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Localization and organization of protein factors involved in chromosome inheritance in *Dictyostelium discoideum*

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Abstract

Heterochromatin protein 1 (HP1) proteins are highly conserved heterochromatin components required for genomic integrity. We have previously shown that the two HP1 isoforms expressed in *Dictyostelium*, HcpA and HcpB, are mainly localized to (peri-)centromeric heterochromatin and have largely overlapping functions. However, they cause distinct phenotypes when overexpressed. We show here that these isoforms display quantitative differences in dimerization behavior. Dimerization preference, as well as the mutant phenotype in overexpression strains, depends on the C-terminus containing the hinge and chromo shadow domains. Both Hcp proteins are targeted to distinct subnuclear regions by different chromo shadow domain-dependent and -independent mechanisms. In addition, both proteins bind to DNA and RNA *in vitro* and binding is independent of the chromo shadow domain. Thus, this DNA and/or RNA binding activity may contribute to protein targeting. To further characterize heterochromatin, we cloned the *Dictyostelium* homolog of the origin recognition complex subunit 2 (OrcB). OrcB localizes to distinct subnuclear foci that were also targeted by HcpA. In addition, it is associated with the centrosome throughout the cell cycle. The results indicate that, similar to Orc2 homologs from other organisms, it is required for different processes in chromosome inheritance.

Keywords: centromere; centrosome; heterochromatin; HP1; mitosis; ORC.

Introduction

In mitosis, genetic information has to be passed to the daughter cells with great accuracy to ensure viability. Defects in the control of chromosome segregation may lead to aneuploidy, which is either lethal or a characteristic hallmark of most solid tumors in humans (Kops et al., 2005).

We analyzed proteins that contribute to chromatin architecture, chromosome inheritance and/or genomic integrity in *Dictyostelium discoideum* to gain further insight into the mechanisms of chromosome inheritance in vegetatively growing cells.

Heterochromatin protein 1 (HP1) proteins are highly conserved throughout evolution and are required for the formation and spreading of heterochromatin at centromeres and telomeres, as well as for gene-specific silencing of euchromatic genes (Hiragami and Festenstein, 2005).

Centromeric heterochromatin is crucial for proper chromosome segregation in various organisms. In *Saccharomyces pombe*, loss of the histone methyltransferase Clr4 or the HP1 homolog Swi6 leads to defects in sister chromatid cohesion and lagging anaphase chromosomes, probably due to merotelic attachment of the spindle microtubules to the kinetochores (Ekwall et al., 1996; Bernard et al., 2001). Similarly, loss of mammalian histone methyltransferases Suv39h1 and Suv39h2 leads to altered pericentric histone methylation patterns and chromosomal instabilities (Peters et al., 2001). Telomeric heterochromatin is required to cap the chromosome ends and protect them from being recognized as DNA damage (Cenci et al., 2005). Consequently, loss of HP1 causes frequent telomeric fusions in *Drosophila*, leading to dicentric chromosomes and mitotic anaphase bridges (Fanti et al., 1998).

In *D. discoideum*, the two HP1 isoforms expressed are largely redundant in function, although expression of at least one isoform appears essential for viability (Kaller et al., 2006). Both isoforms co-localize at centromeric heterochromatin and some other minor sites. Their precise function at these loci is unclear but most likely includes transcriptional silencing of repetitive elements and recruitment of cohesin complexes. Remarkably, separate overexpression of each isoform has distinct effects on growth and differentially affects chromosome dynamics during mitosis. We assume that the distinct phenotypic effects upon overexpression arise from slightly different biochemical properties of the two proteins. To address this issue, chimeric HP1 forms were constructed and tested for function.

We then aimed to identify and characterize possible upstream components that are required for HP1 targeting in *Dictyostelium*. 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 (Beall et al., 2002; Wilmes and Bell, 2002). Upon binding to DNA, it facilitates assembly of the pre-replicative complex (pre-RC) by recruitment of other DNA replication factors such as Cdc6, Cdt1 and the MCM complex. In addition, ORC is required for transcriptional gene silencing at the silent mating type loci in *Saccharomyces cerevisiae* (Shore, 2001) and for position-effect variegation (PEV) in *Drosophila* (Pak et al., 1997). In higher eukaryotes, ORC interacts with HP1 and is associated with heterochromatin. Furthermore, disruption of ORC interferes with HP1 localization to heterochromatin (Pak et al., 1997;

Shareef et al., 2001). In particular, mutants of the Orc2 subunit have impaired HP1 localization in *Drosophila* and human cells, indicating that Orc2 functions in HP1 targeting (Huang et al., 1998; Prasanth et al., 2004). Finally, 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).

The centrosome is the major microtubule-organizing center in the cell and is thus required for proper chromosome segregation during mitosis. Defects in centrosome function cause chromosome missegregation and probably cancer (Doxsey et al., 2005). We attempted to elucidate the role of ORC in heterochromatin formation in *Dictyostelium* and identified all six genes encoding the ORC subunits. To analyze the cellular localization of ORC in *Dictyostelium* with respect to HP1, we cloned the Orc2 homolog (OrcB) and expressed it as a GFP fusion protein.

Results

Regulated depletion of HP1 proteins interferes with vegetative growth

As shown previously, HcpA and HcpB have largely redundant functions, but expression of at least one isoform is essential for viability. This is indicated by the inability to obtain *hcpA*/*hcpB* double null mutants using the Cre/loxP technique (Kaller et al., 2006). However, both endogenous genes can be disrupted in a strain that overexpresses His-HcpA ectopically. In a similar approach, we created a cell line that had the endogenous *hcpA* and *hcpB* genes knocked out (*hcp*$^{AB-}$/GFP-HcpA) and expressed GFP-tagged HcpA (GFP-HcpA) under control of the actin6 promoter. This strain was constructed by first disrupting the *hcpA* gene with a BS^{R} cassette flanked by loxP sites (Figure 1A) and then removing the resistance marker via Cre recombinase (data not shown). The resulting strain was denoted as *hcpA*^{lp} since it contains a single loxP site in the disrupted *hcpA* gene. The *hcpA*^{lp} strain was then transformed with a GFP-HcpA fusion construct on a plasmid containing the G418 resistance cassette, resulting in strain *hcpA*^{lp}/GFP-HcpA. In this strain the *hcpB* gene could be successfully disrupted (Figure 1B). While a double KO failed in the wild-type background (0 out of 292 clones examined, Figure 1C), disruption of the second *hcp* gene was easily obtained when the strain ectopically expressed a copy of the *hcpA* gene. Interestingly, the disruption frequency of *hcpB* was very similar to that in the wild-type background (50% compared to 67%, Figure 1C). Similar disruption frequencies were previously obtained with a *hcpB* knockout in a hcpAlp/His-HcpA strain (Kaller et al., 2006), suggesting that the recombinant gene fusions were fully functional and did not cause any significant growth disadvantage.

