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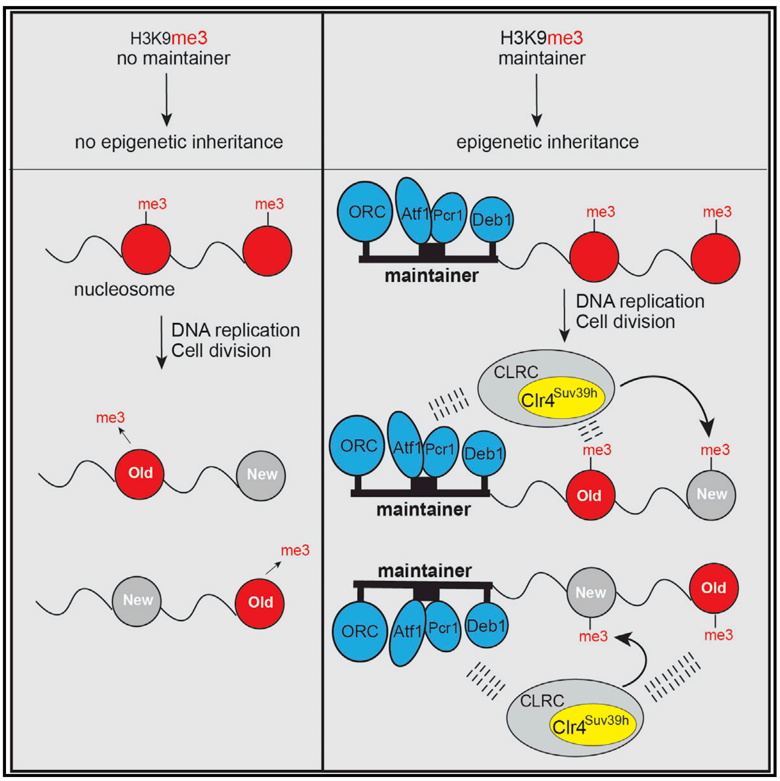
A composite DNA element that functions as a maintainer required for epigenetic inheritance of heterochromatin

Graphical abstract



Highlights

- A composite DNA element acts as a maintainer of epigenetic memory in heterochromatin
- Maintainer contains binding sites for Atf1/Pcr1, Deb1, and the origin recognition complex
- Maintainer functions at native and ectopic loci
- Maintainer works together with pre-existing H3K9me3 to recruit Clr4^{Suv39h}

Authors

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In brief

Wang et al. reveal the mechanism of DNA sequence-dependent epigenetic inheritance by showing that a 130-bp composite DNA element with binding sites for three site-specific DNA binding factors acts as a maintainer of epigenetic memory. This element works together with pre-existing histone H3 lysine 9 methylation to ensure heterochromatin maintenance.

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A composite DNA element that functions as a maintainer required for epigenetic inheritance of heterochromatin

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SUMMARY

Epigenetic inheritance of heterochromatin requires DNA-sequence-independent propagation mechanisms, coupling to RNAi, or input from DNA sequence, but how DNA contributes to inheritance is not understood. Here, we identify a DNA element (termed "maintainer") that is sufficient for epigenetic inheritance of pre-existing histone H3 lysine 9 methylation (H3K9me) and heterochromatin in *Schizosaccharomyces pombe* but cannot establish *de novo* gene silencing in wild-type cells. This maintainer is a composite DNA element with binding sites for the Atf1/Pcr1 and Deb1 transcription factors and the origin recognition complex (ORC), located within a 130-bp region, and can be converted to a silencer in cells with lower rates of H3K9me turnover, suggesting that it participates in recruiting the H3K9 methyltransferase Clr4/Suv39h. These results suggest that, in the absence of RNAi, histone H3K9me is only heritable when it can collaborate with maintainer-associated DNA-binding proteins that help recruit the enzyme responsible for its epigenetic deposition.

INTRODUCTION

Epigenetic inheritance mechanisms play important roles in maintaining gene expression patterns and safeguarding cell identity (Margueron and Reinberg, 2010; Moazed, 2011). Two classes of positive feedback mechanisms, broadly defined as *trans* and *cis* acting, are thought to mediate epigenetic memory (Moazed, 2011). The first class involves transcription factors that regulate the expression of downstream genes and also activate their own expression, thus allowing an initial response to persist in the absence of inducing signals (Ptashne and Gann, 2001). The second class involves changes in covalent modifications of DNA or histones, which are then propagated in *cis* following DNA replication by enzymes that recognize (read) certain parental modifications and catalyze (write) the same modification on newly synthesized DNA strands or newly deposited histones (Margueron and Reinberg, 2010; Moazed, 2011; Probst et al., 2009; Radman-Livaja et al., 2011). *Trans*-acting positive feedback loops rely on specific DNA sequences to which the auto-activating transcription factor binds (Ptashne, 2014; Ptashne and Gann, 2001). By contrast, *cis*-acting chromatin-based mechanisms are generally presumed to function in a DNA-sequence-independent manner through read-write positive feedback. Recent studies have demonstrated that, although the read-write mechanism can propagate meta-stable epigenetic states independently of DNA sequence, stable *cis* epigenetic inheritance requires both the read-write positive feedback loop and specific DNA sequences (Audergon et al., 2015; Coleman and Struhl, 2017; Laprell et al., 2017; Ragunathan et al., 2015; Wang and Moazed, 2017). Despite this profound impact on epigenetic memory of conserved chromatin states in both yeast and *Drosophila*, the nature of DNA sequences that promote *cis*- and allele-specific epigenetic inheritance and how such sequences work together with read-write mechanisms is poorly understood.

Heterochromatic domains of DNA have been shown to have epigenetic inheritance properties in eukaryotes ranging from yeast to mammals (Allshire and Madhani, 2018). In *S. pombe*, heterochromatin is localized at pericentromeric DNA repeats, sub-telomeric regions, and the mating type (*mat*) locus. These domains are associated with histone H3K9 methylation (H3K9me), which is a conserved modification in heterochromatin from fission yeast to mammals. At the *mat* locus, heterochromatin formation requires the parallel activities of the RNA interference (RNAi) machinery, binding sites for the Atf1/Pcr1 transcription factor heterodimer, and other DNA elements, which recruit the Clr4 (Suv39h) H3K9 methyltransferase and other factors (Greenstein et al., 2018; Jia et al., 2004; Thon et al., 1999; Yamada et al., 2005). At an ectopic euchromatic locus, H3K9me domains established by artificial tethering of Clr4 can only be

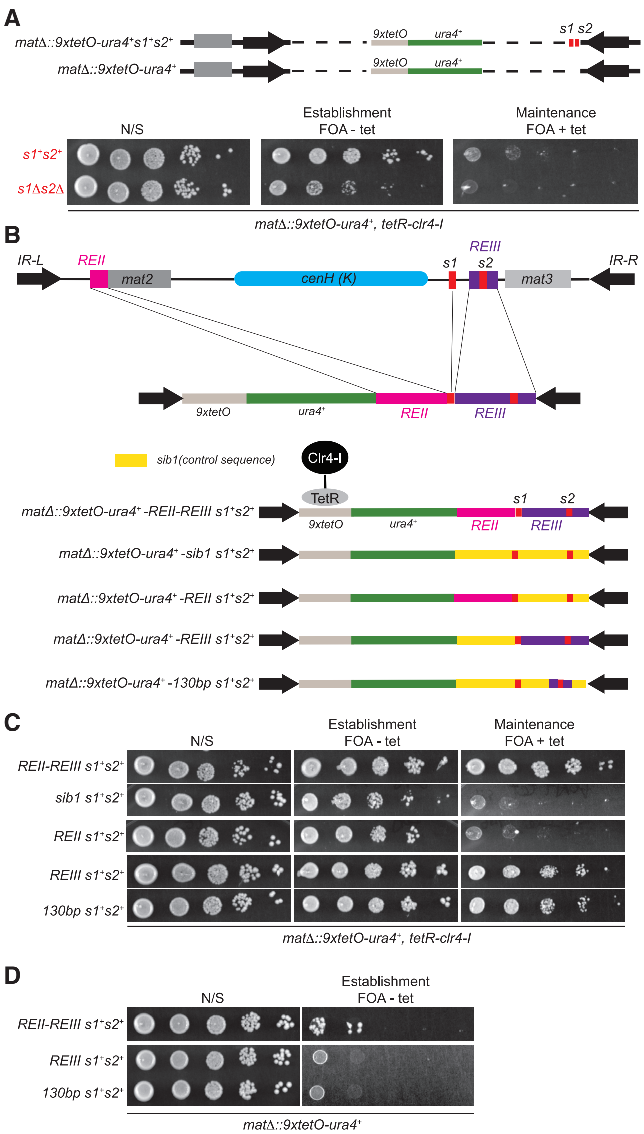

Figure 1. Identification of a minimal maintainer sequence at the *mat* locus

(A) Schematic diagram of the silent mating type (*mat*) locus engineered for testing whether the Atf1/Pcr1 binding sites are sufficient to mediate epigenetic inheritance of H3K9 methylation (top). Growth silencing assays of cells carrying the modified *mat* loci (bottom) are shown.

(B and C) Schematic diagram and silencing assay of modified *mat* loci designed to identify the minimal sequences that are required for epigenetic inheritance of gene silencing.

(D) Silencing assays showing that *REIII* and the 130-bp maintainer sequence lack heterochromatin establishment activity.

epigenetically maintained in cells lacking the Epe1 anti-silencing factor, a member of the Jumonji domain family demethylases, demonstrating that the read-write mechanism could propagate epigenetic information independently of DNA sequence (Audergon et al., 2015; Ragunathan et al., 2015). In contrast, stable *cis* epigenetic inheritance at the *mat* locus and pericentromeric DNA repeats occurs in *epe1*$^+$ cells (Ekwall et al., 1997; Grewal and Klar, 1996), suggesting a role for chromosomal context in stable epigenetic inheritance. Consistent with this expectation, we previously showed that the DNA sequence within the *mat* locus including two binding sites for the Atf1/Pcr1transcription factors is required for epigenetic inheritance of silencing and H3K9me (Wang and Moazed, 2017). However, it has remained unclear whether binding sites for Atf1/Pcr1, which also bind to and activate the transcription of many stress-induced genes across the *S. pombe* genome (Sansó et al., 2011a, 2011b), are sufficient for epigenetic inheritance of heterochromatin.

