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Graphical abstract

Highlights

- Atf1 binding at a single site can broadly modulate heterochromatin establishment

- The effect of Atf1 is greatly enhanced by separating its binding sites from cenH

- Agent-based model recapitulates Atf1 action and other features of heterochromatin

- Nucleosome-based feedback intrinsically connect establishment and maintenance

In brief

Some DNA-bound proteins facilitate heterochromatin formation over large domains. Using single-cell measurements and mathematical modeling, Nickels et al. show how fission yeast Atf1 acts in synergy with other recruitment modes—RNAi and the recruitment of histone-modifying enzymes by modified nucleosomes—to both establish and later maintain a heterochromatic domain.

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The transcription factor Atf1 lowers the transition barrier for nucleosome-mediated establishment of heterochromatin

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SUMMARY

Transcription factors can exert opposite effects depending on the chromosomal context. The fission yeast transcription factor Atf1 both activates numerous genes in response to stresses and mediates heterochromatic gene silencing in the mating-type region. Investigating this context dependency, we report here that the establishment of silent heterochromatin in the mating-type region occurs at a reduced rate in the absence of Atf1 binding. Quantitative modeling accounts for the observed establishment profiles by a combinatorial recruitment of histone-modifying enzymes: locally by Atf1 at two binding sites and over the whole region by dynamically appearing heterochromatic nucleosomes, a source of which is the RNAi-dependent cenH element. In the absence of Atf1 binding, the synergy is lost, resulting in a slow rate of heterochromatin formation. The system shows how DNA-binding proteins can influence local nucleosome states and thereby potentiate long-range positive feedback on histone-modification reactions to enable heterochromatin formation over large regions in a context-dependent manner.

INTRODUCTION

Fission yeast is a powerful model system for quantitative analyses of heterochromatin formation, the process through which histone H3K9 methylation is deposited onto nucleosomes to silence gene expression. This organism employs a single H3K9 methyltransferase encoded by the clr4+ gene. By synchronously re-introducing clr4+ into a number of cells that do not contain it, it is possible to measure the rates of de novo heterochromatin establishment over time. The presence of heterochromatin can be detected at the single-cell level by the silencing of fluorescent reporters. Combined with mathematical modeling, the effects of mutations or perturbations of the system provide unique information on the molecular mechanisms of heterochromatin formation and inheritance. In particular, kinetic analyses have shown that heterochromatin does not propagate in a purely linear manner along the chromatin fiber and that it can be established in an all-or-none manner, in “bursts,” over a large region (Nickels et al., 2021; Oberstiebing et al., 2016).

Here, we used mathematical modeling to conceptualize how very small DNA elements, binding sites for transcription factors, can dynamically affect the chromatin structure of large chromosomal regions when placed in the proper context. While based on the mating-type region and on the Atf1 transcription factor, this is generally relevant to how blocks of heterochromatin might interact with DNA-binding proteins, even at a distance, to mediate heterochromatin formation and gene silencing.

The effects of the fission yeast Atf1 transcription factor vary depending on the chromosomal context. Atf1 acts genome-wide as the main downstream effector of a MAP kinase cascade to activate stress-response genes (Chen et al., 2003; Eshaghi et al., 2010; Sanso et al., 2011; Takeda et al., 1995; Wilkinson et al., 1996). In the mating-type region, Atf1 binds at two sites, s1 and s2, and acts as a silencing factor (Jia et al., 2004; Kim et al., 2004; Thon et al., 1999) (Figure 1). These two functions of Atf1 are possibly related, as Atf1 might participate in the repression of stress-response genes in the absence of stress (Hansen et al., 2005; Kim et al., 2004; Sanso et al., 2008). However, unlike for the mating-type region, large domains of heterochromatin are not observed at stress-response genes (Zofall et al., 2012). Likewise, the Atf1 homolog dATF2 in Drosophila (Seong et al., 2011) and the transcription factors Pax3 and Pax9 in mouse (Bulut-Karslioglu et al., 2012) function both in gene activation in euchromatin and in pericentric heterochromatin formation. Underlying their repressive function, both Atf1 and dATF2 interact with heterochromatic factors. For Atf1, physical interactions have been documented with Clr4, with the chromodomain protein Swi6, and with the histone deacetylases (HDACs) Cir3 and Cir6 (Jia et al., 2004; Kim et al., 2004; Mota-medi et al., 2008; Yamada et al., 2005).
Figure 1. Heterochromatin establishment driven by Atf1 binding and RNAi

(A) S. pombe mating-type region and experimental scheme. Two fluorescent reporters, YFP located within the 4.2 kb cenH element targeted by RNAi and mCherry close to the edge of the silent domain, were used to monitor heterochromatin formation following re-introduction of a functional clr4+ gene into clr4D strains at generation G0. The two binding sites for Atf1, s1 and s2, the repressor element REIII, and the IR-L and IR-R boundaries are shown. Six parallel cultures, one for each genotype, were used to generate this figure.

(B and C) Different establishment patterns in wild type and mutants lacking s1, s2, or REIII or with a defective RNAi pathway (dcr1Δ) visualized in histograms of mCherry cell fluorescence intensities (B) or heatmaps of YFP and mCherry cell fluorescence intensities (C) at selected time points (expressed in generations). On the order of 15,000–20,000 cells were examined at each point.

(D) Different rates of heterochromatic gene silencing in wild type and mutants. The proportions of cells expressing YFP or mCherry were measured from cell fluorescence histograms at the indicated time points (expressed in generations). On the order of 15,000–20,000 cells were examined at each point.