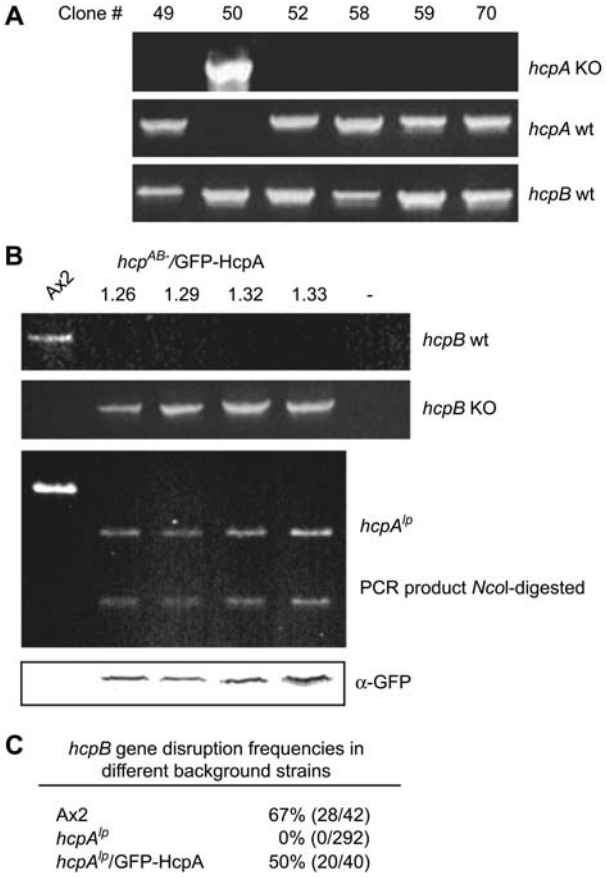

Figure 1 Genotyping of the *hcp*$^{AB-}$/GFP-HcpA strain.
(A) The *hcpA* gene was disrupted by insertion of a floxed BS^{R} cassette via homologous recombination. Clonal isolates were screened for disruptants by PCR analysis with primers specific for homologous recombination at the *hcpA* locus (*hcpA* KO) or for the wild-type gene (*hcpA* wt). A third PCR confirmed that the *hcpB* locus was unaffected (*hcpB* wt). Clone #50 showed the expected KO-PCR product, lacked the wild-type product and displayed an unaffected *hcpB* gene. This clone was used for subsequent Cre-mediated excision of the resistance cassette resulting in *hcpA*^{lp} (not shown). (B) The *hcpA*^{lp} containing strain was transformed with a GFP-HcpA expression vector. In the resulting strain *hcpA*^{lp}/GFP-HcpA, the *hcpB* gene was targeted for disruption. For clones 1.26, 1.29, 1.32 and 1.33, the top row shows that the PCR product specific for the wild-type *hcpB* gene is missing, but that instead the expected PCR product for the *hcpB* KO is detected (second row). Disruption of the *hcpA* gene was confirmed by PCR. Since the disrupted *hcpA*^{lp} gene after removal of the BS^{R} cassette was very similar in size to the wild-type gene, the PCR product was digested with NcoI, which cuts at the remaining loxP site within the disrupted gene, but not in the wild-type gene (third row). The presence of the construct expressing the GFP-HcpA fusion was monitored by Western blot analysis with an anti-GFP-antibody that detected a protein of the expected size (bottom row). (C) Disruption frequencies for the *hcpB* gene in Ax2, *hcpA*^{lp} and *hcpA*^{lp}/GFP-HcpA strains. Whereas high frequencies of *hcpB* gene disruptions were obtained in the Ax2 and *hcpA*^{lp} /GFP-HcpA strains, no *hcpB* gene disruption was obtained in the *hcpA*^{lp} background in multiple independent transformations.

Dynamic association of HP1 with heterochromatin during mitosis

The generation of viable *hcp*$^{AB-}$/GFP-HcpA cells provided genetic evidence of the functionality of the GFP fusion protein and allowed for a more reliable investigation of GFP-HcpA localization. Since the fusion protein compensated for both endogenous proteins, it reflected their dynamics more closely than in the wild-type background, in which it competed with endogenous proteins.

Similar to overexpression of GFP-HcpA in the Ax2 background, localization to heterochromatin was strongly reduced during mitosis (Figure 2). This agrees with findings in, e.g., mammalian cell lines in which HP1 binding during mitosis was strongly diminished. In mitosis, aurora kinase phosphorylates serine-10 adjacent to the methylated lysine-9 residue in histone H3 and its phosphorylation disrupts binding of HP1 (Fischle et al., 2005). Furthermore, HP1 proteins themselves are differentially phosphorylated during mitosis (Minc et al., 1999), which may contribute to dissociation from heterochromatin.

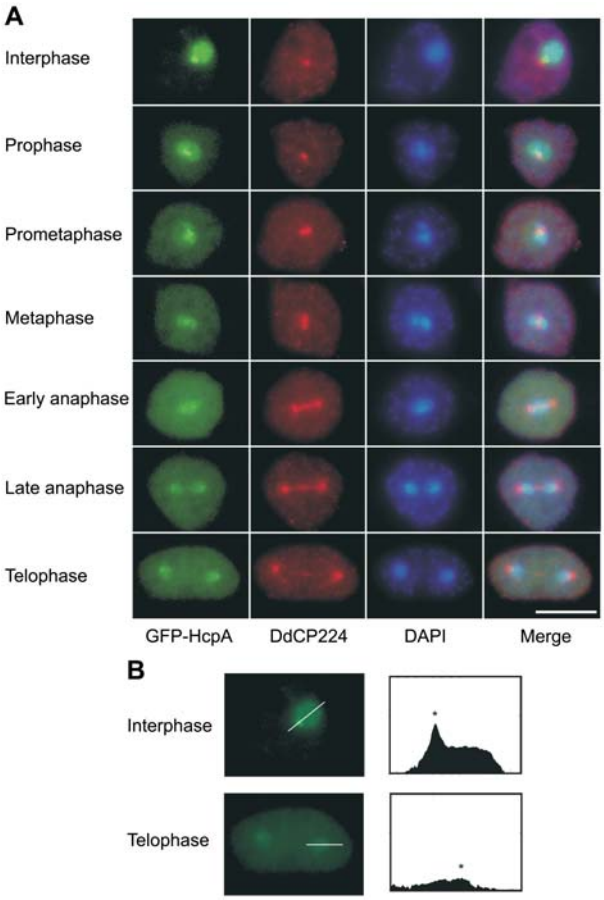

Figure 2 Mitotic dissociation of GFP-HcpA from heterochromatin.
(A) Nuclear and heterochromatic fluorescence of GFP-HcpA in the *hcp*$^{AB-}$/GFP-HcpA cell line is pronounced during interphase, but decreases significantly after metaphase and during later mitotic stages. Immunodetection of the centrosome-specific antigen DdCP224 and DAPI staining was used to visualize mitotic stages, as indicated on the left. Images in vertical rows were acquired with identical exposure times. Identical image processing for all mitotic stages ensured that quantitative differences were genuinely represented. (B) To further quantify differences in fluorescence intensity during interphase and telophase, fluorescence intensity profiling was performed using TINA 2.0 software. Sections used for profiling are indicated by white lines that were placed across the major (centromeric) HP1 spot in the fluorescence images. The y-axis shows fluorescence intensity in arbitrary units, and the x-axis shows the length of the profile in arbitrary units.