In this study, we used an inducible heterochromatin formation system that allowed us to identify native chromosomal sequences that are necessary and sufficient for epigenetic inheritance of H3K9me heterochromatin. We identify an epigenetic maintainer that is composed of closely juxtaposed binding sites for multiple factors, Atf1/Pcr1 and two additional factors. Outside of the heterochromatic *mat* locus, at numerous dispersed binding sites across the genome, each maintainer binding factor performs essential regulatory functions unrelated to heterochromatin formation, suggesting that their close juxtaposition at the *mat* locus gives rise to their ability to act as maintainers. Our findings suggest that site-specific DNA-binding factors act together with pre-existing H3K9me, via multiple weak interactions, to recruit the H3K9 methyltransferase Clr4/Suv39h, ensuring the restoration of heterochromatin following DNA replication and cell division.

RESULTS

Identification of a maintainer of epigenetic information

Previously, we showed that the two Atf1/Pcr1 binding sites *s1* and *s2* are required for epigenetic inheritance of gene silencing and H3K9me at the *mat* locus when other redundant DNA sequences, including the *cenH* and *mat2* regions, which can establish silencing by RNAi-dependent and -independent mechanisms, respectively, are deleted (Wang and Moazed, 2017). Up to 400 Atf1/Pcr1 binding sites are present throughout the *S. pombe* genome (Sansó et al., 2011a, 2011b). To test whether the Atf1/Pcr1 binding sites at the *mat* locus were sufficient to mediate epigenetic inheritance of H3K9 methylation, we generated cells with deletion of the entire region between the inverted repeat-left (*IR-L*) and inverted repeat-right (*IR-R*) boundary elements, with or without insertion of the *s1* and *s2* Atf1/Pcr1 binding sites downstream the *9xtetO-ura4*$^+$ reporter gene (Figure 1A). As expected, tethering the catalytic domain of Clr4 via fusion to tetracycline repressor (TetR-Clr4-I) established silencing at both reporter loci, indicated by growth on 5-fluoroorotic acid (FOA)-containing medium in the absence of tetracycline (Figure 1A). Release of TetR-Clr4-I by the addition of tetracycline resulted in loss of silencing, even in cells with the insertion of the *s1* and *s2* binding sites downstream of the reporter, indicating that the two Atf1/Pcr1 binding sites were not sufficient to maintain silencing at the *mat* locus.

Previous studies have shown that two DNA elements, repressor element II (*REII*) (233 bp) and repressor element III (*REIII*) (424 bp), located immediately upstream of the *mat2* and *mat3* genes, respectively, contribute to gene silencing (Ayoub et al., 2000; Hansen et al., 2011; Thon et al., 1999; Figure 1B). The *REIII* element contains the *s2* Atf1/Pcr1 binding site (Figure 1B). We hypothesized that other sequences within the *REII* and *REIII* work together with the Atf1/Pcr1 binding sites to mediate epigenetic inheritance of silencing and H3K9 methylation. To test this hypothesis, we constructed strains with the *REII*, *REIII* (including *s2*), and *s1* sequences inserted downstream of the *matΔ::9xtetO-ura4*$^+$ reporter gene and tested whether these sequences were sufficient for maintenance of silencing (Figure 1B). As shown in Figure 1C (top row), the combination of *REII*, *REIII*, and *s1* sequences allowed robust growth of the reporter gene on FOA-containing medium, even after the release of TetR-Clr4-I, indicating that the combined sequences were sufficient for maintenance of silencing when located within the boundary elements at the *mat* locus. As a control, replacement of the *REII* and *REIII* with control DNA of equal length from the open reading frame (ORF) of a euchromatin gene, *sib1*$^+$, did not support maintenance (Figures 1B and 1C, second row). These observations indicate that heritable gene silencing at the *mat* locus requires DNA sequences beyond the Atf1/Pcr1 binding sites.

To further analyze the minimal DNA sequences required for epigenetic maintenance, we constructed a series of strains with deletions of the *REII* and *REIII* elements (Figures 1B and S1). Although the combination of *REII s1* and *s2* did not support maintenance (Figure 1C, third row), the *REIII* element together with the *s1* site was sufficient to maintain silencing (Figure 1C, fourth row). We further narrowed down the maintenance function of *REIII* to a minimal 130-bp region, which together with the *s1* site (7 bp) was sufficient to maintain silencing (Figure 1C, row 5). We next deleted one or both *s1* and *s2* sites and found that, although the *s1* site, which is located outside the 130-bp element, was dispensable for maintenance, the *s2* site located within the 130-bp element was essential (Figure S1C). To test whether the full *REIII* element or the 130-bp *REIII* DNA sequence, together with the *s1* site, could also establish heterochromatin at the *mat* locus, we constructed cells with exactly the same genotype as the above-mentioned strains at the *mat* locus but lacking TetR-Clr4-I. Growth silencing assays showed that the *REII*, *REIII*, and *s1* sequences mediated *de novo* establishment of silencing at the *matΔ::9xtetO-ura4*$^+$ reporter locus at a low rate (Figure 1D, top row; ~0.05%). In contrast, *REIII* or the 130-bp minimal sequence plus the *s1* site failed to establish silencing at the reporter locus, as indicated by the absence of growth on FOA-containing medium (Figure 1D, second and third rows). Because the *REIII/s1*, the 137-bp DNA sequence (130 bp plus *s1*), and the minimal 130-bp DNA sequence were able to mediate maintenance, but not establishment, of silencing, we termed the minimal sequence a "maintainer" element.

The mating type maintainer is composed of binding sites for Atf1/Pcr1, Deb1, and origin recognition complex (ORC)

We hypothesized that the 130-bp maintainer recruits other DNA binding protein(s) that work together with Atf1/Pcr1 to maintain silencing. To identify such proteins, we first dissected the 130-bp region by further deletion analysis (Figures 2A and S1). As expected, based on previous results (Wang and Moazed, 2017; Figure S1C), deletion of the *s1* and *s2* Atf1/Pcr1 binding sites abolished epigenetic inheritance (Figure 2B). Furthermore, deletion of a 32-bp region adjacent to the *s2* site (replaced with 32-bp control *sib1* DNA) resulted in loss of epigenetic maintenance (Figure 2B) and led to decreased levels of H3K9me3 at the reporter locus (Figure 2C) after tetracycline-mediated release of TetR-Clr4-I. As a control, H3K9me3 levels at the pericentromeric *dg* regions were not affected by deletion of the 32-bp element (Figure 2D).

To identify the protein(s) that binds to this 32-bp sequence, we used an oligonucleotide pull-down approach in which 5′-biotinylated oligonucleotides bound to streptavidin beads were incubated with cell lysate and the bound proteins were identified by mass spectrometry (Figure 2E). 5′-biotinylated oligonucleotides with the wild-type (*s1*) and mutant (*s1* mutated) Atf1/Pcr1 binding sites served as positive and negative controls, respectively (Figures 2E and S2A). As expected, FLAG-Atf1 was enriched in biotin-s1, but not biotin mutant s1, or other control oligonucleotide pull-downs (Figure S2B). Mass spectrometry analysis identified the Deb1 transcription factor as the most highly enriched protein in the biotin-32-bp oligonucleotide (oligo1) pull-down (Figure 2E). Consistent with this association, chromatin immunoprecipitation (ChIP) experiments showed that FLAG-Deb1 was highly enriched at the *mat* locus and deletion of the 32-bp element abolished this enrichment (Figure 2F). Deb1 is an essential transcription factor and has been previously reported to bind to the promoter region of the *rhp51*$^+$ gene (Shim et al., 2000; Figure 3A). Based on similarity to the Deb1 binding sites at the *rhp51*$^+$ locus, we identified a consensus Deb1 binding site within the 32-bp element (Figure 3A). Mutations within this putative Deb1 binding site abolished its association with the *mat* locus and its ability to maintain silencing (Figures 3B–3E). Together, these results suggest that Deb1 is a second DNA-binding protein that is required for the maintainer function of the 130-bp element.

Further deletion of a 50-bp region in the 130-bp maintainer (50bpΔ), to the left of the Atf1/Pcr1 binding sites, leaving the Atf1/Pcr1 and Deb1 binding sites intact, led to weaker maintenance (Figures 4A, 4B, and S2C). This observation suggested that at least one or more other DNA-binding protein(s) were required for full maintainer function. To identify the potential DNA binding protein(s), we designed two overlapping 49-bp and 53-bp oligonucleotides that contained the additional sequences required for maintainer function (Figure 4C, sequences underlined with green and red lines). We used the oligonucleotide pull-down approach with the same controls described earlier for identification of Deb1. Mass spectrometry analysis of the proteins bound to these oligonucleotides showed that the top enriched protein, which was shared in the two pull-downs, was Orc4 (Figures 4D and 4E). Orc4 is a subunit of the ORC, which contains 6 subunits Orc1–Orc6 (Bell and Stillman, 1992). In *S. cerevisiae*, ORC recognizes a consensus sequence at origins of DNA replication (Bell and Dutta, 2002), but *S. pombe* Orc4, the DNA binding subunit of ORC in this organism, is thought to bind to AT-rich sequences usually spanning several hundred base pairs of DNA (Kong and DePamphilis, 2001). Consistent with these observations, the region of the 130-bp element used in the above pull-downs is highly AT rich (79%; Figure 4C). To test the importance of these AT-rich putative Orc4/ORC binding sites in maintainer function, we generated a series of AT to GC mutations within the region (Figure S2C, highlighted

