Characterization of the role of Chp2/HP1 in heterochromatin assembly in fission yeast

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Introduction

Heterochromatin is a tightly packed form of chromatin that plays an important role in epigenetic gene regulation and maintenance of chromosomal integrity. In general, heterochromatic regions consist of repetitive DNA, including satellite repeats, transposable elements, and retroviruses that must to be transcriptionally silenced. Heterochromatin assembly is associated with changes in post-translational modifications of histone tails and also with RNAi-mediated processes. In particular, trimethylation of histone H3 lysine 9 (H3K9me3) is a hallmark of heterochromatin and serves as a binding site for HP1 family proteins. HP1 family proteins were first identified as one of the factors associated with a phenomenon called position effect variegation (PEV) in *Drosophila* and have subsequently been studied in many organisms, including *Schizosaccharomyces pombe* (*S.pombe*), *Drosophila*, *Xenopus laevis*, plants, and humans. HP1 family proteins contain two evolutionarily conserved domains: an N-terminal chromo domain (CD) and a C-terminal chromoshadow domain (CSD). The CD functions as an H3K9me3-binding module, whereas the CSD functions as a self-dimerization module and provides an interacting surface for other transacting factors. Although a pentapeptide motif, PxVxL, has been identified as the consensus for binding the CSD of human HP1 proteins, it remains unclear whether this motif is recognized by other HP1 proteins in different organisms. The N-terminus and the hinge region connecting the CD and CSD are structurally disordered and evolutionarily less conserved, but these regions contain some clusters of basic amino acid residues and are involved in binding to DNA or RNA. In addition, serine residues in these regions are subject to phosphorylation, which modulates their binding to DNA or RNA and affects the recognition of H3K9me3 by the CD.

The fission yeast *S. pombe* is an excellent model organism to study the molecular mechanisms underlying heterochromatin assembly because its heterochromatin is marked by di- or trimethylation of histone H3K9 (H3K9me2/3) and it expresses two HP1 proteins, Swi6, and Chp2. Clr4, a homolog of mammalian SUV39H, provides H3K9me2/3, which is recognized by Swi6 and Chp2 through their CD. Swi6 is abundantly expressed and plays a dosage-dependent role in heterochromatin formation, whereas the expression level of Chp2 is low compared to Swi6, but it plays a distinct role in heterochromatin assembly. Deletion of *swi6*$^{+}$ or *chp2*$^{+}$ alleviates silencing at the heterochromatic regions, but the defect is not compensated by reciprocal expression under their original promoters, suggesting that Swi6 and Chp2 play distinct roles in heterochromatin assembly. This notion is further supported by studies identifying their interacting partners. While Swi6 interacts with a diverse set of proteins involved in a variety of nuclear processes, Chp2 specifically recruits the Snf2/HDAC repressor complex (SHREC), a family of nucleosome remodeling and deacetylation complexes (NuRDs). SHREC consists of the chromatin remodeler Mit1, the histone deacetylase Clr3, and the MBD-like protein Clr2, which are all linked by the scaffolding subunit Clr1. Among the SHREC components, Chp2 binds the N-terminus of Mit1 with an extensive interface, which is required for Mit1 recruitment to heterochromatin and heterochromatic silencing. In addition, Chp2, but not Swi6, is tightly associated with a chromatin-enriched nuclear subfraction, which is thought to be related to their distinct roles. As shown for other HP1 proteins, Chp2 silencing function is impaired by mutations in the CD or CSD, but the potential role of other domains, particularly the unstructured N-terminus and the hinge regions, in Chp2 function remains elusive. These unstructured regions of HP1

family proteins in other organisms have been implicated in DNA or RNA binding, and a similar activity has been demonstrated for Swi6.