DIRS-1 and *skipper* retrotransposons physically associate with histone H3K9me2

Dictyostelium centromeres are probably composed of complex transposon and retrotransposon arrays (Eichinger et al., 2005). The retroelements *DIRS-1* and *skipper* that preferentially localize in these arrays are targets for DNA methylation and are regulated by the RNAi machinery (Kuhlmann et al., 2005). The clustering of retroelements in one major focus at the nuclear periphery and its splitting into six distinct spots during mitosis (most likely representing the centromeres) (Eichinger et al., 2005) is highly similar to the pattern observed for histone H3K9me2, HcpA and HcpB. We therefore investigated whether the retroelements were targets for chromatin modification, especially methylation of H3K9, which would provide binding sites for HP1 proteins.

Using chromatin immunoprecipitation (ChIP), we showed that both *DIRS-1* and *skipper* sequences are preferentially enriched by precipitation with an antibody recognizing H3K9me2. In contrast, the euchromatic multi-copy *actin* gene family was not associated with H3K9me2 (Figure 3). These results were obtained with Ax2 cells, as well as with strains ectopically expressing myc-HcpA and myc-HcpB. We have thus shown that the retroelements are packaged into chromatin containing dimethylated H3K9. Since HP1 binds to this modification, we conclude that the chromatin on *skipper* and *DIRS-1* also contains HP1.

Different dimerization properties are conferred by the C-termini of HcpA and HcpB

We have previously shown (Kaller et al., 2006) that HcpA and HcpB form homo- and heterodimers *in vivo* and *in vitro*. Although both proteins are capable of dimerization, they do so with different preferences. Since the C-terminal chromo shadow domain is required for dimerization (Cowieson et al., 2000; Kaller et al., 2006), we tested whether exchange of the C-termini of HcpA and HcpB (Figure 4A) would influence the dimerization preference. GFP fusion constructs were introduced into *Dictyostelium* cells and expression was confirmed by Western blotting (Figure 4B). As expected, proteins containing the C-terminus of the *hcpB* gene were slightly larger than those containing the *hcpA* C-terminus. Immunofluorescence analysis showed that the chimeric proteins displayed similar subnuclear localization to their wild-type counterparts (Figure 4C).

For semi-quantitative competitive pull-down experiments, recombinant His-HcpA (Figure 4D, left) or recombinant His-HcpB (Figure 4D, right) was coupled to Ni-agarose beads and incubated with protein mixtures from *Dictyostelium* cells expressing GFP-HcpA, GFP-HcpB or the respective chimeric proteins (combinations indicated in Figure 4D on the right). Western blots of the precipitated proteins showed that the Hcp isoforms that contained the C-terminus of HcpB bound more efficiently to matrix-immobilized His-HcpA or His-HcpB than competitor proteins containing the C-terminus of HcpA. This is best documented by the reversal of the corresponding protein amounts in the fractions eluted compared to the input fractions (Figure 4D): although the input contained

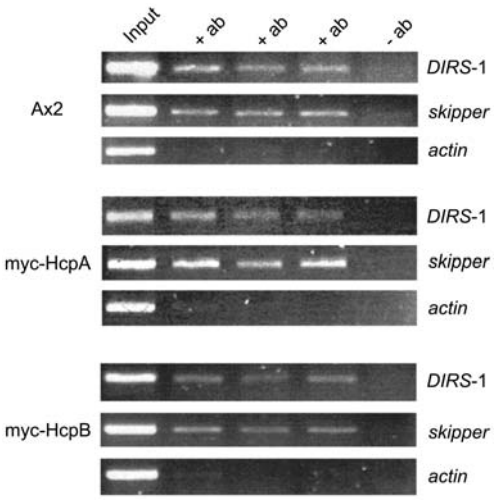

Figure 3 *Skipper* and *DIRS-1* localize to heterochromatic regions.
ChIP was performed with an antibody directed against H3K9me2. PCR was performed in triplicate with immunoprecipitates (+ab) from wild-type Ax2 cells and from two strains Ax2/myc-HcpA and Ax2/myc-HcpB that express Hcp fusions ectopically. Control PCRs were performed on mock precipitates without antibody (-ab). *DIRS-1* sequences were amplified with 26 PCR cycles, *skipper* with 27 PCR cycles and *actin* with 29 PCR cycles to adjust for the genomic copy number. Input fractions were diluted 20-fold before the PCR reaction in comparison to the immunoprecipitated material. In all three strains, H3K9me2 was associated with *DIRS-1* and *skipper* retrotransposon sequences, but not with the euchromatic *actin* genes.

less protein with the HcpB chromo shadow domain, more in comparison to proteins with the HcpA chromo shadow domain (CSD) was recovered in the immunoprecipitates. GFP-tagged proteins were not bound to beads without His-tagged Hcps (data not shown).

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

Exchange of the C-terminus not only switched the dimerization preference, but also conferred the mutant phenotype previously observed for HcpA overexpression. Aberrant anaphase bridges were enriched only in cell lines expressing GFP fusions containing the HcpA C-terminus, i.e., GFP-HcpA or GFP-HcpB_NA_C, but not cell lines expressing GFP-HcpB or GFP-HcpA_NB_C. The increased occurrence of anaphase bridges (Figure 4E) was statistically confirmed by χ^2 test with a significance of α=0.000152 (Table 1).

The C-termini apparently differ in their interaction properties with other chromatin proteins. Overexpression of the A-type C-terminus may thus accumulate complexes that are usually underrepresented and that ultimately result in a higher probability for aberrant mitosis.

Targeting Hcp proteins to chromatin

Previous analysis revealed that subnuclear targeting of HcpA and HcpB is mediated by different CSD-dependent and -independent mechanisms.

Both HcpA and HcpB bind to centromeric heterochromatin independent of the CSD, although the CSD may be required to stabilize chromatin binding by interactions with other chromatin factors (Cheutin et al., 2003; Festenstein and Aragon, 2003).