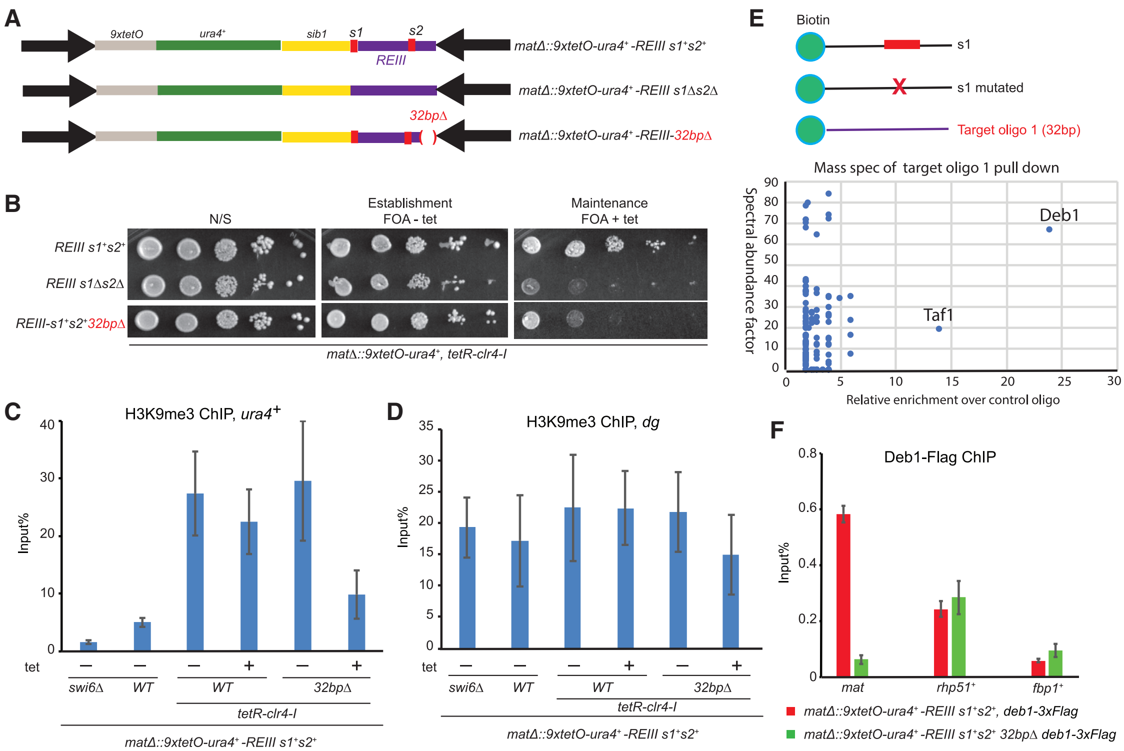

Figure 2. Identification of Deb1 as a maintainer-binding protein

(A) Schematic diagram of the intact maintenance competent *mat* locus (*matΔ::9xtetO-ura4*$^{+}$*-REIII s1*$^{+}$*s2*$^{+}$) versus *mat* loci carrying deletion of the Atf1/Orc1 binding sites (*matΔ::9xtetO-ura4*$^{+}$*-REIII s1Δs2Δ*) or deletion of a 32-bp region located downstream of the *s2* site (*matΔ::9xtetO-ura4*$^{+}$*-REIII 32bpΔ*).

(B) Growth silencing assays showing that the 32-bp deletion results in loss of maintenance. The image of silencing assay is cropped from the image shown in Figure S1B, which also contains data for additional deletions.

(C and D) ChIP experiments showing that the 32-bp deletion results in decreased level of H3K9me at the *ura4*$^{+}$ reporter gene at the *mat* locus (C), but not pericentromeric heterochromatin (D). n = 2 biological replicates. Error bars indicate mean ± standard deviation.

(E) Mass spectrometry identification of proteins enriched in the 32-bp oligonucleotide (oligo 1) pull-down normalized to the *s1* oligonucleotide pull-down. All enriched proteins (peptide count) are shown. n = 1 technical replicates.

(F) ChIP experiment showing enrichment of Deb1-3xFLAG at the *mat* locus and control genes and the effect of deleting the 32-bp sequence, required for maintenance of silencing, on the Deb1-3xFLAG enrichment. *rph51*$^{+}$ and *fpb1*$^{+}$ serve as positive and negative controls, respectively. n = 2 biological replicates. Error bars indicate mean ± standard deviation.

in yellow). As shown by growth-silencing assays, AT to GC mutations within the element phenocopied the effect of deleting the entire 50-bp AT-rich region (Figure 4F). Consistent with these results, ChIP enrichment of the Orc1 subunit of ORC (5xFLAG-Orc1) at the *mat* locus was reduced upon deletion of the *REIII* element (Figures S2E–S2G). Further, if ORC binding sites were required for maintainer function, other ORC binding sites may be expected to functionally replace the putative ORC binding site in the element. To test this possibility, we constructed cells in which the endogenous ORC binding sites in the 130-bp region were replaced by the 251-bp ARS3002 sequence, which has been shown previously to bind to ORC (Kong and DePamphilis, 2001; Figure S2D). The results showed that the ARS3002 sequence rescued the maintenance defect resulting from the deletion of the putative ORC binding site (Figure 4G), suggesting that ORC is the third DNA-binding protein complex that is required for maintainer function.

To further test whether additional DNA sequences within the 130-bp element were required for its maintainer function, we performed a deletion analysis of DNA sequence within the element that was not represented in the oligonucleotide pull-down assays (Figure S3). Replacement of sequences that were 10–20 bp or 20–30 upstream of *s2* site with random sequence had no effect on maintainer function (Figures S3A and S3B). We also replaced the DNA sequences immediately flanking the Atf1/Pcr1 *s2* site (10 bp upstream and 11 bp downstream) with the flanking region of a euchromatin Atf1/Pcr1 binding site localized at SPNCRNA.458 (Figure S3). Replacing the *s2* flanking sequences led to weaker maintenance mediated by the 130-bp element. We then tested whether the deleted sequence itself was important, for maintainer function or the alternative that the addition of euchromatic sequences perturbed maintenance. We inserted the flanking sequence of *SPNCRNA.458* Atf1/Pcr1 binding site immediately upstream and downstream of the *s2* site, without deleting any endogenous maintainer sequence. In support of the latter hypothesis, the insertion of euchromatic sequences weakened maintenance to the same extent as replacing the flanking sequence of *s2* (Figures S3A and S3B). This

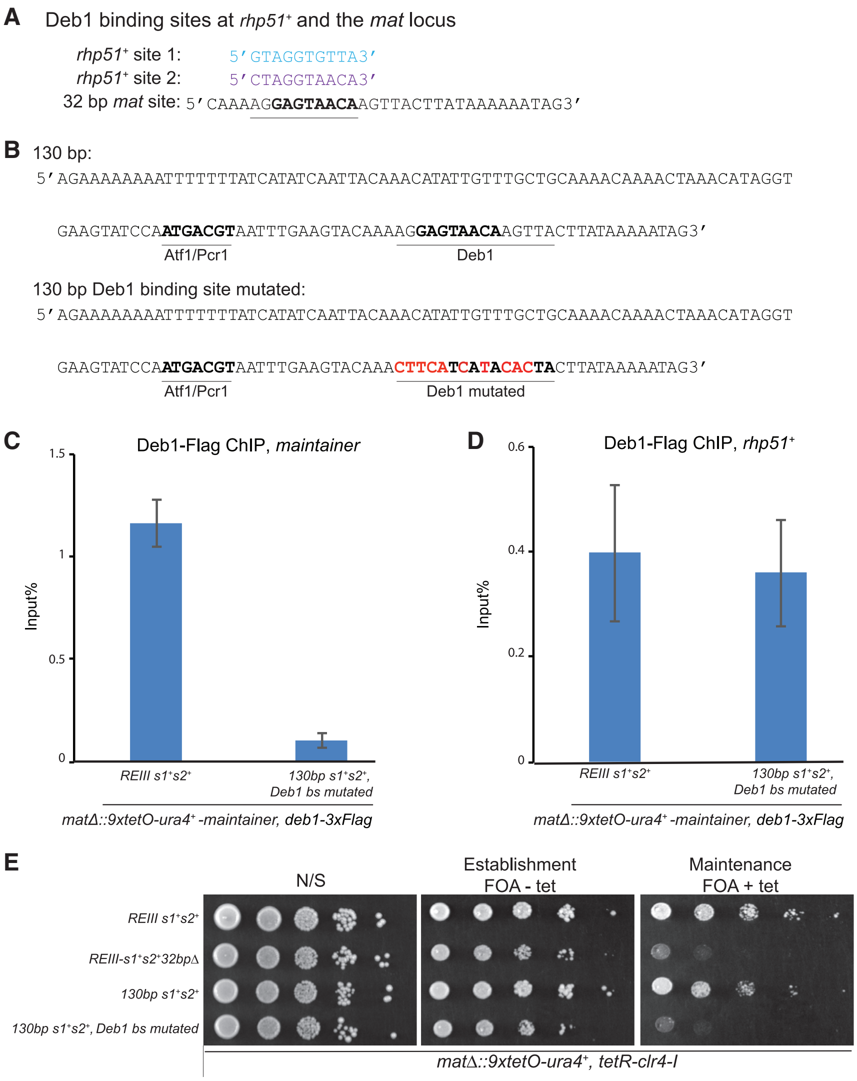

Figure 3. Mutation of the Deb1 binding site in the maintainer abolishes its ability to mediate epigenetic inheritance

(A) DNA sequence comparison of the two previously reported Deb1 binding sites (*rhp51*+ promoter) and the putative Deb1 binding site in the 32-bp DNA sequence at the *mat* locus required for maintenance. Sequences showing similarity with the putative Deb1 binding site in the maintainer are shown in bold letters.

(B) DNA sequence of the wild-type and Deb1 binding-site-mutated 130-bp region are shown. Mutated bases are highlighted in red color.

(C and D) ChIP experiments showing that mutation in the Deb1 binding site abolishes Deb1 localization to the maintainer region (C) but does not affect its binding to the promoter of its euchromatic target, *rhp51*+ (D). n = 2 biological replicates. Error bars indicate mean ± standard deviation.

(E) Silencing assays showing that deletion of the 32-bp element and mutations in the Deb1 binding site (Deb1 bs mutated) have similar loss of maintenance phenotypes.

result suggests that the sequences surrounding the *s2* site contribute to maintainer function but most likely through Atf1/Pcr1 binding rather than recruitment of another DNA-binding protein. Altogether, we analyzed all of the sequences within the 130-bp element by deletion and replacement except for 6 bp upstream and 18 bp downstream of the Deb1 binding site. Although we cannot rule out the possibility that these sequences may contribute to maintainer function by recruiting other factors, we note that they were included in the 32-bp oligonucleotide pull-down experiment (Figure 2E), which did not identify proteins other than Deb1.