Here, I show that the hinge and the N-terminus of the CSD of Chp2 have DNA-binding activity and that the cooperative action of these regions is essential for its silencing function. Although the DNA-binding activity of Chp2 plays a minor role in its tight chromatin binding, it appears to be involved in its stability. The results highlight the importance of the DNA-binding activity associated with HP1 family proteins for their function in heterochromatin assembly.

Materials and methods

Plasmid construction

Plasmids for the expression of recombinant 6×His tagged Swi6 or Chp2 in *E. coli* cells have been described previously. Coding sequences for Chp2 domains (N; 1 – 164 aa, CD; 165 – 244 aa, H; 239 – 313 aa, CSD; 314 – 381 aa), and Swi6 domains (N; 1 – 80 aa, CD; 81 – 139 aa, H; 140 – 260 aa, and CSD; 261 – 329 aa) were PCR amplified and cloned into *Nde*I and *Bam*HI sites of pET15b using NEBuilder HiFi DNA assembly mix (NEB). All mutations in the coding sequences of Chp2 were introduced by site-directed mutagenesis.

Strains and culture media

The media to culture *S. pombe* cells were prepared as previously described. To create a strain carrying $mit1^{I11R}$ the $ura4^{+}$ gene was first introduced into the *mit1* coding region through homologous recombination (*mit1-N::ura4*$^{+}$). The *mit1* coding region was then amplified by PCR, cloned into pBluescript, and subjected to site-directed mutagenesis to generate the $mit1^{I11R}$ mutation. The *mit1* coding region containing the $mit1^{I11R}$ mutation was amplified by PCR, introduced into cells harboring *mit1-N::ura4*$^{+}$, and stable clones in which *mit1-N::ura4*$^{+}$ was replaced by $mit1^{I11R}$ were selected using counter-selective media containing 5-fluorotic acid (5FOA). Strains expressing 3×FLAG-tagged, wild-type and mutant Chp2 were generated as previously described using the $rpl42^{P56Q}$ mutant and an $rpl42^{+}$ cassette to replace wild-type $chp2^{+}$ at the endogenous locus.

Antibodies

The following antibodies were used in this study: anti-FLAG (A8592; Sigma), anti-tubulin (T5168; Sigma), anti-Swi6 (Sadaie et al, 2008), anti-H3K9me2 (m5.1.1, gift of Dr. Takeshi Urano), anti-H3K14Ac (ab52946; Abcam).

Production of recombinant proteins

Recombinant 6×His-tagged Swi6 and Chp2 proteins were expressed in *E. coli* BL21 (DE3) and purified by immobilized metal affinity chromatography (TALON; Clontech) according to the manufacturer's instructions. Recombinant proteins were further purified by anion exchange chromatography (SOURCE 15Q; Cytiva) or cation exchange chromatography (SOURCE 15S; Cytiva). Full-length Chp2 dimers were further purified by size exclusion chromatography (Superdex 200 pg, Cytiva).

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed as previously described (Nishibuchi et al., 2014) with some modifications. Different concentrations of proteins were incubated with 0.19 pmol of 203-bp double-stranded DNA corresponding to pericentromeric repeats in 10 µl of EMSA assay buffer (20 mM Tris-HCl [pH 7.5], 50 mM NaCl, 1 mM dithiothreitol [DTT], and 0.1 mg/ml bovine serum albumin [BSA]) for 15 min at 37°C. After incubation, 1 µl of 30% sucrose was added, and the samples were loaded onto 5% native polyacrylamide gels in 0.5× Tris-borate-EDTA. Gels were run at room temperature for 1.5 h at 100 V. Gels were stained with SYBR Gold (Invitrogen), visualized using a ChemiDoc Imaging System (Biorad), and quantified using ImageJ. Binding curves were fitted with the

following equation: fraction bound = 1 − fraction unbound. Curve fitting was performed using Igor Pro software.

