PNAS RESEARCH ARTICLE | GENETICS

Specialized replication of heterochromatin domains ensures self-templated chromatin assembly and epigenetic inheritance

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Edited by Douglas Koshland, University of California, Berkeley, CA; received September 7, 2023; accepted December 21, 2023

Heterochromatin, defined by histone H3 lysine 9 methylation (H3K9me), spreads across large domains and can be epigenetically inherited in a self-propagating manner. Heterochromatin propagation depends upon a read–write mechanism, where the Clr4/Suv39h methyltransferase binds to preexisting trimethylated H3K9 (H3K9me3) and further deposits H3K9me. How the parental methylated histone template is preserved during DNA replication is not well understood. Here, we demonstrate using *Schizosaccharomyces pombe* that heterochromatic regions are specialized replication domains demarcated by their surrounding boundary elements. DNA replication throughout these domains is distinguished by an abundance of replisome components and is coordinated by Swi6/HP1. Although mutations in the replicative helicase subunit Mcm2 that affect histone binding impede the maintenance of a heterochromatin domain at an artificially targeted ectopic site, they have only a modest impact on heterochromatin propagation via the read–write mechanism at an endogenous site. Instead, our findings suggest a crucial role for the replication factor Mcl1 in retaining parental histones and promoting heterochromatin propagation via a mechanism involving the histone chaperone FACT. Engagement of FACT with heterochromatin requires boundary elements, which position the heterochromatic domain at the nuclear peripheral subdomain enriched for heterochromatin factors. Our findings highlight the importance of replisome components and boundary elements in creating a specialized environment for the retention of parental methylated histones, which facilitates epigenetic inheritance of heterochromatin.

epigenetic | heterochromatin | histone methylation | gene silencing

Significance

Heterochromatin domains defined by specific histone methylation patterns are a major feature of mammalian genomes. Epigenetic pathways ensure stable heterochromatin inheritance through cell division to prevent the expression of lineage-inappropriate genes, and defects in this process can lead to many diseases including cancer. Previous studies have shown that heterochromatin maintenance requires a critical threshold of methylated histones, but how modified parental histones are retained during DNA replication is unclear. We find that heterochromatin domains are specialized replication zones enriched in replisome components, which together with other factors help retain parental histones. This mechanism is further supported by the boundary elements surrounding the heterochromatin domains. DNA replication is thus coordinated with histone preservation to facilitate epigenetic inheritance of heterochromatin.

Gene expression patterns in eukaryotic genomes are tightly regulated to direct differentiation of cell types. To achieve this, the genome is organized into two broad chromatin categories: euchromatin, which contains actively expressed genes, and heterochromatin, which coats silenced regions (1). Euchromatin and heterochromatin domains contain specific posttranslational modifications of histones. In euchromatin, histones are acetylated and methylated on histone 3 lysine 4, while in heterochromatin, histones are generally hypoacetylated and methylated on histone 3 lysine 9 (2–4). Remarkably, heterochromatin domains can be epigenetically inherited, thereby preventing the misexpression of lineage-inappropriate genes and maintaining silencing of repetitive DNA elements (1). However, how heterochromatic structures are stably inherited during cell division is not well understood.

The fission yeast *Schizosaccharomyces pombe* is an excellent model system for studying epigenetic inheritance of heterochromatin (1). Heterochromatin coats extended chromosomal domains associated with pericentromeric repeats, subtelomeric regions, and the well-characterized silent mating-type (*mat*) region (5). These domains contain *dg* and *dh* repeat elements that serve as heterochromatin nucleation centers. Cotranscriptional processing of repeat transcripts by RNAi machinery including Argonaute (Ago1), Dicer (Dcr1), and RNA-dependent RNA polymerase (Rdp1), is linked to the recruitment of Clr4 (6–10), the sole *S. pombe* homolog of the mammalian SUV39H1 and SUV39H2 methyltransferase enzymes (11, 12). As a subunit of the ClrC complex (13), Clr4 methylates H3K9 ("write") to nucleate heterochromatin (11), but also binds H3K9me3 via its chromodomain ("read") (10). Read–write activity allows Clr4/Suv39h to bind preexisting H3K9me3 and further deposit H3K9me, promoting the spreading of heterochromatin (10, 14). At the silent *mat* interval, H3K9me is initially targeted to the *cenH* element sharing homology to *dg/dh* repeats and a nearby site (7, 15, 16), and then spreads across a 20-kb domain surrounded by inverted repeat (*IR*) boundary elements (2). H3K9me allows chromatin association of HP1 proteins (11, 17–19). Besides binding to H3K9me, HP1 proteins engage in low-affinity multivalent interactions (20, 21) to form high-concentration territories, providing an increased interface to concentrate various effector proteins (1), including the histone deacetylase (HDAC) Clr3, a component of the Snf2/HDAC repressor complex (SHREC) (22, 23).

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2315596121/-/DCSupplemental.

Published January 29, 2024.

Remarkably, heterochromatin can be faithfully passed to daughter cells during cell division. Heterochromatin inheritance occurs in cis via a self-templating mechanism (7, 24), and H3K9me3 plays a crucial role as an epigenetic carrier (10, 25). A critical density of H3K9me3 is required to maintain a high local concentration of Clr4/Suv39h on chromatin to support read–write activity (25). Various strategies maintain H3K9me3 levels at different genomic loci. Pericentromeric regions depend on RNAi, targeting multiple copies of *dg/dh* repeats, to maintain H3K9me3, while the silent *mat* region containing a single copy of *dg/dh* element utilizes a distinctive mechanism (1). At *mat*, high levels of the Clr3 HDAC recruited by HP1-dependent and -independent mechanisms safeguard H3K9me3 by preventing nucleosome turnover (26, 27). Robust chromatin association of Clr3, or the absence of the anti-silencing factor Epe1, results in the stabilization of methylated nucleosomes and allows epigenetic inheritance of heterochromatin via H3K9me3 at endogenous and ectopic loci (7, 27–29). Additionally, the RNA processing Rix1 containing complex RIXC, which binds Swi6/HP1 with high affinity and tethers *mat* to the nuclear periphery, facilitates heterochromatin propagation (30).

How H3K9me3 histones are transmitted to sister chromatids during DNA replication, which involves the unraveling of parental nucleosomes, requires further exploration. DNA replication initiates at replication origins distributed across the genome (31). The origin recognition complex (ORC) bound to origins, along with the licensing factors Cdt1 and Cdc6/Cdc18, loads the minichromosome maintenance (MCM) complex to assemble the prereplicative complex (pre-RC) in the G1 phase of the cell cycle (32). Subsequently, origins are activated by the conserved cyclin-dependent kinase (CDK) and Dfp4-dependent kinase (DDK), which are required for the recruitment of Cdc45 and the GINS (Go-Ichi-Ni-San) complex in S phase. This process leads to activation of the MCM helicase, unwinding of parental DNA, and assembly of DNA polymerases for synthesis of the leading and lagging strands (33). Interestingly, replisome components are believed to direct the transfer of parental histones onto daughter DNA strands during DNA replication (34, 35). How these factors coordinate the replication of silenced domains with their role in transmission of parental histones, required for self-propagation of heterochromatin, remains to be explored.

Here, we investigated the requirement for replication factors in heterochromatin propagation in *S. pombe*. We find that heterochromatic domains, such as those at the silent *mat* and pericentromeric regions, are specialized replication zones delimited by boundary elements. Replisome components are preferentially enriched across these zones and Swi6/HP1, a factor previously implicated in the epigenetic inheritance of heterochromatin (7, 36), coordinates replication activation across extended domains. Our analyses identified a crucial role for the replication factor Mcl1 in heterochromatin propagation via a mechanism involving the histone chaperone FACT. Boundary elements facilitate loading of FACT at *mat* by positioning it at the nuclear periphery. Together with our analysis of the histone chaperone function of the MCM complex subunit, Mcm2, these findings underscore the critical requirement of replication proteins in retaining parental methylated histones to transmit epigenetic memory for heterochromatin propagation.

Results

Genetic Screen for Factors Affecting Heterochromatin Propagation. Previous studies of the archetypical mating-type heterochromatin domain comprising the *mat2P* and *mat3M* loci provided important insights into the establishment and maintenance of heterochromatic structures. Genetic screens using silencing reporter systems identified many conserved heterochromatin factors (30, 37–40). We screened for additional factors that affect heterochromatin propagation by exploiting the fact that silencing at *mat* is enforced by two distinct mechanisms. In addition to heterochromatin-mediated silencing, *REII* and *REIII* elements recruit HDACs to locally silence *mat2P* and *mat3M* loci, respectively (27). When the *REII* element is deleted, *mat2P* silencing becomes solely dependent on the spreading of heterochromatin, creating a sensitized region. In nonswitching *mat1M* (*mat1M-smt0*) cells lacking *REII*, defects in heterochromatin propagation cause *mat2P* expression and aberrant sporulation (haploid meiosis) caused by coexpression of both *M* and *P* mating-type information in haploid cells (37). Cells undergoing haploid meiosis stain a dark color upon exposure to iodine vapor, whereas wild-type (WT) colonies stain yellow. A *ura4*$^+$ reporter inserted close to *mat2P* (*mat2P::ura4*$^+$) provides an additional readout for heterochromatic silencing.

