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Charlton, Sebastian Jespersen; Jørgensen, Maria Mønster; Thon, Geneviève

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Integrity of a heterochromatic domain ensured by its boundary elements

Sebastian Jespersen Charlton, Maria Mønster Jørgensen, and Geneviève Thon

*Department of Biology, University of Copenhagen, 2200 Copenhagen N, Denmark

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In fission yeast, the inverted repeats IR-L and IR-R function as boundary elements at the edges of a 20-kb silent heterochromatic domain where nucleosomes are methylated at histone H3K9. Each repeat contains a series of B-box motifs physically associated with the architectural TFIIIC complex and with other factors including the replication regulator Sap1 and the Rix1 complex (RIXC). We demonstrate here the activity of these repeats in heterochromatin formation and maintenance. Deletion of the entire IR-R repeat or, to a lesser degree, deletion of just the B boxes impaired the de novo establishment of the heterochromatic domain. Nucleation proceeded normally at the RNA interference (RNAi)-dependent element cenH but subsequent propagation to the rest of the region occurred at reduced rates in the mutants. Once established, heterochromatin was unstable in the mutants. These defects resulted in bistable populations of cells occupying alternate “on” and “off” epigenetic states. Deleting IR-L in combination with IR-R synergistically tipped the balance toward the derepressed state, revealing a concerted action of the two boundaries at a distance. The nuclear rim protein Amo1 has been proposed to tether the mating-type region and its boundaries to the nuclear envelope, where Amo1 mutants displayed milder phenotypes than boundary mutants. Thus, the boundaries might facilitate heterochromatin propagation and maintenance in ways other than just through Amo1, perhaps by constraining a looped domain through pairing.

Chromatin boundaries | gene silencing | single-cell studies | heterochromatin | fission yeast

Euchromatic and heterochromatic regions alternate along the chromosomes of eukaryotes, often separated by DNA elements with distinctive features and properties. In the fission yeast Schizosaccharomyces pombe, the 2-kb inverted repeats IR-L and IR-R mark the edges of a 20-kb heterochromatic domain that keeps mating-type information silent (SI Appendix, Fig. S1). Sharp transitions occur at these repeats in terms of gene expression (1) and histone modifications, particularly methylation of histone H3K9 precisely found between the two repeats (2).

In other organisms, similarly located DNA elements together with architectural proteins bound to them have been proposed to function as anchors for loop domains to participate in the physical and functional organization of genomes (3, 4). Whether IR-L and IR-R function in such a manner in fission yeast remains unknown. No evidence has been presented for physical or functional interactions between the two repeats, and whether they act as tethers to nuclear structures is unclear as deletion of both repeats has a very minor effect on the localization of the mating-type region at the nuclear periphery, if any (5). Not just the mode of action, but even the biological function of the repeats, is still not well-understood.

Structurally, IR-L and IR-R each contains five B-box motifs bound by the TFIIIC complex. TFIIIC is a multifunctional complex that exerts various effects on gene expression in addition to acting as a transcription factor for RNA polymerase III, including heterochromatin barrier effects and enhancer-blocking effects (6–11). At IR-L and IR-R, TFIIIC binds in the absence of RNA polymerase III (12, 13), similar to extra-TFIIIC binding sites (ETCs) in other organisms (14–16). By analogy with the S. pombe COC3 locus whose localization at the nuclear periphery depends on ETC sites, it has been speculated that TFIIIC might anchor the mating-type region to the nuclear periphery and perhaps in this way contribute to the formation of chromatin boundaries (13); however, direct evidence is lacking.

The nuclear rim protein Amo1 is another proposed anchor of the mating-type region to the nuclear periphery and in this case it has been reported that the peripheral localization of the mating-type region and its tight silencing depends on Amo1 (17). The interaction between Amo1 and the mating-type region is bridged by a protein complex called RIXC that associates along the entire silent region in a heterochromatin-dependent manner, and with the B boxes of the inverted repeats independent of heterochromatin (17). This redundant mode of association opens up the possibility that IR-L and IR-R have an Amo1-dependent tethering function even though deletion of both repeats does not detectably delocalize the mating-type region from the nuclear periphery (5). In the absence of the repeats, Amo1 interactions with heterochromatin maintain the domain at the nuclear envelope. To date, the nature of the interaction between RIXC and the B boxes, and a potential connection with TFIIIC, remains uncharacterized.