See also Figures S1–S5 and Table S2.
Several studies have investigated the effects of Atf1 in heterochromatin in relationship to other pathways such as RNAi. RNAi is a determinant of heterochromatin formation in fission yeast by interacting with the Clr4-containing complex CLRC (Bayne et al., 2010; Gerace et al., 2010; Zhang et al., 2008) that it targets to regions where non-coding RNAs are produced and processed (reviewed by Martienssen and Moazed, 2015). In the mating-type region, RNAi nucleates heterochromatin at the cenH element (Hall et al., 2002). Epistasis analyses have indicated that Atf1 and its binding partner Pcr1 function redundantly with RNAi to establish heterochromatin. Thus, unlike each single mutant, double mutants lacking Atf1 and RNAi, or Atf1 and cenH, are not able to assemble a heterochromatic domain. (Jia et al., 2004; Kim et al., 2004; Yamada et al., 2005). Moreover, cenH and the repressor element REIII—which contains the s2 Atf1-binding site—establish and maintain heterochromatic silencing cooperatively at an ectopic chromosomal location (Hansen et al., 2011). Recent studies have outlined a maintenance function for Atf1 by describing how Atf1 would maintain or stabilize pre-existing heterochromatin nucleated either by cenH (Greenstein et al., 2018) or by tethering the catalytic domain of Clr4 to Tet-operator sequences (Wang and Moazed, 2017; Wang et al., 2021). Collectively, these studies provide somewhat divergent views on the effects of Atf1 in heterochromatin establishment and maintenance, with recent studies stressing more a function in maintenance. Moreover, the intriguing question of how Atf1 would control the chromatin state of a large region when bound at just two sites has not been addressed.

Atf1 binding in the mating-type region provides an excellent system to investigate the interplay of a DNA-bound factor and positive feedback on histone modifications. Positive feedback is widespread in chromatin biology (Zhang et al., 2015) and is excellently well documented in the fission yeast mating-type region for the deposition of heterochromatic nucleosomal modifications. Heterochromatic nucleosomes characterized by their H3K9me state drive both histone deacetylation reactions and methylation of H3K9. Thus, mutant analyses have shown that binding of the Clr4 chromodomain to H3K9me drives widespread association of CLRC over the whole region (Zhang et al., 2008). In a Clr4 chromodomain mutant, CLRC’s association is mostly at the RNAi-dependent cenH element (Zhang et al., 2008). Moreover, binding of another chromodomain protein, Swi6 (HP1 homolog), drives the widespread association of the HDAC Clr3 past nucleation sites (Yamada et al., 2005). In this case, Clr3 is loaded at the Atf1-binding site s2 in the absence of Swi6, but broader association relies on Swi6 (Yamada et al., 2005). This positive feedback is believed to partake in the epigenetic inheritance of chromatin states in the region, whereby alternative euchromatic and heterochromatic states can be inherited for many generations in genetically identical sub-populations (Grewal and Klar, 1996; Thon and Friis, 1997; Hall et al., 2002; Zhang et al., 2008).

To better understand the action of Atf1 in the frame of this feedback, we set up to quantitatively profile heterochromatin formation in Atf1 binding-site mutants in a manner suitable for mathematical modeling. We previously developed models that simulate interconversions between three nucleosomal states responsible for the expression state of the mating-type region (Dodd et al., 2007; Nickels et al., 2021; Obersriebnig et al., 2016; see discussions of these and related models in, e.g., Jost and Vaillant, 2018; Lovkvist and Howard, 2021; Ringrose, 2017; and Sneppen, 2014, 2017). Transitions between the states are facilitated by positive feedback where modified nucleosomes recruit modifying enzymes either indirectly or according to “read/write” reactions where an enzyme recognizes its modified substrate (acting as a “reader”) and modifies other nucleosomes (acting as a “writer”). In the frame of these mathematical models, we previously discovered the need for a global, distance-independent, or near-distance-independent feedback reaction to account for how changing system size affects heterochromatin formation (Nickels et al., 2021). Here, when applied to the establishment profiles experimentally observed in Atf1-binding-site mutants, modeling shows how a very punctual effect on chromatin structure by a DNA-binding protein can facilitate heterochromatin formation in an intuitively disproportionate manner over a large chromosomal domain under the influence of a block of heterochromatin.

**RESULTS**

**Heterochromatin establishment driven by Atf1 binding and RNAi**

To monitor the effects of the s1 and s2 Atf1-binding sites on heterochromatin formation, we created a set of mutant strains with two fluorescent reporter genes, YFP within the RNAi-dependent nucleation center cenH and mCherry close to the IR-R boundary of the mating-type region (Figure 1A). The s1 and s2 Atf1-binding sites were either present or precisely deleted in these strains, and a strain lacking both s1 and the repressor element REIII was also created. REIII is a modular repressor element containing s2 and other elements (Thon et al., 1999) recently shown to consist of binding sites for the DNA-binding protein Deb1 and the origin-recognition complex ORC (Wang et al., 2021). YFP and mCherry expressed from the weak S. pombe ura4 promoter were targeted to the nucleus with an SV40 nuclear localization sequence to facilitate quantification of the lowly expressed proteins, similar to a reporter setup originally developed for S. cerevisiae (Xu et al., 2006) that we adapted to S. pombe (Obersriebnig et al., 2016).

Clr4 reintroduction experiments were conducted as in Charlton et al. (2020), Nickels et al. (2021), and Obersriebnig et al. (2016). In this procedure, starting from a clr4Δ background where no histone H3K9me is present, a clr4* gene is introduced into each strain through a genetic cross. Spores are germinated in a medium selecting for clr4* spores whose mating-type region originated from the clr4Δ parent. Here, the arg12* gene tightly linked to clr4* and the ade6* gene marking the mating-type region with the fluorescent reporters were used for selective spore germination. The selection was subsequently maintained as recombinants were kept in exponential growth for several days to monitor silencing of YFP and mCherry in the cultures.

During the period in which heterochromatin was established, bimodal populations of cells were observed expressing or repressing mCherry (Figures 1B, 1C, S2, and S3). With time, the proportions of cells expressing mCherry decayed exponentially to the silenced “OFF” state. Compared with the relatively fast establishment of mCherry silencing in strains with both s1 and s2, silencing...
of mCherry occurred more slowly in strains lacking s2 (s2Δ or s1Δ s2Δ), while deletion of s1 alone, the site closest to cenH, had no measurable effect in this setup (s1Δ s2Δ strain). Deletion of the entire REII element removing 145 bp (Thon et al., 1999) had an effect similar to deletion of s2 removing 8 bp. This is consistent with the finding by Wang et al. (2021) that deletion of the Atf1-binding sites is sufficient to abrogate REII function in a heterochromatin maintenance assay in spite of the presence of additional binding sites for ORC and Deb1 in REII (Wang et al., 2021). In contrast to silencing of mCherry, silencing of YFP within cenH was fast for both wild-type and Atf1-binding-site mutants (Figures 1B–1D, S2, and S3).

