Regulation of Swi6/HP1-dependent Heterochromatin Assembly by Cooperation of Components of the Mitogen-activated Protein Kinase Pathway and a Histone Deacetylase Clr6
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A study of gene silencing within the mating-type region of fission yeast defines two distinct pathways responsible for the establishment of heterochromatin assembly. One is RNA interference-dependent and acts on centromere-homologous repeats (cenH). The other is a stochastic Swi6 (the fission yeast HP1 homolog)-dependent mechanism that is not fully understood. Here we find that activating transcription factor (Atf1) and Pcr1, the fission yeast bZIP transcription factors homologous to human ATM-2, are crucial for proper histone deacetylation of both H3 and H4. This deacetylation is a prerequisite for subsequent H3 lysine 9 methylation and Swi6-dependent heterochromatin assembly across the rest of the silent mating-type (mat) region lacking the RNA interference-dependent cenH repeat. Moreover, Atf1 and Pcr1 can form complexes with both a histone deacetylase,Clr6, and Swi6, and clr6 deletion mutants affected the H3/H4 acetylation patterns, similar to the atf1 and pcr1 deletion mutant phenotypes at the endogenous mat loci and at the ctn1 promoter region surrounding ATF/CRE-binding sites. These data suggest that Atf1 and Pcr1 participate in an early step essential for heterochromatin assembly at the mat locus and silencing of transcriptional targets of Atf1. Furthermore, a phosphorylation event catalyzed by the conserved mitogen-activated protein kinase pathway is important for regulation of heterochromatin silencing by Atf1 and Pcr1. These findings suggest a role for the mitogen-activated protein kinase pathway and histone deacetylase in Swi6-based heterochromatin assembly.

Methylation of histone H3 lysine 9 (H3 Lys-9) by the conserved H3 Lys-9-specific methyltransferase, Su(var)3-9 in flies, SUV39H1 in human, and Clr4 in the fission yeast Schizosaccharomyces pombe (1–4) correlates with heterochromatin assembly. The methylated Lys-9 residue recruits another conserved heterochromatin protein, which is called Swi6 in S. pombe and HP1 (histone deacetylase 1) in higher eukaryotes (5, 6), leading to regional silencing of chromatin. In the fission yeast, recent studies (6–8) addressing the silencing of the mating-type region provide insights for understanding the regulation of heterochromatin assembly in eukaryotes. Of particular interest, previous work (9) has defined sequential requirements for the establishment and maintenance of regional heterochromatic domains.

Heterochromatin assembly at the mating-type region containing the mat2 and mat3 silent donor loci and an 11-kb interval (K region) between them requires several cis-acting DNA sequences as well as trans-acting factors (8, 10–13). Heterochromatin formation at the centromeres and within the silent mat2/3 interval requires many of the same silencing factors, including Clr3 and Clr6 (H3/H4-specific histone deacetylases), the Clr4-Rik1 complexes, and Swi6 (2, 14–19). The DNA elements involved in silencing within the entire 20 kb of the mat2/3 silent mating-type interval include REII (20), the mat3-M element including putative ATF/CREB-binding sites (21), and the 4.3-kb centromere-homologous repeat (cenH) sequence within the K region (22). The cenH region, which shares strong homology with the dg and dh centromeric elements, is a heterochromatin nucleation center and requires the RNA interference (RNAi) machinery similar to centromeric silencing (9). The cenH-mediated silencing via the RNAi machinery is required for initial formation of heterochromatin but not for its maintenance. In addition, it has been revealed that flanking sequences present in the K3 strain, which lacks the cenH-containing K region, are capable of recruiting and maintaining H3 Lys-9 methylation only in the presence of Swi6. Moreover, heterochromatin formation at the mat2/3 region eventually occurs even without the RNAi-dependent cenH element, suggesting the existence of an additional RNAi-independent Swi6-based mechanism for heterochromatin assembly (9).

ATF/CREB family proteins are among the conserved effector molecules that are regulated by the stress-activated MAP kinase (MAPK) cascade. In fission yeast, Atf1 and Pcr1, members of ATF/CREB family, are phosphorylated by the stress-activated Wis1 (MAPK kinase) and Sty1/Spc1 (MAPK) protein kinases, and this phosphorylation induces transcriptional activation of target genes (23–27). Moreover, many lines of evidence suggest potential roles for ATF/CREB family proteins in chromatin remodeling and gene silencing (28–30). Although deletion of two potential ATF/CREB-binding sites upstream of the mat3-M locus had a slight effect on heterochromatin silencing,
ing (21), there is still no clear evidence addressing the function of Atf1 and Pcr1 in heterochromatin silencing. Here we describe how the ATF/CREB transcription factors regulate a Swi6-dependent heterochromatin assembly in fission yeast. We find that cooperation of the ATF/CREB transcription factors with common silencing factors including histone deacetylase Clr6 and Swi6 protein is important for histone deacetylation and H3 Lys-9 methylation via an additional RNAi-independent, Swi6-dependent mechanism that acts across the repeat of the silent mat locus in the absence of cenH repeats.