Following UV mutagenesis of the reporter strain carrying *REIIΔ* and *mat2P::ura4*$^+$, colonies were screened for haploid meiosis and *mat2P::ura4*$^+$ expression. Dark-stained colonies that grew on medium lacking uracil were backcrossed to a nonmutagenized parental strain (Fig. 1*A*). Whole genome sequencing of mutant and WT segregants revealed mutations in known heterochromatin factors, such as the ClrC components Clr4 and Raf1 (13), and the SHREC components Clr3 and Mit1 (22) (*SI Appendix*, Fig. S1 *A* and *B*). Additionally, mutations in the chromatin remodeler Fft3, implicated in heterochromatin propagation (40, 41), factors linked to DNA replication, including the replisome component Mcl1 (an ortholog of *S. cerevisiae* Ctf4 and mammalian AND1) and the Mediator of Replication Checkpoint Mrc1, were identified (Fig. 1*A* and *SI Appendix*, Fig. S1 *A* and *B*) (42–46).

Although Mrc1 and Mcl1 were found in previous screens (30, 39, 47–49), their exact function was unknown. The mutants showed a variable increase in haploid meiosis and *mat2P::ura4*$^+$ derepression, as indicated by growth on medium lacking uracil (–Ura) and counterselective medium containing 5-Fluoroorotic Acid (FOA) (*SI Appendix*, Fig. S1*A*). The *mrc1*^{R558stop} mutant, which lacks the conserved Mrc1-like domain, and *mrc1Δ* showed only mild silencing defects (*SI Appendix*, Fig. S1 *A* and *C*) (47). By contrast, *mcl1-2* cells bearing a single nucleotide insertion at position 618 showed a more severe silencing defect, and we therefore focused on the role of Mcl1 in heterochromatin assembly.

The slow-growth phenotype of *mcl1-2* limited our analyses. Fortuitously, cells expressing Mcl1 tagged at the carboxy terminus with the HA or FLAG epitope (referred to as *mcl1-3* and *mcl1-4*, respectively) showed a severe silencing defect without affecting growth (Fig. 1*B*). By contrast, Mcl1 tagged with GFP (Mcl1-GFP) did not affect silencing or H3K9me3 levels, indicating that it remained functional (*SI Appendix*, Fig. S1*D*). Chromatin Immunoprecipitation and quantitative PCR (ChIP-qPCR) analyses of H3K9me3 in *mcl1-3* and *mcl1-4* showed a reduction near the *mat2P* locus and no major effect at the *cenH* nucleation site (Fig. 1*C*). Similar changes were observed in the slow-growing *mcl1-2* (Fig. 1*C*). Thus, targeting of heterochromatin to the nucleation site occurs independently of Mcl1, which instead affects the spreading and/or maintenance of heterochromatin.

Mcl1 Associates with the Replisome and the Histone Chaperone FACT. To explore the role of Mcl1 in heterochromatin assembly, we performed mass spectrometry analysis of immuno-affinity purified Mcl1-GFP (Fig. 1 *D* and *E* and *SI Appendix*, Fig. S1*E*). Several replisome factors, including the MCM and GINS holo-complexes, as well as Cdc45, DNA polymerase alpha (Polα), replication factor C (RFC), and RPA components were identified (Fig. 1*E* and *SI Appendix*, Fig. S1*E*). Thus, Mcl1 is a component of the active

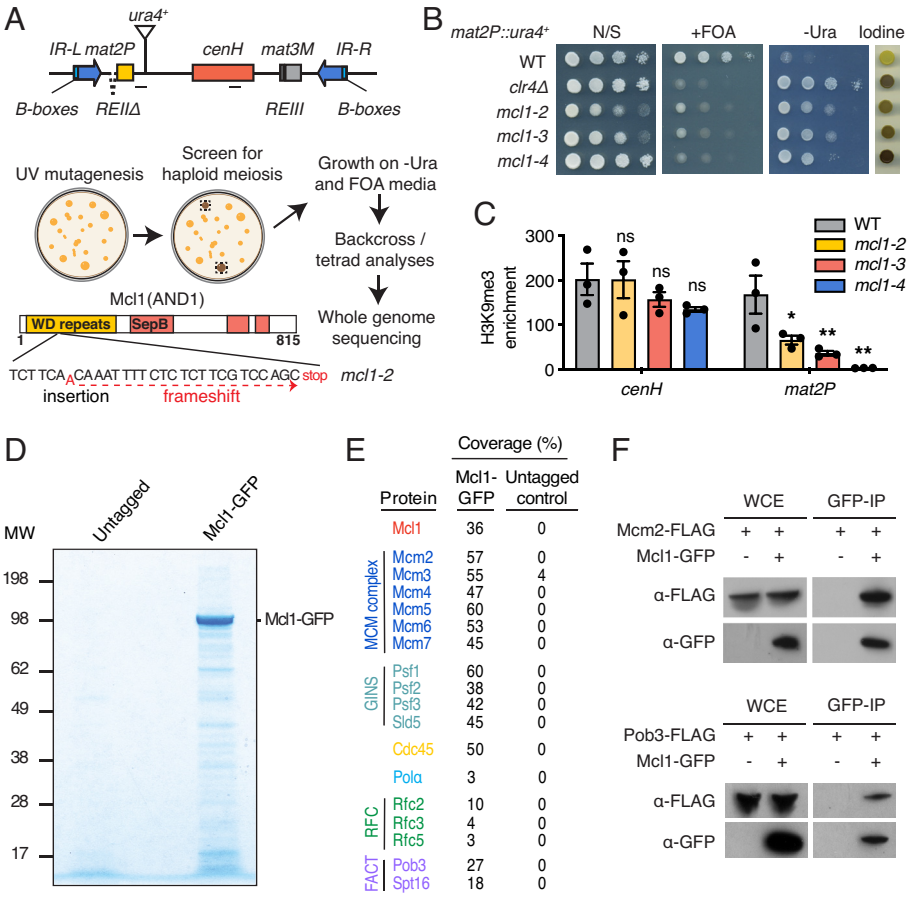

Fig. 1. The replisome component Mcl1 affects heterochromatin assembly. (*A*) Schematic representation of the sensitized mating-type locus (*REIIΔ mat2P::ura4*$^{+}$) reporter used for the genetic screen (*Top*). Step-by-step illustration of the UV mutagenesis screen (*Bottom*). The *mcl1-2* mutant contains a single nucleotide insertion in the *mcl1* gene. (*B*) Ten-fold serial dilutions of the indicated strains were plated on nonselective (N/S) medium, medium lacking uracil (−Ura), or FOA-containing medium (+FOA) to assess derepression of the *ura4*$^{+}$ reporter. Haploid meiosis was assessed by iodine staining. (*C*) H3K9me3 fold enrichment determined by ChIP-qPCR analysis at *cenH* and *mat2P* loci (mean ± SEM; *n* = 3). The location of the oligos used is indicated in (*A*). (*D*) Strains expressing untagged Mcl1 or Mcl1-GFP were subjected to immuno-affinity purification and analyzed by SDS-PAGE. (*E*) The total peptide coverage (%) of the identified Mcl1-interacting proteins was determined by mass spectrometry. (*F*) Co-IP of Mcm2-FLAG or the FACT subunit Pob3-FLAG with Mcl1-GFP. See also *SI Appendix*, Fig. S1.

replisome in *S. pombe*, as in other systems (43, 44, 46). Components of the SCF (Skp1-Cul1-F box) E3 ubiquitin ligase and proteasome components potentially involved in controlling Mcl1 protein levels were also present (*SI Appendix*, Fig. S1*E*) (50). Intriguingly, the Spt16 and Pob3 subunits of FACT (Fig. 1*E*), which is implicated in the propagation of heterochromatin domains (30, 51–54), also copurified. Coimmunoprecipitation (co-IP) experiments confirmed the association of Mcl1 with Mcm2 and FACT (Fig. 1*F*).

Heterochromatin Domains Are Specialized Replication Zones. To assess whether Mcl1 plays a direct role in heterochromatin assembly, we blocked cells carrying the *cdc25-22* temperature-sensitive allele at the G2/M phase boundary, then released them synchronously into S phase, indicated by the presence of cell septa (Fig. 2*A*). The distributions of Mcl1 and the Mcm2 replisome component were determined alongside the incorporation of 5-bromo-2-deoxyuridine (BrdU) by ChIP-sequencing (ChIP-seq). Mcl1 localized to heterochromatic loci in S phase, like Mcm2 (Fig. 2*B*). Moreover, its localization was observed concurrently with BrdU incorporation, suggesting that Mcl1 is involved in DNA replication of these domains. Significantly, the prominent enrichment pattern of Mcl1, Mcm2, and BrdU incorporation across heterochromatin domains at the silent *mat* region and pericentromeric repeats was delimited by their surrounding boundary elements (Fig. 2 *B* and *C*). This distribution contrasted with euchromatin regions, where Mcm2 appeared as distinct peaks corresponding to replication origins around which BrdU was incorporated (*SI Appendix*, Fig. S2*A*).

