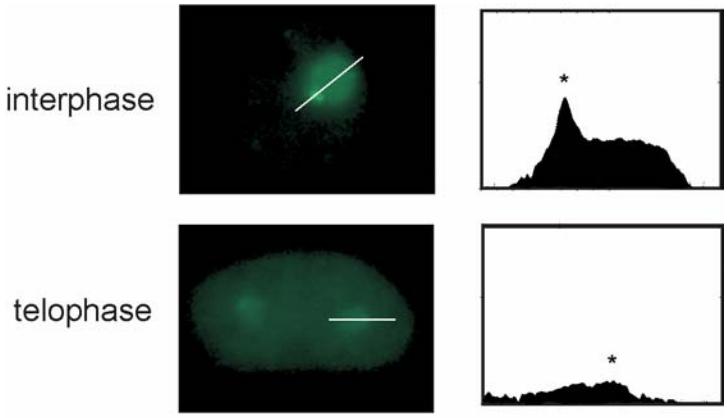

Fig. 3.14: **Mitotic dissociation of GFP-HcpA from heterochromatin.** Nuclear and heterochromatic fluorescence of GFP-HcpA in the *hcp*$^{AB-}$/GFP-HcpA cell line is pronounced during interphase, but decreases significantly after metaphase and especially during late mitotic stages. Images (left) were acquired with identical exposure times. Image processing for mitotic stages was done according to a fixed protocol, ensuring the quantitative differences in the originally acquired images. To further quantify the differences in fluorescence intensity during interphase and telophase, fluorescence intensity profiling was carried out using the TINA 2.0 software (right). The regions used for profiling are indicated by white lines in the corresponding images on the left. The y-axis shows fluorescence intensity (in arbitrary units), the x-axis the length of the profile. The asterisks indicate the positions of pericentromeric heterochromatin.

3.6 A proof-of-principle: Mitotic co-localisation of DdINCENP with HcpA and H3K9me2

Although the data presented demonstrate a compelling correlation between the major heterochromatic cluster and the *Dictyostelium* centromeres, co-localisation of HcpA/HcpB or H3K9me2 with centromere/kinetochore-specific proteins should provide further evidence that *Dictyostelium* heterochromatin is located at the centromeres.

In mammals, INCENP is a component of the chromosomal passenger complex (CPC), that consists of INCENP, Survivin, Borealin and the catalytic core, AuroraB (Vader et al, 2006). AuroraB kinase function is required for the control of mitotic progression, and interaction of AuroraB with INCENP is crucial to guide the AuroraB kinase activity in spatiotemporarily coordinated manner during mitosis. INCENP localizes to centromeres/kinetochores in metaphase, where interaction with the AuroraB and polo-like kinase-1 (Plk1) regulates metaphase/anaphase transition (Goto et al., 2006).

The *Dictyostelium* INCENP homologue, DdINCENP, has recently been described (Chen et al., 2006), but its mitotic localisation to centromeres/kinetochores has not been described in detail due to the lack of proper (endogenous) centromere markers. Therefore, it was tested if DdINCENP would co-localize with HcpA or H3K9me2 in metaphase cells. In interphase, DdINCENP-GFP displayed a punctate nuclear distribution that did not overlap with HcpA (Fig. 3.15A). This finding is consistent with results form other organisms, which did not detect centromeric localisation of INCENP during interphase (Carmena and Earnshaw, 2006). In metaphase, DdINCENP-GFP is targeted to the centromeres/kinetochores, which is indicated by co-localisation with HcpA-RFP. In agreement with previous findings, DdINCENP-GFP then moves towards the central spindle in anaphase (Chen et al., 2006). Furthermore, a significant portion is associated with the spindle poles, and in addition, a minor portion that stays associated with the centromeres, as indicated by co-localisation with HcpA-RFP, was detected. Remarkably, the dual localisation of INCENP-GFP at centromeres and centrosomes only was observed in INCENP / HcpA cotransformants, but not in INCENP-GFP single transformants. Since INCENP has been reported to bind to HP1 (Ainsztein et al., 1998), it remains possible that ectopic overexpression of RFP-tagged HcpA artificially tethers a portion of INCENP to centromeres in late mitosis.

Nearly identical results were obtained for the (co-)localisation of DdINCENP-GFP and H3K9me2 during metaphase and anaphase, although the dual localisation of

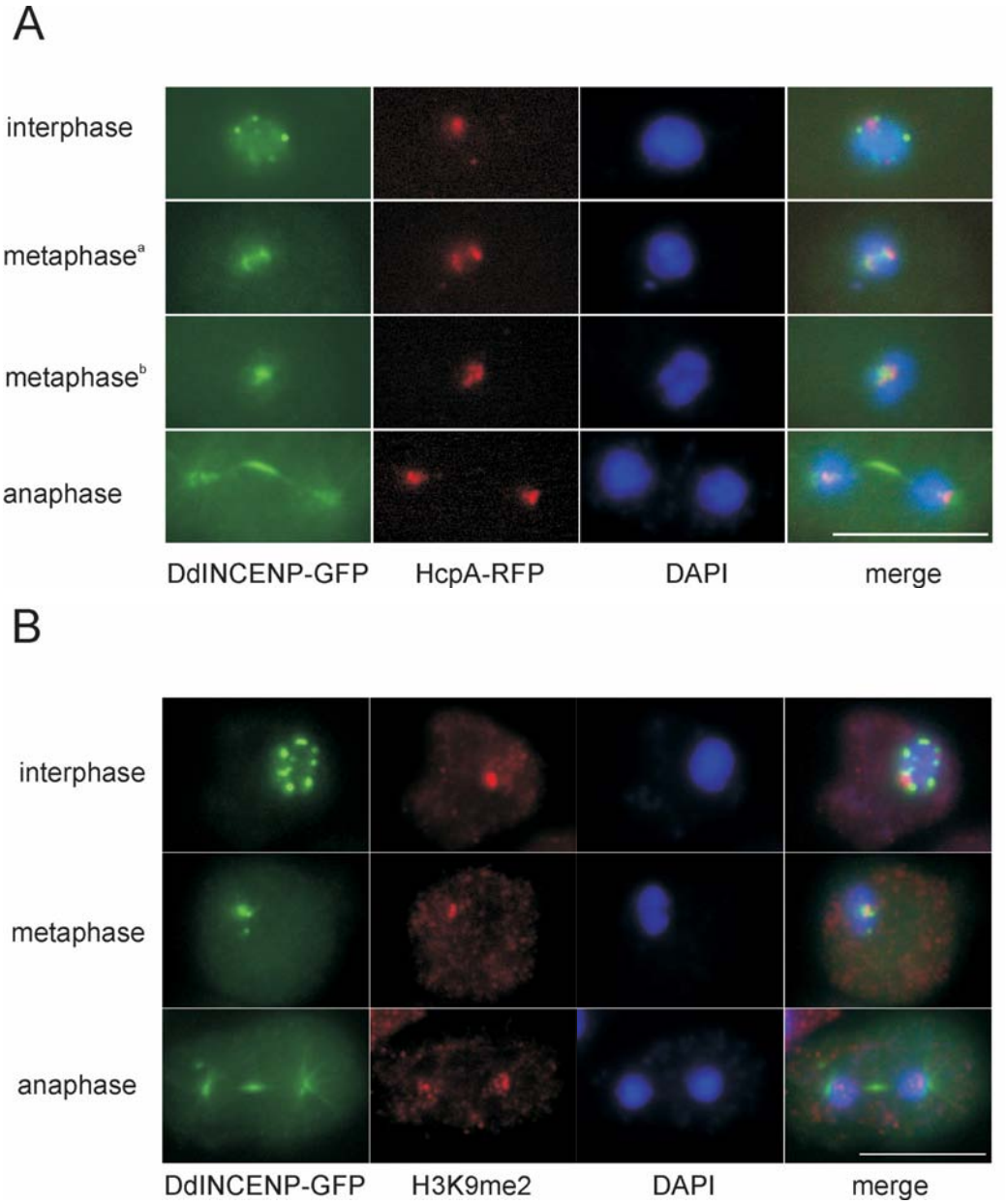

Fig. 3.15: **Mitotic localisation of DdINCENP and HcpA and H3K9me2.** A) Asynchronous cultures co-transformed with DdINCENP-GFP and HcpA-RFP were fixed with methanol and screened for mitotic cells. The cell in the metaphase[a] panel very likely is in the metaphase-anaphase transition, with its centromeres just separating. a) side view of the metaphase spindle b) top view of the metaphase spindle. B) In a similar approach, cell lines transformed with DdINCENP-GFP were stained with the H3K9me2-specific antibody.

INCENP-GFP at centromeres and centrosomes during anaphase was not observed anymore (Fig. 3.15B).

The results support each other in the assumption that DdINCENP-GFP localizes to centromeres/kinetochores during metaphase, and that the centromeres/kinetochores

are indeed found within heterochromatic regions of the genome that are marked by histone H3K9 methylation and HP1.

Intruigingly, known phosphorylation targets of both AuroraA and AuroraB kinases include the serine-10 residue of histone H3. Phosphorylation of S10 on a histone tail that is methylated at K9 drastically lowers the affinity of HP1 for the histone tail, thereby leading to HP1 dissociation from heterochromatin during mitosis (Fischle et al., 2005). Since it was shown that DdINCENP localizes to centromeres during prometa- / metaphase, it is well possible that recruitment of the Aurora kinase activity to centromeric heterochromatin by INCENP contributes to HcpA/HcpB dissociation from heterochromatin during mitosis. Remarkbly, the H3K9me2 signals in the INCENP-GFP overexpression strain during mitosis were significantly weaker than in wild-type Ax2 cells and very often barely detectable, whereas normal signal intensities were observed in interphase cells, indicating that the H3K9me2 epitope is partially blocked in mitosis. This might be due to increased histone H3S10 phosphorylation by Aurora kinase, which might interfere with H3K9me2 recognition by the antibody.

3.7 Knock-out mutants of *hcpA*, but not of *hcpB* show temperature-dependent growth defects

To analyze the function of the two HP1 isoforms *in vivo*, knock-out strains for both *hcpA* and *hcpB* were generated by homologous recombination (Fig. 3.16). Under standard laboratory conditions, none of the single knock-out strains displayed a significant phenotype in axenic suspension culture (Table 3.1), during development or during spore germination (data not shown). However, the *hcpA*$^-$ *,* but not the *hcpB*$^-$ strain displayed a slight growth defect at lower temperatures in suspension culture compared to the parent Ax2 strain (Table 3.1). In *S. pombe*, knock-out of Swi6 causes a similar temperature-dependent growth defect that is characterised by increased chromosome missegregation rates and can be mimicked by microtubule-destabilizing drugs such as thiabendazole (Ekwall et al., 1995; Ekwall et al., 1996). In contrast to that, we could not detect any defects in mitotic progression, as determined by the relative abundance of mitotic stages in asynchronous cultures, or an obvious chromosome missegregation phenotype (data not shown). Furthermore, the temperature-sensitivity of the *hcpA*$^-$ mutant could not be mimicked by thiabendazole treatment, indicating that microtubule (dys-)function was not involved (Table 3.1). Therefore, the reason for the temperature-sensitivity of the *hcpA*$^-$ mutant remains

unclear. However, the lack of any apparent phenotype under standard conditions argued for functional redundancy of the two Hcp isoforms.

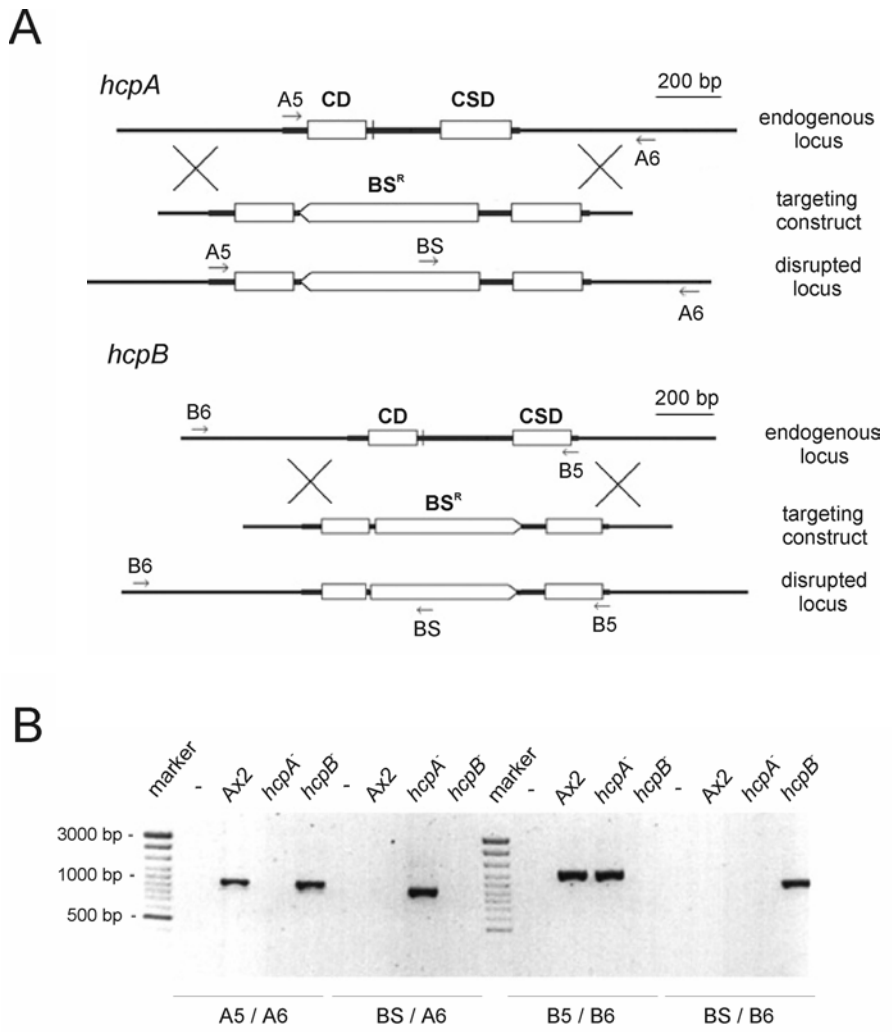

Fig. 3.16: **Strategy for targeted disruption of the *hcpA* and *hcpB* genes.** A) Schematic representation of the genomic *hcpA* and *hcpB* loci, the targeting constructs and the genomic loci after homologous recombination. Chromo domain and chromo shadow domain are shown as boxes. The thin vertical line right of the chromo domain marks the nuclear localisation signal. Coding regions are depicted as thick horizontal lines, the flanking genomic sequences as thin horizontal lines. The recombinogenic arms of the targeting constructs largely consist of flanking genomic sequence, thus ensuring isoform-specific targeting. The primer combination A5/A6 (B5/B6) allows amplification of the wild type *hcpA* (*hcpB*) locus, but not the disrupted *hcpA* (*hcpB*) locus under PCR conditions used. Targeted disruption of the *hcpA* (*hcpB*) locus is verified with the primer combination BS/A6 (B5/BS). The primer-binding sites for primers A6 and B5 are not included in the targeting construct. Primer-binding sites are shown. For primer sequences, see Materials and Methods section. B) PCR analysis to detect isoform-specific gene disruption of the *hcpA* and *hcpB* genes in the indicated strains. Abbreviations: CD: chromo domain; CSD: chromo shadow domain, BS^R: blasticidin resistance cassette.

TABLE 3.1: Generation times of Ax2 and knock-out strains under different growth conditions[a]

growth condition	strain	generation time (± SD)
22°C	Ax2	9,3 (± 0,2)
	hcpA$^-$	8,7 (± 0,1)
	hcpB$^-$	8,8 (± 0,1)
15°C	Ax2	47,0 (± 0,2)
	hcpA$^-$	62,2 (± 5,4)
	hcpB$^-$	44,2 (± 8,1)
22°C / + 10µM TBZ	Ax2	15,9 (± 0,4)
	hcpA$^-$	14,6 (± 0,4)
	hcpB$^-$	15,4 (± 0,3)

[a] Mean generation times (in hours) and standard deviations from at least two independent clones are shown. Growth curves were measured in duplicate in axenic suspension culture in HL5 medium. Thiabendazole (TBZ) treatment was performed as described in the Materials and Methods section.

We therefore attempted to disrupt both endogenous genes to create double knock-out mutants. Since the gene disuption frequencies for the two *hcp* genes were very dissimilar (4% for *hcpA* compared to 67% for *hcpB*), we first created an *hcpA*$^-$ cell line, that was followed by Cre-mediated excision of the blasticidin resistance cassette (Fig. 17A). The resulting cell line, termed *hcpA*^{lp}, was then transformed with the *hcpB* targeting construct. Remarkably, we were not able to isolate any *hcpB*$^-$ clones in the *hcpA*^{lp} background from three independent transformations, indicating that disruption of the second *hcp* gene greatly affects or is essential for cell viability. Ectopic overexpression of a His-HcpA protein in the *hcpA*^{lp} background prior to *hcpB* targeting at least partially restored the disruption frequencies that we had observed in the Ax2 wild-type background (Fig. 3.17D; Table 3.2). Furthermore, no obvious phenotypic differences between the *hcp*$^{AB-}$/His-HcpA and the parental *hcpA*^{lp}/His-HcpA cells could be observed (data not shown).