**EXPERIMENTAL PROCEDURES**

**Media, Strains, and Plasmids—**Fission yeast media and genetic methods have been described previously (31, 32). The *S. pombe* strains used in this study are listed in Table I. Each gene deletion was carried out as described elsewhere. Primer pairs 5′-GGGTTCCCCTATTTCCTACG-3′ and 5′-TCTCCACATCTCTCACAACCA′3′ for *Mat3* and 5′-GAGGAAGAAATCGCAGCG-3′ and 5′-GGGTTCCCCTATTTCCTACG-3′ for *ade6* were used.

**RT-PCR—**RNA was isolated from cells grown at 30 °C in YES, N′, or media and treated with RQ1 DNase (Promega) to remove DNA contaminants. The cDNA was synthesized by using the Superscript reverse transcriptase (Invitrogen) with oligo(dT)17. The cDNAs were PCR-amplified by using primers 5′-GGGTTCCCCTATTTCCTACG-3′ and 5′-TCTCCACATCTCTCACAACCA′3′ for *Mat3* and 5′-GAGGAAGAAATCGCAGCG-3′ and 5′-GGGTTCCCCTATTTCCTACG-3′ for *ade6*.

| Strain | Genotype | Source |
|--------|-----------|--------|
| Hu50   | *h*::otIR (SpbH)::adel6 ura4-D18 ade6-DN/N leu1-32 | K. Ekwall |
| Hu51   | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | K. Ekwall |
| Hu56   | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | K. Ekwall |
| AP136  | mat1-M30::LacZ::adel6 ade6-M210 leu1-32 ura4-D18 | A. Cohen |
| AP144  | mat1-M30::LacZ::adel6 ade6-M210 leu1-32 ura4-D18 | A. Cohen |
| AP137  | mat1-P2::LEU2 leu1-32 ura4-D18 ade6-M201 | A. Cohen |
| AP192  | mat1-M30::K.ade6 ade6-M210 ura4-D18 leu1-32 his2 | A. Cohen |
| JY746  | *h*::ade6-M216 leu1-32 ura4-D18 | M. Yamamoto |
| PG9    | *h*::mat3-Mint::urat4-1 ura4-D18 ade6-M216 | G. Thon |
| FG2154 | *h*::ade6-M216 ura4-D18 sty1TF-12myc(ura4) | K. Shiozaki |
| SPG1013| *h*::ade6-M216 ura4-D18 ort1::urat4 crl1-1 ade6-M216 | S. Grewal |
| HK541  | *h*::ade6::ura4 | This study |
| YK1143 | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
| HS581  | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
| HS701  | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
| HS251  | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
| YK1103 | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
| YK1109 | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
| YK1121 | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
| HS42   | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
| HS801  | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
| HS135  | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
| HS631  | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
| HS651  | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
| YK1129 | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
| YK1176 | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
| YK1178 | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
| YK1185 | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
| HS631  | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
| HS651  | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
| HS651  | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
| HS651  | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
| H5001  | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
| H5001  | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
| H5001  | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
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| H5001  | *h*::mat3EcoRV::adel6 ura4-D18 ade6-DN/N | This study |
anti-acetyl-Lys-9 H3 antibody (07-352, Upstate Biotechnology, Inc.), anti-acetyl-Lys-4 H3 antibody (07-353, Upstate Biotechnology, Inc.), anti-acetyl-Lys-5 H4 antibody (Ab1758, Abcam), anti-acetyl-Lys-8 H4 antibody (Ab1760, Abcam), anti-acetyl-Lys-12 H4 antibody (06-761, Upstate Biotechnology, Inc.), anti-acetyl-Lys-16 H4 antibody (07-329, Upstate Biotechnology, Inc.), and protein A-Sepharose beads. DNA was released from the immunoprecipitates and purified. Recovered DNA was PCR-amplified with specific primers. PCR was carried out with or without −3′PdCTP, and the products were resolved by 6% PAGE and exposed to x-ray film or resolved on 1.8% agarose gels and stained with ethidium bromide.

Glutathione S-transferase (GST)-Pull Down Assay—The plasmids pJL205 (producing only GST moiety) and pREP1-KZ-atf1 were transformed into HS1001 (Clr6-HA) and HS1005 (Clr6-HA) cells, and pJL205 and pREP-GST-Swi6 were introduced into HS801 (Atf1-HA) and HS821 (Per1-HA). Cells were cultured in selective medium with thiamine, and the protein expression was induced by thiamine depletion for 17 h. Protein extracts of cells overexpressing GST, GST-Swi6, and GST-Atf1 were prepared in buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 10% (v/v) glycerol, 0.1% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture) by using the glass bead method (31, 32). GST, SGT-Swi6, and GST-Atf1 proteins were precipitated using glutathione-Sepharose 4B beads for 2 h at 4°C. After intensive washing with binding buffer, proteins bound to Sepharose beads were analyzed by immunoblotting with anti-HA (12CA5; Roche Applied Science).