Swi6/HP1 controls early replication at specific sites within the *mat* and centromeric loci by targeting DDK (55). To determine whether Swi6/HP1 coordinates replication throughout these heterochromatin domains, we mapped Mcm2 and BrdU in cells lacking Swi6/HP1 during S phase (Fig. 2 *D* and *E* and *SI Appendix*, Figs. S2 *A–D* and S3). Mcm2 mapping was performed 60 min after release from the *cdc25-22* block. A similar septation index for WT and *swi6Δ* cells indicated normal progression of *swi6Δ* cells through S phase (*SI Appendix*, Fig. S2*B*). For BrdU, ChIP analysis was performed 60, 70, and 80 min after release in the presence of HU, which blocks cells in early S phase. Indeed, BrdU was absent from the late replicating subtelomeric regions at the time points tested (55) (*SI Appendix*, Fig. S2*C*). Compared to WT, *swi6Δ* cells showed a considerable decrease in BrdU incorporation across *mat*, which persisted through the time course (Fig. 2*D* and *SI Appendix*, Fig. S3). Mcm2 was still detected, although with more defined peaks, likely reflecting licensed, but not activated, replication origins across the domain (Fig. 2*D*). The Mcm2 peaks in *swi6Δ* coincided with sites of preferential enrichment of the ORC subunit Orc1 in cells arrested in G1 phase (Fig. 2*D*). BrdU incorporation was also affected at centromeres (Fig. 2*E*). Mcm2 was still detectable in this case as well (Fig. 2*E*). At subtelomeric regions, despite the absence of BrdU incorporation, which is consistent with their late replication timing (55), ORC and Mcm2 were again heavily

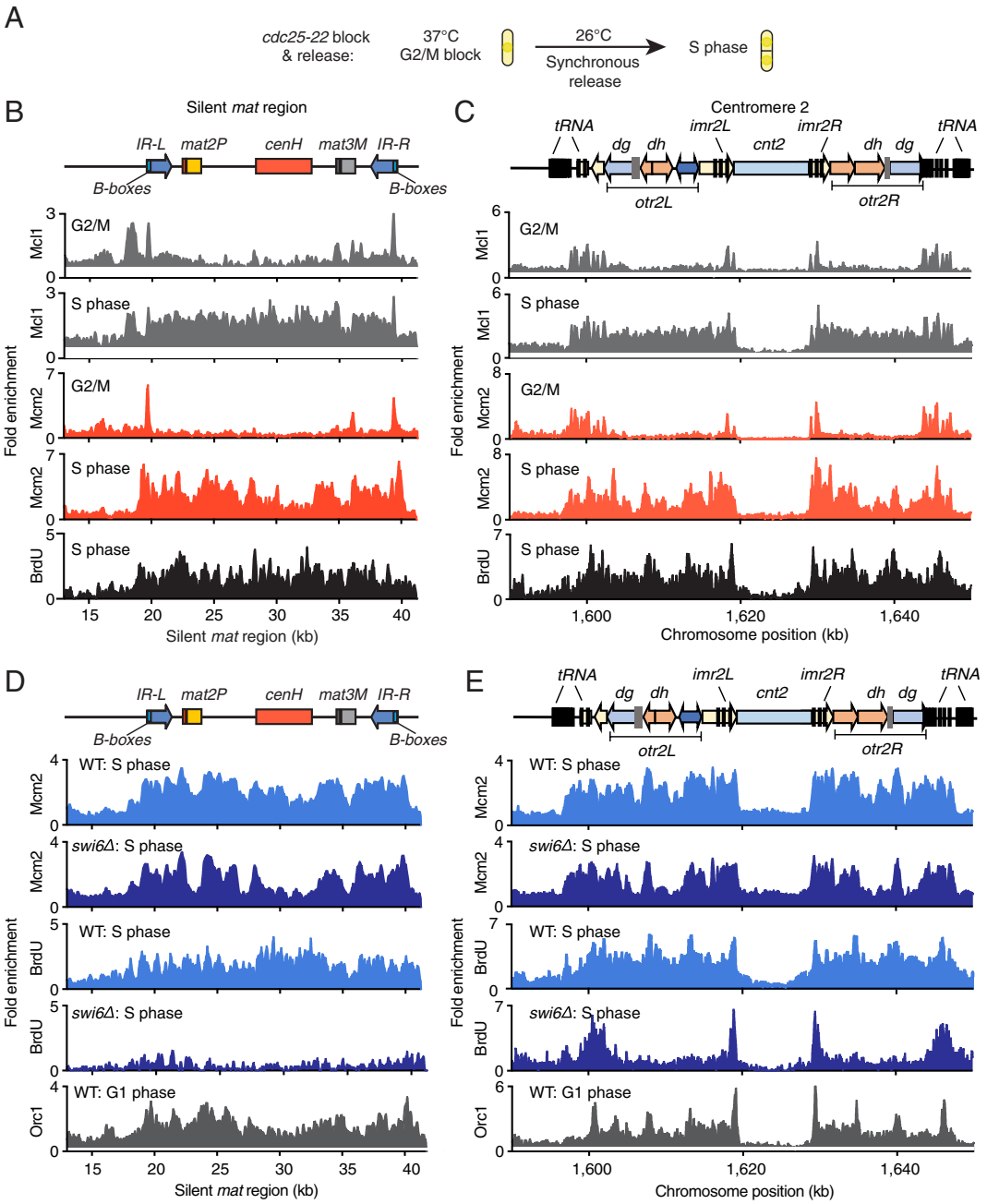

Fig. 2. Replisome components coat heterochromatin domains. (*A*) Illustration of the cell cycle synchronization process using the *cdc25-22* temperature-sensitive mutant. Cells were arrested at the G2/M boundary and then synchronously released into the cell cycle. The septation index was used to follow cell cycle progression. (*B* and *C*) Fold enrichments of Mcl1 and Mcm2 at *mat* (*B*) and centromere 2 (*C*) during G2 and S phases determined by ChIP-seq. S phase entry was confirmed by the incorporation of the thymidine analog BrdU. (*D* and *E*) Fold enrichments of Mcm2 and BrdU in WT and *swi6Δ* cells at *mat* (*D*) and centromere 2 (*E*) during the S phase determined by ChIP-seq. ChIP-seq analysis of Orc1 in WT cells upon *cdc10-v50* block at G1 is also presented. See also *SI Appendix,* Figs. S2 and S3.

enriched, confirming this is as a common feature among heterochromatin domains regardless of their replication timing (*SI Appendix*, Fig. S2*C*). Of note, *swi6Δ* had no apparent effect on replication in other parts of the genome, indicating a specific role for Swi6/HP1 in the replication of heterochromatin domains (*SI Appendix*, Figs. S2*A* and S2*D*).

Collectively, our results suggest that heterochromatic regions are specialized replication zones in which Swi6/HP1 coordinates replication of the entire domain.

Boundary Elements Are Not Necessary for Replication in Heterochromatin Propagation. The boundary elements *IR-L* and *IR-R* surrounding the silent *mat* region are suggested to have replication origin activity, and their deletion affects heterochromatin propagation (56–59). We therefore wondered whether *IRs* affect heterochromatin propagation by impacting the replication of this domain.