In a similar approach, a cell line that ectopically expressed GFP-tagged HcpA (GFP-HcpA) under control of an actin6 promoter, but lacked the endogenous HcpA and HcpB proteins (*hcp*$^{AB-}$/GFP-HcpA), was created. Disruption frequencies of *hcpB* in Ax2, *hcpA*^{lp} and *hcpA*^{lp}/GFP-HcpA strains demonstrated that the GFP-tagged HcpA protein was fully functional and allowed to disrupt the *hcpB* gene in the *hcpA*^{lp} background (Fig. 3.17D; Table 3.2).

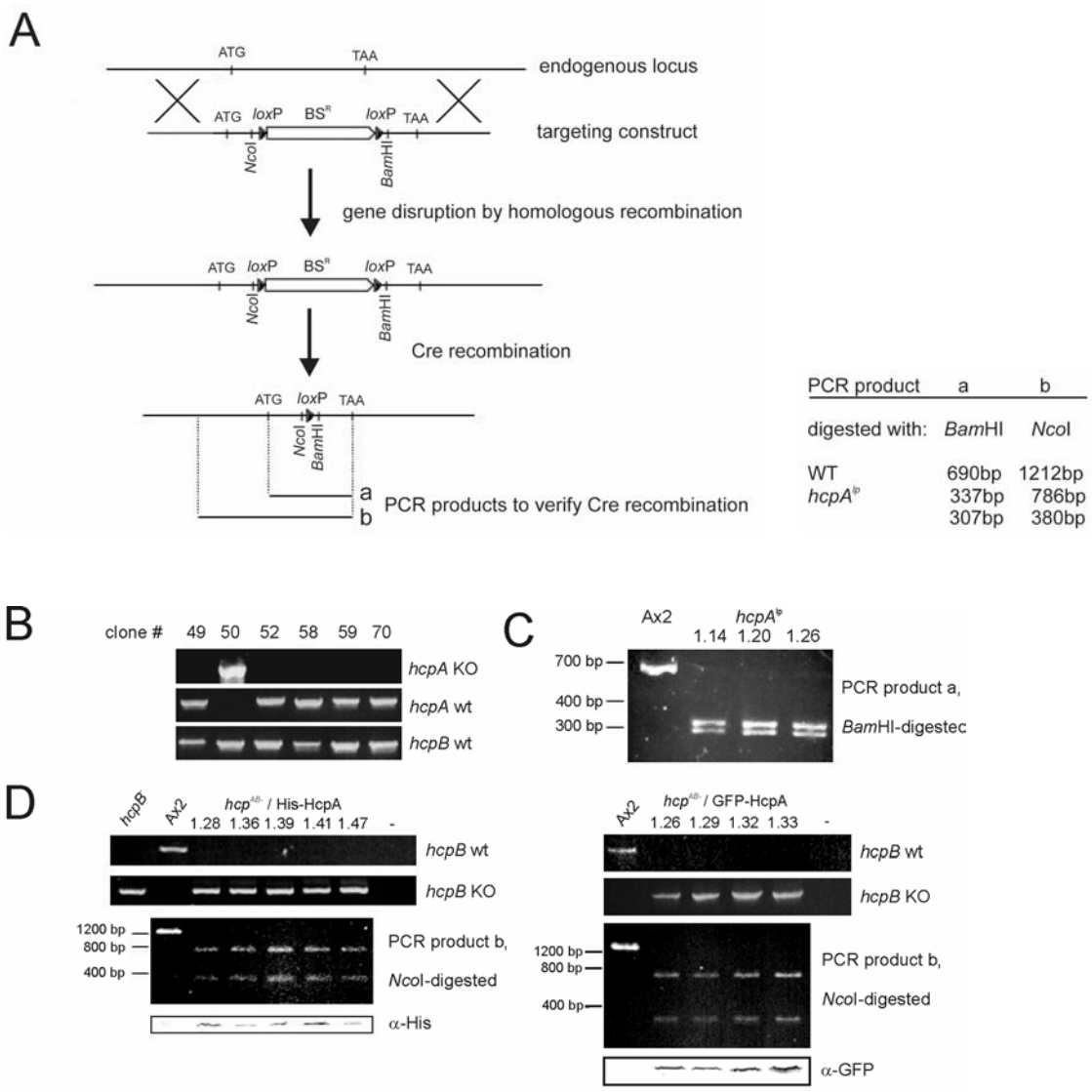

Fig. 3.17: **Disruption of endogenous genes of both *hcp* isoforms.** A) Scheme for *hcpA* gene targeting and subsequent Cre-mediated excision of the BS^R cassette. Gene disruption and Cre-recombination introduces restriction sites flanking the remaining *loxP* site at the endogenous locus. The recombinant *hcpA* allele ($hcpA^{lp}$) can be distinguished from the wild type allele by restriction digestions with enzymes indicated. B) The *hcpA* gene was disrupted by homologous recombination. Disruptants were discovered by PCR analysis with primers specific for the wild type gene (*hcpA* wt) or specific for homologous recombination of the targeting construct into the *hcpA* locus (*hcpA* KO). Clone #50 represents an *hcpA* knock-out. This clone was used for subsequent Cre-mediated excision of the floxed blasticidin resistance cassette. Note that the *hcpB* gene is unaffected (*hcpB* wt). C) Restriction fragment analysis of PCR products derived from either wild type (Ax2) or different recombinant $hcpA^{lp}$ clones after transient Cre-recombinase expression. D) Disruption of the *hcpB* gene after ectopic expression of His-HcpA (left) or GFP-HcpA (right). Clonal isolates were screened by PCR for either homologous recombination of the targeting construct (*hcpB* KO) or an intact *hcpB* gene (*hcpB* WT). Results for several independent clones are shown. The same clones were tested for presence of the Cre-recombinant $hcpA^{lp}$ allele by restriction fragment analysis. A longer PCR product covering the 5´ region

(Fig 3.17 continued)
of the endogenous *hcpA* locus was used in order to distinguish it from the transgenic His-HcpA locus. Clones were also tested for expression of His-HcpA (nuclear extracts) or GFP-HcpA (whole cell extracts) by immunodetection with either with α-His-antibody or α-GFP-antibody.

These findings indicate that the two *hcp* isoforms are largely redundant and that the presence of at least one isoform is essential for cell viability. Furthermore, the findings provided compelling genetic evidence that the GFP-HcpA protein was fully functional and able to replace the endogenous HcpA / HcpB proteins.

TABLE 3.2: Disruption frequencies for the *hcpB* gene in Ax2, *hcpA^{lp}*, *hcpA^{lp}* / His-HcpA and *hcpA^{lp}* / GFP-HcpA strains[a].

strain	disruption frequency
Ax2	67% (28/42)
hcpA^{lp}	0% (0/292)
hcpA^{lp} / His-HcpA	23% (9/40)
hcpA^{lp} / GFP-HcpA	50% (20/40)

[a] Number of homologous recombinations detected and total number of tested clones are given in parentheses.

3.8 Regulated depletion of HP1 proteins interferes with vegetative growth

Due to the inability to obtain *hcpA*/*hcpB* double knock-out mutants, we attempted to knock-down *hcpA* expression in an *hcpB*$^{-}$ strain by RNAi. Therefore, *hcpB*$^{-}$ cells were transformed with a plasmid (pdneo*hcpA*i) that drives transcription of a hairpin construct which forms ~ 500bp of *hcpA* dsRNA. The *hcpA* dsRNA should trigger RNAi-mediated depletion of *hcpA* mRNA, but no significant decrease in *hcpA* mRNA levels could be observed using semiquantitative RT-PCR in several independent subclones (data not shown). Therefore, we aimed to deplete Hcp protein levels in *hcp^{AB-}*/GFP-HcpA cell lines, where ectopically expressed GFP-HcpA compensated for both endogenous Hcp proteins. The *Dictyostelium* actin6 promoter is active in axenic suspension culture, but is down-regulated when *Dictyostelium* cells are cultured in bacterial suspension with *Klebsiella aerogenes* as a food source (Knecht and Loomis, 1987; Liu et al., 2002, Graf et al, 2003). Since the GFP-HcpA fusion construct was driven by an actin6-promoter, cultivation on bacterial lawns was used to down-regulate GFP-HcpA expression and to analyze the effects of HcpA depletion. Cultivation on

bacterial lawns and subsequent Western blot analysis of cell lysates indeed showed a significant decrease in GFP-HcpA protein amounts in both the parental *hcpA*^{lp}/GFP-HcpA and the *hcp*$^{AB-}$/GFP-HcpA cell lines (Fig. 3.18). Since the cell lysates were prepared from axenic suspension culture, where the actin6-promoter is re-activated, the loss of GFP-HcpA expression also reflects a loss of the genome-integrated expression plasmids under non-selective conditions on bacterial lawns. The *hcp*$^{AB-}$/ GFP-HcpA cells displayed growth defects compared to the parental cell line after prolonged cultivation on bacterial lawns, but no obvious developmental defects in the colonies could be detected (Fig. 3.18B).

These data indicated that depletion of Hcp proteins interferes with cell growth in *Dictyostelium*, but due to the diverse functions of HP1 proteins in regulation of chromosome structure and function, probably a multitude of different effects contributed to this growth defect. Furthermore, the observed growth defects were not very strong compared to the lethality of the *hcpA/hcpB* double null mutants, which indicates that even a strong decrease in GFP-HcpA protein levels still provides most of the cells with enough HcpA protein to ensure viability.

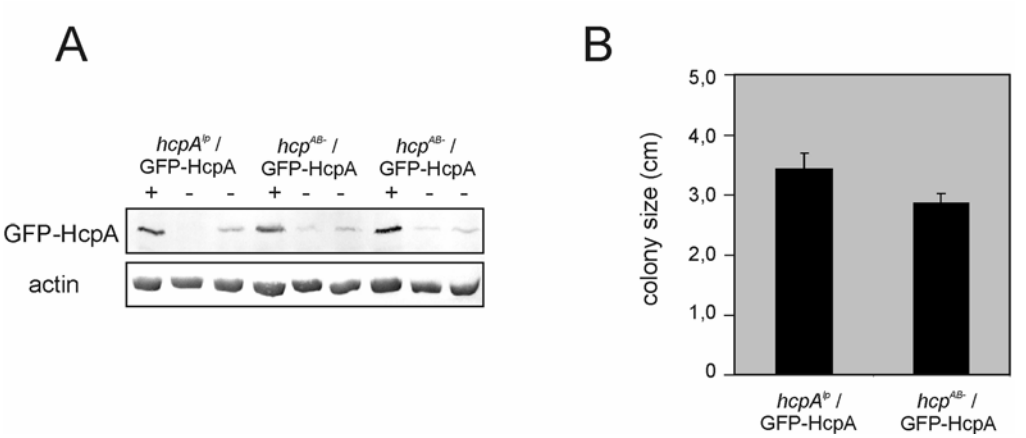

Fig. 3.18: **Regulated depletion of HP1 proteins.** A) Cells from the parental *hcpA*^{lp}/GFP-HcpA cell line and two independent subclones of the *hcp*$^{AB-}$/GFP-HcpA cell line were grown in duplicate on bacterial lawns (-) or under selective (10µg/ml G418) axenic conditions (+). After ~ 15 days on bacterial lawns, cells were re-picked and grown in non-selective axenic suspension culture. Total cell lysates were prepared and assayed for GFP-HcpA expression by a Western blot with an α-GFP-antibody. Actin (detected with a mouse monoclonal antibody 1-11 in a 1:5 dilution) serves as a control for equal loading. B) Growth under non-selective conditions on bacterial lawns resulted in mild growth defects of the *hcp*$^{AB-}$/GFP-HcpA cells compared to the parental *hcpA*^{lp}/GFP-HcpA cell line. Error bars indicate standard deviation.

3.9 Overexpression of GFP-HcpA, but not of GFP-HcpB leads to increased chromosome missegregation

The C-terminal GFP-fusion HcpA-GFP was consistently expressed at much lower levels than HcpB-GFP, with almost no nucleoplasmic fluorescence (Fig. 3.4A). Although both constructs were driven by an actin15-promoter, this difference could also be detected in co-transformants expressing HcpA-RFP and HcpB-GFP fusion proteins (Fig. 3.4B). In contrast, N-terminal GFP-fusions of HcpA and HcpB showed equal expression levels with similar nucleoplasmic background (Fig. 3.19A and 3.21A). Due to the almost identical expression levels, we assumed that the N-terminal GFP-fusions were suited better for functional analysis than the C-terminal GFP-fusions. RT-PCR analysis of the overexpression strains showed that the mRNA levels of the respective Hcp isoforms were substantially higher than in wildtype cells (Fig. 3.19B). Although this may not represent differences at the protein level, it very likely indicates that the protein levels of the respective Hcp isoforms were significantly higher than in wildtype cells.

Interestingly, when studying mitotic heterochromatin dynamics, we observed striking phenotypes that were particularly enriched in cell lines overexpressing GFP-HcpA. We found a more than 4-fold increase in chromosome missegregation events compared to the control transformation (Table 3.3). The most common phenotype were DNA bridges between the separated DNA masses in late mitotic cells. This was reminiscent of anaphase bridges after separation of dicentric chromosomes, for example in *Drosophila* (Fanti et al., 1998). GFP-HcpA signals frequently localized to these DNA bridges (Fig. 3.19C), indicating that heterochromatin was involved in these structures. Furthermore, overexpression of GFP-HcpA caused pronounced growth defects in axenic suspension culture compared to the control transformants (Table 3.4). In contrast, overexpression of GFP-HcpB did not increase chromosome missegregation rates, but still caused growth defects that were even more severe than those observed for GFP-HcpA expressing strains (Tables 3.3 and 3.4). The results were reproduced with several independent clones.

It was shown before that exchanging the C-termini of HcpA and HcpB confers specific dimerisation preferences to the chimeric proteins. Therefore, it was tested if this difference in dimerisation behaviour was causally related to the observed increased frequency of anaphase bridges during late mitosis. Immunofluorescence analysis

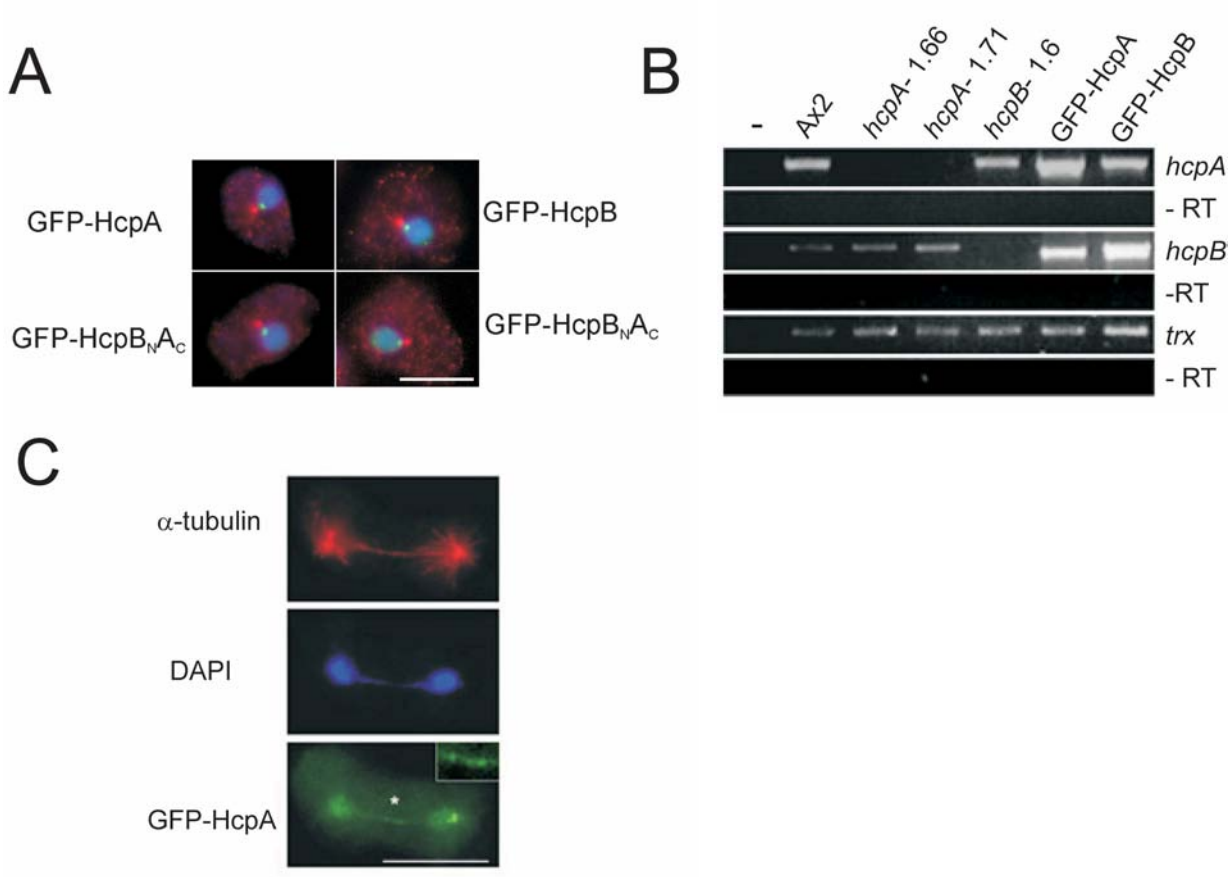

Fig. 3.19: **Distinct phenotypes of cells expressing GFP-HcpA fusion proteins. Exchange of the C-termini of HcpA and HcpB confers distinct properties to the chimeric proteins.** A) Subnuclear localisation of GFP-HcpA and GFP-HcpB. Localisation of chimeras is not altered with respect to the wild type proteins *in vivo*. Green:GFP, red: DdCP224, blue: DAPI. Bar: 5 µm. B) Expression levels of *hcpA*, *hcpB*, and *trx* in different background strains determined by semiquantitative RT-PCR. The unrelated thioredoxin gene (*trx*) was used as a control for equal amounts of template. *hcpA* and *hcpB* transcript levels are significantly enriched in strains overexpressing GFP-HcpA or GFP-HcpB, respectively, but are not detectable in the knock-out strains. Due to partial cross-sensitivity, primers used for *hcpB* can also bind to *hcpA*. Note the increase in signal intensity, but slightly lower size of the PCR product in the GFP-HcpA overexpression strain. Numbers of PCR cycles for amplification were: 32 (*hcpA*, *hcpB*); 27 (*trx*); -RT: minus reverse transcription. C) Chromosome missegregation phenotype in the GFP-HcpA strain. A late mitotic cell, as identified by a long mitotic spindle, is shown. DAPI stain shows a DNA bridge between the main DNA masses. GFP-HcpA is found in small foci on the DNA bridge. The asterisk indicates the area of magnification in the upper right corner. Bar: 5 µm.

showed that the chimeric proteins displayed a similar subnuclear localisation as their wild-type counterparts (Fig. 3.19A).