HP1 proteins bind via their chromo domain to methylated H3K9 (Bannister et al., 2001; Lachner et al., 2001) which is strongly enriched in *Dictyostelium* centromeric heterochromatin. In addition, the H3K9me2 signal is found at various other minor sites at the nuclear periphery that largely overlap with staining by the HcpA- and HcpB-GFP fusions (Kaller et al., 2006). We therefore tested whether Hcp proteins lacking the CSD could still localize to heterochromatin as defined by H3K9me2 staining. Although the major spot was still recognized, no staining of the minor foci was observed with C-terminally truncated HcpA or B proteins (Figure 5A and data not shown). Apparently, H3K9me2 binding by the chromo domain is not the only or not even the major mechanism driving subnuclear HP1 localization. We therefore tested whether other features of HP1 proteins may contribute to centromeric chromatin binding.

DNA and RNA binding of HP1 proteins has been shown for various homologs 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 may be a major force in targeting the protein to structures that are specific for heterochromatin. This may in fact be necessary, since the methylated histone tails are engaged in interactions with the DNA and may thus be primarily inaccessible (Meehan et al., 2003).

Gel retardation experiments showed that HcpA and HcpB can both bind to DNA and to RNA directly, and that this binding is independent of the CSD. Binding was specific in that an eight-fold excess of competitor RNA or a 25-fold excess of competitor DNA could completely reverse complex formation (Figure 5B). The smear at intermediate protein concentrations indicated partial loading of the target nucleic acid with protein, while the distinct band shift at high concentrations probably showed complete occupation with several protein molecules. It should be noted that binding of HcpA to RNA required higher protein concentrations than for HcpB. Overloading with protein resulted in an even larger complex (Figure 5B). The DNA and RNA binding function was not unexpected, yet still remarkable, since the basic amino acid residues within the hinge that are required for

Table 1 Frequencies of anaphase bridges in overexpression strains.

GFP	2/100
GFP-HcpA	14/108
GFP-HcpB	0/104
GFP-HcpA_NB_C	2/102
GFP-HcpB_NA_C	10/105

At least 100 mitotic cells per strain were identified by either tubulin or DdCP224 staining and examined for anaphase bridges. The numbers indicate anaphase bridges per cells counted. Statistical evaluation by χ^2-test resulted in a significance of 0.000152 for increased occurrence of anaphase bridges in strains GFP-HcpA and GFP-HcpB_NA_C.

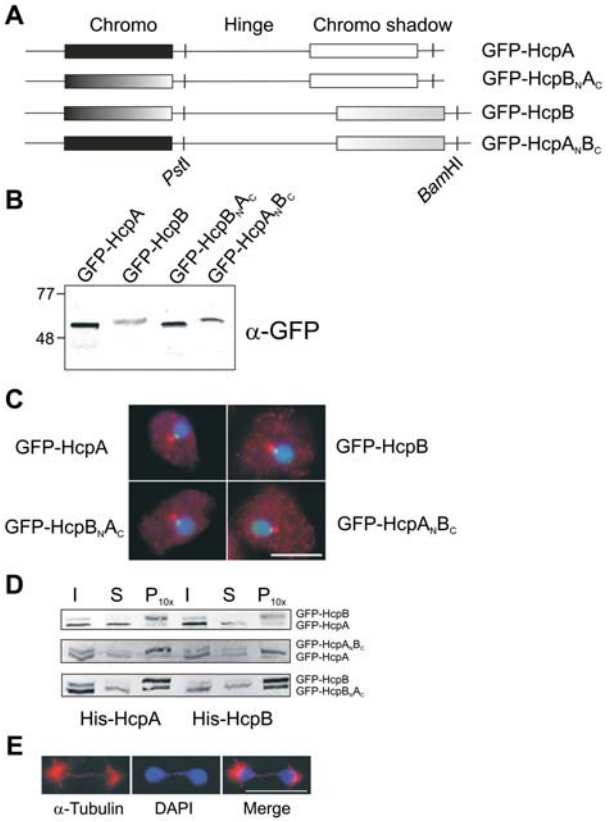

Figure 4 Properties of chimeric Hcp molecules.
(A) Generation of chimeric HcpA and HcpB proteins. The positions of the PstI and BamHI restriction sites that were used for cloning are indicated. (B) Western blot of whole-cell extracts from strains expressing GFP-HcpA, GFP-HcpB and the chimeras. Note that the size differences are due to the longer hinge region in the C-terminal part of HcpB. (C) The subnuclear localization of the chimeric proteins is not altered with respect to the wild-type proteins *in vivo*. Green, GFP; red, DdCP224; blue, DAPI. (D) Competitive pull-down experiments on protein mixtures. I, input; S, supernatant; P, pellet fraction bound to beads (10× concentrated in comparison to input and supernatant fractions). Recombinant His-HcpA (left) or His-HcpB (right) was coupled to Ni-agarose beads and incubated with mixed protein extracts from *Dictyostelium* cells expressing the GFP fusions indicated on the right. Note that the relative amount of isoforms in the input fraction is almost reversed in the bound fractions (P). GFP-HcpB and GFP-HcpA$_N$B$_C$ displayed significantly stronger binding to the His-tagged partners than GFP-HcpA and GFP-HcpB$_N$A$_C$. GFP-tagged proteins could be eluted from beads that were pre-coated with either His-HcpA or His-HcpB, but not from empty beads (data not shown). (E) Anaphase bridge in late mitotic cells of the GFP-HcpA overexpression strain (see Table 1). The scale bar represents 5 μm.

binding are quite differently arranged in the *Dictyostelium* proteins compared to their vertebrate and *Drosophila* counterparts.

Identification of the *Dictyostelium* Orc2 homolog (OrcB)

The ORC interacts with centromeres and HP1. To identify further components that may contribute to chromatin