A radically different pattern was observed for a parallel cross that re-introduced clr4Δ into a strain containing both Atf1-binding sites but in a dcr1Δ background. In this case, both YFP and mCherry were silenced at a low rate, and, unlike for the other strains, the silencing of the two reporters was highly correlated (Figures 1B–1D, S2, and S3).

The experiment was repeated to ensure reproducibility (Figures 2 and S4). Similar patterns of repression were consistently observed. Moreover, a population-wide repression was reached at the end of the establishment courses (Figures 1D, S3, and S4), indicating that a stable heterochromatic repression can be attained in the absence of s1, s2, and REIII. Yet, prior experiments have detected silencing defects in cultures of REIII mutants (Greenstein et al., 2018; Hansen et al., 2011; Thon et al., 1999). As our current observations were made at the relatively low temperature of 30°C, we investigated whether defects could be detected by increasing the temperature. Independent cultures were inoculated with wild-type, s1Δ s2Δ, or s1Δ sREIIIΔ cells with established heterochromatin. When propagated at 30°C, all cultures showed a tight repression of mCherry (Figure S5). At 37°C, a significant derepression of mCherry was detected in Atf1-binding-site mutants, with expression occurring in a fraction of the cell population only rather than uniformly in all cells (Figure S5). These defects occurring at an incubation temperature higher than 30°C reconcile our current observations with previous observations (Greenstein et al., 2018; Hansen et al., 2011; Thon et al., 1999), and they document that Atf1, when bound, allows to overcome a temperature sensitivity inherent to heterochromatin.

The experiments so far thus revealed that the Atf1-binding site s2 facilitates the de novo establishment of heterochromatin in the context of the whole mating-type region, consistent with a previous study (Thon et al., 1999) and with the redundant effects of REII and RNAI for heterochromatin establishment at cenH (Jia et al., 2004). In the absence of s2, the rate of establishment in the 23 kb mating-type region was decreased approximately 2-fold. Moreover, the populations of Atf1-binding-site mutants adopted bimodal distributions of mCherry expression in the establishment period. Heterochromatic silencing, once established, could be stably maintained at 30°C but not at 37°C.

In a nucleosome model, imposing heterochromatic modifications at discrete sites increases the rate of heterochromatin establishment, mimicking the effect of Atf1

Previously, Dodd et al. (2007) formulated a mathematical model to explain the bistable phenotype of ΔK mutants that lack cenH, which underscores three main properties of heterochromatin: (1) implicit cooperativity, a two-step conversion between nucleosomes in an active (A) and a silent (S) state via an intermediate (U) state, (2) positive feedback in the form of read–write enzymes that can bind their own substrate and subsequently modify other nucleosomes (recruitment), and (3) non-local interactions, where nucleosomes can interact across linear distance. In addition to nucleosome-state changes due to recruitment, nucleosomes can also directly change states to mimic direct binding of histone-modifiers to DNA or histone turnover.

This model was extended to explain nucleation and spreading of heterochromatin (Obersriebnig et al., 2016) and the effect of system size on establishment rates of heterochromatin (Nickels et al., 2021). Nucleation is modeled in these studies as a special 31-nucleosome region with a high direct conversion rate, mimicking the RNAi-mediated recruitment of Clr4 to the cenH nucleation region. Larger system sizes result in dramatically lower rates of establishment (Nickels et al., 2021), which suggests two additional properties of the system: (4) a non-local recruitment reaction toward a silent nucleosome state that is (near) independent of linear distance between nucleosomes in the system and (5) three nearest-neighbor-only interactions (local).

To investigate the rate-increasing effect of Atf1 binding on heterochromatin establishment, we simulated heterochromatin establishment using an extended version of the model described above, where we incorporated punctuated Atf1-mediated silencing of the two nucleosomes closest to their respective Atf1-binding sites (nucleosomes at positions 92 and 102 in the 153-nucleosome region; Figures 2A and S8). For simplicity, we modeled Atf1-mediated silencing of both nucleosomes as a conversion of U- to S-nucleosomes using the same rate constant as for the “special” cenH region and simulated the model as a Gillespie-type algorithm as previously described (Figures 2B, 2C, and 2E; Nickels et al., 2021).

To mimic the experimentally observed effect of Atf1-binding-site deletions in simulations, we set either both (s1Δs2Δ) or one (s1Δs2+ or s1+s2Δ) of the Atf1-dependent silencing rates to 0. In simulations where we ran 10,000 cells in parallel, we recorded the proportions of mCherry-ON and -OFF cells before simulating DNA replication. mCherry-OFF cells were defined as cells where at least 7 out of the 14 nucleosomes occupying the mCherry gene were in the S state. The dynamic profiles were very robust to changes, as tested by changing the thresholds to, respectively, 4 and 10 nucleosomes (Figure S9A). The same was true for YFP (Figure S9B).

Our simulations are in line with our experimental observations, showing that silencing of the nucleation region cenH happens fast (within 1–2 generations) and is nearly independent of functional Atf1-binding sites (Figure 2E, top panels). The model could also replicate our experimental finding that deletion of both Atf1-binding sites results in a substantial decrease in heterochromatin-establishment rates by around a factor of 2 (Figure 2E, lower panels).

Interestingly, simulations of the s1Δs2+ system show a much milder rate-decreasing effect than simulations of the s1+s2Δ system, qualitatively recapitulating our experimental findings (Figure 2E, bottom panels). In experiments, the mean lifetimes (b
values of mCherry expression in the s1Δs2Δ and s1Δs2Δ strains are even more similar than in the simulations, and the lifetimes of s1Δs2Δ and s1Δs2Δ strains are almost identical. Despite these slight differences between experiments and simulations, most likely due to simplifications (e.g., both Atf1-mediated direct rates have the same value), we found it interesting that our model could so closely capture the behavior of the experimental system.

Altogether, our modeling shows that punctuated direct U-to-S conversions with a high attempt rate have an unexpectedly large rate-increasing effect on heterochromatin establishment in a 23 kb region (153 nucleosomes), closely matching the in vivo effect of Atf1 binding.