Remarkably, the exchange of the C-terminus did not only switch the dimerisation preference (see Fig. 3.10), but also conferred the property to induce aberrant anaphase bridges from HcpA to HcpB: only cell lines expressing GFP-HcpA or GFP-

TABLE 3.3: Anaphase bridge frequencies in different *Dictyostelium* strains[a].

strain	anaphase bridge frequencies	
Ax2	3,2 %	(3 / 94)
hcpA$^-$	4,1 %	(4 / 98)
hcpB$^-$	1,9 %	(2 / 107)
vector	2,9 %	(3 / 103)
GFP-HcpA	12,9 %	(14 / 108)
GFP-HcpB	0,9 %	(1 / 108)
HcpAΔC-GFP	1,1 %	(1 / 95)
HcpBΔC-GFP	3,3 %	(3 / 90)
GFP-Hcp$A_N B_C$	2,0%	(2 / 102)
GFP-Hcp$B_N A_C$	9,5%	(10 / 105)

[a] Cells were grown on coverslips for 20 to 24 h and fixed. Mitotic cells were identified by anti-DdCP224 or anti-tubulin staining. Late mitotic cells with mitotic spindles of $\geq$ 5 µm were counted (see Fig. 3.19C). In parentheses, absolute numbers of abnormal mitoses per number of counted mitoses are given. Results were reproduced with at least two independent clones of each strain. Chi-square analysis of the obtained data revealed a significance value $P < 0.01$ for the observed enrichment of anaphase bridges in the GFP-HcpA and GFP-Hcp$B_N A_C$ expressing strains.

TABLE 3.4: Generation times of *Dictyostelium* cell lines transformed with different Hcp-expression vectors[a].

Ax2 transformed with	generation time ($\pm$ SD)
vector	9,3 ($\pm$ 1,1)
GFP-HcpA	13,5 ($\pm$ 0,4)
GFP-HcpB	21,8 ($\pm$ 0,6)
HcpAΔC-GFP	*n.d.*
HcpBΔC-GFP	13,2 ($\pm$ 1,7)

[a] Mean generation times (in hours) and standard deviations from several independent clones are shown. Growth curves were measured in axenic suspension culture in HL5 medium supplemented with 20 µg/ml geniticin.

Hcp$B_N A_C$, but not cell lines expressing GFP-HcpB or GFP-Hcp$A_N B_C$ or the vector control displayed increased frequencies of anaphase bridges (Table 3.3).

The C-termini may have quantitative or even qualitative differences in interacting with other chromatin proteins. Overexpression of the A-type C-terminus may thus accumulate protein complexes that are usually underrepresented and alter chromatin

structure in such a way that ultimately leads to the observed phenotype. It should be stressed that although the phenotypic aberrations observed in the GFP-HcpA and GFP-Hcp$B_N A_C$ overexpression strains manifested themselves during mitosis, they did not represent mitotic defects, since mitotic progression, as determined by the relative abundance of individual mitotic stages, was essentially unaffected in all of the strains tested (Fig. 3.20). It was therefore assumed that the defect, which manifested during mitosis, occurred earlier in the cell cycle.

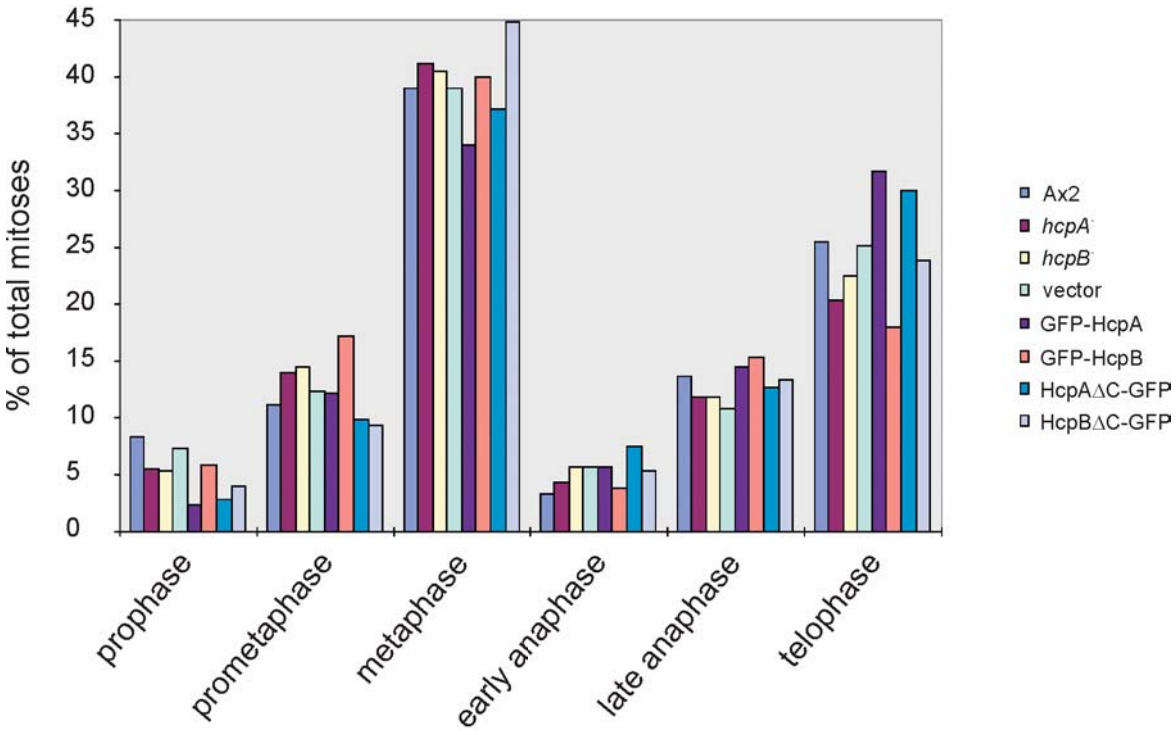

Fig. 3.20: **Distribution of mitotic stages in different *Dictyostelium* strains.** Cells were grown on coverslips for 20 to 24 h and fixed. Mitotic cells were identified by anti-DdCP224 or anti-tubulin staining. More than 100 mitotic cells were counted for each strain.

3.10 Overexpression of C-terminally truncated HcpA impairs cell viability

In order to gain further insight into the function of HP1 proteins in heterochromatin, we overexpressed truncated HcpA or HcpB proteins lacking the C-terminal chromo shadow domain (HcpAΔC-GFP; HcpBΔC-GFP). We assumed that these proteins localize to heterochromatin (Wang et al., 2000), but prevent the formation of higher-order chromatin structures. Since HcpAΔC-GFP and HcpBΔC-GFP were not able to dimerize *in vitro* (Fig. 3.7C), they should not be incorporated into oligomeric Hcp complexes, but rather should disturb formation of these complexes through occupation

of chromatin binding sites and thus result in a partial loss-of-function phenotype. However, it should be stressed that the residence time of the C-terminally truncated proteins at heterochromatin should be shorter than that of the full-length proteins, because the lack of binding opportunities provided by CSD-mediated protein-protein interactions should destabilize chromatin binding (Cheutin et al., 2003).

The truncated proteins were, at least in part, localized to heterochromatin, however, staining of the minor subnuclear foci was strongly reduced (Fig 3.21A), suggesting distinct CSD-dependent and CSD–independent targeting of HP1 proteins in *Dictyostelium*. Overexpression of the truncated HcpA, but not the truncated HcpB protein lead to severe growth defects in axenic suspension culture that did not allow to determine the generation time of this strain (Table 3.4). After ~ 48h in cells in suspension culture microscopic analysis revealed that approximately half of the cells, compared to less than 5% of the control transformant, were abnormally rounded and had aberrantly condensed DNA (data not shown). These cells did not represent prometaphase cells, since mitotic analysis did not show any significant mitotic defects (Fig. 3.20). Moreover, the plating efficiency on bacterial lawns of the HcpAΔC-GFP strain was more than 3-fold reduced compared to the control strain (Fig. 3.21B). The data indicate that overexpression of HcpAΔC-GFP leads to highly elevated rates of cell death in axenic suspension culture, especially when cultures are inoculated at lower cell densities of ~ 3x 10^5 / ml. Similar, but less drastic results were obtained when the mitotic index (i.e. the number of mitoses per 100 cells) of surface-attached cultures (as used for microscopy of mitotic stages) of cell lines transformed with the vector control or with pDd-HcpAΔC-GFP was determined. In this case, the mitotic index of cell lines expressing HcpAΔC-GFP was lower than of the control (data not shown).

Interestingly, overexpression of HcpAΔC-GFP, although being expressed at similar levels as full-length GFP-HcpA, did not elicit chromosome missegregation defects seen for full-length HcpA (Table 3.3). It appears that overexpression of functional HcpA which is able to interact with other proteins via its CSD is required to elicit the chromosome missegregation phenotype.

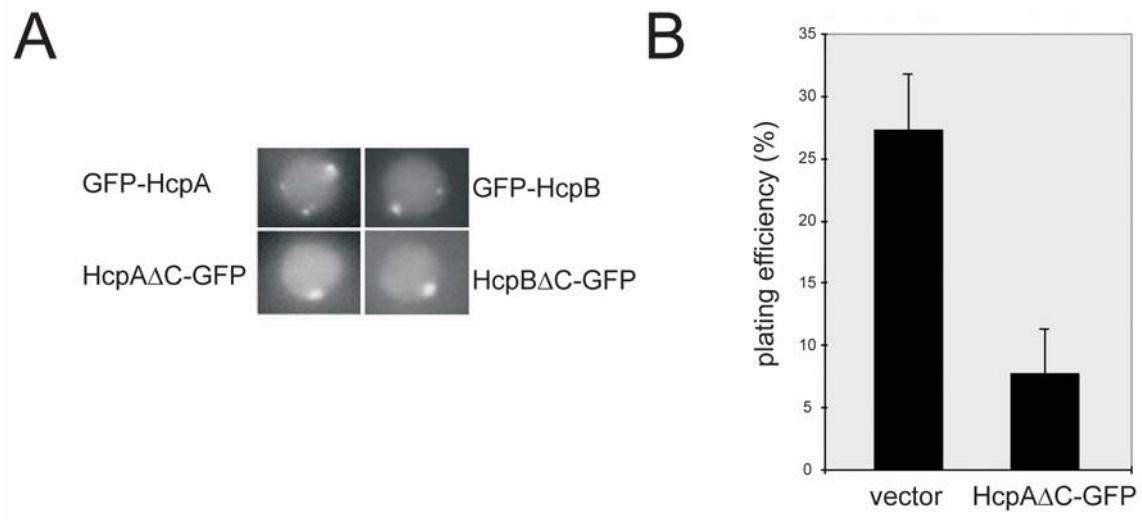

Fig. 3.21: **Overexpression of HcpAΔC-GFP impairs cell viability.** A) Live cell images of GFP-HcpA, GFP-HcpB, HcpAΔC-GFP and HcpBΔC-GFP expressing cells. All images were taken with identical exposure times. Both truncated fusion proteins lacking the CSD still localize to the major heterochromatic cluster, but do not form additional smaller foci. B) Generation times of cell lines overexpressing various Hcp-GFP-fusion proteins or a vector control. C) Cell viability, as determined by plating efficiency, is significantly impaired by overexpression of HcpAΔC-GFP compared to the control transformant.

3.11 CSD-dependent and CSD-independent localisation of HP1 proteins

Previous analysis revealed that subnuclear targeting of HcpA and HcpB is achieved by different CSD-dependent and -independent mechanisms (Fig. 3.21A). Both HcpA and HcpB bind to pericentromeric heterochromatin independently of the CSD, although the CSD may be required to stabilise chromatin binding by mediating interactions with other chromatin factors (Cheutin et al., 2003; Festenstein et al., 2003). HP1 proteins bind to histone H3 methylated at K9, which is strongly enriched in *Dictyostelium* centromeric heterochromatin, by their CD, but this interaction is rather weak *in vitro* (Bannister et al., 2001; Lachner et al., 2001). Furthermore, the H3K9me2 signal is found at various other sites at the nuclear periphery that largely overlap with staining by the HcpA- and HcpB-GFP fusions. However, foci formation of C-terminally truncated HcpA/B proteins cannot be detected at these sites (Fig. 3.22A). Therefore, it is very likely that H3K9me2 binding by the CD is not the only or even major mechanism driving subnuclear HP1 localisation, which is in agreement with findings in other organisms (Stewart et al., 2005). We therefore tested if other features of HP1 proteins may contribute to centromeric chromatin binding.

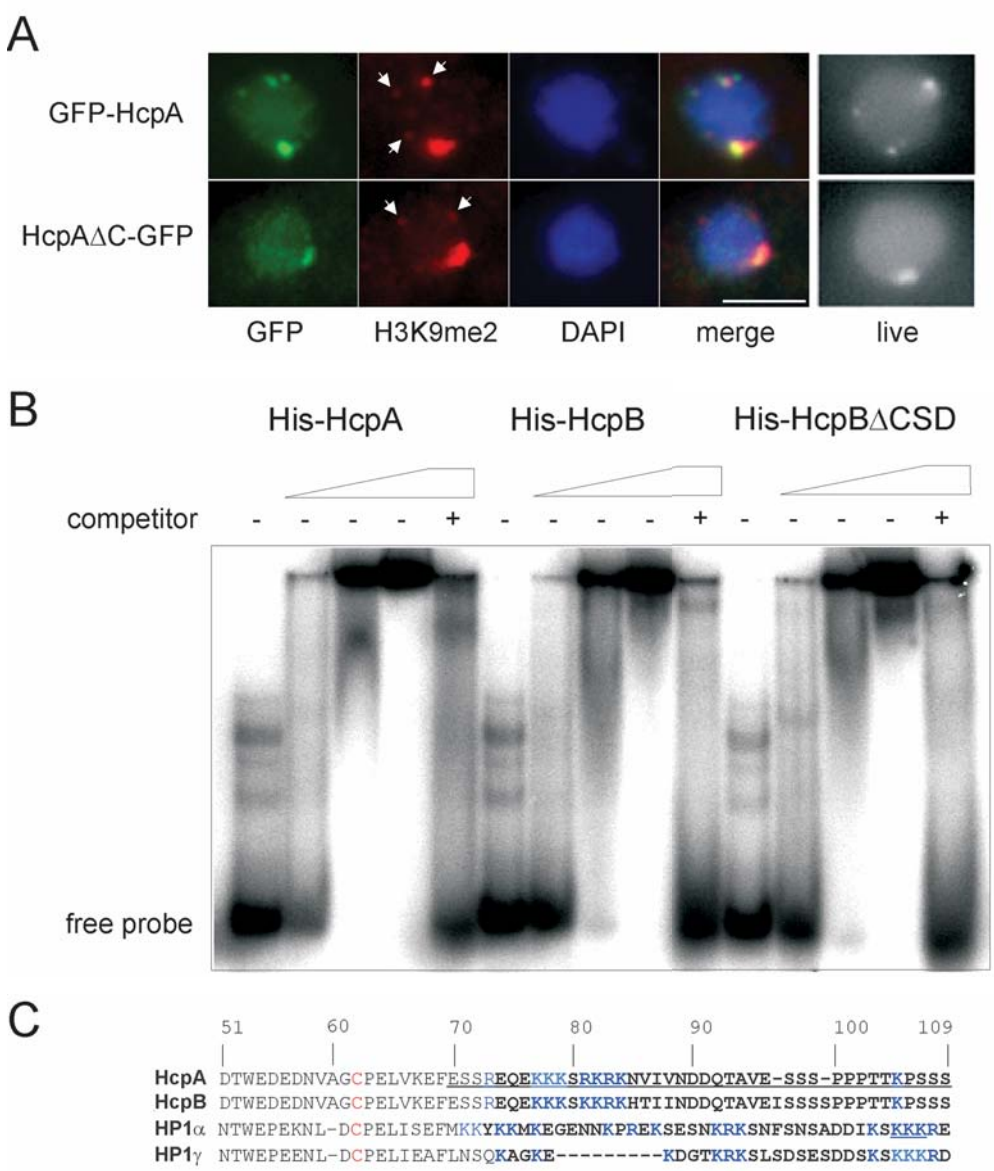

Fig. 3.22: **CSD-dependent and CSD-independent localisation of HP1 proteins.** (A) While GFP-HcpA stains the major heterochromatic cluster and additional smaller foci at the nuclear periphery, the C-terminally truncated HcpAΔC-GFP lacking the CSD only localizes to the main heterochromatic cluster without formation of additional foci. H3K9dimethylation is present both at the major heterochromatic cluster as well as at minor foci at the nuclear periphery (arrows). Similar data were obtained with GFP-HcpB and HcpBΔC-GFP (not shown). Since the C-terminally truncated proteins were highly sensitive towards fixation and fluorescence intensity significantly decreased in the fixed material, a live cell image is shown on the right to illustrate the different subnuclear localisation. Bar: 2 µm. B) Gelshifts with His-HcpA, His-HcpB and His-HcpBΔC. All three proteins bind to DNA, indicating that DNA binding is independent of the CSD. The His-HcpBΔC has been described elsewhere (B. Földesi, Diploma thesis, Kassel University, 2005). C) Amino acid alignment of the CD and hinge regions of HcpA, HcpB, HP1α and HP1γ. Numbering is for HcpB. Bold letters mark the hinge region. Basic residues within the hinge are in highlighted in blue. The conserved C-62 residue required for DNA binding is in red. Sequences underlined were used for pK_I calculation. The KKK motif in the HP1α sequence required for RNA binding (Muchardt et al., 2002) is underlined.