Immunoblotting

Harvested cells (~2×10^7) were washed once with ice-cold water, resuspended in 75 µl of alkaline 2ME solution (1.85N NaOH and 1.07 M 2-mercaptoethanol) and incubated on ice for 5 min. The cell suspension was mixed with 75 µl of 50% TCA and further incubated on ice for 5 min. Cellular proteins were precipitated by centrifugation, and the protein pellets were resuspended in SDS sample buffer, followed by boiling at 95°C for 5 min. Protein samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and subjected to Western blotting.

Silencing assays

Cells carrying a silencing marker (*mat3M::ura4*$^+$) were grown, collected by centrifugation, and resuspended in water. Serial dilutions (10-fold) were prepared and spotted on non-selective medium (YEA; yeast extract with adenine), minimal medium lacking uracil (PMG-ura), and minimal medium with 5FOA (PMG+5FOA). Plates were then incubated at 30°C for 2 – 4 days.

Chromatin fractionation assays

The chromatin fractionation assay was performed as described previously (Sadaie et al., 2008) with some modifications. Briefly, cells (2.5×10^8 cells) were harvested, washed once with stop buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM NaN_3), and placed on ice for 5 min. The cells were resuspended in PEMS (100 mM PIPES [pH 6.9],

10 mM EGTA, 10 mM $MgSO_4$, 1.2 M sorbitol) containing 1 mg/ml lysing enzyme and 1 mg/ml Zymolyase 100T, and incubated at 37°C for 20 min. The cell suspensions were spun at 2,000 rpm for 5 min at 4°C, and the resulting cell pellet was washed twice with 1.2 M sorbitol and then lysed with HBS buffer (25 mM MOPS [pH 7.2], 60 mM β-glycerolphosphate, 15 mM $MgCl_2$, 15 mM EGTA, 15 mM p-nitrophenylphosphate, 0.1 mM sodium vanadate) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), protease inhibitor (Complete^{TM} [Roche]), and 0.5% Triton X-100. The resulting lysate, whole cell extract, was spun at 15,000 rpm for 15 min at 4°C to obtain supernatant and pellet fractions. The whole cell extract, supernatant and pellet fractions were resolved by SDS-PAGE and subjected to Western blotting.

Chromatin immunoprecipitation (ChIP) and quantitative PCR (qPCR)

For all ChIP experiments, the cells were grown at 30°C in YEA until the cell density reached 1×10^7 cells/ml. For Swi6 and Chp2 ChIP experiments, cells were incubated for 2 h at 18°C and fixed with 1% formaldehyde for 30 min at 18°C. For H3K9me2 and H3K14ac ChIP experiments, the cells were immediately fixed with 1% formaldehyde for 20 min at 25°C. The fixed cells were harvested by centrifugation, washed twice with ice-cold PBS, transferred to a 1.5-ml tube, frozen in liquid nitrogen, and stored at -80°C before use. Antibodies were preincubated with 20 µl IgG- or Protein A-conjugated magnetic beads (Dynabeads; Thermo Fisher Scientific). Cells were lysed with ChIP lysis buffer (50 mM HEPES-KOH [pH 7.5], 140 mM NaCl, 1 mM EDTA, 1% Triton-X, 0.5% Na-deoxycholate) containing protease inhibitor (Complete^{TM}; Roche) and 1mM PMSF, and disrupted with Multi-Beads Shocker (Yasui Kikai). Chromatin was sheared by sonication using Bioruptor (Cosmobio) and incubated with antibody-bound magnetic

beads overnight at 4°C. Beads were washed twice with ChIP lysis buffer, twice with ChIP lysis buffer containing 0.5 M NaCl, twice with wash buffer (10 mM Tris-HCl [pH 8.0], 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.1% Na-deoxycholate), and twice with TE buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA). Precipitated chromatin was incubated with RNase A, eluted from the beads by incubation with TES buffer (50 mM Tris-HCl [pH 8.0] and 10 mM EDTA, 1% SDS), and reverse cross-linked by incubation at 65°C overnight. DNA samples were purified by NucleoSpin gel and PCR clean-up (Macherey-Nagel). qPCR was performed using Luna qPCR master mix (NEB) and a real-time PCR machine (StepOnePlus™; ABI). Primers used for qPCR are listed in Supplementary Table S1.