We used CRISPR to simultaneously delete sequences in both *IR-R* and *IR-L* and then looked for effects on heterochromatin propagation using the sensitized *REIIΔ mat2P::ura4*$^{+}$ reporter. We targeted regions containing *B*-boxes, which are critical for their function (56, 57). *IR-Δ1* removed a T-rich region and an adjacent DNAase I hypersensitive site, *IR-Δ2* deleted *B*-boxes 3 to 5 and a DNAase I hypersensitive site, and *IR-Δ3* eliminated *B*-boxes 1 and 2 (Fig. 3*A*). While *IR-Δ1* and *IR-Δ3* had minor effects, *IR-Δ2* showed a variegated pattern of derepression, indicated by dark iodine staining from

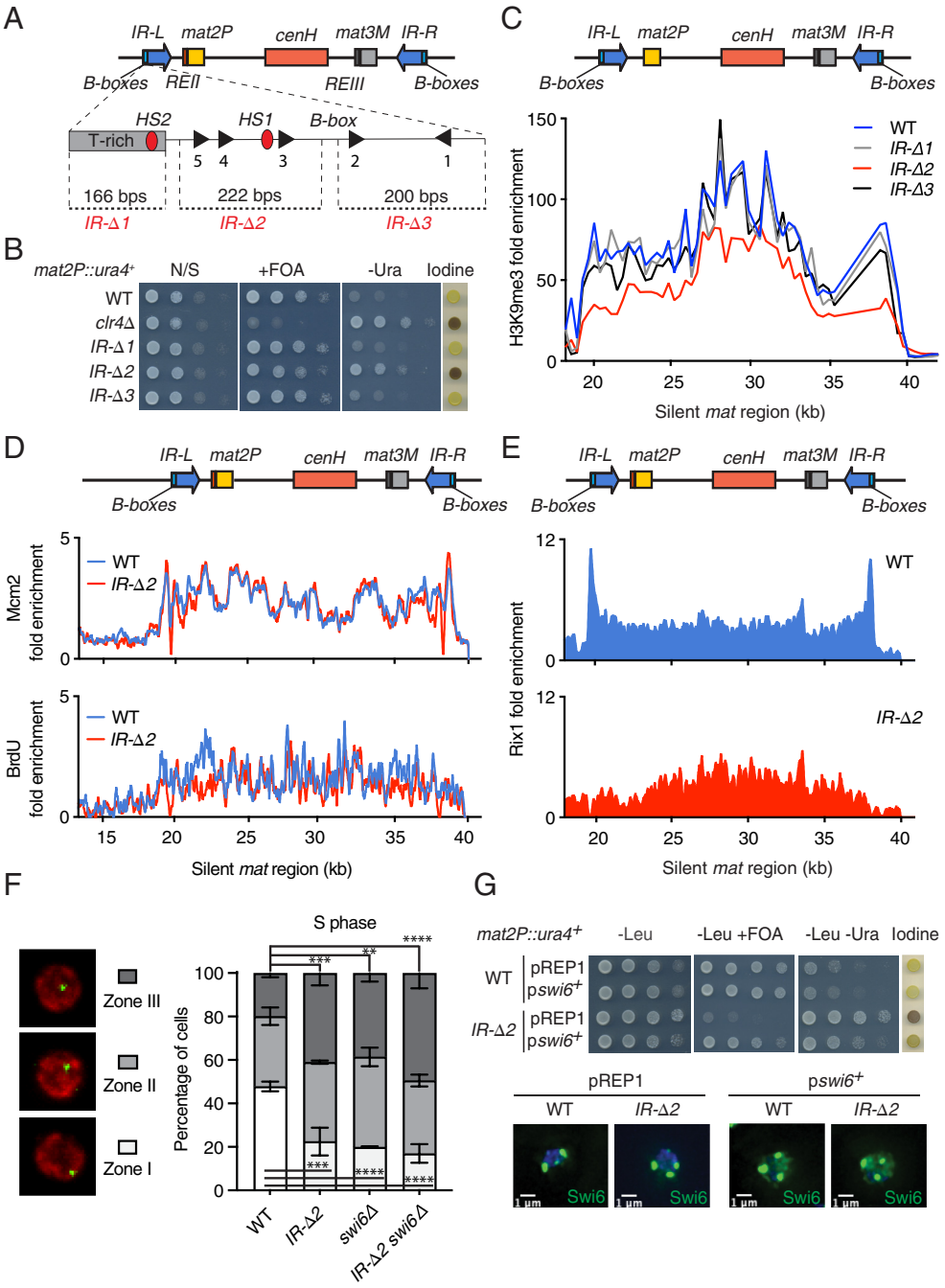

Fig. 3. Boundary sequences required for heterochromatin propagation are dispensable for replisome recruitment. (*A*) Schematic showing deletion constructs *IR-Δ1*, *IR-Δ2,* and *IR-Δ3*. (*B*) Ten-fold serial dilutions were plated on the indicated media to assess derepression of *ura4*$^+$. (*C*) H3K9me3 fold enrichments determined by ChIP-chip. (*D*) Mcm2 and BrdU fold enrichments at *mat* determined by ChIP-seq in cells synchronized in the S phase. (*E*) Fold enrichment of Rix1 determined by ChIP-seq. (*F*) Representative images showing localization of *mat* in three distinct nuclear zones (*Left*). *IR-R>lacO*/LacI-GFP marks *mat*. Nup40-mCherry marks the nuclear envelope. The percentage of cells in each zone is plotted for WT (*n* = 183), *IR-Δ2* (*n* = 181), *swi6Δ* (*n* = 192), and *IR-Δ2 swi6Δ* (*n* = 167). *n* represents the combined number of cells from two independent experiments. Pairwise comparisons between the WT and each mutant were performed using two-way ANOVA (mean ± SEM). *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. (*G*) Ten-fold serial dilutions were plated to assess derepression of *ura4*$^+$ in WT or *IR-Δ2* cells carrying an empty vector (pREP1) or a plasmid containing *swi6*$^+$. Representative immunofluorescence images against Swi6 are shown (*Bottom*). See also *SI Appendix,* Fig. S4.

elevated levels of haploid meiosis and growth analysis on –Ura and FOA medium (Fig. 3*B*). Consistently, defects in H3K9me3 spreading from the nucleation site to surrounding sequences were observed in *IR-Δ2*, but not *IR-Δ1 or IR-Δ3* (Fig. 3*C*).

We then tested whether silencing defects are related to defects in replication across the silent *mat* region and found that *IR-Δ2* showed neither major changes in Mcm2 localization nor significant changes in BrdU incorporation across the domain (Fig. 3*D*). Therefore, the heterochromatin propagation function of the boundary elements is not linked to dramatic changes in the replication program of the *mat* region, which most likely utilizes origins within the domain to replicate.

IR boundaries recruit the RNA processing complex RIXC, which also binds Swi6/HP1 with high affinity, and tethers the *mat* region to a nuclear peripheral subdomain enriched for silencing proteins (30). This facilitates the loading of FACT, which prevents the turnover of histones to maintain heterochromatin (30, 53). Unlike *IR-Δ1* and *IR-Δ3, IR-Δ2* showed severe disruption of Rix1

localization at *IR*s (Fig. 3*E* and *SI Appendix*, Fig. S4*A*), although Rix1 was still detected internally at the silent *mat* domain where Swi6/HP1 loads RIXC (Fig. 3*E*) (30). *IR-Δ2* cells presented delocalization of *mat,* marked with *lacO/lacI-GFP,* from the periphery during S phase (Fig. 3*F*). Loss of Swi6/HP1 that removes RIXC from the silent *mat* interval further affected its peripheral localization (Fig. 3*F*). Swi6 overexpression, which causes a pan-nuclear localization, rescued the silencing defects in *IR-Δ2* cells resulting from delocalization of *mat* from the periphery (Fig. 3*G*). Thus, boundaries are important for the peripheral localization of the *mat* domain during S phase.

We asked whether delocalization of *mat* from the periphery in *IR-Δ2* cells is linked to a decrease in FACT loading. During S phase in *IR-Δ2* cells, the Pob3 subunit of FACT was significantly reduced, while the anti-silencing factor Epe1 was increased at the silent *mat* domain (*SI Appendix*, Fig. S4 *B* and *C*). Loss of FACT and elevated levels of Epe1 can lead to increased histone turnover (26, 30, 53), ultimately affecting the H3K9me3 density required to support Clr4/Suv39h read–write activity (25). Cells carrying *IR-Δ2* alone or in combination with a deletion of the HDAC *clr3* showed increased histone H3 turnover compared to WT (*SI Appendix*, Fig. S4 *D–F*). Additionally, the heterochromatin defects in *IR-Δ2* could be rescued by expressing the chimeric $Clr4^{Chp1CD}$ protein (25), in which the Clr4 chromodomain (CD) is replaced by the Chp1 CD, which restores a high local concentration of Clr4 through the high-affinity binding of Chp1 CD to H3K9me2 (*SI Appendix*, Fig. S4*G*).

These results show that the function of boundaries in heterochromatin propagation is closely associated with RIXC association and its downstream effects, such as the loading of FACT, which is required to maintain methylated histones and support read–write.

Mcm2 Affects Heterochromatic Silencing through Histone Binding. Mcm2 is believed to transfer parental histones to daughter strands during DNA replication in other species (60–63). To determine whether Mcm2 also contributes to heterochromatic silencing *in S. pombe*, we used an error-prone PCR technique to generate *mcm2* mutants affecting *mat* silencing using the *REIIΔ mat2P::ura4*$^+$ reporter (Fig. 4*A*). We isolated 3 mutants: *mcm2-1, mcm2-2,* and *mcm2-6*. Interestingly, the mutations in *mcm2-1* (D87G and D112G) and *mcm2-2* (D79V and D112G) correspond to conserved residues that are required for the H3-H4 histone binding activity of the human MCM2 (62) (Fig. 4*B* and *SI Appendix*, Fig. S5*A*), while the third mutant *(mcm2-6)* contains a mutation (S218F) near the MCM box (*SI Appendix*, Fig. S5*B*). The *mcm2-6* mutant showed sensitivity to hydroxyurea (HU) (*SI Appendix*, Fig. S5*C*), indicating defective replication, and was not further analyzed. The other two mutants showed defective silencing of the *mat2P::ura4*$^+$ reporter and haploid meiosis (Fig. 4*C*), as well as detectable levels of the *mat2P* transcript (Fig. 4*D*).

