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Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain

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Heterochromatin protein 1 (HP1) is localized at heterochromatin sites where it mediates gene silencing[1,2]. The chromo domain of HP1 is necessary for both targeting and transcriptional repression[3,4]. In the fission yeast *Schizosaccharomyces pombe*, the correct localization of Swi6 (the HP1 equivalent) depends on Clr4, a homologue of the mammalian SUV39H1 histone methylase[5,6]. Both Clr4 and SUV39H1 methylate specifically lysine 9 of histone H3 (ref. 6). Here we show that HP1 can bind with high affinity to histone H3 methylated at lysine 9 but not at lysine 4. The chromo domain of HP1 is identified as its methyl-lysine-binding domain. A point mutation in the chromo domain, which destroys the gene silencing activity of HP1 in *Drosophila*[3], abolishes methyl-lysine-binding activity. Genetic and biochemical analysis in *S. pombe* shows that the methylase activity of Clr4 is necessary for the correct localization of Swi6 at centromeric heterochromatin and for gene silencing. These results provide a stepwise model for the formation of a transcriptionally silent heterochromatin: SUV39H1 places a 'methyl marker' on histone H3, which is then recognized by HP1 through its chromo domain. This model may also explain the stable inheritance of the heterochromatic state.

The histone code hypothesis predicts the existence of domains that specifically recognize modified histone residues[7]. One such domain is the bromodomain, which allows transcription factors such a P/CAF and $TAF_{II}250$ to recognize histone tails only when they are acetylated at lysine residues[8,9]. To identify an equivalent domain that recognizes methyl-lysines, we examined a number of chromatin-associated factors for their ability to recognize specifically a histone H3 peptide (residues 1–16) tri-methylated at lysines 4 and 9.

Figure 1b shows that one protein, HP1, was able to bind to the methylated peptide (red line), but not to the unmethylated peptide (data not shown), using surface plasmon resonance (SPR) analysis. Other chromatin-associated proteins, such as Mi2, MeCP2 and P/CAF did not bind in this assay (data not shown). Of the two conserved domains in HP1 (Fig. 1a), the chromo domain was able to bind (Fig. 1b, green line) whereas the chromo-shadow domain was not (blue line). Figure 1c shows that the on-rate for the chromo domain (red line) is not affected by the addition of unmethylated histone H3 peptide (green line), but binding is completely abolished in the presence of the methylated H3 peptide (blue line). The dissociation constant (K_d) of the HP1 chromo domain for the methylated H3 peptide (methyl-lysine) is about 70 nM, as determined by SPR (data not shown). The affinity of the HP1 chromo domain for methyl-lysine compares favourably with the affinity of the $TAF_{II}250$ and P/CAF bromodomains for acetyl–lysine[8], although a more definitive comparison will be possible with ITC analysis of the HP1/methyl-lysine interaction. Our analysis of other chromo domain proteins such as polycomb (M33), Mi2 and

SUV39H1 has not identified another chromo domain with methyl-lysine-binding ability (data not shown).

The specificity of the HP1 chromo domain for methyl-lysine can also be shown using a 'pull-down' assay with H3 peptide linked to beads (Fig. 2a). Full-length wild-type HP1 that is bacterially expressed, labelled with ^{35}S-labelled methionine (input), bound the methylated H3 peptide but not the unmethylated peptide. A large proportion of the input protein is bound, which is consistent with the low K_d measured by SPR analysis.

Mutations of conserved residues in the HP1 chromo domain (V23M and KW41/42AA) abolish recognition of methylated H3, whereas mutation of a non-conserved residue (L68P) has little effect on binding (Fig. 2a). The chromo domain of Mi2 does not bind the methylated H3 peptide in this assay. These results indicate the high affinity and specificity of the HP1 chromo domain structure in the recognition of methylated lysines. One of the chromo domain mutants (V23M) disrupts the gene silencing functions of *Drosophila* HP1 (ref. 3), suggesting a link between the ability of HP1 to repress transcription and its ability to bind methyl-lysine of histone H3.