RESULTS

Atf1 and Pcr1 Proteins Are General Silencing Regulators at the Silent Mating-type Loci and Their Deficiencies Cause Silencing Defects via Changes in Histone Acetylation Patterns—Deletion of two putative ATF/CREB-binding motifs downstream of the cenH repeat caused a partial derepression of the endogenous Mc gene from mat3-M locus (21). To identify factors important for the stochastic heterochromatin assembly in an RNAi-independent, Swi6-dependent manner, we investigated the roles of bZIP transcription factors in heterochromatin silencing at the mating-type region. To test whether Atf1 is involved in heterochromatin silencing at centromeres and telomeres, in addition to the silent mat locus, we investigated the effect of atf1 deletion on silencing of reporter genes inserted within each heterochromatid domain. An atf1 deletion strain carrying the ade6" reporter gene inserted at the cenH repeat caused a partial derepression of the reporter gene (Fig. 1A). In contrast, the atf1 deletion resulted in increased transcriptional repression at both centromeres and telomeres (Fig. 1A). Moreover, the absence of atf1 disrupted silencing of another reporter gene, ura4Δ, inserted at the silent mat3-M locus (data not shown). These data indicate that Atf1 is required for silencing RNA pol II-transcribed genes inserted into the silent mat3 region. In summary, this indicates that Atf1 acts as a positive regulator of mat3-M silencing but as a negative regulator of heterochromatin formation at other regions.

A previous report (25) demonstrated that Pcr1 binds to the ATF/CREB-binding sequences in vitro and that the presence of Pcr1 was critical for nuclear localization of Atf1 (23). Thus, we examined whether Pcr1, a binding partner of Atf1, is also required for mat3-M silencing. Similar to atf1 mutants, Δpcr1 showed reduced silencing, but the effect was weaker than that of Δatf1 or Δatf1Δpcr1 (Fig. 1B). These results suggest that heterodimerization of Atf1 and Pcr1 is important for the mat3-M silencing but that to some extent Atf1 can compensate for silencing defects resulting from the absence of Pcr1.

To assess whether the position effect on ade6" was directly related to its transcriptional expression, we performed RT-PCR to measure levels of mRNA from wild-type ade6" inserted within the mat3-M locus and the corresponding mini-gene ade6-DN/N at the endogenous locus on wild type (WT), atf1, pcr1, and swi6 deletion derivatives (Fig. 1C). The relative expression of the ade6" marker can be determined by the ratio of the full-length ade6" to the ade6-DN/N products in a competitive RT-PCR. The ratio in the Δatf1 and Δpcr1 mutants was considerably higher than that in the isogenic parental strain (WT) but much lower than that in a swi6 deletion mutant. Thus, our data demonstrate that transcriptional derepression of ade6" at the inactive mat locus occurred via a disruption of silencing.

We next performed a transition assay to determine the effect of the Δatf1 and Δpcr1 mutations on the epigenetic inheritance of the repressed state of ade6" expression (Fig. 1D). In this system, ade6-off cells in the repressed state were distinguished by red coloration, whereas cells expressing ade6", ade6-on cells were white. We found a striking increase in ade6-off to ade6-on conversion in Δatf1, Δpcr1, and Δatf1Δpcr1 mutants, to levels that were significantly higher than that of the wild-type background (HU51 strain) (Fig. 1D). This indicates that Atf1 and Pcr1 are required for stable epigenetic inheritance of the heterochromatin state at the mat locus.

To address whether Atf1 and Pcr1 are involved in silencing at another interval between mat1 and mat2, called the L region, we investigated the effect of Δatf1 or Δpcr1 null mutations on the silencing of ade6" reporter genes integrated at the BgIII and SacI sites of the L region. Despite the fact that the L region has no apparent ATF/CREB-binding motifs, the Δatf1 and Δpcr1 deletion strains had uniformly white colonies on low adenine medium and higher cell viability on adenine-free selective medium relative to the parental strains (AP136 and AP144), demonstrating full derepression of silencing at the L region (Fig. 2A). Furthermore, ChIP analysis showed that the absence of atf1 and pcr1 resulted in a considerable increase in histone H3/H4 acetylation and euchromatic-specific H3 Lys-4 methylation of the selected region (BglII-R) around the BglII site upstream of the mat2-P locus compared with that of wild type. The heterochromatin-specific H3 Lys-9 methylation was unlikely to be affected (Fig. 2B), perhaps because of the presence of other redundant nucleation mechanisms of heterochromatin assembly via the REEl element (20) or cenH repeat (9, 22). This indicates that the silencing defects observed in Δatf1 and Δpcr1 mutants were caused by the change in histone acetylation pattern and subsequent H3 methylation pattern. Furthermore, we found that atf1 and pcr1 deletions affect endogenous mat3-M expression in addition to altering the expression of the ade6" and ura4Δ reporter genes adjacent to the silent mat3-M locus (Fig. 2C). Thus, our results suggest that Atf1 and Pcr1 might act as general silencing factors of the inactive mat2/3 interval and nearby regions.