DNA- and RNA binding of HP1 proteins has been shown for various homologues from other organisms (Zhao et al., 2000; Muchardt et al. 2002; Meehan et al., 2003), indicating that it is a conserved function of HP1 proteins. It has been suggested that direct HP1 binding to DNA in certain unusual chromatin structures specific for heterochromatin might be a major force of heterochromatic targeting, since under physiological conditions, the methylated histone tails (which are bound *in vitro*) themselves are engaged in interactions with the DNA and thus might be inaccessible (Meehan et al., 2003).

Gel retardation experiments showed that HcpA and HcpB can both bind to DNA directly, and that this binding is independent of the CSD. Binding was specific in that addition of competitor DNA could completely reverse complex formation (Fig. 3.22B). The DNA binding function was, though not surprising, quite remarkable, since the basic amino acid residues within the hinge that are required for binding are arranged in a very different manner in the *Dictyostelium* proteins compared to their mammalian counterparts (Fig. 3.22C). Nevertheless, calculation of the pK_I values of the amino acid sequences within the hinge of the *Dictyostelium* HcpA/B proteins gave similarly basic values, compared to their mammalian counterparts (HcpA: 9,70; HcpB: 9,60; HP1α: 10,12; HP1γ: 9,82). Furthermore, additional conserved residues required for DNA binding, e.g. C-62 are conserved in the *Dictyostelium* proteins (Fig. 3.22C).

It should be stressed that the hinge regions of HcpA and HcpB, which are likely to be required for nucleic acid binding, contain a high number of putative phosphorylation sites. Furthermore, the distribution of putative phosphorylation sites differs significantly between HcpA and HcpB (Fig. 3.23). It is conceivable that phosphorylation, by alteration of the charges within the nucleic-acid binding region, is likely to affect DNA- (and RNA-) binding activity *in vivo*. Phosphorylation of the hinge has been reported for HP1 proteins from various organisms (Huang et al., 1998b; Minc et al., 1999) and thus seems to be conserved mode of functional regulation of HP1 proteins.

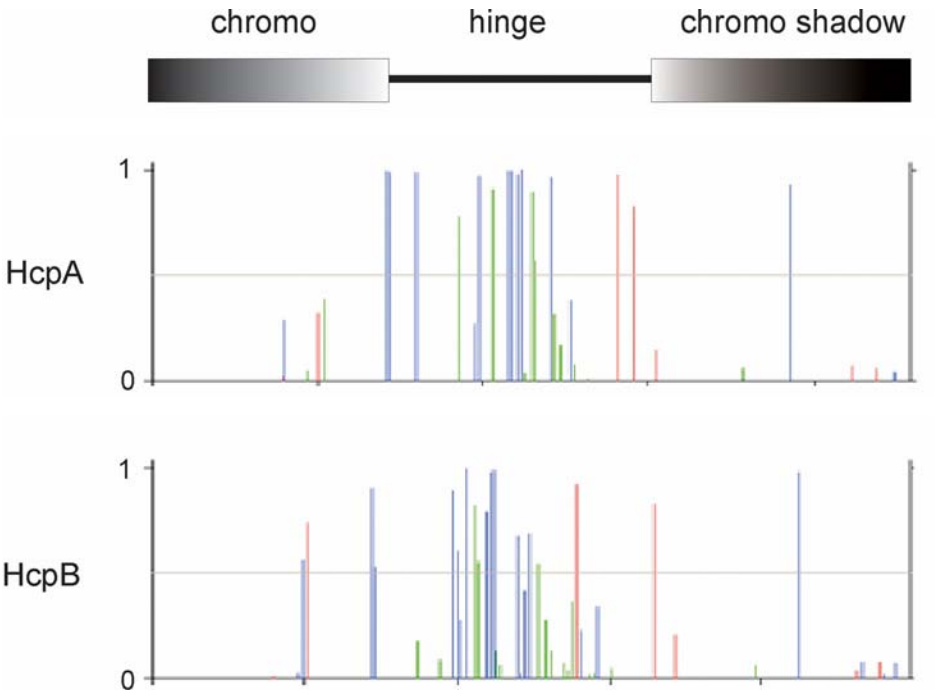

Fig. 3.23: **Distribution of putative phosphorylation sites in the HcpA and HcpB amino acid sequences**. Height of vertical bars represents probability (ranging from 0 to 1) for phosphorylation (green: serine; blue: threonine; red: tyrosine). Phosphorylation sites were determined at www.cbs.dtu.dk/services/NetPhos/ (Blom et al., 1999).

3.12 *DIRS*-1 and *skipper* retrotransposons physically associate with histone H3K9 dimethylation

Though ill-defined until now, *Dictyostelium* centromeres are very likely composed of complex arrays of transposons and retrotransposons located at the chromosome ends (Eichinger et al., 2005). Since the most prominent retrotransposons in *Dictyostelium* that are preferentially located in these arrays, *DIRS*-1 and *skipper*, have been shown to be targeted by DNA methylation and, in addition, are regulated by the RNAi machinery (Kuhlmann et al., 2005), it was assumed that they also might be subjected to additional mechanisms of epigenetic gene silencing, e.g. histone H3K9 methylation and assembly into heterochromatin via HP1-mediated chromatin compaction.
Furthermore, the clustering of these complex arrays during interphase (Eichinger et al., 2005) strikingly resembles the subnuclear pattern observed for histone H3K9 dimethylation and HcpA/HcpB; therefore, it was tested if histone methylation and HcpA/HcpB physically associate with these arrays. For this purpose, *Dictyostelium* cell lines stably expressing myc-HcpA and myc-HcpB were generated (Fig. 3.24A).

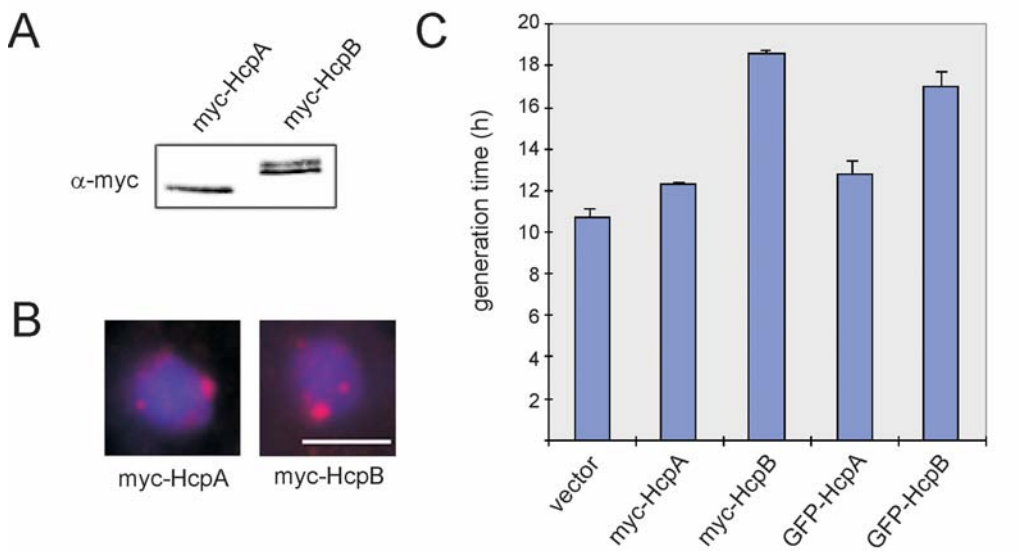

Fig. 3.24: **Generation of myc-HcpA and myc-HcpB expressing cell lines.** A) Western blot with α-myc-antibody to verify myc-HcpA and myc-HcpB expression. B) Immunofluorescence of myc-HcpA and myc-HcpB expressing cells. Bar: 2µm. C) Generation times in hours (h) of *Dictyostelium* cell lines expressing myc-HcpA, myc-HcpB, GFP-HcpA and GFP-HcpB. Generation times of at least two independent clones from each transformation were determined. Cell growth was measured in duplicate for each clone. Error bars indicate standard deviations from the mean.

Similarly to the GFP-tagged proteins, myc-HcpA and myc-HcpB localized predominantly to one major cluster at the nuclear periphery (Fig. 3.24B). Overexpression of myc-tagged proteins had largely similar effects on vegetative growth compared to the GFP-tagged proteins (Fig. 3.24C), indicating that myc-HcpA and myc-HcpB were functionally identical to their GFP-tagged counterparts. Furthermore, the proteins could be immunoprecipitated from *Dictyostelium* cell extracts (Fig. 3.25A), indicating that the myc-epitope is accessible to the antibody. Having characterised the myc-HcpA/B overexpression strains, they were used to demonstrate heterochromatic association of *DIRS*-1 and *skipper* retrotransposons by chromatin immunoprecipitation (ChIP).

Shearing of the formaldehyde-cross-linked chromatin into small fragments before immunoprecipitation is crucial for the spatial resolution of chromatin binding sites of the immunoprecipitated proteins. The cross-linking and shearing efficiency of genomic DNA from *Dictyostelium* was verified by gel electrophoresis and showed a low molecular weight smear, compared to the unsonified high molecular weight DNA (Fig. 3.25B). Unfortunately, chromatin immunoprecipitation (ChIP) with a monoclonal α-myc-antibody did not result in target-specific precipitation of retrotransposon

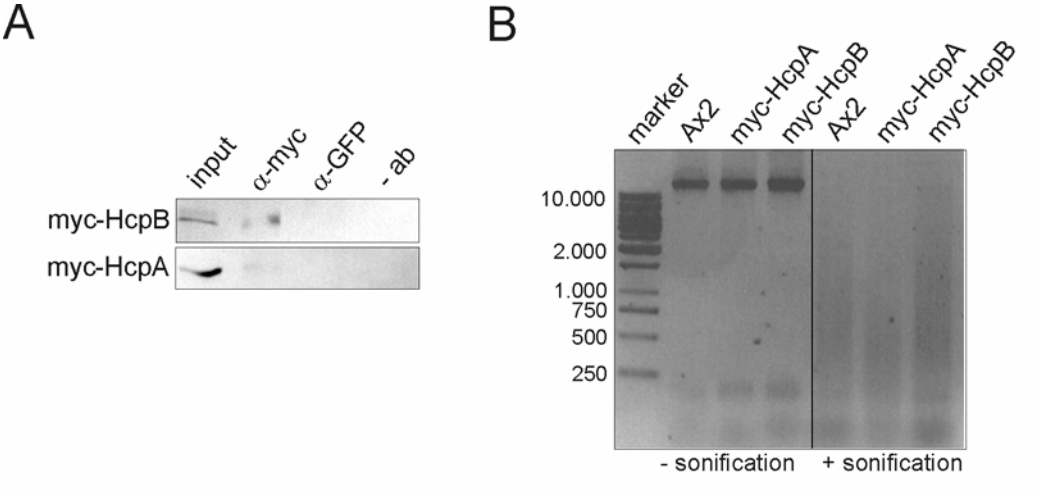

Fig. 3.25: **Preparation of cross-linked *Dictyostelium* chromatin and immunoprecipitation of myc-tagged HcpA/B from native cell lysates.** A) Control of sonification conditions for ChIP. After sonification, DNA is sheared to a preferential length of 250-2000bp, indicated by the DNA smear in the sonified fractions. B) Immunoprecipitation of myc-HcpA and myc-HcpB. Both myc-HcpA and myc-HcpB can be precipitated with an α-myc-, but not with an α-GFP-antibody. –ab: no antibody-control.

sequences in myc-HcpA or myc-HcpB overexpressing strains. Although much lesser amounts of DNA were precipitated in the Ax2 strain (which lacks the myc-epitope), or the minus-antibody controls, no specific enrichment of *DIRS*-1 and *skipper* sequences compared to the actin control could be detected (Fig. 3.26B).

However, it could be shown that both *DIRS*-1 and *skipper* sequences can be preferentially enriched by precipitation with an H3K9me2-specific antibody. In contrast, the euchromatic multi-copy actin gene family, which was chosen as a negative control, is not associated with H3K9me2, resulting in significantly lesser enrichment in the precipitated fraction (Fig. 3.26C). The results were reproducible with all three cell lines tested (Ax2, myc-HcpA and myc-HcpB). Similar results were obtained with different primer pairs (see Materials and Methods section). It is possible that ectopic overexpression of HcpA or HcpB causes changes in H3K9 methylation levels at these loci due to mutual reinforcement of HP1 binding and histone methyltransferase activity. This would lead to a preferential enrichment of precipitated *DIRS*-1 or *skipper* sequences in myc-HcpA/B overexpression strains; however, the current data do not allow an estimation about changes in histone methylation levels upon HcpA/B overexpression.

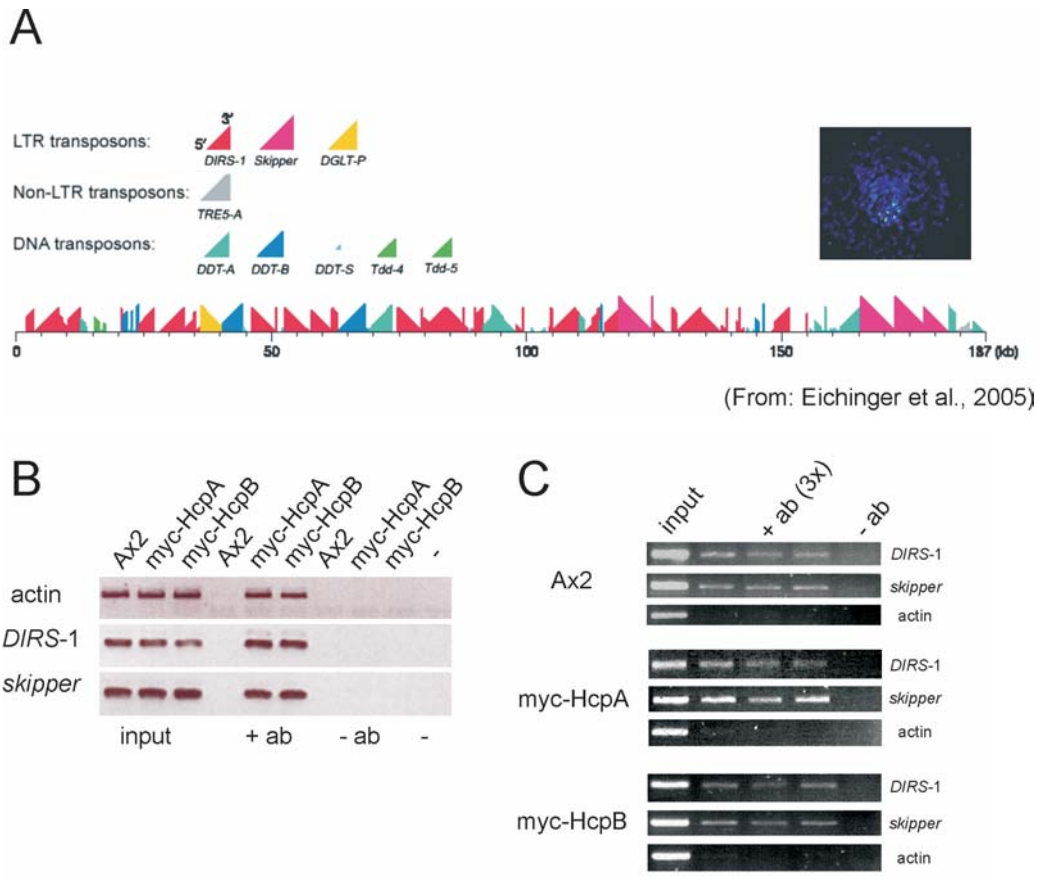

Fig. 3.26: ***DIRS*-1 and *skipper* retrotransposons are heterochromatin-associated.** A) Physical map of one end of chromosome 1. Several types of DNA transposons and retrotransposons preferentially cluster in this genomic region. Inset: FISH with a *DIRS*-1-specific probe (green) on interphase chromosomes (blue). Note the clustering of the FISH-signal. B) ChIP with α-myc-antibody. Although DNA is highly enriched in the immunoprecipitated fractions of cell lines expressing myc-HcpA or myc-HcpB, but not in the Ax2 or minus-antibody controls, no specific enrichment of *DIRS-1* or *skipper* sequences compared to the euchromatic actin control could be detected (+/- ab : plus/minus antibody; - PCR negative control). C) ChIP with a-H3K9me2-antibody. In all three strains tested, H3K9me2 is associated with *DIRS-1* and *skipper* retrotransposon sequences, but not with the euchromatic actin genes. PCRs on immunoprecipitated fractions were run in triplicate (3x).