We next tested whether the HP1 chromo domain had a preference

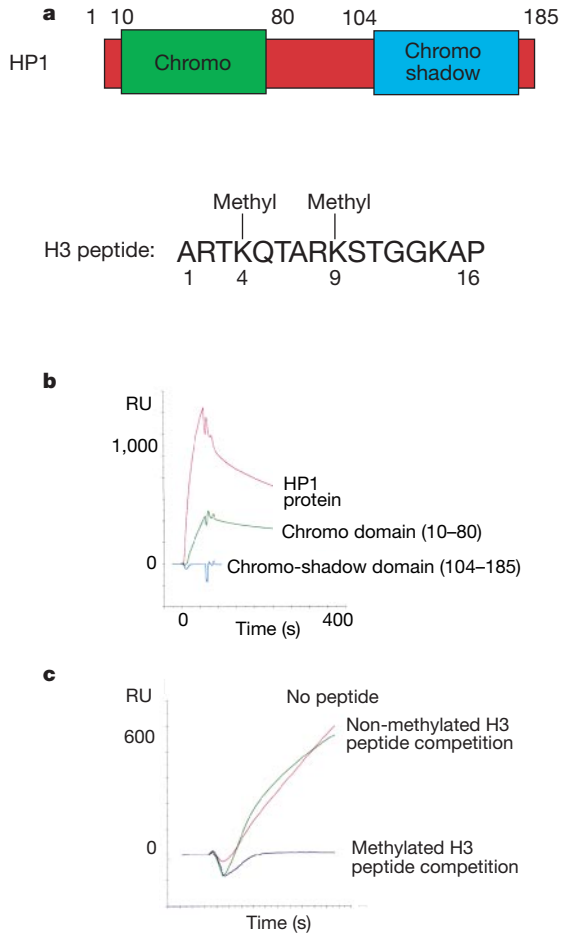

Figure 1 HP1 chromo domain binds methylated H3. **a**, Schematic representation of HP1 and histone H3 peptide used in this study. Peptides were either unmodified, tri-methylated at both Lys 4 and Lys 9, or tri-methylated at Lys 4 or Lys 9. **b**, Equimolar amounts of his-HP1 full length (red line), his-chromo domain (green line) and the his-chromo-shadow domain (blue line) were tested for binding to the Lys 4 and Lys 9 tri-methylated histone H3 peptide using a BIAcore J instrument. The *y* axis units are resonance units (RU), which are proportional to the mass of the protein binding to the peptide on the surface of the chip. **c**, His-HP1 was tested as described for **b** except that it was pre-incubated with either no peptide (red line), unmethylated peptide (green line) or Lys 4 and Lys 9 tri-methylated peptide (blue line).