The rate enhancement by Atf1 rises when separating Atf1-binding sites from cenH

To probe the model experimentally while also investigating biological situations where factors would bind farther from nucleation centers, we simulated the effect of Atf1 binding when the s1 and s2 sites are moved away from cenH in an extended mating-type region. The profile of heterochromatin establishment in extended regions discriminates between various modes of propagation of nucleosomal modifications that are differentially affected by domain size (Nickels et al., 2021), but the effect of DNA-bound factors has not been investigated. We thus proceeded to simulate Atf1 binding in a 27.5 kb region (182 nucleosomes) with an extension between cenH and the s1 binding site, such that s1 would be moved to nucleosome 122 and s2 to nucleosome 132 (Figure 3A). All other parameters were the same as for the wild-type 23 kb system (Figure 2C).

In addition to predicting a reduced rate of establishment when deleting both s1 and s2, the simulations predicted a stronger effect of deleting s1 alone than when s1 is very close to cenH in the 23 kb region (Figure 3B). Moreover, space-time plots suggested how Atf1 binding might co-operate with the read/write activities of histone-modifying enzymes to establish heterochromatin over the whole region (Figure 3C), as discussed further down.

We then repeated the clr4+ re-introduction experiments in strains with an intervening 4.5 kb added between cenH and s1 (Figures S3, S6, and S7) and tested the effect of deleting s1 or s1 and s2. As previously observed for a mating-type region with
intact elements (Nickels et al., 2021), the establishment of heterochromatic silencing at the population scale was slowed down by the 4.5 kb extension. Moreover, populations of cells expressing or repressing (EcoRV)::mCherry co-existed during the establishment period both for the strain with intact elements and for the mutants lacking Atf1-binding sites (Figures 3Da and S6). According to model predictions, the mCherry-ON state decayed exponentially in all cases (Figure 3B). Heterochromatin was established at the fastest rate for the strain containing both \( s_1 \) and \( s_2 \) and at the slowest rate for the strain lacking both binding sites, with a 4-fold difference in rate between the two strains (Figure 3B). Lack of just \( s_1 \) had an intermediate effect, with a 2-fold rate reduction, compared with when both sites were present. Silencing of Kint2::YFP occurred in all cases with much faster kinetics than silencing of EcoRV::mCherry (Figures 3B, 3D, and S6) consistent with fast, RNAi-dependent nucleation at cenH.

These measurements were corroborated experimentally by the ability of cells to switch mating type and sporulate. Efficient mating-type switching results from gene conversions of the mat1 locus by the mat2-P and mat3-M loci when they are in a heterochromatic state (Thon et al., 2019). The ability to switch was re-acquired fastest when both Atf1-binding sites were present (Figure 3E). Thus, twelve generations after the re-introduction of Clr4, cells with wild-type elements formed uniformly sporulating colonies that were stained darkly by iodine vapors due to their high spore content (\( s_1^+ s_2^+ \) in Figure 3E). At the same time point, cells lacking both binding sites existed in two states that produced, respectively, uniformly dark (diagnostic of stable heterochromatin in the region) and light (diagnostic of euchromatin) colonies upon iodine staining (\( s_1^+ s_2^+ \) in Figure 3E). Cells with just \( s_2 \) formed dark and streaky colonies indicative of stably established heterochromatin in dark colonies or heterochromatin establishment during colony growth for the others (\( s_1 s_2^+ \) in Figure 3E). These phenotypes stress the bimodality of epigenetic states in establishment periods and the stability of each state at the time scale of colony growth in the absence of Atf1-binding sites.
Collectively, the experimental results obtained with extended mating-type regions agreed almost perfectly with the simulations. Among others, they showed that binding of Atf1 at s1 has a stronger effect on heterochromatin establishment when being linearly farther away from cenH.

The concordance between experiments and modeling indicated that simulations could be used to ask how Atf1 co-operates with nucleosomal modifications to mediate heterochromatin establishment. Figure 3C shows two examples of time-space plots for the 27.5 kb system with global S-mediated A-to-U feedback and both Atf1 sites present. The right panels show a finer resolution of the transition from the active to the silent state of the region. Before the transition to the silent state, small islands of S-nucleosomes form in addition to the S-nucleosomes already present in the cenH region. Since the nucleosomes closest to the Atf1 sites are converted to S-nucleosomes at a high rate, the probability of forming an island at this location is increased. Once they form, the S-nucleosomes can protect themselves against A-mediated feedback and U-nucleosomes arising from noise and DNA replication. Two small islands surrounding their respective Atf1 site extend outward and occasionally merge to form a larger island that actively contributes to heterochromatin establishment by strengthening the S-mediated A-to-U global feedback reaction.

All in all, when the heterochromatic part of the mating-type regions occupies, in total, 27.5 kb, just 8 nucleotides 5–6 kb away from cenH are effective in dramatically decreasing the time necessary to silence the region at the population scale. Modeling shows how the Atf1-binding sites help in forming small islands of silent nucleosomes that greatly increase the probability of switching to the silent state and thus speed up the silencing dynamics in the entire domain.

The same model and parameter values recapitulate heterochromatin establishment in a broad range of mutants with alterations in the mating-type region or RNAi pathway

To determine whether our three-state model could recapitulate the effect of Atf1 on heterochromatin establishment in the wild-type (23 kb) and 27.5 kb systems together with other characteristic properties of the mating-type region, we scanned the model for the 27.5 kb system for different parameter values (see Figure S8). For parameter values that could recapitulate the experimental results of the 27.5 kb system in simulations, we simulated the system again under slightly different conditions by changing only one relevant parameter value and leaving all other parameter values unchanged. To simulate the effect of Atf1 in the 23 kb system, we thus only changed the system size from N = 182 to N = 153. The effect of system size was simulated for experimentally characterized sizes (23, 27.5, 29, and 31 kb; Nickels et al., 2021), and the dcr1Δ mutant was simulated in the 23 kb system by only changing the special rate (direct conversion attempt rate at cenH). Interestingly, we found parameters (Figure 4, center) at which the model simultaneously explains our current observations as well as previously documented properties of heterochromatin in the mating-type region, such as the effect of Atf1-binding-site deletions in (1) the wild-type 23 kb system and (2) the extended 27.5 kb system, (3) the reduced rate of heterochromatin establishment and highly correlated switch-off dynamics of YFP and mCherry in the dcr1Δ mutant, (4) the rate-reducing effect of system size on heterochromatin formation (Nickels et al., 2021), and (5) the bistable behavior of ΔK mutants (Grewal and Klar, 1996; Thon and Friss, 1997). All simulations were made with the same parameter constants as shown in Figure 4 (center) except for the simulation of the dcr1Δ strain, where the special rate constant was reduced by >50% (from 250 to 115) to mimic the loss of RNAi-dependent nucleation at cenH. This new special rate constant of 115 was chosen to reflect the fact that the processing of non-coding RNAs produced at cenH can mediate the recruitment of Ctr4 even in the absence of RNAi, albeit less efficiently than when RNAi also operates (Chalamcharla et al., 2015; Marina et al., 2013; Reyes-Turcu et al., 2011). Interestingly, simulations show highly correlated switch-off dynamics of cenH and the periphery (Figure 4) and thus agree well with our experimental observations (Figure 1D).