3.13 Transcriptional activities of (pericentromeric) retrotransposons in different background strains

Since the role of HP1 proteins in gene silencing mechanisms is well established, we tested if retrotransposon activities were affected in different HP1 mutant strains. No influence on transcription of *DIRS*-1 and *skipper* retrotransposons could be detected in any of the knock-out strains or the GFP-HcpA and GFP-HcpB overexpression strains (Fig. 3.27A). Remarkably, overexpression of HcpAΔC-GFP caused significant

upregulation of *skipper* transcription compared to the control transformant (Fig. 3.27B). The data indicate that *skipper* retrotransposons are at least partially transcriptionally regulated, although we observed relatively high mRNA levels in Ax2 cells. As we also detected a decrease in cell viability in the same strain (Fig. 3.21), we assume a dominant-negative function of the (overexpressed) truncated HcpAΔC-GFP protein, which is, however, not as severe as a complete loss-of-function mutation in both Hcp isoforms, since it is not lethal to the cell.

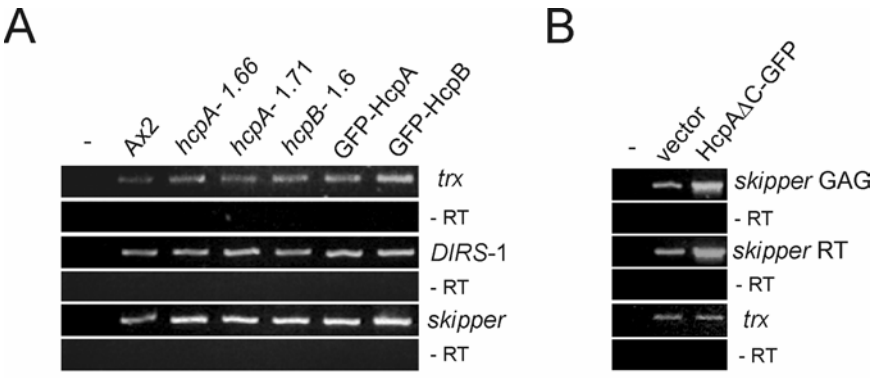

Fig. 3.27: **Influence of Hcp proteins on transcriptional activity of retrotransposons.** A) Expression levels of *DIRS*-1, *skipper* and *trx* in different background strains. Neither loss nor overexpression of HcpA or HcpB affects *DIRS*-1 or *skipper* transcript levels. Numbers of PCR cycles for amplification were: 27 (*trx*); 25 (*DIRS*-1); 28 (*skipper*). B) Overexpression of HcpAΔC-GFP causes increased transcription of the *skipper* retrotransposon. Two open reading frames covering different regions, e.g. open reading frames, of the retrotransposon sequence (GAG and RT), were amplified by PCR. Both open reading frames are transcribed at significantly higher levels than in the control transformant. Due to the strong growth defect of the HcpAΔC-GFP strain in axenic suspension culture, RNA was prepared from cells cultured in petri dishes. Due to different culture conditions, transcript analysis was done in a separate experiment. (*trx* : thioredoxin control ; -RT: minus reverse transcription)

3.14 RNAi-mediated heterochromatin formation in *Dictyostelium* ?

In *Drosophila* (Schotta et al., 2002) and *S. pombe* (Hall et al., 2002), HP1 loss-of-function-mutants influence H3K9 methylation levels and their subnuclear distribution. In *Dictyostelium*, the knock-out mutants of individual HP1 genes did not cause significant changes in H3K9me2 levels or their subnuclear distribution, as judged by immunofluorescence (Fig. 3.28). This indicated that either the two isoforms are redundant for recruiting histone methyltransferases (HMTs) or that HMTs act upstream of HP1.

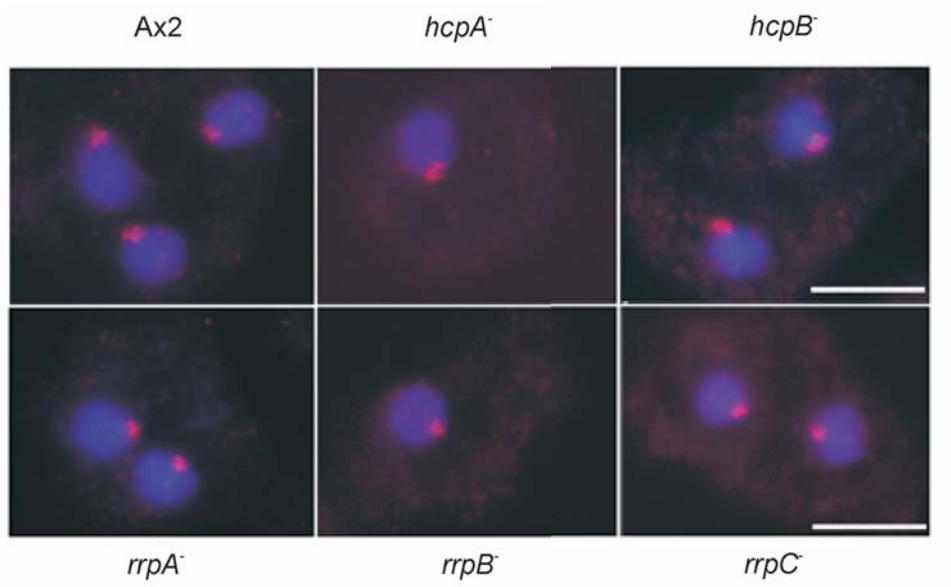

Fig. 3.28: **H3K9me2 localization in *hcp* and *rrp* knock-out mutants.** Knock-out of either *hcpA* or *hcpB* has no effect on H3K9me2 levels or its subnuclear distribution. Similarly, knock-out of the RNA-dependent RNA polymerase genes *rrpA*, *rrpB* or *rrpC* that are involved in RNA-mediated post-transcriptional gene silencing does not affect H3K9me2 level and distribution; DAPI (blue) and H3K9me2 (red).

Mutations in components of the RNAi machinery affect heterochromatin formation and its subnuclear localisation in various organisms (Hall et al., 2003; Zilberman et al., 2003; Pal-Bhadra et al., 2004; Kanellopoulou et al., 2005). We therefore examined if RdRP activity, which is required for functional RNA interference, was required for heterochromatin formation in *Dictyostelium*. No obvious defects in H3K9me2 localisation (Fig. 3.28) or localization of the HP1 proteins (data not shown) in any of the three RdRP knock-out strains could be detected. Although we cannot rule out that in *Dictyostelium*, RdRPs play a minor or a redundant role in RNAi-mediated heterochromatin formation, it is well possible that, like in other organisms, there are parallel pathways for heterochromatin formation, including RNAi-independent pathways (Jia et al., 2004).

3.15 Loss of histone H3K4 methylation has no influence on histone H3K9 methylation patterns

Methylation of the histone H3K4 and K9 residues are considered to be mutually exclusive and mark different chromatin states. While H3K4 methylation is mainly found in euchromatic and transcriptionally active regions (Santos-Rosa et al., 2002), K9

methylation is a marker for transcriptionally silent heterochromatin. Genome-wide analysis of histone H3 methylation patterns of lysines K4 and K9 in the coding regions of human genes by using chromatin immunoprecipitation linked to cDNA arrays revealed that both histone modifications are mutually exclusive (Maio and Natarajan, 2005). The boundaries between heterochromatin and euchromatin at the fission yeast mating type locus are marked by a sharp transition between these two different methylation marks (Noma et al., 2001). Loss of the *Dictyostelium* SetA protein has been shown to abolish all histone H3K4 methylation, indicating that SetA is the sole histone H3K4-specific methyltransferase in *Dictyostelium* (Chubb et al., 2006). It was tested if loss of histone H3K4 methylation, which is found in the entire nucleus in wild-type Ax2 cells, causes a redistribution and spreading of heterochromatin markers such as histone H3K9 methylation and HP1. Surprisingly, no effects on heterochromatin localisation and subnuclear distribution could be detected in *setA*$^-$ cells (Fig. 3.29).

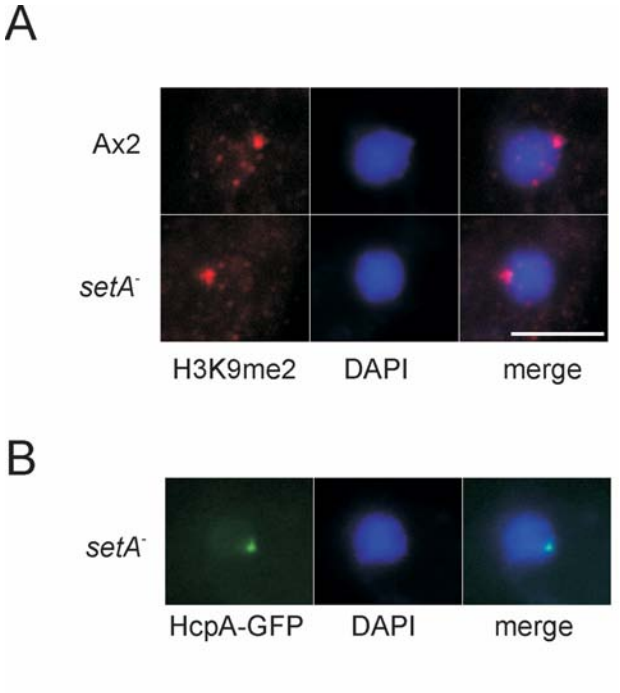

Fig. 3.29: **Loss of the setA histone H3K4 methyltransferase does not affect localisation of histone H3K9 methylation or HcpA-GFP.** A) Ax2 and *setA*$^-$ cells were immunostained with an α-H3K9me2 antibody. B) *setA*$^-$ cells were transformed with pDd-HcpA-GFP. No influence on HcpA-GFP localisation compared to Ax2 wild-type cells (cross check Fig. 3.4A) could be detected. DNA is stained with DAPI.

The results indicate that histone H3K9 methylation is not restricted by the K4-methyl mark, but instead is strictly localized to centromeric heterochromatin by other,

unknown mechanisms.

3.16 Identification of the *Dictyostelium* Orc2 homologue (OrcB)

In higher eukaryotes such as *Drosophila*, *Xenopus* and mammals, ORC interacts with HP1 and is associated with heterochromatin. Furthermore, disruption of ORC interferes with HP1 localisation to heterochromatin (Pak et al., 1997; Shareef et al., 2001). Especially mutants of the Orc2 subunit of ORC have impaired HP1 localisation into heterochromatin in *Drosophila* and human cells, indicating that Orc2 functions in HP1 targeting (Huang et al., 1998b; Prasanth et al., 2004). Therefore, it we aimed to test if Orc2 might have a similar function in HP1 targeting in *Dictyostelium*. Since the role of ORC in DNA replication initiation is highly conserved, we tested if all ORC subunits exist in *Dictyostelium*. Consistent with the ubiquituous presence of ORC in eukaryotes, a detailed BLAST search in the *Dictyostelium* genome database with the six human ORC subunits showed that homologues for all of them are present in the *Dictyostelium* genome (Table 3.5).

TABLE 3.5: Identification of the *Dictyostelium* ORC subunit homologues[a]

human Orc subunit	*Dictyostelium* homologue
Orc1	DDB0218435
Orc2	DDB0190652
Orc3	DDB0216767
Orc4	DDB0168430
Orc5	DDB0191826
Orc6	DDB0186183

[a] BLAST searches (Altschul et al., 1997) were performed with a BLASTp algorithm against the *Dictyostelium* genome database (www.dictybase.org). The *Dictyostelium* homologues of all ORC subunits are specified by their Dictybase entries.

A tBLASTN search with the human Orc2 amino acid sequence revealed the presence of only one gene with significant homologies to Orc2. Alignment with several different Orc2 homologues from different species showed that the conserved C-terminal portion is present in the *Dictyostelium* homologue (Fig. 3.30), but that the N-terminal portion is absent, resulting in a significantly smaller protein (391 amino acids compared to 577 amino acids for the human Orc2 protein). In the conserved C-terminal part of the

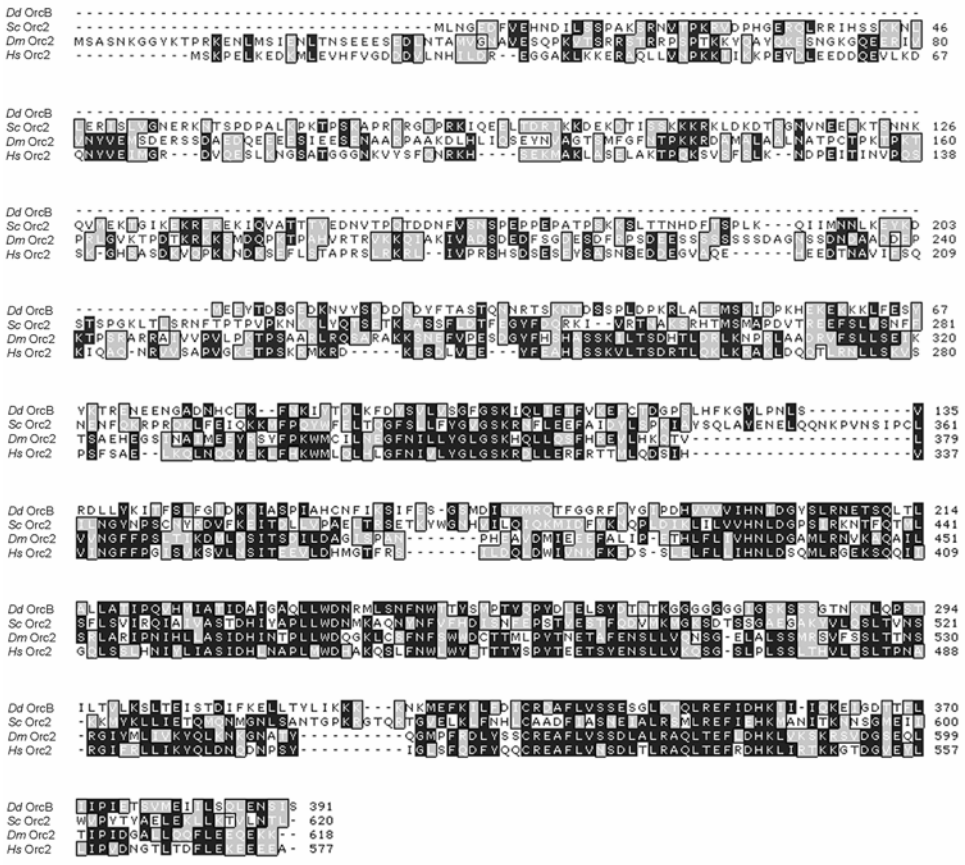

Fig. 3.30: **Alignment of *Dictyostelium* (*Dd*) OrcB with Orc2 homologues from *S. cerevisiae* (*Sc*), *Drosophila melanogaster* (*Dm*) and *Homo sapiens* (*Hs*).** Identical amino acids are in black, similar amino acids in grey.

protein, *Dictyostelium* OrcB is 29% identical (49% similar) to both human and *Drosophila* Orc2. The *orcB* gene could be amplified from cDNA, indicating that it is actively transcribed in vegetative cells, but it is expressed at low levels compared to the housekeeping gene thioredoxin (data not shown). To further analyze its function, OrcB was expressed in *Dictyostelium* as a GFP-fusion protein. Western blot analysis using an α-GFP antibody confirmed the presence of a protein in *Dictyostelium* whole cell extracts, the size of which corresponds to the calculated MW of 72,7 kDa for GFP-OrcB (Fig. 3.31A).

3.17 Partial colocalisation of OrcB with HcpA

Co-transformation with HcpA-RFP showed that GFP-OrcB did not localize to the major heterochromatin cluster, which represents *Dictyostelium* centromeric heterochromatin

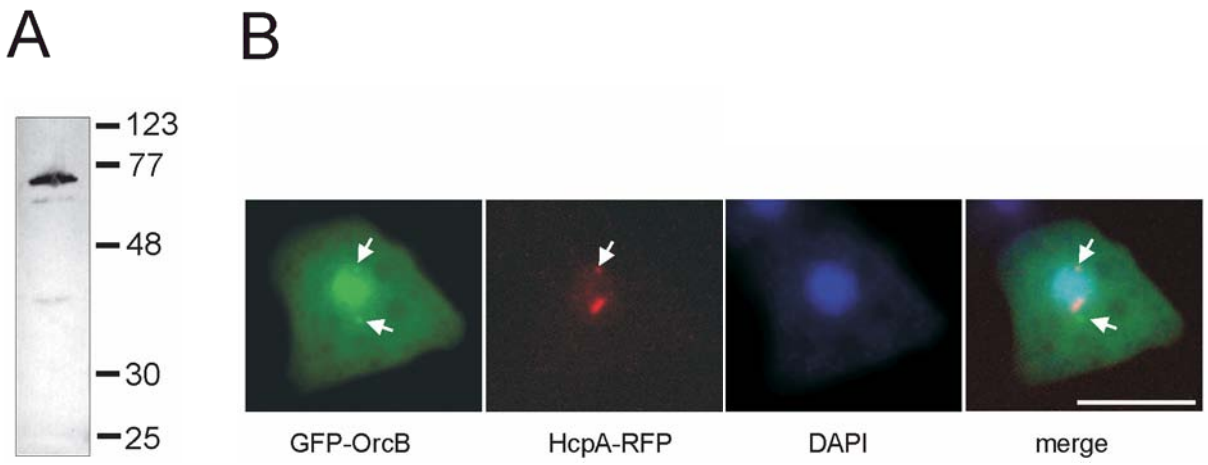

Fig. 3.31: **Partial colocalisation of GFP-OrcB with HcpA-RFP.** A) Western blot analysis with an anti-GFP-antibody showed the expression of a ~ 73kDa protein in transformed *Dictyostelium* cells. B) In interphase, GFP-OrcB localizes to the nucleus, but also shows strong cytoplasmic staining. Nuclear foci co-localize with HcpA-RFP, but do not represent major heterochromatin. In addition, a cytoplasmic signal in close vicinity to the nucleus and to centromeric heterochromatin can be detected. The upper arrow indicates co-localisation with HcpA-RFP in minor subnuclear foci. The lower arrow indicates centrosomal localisation.