To determine whether WT and mutant Mcm2 bind to histones H3 and H4, we purified the histone binding domains (HBD). Both H3 and H4 histones copurified with the WT HBD, but H3 did not copurify with either the *mcm2-1* or *mcm2-2* mutant HBDs (Fig. 4 *E* and *F*). Furthermore, the *mcm2-1* HBD was severely compromised in its ability to bind histone H4 (Fig. 4 *E* and *F*). Notably, the addition of the WT Mcm2 HBD during the purification of the H3 and H4 histones promoted their solubilization, highlighting its role as a histone chaperone (*SI Appendix*, Fig. S5*D*). In vivo co-IP experiments confirmed defective binding of H3 in the *mcm2-1* and *mcm2-2* mutants (Fig. 4*G*). These analyses demonstrate that Mcm2 binds histone H3 and H4 via its HBD and that mutations that affect this interaction cause silencing defects.

***mcl1* and *mcm2* Mutants Show Progressive Loss of Heterochromatic Silencing.** Mutations in replisome components might cause progressive loss of heterochromatic silencing as cells undergo successive replication cycles. To assess this, we employed a fluorescent reporter system consisting of a *YFP* gene inserted within the *cenH* nucleation element (*Kint2::YFP*) and a *mCherry* gene inserted near *mat3M* (*mat3M::mCherry*) (56) (Fig. 5*A*). This system allows simultaneous monitoring of silencing at *cenH* and a site distal to the nucleation center within the *mat* region at the single cell level, and quantitative fluorescence imaging of the cassettes determines derepression at each position.

We monitored the silencing of *Kint2::*YFP and *mat3M::mCherry* reporter genes in WT*, mcm2-1,* and *mcl1-4* cells that were allowed to divide through 6 generations. Similar doubling times for both WT and mutant cells ruled out any impact from different generation times between the strains (*SI Appendix*, Fig. S6*A*). In WT cells both cassettes remained repressed through six cell divisions. However, *mcm2-1* and *mcl1-4* showed derepression of *mat3M::mCherry* beginning at generation 2 to 3, and the number of mCherry positive cells increased with cell divisions (Fig. 5 *B* and *C*). Interestingly, *mcl1-4* caused derepression of *mat3M::mCherry* in a wider cell population compared to *mcm2-1* (Fig. 5*C* and *SI Appendix*, Fig. S6*B*). Quantification showed that successive cell divisions led to cells with higher mCherry intensities, reflecting increasingly severe silencing defects (Fig. 5*D*).

Heterochromatin Propagation at an Ectopic Site Requires Mcl1 and Mcm2. We next tested whether Mcl1 and Mcm2 are required for maintenance of heterochromatin at an ectopic site. We inducibly expressed Clr4/Suv39h fused to a $TetR^{off}$ DNA-binding domain (TetR-Clr4) in cells harboring an *ade6*$^+$ reporter located downstream of six tetracycline operators (*6xtetO-ade6*$^+$) (28, 30). The strains also expressed Clr3 HDAC fused to two chromodomains (Clr3-CDx2) (27). TetR-Clr4 recruitment to *tetO* causes accumulation of H3K9me3 and silencing of the *ade6*$^+$ reporter. Clr3-CDx2 binds to methylated nucleosomes with high affinity, leading to histone deacetylation and stabilization of nucleosomes by preventing their turnover, supporting stable propagation of heterochromatin (27). Indeed, upon release of TetR-Clr4 in cells expressing Clr3-CDx2, the read–write activity of endogenous Clr4/Suv39h maintains heterochromatin for multiple generations (27) (*SI Appendix*, Fig. S7). Tethering of TetR-Clr4 established heterochromatic silencing of *ade6*$^+$ in *mcm2-1* and *mcl1-4* mutant cells, as indicated by the red coloration of colonies, and H3K9me3 levels were comparable to WT (*SI Appendix*, Fig. S7). However, following the release of TetR-Clr4, *mcm2-1* and *mcl1-4* cells failed to maintain heterochromatin, as indicated by white colony color and loss of the H3K9me3 mark (*SI Appendix*, Fig. S7). Thus, both Mcl1 and Mcm2 are required for propagation of heterochromatin at an ectopic site.

Mcl1 Is More Critical Than Mcm2 for the Retention of Parental Histones. Defects in heterochromatin propagation in *mcl1* and *mcm2* mutant cells led us to explore their involvement in the retention of parental histones in cycling cells. To test whether the "dilution" of parental histones coupled to DNA replication occurs more readily in *mcl1* or *mcm2* mutant cells, we employed an assay that directly measures the retention of histones at heterochromatic loci in cycling cells. Flag-tagged histone H3 (H3-FLAG) under the control of the inducible promoter *inv1* allows the rapid switch between H3-FLAG expression or repression simply by using sucrose or glucose as a carbon source for growth, respectively (64). Cells grown overnight in sucrose-containing medium to express FLAG-tagged H3 were shifted to glucose-containing medium to turn off H3-FLAG expression (*SI Appendix*, Fig. S8*A*). Time

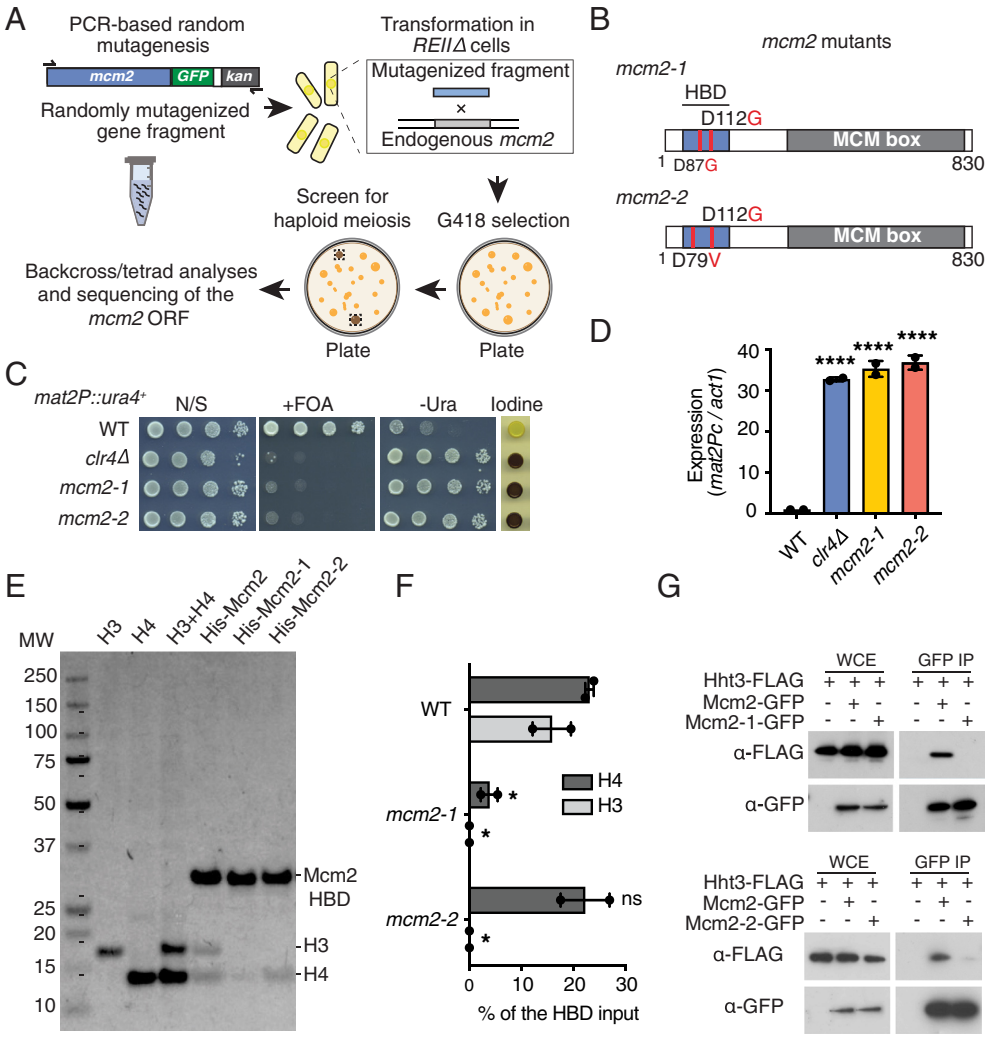

Fig. 4. Mutations in the histone binding domain (HBD) of Mcm2 cause silencing defects. (*A*) The random mutagenesis screen used to isolate *mcm2* mutants defective in heterochromatic silencing. (*B*) The *mcm2-1* and *mcm2-2* mutants isolated from the screen. The blue solid box represents the HBD of Mcm2. The identified amino acid substitutions are indicated by red lines. (*C*) Ten-fold serial dilutions were spotted on nonselective, −Ura or +FOA medium to assess derepression of *ura4*$^{+}$. (*D*) RT-qPCR analysis of *mat2P* expression (mean ± SD; *n* = 2). (*E* and *F*) In vitro histone binding assay using cells expressing the WT or mutated HBD of Mcm2. The ability of WT or mutant HBDs to bind H3/H4 histones was assessed by affinity chromatography (*E*). The fraction of H3/H4 histones pulled down is plotted as the percentage of the HBD input signal in each lane (*F*). (*G*) Co-IP analysis of histone H3 with Mcm2 in WT and *mcm2-1* cells (*Top*) or *mcm2-2* cells (*Bottom*). See also *SI Appendix*, Fig. *S5*.

course analysis of H3-FLAG dilution at the silent *mat* region showed more rapid loss in *mcm2-1* and *mcl1-3* compared to WT, and *mcl1* mutant cells lost H3-FLAG considerably faster than the *mcm2-1* mutant cells, defective in binding to histones (Fig. 5*E*). Thus, although both mutants affect the retention of parental histones, Mcl1 plays a more critical role in the process.