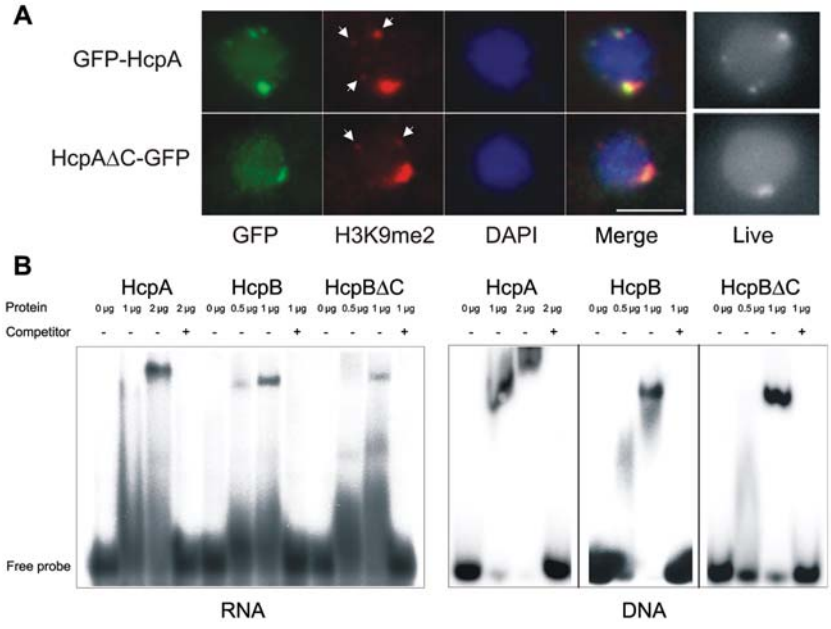

Figure 5 Influence of the chromo shadow domain (CSD) on Hcp localization and binding properties to nucleic acids.
(A) CSD-dependent and -independent localization of HP1 proteins. While GFP-HcpA stained the major heterochromatic cluster and additional smaller foci at the nuclear periphery, the C-terminally truncated HcpAΔC-GFP lacking CSD only localized to the main heterochromatic cluster. H3K9me2 was present both at the major heterochromatic cluster and at minor foci at the nuclear periphery (arrows). Similar results were obtained for GFP-HcpB and HcpBΔC-GFP (data not shown). Since fluorescence intensity for the C-terminally truncated proteins significantly decreased after fixation, an additional live-cell GFP image is shown on the right. The scale bar represents 2 μm. (B) Gel-shift assays with His-HcpA, His-HcpB and His-HcpBΔC. The three proteins bind to the labeled RNA and DNA fragments. Binding was specifically competed out with an eight-fold excess of unlabeled competitor RNA or a 25-fold excess of unlabeled competitor DNA (+ lanes). For binding, 0, 1 and 2 μg of HcpA and 0, 0.5 and 1 μg of HcpB or HcpBΔC were used (- lanes).

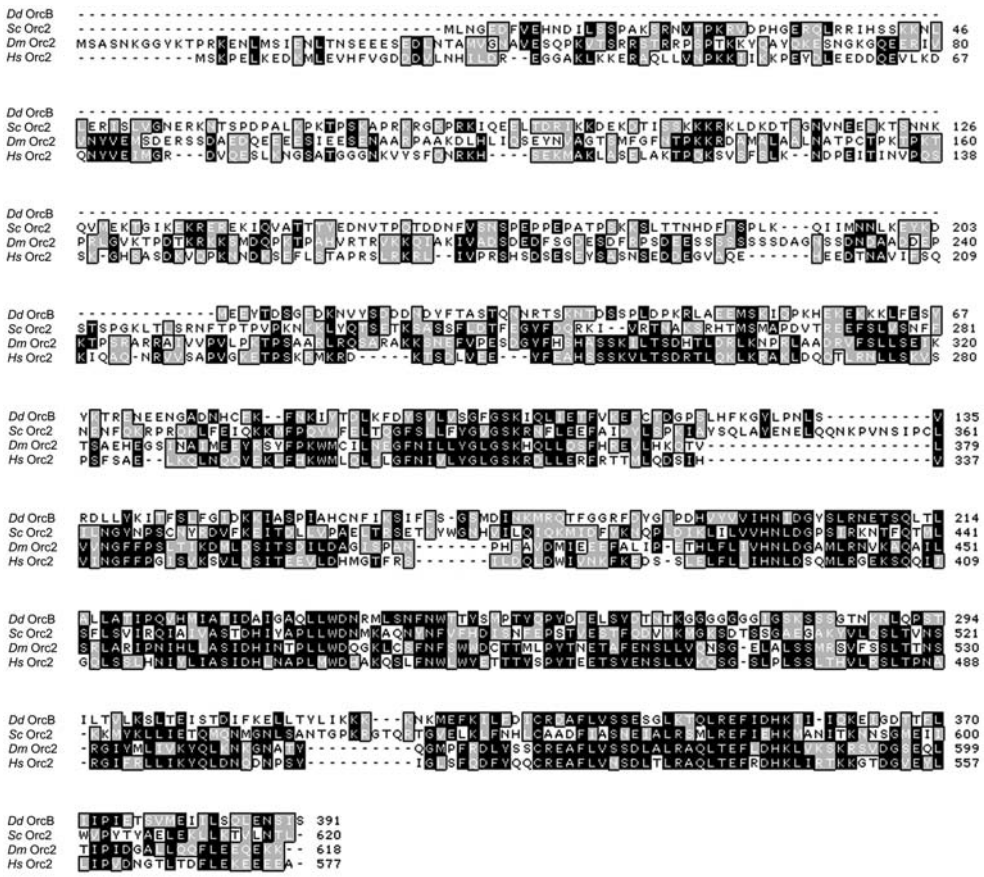

Figure 6 Alignment of *Dictyostelium* OrcB with Orc2 homologs from *S. cerevisiae* (Sc), *Drosophila melanogaster* (Dm) and *Homo sapiens* (Hs).
Identical amino acids are indicated by black boxes and similar amino acids by gray boxes.

structure and specifically to centromere function, we searched the *Dictyostelium* database for Orc homologs. A tBLASTN search of the amino acid sequence of subunit 2 of the human origin recognition complex (Orc2) revealed the presence of a single gene with significant homologies. Alignment with Orc2 homologs from different species showed that the conserved C-terminal portion was present in the *Dictyostelium* protein (Figure 6), but that the N-terminal portion was missing. This results in a significantly smaller protein of 391 aa compared to 577 aa in the human counterpart. We denoted the gene as *orcB*. In the conserved C-terminal part of the protein, OrcB is 29% identical (49% similar) to both human and *Drosophila* Orc2.

Since the role of ORC in DNA replication initiation is highly conserved, we examined whether all ORC subunits exist in *Dictyostelium*. A detailed BLAST search with the six human ORC subunits showed that homologs for all of them are present in the *Dictyostelium* genome (Table 2).

Table 2 Identification of the *Dictyostelium* ORC subunit homologs.

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

Orc subunits were identified by BLAST search with the human homologs and were annotated in DictyBase.

Amplification of the *orcB* gene from cDNA indicated that it was actively transcribed in vegetative cells. Compared to the housekeeping gene *thioredoxin*, transcription levels were, however, very low (data not shown).

To further analyze its function, OrcB was expressed in *Dictyostelium* as a GFP-fusion protein. Western blot analysis using an anti-GFP antibody confirmed the presence of a 72.7-kDa protein in *Dictyostelium* whole-cell extracts, which corresponded to the calculated size of GFP-OrcB (Figure 7A).

Partial co-localization of OrcB with HcpA

Co-transformation with HcpA-RFP showed that GFP-OrcB did not localize to the major centromeric heterochromatin cluster (Figure 7B). This was in strong contrast to the observations in *Drosophila* and mammalian cells. However, owing to the strong nucleoplasmic background fluorescence, we cannot exclude a minor association with centromeres. Nevertheless, GFP-OrcB co-localized with at least some of the minor HcpA-RFP foci at the nuclear periphery (Figure 7B, upper arrow). Although localization to these spots may merely be caused by protein overexpression, it appears that distinct heterochromatin domains contain this protein, while others (e.g., the major HP1 spot at centromeres) do not.