The above results suggest that a functional maintainer contains binding sites for Atf1/Pcr1, Deb1, and ORC. Certain mutations in these DNA binding proteins would therefore be expected to abolish maintainer function. Cells with deletions of either Atf1 or Pcr1 grow very poorly, and Deb1, as well as every subunit of ORC, is essential for viability (Bell, 2002; Shim et al., 2000; Takeda et al., 1995). We therefore designed a strategy to isolate viable alleles in these factors that may specifically disrupt epigenetic maintenance. We constructed a strain in which the *9xtetO-ura4*+ reporter gene at the *mat* locus was replaced with a *9xtetO-ade6*+ reporter gene, followed by the *REIII* maintainer. Cells in which *9xtetO-ade6*+ is silenced form red colonies, allowing us to use an assay based on colony color to perform a genetic screen for viable mutations in the above factors that abolish maintainer function. We first modified the endogenous copy of the *atf1*+ gene to generate cells expressing a series of different Atf1 truncation, which left its DNA-binding domain intact and had wild-type growth rates (Figure S4). The results showed that a region of Atf1 located between amino acids 106 and 202 (epigenetic maintenance domain [EMD]) was required for maintainer-dependent epigenetic inheritance of silencing (Figure S4). Furthermore, using PCR mutagenesis, we obtained point mutations in Deb1 (W177R and M213V) and Orc1 (E129A) subunit of the ORC complex that abolished or reduced epigenetic maintenance but did not affect growth (Figures S5B and S5C). Interestingly, the Orc1-E129A mutation with severe maintenance defects localized to the Bromo adjacent homology (BAH) domain (Figure S5C), which is also required for the silencing activity of ORC in budding yeast (Zhang et al., 2002). Furthermore, combining the Orc1-E129A mutation with 50-bp deletion of the ORC binding site in the maintainer (130b *s1*+ *s2*+ *50bpΔ*; Figure 4F) resulted in a stronger maintenance defect for the *matΔ::9xtetO-ura4*+ reporter than either single mutation (Figure S5D). This result is consistent with the possibility that the 50-bp deletion does not completely abolish ORC binding to the maintainer because it does not remove all AT-rich sequences, although we cannot rule out the possibility that Orc1-E129A mutation has a general effect on

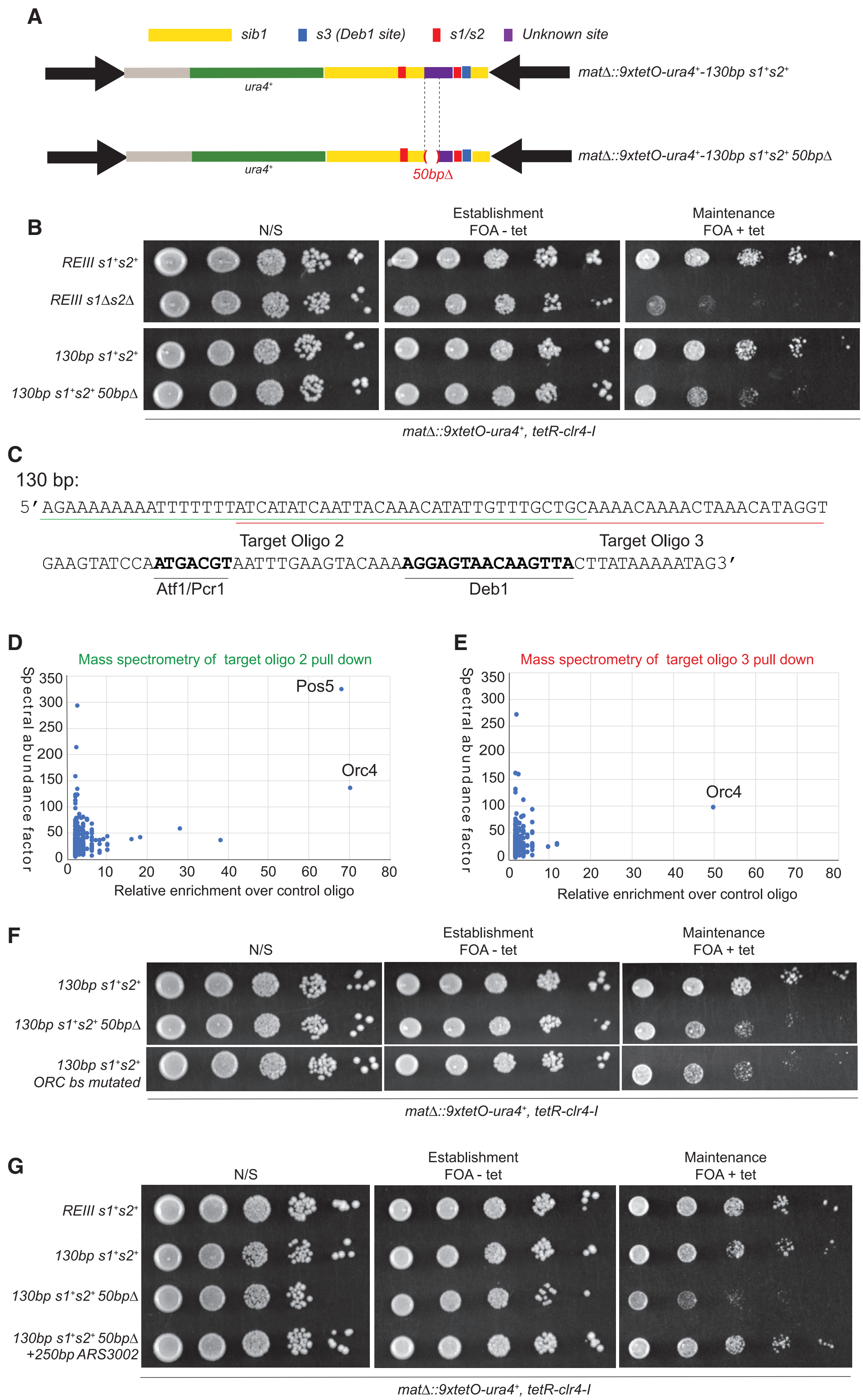

Figure 4. Identification of ORC as a maintainer-binding protein complex

(A) Schematic diagram of the mating type (*mat*) locus showing the location of a 50-bp deletion upstream of the *s2* site (*mat*Δ*::9xtetO-ura4*$^+$*-REIII s1*$^+$*s2*$^+$ versus *mat*Δ*::9xtetO-ura4*$^+$*-REIII s1*$^+$*s2*$^+$ *50bp*Δ).

(B) Growth silencing assays showing that deletion of the 50-bp sequence results in defective maintenance.

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epigenetic inheritance of silencing. Altogether, these results indicate that specific mutations in Atf1/Pcr1, Deb1, and ORC, which are located outside of their known DNA-binding domains, compromise maintainer function.

To test whether maintainer-binding proteins interact directly with components of the CRL4-like Clr4 complex (CLRC), we performed yeast two-hybrid assays. Although the full-length Deb1 fusion protein is toxic to yeast cells, we could express the N-terminal fragment of Deb1(1–200) and found that it interacted with the Raf1 and Rik1 subunits of CLRC (Figure S6). Moreover, these interactions were weakened by the Deb1-W177R mutation, which abolishes maintainer function *in vivo* (Figures S6A and S6B), suggesting that the ability of Deb1 to interact with CLRC is critical for maintainer function. We also analyzed immune-purified Deb1-FLAG using mass spectrometry and failed to detect Raf1, Rik1, or other subunits of the CLRC complex, suggesting that the interaction of isolated maintainer-binding proteins with the CLRC complex may be too weak to detect in whole-cell extracts.

Maintainer is sufficient for epigenetic inheritance

To investigate whether the epigenetic maintainer could act in a locus-independent manner, we generated cells with the minimal 130-bp element inserted at an ectopic locus. We previously showed that the *IR-L* and *IR-R* boundary sequences that flank the *mat* locus are necessary for epigenetic maintenance, but the nature of this requirement had remained unknown. Consistent with the requirement for the boundary elements in epigenetic maintenance at the native *mat* locus, the minimal maintainer alone mediated only weak maintenance of silencing when we inserted the *9xtetO-ura4*$^+$ reporter at the euchromatin *ura4*$^+$ locus, unless it was flanked by the boundary sequences (Figure 5A, compare rows 1 and 2 with rows 5 and 6). Therefore, together with the boundary elements, the 130-bp element was sufficient for epigenetic maintenance independently of chromosomal location. We next asked whether the boundary sequences were absolutely essential for maintenance or could be bypassed by increasing the copy number of the 130-bp element. We found that 2 and 3 copies of the element showed increasing maintenance activity at the ectopic locus, whether the boundary elements were present or not (Figures 5A, compare rows 3 and 4 with rows 7 and 8, and 5E). The boundary elements therefore play a potentiating, rather than essential, role in maintainer function. Moreover, the *IR-L* and *IR-R* boundary elements were fully effective in promoting epigenetic maintenance when both were inserted at the centromere-proximal side of the reporter locus, but not when inserted at the telomere-proximal side (Figure S7C), indicating that the 130-bp element did not need to be flanked by the boundary elements to be functional. At the ectopic locus, the boundary elements are likely to protect the H3K9me domain from anti-silencing effects of adjacent euchromatin, as is the case with their roles at the *mat* locus (Charlton et al., 2020; Thon et al., 2002). In the absence of the TetR-Clr4-I initiator, even at increased copy number, with or without the boundary elements, the 130-bp element showed little or no *de novo* silencing establishment activity, indicating that it could not function as a conventional silencer (Figure 5B). Consistent with the growth-silencing assays, ChIP-qPCR experiments showed that 3 copies of the element inserted upstream of the reporter locus were sufficient for maintenance of H3K9me3 72 h after tetracycline-mediated release of TetR-Clr4-I (Figure 5C). As controls, the presence of the 130-bp element and Tetr-Clr4-I at the *ura4*$^+$ locus had little or no effect on H3K9me levels at the pericentromeric *dg* repeats (Figure 5D). Together, these results demonstrate that the element, on its own, is sufficient for epigenetic inheritance of H3K9 methylation.