Results

Chp2 is tightly associated with a chromatin-enriched fraction independent of Clr4 and Mit1

Although Swi6 and Chp2 localize to heterochromatic regions through Clr4-mediated H3K9me2/3, Chp2 is tightly associated with chromatin-enriched fractions, and this association persists even in Clr4-depleted cells, suggesting that an additional mechanism(s) beyond H3K9me2/3 recognition contributes to Chp2 chromatin association. Since Chp2, like other HP1 proteins, acts as an adaptor to recruit chromatin regulatory factors, Chp2 may associate with the chromatin-enriched fraction through interaction with other binding partner(s). Chp2 has been shown to interact specifically with SHREC, a family of nucleosome remodeling and deacetylation complexes (NuRDs). Among the SHREC components, Chp2 directly binds the N-terminus of the chromatin remodeler Mit1 with an extensive interface, and this interaction is important for the function of Chp2 in recruiting SHREC to heterochromatic regions. To test whether the tight chromatin association of Chp2 is mediated by its interaction with Mit1, I generated a strain expressing Mit1 with the I11R mutation ($Mit1^{I11R}$), which has been shown to disrupt the interaction with Chp2, and performed a chromatin fractionation assay.

As previously reported, in wild-type cells, Swi6 was present in both the soluble (S) and chromatin-enriched pellet (P) fractions, with approximately 40% of the total Swi6 protein detected in the pellet fraction, and most of the Swi6 in the pellet fraction was redistributed to the soluble fraction in *clr4*Δ cells. In contrast, Chp2 was preferentially present in the chromatin-enriched pellet fraction in wild-type cells, and the Chp2 in this fraction was not altered by the $clr4^{+}$ depletion. Interestingly, I found that Chp2 in the

chromatin-enriched pellet fraction was not affected by the Mit1^{I11R} mutation). These results suggest that Chp2 is tightly associated with a chromatin-enriched fraction independent of Clr4 and Mit1, and uses a different mechanism(s) for this association.

Chp2 binds to DNA through its hinge and chromoshadow domains

HP1 proteins bind to DNA or RNA via the hinge region, and this activity is thought to be involved in their stable chromatin association. While a similar activity has been demonstrated for Swi6, it has not been clear whether Chp2 has DNA-binding ability and, if so, which domain contributes to the binding. To investigate the relationship between the tight chromatin association of Chp2 and its DNA binding ability, I prepared recombinant Chp2 and Swi6 as a control to perform electrophoretic mobility shift assays (EMSAs).

A previous study using size exclusion chromatography showed that recombinant Swi6 eluted as a single peak, whereas recombinant Chp2 eluted as two distinct peaks; the second peak corresponded to that of mutant Chp2 (Chp2^{L370E}), which is defective in dimer formation. From these results, previous study concluded that Swi6 forms stable dimers, whereas Chp2 dimers are less stable. To prepare stable dimer-formed Chp2 for EMSAs, I separated Chp2 by the size exclusion chromatography and pooled Chp2 in the first peak. Interestingly, I noticed that, in the fractions for the second peak, peptides with molecular mass less than 10 kDa were eluted with a similar profile, and mass spectrometric analysis revealed that these peptides corresponded to Chp2 CSD. This result suggests that Chp2 was detected in two distinct peaks in size exclusion chromatography, not because the monomeric form of Chp2 was detected due to the instability of the Chp2 dimer, but because Chp2 was degraded in the region near the CSD,

and a heterodimer of full-length Chp2 and CSD alone was detected as the second peak. The labile nature of Chp2 revealed in this *in vitro* experiment may be related to the *in vivo* function of Chp2 (see below).