Phosphorylation Mediated by Sty1/Spc1 MAP Kinase Is Important for Atf1-dependent Regulation of Heterochromatin—Most surprisingly, the Wis1-Sty1/Spc1 kinase cascade is linked to the G2/M cell cycle control in optimal growth conditions (24, 37), indicating that the role of the pathway is not restricted to stress-inducible responses. Atf1 and Pcr1 are required for heterochromatin silencing at the mat locus under optimal growth conditions, and we wondered whether a deficiency in other components of the Wis1-Sty1/Spc1 cascade could affect mating-type silencing. Most interestingly, we observed that unlike Δatf1 and Δpcr1 mutant strains, silencing at the mat region was comparable in wild-type, Δsty1, and Δwis1 mutant strains (Fig. 3A). Moreover, the ade6-off to ade6-on conversion in Δsty1 and Δwis1 mutants was significantly reduced relative to wild-type cells, indicating that sty1 and wis1 deletions enhanced stabilization of the epigenetic inheritance of the silent states (Fig. 3B). Furthermore, a loss of variegation of ade6" expression and the stabilized inheritance of the silenced state in the Δsty1 mutant strain were reversed in Δatf1ΔΔsty1 double mutants (Fig. 3, A and B). This might be explained by previous work showing that the repressor activity of Atf1 protein is converted to activator activity via phosphorylation by Sty1/
Spc1 kinase, in the case of the UV-dependent catalase gene (ctt1/H11001) induction (38). Specifically, the absence of Sty1/Spc1 MAP kinase might strengthen the repressor activity of the Atf1 protein, leading to increased silencing at the mat region. Thus, we predict that as in general stress responses, Atf1 acts as a downstream effector of the Wis1-Sty1/Spc1 kinases for regulation of heterochromatin silencing.

The results presented above suggest that the kinase activity of Wis1 and Sty1/Spc1 is required for proper control of heterochromatin assembly by Atf1 and Pcr1. The Sty1/Spc1 kinase possesses conserved threonine 171 and tyrosine 173 residues that are phosphorylated by its cognate MEK Wis1 (23). Three mutants in which these conserved amino acids are modified, sty1AY (T171A), sty1TF (Y173F), and sty1AF (T171A, Y173F), displayed an elongated morphology phenotype that was identical to the phenotype of Δsty1 deletion cells (23). We used the sty1TF (originally designated spc1TF) mutant to elucidate the role of the phosphorylation state in silencing. To verify that phosphorylation of Sty1/Spc1 kinase by Wis1 is important for silencing of reporter genes inserted within the mat locus, we introduced the mat3-M::ade6/H11001 reporter into the Spc1/Sty1 phosphorylation mutant background (sty1TF) with genetic crosses. Most interestingly, silencing and maintenance of epigenetic inheritance of the silenced state at the mat3 locus were comparable in the ΔH9004 sty1 deletion and the phosphorylation mutant strains (Fig. 3, A and B). These analyses suggest that indeed phosphorylation signaling via the Wis1-Sty1/Spc1 kinase cascade is important for regulation of heterochromatin by Atf1 and Pcr1.

Atf1 and Pcr1 Are Indispensable for Heterochromatin Assembly at the mat2/3 Region in a cenH Deletion Background—Our data presented above demonstrated that Atf1 and Pcr1 are indispensable for heterochromatin assembly at the mat2/3 region in a cenH deletion background, indicating that Atf1 and Pcr1 are essential for proper heterochromatin assembly at the mat loci. However, the specific role of Atf1 and Pcr1 in heterochromatin assembly remains to be elucidated. The results presented above suggest that the kinase activity of Wis1 and Sty1/Spc1 is required for proper control of heterochromatin assembly by Atf1 and Pcr1. The Sty1/Spc1 kinase possesses conserved threonine 171 and tyrosine 173 residues that are phosphorylated by its cognate MEK Wis1 (23). Three mutants in which these conserved amino acids are modified, sty1AY (T171A), sty1TF (Y173F), and sty1AF (T171A, Y173F), displayed an elongated morphology phenotype that was identical to the phenotype of Δsty1 deletion cells (23). We used the sty1TF (originally designated spc1TF) mutant to elucidate the role of the phosphorylation state in silencing. To verify that phosphorylation of Sty1/Spc1 kinase by Wis1 is important for silencing of reporter genes inserted within the mat locus, we introduced the mat3-M::ade6/H11001 reporter into the Spc1/Sty1 phosphorylation mutant background (sty1TF) with genetic crosses. Most interestingly, silencing and maintenance of epigenetic inheritance of the silenced state at the mat3 locus were comparable in the ΔH9004 sty1 deletion and the phosphorylation mutant strains (Fig. 3, A and B). These analyses suggest that indeed phosphorylation signaling via the Wis1-Sty1/Spc1 kinase cascade is important for regulation of heterochromatin by Atf1 and Pcr1.
Fig. 2. Δatf1 and Δpcr1 deletions derepress mat2-P silencing and increase endogenous mat3-M gene expression. A, effects of Δatf1 and Δpcr1 deletions on mat2-P silencing. A schematic representation of the mating-type locus where the ade6- marker genes were inserted (top). Comparisons of colony growth ability (middle) and color (bottom) on selective (−Ade) or nonselective (N/S) or low adenine medium are shown. Strains used are as follows: SacI plate: WT (AP136), Δatf1 (YKJ172), and Δpcr1 (YKJ177); BglII plate: WT (AP144), Δatf1 (YKJ176), and Δpcr1 (YKJ178). B, comparison of H3/H4 acetylation and H3 Lys-4 and Lys-9 methylation levels in atf1 and pcr1 deletion derivatives of the API4 strain. The bar located downstream of BglII site (A, indicated as BglII-R in B) indicates location of the primer set used in PCR. The levels of histone acetylation and euchromatin-specific H3 Lys-4 methylation were significantly increased, suggesting that atf1 and pcr1 deletions affect the acetylation patterns and subsequently caused the change of methylation patterns at the silent mat loci. The relative fold enrichment shown below each lane was calculated by dividing the ratio of the selected loci/leu1 PCR products in the ChIP sample with that in the input sample. Ac, acetylation; K, lysine; Me, methylation. C, effects of Δatf1 and Δpcr1 deletions on expression of the endogenous mat3-M gene. Cells were grown in nitrogen-rich (+) and starved (−) media. Total RNA was prepared from the cultures and subjected to RT-PCR. Competitive PCR for the expression of the Mc and the act1+ genes was carried out. Strains used are as follows: WT (AP137), Δatf1 (YKJ135), Δpcr1 (HS631), and Δswi6 (YKJ129).