To simulate the effect of domain size on heterochromatin-establishment dynamics, we ran simulations with the same set of parameters but for four different system sizes (153, 182, 191, and 203 nucleosomes, respectively). Essentially, simulations were performed in the same manner as previously (Nickels et al., 2021) but with s1 and s2 being present. Since in Nickels et al. (2021), DNA was introduced between s1 and s2, at the Kint3 site, s1 was placed at position 92 for all system sizes, while s2 was located at different positions, depending on system size (length(system)-42). The simulations qualitatively agree with Nickels et al. (2021), showing drastically slower establishment of heterochromatin in larger systems. Nevertheless, establishment is systematically a bit faster here for all system sizes than was observed in Nickels et al. (2021). These discrepancies could be easily explained by simplifications in the modeling. For example, we did assign the same parameter constants to the Atf1-binding sites s1 and s2 even though we do not know whether Atf1 binds each site equally. Moreover, the range of effect of the cenH region is not exactly defined in the literature, leaving the possibility that s1, which is only three nucleosomes away from the centromere homology in the wild-type region, falls within that range. Relaxing both requirements for simulations, where we give s1 and s2 different special rate constants while still preserving the same sum and where we place s1 within the influence of cenH (Figure S11), gives a very good agreement with the previous modeling results while preserving all other simulation results shown in Figure 4. Lastly, point 5 in Figure 4 shows simulation results of the 76-nucleosome system, where the fraction of time of a single cell simulated for a total of 100,000 generations was plotted over the number of S-nucleosomes. The system started with only A-nucleosomes and was simulated for 50,000 generations. After 50,000 generations, all nucleosomes were replaced with S-nucleosomes, and the simulation proceeded for another 50,000 generations. The histogram illustrates that mainly extreme active or silent states are favored. Altogether, this simple three-state model explains many of the observed behaviors associated with heterochromatin in the mating-type region with the same set of parameters simultaneously and thus proves itself to be a powerful, predictive tool that provides a simple, unifying explanation for many seemingly disconnected phenomena.
DISCUSSION

Our experiments and modeling on the effects of Atf1 in the mating-type region provide insight into how a transcription factor can facilitate the de novo formation of a large heterochromatic domain. They show how the effect can be brought about by a combination of local recruitment of histone-modifying enzymes by the DNA-bound transcription factor together with positive feedback on enzyme recruitment by modified nucleosomes operating over a broader region. These simple parameters help understand the propagation of chromatin states and emergent, context-dependent properties of transcription factors.

Transcription factors such as Atf1 have been proposed to maintain epigenetic information upon DNA replication. In line with this view, the s1, s2, and REIii elements bound by Atf1 are means of maintaining pre-established heterochromatin when cenH and the bulk of the mating-type region are replaced with conditional tethering of Clr4 (Wang et al., 2021). In this synthetic system, the fact that REIii does not effectively establish heterochromatin in the absence of Clr4 tethering has led to emphasize maintenance as a predominant function of REIii and Atf1 (Wang et al., 2021). Our observations in the context of the full mating-type region provide a different perception by highlighting instead the role of Atf1 in heterochromatin establishment. In the absence of the s1 and s2 Atf1-binding sites, the rates of silencing establishment were reduced (Figures 2E and 3C), leading to considerable delays in silencing establishment in cell populations. Such a role is in keeping with prior observations: first, the synergy between REIii and cenH in establishing heterochromatin at an ectopic location (Hansen et al., 2011) and, second, the fact that heterochromatin can be established in a cenH-independent manner in the mating-type region in ΔK mutants (Grewal and Klar, 1996; Thon and Friis, 1997), dependent on Atf1 (Jia et al., 2004). The same molecular mechanisms employing Atf1 and the histone-modifying enzymes that it recruits are likely to facilitate both the de novo formation of heterochromatin and heterochromatin maintenance.

Rather than distinguishing between separate establishment and maintenance steps, our models distinguish between different enzyme-recruitment modes. Thus, modifying enzymes are recruited by RNAi over the entire cenH element by Atf1 punctually at s1 and s2 and by modified nucleosomes as they appear over the whole region. The various recruitment modes are not temporally separated, although their relative contribution changes over time with the evolution of nucleosomal modifications in the system. The approach accounts for many known properties of the mating-type region including the bistable state of ΔK mutants (Figure 4). It provides a simple, integrated
Mechanistically, our model accounts for the apparently disproportionate effects of a locally bound factor over a large chromosomal region. It has been suggested that proteins bound at the 130 bp REII core element permit heterochromatin maintenance by contributing weak interactions with CLRC to strengthen other weak interactions of CLRC with modified histones in the vicinity (Wang et al., 2021). However, how these interactions would affect chromatin structure at a distance from the 130 bp element has not been addressed. Our simulations support a mechanism during heterochromatin establishment where local nucleosomal modifications are induced close to the Atf1-binding sites independent of pre-existing H3K9 methylation (Figure 3D). These local modifications would then function in synergy with cenH—or an equivalent element in other systems—through positive feedback reactions. Supporting such a mechanism where Atf1 would act early on in the process of heterochromatin formation, local tri-methylation of histone H3K9 is detected at the Atf1-binding sites independent of the absence of Cln4 tethering (Wang et al., 2021).