Using purified full-length, dimerized Chp2 and Swi6, I performed EMSAs using pericentromeric DNA as a probe. Consistent with previous results, Swi6 bound DNA efficiently, and Chp2 also showed similar DNA binding activity. To investigate which domain of Chp2 and Swi6 is responsible for the DNA binding, I purified each domain of Chp2 and Swi6 and examined their ability to bind DNA. As previously reported, the hinge region of Swi6 (Swi6-H) bound strongly to DNA, and the activity appeared to be higher than that of full-length Swi6. The DNA binding of Swi6-H might be suppressed by other domains as shown for human HP1α. The N-terminal disordered region of Swi6 (Swi6-N) bound weakly to DNA, whereas no DNA binding activity was detected for Swi6-CD or Swi6-CSD. The hinge and the N-terminal disordered regions of Chp2 (Chp2-H and Chp2-N) also bound DNA, and no detectable DNA-binding activity was observed for Chp2-CD. Interestingly, I found that Chp2-CSD exhibited a robust DNA binding activity, in stark contrast to Swi6-CSD. Since DNA-binding activity associated with CSD has not been so far described in other HP1 proteins, this activity involving Chp2-CSD may contribute to Chp2-specific function.

Basic residues in the hinge and the N-terminus of CSD are involved in the DNA binding activity of Chp2

To gain insight into the amino acid residues involved in the DNA binding activity of Chp2, I first introduced amino acid substitutions in the hinge and CSD domains, respectively. Previous studies using human HP1α have shown that a cluster of basic

amino acid residues is critical to the DNA binding activity of the hinge region. In the Chp2 hinge region, there is a cluster of basic amino acid residues, KRRRSR (271–276 aa), and when these were replaced by alanine, the resulting Chp2-H mutant (Chp2-H^{5A}) no longer bound to DNA, suggesting that these positively charged residues are involved in the DNA binding of the Chp2 hinge.

The CSD is an evolutionarily conserved module consisting of three β-sheets followed by two α-helixes. The overall structure of the Swi6-CSD and the Chp2-CSD is similar, but the predicted structure of their N-terminal region is different. In addition, while there is no obvious cluster of basic amino acid residues in the Chp2-CSD, there are two lysine residues in the N-terminal region of the CSD (321–322 aa) that are not present in the corresponding region of Swi6. When these lysine residues in Chp2-CSD were replaced by alanine, the mutant Chp2-CSD (Chp2-CSD^{2A}) exhibited only a very weak DNA binding activity compared to wild-type Chp2-CD. When an additional alanine substitution was introduced into the neighboring glutamine residue (Q320), the mutant Chp2-CSD (Chp2-CSD^{3A}) no longer bound DNA, suggesting that these three residues are involved in the DNA binding activity associated with Chp2-CSD.

To confirm the involvement of these residues in the DNA-binding activity of full-length Chp2, I generated mutant Chp2 proteins with amino acid substitutions in either the hinge or the CSD, or both, and examined their DNA-binding activity by EMSAs. I found that full-length Chp2 with H^{5A} mutation in the hinge (Chp2-mut1) or with either CSD^{2A} or CSD^{3A} mutation in the CSD (Chp2-mut2 and Chp2-mut4) exhibited weaker DNA-binding activity compared to wild-type Chp2. Interestingly, when amino acid substitutions in the hinge and CSD were combined, the resulting Chp2 mutants (Chp2-mut3 and Chp3-mut5) no longer bound DNA. I confirmed that mutant Chp2 proteins with

these amino acid substitutions form a stable dimer, as judged by elution profiles in the size exclusion chromatography. These results clearly indicate that basic residues in the hinge and the N-terminal region of the CSD cooperatively contribute to the DNA-binding activity of full-length Chp2.