general silencing factors acting on the entire silent mat2/3 region. Moreover, deletion of the mat region containing the potential ATF/CREB-binding sequences caused a partial derepression of endogenous Mc expression at the mat3-M locus (21). Thus, these findings raised the possibility that Atf1 and Pcr1 are associated with an additional RNAi-independent Swi6-based mechanism for heterochromatin assembly. To test whether Atf1 and Pcr1 are involved in an alternative Swi6-based mechanism, we used a KΔ:ade6+ reporter strain, in which a part of the K region containing the cenH element was replaced by an ade6+ marker (Fig. 4A). The KΔ:ade6+ strain predominantly exhibited the silent state (designated as ade6-off), showing red coloration (Fig. 4B). Moreover, we confirmed that the deletion of an RNAi component named rdr1+ (RNA-dependent RNA polymerase) in KΔ:ade6+ ade6-off cells did not influence the silencing phenotype (Fig. 4B). This supports the model of a Swi6-dependent mechanism for the residual silencing capability in the KΔ:ade6+ reporter strain lacking the RNAi-depend-
ent cenH region. Thus, we investigated the effects of atf1 and pcr1 deletions on the silencing phenotype of \( \text{K} \Delta_{\text{ade}6} \text{ade}6\text{-off} \) cells. Most surprisingly, the silencing defects at the mat2/3 region were comparable in the \( \text{K} \Delta_{\text{ade}6} \text{atf1}, \text{K} \Delta_{\text{ade}6} \text{pcr1}, \text{K} \Delta_{\text{ade}6} \text{swi6} \), and \( \text{K} \Delta_{\text{ade}6} \text{rdr1} \) mutants (Fig. 4B). Moreover, our ChIP analysis revealed that the deficiencies of atf1, pcr1, or swi6 completely abolished heterochromatin-specific H3 Lys-9 methylation, concomitant with a significant increase of euchromatin-specific H3 Lys-4 methylation at the selected chromosomal loci across the rest of mat2/3 region lacking the cenH element (Fig. 4, C and D). This demonstrates that in \( \text{K} \Delta_{\text{ade}6} \) cells lacking the cenH repeat, the presence of H3 Lys-9 methylation strictly depends on Atf1 and Pcr1, in a manner similar to its dependence on Swi6. Furthermore, ChIP analysis using histone acetylation-specific antibodies showed that deficiencies of atf1, pcr1, and swi6 resulted in a significant increase of histone H3/H4 acetylation at all positions tested at the mat loci indicated as ade6-R. PCR was performed using primer sets of ade6-R and leu1. Relative fold enrichment in each graph was calculated by dividing the ratio of the ade6-R locus/leu1 PCR products in the ChIP sample with that in the input sample.