(Fig. 3.31B). However, we cannot exclude that a minor fraction of GFP-OrcB localized to centromeres, but could not be detected due to the strong nucleoplasmic background fluorescence. Nevertheless, GFP-OrcB co-localizes with minor HcpA-RFP foci at other sites at the nuclear periphery (Fig. 3.31B, upper arrow). Although we cannot entirely rule out that formation of these foci was an effect of protein overexpression, it seems that there are distinct heterochromatin domains that can be distinguished cytologically and that differ in their protein composition.

ORC has been shown to interact with HP1 in *Drosophila, Xenopus* and mammals. We therefore tested if the OrcB subunit can directly bind to either HcpA or HcpB, but pull-down analysis did not show any interaction between GFP-OrcB and His-tagged HcpA or HcpB (data not shown). However, it is well possible that interaction of HcpA/B with ORC depends on distinct phosphorylation states of either HcpA/B (Badugu et al, 2005), which were not present in the bacterially expressed proteins, or interaction of HcpA/B with ORC is mediated by another ORC subunit. Accordingly, in *Drosophila* and humans, HP1 does not interact with the Orc2, but rather the Orc1 subunit of ORC (Pak et al., 1997; Lidonnici et al., 2004).

3.18 OrcB is associated with the centrosome throughout the cell cycle

Overexpressed GFP-OrcB localized to the nucleus as expected, where it formed a small number of foci at the nuclear periphery (Fig. 3.30B). In addition, a large portion of the protein remained cytosolic, and unexpectedly localized to the centrosome in *Dictyostelium* cells (Fig. 3.31B (lower arrow) and 3.32). Centrosomal association, though not confirmed by other means, is very likely specific, since control transformations with the empty vector which only expressed GFP never showed foci formation at any place in the cell (data not shown). The association with the centrosome was stable throughout the cell cycle, whereas nuclear localisation was diminished during mitosis (see telophase, Fig. 3.32). The loss of nuclear and chromatin-associated Orc2 during M phase resembles the situation in *X. laevis* and

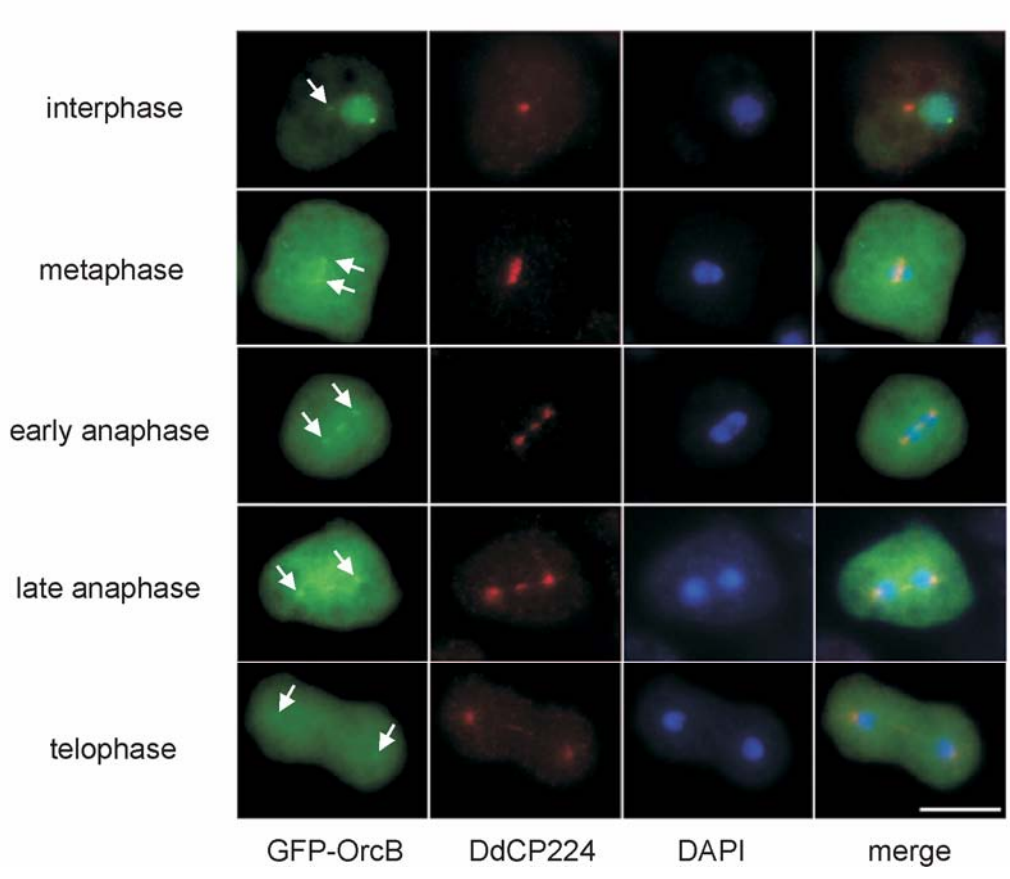

Fig. 3.32: **Localisation of GFP-OrcB during the cell cycle.** The cytoplasmic GFP-OrcB signal (see Fig. 3.31B) co-localizes with DdCP224, a known centrosomal marker protein (arrows). Co-localisation with DdCP224 was observed during all mitotic stages, whereas overall nuclear localisation was lost during late mitotic stages.

human cells (Romanowski et al., 1996; Prasanth et al., 2004), but is in contrast to the situation in the yeasts, where ORC stays associated with chromatin throughout the cell cycle (Diffley et al., 1994; Lygerou and Nurse, 1999). Human Orc2 has recently been shown to localize to centromeres and centrosomes, where it fulfills multiple roles in chromosome inheritance during mitosis (Prasanth et al., 2004). Unlike human or *Drosophila* Orc2 (Pak et al., 1997), we did not observe obvious association with centromeric heterochromatin at any stage of the cell cycle. However, our localisation data suggest that, similar to human Orc2, the *Dictyostelium* Orc2 homologue also serves several functions apart from replication initiation. Further analysis will be required to elucidate the centrosomal function of OrcB.

3.19 Overexpression and nuclear targeting of the sole *Dictyostelium* DNA methyltransferase DnmA

DIRS-1 and *skipper* retrotransposons have been shown to be methylated by the sole *Dictyostelium* DNA methyltransferase DnmA (Kuhlmann et al., 2005). However, DNA methylation by DnmA seems to be dispensable for heterochromatin formation, since RNAi-mediated depletion of DnmA does not change H3K9me2 (M. Kaller, Diploma thesis, Kassel University, 2002). To analyze if DnmA localisation and / or activity in turn depends on functional histone H3K9 methylation and/or HP1, myc- and GFP-tagged DnmA proteins were overexpressed in *Dictyostelium* (Fig. 3.23). Although both fusion proteins localized to the nucleus (Fig. 3.23A and Kuhlmann et al., 2005), no specific subnuclear localisation that correlated with the known H3K9me2 or HP1-localisation patterns could be observed. Furthermore, capillary electrophoretic analysis of genomic DNA from these cell lines did not show significant increase in genomic 5-methyl cytosine levels (C. Mund and F. Lyko, personal communication; data not shown).

Due to the lack of mutants defective in H3K9 methylation, HP1 targeting, and the redundancy of HcpA/B proteins, a possible signalling pathway for DNA methylation could not be dissected further.

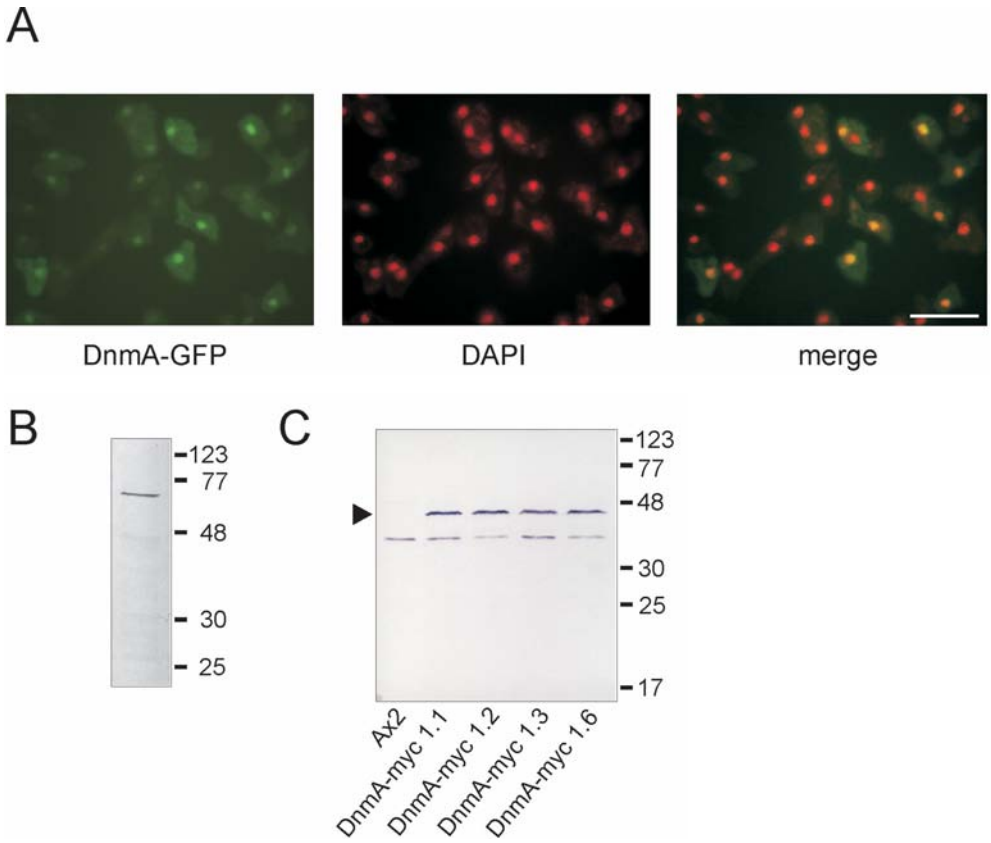

Fig. 3.33**: Expression of DnmA-fusion proteins in *Dictyostelium*.** A) DnmA localizes to the nucleus as a C-terminal-GFP fusion protein. DAPI is pseudocoloured red. Bar: 20µm. B) Western blot of *Dictyostelium* cell lysates with an α-GFP-antibody. The protein detected corresponds in size to the *in silico* calculated MW of DnmA-GFP (~ 71 kDa). C) Overexpression of DnmA-myc. A Western blot with an α-myc-antibody and several independent clones is shown. The arrow marks DnmA-myc. The lower band is derived from unspecific interaction with an unknown protein, which is also detected in the Ax2 wild-type strain.

4 Discussion

4.1 Characterisation of *Dictyostelium* heterochromatin

The centromere is a unique feature of each chromosome that mediates movement of the chromosomes towards the spindle poles in mitotic anaphase. Despite this conserved and crucial function of the centromere for the stable transmission of genetic information to subsequent cell generations, the mechanisms that determine centromere positioning on the chromosomes are still enigmatic. In *S. cerevisiae*, a ~ 125 bp DNA sequence specifies the centromere, but such unique sequence features defining the centromere are lacking in almost all eukaryotic organisms (Cleveland et al., 2003).

In most cases, centromeres reside within highly repetetive satellite sequences, which, however, do not show any obvious evolutionary conservation. The lack of sequence conservation is also illustrated by neocentromere formation from widely different genomic sequences in humans, some of which lack satellite repeats completely (Saffery et al., 2003). Despite the lack of a common underlying DNA sequence, centromeric chromatin from most organisms is composed of nucleosomes that contain homologues of centromere-specific histone H3 variants such as CENP-A; however, the mechanims that drive centromere-specific localisation of these histone variants are unclear (Henikoff and Dalal, 2005).

A common feature of centromeres from most organisms is that they are embedded within regions of constitutive heterochromatin. This pericentromeric heterochromatin is thought to prevent recombination events between the underlying repetetive DNA sequences and to repress transcription of pericentromeric (retro-)transposon sequences (Grewal and Moazed, 2003). Therefore, one common feature of centromeres is their epigenetic definition by the surrounding heterochromatin. Accordingly, epigenetic markers such as histone H3K9 methylation and HP1 proteins can be used to identify pericentromeric sequences.

In *Dictyostelium*, *DIRS*-1 and *skipper* retrotransposons, together with some DNA transposons, form large irregular arrays at the ends of all six *Dictyostelium* chromosomes. Since no other chromosomal regions rich in repetetive sequences could be identified, the retrotransposon clusters were suggested to represent the

Dictyostelium centromeres. The clusters from all chromosomes concentrate in one subnuclear area during interphase and mitosis (Eichinger et al., 2005).

Association of *DIRS*-1 and *skipper* retrotransposons with H3K9me2 could be shown by chromatin immunoprecipitation (ChIP) with α-H3K9me2-specific antibodies, which preferentially precipitated retrotransposon DNA, but not euchromatic DNA from the actin genes, which are also present as a multi-copy gene family that was entirely covered by the chosen PCR primers due to their high sequence conservation. To confirm heterochromatic localisation of *DIRS*-1 and *skipper* retrotransposons in an independent approach, two cell lines stably expressing myc-tagged HcpA and HcpB were established. Unfortunately, ChIP with monoclonal α-myc antibodies did not result in target specific enrichment of retrotransposon sequences. Since both myc-HcpA and myc-HcpB have been shown to localize to heterochromatin, this is likely due to technical problems, as detailed below.

It is not known if the monoclonal antibody can recognize the myc-epitope from the HcpA/B proteins in formaldehyde-cross-linked heterochromatin, in which the myc-epitope may be inaccessible. Nevertheless, at least some of the myc-epitope should be principally accessible in the cross-linked material, since much more DNA was reproducibly precipitated from myc-HcpA or myc-HcpB expressing cells than in the Ax2 wild-type controls, which lack the myc-epitope. Overexpression of HcpA and HcpB by an actin6-promoter results in a large chromatin-unbound nucleoplasmic fraction, which might be randomly cross-linked to chromatin during fixation. Thus, precipitation might cause an unspecific enrichment of DNA from all genomic locations, but not preferentially from heterochromatic regions. In that respect, it may be possible that too much antibody was used, which would even enhance unspecific precipitation of cross-linked chromatin of euchromatic origin, especially given the strong overexpression of myc-HcpA/B. However, given that HcpA and HcpB largely colocalize with H3K9me2, it can be assumed that they are associated with *DIRS*-1 and *skipper* retrotransposons sequences. Furthermore, overexpression of truncated HcpA proteins (which were assumed to display a dominant negative effect) showed increased rates of *skipper* retrotransposon transcription, indicating a role for HcpA/B in regulating the activity of these retroelements, probably in the context of heterochromatin.

Both H3K9me2 and HcpA/B localize in one major subnuclear cluster, and several minor foci, at the nuclear periphery. The observed subnuclear localisation is strikingly

similar to that seen for *DIRS*-1 retrotransposons (Eichinger et al., 2005), providing further support that the *DIRS*-1/*skipper* retrotransposon arrays are associated with H3K9me2 and HcpA/B.

The mitotic behaviour of the H3K9me2 / HcpA/B cluster and its localisation next to the centrosome strongly support the conclusion that it represents pericentromeric heterochromatin. Furthermore, the *Dictyostelium* inner centromere protein (INCENP) homologue, DdINCENP, co-localizes with this heterochromatic cluster in metaphase. Since INCENP, as part of the chromosomal passenger complex, is targeted to centromeres / kinetochores during prometa-/metaphase in other organisms, this finding provides further indications that the heterochromatin cluster indeed represents the centromeres.

Another chromosomal domain known to be heterochromatic are telomeres, which thus might be represented in the observed heterochromatic structure. In mitotically cycling cells of *S. pombe* (Funabiki et al., 1993), *S. cerevisiae* (Gotta et al., 1996) and *P. falciparum* (Freitas-Junior et al., 2000), telomeres form clusters at the nuclear periphery. However, telomeres do not necessarily form clusters near the spindle pole body in mitotically cycling cells, but rather during meiotic prophase in the so-called bouquet stage, when they transiently cluster at a limited sector of the nuclear envelope (Scherthan, 2001). Since centromeres are probably subtelocentric in *Dictyostelium*, the main heterochromatic cluster may also contain the proximal telomeres. The distal telomeres may be located elsewhere at the nuclear periphery and could represent the additional minor heterochromatic foci.

Although this work provides compelling evidence that the *Dictyostelium* centromeres are epigenetically defined by their heterochromatic location, nothing is known about their precise structural organisation. The location of the centromere core, e.g. the site of kinetochore formation, and the surrounding (pericentromeric) heterochromatin is still unclear. Furthermore, it is unclear whether the entire arrays of *DIRS*-1 and *skipper* retrotranspons are uniformly epigenetically modified by H3K9me2 and/or HcpA/B deposition.