Mcl1, but not Mcm2, Is Vital for Endogenous Heterochromatin Propagation. We next assessed propagation of an endogenous heterochromatin domain in *mcl1* and *mcm2* mutants. Heterochromatin at the silent *mat* region, initially established by an RNAi-dependent pathway, can propagate for multiple generations in the absence of RNAi via the Clr4/Suv39h read–write mechanism (7, 10). This process requires factors such as Clr3 HDAC and FACT, which maintain high H3K9me3 density (26, 30, 53). When Clr3 and FACT are mutated, heterochromatin can be nucleated at *cenH* via RNAi, but it cannot spread due to failure to preserve H3K9me3.

ChIP-seq analysis of H3K9me3 distribution was performed in *mcl1-4* and *mcm2-1* single mutants, and in double mutants that also contained *ago1Δ*, which disrupts heterochromatin nucleation at *cenH* (7). In *mcl1-4*, H3K9me3 was detected at the *cenH* nucleation site but failed to spread (Fig. 5*F*), similar to mutants defective in heterochromatin propagation. Moreover, unlike *ago1Δ*, which can maintain heterochromatin at *mat* via the read–write mechanism (7, 10), *mcl1-4 ago1Δ* showed complete loss of H3K9me3 throughout *mat* (Fig. 5*F*). Cells expressing *mcm2-1* defective in binding to histone H3 showed only a moderate reduction in H3K9me3 across *mat* (Fig. 5*F*), suggesting that heterochromatin propagation can occur, albeit slightly less efficiently. Further supporting this argument, a substantial level of H3K9me3 was retained throughout the silent *mat* interval in *mcm2-1 ago1Δ* cells (Fig. 5*F*). The retention of H3K9me3 required the read–write activity of Clr4/Suv39h. A mutation in Clr4 chromodomain (Clr4^{W31G}), which disrupts its binding to methylated H3K9 (10), abolished H3K9me3 in *mcm2-1 ago1Δ* cells (Fig. 5*F*). Based on these results, the read–write mechanism can still maintain H3K9me3 across an endogenous heterochromatin domain in cells with defects in the histone chaperone function of Mcm2. On the other hand, Mcl1 is vital for this process.

We also investigated the role of Mcl1 and Mcm2 at pericentromeric repeats, where factors such as Clr3 HDAC, critical for read–write heterochromatin propagation, are not enriched (27). Pericentromeric regions contain multiple copies of *dg/dh* repeats

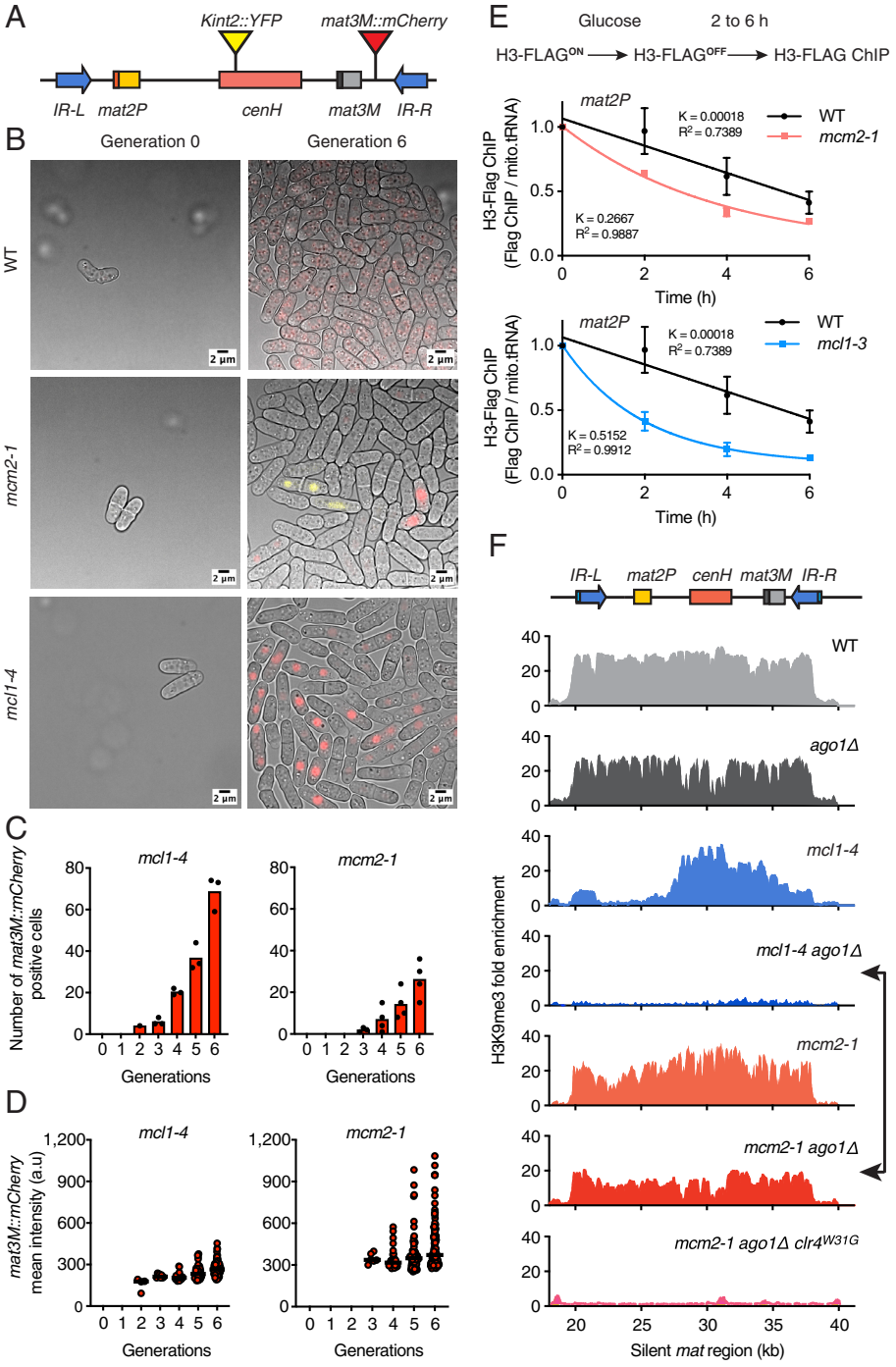

Fig. 5. Effects of mutations in Mcl1 and Mcm2 on retention of parental histones and heterochromatin propagation. (*A*) Schematic of the reporter cassettes used to assay silencing by time-lapse microscopy. *YFP* is embedded in the *cenH* (*Kint2::YFP*), while *mCherry* is embedded close to the distal *mat3M* gene (*mat3M::mCherry*). (*B*) Representative images from the time-lapse analysis of WT, *mcm2-1,* and *mcl1-4* for generations 0 and 6. (*C*) The numbers of *mcm2-1* and *mcl1-4* cells expressing mCherry per generation are plotted. The numbers of cells from independent fields followed using time-lapse microscopy are presented for each case (*mcm2-1 n* = 4; *mcl1-4 n* = 3). (*D*) Mean intensities of mCherry positive *mcm2-1* and *mcl1-4* cells per generation. Cumulative numbers are plotted. mCherry and YFP expression were absent in WT cells in all fields tested. (*E*) Schematic representation of the histone retention assay (*Top*). H3-FLAG enrichment at *mat2P* relative to mitochondrial *tRNA* was quantified by qPCR at the indicated time points after H3-FLAG suppression for *mcm2-1 and mcl1-4* (mean ± SEM; *n* = 2). (*F*) Fold enrichment of H3K9me3 at the silent *mat* interval was determined by ChIP-seq. See also *SI Appendix,* Figs. S6–S8.

and rely on persistent targeting of Clr4/Suv39h via RNAi to maintain H3K9me3 (9). Neither *mcl1-4* nor *mcm2-1* showed major changes in H3K9me3 at centromeres, except when combined with *ago1Δ* (*SI Appendix*, Fig. S8*B*).