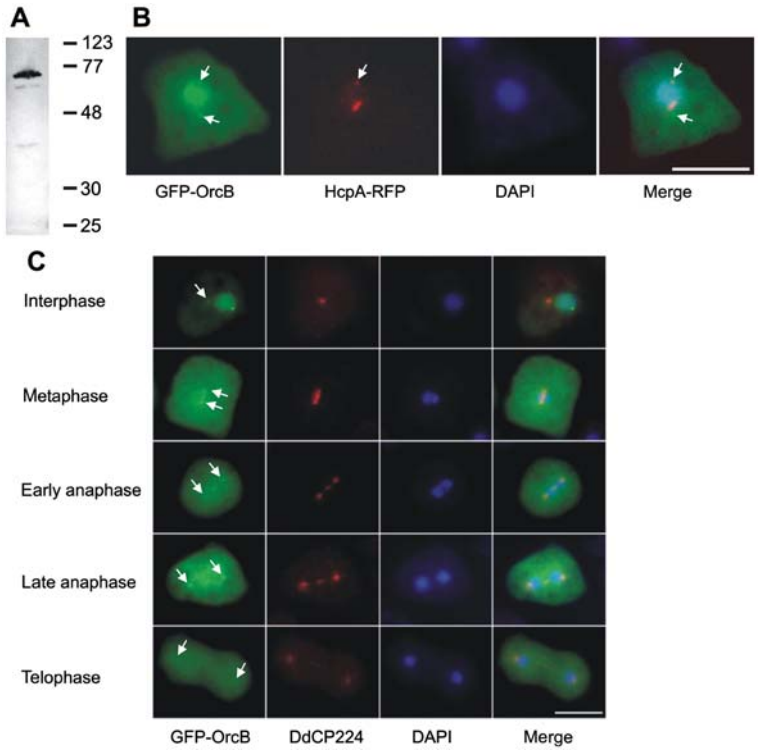

Figure 7 Localization of GFP-OrcB during the cell cycle.
(A) Western blot analysis with an anti-GFP-antibody detected an approximately 73-kDa protein in whole-cell lysates of transformed *Dictyostelium* cells. (B) In interphase, GFP-OrcB localized to the nucleus, but also showed strong cytoplasmic staining. Nuclear foci co-localized with a minor HcpA-RFP signal (upper arrow), but not with the major centromeric heterochromatin. In addition, a cytoplasmic signal in close vicinity to the centromeric heterochromatin was detected (lower arrow). (C) The cytoplasmic GFP-OrcB spot co-localized with the centrosomal marker protein DdCP224 throughout the cell cycle (arrows). Preferential nuclear localization was almost completely lost during late mitotic stages.

ORC interacts with HP1 in *Drosophila* and in mammals. In particular, in *Drosophila* it has been shown that a hypophosphorylated fraction of HP1 co-localizes with ORC (Huang et al., 1998). Immunostaining shows, however, that there are also chromatin regions that contain either HP1 or ORC (Pak et al., 1997). We therefore tested whether the OrcB subunit could bind to either HcpA or HcpB *in vitro*. Pull-down analysis did not show any direct interaction between GFP-OrcB and His-tagged HcpA or HcpB (data not shown). It is quite possible that interaction of HcpA and HcpB with ORC depends on distinct phosphorylation states of either HP1 proteins (Badugu et al., 2005) or OrcB that are not present in the bacterially expressed proteins. Alternatively, interaction of HcpA and HcpB with ORC may be mediated by another ORC subunit. Human HP1α, for example, interacts with Orc1, but not with the Orc2 subunit (Lidonnici et al., 2004).

OrcB is associated with the centrosome throughout the cell cycle

Overexpressed GFP-OrcB localized to the nucleus, where it formed a small number of foci at the nuclear periphery (Figure 7B,C). In addition, a large proportion of the protein remained cytosolic, and unexpectedly, localized to the centrosome in *Dictyostelium* cells, as indicated by co-localization with the DdCP224 antigen (Figure 7C). The association with the centrosome was stable throughout the cell cycle, whereas nuclear localization was reduced during mitosis (Figure 7C). The reduction of nuclear and chromatin-associated Orc2 during M phase resembles the situation in *Xenopus laevis* and human cells (Romanowski and Madine, 1996; Prasanth et al., 2004), but is in contrast to the situation in the yeasts, where ORC remains 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). In contrast to 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 localization data suggest that, similar to the human counterpart, *Dictyostelium* Orc2 also serves several functions apart from replication initiation. Further analysis will be required to elucidate a potential centrosomal function of OrcB.

Discussion

Dictyostelium is a well-established model system for studying centrosome structure and function; however, only very few centrosomal proteins have been identified and studied to date (Reinders et al., 2006). Here we provide evidence that the *Dictyostelium* homolog of Orc2, one of the six subunits of the ORC, co-localizes with the centrosome throughout the cell cycle. Intriguingly, human Orc2 has also been shown to reside at the centrosome throughout the cell cycle (Prasanth et al., 2004), indicating that this function of Orc2, though not highly con-

served in evolution, is present in different evolutionary lineages.

Orc2 could be part of a signaling pathway from DNA replication to centrosome duplication, thus ensuring that entry into mitosis only occurs when the DNA is fully replicated. Orc2 in *Dictyostelium* may also be a structural component of the centrosome. Replication initiator proteins can serve multiple functions in chromosome segregation; e.g., ORC subunit 6 (Orc6) of *Drosophila* associates with the cell membrane and the cleavage furrow during cytokinesis (Chesnokov et al., 2003). Other ORC subunits also appear to have adopted additional functions in various cellular processes that appear unrelated to DNA replication. Recently, the Orc3 and Orc4 subunits have been shown to localize to the membrane and are required for dendritic growth in post-mitotic neurons (Huang et al., 2005).

In *Drosophila*, HP1 associates with ORC and the Orc2 and Orc6 subunits are co-precipitated with HP1. It appears that mainly the underphosphorylated fraction of HP1 co-localizes with ORC on interphase chromatin. It is therefore assumed that ORC may recruit HP1 to these sites (Shareef et al., 2003). However, in immunofluorescence experiments, only a fraction of ORC overlapped with HP1, suggesting that there are common and different chromatin targets (Pak et al., 1997). Similarly, we found that in *Dictyostelium* some of the minor HP1 foci were stained by Orc2, while the major spot was not.

GFP-tagging of OrcB did not appear to interfere with protein function, since we did not observe obvious defects in centrosome duplication or spindle formation. These phenotypes had been observed upon depletion of human Orc2 (Prasanth et al., 2004) and would be expected if GFP-OrcB had a dominant negative effect.

HP1 proteins are crucial for cell viability in *Drosophila*, mammals and *Dictyostelium* (Eissenberg et al., 1990; Filesi et al., 2002; Kaller et al., 2006).