Although association of histone H3K9 methylation with both *DIRS*-1 and *skipper* sequences could be demonstrated, both retrotransposons accumulate high (*DIRS*-1) and medium (*skipper*) levels of steady-state mRNA. How can the high levels of retrotransposon RNA be explained in the context of their likely heterochromatic organisation ? Similarly to pericentromeric repeats in *S. pombe* (Volpe et al., 2002;

Kato et al, 2005; Djupedal et al., 2005), *Dictyostelium* heterochromatic DNA sequences may very well be transcribed, and this transcription then in turn would lead to heterochromatin formation at these loci by RNAi-mediated transcript degradation and siRNA-dependent targeting of H3K9me2 and/or HP1 (Noma et al., 2004; Verdel et al., 2004). Evidence for RNAi-mediated targeting of *DIRS*-1 comes from analysis of cloned siRNA libraries, where high numbers of siRNAs with homologies to *DIRS*-1 can be found (Kuhlmann et al., 2005). However, the high steady-state levels of *DIRS*-1 mRNA imply that their turnover-rate by the RNAi machinery is rather low.

Furthermore, some *DIRS*-1 copies may escape *cis*-acting transcriptional silencing through a different chromosomal location or through protection by boundary elements that prevent them from being incorporated into heterochromatin. This might explain why no significant down-regulation of retrotransposon transcription is observed upon overexpression of HcpA or HcpB, which is thought to cause heterochromatin spreading and transcriptional silencing of adjacent chromosomal regions.

It should be stressed that transcriptional competency within centromeric and pericentromeric sequences from other organisms varies significantly. Insertion of marker genes inserted into the heterochromatic outer repeats of *S. pombe* centromeres results in robust silencing, whereas insertion of the same marker genes into the central core or the inner repeats results in much weaker silencing. Remarkably, specific loci between two centromeric tRNA genes within the inner repeats display high rates of transcription (Pidoux and Allshire, 2004). In addition, transcription of genes within the centromeric core region of human neocentromeres has been reported (Saffery et al., 2003). Similarly, the observed high levels of transcription for *DIRS*-1 may be due to transcriptional competency of centromeric chromatin in *Dictyostelium*.

Alternatively, a portion of the observed transcripts may be derived from the telomeric or subtelomeric regions of the chromosomes that are constituted of complex and irregular arrays of both retrotransposons and rDNA sequences that are not sequenced so far (Eichinger et al., 2005). Since telomeres are likely maintained by a recombination mechanism, it may be possible that some of these copies are driven by a strong heterologous promoter that was fused to the retrotransposon sequence after recombination.

DIRS-1 and *skipper* retrotransposon sequences have been shown to be targets of DNA methylation, and loss of the sole *Dictyostelium* DNA methyltransferase DnmA

leads to deregulation of *skipper* retrotransposon activity. Since bisulfite sequencing and chromatin immunoprecipitation showed that histone H3K9me2 and DNA methylation occur on the same genomic loci (Kuhlmann et al., 2005; this work), it should be possible to dissect a hierarchy of events that lead to epigenetic silencing at these loci in further studies. It was shown previously that dsRNA-mediated depletion of DnmA did not lead to significant changes in histone H3K9 methylation levels and patterns, as judged by immunofluorescence data (M. Kaller, Diploma thesis, 2002). Unfortunately, the reciprocal experiment to analyze DNA methylation patterns in a H3K9me2-deficient strain will be difficult to achieve, because the prime candidate gene required for H3K9me2 in *Dictyostelium*, is essential (M. Essid, Diploma thesis, 2003). Although it is likely that H3K9me2 acts upstream of DnmA-mediated DNA methylation, fine-scale mapping of known loci targeted by both epigenetic modifications would be required to further prove this hierarchy of silencing events, since there might be different subdomains of heterochromatin, which are differentially regulated by histone and/or DNA methylation.

During this work, H3K9me2 was found to be restrictively targeted and not to spread significantly if opposing histone modifications such as histone H3K4 methylation, were missing. This could mean a strong selective pressure against heterochromatin spreading into adjacent chromosomal regions, or indicate the presence of some kind of boundary element that separates H3K9-methylated regions, thereby inhibiting heterochromatin spreading.

No mutants could be identified that displayed a significant loss in histone H3K9 methylation levels or altered histone H3K9 methylation patterns, or altered HcpA/B localisation, indicating that proper organisation of heterochromatin is crucial in *Dictyostelium*. Therefore, the mechanisms targeting both histone H3K9 methylation and HcpA/B to pericentromeric heterochromatin remain largely unclear.

4.2 Functions of HP1-like proteins in *Dictyostelium*

HP1 isoforms in *Dictyostelium* are largely redundant

Both HP1 isoforms, when expressed as GFP fusion proteins, are very similar with regard to subnuclear localisation and association with heterochromatin during the cell cycle. Despite of their likely centromeric localization, no mitotic defects in either knock-out strain that could be assigned to centromere/kinetochore function could be

detected. This suggests that the HP1 isoforms are either redundant or, though highly unlikely, not at all required for centromere function. Null mutants of a single HP1 isoform are fully viable under standard growth conditions and only display subtle growth phenotypes at lower temperatures. This was rather surprising since single HP1 isoforms in other organisms such as *Drosophila* are essential for viability, or have at least distinct important functions that cannot be fully compensated by the other isoforms (Eissenberg et al., 1992; Filesi et al., 2002).

It was impossible to generate viable double knock-out strains in the wild type background, but disruption of both endogenous *hcp* genes was obtained, when a rescue construct ectopically expressing HcpA had been introduced into the cells before. This strongly suggested that HcpA and HcpB were redundant in terms of viability. This is further supported by the observation that overexpression of a C-terminally truncated HcpA (HcpAΔC-GFP), assumed to display a dominant negative effect, lead to severe growth defects and significantly reduced cell viability. Consistent with these results, regulated depletion of the rescuing GFP-HcpA in an hcp^{AB-} background was found to interfere with vegetative cell growth.

Formally, it cannot be ruled out that the inability to obtain an *hcpA* / *hcpB* double null cell line is due to a general defect of DNA repair mechanisms, e.g. homologous recombination, in the $hcpA^{-}$ cell line, which is required for targeted gene disruption. This idea is compelling, since cohesin complexes are thought to facilitate recombination between homologous sequences through sister chromatid pairing (Lehmann, 2005). Since mitotically cycling *Dictyostelium* cells are mainly in G2 phase, homologous recombination likely is the major mechanism to repair DNA double-strand breaks (Hudson et al., 2005). Similar to *S. pombe*, loss of *hcpA* may lead to (partial) loss of chromatid cohesion (Bernard et al., 2001; Nonaka et al., 2001), thus impairing DNA repair. The idea could be tested by trials to obtain secondary knock-outs of unrelated *Dictyostelium* genes, which have been shown to be subjected to targeted gene disruption with high frequencies in the Ax2 wild type strain. Alternatively, DNA double-strand breaks could be induced artificially, either by ionizing radiation, or favourably by treatment with bleomycin, which has been shown to cause DNA double-strand breaks in *Dictyostelium* (Hudson et al., 2005). By this it would be possible to assay for a possible hypersensitivity towards DNA damaging agents in any of the *hcp* single knock-out strains.

However, overexpression of GFP-HcpA in an *hcpA*$^-$ knock-out background allowed to obtain a secondary *hcpB* knock-out and thus provided compelling genetic evidence that the GFP-HcpA protein is fully functional, which was important to assess the phenotypic effects upon overexpression of GFP-HcpA/B (see below).

HcpA and HcpB form homo- and heterodimers *in vitro* and *in vivo*

Homo- and heterodimerisation of HP1 isoforms is a conserved hallmark feature of HP1 proteins, which is believed to contribute to the establishment of higher order chromatin structure by formation of large oligomeric protein complexes. In line with that, homo- and heterodimerisation of HcpA and HcpB could be demonstrated both *in vitro* and *in vivo*, indicating that this function is conserved in the *Dictyostelium* proteins. It is largely unclear, if there are biochemical differences between oligomeric HP1 complexes that are composed of HP1 isoforms in different stochiometries, and if these biochemical differences have functional implications (Hiragami and Festenstein, 2005). Here we provide semi-quantitative evidence that HP1 isoforms in *Dictyostelium* have differential affinities for homo- or heterodimerisation. These differential affinities are conferred by the C-termini of the HP1 isoforms, since exchange of the C-termini specifically alters the behaviour of the chimeric proteins. These results are new in that they, for the first time, provide evidence that, although all HP1 isoforms are basically capable of homo- and heterodimerisation, they do so with quantitatively distinct, isoform-specific preferences. These results also may help to explain why HP1 interactions with other proteins are very often isoform-specific (see Introduction, Table 1.1).

Remarkably, the differences detected in *in vitro* assays correlate with specific phenotypic effects in late mitotic stages, where overexpression of proteins containing the C-terminus of HcpA caused increased frequencies of anaphase bridges.

Recently, it has been shown that mammalian HP1 isoforms are differentially postranslationally modified by phosphorylation, acetylation, arginine methylation, ubiquitination, and SUMOylation, a finding that has been interpreted as the existence of an HP1-mediated subcode, which contributes to the differential read-out of the histone H3K9 methyl mark (Lomberk et al., 2006). Intruigingly, in *Dictyostelium* there is preliminary evidence for similar, isoform-specific posttranslational modification, e.g. phosphorylation of HP1 proteins (B. Földesi, Diploma thesis, 2005). Although the

nature of these posttranslational modifications has not been studied in detail yet, their isoform-specific patterns of posttranslational modifictions might represent a (maybe reversible) functional switch in the proteins, which ultimately leads to distinct phenotypic results after protein overexpression. Alternatively, there may be intrinsic biochemical differences between the two isoforms that are determined by the primary amino acid sequence, and not by any postranslational modification. To dissect these different possibilities, it would be required to bacterially express Hcp proteins with a different tag than the already used His-tag (for example, GST- or strep-tag), which would lack any posttranlational modifications, and again perform competetive pull-down experiments as described in the current thesis, or perform a more quantitative assays, for example using surface plasmon resonance (SPR).

Remarkably, *Dictyostelium* HP1 proteins do not interact with their mammalian counterparts, although they are structurally and functionally conserved. This might be explained by functional co-evolution of protein interaction partners and it can be speculated that *Dictyostelium* HP1 proteins do not interact with mammalian HP1 interaction partners such as CAF-1 or members of the TIF family; nevertheless, the *Dictyostelium* HP1 proteins would possibly interact with functional homologues of the mammalian interaction partners from *Dictyostelium*. These would not need the same interaction interface like the consensus PxVxL motif described for most binding partners of mammalian HP1 proteins, but would use a functionally equivalent sequence that allows for interactions with the *Dictyostelium* HP1 proteins.

Overexpression of HcpA and HcpB has distinct phenotypic effects

Although the two HP1 isoforms are very similar in sequence and single knock-outs did not display obvious phenotypes, overexpression of full-length GFP-HcpA and GFP-HcpB had distinguishable effects on growth of mitotically cycling cells. Overexpression of GFP-HcpA, but not GFP-HcpB caused a ~ 4fold increase in aberrant DNA bridges between the separated DNA masses in late mitotic cells. Intruigingly, increased rates of anaphase bridges were also found in strains that overexpressed a chimeric Hcp protein containing an HcpB-N-terminus and an HcpA-C-terminus ($HcpB_NA_C$), indicating that the C-terminus of HcpA elicits the observed phenotype. In contrast, overexpression of full-length GFP-HcpB or its chimeric counterpart GFP-$HcpA_NB_C$, which contains the HcpA-N-terminus and the HcpB-C-terminus, did not have any

effect on chromosome segregation. The observed phenotype thus also correlates with the low *in vitro* dimerisation activity of both GFP-HcpA and GFP-HcpB_NA_C proteins with column-immobilized His-HcpA or His-HcpB. Since His-HcpA is likely to be engaged in homotypic interactions on the column, the low *in vitro* dimerisation activity can be explained by a low exchange rate between column-bound His-HcpA and soluble GFP-HcpA in the lysate. The column-bound HcpA-dimers thus would be comparably stable and would bind few GFP-HcpA proteins, which likewise are engaged in stable interactions in the lysate. If one assumes that the stability of HcpB interactions is lower, GFP-HcpB would form more rapidly heterodimers with column-bound His-HcpA, and even more with column-bound His-HcpB, which would have a higher exchange rate.

The aberrant chromosome configurations found in these two overexpression strains resembled those in HP1 null strains of *Drosophila* (Fanti et al., 1998), where they are caused by telomere fusions. In mammals, overexpression of HP1 isoforms as N-terminal GFP-fusions has been reported to cause telomere fusions and changes in telomere length by disturbed interaction of telomerase with telomeric sequences (Sharma et al., 2003). *Dictyostelium* telomeres appear not to be composed of specific hexanucleotide repeats and a homolog for a telomerase gene was not found in the genome. Intruigingly, rDNA sequences from the extrachromosomal rDNA copies can be found at the ends of *Dictyostelium* chromosomes (Eichinger et al., 2005). It is therefore likely that telomeres in *Dictyostelium* are maintained by a DNA recombination mechanism that fuses rDNA sequences to the chromosome ends. We propose that overexpression of HcpA or its chimeric counterpart HcpB_NA_C lead to alterations in chromatin structure at targeted loci, at telomeres, these may impair proper telomere maintenance by interfering with DNA recombination or with the resolution of aberrant recombination intermediates by changing the chromatin accessibilty for other proteins required for these processes. Although the *in vitro* dimerisation characteristics of both HcpA and HcpB cannot be causally related to the observed *in vivo* phenotypes upon overexpression, they provide indications that the two proteins specifically alter chromatin structure at targeted loci by changing the accessibility for other proteins. This may be due to a steady-state residence time at chromatin which is different from that of HcpB or its chimeric counterpart HcpA_NB_C. Furthermore, all overexpressed Hcp fusion proteins are present as a chromatin-bound fraction and an additional nucleoplasmic fraction (indicated by the overall nuclear

fluoresence in the overexpression strains). Therefore, the overexpressed proteins may deplete other protein factors from chromatin due to protein-protein interactions in the nucleoplasm. Since these interactions may be isoform-specific, overexpression of the isoforms may have distinct phenotypic effects.

Since the anaphase-bridge phenotype cannot be observed in cells expressing C-terminally truncated forms of HcpA, only overexpression of functional HcpA can elicit the defect in chromosome distribution. The functionality of GFP-tagged HcpA protein was supported by the observation that ectopic expression of the protein allowed to disrupt the *hcpB* gene in an *hcpA*$^-$ background strain and restored the *hcpB* disruption frequencies obtained in the Ax2 wild-type strain (see above). Furthermore, all fusion proteins maintained their ability to specifically form homo- and heterodimers *in vitro* and *in vivo* and showed correct subnuclear localisation. In addition, it has been shown that N-terminal GFP fusions of HP1 maintain their functions in various other organisms (Kirschmann et al., 2000; Wang et al., 2000; Cheutin et al., 2003; Cheutin et al., 2004).

However, the observed phenotype of GFP-HcpA or GFP-HcpB_NA_C overexpression strains can also be explained by additional binding of HcpA at sites that are not covered in the wild type. This could be either due to spreading out of heterochromatic loci as observed in position effect variegation. Alternatively, other parts of chromatin may be aberrantly targeted by excess HP1, since overexpression resulted in increased nuceoplasmic staining.

Why does overexpression, but not gene disruption of the two isoforms cause different phenotypic effects? One possibility is that both isoforms acquire distinct functional properties upon differential post-translational modifications. In the overexpression strains, a differential modification pattern of the overexpressed isoform may confer distinct functional properties to this isoform, e.g. distinct preferences for protein-protein-interactions, which lead to distinct changes in chromatin dynamics. In the single knock-out strains, a switch in the modification state may shift the remaining isoform into another functional state, where it can compensate for the loss of the other isoform, resulting in no detectable phenotypes.

Alternatively, strong overexpression of one isoform shifts the stoichiometric balance in Hcp oligomeric complexes in a way that these complexes acquire different biochemical properties. These different biochemical properties would not have drastic effects in the single knock-out strains, where only one isoform is present, but may

cause problems upon overexpression, as by increasing the overall Hcp dosage in the cell, which would then cause heterochromatin spreading and/or aberrant protein targeting.

CSD-dependent and CSD-independent localisation of HP1 proteins.

HP1 shows different mechanisms of targeting to chromatin, which largely dependent on chromosomal location. Especially at euchromatic loci, HP1 is targeted by interactions with other proteins such as the Rb or members of the TIF-family through the CSD (Lechner et al., 2000; Nielsen et al., 2001). At heterochromatin, other mechanisms contribute to correct localisation, since C-terminally truncated proteins lacking the CSD still localize to heterochromatin (Wang et al., 2000; Cheutin et al., 2003; this work). These other mechanisms may include binding of methylated H3K9, binding to other proteins through the chromo domain / hinge, or targeting by binding either directly to DNA or to RNA.