The position of the inverted repeats precisely at the edges of the heterochromatic domain is strongly suggestive of boundary activity. Yet, studies aimed at detecting heterochromatin expansion in mutants lacking the repeats or B boxes have identified only weak phenotypes unless conditions that foster heterochromatin formation are employed such as overexpressing the chromodomain protein Swi6, homolog of the major heterochromatin protein HP1 in other eukaryotes (1, 2, 13), or mutating the antisilencing factor Epe1 (18). However, early studies had suggested

Significance

The fission yeast silent mating-type region provides an excellent system to ask how chromatic domains with opposite effects on gene expression coexist side by side along chromosomes and to investigate roles played by DNA elements and architectural proteins in the phenomenon. By showing that the IR-L and IR-R chromatin boundaries favor heterochromatin formation in the domain that separates them, dependent on each other and on binding sites for the architectural factor TFIIIC, our work brings to light an important function of these elements and supports the notion that similar types of interactions between boundaries might in other organisms as well stimulate heterochromatin formation in intervening chromosomal loops to actively shape gene expression landscapes.

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The authors declare no competing interest.

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1To whom correspondence may be addressed. Email: gen@bio.ku.dk.

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that the inverted repeats might have an additional function, in preventing encroachment of the heterochromatic domain by euchromatin (1, 19). In the mating-type region, RNA interference (RNAi) initiates heterochromatin formation at the cenH element located centrally (20). Heterochromatin formation catalyzed by the histone H3K9 methyltransferase Clr4 occurs rapidly within cenH and, in a subsequent step, the heterochromatic state propagates to the rest of the domain with slower kinetics (21). Once established, heterochromatin is maintained in an RNAi-independent manner (20) to control mating-type switching and to silence the mating-type donor loci (22). The aspect of heterochromatin formation or stability that would be affected by IR-L and IR-R is not known.

To better our understanding of boundary function, we created a series of IR-R mutants with which we performed multiple phenotypic characterizations. We present evidence that the boundary elements, not just their B boxes but other parts as well, participate in de novo heterochromatin establishment within the silenced domain and subsequently stabilize established heterochromatin in the following generations. Our experiments further uncover functional interactions between IR-L and IR-R that are essential to the robustness of the heterochromatic domain and that occur at least in part independent of tethering to the nuclear envelope by Amo1.

Results

Suppression of Gene Expression by the B Boxes and Other Parts of the IR-R Element. To evaluate the function of the inverted repeats and B boxes in heterochromatic gene silencing, we first examined expression of the S. pombe ade6+ gene inserted near the mat3-M mating-type cassette in strains lacking IR-R or portions of IR-R (SI Appendix, Fig. S1). Seven partial deletions were generated by removing incrementally ~500 bp of the 2.1-kb element starting from either end or by precisely excising the B boxes (Fig. 1A and SI Appendix, Fig. S1).

The reporter used, (EcoRV)::ade6+, is normally silenced by heterochromatin (23). This results in adenine auxotrophy and in the accumulation of a red pigment in colonies grown on medium with a low concentration of adenine (Fig. 1 B and C). Here, all strains lacking the IR-R B boxes (B-boxΔ, DR05, DR10, DR15, and IR-RΔ) produced light pink or white colonies on low-adenine medium and grew well in the absence of adenine, indicative of (EcoRV)::ade6+ derepression. The smallest deletions (B-boxΔ and DR05) had less of an effect than the larger deletions. In addition, nested deletions from the side of IR-R adjacent to the heterochromatic domain (DL05, DL10, and DL15) gradually derepressed (EcoRV)::ade6+, in a variegated manner, even when the five B boxes remained (Fig. 1B and C). In particular, DL15 removing 1,622 bp of IR-R+ but not the B boxes caused a pronounced derepression. The greatest loss of silencing was observed when the whole of IR-R was deleted (IR-RΔ). These observations suggested that the B boxes are essential for tight silencing, but also that the whole of IR-R is required for optimal silencing.