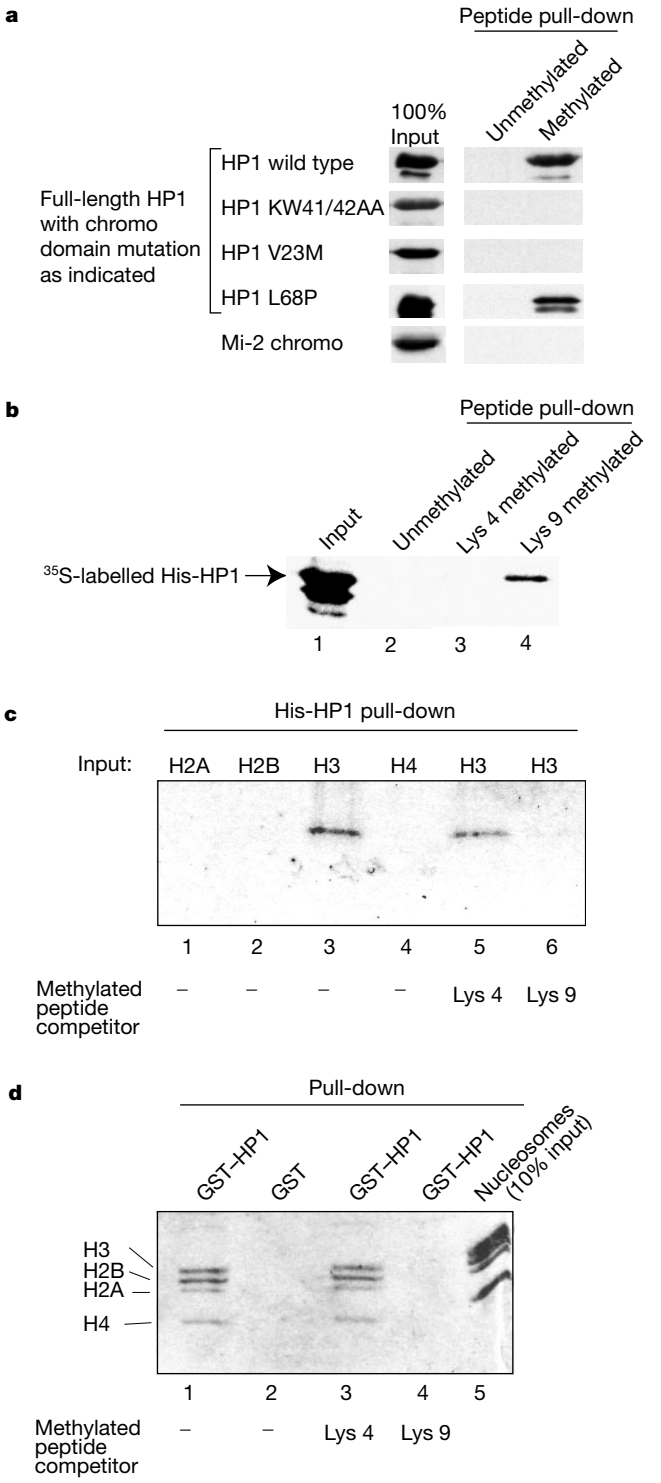

Figure 2 HP1 chromo domain specifically binds methylated Lys 9 of histone H3. **a**, Recombinant, radiolabelled proteins were tested for binding to either unmodified or Lys 4 and Lys 9 tri-methylated H3 peptide (see Fig. 1a) immobilized on beads. **b**, His-HP1 full length was tested for binding to unmethylated peptide (lane 2), Lys 4 methylated peptide (lane 3) or Lys 9 methylated peptide (lane 4). Lane 1 shows 100% input. **c**, Each core histone (5 μg) was assessed for binding to his-HP1 in a pull-down experiment without competitor peptide (lanes 1–4), or in the presence of histone H3 peptide tri-methylated at Lys 4 (lane 5) or Lys 9 (lane 6). After SDS–PAGE, bound histone was visualized by Coomassie staining. **d**, Mono-nucleosomes (200 μg) were assessed for binding to GST/HP1 or GST without competitor peptide (lanes 1 and 2), or in the presence of histone H3 peptide tri-methylated at Lys 4 (lane 3) or Lys 9 (lane 4). Ten per cent input is shown (lane 5). After SDS–PAGE, nucleosome-binding was determined by Coomassie staining of nucleosomal histones.

for a particular methyl-lysine within histone H3. H3 peptides methylated at either Lys 4 or at Lys 9 were individually tested for HP1 binding (Fig. 2b). HP1 shows clear selectivity for methylated Lys 9 as it does not bind to methylated Lys 4. HP1 recognized di-methylated Lys 9 as efficiently as tri-methylated Lys 9, and all mammalian forms of HP1 (α, β and γ) show the same selectivity for methylated Lys 9 (data not shown).

To test whether HP1 could recognize specifically full-length methylated histone H3, we added Ni^{2+}-agarose-bound his-HP1 to individually purified histones (Roche) and performed a pull-down assay. Figure 2c shows that his-HP1 only binds to histone H3 (lane 3) and not to histones H2A, H2B or H4 (lanes 1, 2 and 4). The H3 recognized by HP1 is methylated at Lys 9 because the HP1/H3 interaction can be disrupted by excess methylated Lys 9 H3 peptide (lane 6), but not by methylated Lys 4 H3 peptide (lane 5).