To gain insight into the genome-wide localization of maintainer-binding proteins and in particular their distribution at the *mat* locus, we performed a ChIP sequencing (ChIP-seq) experiment of FLAG-tagged Atf1, Pcr1, Deb1, and Orc1. At the *mat* locus, consistent with the ChIP-qPCR results and previous reports, ChIP-seq showed two peaks for each Atf1-FLAG and Pcr1-FLAG, which were lost when we deleted the Atf1/Pcr1 consensus sites (Figures 6A and S7A). Although the ChIP-seq of Atf1 and Pcr1 was performed in *cenH*Δ background, previous ChIP-qPCR experiments did not detect Atf1 or Pcr1 localization at *cenH* (Jia et al., 2004). Deb1-FLAG showed only one peak of binding, which localized at the *REIII* region (Figure 6A), consistent with its association with the 30-bp fragment of the maintainer element (Figure 2). For FLAG-Orc1, we only observed very weak enrichment at *REIII* and the boundary sequences, suggesting that ORC associates with the AT-rich region of the 130-bp element weakly (Figure 6A). In contrast, we observed stronger peaks of FLAG-Orc1 localization to known ARS regions, such as ARS2003 and ARS2004 (Figures 6B and S7B; Okuno et al., 1997). Despite this apparently weak FLAG-Orc1 localization, it has previously been reported that sequences flanking the *mat*3 gene or the boundary sequence are both sufficient to drive the replication of a plasmid in *S. pombe* (Thon et al., 1999, 2002), indicating that sequences at the *REIII* region can act as functional ORC binding sites.

At the genome-wide level, we observed numerous binding sites for each Atf1-FLAG, Pcr1-FLAG, Deb1-FLAG, and FLAG-Orc1 and at least 49 instances in which their binding sites were co-localized (Figures 6C and 6D; Table S4). Consistent with our findings that the 130-bp element cannot establish *de novo* H3K9 methylation, none of the co-localization regions were associated with H3K9 methylation (Figures 6C and 6D). Furthermore, the requirement for either oligomerization of the 130-bp element or its association with boundary elements (Figure 5), to maintain silencing, suggests that the euchromatic binding sites will lack maintainer function.

(C) DNA sequence of the 130-bp maintainer region and the oligonucleotides used for pull-down experiments. Target oligo 2 corresponds to the first 49 bp of the 50-bp deletion and is underlined with green; target oligo 3, underlined red. The binding sites for Atf1/Pcr1 and Deb1 are indicated for reference.

(D and E) Mass spectrometry identification of proteins enriched in oligo 2 (D) and oligo 3 (E) pull-downs normalized to the s1 oligo. n = 1 technical replicates.

(F) Silencing assays showing that the mutated 50-bp putative ORC binding site phenocopies deletion of the entire region. See Figure S3A for substitution in the ORC binding site.

(G) Silencing assays show that the maintenance defect resulting from deletion of the 50-bp ORC binding site could be rescued by insertion of a 251-bp euchromatic autonomously replicating sequence (ARS) sequence with known ORC recruitment activity.

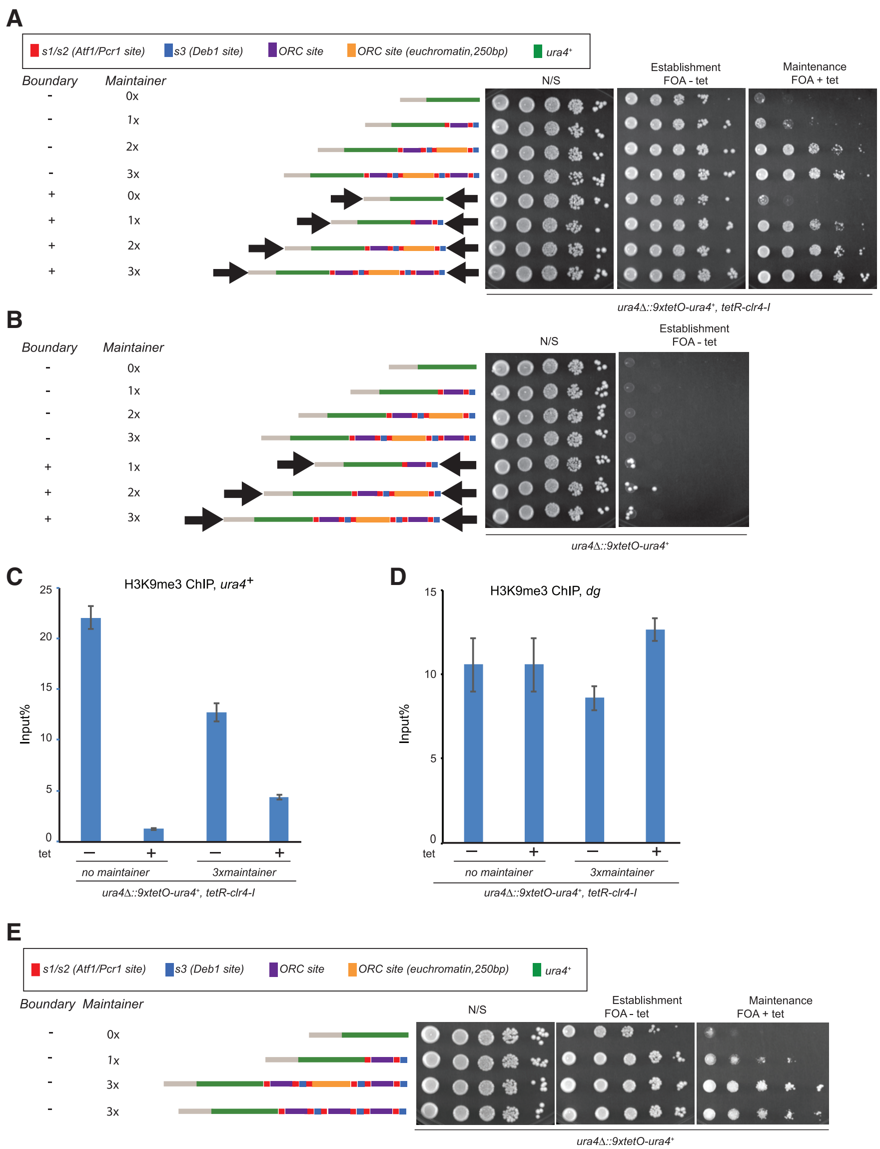

Figure 5. The role of boundary sequences and maintainer copy number in epigenetic inheritance of gene silencing

(A) Silencing assays showing that increasing the copy number of the maintainer bypasses the requirement for boundary sequences and is associated with stronger maintenance of silencing. Red, Atf1/Pcr1 binding site; blue, Deb1 binding site; purple, ORC binding site; orange, euchromatic ORC binding site (250-bp ARS3002 sequence).

(B) Silencing assays showing that increasing the copy numbers of the maintainer does not convert it into a silencer that can establish silencing.

(C) ChIP experiments showing that the 3× maintainer sequence is sufficient to mediate epigenetic inheritance of H3K9me at an ectopic locus.

(D) Control H3K9me ChIP results for pericentromeric heterochromatin for the same strains as in (C). n = 2 biological replicates. Error bars indicate mean ± standard deviation.

(E) Silencing assay showing that the 3× maintainer remained functional when one of the AT-rich ORC binding sites was replaced with a euchromatic ORC binding.

Evidence that the 130-bp element can initiate H3K9me

We next addressed the mechanism of maintainer action. Two mutually non-exclusive models can explain maintainer function (Wang and Moazed, 2017). In the first model, maintainer-binding proteins together with pre-existing H3K9me may act cooperatively to recruit Clr4 (Moazed, 2011). In the second model, the element forms a permissive chromatin or nuclear environment for the Clr4 read-write to copy pre-existing H3K9me. If the first model were correct, it may be possible to convert a maintainer from a functionally inert state into a state in which it could act as a silencer, which would only be possible if the element could recruit Clr4 *de novo*, directly or indirectly, to initiate H3K9me. In an attempt to distinguish between these possibilities, we deleted the gene encoding the anti-silencing factor *epe1*$^{+}$ because previous studies have demonstrated that Epe1 promotes H3K9 demethylation and increases the threshold Clr4 activity required to initiate ectopic