Fig. 4. Deficiencies in atf1\(^{-}\), pcr1\(^{-}\), and swi6\(^{-}\) affect heterochromatin-specific histone modification patterns and subsequent silencing across the rest of the silent mat region lacking the cenH repeat. A, schematic representation of \( \text{K} \Delta_{\text{ade}6} \), in which the cenH region was replaced with ade6\(^{-}\). The bars marked by numbers or names indicate location of primer sets used for PCR amplification. B, three genes, atf1\(^{-}\), pcr1\(^{-}\), and swi6\(^{-}\), are required to maintain silencing, whereas the RNA-dependent RNA polymerase gene, rdr1\(^{-}\), is dispensable for maintenance of heterochromatin silencing in the \( \text{K} \Delta_{\text{ade}6} \) ade6-off strain. Comparison of each colony growth and color on selective (-Ade) or low adenine medium is shown. Strains used are as follows: WT (AP152), \( \Delta_{\text{atf1}} \) (HS3001), \( \Delta_{\text{pcr1}} \) (HS3051), \( \Delta_{\text{swi6}} \) (HS3072), and \( \Delta_{\text{rdr1}} \) (HS3091). C, levels of H3 Lys-9 methylation in \( \Delta_{\text{atf1}}, \Delta_{\text{pcr1}}, \Delta_{\text{swi6}} \), and \( \Delta_{\text{rdr1}} \) derivatives from Fig. 3B were determined by ChIP analysis. DNA extracted from ChIP or input was amplified by competitive PCR by using the primer sets shown in A. An endogenous leu1 fragment served as an internal control. Relative fold enrichment shown below each lane was calculated by dividing the ratio of the selected loci/leu1 PCR products in the ChIP sample with that in the input sample. The absence of atf1 and pcr1 reduced H3 Lys-9 methylation to a level comparable with swi6. D, levels of H3 Lys-4 methylation in the strains used in C were determined by ChIP analysis. The absence of atf1 and pcr1 resulted in a significant increase of euchromatin-specific H3 Lys-4 methylation at the mat loci indicated as ade6-R. PCR was performed using the primer set of ade6-R and leu1. E, levels of H3/H4 acetylation in the same strains used in C and D were determined by ChIP analysis. The absence of atf1 and pcr1 resulted in a significant increase of histone H3/H4 acetylation at all positions tested at the mat loci indicated as ade6-R. PCR was performed using primer sets of ade6-R and leu1. Relative fold enrichment in each graph was calculated by dividing the ratio of the ade6-R locus/leu1 PCR products in the ChIP sample with that in the input sample. Ac, acetylation; K, lysine; Me, methylation.
deacetylation at H3 Lys-9 and H3 Lys-14. Taken together, these findings suggest that flanking sequences present in the $K\Delta:ade6^{\text{on}}$ strain are capable of establishing heterochromatin-specific histone modification patterns such as H3/H4 deacetylation and H3 Lys-9 methylation only in the presence of Atf1, Pcr1, and Swi6.

The Swi6 andClr4 proteins were previously known as limiting chromatin components that convert the epigenetic imprint from the expressed state to the silenced state when overexpressed, emphasizing their dosage-critical roles in heterochromatin formation (39). To evaluate the establishment of a heterochromatin-like structure in the $K\Delta:ade6^{\text{on}}$ (white color colony), we quantified the ade6-on to ade6-off conversion following overexpression of Swi6 and Clr4. We observed increases in ade6-on to ade6-off conversion by overexpressed Swi6 and Clr4 (Fig. 5A, left panel), consistent with previous work (9). To delineate the role of Atf1 and Pcr1 in a Swi6-based silencing mechanism in the absence of the RNAi-dependent cenH repeat, we investigated whether atf1 deletion affects the ade6-on to ade6-off conversion in $K\Delta:ade6^{\text{on}}$ ade6-on when Swi6 or Clr4 is overexpressed. Most surprisingly, the absence of atf1 abolished the dosage-dependent effect of Swi6 and Clr4 on the ade6-on to ade6-off conversion (Fig. 5A, right panel). More importantly, only re-introduction of a wild-type atf1+ copy recovered the ade6-on to ade6-off conversion to a level similar to the isogenic parental strain in $K\Delta:ade6^{\text{on}}$ ade6-on cells bearing a null allele of atf1 (Fig. 5A). This demonstrates that the residual capacity for ade6-on to ade6-off conversion found in $K\Delta:ade6^{\text{on}}$ ade6-on cells was completely dependent on the presence of Atf1 (Fig. 5A). This suggests that Atf1 and Pcr1 are indispensable for the initiation of heterochromatin formation via an alternative Swi6-based mechanism in the absence of the RNAi-dependent cenH element.

We next measured the levels of H3 Lys-9 methylation in Swi6- or Clr4-overexpressing derivatives of $K\Delta:ade6^{\text{on}}$ ade6-on cells using a ChIP assay. Consistent with the silencing phenotypes (Fig. 5A), ChIP analysis showed that overexpression of Swi6 or Clr4 recovered H3 Lys-9 methylation in $K\Delta:ade6^{\text{on}}$ ade6-on cells with an atf1+ wild-type copy but not in an atf1-null background (Fig. 5B). The overexpressed protein levels induced in thiamine-depleted media were confirmed by Western blot analysis using anti-HA antibodies (Fig. 5C). Taken together, these results suggest that Atf1 and Pcr1 are key regulators of Swi6-dependent heterochromatin formation and silencing at the silent mat region in the absence of the RNAi-dependent cenH element.