The ability of HP1 to recognize methylated histone H3 caused us to investigate whether this recognition allows HP1 to associate with methylated mono-nucleosomes. Glutathione *S*-transferase (GST)/HP1 can bind a proportion of purified nucleosomes from chicken erythrocytes (see Methods), whereas GST does not (Fig. 2d, compare lanes 1, 2 and 5). The association between HP1 and nucleosomes is not disrupted by the addition of excess methylated Lys 4 H3 peptide (lane 3) but is completely disrupted by the addition of excess methylated Lys 9 peptide (lane 4). Thus, HP1 can recognize mono-nucleosomes, at least partly, through its recognition of Lys 9-methylated H3 tails.

We next assessed whether HP1 was tethered to cellular chromatin through its specific recognition of methylated histone H3. We prepared nuclei from human U2OS cells such that they were depleted of most soluble protein. They were then challenged with methylated or unmethylated H3 peptide, and after the insoluble (chromatin) fraction was pelleted, the supernatant was western blotted for HP1 using an HP1 antibody that recognizes all mammalian HP1 forms[10]. Figure 3a shows the proportion of HP1 in total nuclear fraction (lane 1), compared with the soluble fraction after incubation (lane 2). Challenging with excess methylated H3 peptide increases the proportion of HP1α, -β and -γ in the soluble fraction (lane 4), whereas competition with the unmethylated H3 peptide had no effect (lane 3). Thus, methylated H3 peptide can displace HP1 from the chromatin fraction of U2OS nuclei *in vitro*.

We next asked whether competition with the methylated H3 peptide could disturb the localization of HP1 in U2OS cells. Cell fixation and permeabilization conditions were established whereby a peptide could be delivered into cells, and then immunofluorescence was used to detect HP1. Figure 3b shows the nuclear localization of HP1 when no peptide was added; this did not change when an unmethylated H3 peptide was added (Fig. 3c). However, addition of the methylated H3 peptide resulted in displacement of HP1 from the nucleus, where it is otherwise held by association with chromatin (Fig. 3d), and accumulation in the cytoplasm. Similar results were obtained in a distinct cell line, CH1 (data not shown).

The fission yeast *S. pombe* has an HP1 homologue Swi6, which contains a chromo domain that is closely related to those of HP1 family members[1]. Figure 4a shows that the Swi6 chromo domain is able to bind H3 peptide methylated at Lys 9, whereas its chromo-shadow domain has no methyl-binding activity. *S. pombe* also contains an SUV39H1 homologue, Clr4, which possesses methylase activity that is specific for histone H3 Lys 9 (ref. 6). To investigate whether the methylase activity of Clr4 is necessary for the correct localization of Swi6 in yeast, we examined a point mutant (G341D) in the SET domain of Clr4, which has lost the ability to methylate histone H3 (Fig. 4b). When the localization of Swi6 is monitored in Clr4-G341D mutant yeast cells, its distribution is different from that in wild-type cells. Swi6 is localized to two to four foci in wild-type