Mcl1 Promotes FACT Association with the Replisome for Heterochromatin Propagation. The above results prompted us to investigate how Mcl1 contributes to the retention of parental histones. A critical clue was provided by our finding that Mcl1 copurifies with both subunits of FACT (Fig. 1 *E* and *F*). Furthermore, FACT associates with the replisome in *S. cerevisiae,* although how this happens remains to be fully investigated (60, 65). We asked whether heterochromatin maintenance defects in *mcl1-4* and *mcm2-1* mutants are related to defective association of FACT with the replisome. FACT associates with subunits of MCM (Mcm2) and GINS (Psf3) when Mcl1 is present, but these interactions

are lost in cells lacking Mcl1 (Fig. 6*A*), suggesting that Mcl1 is required for the association between FACT and the replisome. Importantly, FACT was able to associate with the replisome, albeit to a lesser extent, in cells carrying the mutation in the histone binding domain of Mcm2 (Fig. 6*A*).

To explore the functional relationship between replisome components and FACT in heterochromatin propagation, we performed ChIP-seq analysis of H3K9me3 distribution in single and double mutants. Compared to WT cells, the *mcl1-4* and *pob3Δ* single mutants showed loss of H3K9me3 at regions distal to the *cenH* nucleation center within the silent *mat* interval. Interestingly, the *mcl1-4 pob3Δ* double mutant did not show an additive effect on H3K9me3 levels (Fig. 6*B*). On the other hand, *mcm2-1 pob3Δ* showed a cumulative loss of H3K9me3, including at the *cenH* nucleation site (Fig. 6*C*), suggesting that Mcm2 and FACT might have additional roles beyond their shared function. Moreover, the single and double mutants showed no major changes in H3K9me3 at pericentromeric repeats where RNAi machinery targets Clr4/Suv39h to maintain H3K9me3 (*SI Appendix*, Fig. S9 *A* and *B*).

Collectively, our work reveals that replisome components such as Mcl1 and Mcm2 are preferentially enriched across specialized replication zones at heterochromatic regions and help retain parental methylated histones to support heterochromatin propagation via a mechanism involving the histone chaperone FACT.

Discussion

Stable heterochromatin propagation preserves cell type-specific gene expression patterns and silences repetitive DNA elements. We showed previously that heterochromatin is inherited via the read–write mechanism in a self-templating manner, whereby H3K9me3 locally concentrates Clr4/Suv39h on chromatin to further deposit H3K9me and promote heterochromatin propagation (10, 25). Understanding how methylated histones are transferred to daughter DNA strands during replication is of fundamental importance. Here, we describe the involvement of replication machinery in the retention of parental histones, which transmit epigenetic memory to support heterochromatin propagation (Fig. 7).

We find that in *S. pombe*, major heterochromatin domains are replicated as specialized zones. These regions are heavily coated with replication factors, such as ORC and MCM, distinguishing them from the rest of the genome. Moreover, we show that Swi6/HP1 coordinates replication throughout heterochromatin domains such as the silent *mat* region and pericentromeric repeats, in addition to its previously described role in early replication by recruiting DDK (55). Cells lacking Swi6/HP1 exhibit defects in replication initiation specifically within heterochromatin. In *swi6Δ* cells, Mcm2 protein is observed in multiple peaks across heterochromatin

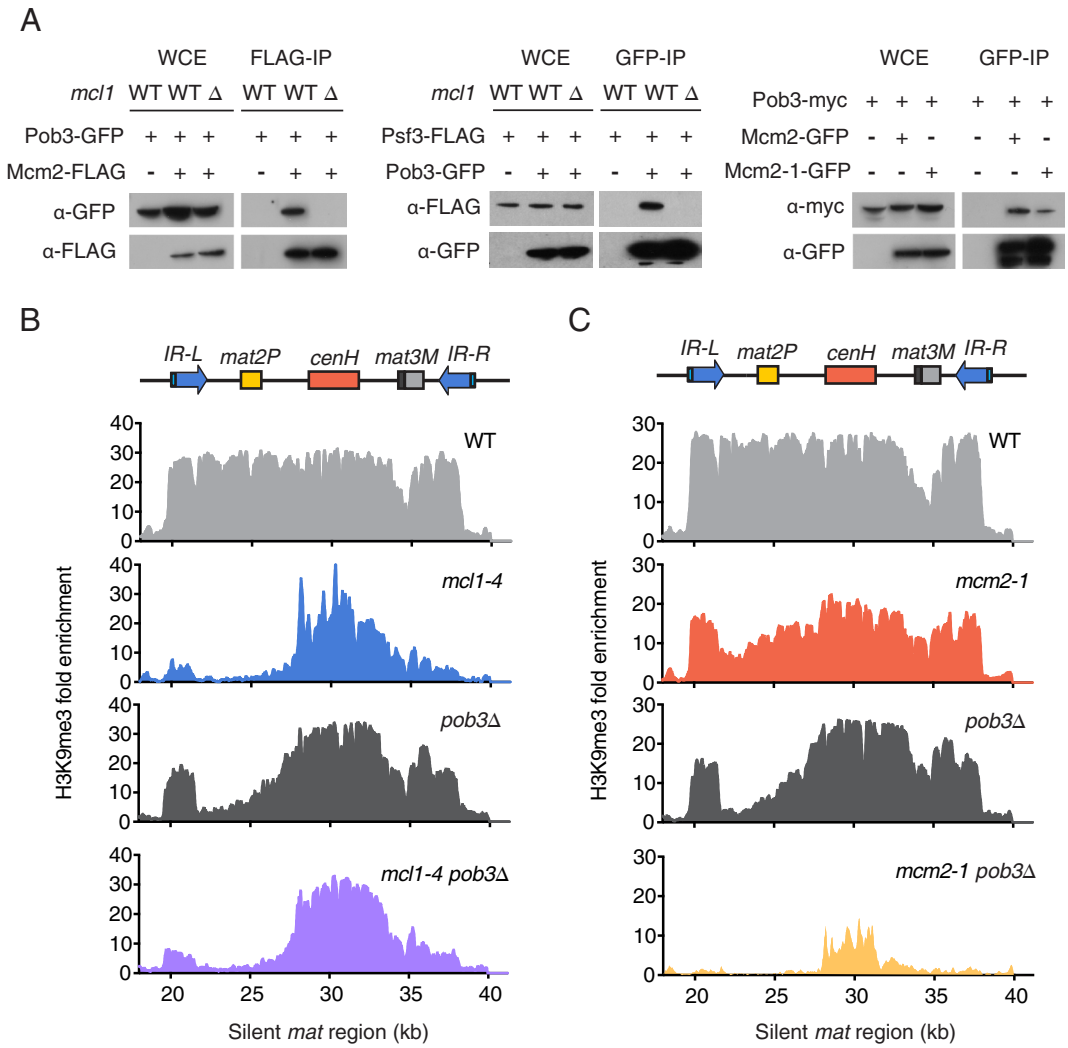

Fig. 6. Mcl1 affects heterochromatin propagation by controlling the association of FACT with the replisome. (*A*) Co-IP analyses of FACT (Pob3) and MCM (Mcm2) or GINS (Psf3) in the presence or absence of Mcl1 (*Left* and *Middle*). Co-IP analysis of FACT and WT or mutated Mcm2 (*mcm2-1*) (*Right*). (*B* and *C*) Fold enrichment of H3K9me3 at *mat* was determined by ChIP-seq. See also *SI Appendix,* Fig. S9.