Relatively uncharacterized determinants of chromatin dynamics are system size and long-range positive feedback. Heterochromatic patches or domains vary widely in size. In S. pombe, they vary from <1 to >100 kb (Zofall et al., 2012). For the mating-type region, increasing system size results in slower establishment rates at the population level (compare Figure 1 or 2 with 3; see also Nickels et al., 2021) in a manner that, together with other properties of the region, suggests the existence of a long-range, distance-independent or near-distance-independent positive feedback (Figure 4). In the nuclear space, rather than relying solely on the linear arrangement of nucleosomes along DNA, positive feedback on enzyme recruitment must be affected by subnuclear compartmentalization, supercoiled loops, or biomolecular condensates, among other factors that altogether constitute the context in which transcription factors operate. These environments might fluctuate with temperature and so might the efficiency of feedback that depends on them, perhaps accounting for the partial loss of silencing observed at higher temperatures in Atf1-binding-site mutants. We envision that temperature-sensitive feedback could participate in regulatory functions, such as potentially in the sensing of temperature during vernalization (Antoniou-Kourounioti et al., 2018). In the future, these phenomena can be documented through experiments and modeling to improve our knowledge of how the nuclear environment, in combination with DNA-bound proteins, impacts heterochromatin domains and epigenetic inheritance.

**Limitations of the study**

The model assumes four positive feedback. While positive feedback operating toward the heterochromatic state is well documented in vivo (Yamada et al., 2005; Zhang et al., 2008), this is less the case in the other direction. Regarding the A-stimulated U→A reaction, the Pdp3 protein bound to active nucleosomes might recruit the H3K14 acetyltransferase Mst2 (Flury et al., 2017). Regarding the A-stimulated S→U reaction, the putative H3K9 demethylase Epe1 (Ayoub et al., 2003) might be recruited through the bromodomain protein Bdf2 bridging acetylated nucleosomes to Epe1 (Wang et al., 2013) or the Lsd1/Lsd2 H3K9 demethylases (Lan et al., 2007) might be recruited by nucleosomes in the A state (Gordon et al., 2007). However, the relevance of this potential feedback from the A state remains to be confirmed. For instance, it has been suggested that Epe1 recruits Bdf2 rather than the other way around (Wang et al., 2013). Moreover, our model did not attempt to incorporate an entirely different type of feedback, S-stimulated S→U transitions, that might be induced by physical interaction between Swi6 and Epe1 to curb heterochromatin formation (Zofall and Grewal, 2006). Changes in nucleosome turnover (Taneja et al., 2017) or local remodeling of chromatin structure (Sugiyama et al., 2007) that might occur during heterochromatin establishment were not taken into consideration by our model either. Finally, our measurements relied on the expression of fluorescent reporter genes as a proxy for heterochromatin formation to obtain single-cell data; however, we acknowledge that single-cell measurements of nucleosomal modifications might in the future become a more direct way to follow heterochromatin formation, as recently pioneered for S. cerevisiae (Brothers and Rine, 2022).

**STAR★ METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2022.110828.

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AUTHOR CONTRIBUTIONS
J.F.N.: conceptualization, software, formal analysis, visualization, writing — original draft preparation; M.-E.D.R.: investigation, formal analysis, writing — original draft preparation; I.M.-G.: investigation, formal analysis, visualization; K.S.: conceptualization, supervision, writing — original draft preparation; I.M.-G.: investigation; S.J.C.: investigation, formal analysis, writing — original draft preparation; M.-E.D.R.: investigation, formal analysis, writing — original draft preparation, funding acquisition; G.T.: conceptualization, investigation, analysis, visualization; K.S.: conceptualization, supervision, writing — original draft preparation; I.M.-G.: investigation; S.J.C.: investigation, formal analysis, writing — original draft preparation, funding acquisition.

DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES


## STAR METHODS

### KEY RESOURCES TABLE

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Genevieve Thon (gen@bio.ku.dk).

Materials availability
All unique/stable reagents generated in this study are available from the Lead Contact without restriction.

Data and code availability
- Microscopy data reported in this paper will be shared by the lead contact upon request. Values extracted from the microscopy data are reported in Tables S2 and S3.
- All original code has been deposited at Zenodo:https://zenodo.org/badge/latestdoi/349075339 and is publicly available as of the date of publication.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Experimental model organism
Experiments were conducted with the fission yeast Schizosaccharomyces pombe. Strains were constructed by transformation (Murray et al., 2016) or crosses (Ekwall and Thon, 2017). A strain list is presented in the Key Resources Table.
Growth conditions
Cells were propagated vegetatively in rich medium (Yeast Extract with Supplements, YES). Mating-mixes were set up on Minimum Sporulation Agar (MSA) with arginine as nitrogen source and supplemented as required with leucine at 200 mg/L, adenine at 100 mg/L and uracil at 100 mg/L. Liquid cultures for re-establishment experiments were in Edinburgh Minimum Medium (EMM2) supplemented with leucine at 200 mg/L when required. The EMM2 medium was freshly prepared and filter sterilized rather than autoclaved. Cultures were at 30 °C unless specified otherwise. Media composition can be found in (Petersen and Russell, 2016).

METHOD DETAILS

Oligonucleotides
Oligonucleotides were obtained from IDT. Their sequences are in Table S1.

Plasmid constructions
The plasmid pSM10 (Kelly et al., 1988) contains a 4.2 kb HindIII insert of S. pombe genomic DNA that includes the mat3- M mating-type cassette. pSM10 was digested with EcoRI and religated to produce plasmid pGT251 containing mat3-M centromere-proximal DNA and part of the mat3-M cassette up to its EcoRI site. Plasmid pGT609 was made by religating pGT251 amplified with GTO-1477 and GTO-1478 to replace the s2 Atf1 binding site, ATGACGTA, with an XhoI site, CTCGAG, (s2Δ::XhoI), and plasmid pGT610 was made by religating pGT251 amplified with GTO-1477 and GTO-1478 to replace the REIII element including the s2 Atf1 binding site with an XhoI site (REII::XhoI). To delete both Atf1 binding sites, pGT609 and pGT610 were each amplified with GTO-1475 and 1476 and religated creating respectively pGT611 (s1Δ::PstI s2Δ::XhoI) and pGT612 (s1Δ::PstI REII::XhoI). NotI sites were added at the end of the inserts in pGT251, pGT609, pGT610, pGT611 and pGT612 by cloning PCR products obtained with GTO-1504 and GTO-1505 into pJet1.2 (ThermoFisher) creating respectively pGT613, pGT615, pGT616, pGT617, and pGT618. To extend the region, a 7.8 kb DNA amplicon containing the S. pombe leu1 gene was obtained by PCR with oligonucleotides GTO1617 linearized at the Kint4 site by PCR amplification with GTO-1504 and GTO-1505 produced pGT619 and assembly with pGT613 produced pGT621. The leu1 insert was reduced to about 4.5 kb by inverse PCR with pGT-1351 and GTO-1507 and ligation. Thus, pGT625 (Kint4::leu1 (4.5kb) s1Δ::PstI s2Δ::XhoI) was obtained from pGT619 and pGT627 (Kint4::leu1 (4.5kb) with intact elements) was obtained from pGT621.