**Atf1 and Pcr1 Can Form Complexes with Clr6 and Swi6—** For silencing at the mat2/3 region, Atf1 and Pcr1 proteins might cooperate with common silencing factors. In particular, we reasoned that Clr3, Clr6, and Swi6 are potential candidates because atf1 deletion affected the histone deacetylation and methylation patterns responsible for establishment and maintenance of heterochromatin (Fig. 2B and Fig. 4, C–E). When cells were treated with tricostatin A, a histone deacetylase inhibitor, the basal level of ctt1+ expression in the $\Delta sty1$ deletion background was equivalent to that of $\Delta atf1$ mutant cells. To find out whether Atf1 and Pcr1 interact with common silencing factors such as Clr3, Clr6, and Swi6, we performed GST-pull-down assays. Protein extracts from cells expressing each protein were used for GST-pull downs followed by Western blotting. Most interestingly, GST-Atf1 could form complexes with Clr6-HA, but interactions with Clr3-HA were minimal (Fig. 6A, left top panel). GST-Swi6 binds to Atf1-HA and Pcr1-HA (Fig. 6A, left bottom). Expression of the GST-tagged proteins was confirmed by Western blot by using an anti-GST antibody (Fig. 6A, right). Thus, these data suggest that complex formation between Atf1, Pcr1, Clr6, and Swi6 is important for heterochromatin assembly at the mat2/3 region.

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2 H. S. Kim, E. S. Choi, J. A Shin, Y. K. Jang, and S. D. Park, unpublished data.

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**FIG. 5.** Atf1 and Pcr1 are critical for silencing and H3 Lys-9 methylation via a Swi6-dependent mechanism across the silent mat region in the absence of the RNAi-dependent cenH element. A, overexpression of Swi6 and Clr4 caused increases in the ade6-on to ade6-off conversion in wild type but not in $\Delta atf1$ mutants. After introduction of pREP41 plasmid-derived overexpression cassettes for HA-tagged Swi6 and HA-tagged Clr4 or HA-Clr4 proteins (or vector only) into $K\Delta:ade6^{\text{on}}$ ade6-on strain (AP152) or $K\Delta:ade6^{\text{on}}$ $\Delta atf1$ (HS3011) cells, white (transformant) colonies were selected and plated onto adenine-limiting minimal medium without thiamine. After a 5-day incubation, red (ade6-off) colonies were counted, and the frequency of red to total colonies was calculated. The ade6-on to ade6-off conversion in Swi6- or Clr4-overexpressing strains increased 6–15-fold relative to that of the wild type strains carrying vector only (left). The absence of atf1 abolished the effect of Swi6- or Clr4-overexpression on ade6-on to ade6-off conversion (right). B, ChIP analysis of H3 Lys-9 methylation in Swi6- or Clr4-overexpressing derivatives of $K\Delta:ade6^{\text{on}}$ ade6-on (right panel).
Clark6 is a Major Histone Deacetylase Essential for Regulation of Heterochromatin Assembly and Gene Silencing by Atf1 and Pcr1—To explain the biological significance of this physical interaction between Atf1, Pcr1, and Clark6, the effect of clark6 mutations on histone acetylation and methylation patterns was examined. As shown in Fig. 6B, under semi-permissive growth conditions (30°C), mutations in clark6 essential for cell growth resulted in an increased level of histone acetylation at all positions tested, a phenotype similar to that of clark6 mutants (see Fig. 4). In addition, the clark6 mutations showed a moderate but consistent decrease in heterochromatin-specific H3 Lys-9 methylation, concomitant with a moderate increase of euchromatin-specific H3 Lys-4 methylation at the selected chromosomal loci (ade6-R). This functional interaction between Atf1 and Clark6 was also supported by the findings that both Δclark6-clark6-1 and Δatf1-Δclark6 double mutants showed synergistic effects on the silencing and histone acetylation patterns at the mating-type region (data not shown; see Ref. 17).

To investigate whether the functional interaction between Atf1 and Clark6 is required for euchromatic gene silencing, we performed ChIP analysis for localization of Atf1 and histone acetylation patterns at the stress-inducible ctt1 promoter region in clark6 mutants. ChIP analysis revealed that Atf1-HA was highly enriched at the promoter region encompassing the ATF/CREB-binding site (Fig. 6C, left). Moreover, the ChIP assay using histone acetylation-specific antibodies showed that, similar to Δatf1 and Δpcr1 deletion mutants, the clark6 mutation resulted in a moderate but consistent increase (about 2-fold) in histone H3/H4 acetylation at the ctt1 promoter region (Fig. 6C, right). In contrast, the clark3 deletion mutant showed little effect on histone acetylation (Fig. 6C, right).
6C, right). Thus, these findings suggest that Clr6 is a key histone deacetylase that regulates heterochromatin assembly and gene silencing by the Atf1-Pcr1 heterodimer.