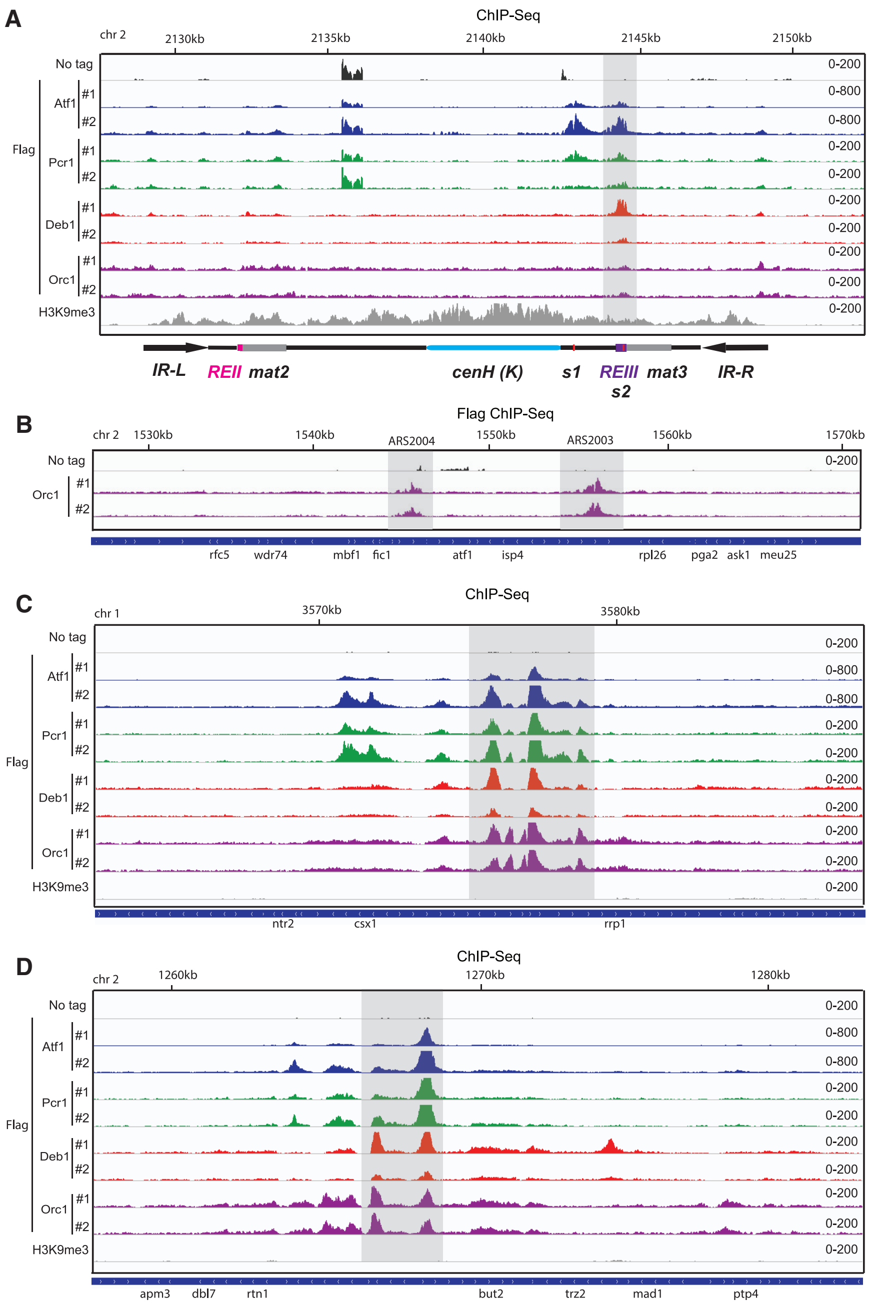

Figure 6. Genomic localization of Atf1, Pcr1, Deb1, and Orc1

(A) Atf1-FLAG, Pcr1-FLAG, Deb1-FLAG, and FLAG-Orc1 ChIP-seq reads mapped to the mating type locus. Note that the Atf1-FLAG and Pcr1-FLAG ChIP was performed in *cenH*Δ cells. H3K9me3 ChIP-seq data are from Iglesias et al. (2018). Top: chromosomal coordinates are shown. Bottom: genes are shown. Numbers of reads per million were shown on the right of the tracks.

(B) FLAG-Orc1 ChIP-seq reads mapped to known ORC binding sites ARS2003 and ARS2004.

(C and D) ChIP-seq showing examples of co-localization of Atf1, Pcr1, Deb1, and ORC at euchromatin loci.

H3K9me (Iglesias et al., 2018; Wang et al., 2015). As shown in Figure 7A, deletion of *epe1*$^+$ allowed both the *REIII* element and the 137-bp maintainer to initiate *de novo* silencing, acting as silencers. This silencing correlated with increased levels of H3K9me3 at the 130-bp element, but not a control locus, in *epe1*Δ cells (Figures 7B and 7C). Interestingly, low levels of H3K9me were present at the 130-bp region, even in *epe1*$^+$ cells, but likely at levels that were too low to promote silencing of the *ura4*$^+$ reporter gene (Figure 7B). To further test whether the element alone without the boundary sequences could be converted into a silencer, we deleted *epe1*$^+$ in 3× maintainer cells in which the maintainer and the *9xtetO-ura4*$^+$ reporter were inserted at the euchromatic *ura4*$^+$ locus. Deletion of *epe1*$^+$ resulted in only a low frequency of FOA-resistant cells (~0.0005%), suggesting weak establishment (Figure S7D). However, the resulting FOA-resistant cells could be stably propagated following replating, indicating that, once established, the silent state was stably maintained (Figure S7D). This could represent a low frequency of heterochromatin establishment or a genetic mutation in the *ura4*$^+$ gene. To distinguish between these possibilities, we isolated FOA-resistant colonies and tested whether the silencing of *ura4*$^+$ was dependent on heterochromatin proteins. Deletion of either *swi6*$^+$ or *clr4*$^+$ abolished maintainer-induced FOA resistance, indicating the observed FOA-resistant colonies resulted from maintainer-dependent establishment of silencing (Figure S7D). In support of the direct cooperativity model, these results demonstrate that the maintainer can act as a silencer when the anti-silencing factor *epe1*$^+$ is removed, indicating that it can initiate *de novo* Clr4 recruitment and H3K9 methylation.

DISCUSSION

Our findings reveal a minimal DNA element, termed maintainer, that is sufficient for epigenetic maintenance of H3K9me and gene silencing. Like silencers and enhancers, this epigenetic maintainer is a combinatorial DNA element containing binding sites for multiple site-specific DNA-binding factors, which are located within a 130-bp region. However, unlike enhancers and silencers, in wild-type cells, this maintainer element lacks *de novo* activity and cannot initiate H3K9me and silencing. Notably, deletion of *epe1*$^+$, which encodes an anti-silencing and anti-inheritance protein (Audergon et al., 2015; Bao et al., 2019; Ragunathan et al., 2015; Raiymbek et al., 2020; Trewick et al., 2007), allows the maintainer to act as a silencer, revealing its inherent ability to recruit the Clr4/Suv39h H3K9 methyltransferase. A maintainer can

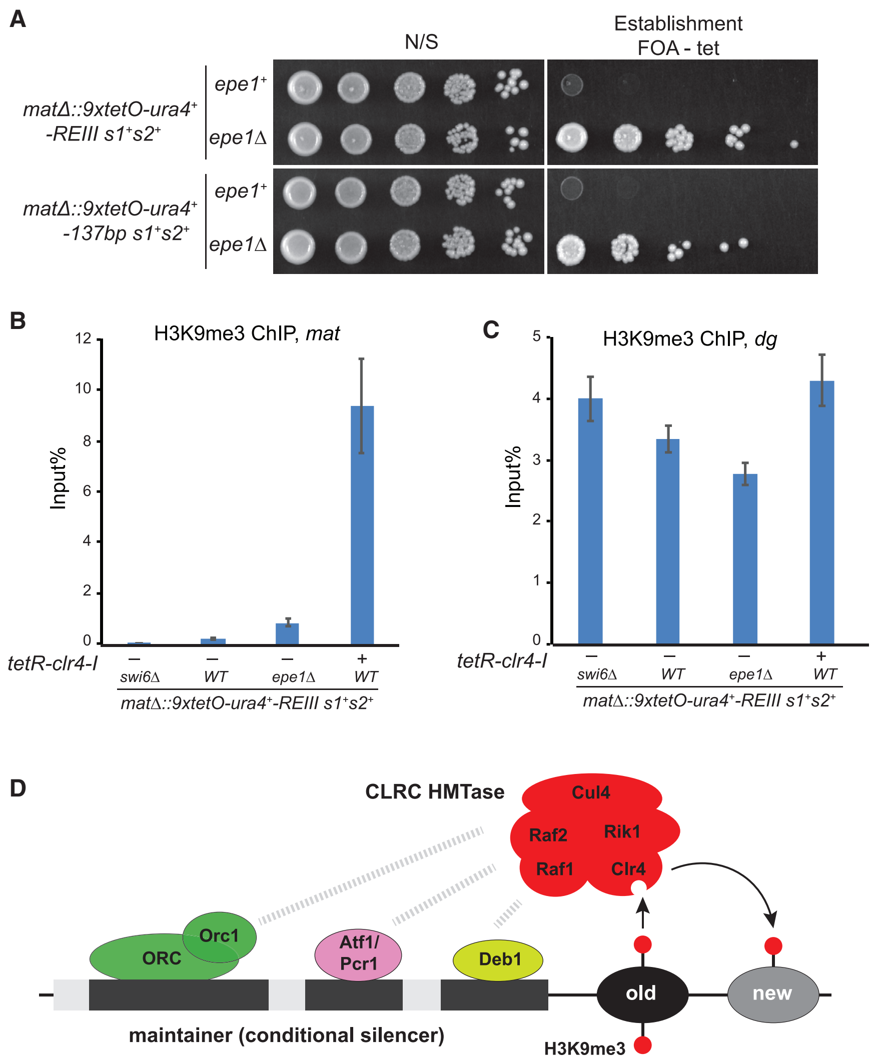

Figure 7. Deletion of Epe1 converts the maintainer to a silencer

(A) Silencing assays showing that, in *epe1Δ* cells, the maintainer can establish *de novo* gene silencing.

(B and C) ChIP experiments showing that, in *epe1Δ* cells, the maintainer can mediate *de novo* establishment of H3K9me3 at the *ura4*+ reporter gene inserted in the *mat* locus (B) without affecting H3K9me3 at the control pericentromeric heterochromatin locus (C). n = 2 biological replicates. Error bars indicate mean ± standard deviation.

(D) Schematic diagram of the minimal maintainer and model for the recruitment of the Clr4-containing CLRC complex and methylation of H3K9 on newly deposited nucleosomes (new). CLRC recruitment requires input from maintainer-binding proteins and H3K9me on pre-existing or inherited H3K9me3 (old). Maintainer-binding proteins may also participate in recruitment of other proteins that facilitate heterochromatin formation.

thus be thought of as a conditional silencer whose activity can be regulated by anti-silencing factors, such as Epe1. In the context of a wild-type mating-type locus, the maintainer may also promote the RNAi-nucleated spreading of heterochromatin and gene silencing (Greenstein et al., 2018). The maintainer defines a new type of DNA sequence that becomes functional in the context of specific histone post-translational modifications, in the case of *S. pombe* heterochromatin, methylation of histone H3K9 that is inherited following DNA replication and cell division.