Homo- and heterodimerization of HP1 isoforms are conserved features and are believed to contribute to the establishment of higher order chromatin structures. However, it is unknown whether the stoichiometry of HP1 complexes composed of different isoforms has functional implications (Hiragami and Festenstein, 2005). Here we provide semi-quantitative evidence that the isoforms have different properties in homo- and heterodimerization and that these are conferred by the C-termini of the proteins. Remarkably, only the overexpression of isoforms containing the C-terminus of HcpA caused increased frequencies of anaphase bridges.

Why does overexpression but not disruption of either of the two isoforms cause different phenotypic effects? HP1 proteins are subject to extensive differential modifications that may present an additional dynamic regulation beyond the histone code (Lomberk et al., 2006). The two isoforms in *Dictyostelium* acquire distinct post-translational modifications (unpublished data) that probably result in different functional properties. In the single knockout strains, the remaining isoform may be modified in both ways and thus compensate for the form knocked out, resulting in no detectable phenotype. In contrast, an overexpressed isoform may exhibit, for example, distinct preferences for protein-protein interactions and thus lead to changes in chromatin dynamics. Alternatively, an overexpressed isoform may shift the equilibrium to an otherwise underrepresented HP1 dimer that disturbs chromatin assembly when present in excess.

HP1 shows different mechanisms of targeting to chromatin. Especially at euchromatic loci, HP1 interacts with proteins such as the retinoblastoma protein (Rb) or other co-repressors through the CSD and participates in epigenetic gene silencing (Nielsen et al., 2001; Ayyanathan et al., 2003). In heterochromatin, other mechanisms contribute to correct localization, since C-terminally truncated proteins lacking the CSD still localize to heterochromatin (Wang et al., 2000). These may include binding to H3K9me, binding to other proteins via the chromodomain/hinge, or direct binding to either DNA or RNA.

Although an as yet unidentified RNA species contributes to HP1 binding to chromatin (Maison et al., 2002), it is largely unclear whether the RNA-binding activity of HP1 only stabilizes its binding to chromatin or if it actively recruits HP1 to chromatin. No specific sequence elements appear to be required for nucleic-acid binding *in vitro*, but it will be interesting to determine if there is a specific type of RNA that is bound by HP1 proteins *in vivo*.

Since siRNAs, the effector molecules of the RNAi machinery, target chromatin-modifying activities to the respective genomic loci in various organisms (Matzke et al., 2004), it is quite possible that an RNA may recruit HP1 proteins directly to chromatin.

HP1 proteins may also be recruited to heterochromatin by interaction with other centromere-specific proteins, such as INCENP (Ainsztein et al., 1998). The *Dictyostelium* INCENP homolog, DdINCENP, has recently been described (Chen et al., 2006), but its mitotic localization to centromeres/kinetochores has not yet been elucidated in detail owing to a lack of proper (endogenous) centromere markers. In mammalian cells, INCENP localizes to centromeres/kinetochores in metaphase, where interaction with the Aurora-B and polo-like kinase-1 (Plk1) regulates metaphase/anaphase transition (Goto et al., 2006). Phosphorylation of serine 10 in histone H3 by Aurora-B causes HP1 to dissociate from heterochromatin (Fischle et al., 2005; Hirota et al., 2005). This is in agreement with our observations on HP1 dissociation from heterochromatin during prometa-/metaphase.

The function of HP1 dissociation is largely unknown. Since HP1 recruits cohesin complexes to heterochromatin, dissociation could support detachment and proteoloytic cleavage of cohesin complexes at the onset of anaphase by the anaphase promoting complex (Uhlmann et al., 2000; Waizenegger et al., 2000). The minor fraction of HP1 that remains associated with pericentromeric heterochromatin may help to re-establish the pericentromeric imprint after mitotic exit. *Dictyostelium* cells lack the G1 phase of the cell cycle and immediately enter S phase after mitosis. Since modified histones may be diluted out during chromatin reassembly, it is probably of particular importance to stably mark and maintain pericentromeric heterochromatin.

Our findings are of particular importance for further exploration of the functions of known *Dictyostelium* proteins in mitotic chromosome segregation, for assessment

of the localization of newly identified *Dictyostelium* proteins involved in centromere/kinetochore function, and for understanding the dynamics of heterochromatin remodeling.

Materials and methods

Strains

The strains used were Ax2::GFP-HcpA, Ax2::GFP-HcpB, Ax2::GFP-HcpA$_N$B$_C$, Ax2::GFP-HcpB$_N$A$_C$, Ax2::GFP-OrcB, Ax2::GFP-OrcB/HcpA-RFP, Ax2::myc-HcpA, Ax2::myc-HcpB, *hcpA^{lp}*, *hcpA^{lp}*::GFP-HcpA, and *hcpAB$^-$*::GFP-HcpA.

Genotyping of the *hcpA^{lp}*, *hcpA^{lp}*/GFP-HcpA and *hcpAB$^-$*/GFP-HcpA strains was performed as described previously (Kaller et al., 2006). All transformations, co-transformations and gene disruptions by homologous recombination were carried out as previously described (Nellen and Firtel, 1985; Nellen et al., 1987; Witke et al., 1987; Kaller et al., 2006).

Pull-down analysis

Pull-down analysis was performed as previously described (Kaller et al., 2006). For competitive pull-down assays, mixtures of cells expressing either GFP-HcpA or GFP-HcpB, or the respective chimeric proteins were lysed and processed as described (Kaller et al., 2006).

Fluorescence microscopy and statistical evaluation

Fluorescence microscopy was performed as previously described (Kaller et al., 2006). Image acquisition was performed using a Leica DMIRB microscope with a CCD camera. Images were processed using Adobe Photoshop.

Mitotic analysis of cells and statistical data evaluation were performed as described by Kaller et al. (2006). Briefly, we adjusted the data to 100 counted cells per strain, resulting in 13 anaphase bridges (events) for GFP-HcpA, and 9 for GFP-HcpB$_N$A$_C$, with all other numbers fixed. χ^2 statistics were computed and tested using the Monte Carlo method for the probability of random distribution of events in the five strains.

Plasmids

The *orcB* coding sequence was amplified with primers 5′-GTC GAC ATG GAA GAA TAT ACA GAT TCA GG-3′ (forward) and 5′-CTC GAG TTA TGA TAT TGA ATT TTC TAA TTG TG-3′ (reverse) from oligo-dT-primed cDNA, and cloned into pGEM-T-Easy (Promega, Mannheim, Germany). The *orcB* sequence was excised with SalI and BamHI and cloned into pdneoGFP (Rauchenberger et al., 1997).

To create N-terminal myc-fusions of the Hcp proteins, the corresponding coding sequence was excised from pdneoGFP-HcpA or -HcpB with SalI and SacI, and cloned into pdneo-myc-Cofilin (Drengk et al., 2003), where the cofilin coding sequence had been completely deleted by SalI/SacI digestion.