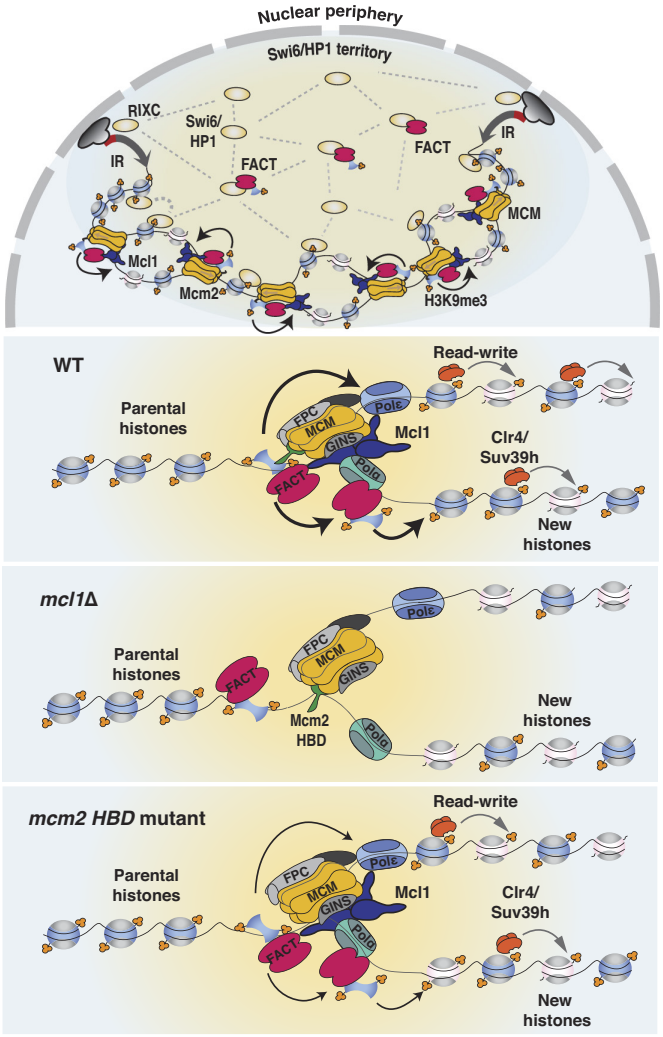

Fig. 7. Model showing specialized replication of the silent *mat* region and its role in heterochromatin propagation. (*Top*) Anchoring of *mat* at the nuclear periphery via RIXC and the boundary elements spatially constrains *mat* at a peripheral subdomain enriched for the heterochromatin protein Swi6/HP1. During S phase, Swi6/HP1 promotes the efficient activation of origins, leading to the enrichment of replisome components, which together with FACT help retain parental histones during DNA replication. The ability of Swi6/HP1 to engage in low-affinity interactions (straight dotted lines) and to form dimers (curved dotted lines) creates a high-concentration microenvironment that might help retain FACT and released parental histones within the vicinity of the replication fork to augment histone recycling during replication. (*Bottom*) Replication-coupled histone transfer in WT, *mcl1* and *mcm2* mutant cells. In WT cells, FACT and replisome components including Mcm2 facilitate the transfer of parental histones to daughter strands. Full maturation of chromatin behind the replication fork is achieved through the "read–write" activity of Clr4/Suv39h (*Top*). Loss of Mcl1 disrupts replisome and the transfer of parental histones by FACT, leading to defects in heterochromatin propagation (*Middle*). FACT can still associate with the replisome when the HBD of Mcm2 is mutated. This allows the transfer of sufficient parental histones to support the read–write activity of Clr4/Suv39h, which compensates for the loss of modified histones during DNA replication and allows epigenetic inheritance of heterochromatin (bottom).

domains, corresponding to ORC localization during G1, likely representing inactive but licensed replication origins. These observations, along with evidence showing that features predictive of *S. pombe* origins are enriched at the *mat* locus, centromeres, and subtelomeric regions (66), further support the presence of clustered origins within heterochromatin domains. The clustering of replication origins, typically found in efficiently replicated parts of the genome (67), together with the activating role of Swi6/HP1, may help overcome challenges in replicating these repressive domains. This specialized replication environment leads to a high local concentration of replisome components, which overcomes yet another challenge-the need to retain parental histones to maintain heterochromatin.

Disassembly of nucleosomes and rapid reassembly at the back of the replication fork preserves chromatin structure (68–70) and requires the coordinated action of factors including replication proteins (34, 35, 60, 61, 63, 71). Replication proteins localized across heterochromatin facilitate the retention of parental histones and likely other factors. As in other systems, *S. pombe* Mcm2 possesses histone chaperone activity, and we find that the *mcm2* HBD mutant cells, that are defective in binding histones, fail to propagate an artificially assembled heterochromatin domain at an ectopic site. However, the same mutant presents only modest defects in the retention of parental histones and the self-propagation of endogenous heterochromatin. Other replication factors localized in high abundance at endogenous heterochromatic loci may act in an overlapping manner with Mcm2 to mediate histone transfer. Supporting this, mutations in replication components, such as Mrc1, Polα, and the fork protection complex, impair heterochromatin propagation (39, 61, 71, this study). Importantly, besides chaperoning histones, replication factors engage other factors involved in retention of parental histones. In this regard, we show that Mcl1 is required for connecting the replisome to the histone chaperone FACT, implicated in heterochromatin propagation (30, 53). By contrast, mutation of the Mcm2 HBD compromises but does not completely disrupt FACT association with the replisome. This differential effect correlates with the heterochromatin propagation defects, such that mutations in Mcl1 severely affect the retention of parental histones in cycling cells and result in defective heterochromatin propagation at both ectopic and endogenous loci.

The ortholog of Mcl1, Ctf4, exists as a homotrimeric complex and connects multiple factors to the replication fork, such as the Cdc45-MCM-GINS (CMG) to polymerases, like Polα (43, 44, 46). Mcl1/Ctf4 might provide a platform for directly engaging FACT with the replisome or may simply maintain the structural integrity of the replisome to ensure effective cooperation with FACT. In either case, FACT likely captures histones released during replication and redeposits them onto daughter strands to preserve H3K9me3 (51, 54, 72, 73). This transfer is possibly guided by the histone chaperone function of replication factors and may require other factors like the SMARCAD1 homolog Fft3 that also associates with replication machinery (40). Importantly, both Fft3 and FACT facilitate the retention of parental histones, and their loss causes similar defects in the epigenetic propagation of heterochromatin domains (30, 40, 53).

We find that boundary elements, specifically those surrounding the silent *mat* domain, are crucial for loading FACT to maintain heterochromatin (Fig. 7), while not essential for DNA replication per se. This boundary function in loading FACT and heterochromatin propagation is tightly linked to the recruitment of RIXC. Among other functions, RIXC positions the *mat* domain at the nuclear periphery (30), which helps form high-concentration Swi6/HP1 territories. This process involving nonstoichiometric multivalent HP1 interactions is thought to create a microenvironment for efficient recruitment of effectors (1). To this end, DDK targeted by Swi6/HP1 might allow effective coupling of replisome abundance with FACT engagement for the retention of parental histones. We envision that high-concentration Swi6/HP1 territories facilitate the local retention of FACT and its associated parental histones and their transfer to daughter strands, promoting the self-propagation of heterochromatin (Fig. 7).

Specialized replication for heterochromatin propagation is likely conserved in other eukaryotes. In *S. cerevisiae*, silencer elements, which possess replication origin activity, are bound by ORC, which ensures silencing of *mat* loci by directly recruiting

Sir2 (74–76). Similarly, in *S. pombe,* ORC copurifies with Swi6/HP1 (77), suggesting a more direct role for high levels of pre-RC for the propagation of heterochromatin domains. Regardless, in addition to directly recruiting silencing factors, ORC might help establish a high local concentration of replisome components required for retaining parental histones during replication in yeast and higher eukaryotes. In human cells, the ORC-associated protein ORCA stabilizes ORC binding on chromatin (78). Interestingly, ORCA binds to H3K9me3 that coats heterochromatin domains (79). ORCA may stabilize ORC binding to promote efficient replication of heterochromatin regions. This may enrich the replisome components, ultimately facilitating efficient DNA and chromatin replication. All of the above observations support that targeting replication factors in high concentrations across heterochromatin domains has evolved as a general strategy across species, aiding the stable inheritance of heterochromatin domains.

Materials and Methods

Strains are listed in *SI Appendix*, Table S1. Standard techniques were used to culture *S. pombe*. All experiments were performed in yeast extract–rich medium supplemented with adenine (YEA) at 30 °C unless indicated otherwise. For BrdU chromatin immunoprecipitation experiments, Edinburgh's Minimal Medium (EMM) was used. Strains were constructed by genetic crosses, followed by tetrad dissection or random spore analysis, and by CRISPR (*SI Appendix*). Standard methods for cell cycle synchronization were used (*SI Appendix*). The Mcl1-GFP purification and mass spectrometry were performed following standard procedures (30) (*SI Appendix*), and the Mcl1 interactors are listed in *SI Appendix*, Table S2. The silencing assay at the ectopic site was performed as previously described (27) (*SI Appendix*). Histone turnover and histone retention assays were previously described (40) (*SI Appendix*). A description of random and targeted genetic screens, co-IP analysis, ChIP-chip, ChIP-seq, qPCR, RT-qPCR, FACS, and western blot procedures can be found in SI *Appendix*. Primers used in this study are listed in *SI Appendix*, Table S3.

Data, Materials, and Software Availability. ChIP-seq and microarray data are deposited in the Gene Expression Omnibus (GEO) under accession numbers GSE242353 (80), GSE242429 (81), and GSE242430 (82). All other data are included in the manuscript and/or *SI Appendix*.

ACKNOWLEDGMENTS. We thank members of the Grewal lab for stimulating discussions, Amar Klar and Genevieve Thon for providing strains, and Jemima Barrowman for editing the manuscript. We also thank Diego Folco for comments. This study used the Helix Systems and Biowulf Linux cluster at the NIH. This work was supported by the Intramural Research Program of the NIH, National Cancer Institute.

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