S. pombe strain constructions - Recipient strains
S. pombe strain PA76 was produced by transforming SOM62 that contains the Kint2::YFP and (EcoRIV):mCherry reporter genes (Obersriebnig et al., 2016) with pGT248 (Thon et al., 2002) digested with NotI to introduce the S. pombe ade6+ gene at the SpeI site immediately centromere-distal to the IR-R boundary, in the euchromatic domain ((SpeI)::ade6+). Strain PA89 was produced by transforming PA76 with pGT253 (Thon et al., 2002) digested with a mix of HindIII and EcoRI to introduce (Swal)::ura4+ between cenH and mat3-M (between s1 and s2). Correct integration of the transforming DNA in PA76 was checked by PCR with primer pairs CHIP-37 and GTO-219, and CHIP-37 and GTO-156. Correct integration of the transforming DNA in PA89 was checked by PCR with primer pairs TJO-36 and OKR-50. In addition, correct integration in PA89 was verified by Southern blot of genomic DNA digested with HindIII and hybridized to a probe made from the HindIII fragment containing mat3-M. A leu1-32 derivative of PA89 was obtained through a cross, PIM1. PA76, PA89, and PIM1 were used as recipient strains to mutategen the s1 and s2 Atf1 binding-sites and REIII element through transformation.

Strains with 23 kb mating-type region
The strains ME44, ME46, ME48 and ME52 were generated by transforming the S. pombe strain PA89 with plasmids pGT615 (ME48), pGT617 (ME46 and ME44) and pGT618 (ME52) digested with NotI. Transformants were selected as FOA-resistant colonies since correct integration of the transforming DNA was expected to replace (Swal)::ura4+ present in PA89. To avoid growth of untransformed PA89 cells with mutations in ura4+ on 5-FOA plates, the transformation was carried out by co-transforming with a plasmid containing an arg12+ gene which allowed a first selection for cells prone to take-in exogenous DNA on plates lacking arginine, followed by screening for transformants that lacked the (Swal)::ura4+ gene on 5-FOA plates. Clonal populations where the arg12+ plasmid had been lost were subsequently isolated. The strains were verified by diagnostic PCR and the purified PCR products were digested with PstI and XhoI. The resolution of the homologous recombination resulted in strains with one Atf1 binding-site: ME44 (s1Δ::PstI); ME46 (s2Δ::XhoI); deletion of both Atf1 binding-sites: ME46 (s1Δ::PstI s2Δ::XhoI); or deletion of both Atf1 binding-sites and REIII: ME52 (s1Δ::PstI REII::XhoI).

Strains with 27.5 kb mating-type region
The leu1-32 (Swal)::ura4+ recipient strain PIM1 was used for transformation with respectively pGT625 and pGT627 digested with NotI and selection of Leu+ FOA-resistant transformants in which the leu1+ containing insert of pGT625 or pGT627 had replaced (Swal)::ura4+. Transformation of PIM1 with pGT625 produced strain PIM18 with deletion of both Atf1 binding-sites s1 and s2, as well as strain PIM19 with deletion of just the s1 binding-site (due to positioning of cross-over between transforming and chromosomal DNA); transformation of PIM1 with pGT627 produced strain PIM16. PIM16, PIM18, and PIM19 were tested by Southern blot where
genomic DNA digested with a mix of PstI and Xhol was hybridized to mat3-M HindIII probe. The ura4+ allele was reintroduced into PIM16, PIM18, and PIM19 by a cross prior to a series of establishment experiments, and the ura4+ strains were called ME18, ME19 and ME20, respectively.

Re-introduction of clr4+ gene into clr4Δ cells

The clr4+ allele was re-introduced into clr4Δ cells through genetic crosses and selective spore germination essentially as described previously (Obersniebig et al., 2016). A small difference was that the strains with 23 kb and 27.5 kb mating-type regions created here had an ade6+ gene adjacent to the mating-type region (Xspel::ade6+) instead of the previously used (Xbal)::ura4+. As before, the strains were clr4Δ arg12Δ. They were crossed with S. pombe h+ strains where the endogenous clr4+ gene is tightly linked to arg12+. The S. pombe h+ clr4+ arg12+ ade6-DN/N strain PG3715 was crossed respectively with PA76, ME44, ME46, ME48 and ME52. The S. pombe h+ clr4+ arg12+ ade6-DN/N leu1-32 strain PG3714 was crossed with PIM16, PIM18, PIM19, ME19 and ME20. The dcr1Δ strain PG3884 was crossed with the dcr1Δ strain PG3879, selecting in this case for (XbaI)::ura4+. A parallel control cross with the same selection was conducted with the dcr1Δ strains PG3716 and PG3783. In all cases, isolated colonies were patched on YES prior to setting up mating mixes. Mating mixes were set up on supplemented MSA medium, incubated 48 h at 30 °C and collected in 1 mL 1% glusulase (Roche) solution in H2O. Vegetative cells and asci walls were digested overnight at 30 °C. Free spores were washed three times in 1 mL H2O and inoculated into 1 mL EMM2 (or EMM2 supplemented with leucine) to selectively permit the germination of clr4+ arg12+ spores containing also the (Xspel)::ade6+ (or (XmnI)::ura4+) mating-type region. The cultures were then kept in exponential growth in the same medium over the course of the experiment, for one or two weeks. Culture volumes were kept at 1 or 2 mL in culture tubes and the cultures were vigorously agitated at a constant temperature of 30 °C in an Innova S44i shaker (Eppendorf). In parallel with setting up cultures, as a mean of monitoring each cross, spores were plated on YES plates, allowed to form colonies, and 100 spore-colonies were arrayed on YES plates and tested for all markers by replica-plating onto selective plates.