A key question regarding the role of maintainer DNA sequences in epigenetic inheritance is how such sequences can help maintain pre-existing histone modifications while lacking the ability to initiate *de novo* modification events. This feature is fundamental to the ability of a maintainer to act as an epigenetic memory module whose activity is history dependent. The composite architecture of the maintainer provides a possible explanation for its H3K9me-dependent function in maintaining heterochromatin. The maintainer contains binding sites for Atf1/Pcr1, Deb1, and ORC, all of which have numerous binding sites outside of the heterochromatic *mat* locus and play important or essential roles that do not involve heterochromatin formation. The unique property of these factors in the maintainer is likely due to their close juxtaposition, which allows them to make multiple weak interactions with the Clr4 complex or other heterochromatin factors (Figure 7D). Although these weak interactions, together with input from pre-existing H3K9me, are sufficient to recruit Clr4, they cannot efficiently recruit Clr4 in the absence of pre-existing H3K9me. We note that the ORC has been identified with heterochromatin-associated functions as a maintainer-binding protein in *S. pombe* (this study), a silencer binding protein in *S. cerevisiae* (Bell et al., 1993), and an HP1-interacting protein in human cells (Prasanth et al., 2010). In mammalian cells, HP1 proteins physically associate with the Clr4 homolog, SUV39H1 (Wang et al., 2019). It will be interesting to investigate whether ORC functions in heterochromatin maintenance in mammals by providing a bridge between maintainer-like sequences and HP1/SUV39H.

Fission yeast appears to have the capability to maintain epigenetic information using multiple strategies. First, the read-write ability of the Clr4 methyltransferase can mediate DNA-sequence-independent epigenetic inheritance of H3K9me and silencing (Audergon et al., 2015; Ragunathan et al., 2015). However, this sequence-independent inheritance rapidly decays within a few cell divisions and the inheritance of silencing can be more readily observed in cells lacking the anti-silencing factor Epe1. Second, in wild-type *epe1*+ cells, a small interfering RNA (siRNA) amplification loop can be coupled to Clr4 read-write-mediated H3K9me to promote epigenetic inheritance (Yu et al., 2018). In this case, siRNAs act in a similar manner to the maintainer described here. In the absence of pre-existing H3K9me, the ability of siRNAs to initiate H3K9me at euchromatic loci is opposed by RNA processing mechanisms that promote mRNA polyadenylation and export from the nucleus (Kowalik et al., 2015; Yu et al., 2014, 2018). However, when siRNA amplification is coupled to H3K9me, the two positive feedback loops reinforce each other and maintain silencing for multiple generations. Finally, our identification of a DNA maintainer element demonstrates that specific DNA sequences can define chromosomal regions within which, once established, H3K9me can be epigenetically maintained.

Because histone modification turnover and heterochromatin properties are highly conserved, cooperation between maintainer-like elements and pre-existing modifications is likely to govern the heritability of chromatin states in other organisms. In *Drosophila*, specific DNA sequences called polycomb response elements (PREs) are continuously required for epigenetic inheritance of H3K27 methylation (Coleman and Struhl, 2017; Laprell et al., 2017). Similarly, in the budding yeast *S. cerevisiae*, silencer DNA elements are continuously required for maintenance of gene silencing (Cheng and Gartenberg, 2000; Holmes and Broach, 1996). Maintainer-based mechanisms can, in principle, act as temporal and spatial sensors that determine whether histone modifications, such as H3K9me and H3K27me, associated with developmentally regulated genes are epigenetically maintained or not. For example, in the absence of pre-existing H3K9me, deposited early during development, genes with maintainers will be expressed later in development. However, when H3K9me is deposited at maintainer-containing genes during early development, the genes will retain H3K9me and will be kept silenced for multiple cell divisions. In this way, DNA maintainers may provide a potential mechanism that determines which subsets of histone modifications, established as a result of transient spatial cues early in development, are faithfully inherited later in development.

Limitations of the study

Our findings suggest that the maintainer is a composite DNA element that can recruit the Clr4 methyltransferase to initiate H3K9 methylation on newly deposited nucleosomes only in the presence of pre-existing H3K9 methylation. We have proposed that the maintainer-binding proteins and pre-existing H3K9 methylation work together to cooperatively recruit Clr4. The demonstration of such a cooperativity-based conditional recruitment mechanism requires *in vitro* biochemical experiments using purified components. Furthermore, additional studies are required to determine whether the arrangement or distance between the binding sites in the maintainer is critical for its function. Our results also do not rule out the possibility that the mating type maintainer may recruit factors other than the Clr4 complex that may create a chromatin environment that is permissive for epigenetic inheritance of heterochromatin. Future studies are required to address this issue. Finally, our identification of the maintainer required the deletion of other DNA elements at the mating type locus that act redundantly with the *REIII* element to establish silencing. In the wild-type context, the maintainer therefore acts as a sub-element that ensures the robust establishment of silencing. Our findings identify potential routes for the evolution of maintainers from silencers and vice versa, but additional studies in organisms that use epigenetic memory to maintain distinct gene expression patterns are required to determine whether and how evolved maintainer elements participate in propagation of epigenetic states.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.molcel.2021.07.017.

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AUTHOR CONTRIBUTIONS

X.W. and D.M. conceived the project. X.W. performed all experiments except for the ChIP-seq for Deb1 and Orc1, which was performed by H.Z. and J.Y. J.A.P. performed mass spectrometry and data analysis. X.L. performed all the bioinformatics analysis. X.W. and D.M. wrote the manuscript with input from all authors. D.M. and S.P.G. supervised research.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-Flag M2	Sigma-Aldrich	Cat# F1804; RRID:AB_262044
Monoclonal recombinant H3K9me3	Diagenode	Cat# C15500003
Mouse monoclonal anti-Flag M2-Peroxidase	Sigma-Aldrich	Cat# A8592; RRID:AB_439702
Chemicals, peptides, and recombinant proteins		
Pierce Silver Stain SNAP Kit	Thermo Fisher Scientific	Cat# 24612
PMSF	Thermo Fisher Scientific	Cat# 36978
cOmplete, EDTA-free Protease Inhibitor Cocktail Tablets	Sigma-Aldrich	Cat# 05056489001
0.5mm glass beads 10 lbs bulk container	BioSpec	Cat# 11079105
Dynabeads Protein G	Invitrogen	Cat# 1004D
Dynabeads Protein A	Invitrogen	Cat# 100-02D
Formaldehyde, 37%	Thermo Fisher Scientific	Cat# F79-500
Proteinase K	Sigma-Aldrich	Cat# 03115828001
Phenol:Chloroform:Isoamyl Alcohol 25:24:1 Saturated	Sigma-Aldrich	Cat# P2069-100ML
Glycogen	Sigma-Aldrich	Cat# 10901393001
Acid-Phenol: Chloroform, pH 4.5	Thermo Fisher Scientific	Cat# AM9720
Chloroform	Thermo Fisher Scientific	Cat# BP1145-1
DNase I, RNase free	Sigma-Aldrich	Cat# 04716728001
Superscript III reverse transcriptase	Invitrogen	Cat# 18080-044
T4 DNA Polymerase	NEB	Cat# M0203L
T4 Polynucleotide kinase	NEB	Cat# M0201L
DNA Polymerase I, Large (Klenow) Fragment	NEB	Cat# M0210S
Klenow Fragment (3′/5′ exo–)	NEB	Cat# M0212L
Quick Ligase	NEB	Cat# M2200L
T5 Exonuclease	NEB	Cat #M0663
Phusion High-Fidelity DNA Polymerase	NEB	Cat #M0530S
Taq DNA Ligase	NEB	Cat #M0208S
DNA Size Selector I	Thermo Fisher Scientific	Cat# NC1148310
G418 sulfate	Thermo Fisher Scientific	Cat# 11811031
Hygromycin B	Sigma-Aldrich	Cat# 10843555001
5-Fluoroorotic acid monohydrate	Goldbio	Cat#F-230-10
Dynabeads M-280 Streptavidin	Thermo Fisher Scientific	Cat# 11206D
Dynabead MyOne Streptavidin C1	Thermo Fisher Scientific	Cat# 65001
Critical commercial assays		
QIAquick PCR Purification Kit	QIAGEN	Cat# 28106
Qubit dsDNA HS Assay Kit	Invitrogen	Cat# Q32854
NucleoSpin_ Extract	Takara/Clontech	Cat# 740609.250
MiniElute Reaction Cleanup kit	QIAGEN	Cat# 28206
SuperSignal West Pico PLUS Chemiluminescent Substrate	Thermo Fisher Scientific	Cat# 34577

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Supersignal West Femto maximum sensitivity substrate	Thermo Fisher Scientific	Cat# 34095
Matchmaker Gold Yeast Two-Hybrid System	Takara/Clontech	Cat# 630489
Deposited data		
ChIP-seq data	This study	GEO169537
Mass spectrometry	Tables S5 and S6	PXD027214
Experimental models: organisms/strains		
S. pombe strains	Table S1	N/A
Oligonucleotides		
Primers and biotinylated oligos	Table S2	N/A
Recombinant DNA		
Plasmids	Table S3	N/A
Software and algorithms		
Bowtie	N/A	RRID:SCR_005476
IGV	N/A	RRID:SCR_011793, https://www.broadinstitute.org/igv/
macs2-2.1.2	N/A	RRID:SCR_013291, https://pypi.org/project/MACS2/
BEDTools	N/A	RRID:SCR_006646
Microsoft Excel	N/A	RRID:SCR_016137, https://www.microsoft.com/en-gb/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for reagents should be directed to and will be fulfilled by the lead contact, Danesh Moazed (danesh@hms.harvard.edu).

Materials availability

The materials generated in this study would be shared without restriction.

Data and code availability

The accession number for the raw and processed high-throughput sequencing data is GEO: GSE169537. The Python script used in this study is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Yeast strains

Schizosaccharomyces pombe strains used in this study are listed in Table S1. *S.pombe* cells were grown at 32°C either in liquid YES or YEA media at 220rpm or on solid agarose plates. YEA or YES plates were used to grow and maintain *S. pombe*.

METHOD DETAILS

Plasmids

Plasmids were constructed by restriction enzyme digestion followed by Gibson ligation, as previously reported (Wang and Moazed, 2017). Briefly, potential maintainer sequences were synthesized by IDT as gene blocks, which were then assembled to SalI and PacI digested pFA6A plasmid backbone with *ura4*$^{+}$, *9xtetO* sequence and a ~350 bp *mat* sequence used for homologous recombination by Gibson ligation (Gibson et al., 2009). Plasmids with Atf11 truncations and Flag tagged Deb1 were also constructed by Gibson ligation.