Chimeric proteins of HcpA and HcpB were derived from the previously described plasmids pdneoGFP-HcpA and pdneoGFP-HcpB. Both plasmids were digested with PstI and BamHI, thereby excising the sequences encoding for most of the hinge and the entire CSD of HcpA and HcpB. The excised fragments were exchanged between the two plasmids (see Figure 3A), 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).

Electrophoretic mobility shift assay

For DNA/RNA binding assays, His-tagged recombinant proteins were expressed in *E. coli* and purified using Ni-Sepharose. Before adding the nucleic acids, ca. 1 and 2 μg of freshly purified HcpA or ca. 0.5 and 1 μg of HcpB/HcpBΔCSD were incubated on ice for 15 min in RNA- (20 mM HEPES, pH 7.6, 100 mM KCl, 2 mM EDTA, 0.01% Nonidet P-40) or DNA-binding buffer (11 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 5 mM $MgCl_2$) (Zhao et al., 2000; Muchardt et al., 2002).

The DNA probe was produced by PCR amplification of a 94-bp fragment of the *dnmA* gene (DictyBase DDB0231095, NCBI: XM_631863) with the following sequence: 5′-GGA AAA TTT ATT GAA GGT ACA GGA ATC ACT AGT GAA TTC GCG GCC GCC TGC AGG TCG ACC ATA TGG GAG AGC TCC CAA CGC GTT GGT ACC AAT A-3′. The product was end-labeled using [γ-^{32}P]ATP and T4 PNK in a forward reaction. The 43-nt RNA probe was prepared by linearizing pGEM® -7z with EcoRI, followed by *in vitro* transcription using T7 RNA polymerase in the presence of [α-^{32}P]UTP. The DNA or RNA probes were added to the protein samples and incubated for 30 min on ice. Separation was carried out by native PAGE on a 6% gel in 0.25× TBE for 5 h at 130 V. The gels were exposed to an imager plate and visualized in a Fuji Phosphorimager (Düsseldorf, Germany).

Chromatin immunoprecipitation

Aliquots of 100 ml of *Dictyostelium* cell suspension (3×10^6 cells/ml) were harvested 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, to which 400 μl of 10× PBS and 500 μl of a fresh 8% paraformaldehyde solution were added. Paraformaldehyde solution was prepared by mixing 0.08 g of paraformaldehyde (Merck, Darmstadt, Germany) with 900 μl of distilled water, incubation on a heating block at 40°C, addition of 100 μl of 1 M NaOH solution and further incubation 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 harvested for 3 min at 150 *g*, washed four times with 4 ml of 1× TBS (50 mM Tris, pH 8.0, 150 mM NaCl) and once with 1× PBS. The pellet was resuspended in 1.5 ml of 1× TBS. Then 1.5 ml of 2× TTS/1× TBS (50 mM Tris, pH 8.0, 150 mM NaCl, 2% Triton X-100, 4 mM EDTA, 0.1% SDS) and 15 μl of 200 mM PMSF were added and incubated for 15 min on ice, followed by sonication using a UP 200 S sonifier (Dr. Hielscher GmbH, Stansdorf, Germany) at 40% intensity and duty cycle 1 using an S3 microtip. Cells were sonified five times for 10 s at 30-s intervals. Then each sample was again sonified twice for 10 s at a 30-s interval. The lysate was centrifuged for 10 min at 20 000 *g* at 4°C, and the supernatant was transferred to a fresh tube and centrifuged again. A 500-μl portion of the supernatant was diluted with 1 ml of 1× TTS (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 0.05% SDS). To eliminate unspecific protein interactions with protein A-Sepharose, the extract was pre-cleared with 40 μl of protein A-Sepharose CL4B (Amersham) (1:3 slurry equilibrated in 1× PBS) for 2 h at 4°C on a spinning wheel. Beads were briefly spun down at 2000 *g*. Then 20 μl of the supernatant was kept as the input fraction, while 120 μl was further diluted with 680 μl of 1× TTS, and 5 μl of 200 mM PMSF and 3 μl of anti-(histone H3) dimethyl-K9-antibody (Upstate Biotechnology, Lake Placid, USA) were added. As a control, samples without addition of antibody were prepared in parallel. The protein amounts used for immunoprecipitation were thus equivalent to approximately 5×10^6 cells. The mixture was incubated overnight on a spinning wheel at 4°C. Then 70 μl of protein A-Sepharose was added

and incubated for 1–2 h. The beads were spun down for 1 min at 2000 *g*, the supernatant was carefully removed and the beads were washed four times with 1 ml of 1× TTS and twice with 1 ml of LiDN buffer (10 mM Tris pH 8.0, 250 mM LiCl, 1% deoxycholate, 1% Nonidet P-40, 1 mM EDTA). Washing of the beads was performed for 3 min on a rocking platform. After removal of the supernatant, the antibody-protein-DNA complex was eluted with 100 µl of buffer E (50 mM Tris, pH 7.5, 1% SDS, 1 mM EDTA) for 15 min at 37°C on a rocking incubator. Beads were spun down briefly at 2000 *g* and the supernatant was stored. The elution step was repeated once. Both eluted fractions were pooled, and 8 µl of 5 M NaCl was added. In parallel, 20 µl of the input fractions was diluted with 180 µl of buffer E and 8 µl of 5 M NaCl. Cross-linking was reversed by overnight incubation at 65°C. Then 1 µl of RNase A solution (1 mg/ml) was added and the mixture was incubated for 1 h at 37°C. Subsequently, 1.5 µl of proteinase K (25 mg/ml; Roth, Karlsruhe, Germany) was 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 of TE buffer. Samples were stored at -20°C until further use.

Quantification of precipitated DNA sequences

For quantification of precipitated DNA, input fractions, precipitated fractions and minus-antibody-controls were analyzed by PCR. The input fractions were diluted 1:20 in TE buffer. PCR cycle numbers had to be determined empirically to avoid saturating PCR conditions. The number of PCR cycles was adjusted to the copy number of the chosen target genes in the genome. PCR cycles were 26 (*DIRS-1*), 27 (*skipper*) and 29 (*actin*) for ChIPs with the α-H3K9me2-antibody.

PCR primers were: *DIRS-1* forw, 5′-GTA TGC CCT GTT CGC CAC CTT GC-3′; *DIRS-1* rev, 5′-CGT AGA AGG TAT CTA CAG TAT C-3′; *skipper* RT forw, 5′-CTG TTA CCT TAG TGA AGA TGGG-3′; *skipper* RT rev, 5′-GGG CAT CTA TTG TCT TAT GAC ATG G-3′; Act forw, 5′-GAT AAC GGT TCT GGT ATG TG-3′; Act rev, 5′-CCT GAA TCC ATA ACG ATA CC-3′. Similar results were obtained with independent primer sets for each target sequence (data not shown).

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