DNA-binding activities of Chp2 are required for heterochromatic silencing

To investigate the importance of DNA binding in Chp2 function *in vivo*, the above mutant Chp2 proteins were expressed from the endogenous *chp2*$^{+}$ locus with N-terminal FLAG tag, and their effects on the mating-type *mat3M::ura4*$^{+}$ silencing were examined by using *chp2*Δ cells as control. Immunoblotting assay showed that the levels of mutant Chp2 protein were comparable to that of wild-type Chp2. Cells expressing mutant Chp2 containing either the hinge mutation (mut1) or one of the mutations in the N-terminus of CSD (mut2 or mut4) showed silencing equivalent to that of the cells expressing wild-type Chp2. However, I found that cells expressing mutant Chp2 containing combined mutations of the hinge and one of the mutations in the N-terminus of CSD (mut3 and mut5) showed a silencing defect. Although the cells expressing Chp2-mut3 or -mut5 grew well on the PMG-Ura4 plates with comparable efficiency to the *chp2*Δ cells, they also grew robustly on the PMG+5FOA plates, indicating that the heterochromatic silencing is partially impaired in the cells expressing Chp2-mut3 and -mut5. Taken together, these results suggest that the DNA-binding activities of the hinge and the N-terminus of CSD are important for the silencing at the mating-type locus and presumably at other heterochromatic regions, and that these activities cooperate to maintain the silencing function of Chp2 *in vivo*.

DNA-binding activities of Chp2 are involved in its *in vivo* stability

Next, I tested whether the DNA binding activities of Chp2 are involved in its tight chromatin binding by the chromatin fractionation assay. I found that mutant Chp2 proteins lacking DNA binding activities associated with both the hinge and the N-terminus of CSD (mut 3 and mut 5) were predominantly detected in the chromatin-enriched pellet fraction (P). These results suggest that, on the contrary to our initial prediction, the DNA-binding activity of Chp2 plays a minor role in its tight binding to the chromatin-enriched fraction. Interestingly, however, when immunoblotting mutant Chp2 (mut3 and mut5), I routinely detected faster-migrating Chp2 bands in both whole-cell lysates (W) and chromatin-enriched pellet (P) fractions, which were not present in the immunoblotting of wild-type Chp2 and were only weakly detected in the immunoblotting of samples prepared with alkaline and TCA. Since I introduced the FLAG tag at the N-terminus of Chp2, the smaller Chp2 protein appeared to be a C-terminally truncated Chp2 species.

To further characterize this faster-migrating Chp2 species, I generated a strain expressing mutant Chp2 with CSD deletion and compared its migration with that of Chp2-mut3 and -mut5. Immunoblotting results showed that the faster-migrating Chp2 species migrated slightly faster than that of Chp2ΔCSD, suggesting that mutant Chp2 lacking DNA-binding activities associated with both the hinge and the N-terminus of CSD (mut 3 and mut 5) were susceptible to cleavage at a specific position just upstream of CSD, corresponding to a region sandwiched between two regions with mutations. Regarding the stability of Chp2 proteins, the hinge mutation (mut1) caused an increased amount of truncated Chp2 products, although the effect was milder than that of mut3 or mut5. On the other hand, one of the mutations in the N-terminus of the CSD (mut2 or

mut4) had a minor effect on the stability of the mutant proteins; the banding patterns are comparable to those of wild-type Chp2. Thus, the stability of Chp2 is also determined by the cooperative action of the hinge and the N-terminus of the CSD. Although the physiological significance of the cleavage of the C-terminus of Chp2 is not yet clear, these results appear to be related to the behavior of recombinant Chp2 in size exclusion chromatography and suggest that DNA-binding activities of Chp2 are involved in its *in vivo* stability.