**DISCUSSION**

Most interestingly, we showed that disruption of Atf1 and Pcr1 resulted in a marked decrease of gene silencing at the silent mat region, but increased repression at centromeres and telomeres, suggesting that this silencing is locus-specific (Fig. 1). The phenotype could be explained by a competition model based on the functional interaction of Atf1 with common silencing factors such as Clr6 and Swi6 (Fig. 6). This phenotype is reminiscent of the relocalization of Sir3 from telomeres to rDNA loci and the subsequent increase of rDNA silencing in Sir4-null mutant cells of *S. cerevisiae* (40). Likewise, in the ∆atf1 mutant cells, general silencing factors such as Clr6, Clr4, and Swi6 normally associated with Atf1 might be released from specific genomic loci including the *mat* loci and potential Atf1-repressive promoter regions (41), and then accumulate in other regions of heterochromatin such as centromeres and telomeres, leading to their increased silencing.

The findings presented here suggest that phosphorylation by the Wis1-Stmt1/Spc1 kinase cascade is important for regulation of heterochromatin by Atf1 and Pcr1. How phosphorylation can influence Atf1-dependent heterochromatin silencing might be explained by an activator-repressor model for Atf1 activity, as proposed by Degols and Russell (38). This hypothesis suggests that Atf1 has an intrinsic repressor activity, which is converted to an activator upon phosphorylation by the Spc1/Stmt1 kinase. This model was supported by the previous findings that Atf1 negatively regulates *ctt1* expression in the absence of Spc1/Stmt1 kinase activity (38), and Spc1/Stmt1 directly regulates Atf1 activity through phosphorylation (24, 26). In addition, the model is supported by reports that the budding yeast Hog1 kinase converts a repressor complex containing the ATF/CREB repressor Sko1 (Acr1) into an activator in response to osmotic stress (42). What is the biological relevance of interconversion between an activator activity and a repressor activity of Atf1? Swi6, one of the common silencing factors, physically interacts with several proteins involved in mating-type switching (10), suggesting that Swi6 provides an interface between heterochromatin silencing and mating-type interconversion by establishing a chromatin structure favorable to both processes (10). Similarly, we propose that regulation of Atf1 activity by the MAP kinase cascade may be required to establish a dynamic chromatin structure favorable to both silencing and switching processes at the mating-type region.

As indicated previously (7, 9), the precise mechanism of the Swi6-dependent heterochromatin assembly at the silent *mat* regions in the absence of RNAi-dependent cenH is still not clear. Our present findings demonstrate that Atf1 and Pcr1 regulate Swi6-dependent silencing at the *mat* region independently of cenH-mediated silencing. In our current model, Atf1 and Pcr1 bind specifically to putative ATF/CREB-binding sites upstream of *mat3-M* (21), as a result of their sequence-specific binding properties (25, 30, 33); subsequently, they recruit Clr6 histone deacetylase and Swi6 to the *mat* locus. This hypothesis is consistent with our findings that Atf1 and Pcr1 can form complexes with Clr6 and Swi6 (Fig. 6) and that histone acetylation patterns found in *clr6-1* mutant cells were similar to those of ∆atf1 and ∆pcri deletion mutant cells (Figs. 4E and 5B). In the absence of the main nucleation site of the cenH element, the establishment of histone deacetylation patterns at both H3 and H4 is initiated by Atf1 and Pcr1. These events nucleate heterochromatin by subsequently targeting H3 Lys-9 methylation and creating Swi6-binding sites around the ATF/CREB-binding sequence. Subsequently, the spread of heterochromatin across the whole *mat2/3* region occurs in a Swi6-dependent, self-propagating manner (6, 9). These Swi6-based activities initiated by Atf1 and Pcr1 might function in an inefficient and highly stochastic manner, as proposed previously (9), because the dosage-critical role of Swi6 and Clr6 in silencing is strictly dependent on the presence of Atf1 (Fig. 5, A and B).

This proposed mechanism is reminiscent of mammalian gene silencing by the retinoblastoma-SUV39H1-HP1 complex (43), mating-type, and telomeric silencing by the budding yeast Rap1-Sir protein complexes (44, 45) and centromeric heterochromatin assembly by the CENP-B-Sw6 complex (46). A tumor suppressor protein retinoblastoma recruits histone-modifying factors and HP1 protein to the cyclin E promoter, leading to H3 Lys-9 methylation and gene silencing (43). In addition, it has been proposed that both Sir3 and Sir4 can directly and independently bind to Rap1 at mating-type silencers and telomeres, suggesting that Rap1-mediated recruitment of Sir proteins operates through multiple cooperative interactions (44, 45).

Collectively, our findings suggest that Atf1 and Pcr1 are key regulators that are involved in an early step for nucleation of a Swi6-dependent, RNAi-independent heterochromatin assembly at the mating-type region through cooperation with the histone deacetylase Clr6 in the fission yeast. Moreover, cooperation between Atf1-Pcr1 heterodimers and Clr6 is also required for euchromatic gene silencing of transcriptional targets of Atf1, such as the stress-inducible *ctt1* gene. Although the conserved role of ATF-2, the mammalian counterpart to Atf1, in heterochromatin silencing remains to be demonstrated experimentally, our data provide novel insights into the roles of members of the ATF/CREB family in heterochromatin formation and gene silencing in higher eukaryotes.

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Atf1-Pcr1 and Clr6 Cooperate to Nucleate Heterochromatin

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