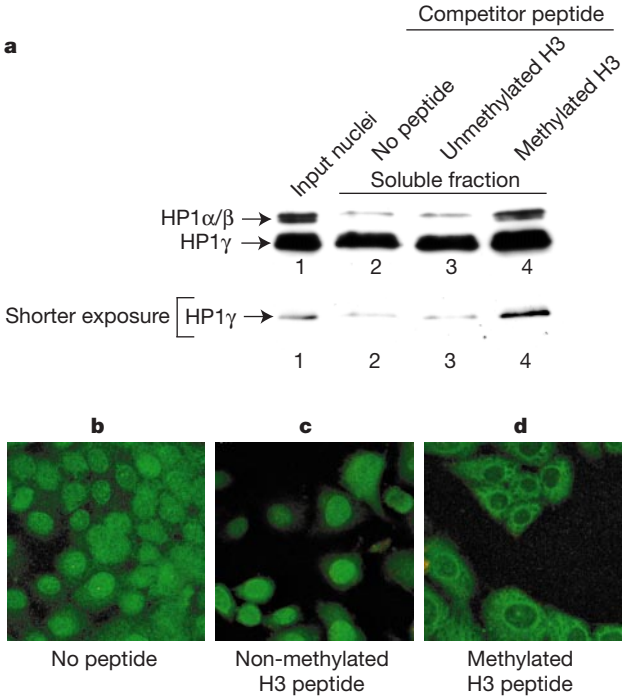

Figure 3 HP1 chromatin localization is dependent on binding to methylated K9 of H3. **a**, Purified and permeabilized U2OS cell nuclei were challenged with no peptide (lane 2), unmethylated peptide (lane 3) or the Lys 4 and Lys 9 methylated peptide (lane 4). After incubation, supernatant was western blotted for HP1 using an antibody specific for HP1α, -β and -γ (long exposure, top; shorter exposure, bottom). Lane 1 shows 50% input. **b**, HP1 immunofluorescence in U2OS cells without competitor peptide. **c**, **d**, Immunofluorescence as in **b**, except that unmethylated H3 peptide or the Lys 4 and Lys 9 methylated peptide was added, respectively. The peptide did not affect the specificity of the antibody (data not shown).

cells, corresponding to silent chromatin domains at the nuclear periphery[11], whereas the Clr4-G341D strain shows loss of localization from the nuclear periphery and accumulation of more diffuse staining over the nucleolus (Fig. 4c). The Clr4-G341D strain shows a very similar pattern of Swi6 distribution to that of the Clr4Δ strain lacking Clr4 (ref. 5). The specificity of the Swi6 antibody is demonstrated using a Swi6Δ strain (Fig. 4c).

To confirm that Swi6 association with silent chromatin is dependent on SET domain function, we performed chromatin immunoprecipitation with anti-Swi6 antibodies (Fig. 4d). Centromeric chromatin, known to be associated with Swi6, was enriched relative to the control (euchromatin) locus in the immunoprecipitated sample compared with the total extract[12]. In both the *clr4-G341D* and *clr4Δ* strains, enrichment of this centromeric sequence was lost. Thus, a mutation in the Clr4 SET domain that abolishes histone H3 methylase activity disrupts recruitment of Swi6 to silent chromatin. Loss of association of Swi6 with centromeres should result in expression of a normally silent marker gene embedded in centromeric chromatin. Figure 4e shows that this is indeed the case. On indicator plates, wild-type strains silence the centromeric *ade6*$^+$ marker, which results in red, repressed colonies[13]; however, in strains lacking Clr4 (Δ) or strains defective in Clr4 methylase activity (G341D), this *ade6*$^+$ gene is clearly expressed, resulting in the formation of white colonies. Collectively these results show that methylase activity of Clr4 is required for the recruitment of Swi6 to silenced centromeric heterochromatin and for transcriptional silencing.

The experiments described here fit well with existing data showing that SUVAR39H1 and HP1 colocalize at heterochromatic sites and interact biochemically[5]. We show that HP1 recognizes histone H3 only when Lys 9 has been methylated by SUVAR39H1. In *S. pombe*, Lys 9 methylase activity is required for transcriptional repression and HP1 localization. Thus, these data make a direct correlation between methylated, HP1-bound histones and transcriptionally silent heterochromatin.