DNA-binding activities of Chp2 are required for its stable association with heterochromatic regions

To investigate the effect of the DNA-binding activities of Chp2 on its localization to heterochromatic regions, I performed chromatin immunoprecipitation (ChIP) assays. Chp2 mutants lacking DNA-binding activities associated with both the hinge and the N-terminus of CSD (mut 3 and mut 5) exhibited reduced heterochromatin association compared to wild-type Chp2 at representative heterochromatic regions (centromeric *dg*, the mating-type *cenH*, telomere, and *mat3-M::ura4*$^{+}$), and the reduction was more severe for Chp2-mut3 compared to Chp2-mut5. These results suggest that the DNA-binding activities of Chp2 are required for its stable association with heterochromatic regions. Swi6 localization was moderately reduced at some of the heterochromatic regions in the cells expressing Chp2-mut3, but not evident in the cells expressing Chp2-mut5. Interestingly, the reduction of Swi6 at some of the heterochromatic regions seems to have a similar trend to that of Chp2. This result may indicate their cooperative function in localizing to these heterochromatic regions. Although Chp2 has been shown to bind the chromatin remodeler Mit1 and recruit the SHREC complex containing the H3K14

deacetylase Clr3, the levels of H3K14ac were not noticeably changed in the ChIP experiments in cells expressing Chp2-mut3 and -mut5. This result is likely due to the fact that the SHREC complex could be recruited to the heterochromatic regions via an alternative mechanism involving the Clr2 component. In addition, although the levels of H3K9me2 were slightly reduced at the examined heterochromatin regions examined in cells expressing Chp2-mut5, this reduction is not statistically significant. Taken together, these results suggest that the silencing defect observed in cells expressing Chp2-mut3 and -mut5 is primarily due to the reduced heterochromatin localization of Chp2.

Discussion

Although many eukaryotic cells express HP1 isoforms, the distinct and/or overlapping roles of these isoforms have not been fully elucidated. In this study, I focus on Chp2, one of the two HP1 isoforms in the fission yeast *S. pombe*, and demonstrate that DNA binding activities mediated by the hinge and the N-terminus of the CSD are critical for the Chp2 function in heterochromatic silencing. Previous studies have shown that HP1 proteins have the ability to bind DNA or RNA primarily through their unstructured hinge regions, and several studies have also shown that the unstructured N-terminal region also contributes to the binding. Similar to other HP1 proteins, Chp2 also binds to DNA through its hinge and the N-terminal regions, but this study showed that it has a unique DNA-binding activity through the N-terminus of the CSD, which has not been described for other HP1 proteins. Importantly, I also showed that mutant Chp2 containing either the hinge or the N-terminus of the CSD retains silencing function, but mutant Chp2 with combined mutations (Chp2-mut3 and -mut5) showed clear silencing defects and impaired heterochromatin localization, suggesting that DNA binding activities involving these regions contribute to proper Chp2 function in heterochromatin assembly.

Both Chp2-mut3 and -mut5 showed a similar silencing defect, but the effect of mut5 on Chp2 heterochromatic localization was milder than that of mut3. The reason for this effect is currently unclear, but the N-terminus of the Chp2 CSD contributes to the interaction with Mit1: the structural analysis of Chp2-CSD and Mit1 revealed the interaction of Chp2F319, the neighboring residue of Q320, with Mit1A49. Although Chp2Q320 does not appear to be directly involved in the interaction with Mit1, it is possible that Q320A increases regional hydrophobicity to support the interaction with

Mit1, thereby rescuing the heterochromatic localization of Chp2 via the enhanced interaction with Mit1.

Interestingly, I also found that the heterochromatic localization of Swi6 was reduced by Chp2-mut3 expression, and the reduction of Swi6 seems to have a similar trend to that of Chp2. This result may indicate their cooperative function in localizing to these heterochromatic regions. However, previous study showed that the heterochromatin localization of Swi6 is not significantly altered by simple deletion of Chp2 and also that overproduction of Chp2 leads to a decrease in the level of Swi6 levels at the mating type locus. Therefore, the reduction of Swi6 levels at the heterochromatin regions appears to be a Chp2-mut3 specific effect. It is possible that combined amino acid substitutions could alter the chromatin/nucleosome binding of Chp2 and that such Chp2 dysfunction results in the localization defect of Swi6. How Chp2's DNA-binding activities are coordinated with the recognition by the CD and/or interaction with other binding partners by dimerized CSD in the nucleosomal context remains to be elucidated by further structural analysis.