Strain construction

S. pombe strains were constructed as previously described (Wang and Moazed, 2017). Briefly, all strains are either constructed by transforming a PCR based targeting DNA fragment into cells by using lithium acetate protocol or by genetic crossing. To insert the reporter gene with maintainer sequences at the *mat* locus, *swi6*$^+$ were first deleted and *cenH* was replaced with the *ade6*$^+$ gene. The *9xtetO-ura4*$^+$ reporter, with different maintainer sequences, was then introduced to replace *ade6*$^+$. *swi6*$^+$ and TetR-Clr4 were then introduced by genetic crosses. To construct strains with the boundary sequences at *ura4*$^+$ locus, the *IR-L* or *IR-R* boundary elements were first inserted into pFA6a plasmids containing hygromycin B (hph) or blasticidin (bsd), transformed into yeast cells and plated on medium containing hph or bsd to select for insertion into the *ura4*$^+$ locus. *9xtetO- ura4*$^+$- maintainer cassette were introduced by transformation following insertion of the boundary sequences. Proper insertion was further verified by colony PCR.

Silencing assays

Cell with the *ura4*$^+$ reporter gene were harvested by centrifugation after overnight growth at 32°C in yeast extract+adenine (YEA) medium (Forsburg and Rhind, 2006) followed by 5-fold serial dilution in H_2O before plating on yeast extract+supplements (YES), and YES+FOA(2ug/ml) with or without tetracycline (5μg/ml). For cells with the *ade6*$^+$ reporter gene, cells were spotted on yeast extract (YE) medium containing low adenine (Low Ade) or YE Low Ade with tetracycline (5μg/ml) for 3 days in 32°C and one day in 4°C before being photographed.

Quantitative Chromatin Immunoprecipitation (ChIP-qPCR)

ChIP experiments were performed as described previously (Wang and Moazed, 2017). For H3K9me3 ChIP experiments, cells from a 50 mL overnight (24 hours) culture at optical density (OD) of 1-2 (24hours), with or without tetracycline (5μg/ml), were crosslinked with 1% formaldehyde at room temperature for 15 minutes. Cells were quenched with 130 mM glycine for 5 minutes and pelleted before lysate preparation. For Flag ChIP, cells were first grown at 18°C for 2 hours before crosslinking with 1% formaldehyde at 18°C for 30 minutes. The following steps were performed as previously described (Egan et al., 2014). Specifically, the pellets were washed twice with 1xTBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) and then re-suspended in 600 μl lysis buffer (50 mM HEPES-KOH, pH 7.5, 500 mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% SDS, and protease inhibitors) in a 2 mL screw-cap tube. Cells were then lysed by bead beating with 1 mL acid-washed 0.5 mm glass beads with 3 cycles of 90 s at 4500 rpm and 1 cycle of 30 s at 5000rpm on a MagNA Lyser Instrument (Roche). For preparation of ChIP-Seq samples, cells were lysed by bead beating on the same instrument with 6 cycles of 30 s at 5000 rpm. Extracts were then sonicated for 3x20 s at 50% amplitude using a sonicator (Branson Digital Sonifier), centrifuged at 13000 rpm for 10 mins in a microfuge, and the supernatant was incubated with antibody. Antibodies used for ChIP experiments (per sample): 1 μg anti-H3K9me3 (Diagenode C15500003) and 4 μg anti-Flag (M2, Sigma) antibody for FLAG-tagged Atf1, Deb1 or Orc1. Immunoprecipitated DNA was then quantified by q-PCR using an Applied Biosystems 7900HT Fast Real-Time PCR system.

Sample preparation and analysis of ChIP-Seq

Library for illumine sequencing was performed as previously described following standard manufacture protocols, starting with 1-10ng immunoprecipitated DNA (Yu et al., 2018). Each library was generated by ligating with unique custom made adaptors. Libraries were pooled together and sequenced with Illumina HiSeq2500. Data analysis was also performed as previously described (Yu et al., 2018), reads mapped to repeat regions (*IR-L, IR-R*) were randomly assigned. The raw and processed reads can be accessed via accession number GSE169537. To call peaks of different DNA binding proteins, we used MACS2 and modified the parameters according to the yeast genome. Specifically, the following parameter was used to call peak: genome size:1.38e7 bp, bind width: 150bp, upper mfold: 200, lower mfold:3. To identify genomic locations that is bound by all four factors, regions with more than 40% overlap of peaks from all four proteins were calculated and are presented in Table S4.

Oligonucleotide Pull down

For each oligonucleotide (oligo) pulldown experiment, 40 μg of biotinylated double strand DNA oligos (IDT) were mixed with 200 μL Streptavidin myOne C1 magnetic beads (Thermo Fisher Scientific) in the binding buffer (5 mM Tris-HCl [pH 7.5], 0.5mM EDTA, 1 M NaCl) and incubated at room temperature for 15 minutes. Beads with immobilized DNA oligos were incubated with 3 mL of whole cell lysate (~20 mg/ml), prepared by using the following protocol. 1L cultures of cells were grown in YEA to OD600 of 1. Cell were pelleted and resuspended in lysis buffer (20 mM HEPES [pH 7.5], 100 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 10% glycerol, 0.5 mM DTT, 1 mM PMSF, and complete EDTA-free protease inhibitor cocktail [Roche)) and lysed by bead beating at 5,000 rpm for 6 × 45 s using a MagNa Lyser (Roche). Beads with DNA and cell lysates were incubated for 3 hr at 4°C. After the incubation, beads were washed 3 times with lysis buffer and incubated in a shaker with 500 mM NH_4OH at 37°C for 20 minutes to elute bound proteins, which were vacuum dried with centrifugation. For western blot analysis, dried elutes were resuspended in SDS sample buffer. 0.5% of input and 10% of bound proteins were run on a 4%–12% gradient SDS-PAGE gel, transferred to nitrocellulose membrane, and blotted with mouse anti-Flag conjugated to HRP (Sigma A8592) at 1: 5,000 dilution. 90% of the bound protein was used for mass spectrometry analysis.

Mass spectrometry

Protein was eluted from beads using 500 mM ammonium hydroxide and the eluate was dried via vacuum centrifugation. We added 20 μl of 8 M urea, 100 mM EPPS pH 8.5 to the bead. We added 5mM TCEP and incubated the mixture for 15 min at room temperature. We then added 10 mM of iodoacetamide for 15min at room temperature in the dark. We added 15 mM DTT to consume any unreacted iodoacetamide. We added 180μl of 100 mM EPPS pH 8.5. to reduce the urea concentration to < 1 M, 1 μg of trypsin, and incubated at 37°C for 6 hr. The solution was acidified with 2% formic acid and the digested peptides were desalted via StageTip, dried via vacuum centrifugation, and reconstituted in 5% acetonitrile, 5% formic acid for LC-MS/MS processing.

All label-free mass spectrometry data were collected using a Q Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA) coupled with a Famos Autosampler (LC Packings) and an Accela600 liquid chromatography (LC) pump (Thermo Fisher Scientific). Peptides were separated on a 100 μm inner diameter microcapillary column packed with ~20 cm of Accucore C18 resin (2.6 μm, 150 Å, Thermo Fisher Scientific). For each analysis, we loaded ~2 μg onto the column. Peptides were separated using a 1 hr method from 5 to 29% acetonitrile in 0.125% formic acid with a flow rate of ~300 nL/min. The scan sequence began with an Orbitrap MS^1 spectrum with the following parameters: resolution 70,000, scan range 300−1500 Th, automatic gain control (AGC) target 1×10^5, maximum injection time 250 ms, and centroid spectrum data type. We selected the top twenty precursors for MS^2 analysis which consisted of HCD high-energy collision dissociation with the following parameters: resolution 17,500, AGC 1×10^5, maximum injection time 60 ms, isolation window 2 Th, normalized collision energy (NCE) 25, and centroid spectrum data type. The underfill ratio was set at 9%, which corresponds to a 1.5×10^5 intensity threshold. In addition, unassigned and singly charged species were excluded from MS^2 analysis and dynamic exclusion was set to automatic.

Mass spectrometric data analysis

Mass spectra were processed using a Sequest-based in-house software pipeline. MS spectra were converted to mzXML using a modified version of ReAdW.exe. Database searching included all entries from the *S. pombe* uniprot database which was concatenated with a reverse database composed of all protein sequences in reversed order. Searches were performed using a 50 ppm precursor ion tolerance. Product ion tolerance was set to 0.03 Th. Carbamidomethylation of cysteine residues (+57.0215Da) were set as static modifications, while oxidation of methionine residues (+15.9949 Da) was set as a variable modification

Peptide spectral matches (PSMs) were altered to a 1% FDR (Elias and Gygi, 2007, 2010). PSM filtering was performed using a linear discriminant analysis, as described previously (Huttlin et al., 2010). Briefly, target-decoy filtering was applied to control false discovery rates, employing a linear discriminant function for peptide filtering and probabilistic scoring at the protein level (Huttlin et al., 2010). We considered the following parameters: XCorr, ΔCn, missed cleavages, peptide length, charge state, and precursor mass accuracy. As such, peptide-spectral matches were identified, quantified, and collapsed to a 1% FDR and then further collapsed to a final protein-level FDR of 1%. Furthermore, protein assembly was guided by principles of parsimony to produce the smallest set of proteins necessary to account for all observed peptides. For quantification of differences between samples, we assigned a value of 0.5 to proteins with 0 peptides.

Yeast two hybrid assay

Yeast two hybrid (Y2H) assays were performed by using the Matchmaker Gold Yeast Two-Hybrid System (Takara). Briefly, proteins of interest were cloned into vectors expressing the bait (GAL4 DNA-BD) and prey (GAL4 AD) as fusion proteins. Yeast cells expressing different bait and prey proteins were crossed and diploid cells were collected by picking colonies growing on selective medium (-Leu-Trp). Expression of the reporter genes reflected by growth in -Leu-Trp-Ade-His dropout medium indicated interaction between specific bait and prey pairs of proteins.

QUANTIFICATION AND STATISTICAL ANALYSIS

For ChIP-qPCR, statistical analysis was performed using the two-tailed, two-sample unequal variance Student's t test. Error bars show standard deviation (s.d.) and three replicates were performed for each experiment. Microsoft Excel was used to perform the statistical analysis.