The fact that HP1 is associated with the enzyme that methylates and 'marks' histones for HP1 binding suggests a self-maintenance model for how HP1 spreads over chromatin to form heterochromatically repressed regions (Fig. 5). An extension of this model could also explain how silenced heterochromatin could be passed on during DNA replication. The SUVAR39H1 methylase, bound to methylated histones via HP1, could direct the methylation of newly deposited histones. This self-maintaining mechanism would then

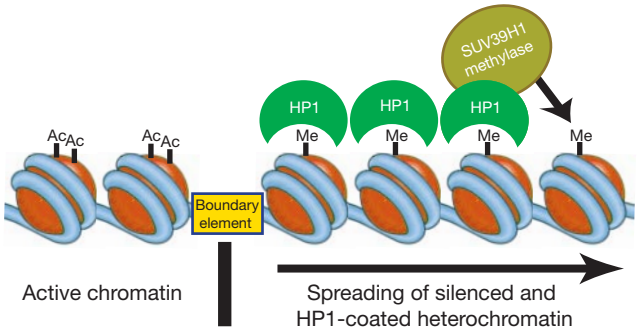

Figure 5 A model of heterochromatic self-maintenance by the SUVAR39H1/HP1 complex.

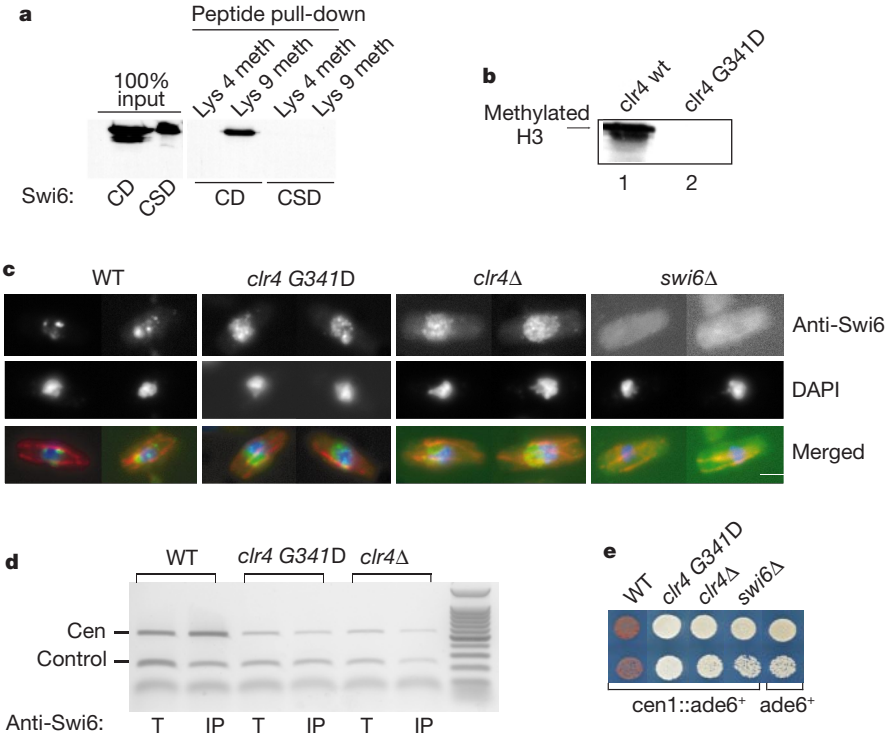

Figure 4 Swi6 localization is dependent on the methylase activity of the Clr4 SET domain. **a**, Swi6 chromo domain (CD) or chromo-shadow domain (CSD) were tested for binding to H3 peptide methylated at either Lys 4, or Lys 9. **b,** Recombinant Clr4 SET-domain protein (or a mutant version, G341D) were tested for histone methylase activity as described[6]. **c**, Swi6 immunolocalization in strains bearing wild-type *clr4*$^+$ (WT), a complete deletion of *clr4* (*clr4Δ*), or a missense mutation in the Clr4 SET domain (*clr4* G341D). Swi6 immunolocalization (top), DAPI staining of chromosomal DNA (middle) and a merged image of α-tubulin staining (red), DAPI (blue) and Swi6 (green) (bottom) are shown. Scale bar, 5 μM. **d**, Chromatin immunoprecipitation using Swi6 antibodies. T, total extract input; IP, immunoprecipitated sample. Multiplex PCR assessed enrichment of centromeric (cen) sequences over an euchromatic control in the different strain backgrounds. **e**, Strains bearing mutations in *clr4* and *swi6* were tested for their ability to transcriptionally silence the centromeric *ade6*$^+$ marker on media with limiting adenine (red colonies, repressed; white colonies, expressed). An *ade6*$^+$ strain (white) provides a control.