Although it is well possible that HP1 proteins interact with other centromere-specific proteins through the chromo domain / hinge, and are thus recruited to heterochromatin, it is intriguing to speculate that HP1 proteins might be directly targeted to (peri-)centromeric DNA. This might be realised either by direct binding due to (unknown) features of this DNA, or by being targeted by certain RNA species. It is unlikely that pericentromeric DNA is bound in a sequence-specific manner, because the hinge region, which is required for binding, does not code for any known sequence-specific DNA binding motifs, but contains patches of basic amino acid residues that are required for interactions with negatively charged nucleic acids. In line with that, *in vitro* DNA binding is observed with randomly chosen DNA probes. However, it cannot be ruled out that specific sequence motifs within pericentromeric DNA are preferentially bound by HP1 proteins *in vivo*. Another possibilty is that (peri-) centromeric DNA is folded into an unusual higher-order structure that somehow exposes stretches of DNA that would otherwise be buried in the chromatin fiber, and thus are recognized by HP1 proteins.

Finally, it is possible that the major pathway of targeting HcpA/B to heterochromatin is achieved by protein-protein interactions with upstream factors, and that to DNA binding rather represents one out of several possible binding possibilities that stabilize overall binding of HP1 to chromatin. It should be stressed that overexpression of C-

terminally truncated forms of HcpA/B was performed in an Ax2 wild-type background. Therefore, protein targeting was achieved in the presence of endogenous HP1 proteins and moreover, on preformed heterochromatin. Formation of the latter thus may be a prerequisite for proper targeting of the truncated proteins.

A similar scenario might hold true for HP1 binding to RNA. Although an (unidentified) RNA species contributes to HP1 binding to chromatin in mammals (Maison et al., 2002), it is largely unclear if the RNA-binding activity of HP1 stabilises its binding to chromatin, or if it acts in recruiting HP1 proteins to chromatin. Although there are apparently no specific sequence requirements for nucleic-acid binding *in vitro*, it will be interesting to determine if there is a specific type of RNA which is bound to HP1 proteins *in vivo*. Since siRNAs, the effector molecules of the RNAi machinery, have been shown to target chromatin-modifying activities to the respective genomic loci in various organisms (Bernstein and Allis, 2005), one could assume that some kind of RNA species might recruit HP1 proteins in a similar fashion directly to chromatin. If this RNA species are the siRNAs themselves, remains to be determined.

Thus, transcription of (peri-)centromeric sequences might stabilize HP1 binding to these regions either by direct binding to the transcribed RNAs. These RNAs may further be converted into siRNAs by the RNA machinery, which could be bound by HP1. Alternatively, the siRNAs might target histone modifying activities and thus cause transcriptional silencing of these regions in a parallel pathway.

Several reports have demonstrated that the mammalian HP1γ isoform localizes to actively transcribed euchromatic regions, where it is associated with transcription elongation (Vakoc et al, 2005; Lomberk et al., 2006). Intruigingly, RNA binding activity for HP1γ has been demonstrated (Muchardt et al., 2003; Schmiedeberg et al., 2004). Thus, the RNA binding activity of HP1 may represent a general mechanism of HP1 chromatin targeting and may be involved in both activating and repressing pathways, which would be defined by additional protein factors and their respective chromosomal location (Kellum, 2003).

4.3 Evolutionary conservation of the histone H3K9methylation / HP1 pathway

Histone H3K9 methylation is an evolutionarily conserved histone modification that in most organisms marks silenced chromatin domains. Furthermore, the association of H3K9 methylation and HP1 proteins also seems to represent an evolutionarily conserved pathway: Apparantly all organisms that show histone H3K9 methylation

also encode for HP1 proteins in their genomes. Intruigingly, in *S. cerevisiae*, both histone H3K9 methylation and HP1 proteins are absent, and instead, histone H3K4 methylation (in combination with histone deacetylation) is used to mark transcriptionally silent regions (Bryk et al., 2002). Although evolutionarily conserved, the histone H3K9 methylation system is flexible in that it can be used for biological purposes different from heterochromatin formation or epigenetic gene silencing. Furthermore, different methylation states of the H3K9-residue are used to mark specific chromatin domains in different organisms.

In mammals, H3K9me3 (catalyzed by Suv39h1/2) marks pericentromeric heterochromatin, whereas silenced domains within euchromatin are marked by mono- and dimethylation, which is mainly catalyzed by G9a (Rice et al., 2003; Peters et al., 2003). The HP1α and HP1β isoforms show largely overlapping localisation with pericentromeric histone H3K9 methylation, whereas the HP1γ isoform is euchromatic (Fischle et al, 2005; Maison et al., 2002; Minc et al., 1999). An HP1 knock-out of any of the isoforms in mammals (or even vertebrates) has not been described, presumably due to the lethality of the knock-out (Filesi et al., 2002).

In *Drosophila*, both H3K9me2 and H3K9me3 (catalyzed by Su(var)3-9) are found in chromocenters of salivary gland polytene chromosomes, with H3K9me3 being particularly enriched at the chromocenter core that represents centromeric heterochromatin (Ebert et al., 2004). In addition, H3K9me2 is associated with some euchromatic bands and telomeres. HP1 in *Drosophila* is also associated with chromocenters, telomeres and many euchromatic regions, the latter partially overlapping with histone H3K9 methylation (Schotta et al., 2002; Perrini et al., 2004; Greil et al., 2003). HP1 is essential for viability in *Drosophila*; however, due to a supply of the embryo with maternal HP1 mRNA, the HP1 null embryos only die at late embryonic stages (Eissenberg et al., 1992), when the maternal HP1 mRNA stores become depleted.

In contrast, heterochromatic chromocenters in *Arabidopsis* (which contain centromeric sequences) are marked by histone H3K9me2, whereas H3K9me3 is exclusively found within euchromatin (Lindroth et al., 2004; Jackson et al., 2004; Fischer et al., 2006). Furthermore, the *Arabidopsis* HP1 homologue *LHP1* has adopted specialized functions in silencing of floral homeotic genes (Gaudin et al., 2001; Kotake et al., 2003) and is mainly localized to euchromatic regions (Libault et al., 2005; Nakahigashi et al., 2005).

In *Tetrahymena*, histone H3K9 methylation has adopted a rather specialized function in marking internal excision sequences (IESs) during DNA elimination (Taverna et al., 2002). An HP1 homologue in *Tetrahymena* has been described, however, its involvement in DNA elimination and histone methylation has not been analyzed (Huang et al., 1998a; Huang et al., 1999). Intriguingly, two other chromodomain proteins, Pdd1p and Pdd3p are involved in the programmed DNA elimination and bind to methylated methylated H3K9 *in vitro* (Taverna et al., 2002).

In *C. elegans*, histone H3K9 methylation serves to silence unpaired DNA sequences during meiosis, which is the case for the male X chromosome, but also for transposable elements (Bean et al., 2004; Maine et al., 2005). Two HP1 homologues exist in *C. elegans*, which have specialized functions in Rb-mediated gene silencing during germ line and vulval development (Couteau et al., 2002; Cardoso et al., 2005).

In *Neurospora crassa*, histone H3K9 trimethylation and HP1 recruitment are key players of the “canonical” gene silencing pathway that also applies DNA methylation to silence transposable elements, likely by the RIP (repeat-induced point mutation) pathway (Tamaru et al., 2003; Freitag et al., 2004).

A great contribution to our understanding of how heterochromatin formation contributes to epigenetic gene silencing has been made by numeruos studies in *S. pombe*, which also involves histone H3K9 dimethylation and HP1/Swi6 recruitment Both H3K9me2 and Swi6 are found at centromeres, telomeres and the silent mating-type locus, where they are involved in transcriptional silencing of these loci (Nakayama et al., 2000; Hall et al., 2003). At the centromeres, heterochromatin marked by H3K9me2 and Swi6 are also required for proper kinetochore function (Bernard et al., 2001; Nonaka et al., 2002).

In *Dictyostelium*, both histone H3K9 dimethylation and HP1 proteins are used to mark (peri-)centromeric and possibly telomeric DNA sequences, whereas H3K9me3 is not detectable using methylation-state specific antibodies. Furthermore, histone H3K9 methylation is used to mark retroelements within these chromosomal regions. Two HP1 isoforms are expressed, which have largely overlapping functions, yet display quantitative biochemical differences. Both histone H3K9 methylation and HP1 appear to be essential in *Dictyostelium*. Comparing these characteristics with those from the aforementioned organisms, *Dictyostelium* appears to be an organism in which histone H3K9 methylation / HP1 function is much closer related to its mammalian

counterparts, than for example in *Arabidopsis*, *Tetrahymena* or *C. elegans*, where HP1 proteins have adopted rather specialized functions in gene silencing.
Thus, using the term “model organism”, *Dictyostelium* may indeed be considered as such to study chromatin-mediated mechanisms of gene silencing.

4.4 Identification of the *Dictyostelium* Orc2 homologue (OrcB)

HcpA and HcpB appear to have the intrinsic ability to target centromeric sequences (probably by their definition of being heterochromatic, not by sequence definition), which likely is mediated by H3K9me2 binding, but also direct nucleic acid interactions. However, this does not necessarily mean that HP1 targeting is independent from upstream factors, because several pathways may act in parallel to ensure heterochromatin maintenance. One possible protein factor involved in HP1 targeting to centromeric heterochromatin is the origin recognition complex (ORC). ORC is preferentially associated with heterochromatin in *Drosophila* and humans, and disruption of the Orc2 subunit of ORC interferes with HP1 localisation to heterochromatin (Pak et al., 1997; Huang et al., 1998; Shareef et al., 2001; Prasanth et al., 2004). Therefore, it was investigated if ORC could serve as an upstream factor required for HP1 targeting in *Dictyostelium*. According to its probably conserved function in initiation of DNA replication (Bell, 2002), the *Dictyostelium* Orc2 homologue OrcB, when overexpresssed as N-terminally tagged GFP-OrcB, was found to be preferentially enriched in the nucleus, although a cytoplasmic pool of GFP-OrcB was commonly detected.
Subnuclear colocalisation of GFP-OrcB and HcpA-RFP was found in some minor foci at the nuclear periphery, but no colocalisation of OrcB and HcpA could be detected at pericentromeric heterochromatin. Localisation to heterochromatin is likely dependent on incorporation into the ORC holo complex, thus the (comparably low) amount of all other subunits may limit the localisation of GFP-OrcB to heterochromatin. In addition, some specific localisation may be covered by the strong nucleoplasmic background of chromatin-unbound GFP-OrcB and be difficult to detect. This problem could be circumvented by either using weaker promoters for ectopic overexpression or a knock-in strategy for tagged expression of OrcB under its endogenous promoter. Nevertheless, GFP-OrcB obviously does not preferentially localize to pericentromeric sequences.

Another possibility is that the GFP-tagging of OrcB interferes with OrcB incorporation into the ORC holo complex. This problem may be circumvented by using smaller tags (e.g. myc), which would interfere sterically much less than the comparably big GFP-tag. However, GFP-tagged human Orc2 has been shown to maintain its ability to interact with Orc1 in *in vivo* FRET experiments (Lidonnici et al., 2004). Furthermore, assuming that GFP-OrcB is incorporated into the ORC holo complex, GFP-tagging OrcB does not appear to interfere with OrcB function, and thus GFP-OrcB does not act as a dominant-negative factor, since we did not observe any obvious defects in centrosome duplication or spindle formation. These phenotypes are observed upon Orc2 depletion in human cells, where Orc2 is essential for viability (Prasanth et al., 2004).

In contrast to the described function of ORC in centromeric heterochromatin organisation in *Drosophila* and humans, preferential localisation to centrosomes was observed of OrcB. Although it cannot be ruled out that the observed localisation is an overexpression artefact, it is tempting to speculate that the centrosomal localisation might reflect that of the endogenous OrcB protein. Intruigingly, human Orc2 also has been shown to reside at the centrosome throughout the cell cycle (Prasanth et al., 2004), indicating that this property of Orc2, though not fully conserved, is present in different evolutionary lineages. *Dictyostelium* is a well established model system to study centrosome structure and function, however, only very few centrosomal proteins have been identified and studied to date (Reinders et al., 2006). The finding that OrcB localizes to centrosomes therefore may be of interest to study the functional implications of this localisation and to gain further insight into the protein repertoire required for proper centrosome function.

One explanation for this finding is that Orc2 is part of a signalling pathway from DNA replication to centrosome duplication, ensuring that centrosome duplication and entry into mitosis only occurs when the DNA is fully replicated. Alternatively, Orc2 in *Dictyostelium* might be a structural component of the centrosome and serve a different function.

In line with that, other ORC subunits have adopted additional functions in various cellular processes, which are largely independent of their core function during DNA replication, further stressing the multifunctionality of replication initiator proteins. The Orc6 subunits of both *Drosophila* and human ORC associate with the cell membrane and the cleavage furrow during cytokinesis (Prasanth et al., 2002; Chesnokov et al.,

2003). Recently, the mammalian Orc3 and Orc4 subunits have been shown to localize to the cell membrane and to be required for dendritic growth in post-mitotic neurons (Huang et al., 2005).

4.5 A proof-of-principle: Mitotic co-localisation of DdINCENP with HcpA and H3K9me2

One aim of this work was to provide molecular tools to analyze heterochromatin function in *Dictyostelium*. In other organisms, pericentromeric heterochromatin is required for transcriptional silencing of repetetive elements, but also serves to maintain centromere/kinetochore function, for example by promoting sister chromatid cohesion. Thus, pericentromeric heterochromatin is linked to a variety of different processes apart from epigenetic gene regulation, such as mitotic chromosome segregation.

Dictyostelium is widely used to analyze cell biological questions, such as centromere function, mitosis and cytokinesis. The knowledge about centromere organisation and localisation is in many ways crucial for these investigations; however, many of these studies lacked evidence for localisation of the *Dictyostelium* centromeres, which remained speculative. The current work at least partly resolves this problem by providing an epigenetic definition of centromeric chromatin that manifests in histone H3K9 methylation and HP1 targeting and thus spatiotemporarily resolves its cell-cycle related localisation. As an example, the mitotic localisation of the *Dictyostelium* inner centromere protein DdINCENP, could be further characterised. It was demonstrated that DdINCENP localizes in numerous foci at the nuclear periphery during interphase, but then moves to the centromeres/kinetochores during metaphase, and passages along the microtubules towards the central spindle during anaphase.

These findings are not only important for the characterisation of DdINCENP function, but furthermore have a direct impact on the understanding of HcpA/HcpB dynamics during mitosis. INCENP is part of the chromosomal passenger complex (CPC), which recruits Aurora-B kinase activity to the kinetochores during prometa- / metaphase, when Aurora is required to regulate microtubule-kinetochore interactions (Murata-Hori and Wang, 2002). One substrate of Aurora-B is the S-10 residue of histone H3, and S-10 phosphorylation of K-9 methylated histone H3 tails causes HP1 to dissociate from heterochromatin (Fischle et al., 2005; Hirota et al., 2005). Thus, Aurora kinase is responsible for HP1 dissociation from heterochromatin during prometa-/metaphase, as

it was observed for the *Dictyostelium* HP1 homologues. It thus can be speculated that *Dictyostelium* uses similar “binary modification switches” on the histones (Fischle et al., 2003), as they have been proposed and begun to be demonstrated for mammalian systems.
The results presented here are likely to facilitate research on the mitotic regulation of Aurora kinase activity in *Dictyostelium* and on related subjects such as kinetochore capture by microtubules and the role of microtubule plus-end stabilizing proteins such as EB1, Lis1 or the XMAP215 homologue DdCP224 in these processes (Rehberg and Graf, 2002; Rehberg et al., 2005; Graf et al., 2000).

4.6 Outlook

Since loss of HP1 and possibly also loss of SuvA activity is lethal to *Dictyostelium* cells, one may speculate that it will be difficult to obtain mutants of components that act upstream in the heterochromatin formation pathway and cause a significant loss of pericentromeric H3K9me2 and/or HP1. Indeed, all available mutant strains tested so far do not display significant differences in H3K9me2 levels and patterns, or altered HcpA/B localisation.

Using ChIP, it should be possible to identify genes that are differentially epigenetically modified during vegetative growth and development. A starting point would be to analyze genes, which are known to be differentially regulated during vegetative growth and development, as it has successfully been done with histone H3K4 methylation for a number of developmentally regulated genes such as *acaA* or *rasG* (Chubb et al., 2006). In a more sophisticated approach, it may possible to combine the ChIP technique with the microarray technology (ChIP-on-chip) to identify genes whose epigenetic modification pattern is dynamically regulated during development.
Thereby, target genes could be identified, and the sequence of events upon either gene activation or repression of these genes could be analyzed in more detail.

Furthermore, HP1 interaction partners could be identified, either by yeast-two-hybrid analysis or by purification of (TAP-)tagged HcpA/B by affinity-chromatography and mass spectrometric analysis of co-purified proteins. This would allow to analyze

possible upstream components required for HP1-mediated mechanisms of gene silencing and heterochromatin formation.

In order to gain further insight into epigenetic mechanisms of gene regulation by site-specific histone modifications, it may be useful to identify all histone modifications present in *Dictyostelium* by mass spectrometry. First, this would allow to assess which “histone-encoded” pathways are present in *Dictyostelium*. For example, the K27 residue is present in the *Dictyostelium* histone H3, but the genome does not seem to encode for any K27-specific histone methyltransferases (see introduction), indicating the absence of any Polycomb-related pathways of gene regulation in *Dictyostelium*. It should be noted that histone H3K27 methylation has not been reported in the yeasts (see introduction, Fig. 1.3), nor in any other unicellular eukaryote, indicating that K27 methylation emerged as a silencing module in metazoan development.
Second, having established this platform technology of histone preparation and mass spectrometric analysis, it would allow to address the effects of certain mutants on specific histone modification levels independently from antibody-based strategies like western blotting or immunocytochemistry.

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