The DNA binding activity associated with HP1 proteins has been shown to be modulated by phosphorylation. A previous study showed that Chp2 is phosphorylated in *S. pombe* cells and that depletion of Ckb1, the catalytic subunit of casein kinase II, results in decreased levels of Chp2 phosphorylation, suggesting that Chp2 is phosphorylated by CK2 *in vivo*. However, recombinant CK2 failed to phosphorylate Chp2 *in vitro*, and mutant Chp2 with amino acid substitutions at candidate phosphorylation sites showed marked instability *in vivo*. Therefore, the role of CK2 in Chp2 phosphorylation and the exact phosphorylation sites of Chp2 remain unclear. Interestingly, a previous phoshoproteomic study has identified Chp2 phosphorylation sites (S288, S291, and

S293), and these are located between the basic amino acid cluster in the hinge and the N-terminus of the CSD. It is therefore possible that phosphorylation at some or all of these serine residues is involved in the DNA-binding activity of Chp2, as previously shown for Swi6 and human HP1α, and also in the stability of Chp2 *in vivo*.

In the course of the experiment to analyze the DNA binding activity of the Chp2 CSD, I identified three residues, Q320, K321, K322, that are critical for its DNA binding activity. These three residues are followed by four evolutionarily conserved hydrophobic resides, F315, P317, P318, and F319, and in a predicted 3D structural model, these hydrophobic residues interact with hydrophobic patches of the CSD, which makes the resides of Q320, K321, and K322, face the solvent region and allows side chains to behave flexibly. On the other hand, in a predicted 3D structure model, the N-terminal region of the Swi6 CSD has a short α-helix in the corresponding region, which appears to be similar to that of three human HP1 proteins. Thus, it is likely that the N-terminal region of Chp2 CSD has a unique structural property and that the flexible side chains contribute to DNA binding. In addition, such a unique structure may also be related to the unstable nature of full-length Chp2 *in vitro* and *in vivo*. When I replaced the Chp2 residues PPFQKK (317–322 aa) with the residues of the corresponding region of Swi6, QVENYD (262-267 aa), the mutant Chp2 consequently became unable to bind DNA *in vitro*, but it also became highly unstable in the cell. A previous study has also showed that mutant Chp2 with an amino acid substitution at S323, next to the residues required for the DNA binding, showed marked instability *in vivo*. These results also support the idea that the N-terminal region of Chp2 CSD has unique structural property that is closely related to its protein stability.

In agreement with a previous report, I confirmed that in the chromatin fractionation assay, Chp2 is tightly associated with the chromatin-enriched fraction, and this association is maintained in Clr4 depleted cells or cells expressing mutant Mit1 ($Mit1^{I11R}$), which is defective in interaction with Chp2 CSD. Based on these observations, I focused on the DNA binding activity associated with Chp2, but I showed that mutant Chp2 defective in DNA binding is still tightly associated with the chromatin enriched fraction. How does Chp2 bind tightly to the chromatin enriched fractions? It is possible that Chp2 uses multiple modes in addition to the H3K9me2/3 recognition, DNA binding activity, and the interaction with Mit1 to maintain stable association with chromatin. According to this scenario, it would be interesting to identify Chp2 interacting partners other than Mit1. It is also possible that Chp2 dissociated from the chromatin is susceptible to degradation, which may correlate with the observations that Chp2 expression levels appear to be kept in low and that overexpression of Chp2 results in a silencing defect. Further studies are needed to explain how DNA binding activities, protein stability, and tight chromatin association cooperatively contribute to the function of Chp2 in heterochromatin assembly.