allow the spread of HP1 on new histones, re-establishing the silent heterochromatic state in the next generation. □

Methods

Peptides and biophysical techniques (SPR)

H3 peptide: NH_2-ARTKQTARKSTGGKAPGGC-COOH. We incorporated methyl-lysines where required. Peptides were immobilized on BIAcore CM5 chips, and interactions were measured in HBS-EP buffer (10 mM HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% v/v surfactant P20) using a BIAcore J Instrument.

Pull-down assays

Recombinant proteins were expressed in and purified from *Escherichia coli* as described[14]. We produced radiolabelled protein in bacteria by growing them in the presence of ^{35}S-labelled methionine. GST fusions of the Swi6 chromo domain (residues 80–133) and chromo-shadow domain (residues 261–328) have been described[15]. Mouse full-length HP1 was expressed either as a GST fusion protein or as a his-tagged protein. Mouse HP1 KW41/42AA and V23M mutants were expressed as GST fusions and L68P as a his-tagged protein. The Mi-2 chromo domain was expressed as a his-tagged protein. Where appropriate, we eluted recombinant proteins from beads and dialysed them against HBS-EP.

Peptides, linked to SulfoLink Coupling Gel (Pierce), were used in pull-down assays with eluted GST or his-tagged fusion proteins in HBS-EP. Pull-down assays with histones were done in 600 mM NaCl HBS-EP, whereas nucleosomes were analysed in 400 mM NaCl HBS-EP.

Preparation of nuclei and peptide challenge

U2OS nuclei were purified[16] and permeabilized[17] as described, except that 0.25% Triton X-100 was used. Permeabilized nuclei were diluted into PBS and incubated with or without peptide for 2 h on ice. We pelleted nuclei and analysed the supernatant by western blotting.

Immunofluorescence of mammalian cells

U2OS cells were fixed for 2 min in ice-cold methanol (containing 10 $\mu g\,ml^{-1}$ peptide, where used) and then blocked for 15 min in 3% bovine serum albumin, 0.6% Triton-X-100 in PBS (containing 10 $\mu g\,ml^{-1}$ peptide, where used). We performed staining using anti-HP1 antibodies[10] (1:1000). Antibody incubations contained 20 $\mu g\,ml^{-1}$ peptide (where used).

Schizosaccharomyces pombe strains and media

All strains had a centromeric insertion of the *ade6*$^+$ marker (*otr1R-Sph1::ade6*$^+$), and carried a genomic mutation in *ade6* (*ade6-210*), with the exception of the *ade6*$^+$ strain used as a control in the silencing assay. *swi6Δ::his1*$^+$ was described previously[18], and *clr4 Δ::ura4*$^+$ was generated by replacement of the *clr4*$^+$ open reading frame with the *ura4*$^+$ marker. The *clr4* G341D allele was isolated as a mutant that alleviated telomeric silencing[19]. We assessed centromeric silencing as described[20].

Protein methyltransferase assay

Wild-type and mutant (G341D) *clr4* SET-domain regions (residues 127–490) were cloned into pMAL (NEB) and expressed as maltose-binding protein fusions. Methyltransferase assays were done as described[6].

Yeast immunostaining

We performed immunostaining as described[5], except that we used Texas Red-conjugated anti-mouse, and FITC-conjugated anti-rabbit antibodies. Cells were simultaneously stained with mouse monoclonal antibodies against α-tubulin (TAT1) and rabbit anti-Swi6 antibodies.

Chromatin immunoprecipitation

Swi6 antibodies immunoprecipitated fixed chromatin as described[12]. Immunoprecipitated DNA was analysed by PCR using primers that detect the imr/otr junction of the outer repeats of the centromere and the euchromatic *fbp1*$^+$ locus (control).

Preparation of mono-nucleosomes

We isolated chicken erythrocyte mono-nucleosomes as described[21].

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