Data acquisition with XCyto-10 Quantitative Cell Imager

Cells in the process of establishing heterochromatin were periodically imaged to quantify YFP and mCherry fluorescence. 50 μL of cell culture were placed in disposable glass chambers for imaging with an XCyto-10 Quantitative Cell Imager (ChemoMetc). The LEDs35 lamp and filter 582–636 were used for mCherry, LED488 lamp and filter 513–555 for YFP, with exposure times of 1 s in both cases. Cells were identified by the XCytoView software package in the bright field channel and fluorescence intensities were measured for single cells, between 15,000 and 30,000 cells per measurement. Histograms were used to determine the proportions of OFF and ON cells by setting intensities thresholds of 150 a.u. for YFP and 100 a.u. for mCherry based on clr4Δ and clr4+ histograms.

Experimental sets for clr4+ re-introduction experiments

For the 23 kb mating-type region, the experimental set ‘exp1’ was obtained by crossing an isolate of strain PG3715 with isolates of each PA76, ME44, ME46, ME48 and ME52; and an isolate of PG3879 with an isolate of PG3884 for the ade6-DN/N background. The experimental set ‘exp2’ was obtained in parallel starting from independent colonies. The experimental set ‘exp3’ was obtained at a different time from independent colonies of the same strains; ‘exp4’ refers to a cross between PG3716 and PG3876 performed in parallel to ‘exp3’. The measured proportions of mCherry ON cells and YFP ON cells at the experimental timepoints are reported in Table S2 and Figure S4.

For the 27.5 kb mating-type region, the experimental set ‘exp1’ was obtained by crossing an isolate of strain PG3714 with isolates of each ME18, ME19 and ME20. The experimental set ‘exp2’ was obtained in parallel starting from independent colonies. The experimental set ‘exp3’ was obtained at a different time by crossing an isolate of strain PG3714 with isolates of respectively PIM16, PIM18 and PIM19. The measured proportions of mCherry ON cells and YFP ON cells at the experimental timepoints are reported in Table S3 and Figure S6.

Modeling

The model was implemented as an agent-based model with $N (N = 153$ for the 23 kb system and $N = 182$ for the 27.5 kb system) units on a line, simulated as previously described ((Nickels et al., 2021), Supplemental information) but with two additional direct conversion reactions (Y6 and Y7), one for each Atf1 binding-site. Simulation profiles were obtained by simulating 10,000 cells in parallel for 150 (or 50 for the wild type) generations and recording the fraction of silenced cells after each generation (before simulating DNA-replication). A cell was counted as silent at cenH if at least 7 out of 14 nucleosomes corresponding to the Kint2::YFP reporter (nucleosomes at positions: 65 to 79) were in S-state. Analogously, a cell was counted as silent in the periphery if at least 7 out of 14 nucleosomes corresponding to the (EcoRV)::mCherry reporter (nucleosomes at positions: length(system) - 35 to length(system) - 21) were in S-state. Changing the threshold to 4 or 10 essentially gave the same results (Figures S9).

QUANTIFICATION AND STATISTICAL ANALYSIS

Curve-fitting and confidence bands of the fit

Lifetime (b) values were obtained by fitting experimental and simulation data points using the curve_fit function of the Scipy.optimize Python package, which uses the method of nonlinear least squares, to the exponential function $f(x) = ae^{-x/b}$ where $x$ represents...
generations and \( f(x) \) the fraction of “ON” cells at generation \( x \). The 95% confidence bands of the fit were determined with the uncertainties Python package. The outputs of the curve_fit function are the estimated parameter values \( a \) and \( b \) (\( \text{popt} \)) and the corresponding covariance matrix (\( \text{pcov} \)). We used \( \text{popt} \) and \( \text{pcov} \) to calculate and attach the uncertainty of \( a \) and \( b \) using the correlated_values function of the uncertainties package. This allowed us, for each fitted curve, to derive a list containing 200 estimated \( f(x) \) values (corresponding to generations 1 to 200) each with an estimated error (\( \text{std} \)). Lastly, we calculated the 95% confidence bands of the fit using the formula \( f(x) \pm 2 \text{std}(x) \). For each reporter and each strain, \( n \) was defined as the number of pooled data-points used for fitting. Figure 2E (right panels) shows \text{mCherry} \) and \text{YFP} \) data for various 23 kb region strains. The lower panel shows \text{mCherry} \) data for the \( s1^+s2^+ \) (\( n = 31, 4 \) independent experiments), \( s1^+s2\Delta \) (\( n = 31, 3 \) independent experiments), \( s1\Delta s2^+ \) (\( n = 21, 3 \) independent experiments), and \( s1\Delta s2\Delta \) (\( n = 33, 3 \) independent experiments) strains. The top panel shows \text{YFP} \) data for the \( s1^+s2^+ \) (\( n = 14, 2 \) independent experiments), \( s1^+s2\Delta \) (\( n = 7, 1 \) experiment), \( s1\Delta s2^+ \) (\( n = 6, 1 \) experiment), and \( s1\Delta s2\Delta \) (\( n = 5, 1 \) experiment) strains. Figure 3B (right panels) shows \text{mCherry} \) and \text{YFP} \) data for various 27.5 kb strains. The lower panel shows \text{mCherry} \) data for the \( s1^+s2^+ \) (\( n = 23, 3 \) independent experiments), \( s1\Delta s2^+ \) (\( n = 29, 3 \) independent experiments), and \( s1\Delta s2\Delta \) (\( n = 48, 3 \) independent experiments) strains. The top panel shows \text{YFP} \) data for the \( s1^+s2^+ \) (\( n = 12, 3 \) independent experiments), \( s1\Delta s2^+ \) (\( n = 14, 3 \) independent experiment), and \( s1\Delta s2\Delta \) (\( n = 11, 3 \) independent experiments) strains. Complete experimental datasets are reported in Table S2 for strains with the 23 kb mating-type region and in Table S3 for the 27.5 kb region and Figure legends indicate the specific datasets used in each case.