As the deleted DNA was not replaced by exogenous fragments, one effect of the deletions is to reduce the distance between the (EcoRV)::ade6+ reporter and euchromatin. This raises the possibility that the difference between B-boxΔ and IR-RΔ cells stems from the reduced distance. However, in a slightly different setting in a previous study, ura4+ placed on the heterochromatic side of IR-R was derepressed to the same extent by deletion of IR-R or by replacement of IR-R with 2 kb of λ DNA (1). The fact that a level of silencing comparable to what remains here in the B-boxΔ mutant was not attained with just λ DNA argues for a B box-independent sequence-specific effect by IR-R.

For a more quantitative evaluation of (EcoRV)::ade6+ expression, qRT-PCR was performed to measure transcript levels in some of the strains (Fig. 1D). As expected from the plating...
assays, expression of \((\text{EcoRV})::\text{ade}6^+\) was greatly enhanced by deleting IR-R, resulting in a \(>15\)-fold increase in messenger RNA. \((\text{EcoRV})::\text{ade}6^+\) expression was enhanced to a lower degree by deleting just the B boxes (Fig. 1D). This suggests that molecular differences at the mating-type region affecting transcriptional activity exist between these two strains, but that the Ade6 protein concentration in the strains lacking the B boxes is sufficient for some degree of adenine prototrophy.

The growth assays and transcript analysis of the \((\text{EcoRV})::\text{ade}6^+\) reporter thus reveal a role for the B boxes in heterochromatic silencing at a population level and furthermore indicate that the B boxes operate synergistically or in parallel with the rest of IR-R to achieve full silencing.

**Single-Cell Measurements Detect Two Subpopulations with Distinct Expression States in IR-R Mutants.** We turned to fluorescent reporters, a YFP (yellow fluorescent protein) gene at the \(\text{Kint2}\) site within cenH and mCherry at the \((\text{EcoRV})\) site near \(\text{mat3}-M\) (Fig. 1E), to visualize silencing defects in IR-R mutants at a single-cell level. Similar to previous experiments (21), expression of both YFP and mCherry was controlled by the \(S.\, \text{pombe uaf4}^+\) promoter and terminator of transcription, and the fluorescent proteins were targeted to the nucleus by the SV40 nuclear localization sequence. Quantitative fluorescence microscopy was carried out with the IR-\(R^\Delta\), B-box\(\Delta\), and IR-R\(\Delta\) strains (Fig. 1F–H and SI Appendix, Figs. S2–S4). Images were acquired for 10 independent cultures of each strain and fluorescence intensities were measured for 1,500 to 15,000 cells in each culture (Table 1 and SI Appendix, Fig. S1). \(\text{clr4}\Delta\) mutants served as reference for the expressed state (SI Appendix, Fig. S2).

Silencing of \(\text{Kint2}:\text{YFP}\) was very stringent in the three \(\text{clr4}\Delta\) strains examined, independent of changes at the IR-\(R^+\) element (Fig. 1 G and H). Silencing of \((\text{EcoRV})::\text{mCherry}\), on the other hand, was stringent in the wild type but lost in a fraction of the cell populations for the B-box\(\Delta\) and IR-R\(\Delta\) mutants (Fig. 1 F–H and SI Appendix, Figs. S3 and S4). At least for the IR-R\(\Delta\) mutant, where more cells were derepressed and the pattern thus more easily seen, the distribution of fluorescence intensities was bimodal with a subpopulation expressing \((\text{EcoRV})::\text{mCherry}\) to a level similar to the \(\text{clr4}\Delta\) mutant and the other subpopulation tightly repressing the reporter similar to the IR-\(R^+\) strain (Fig. 1G and SI Appendix, Figs. S2 and S3).

The proportions of ON and OFF cells were determined in each culture by setting arbitrary intensity thresholds of \(\sim 150\) for YFP and \(\sim 100\) for mCherry based on \(\text{clr4}\Delta\) and \(\text{clr4}^+\) histograms accompanied by visual examination of all micrographs to verify the calls (Fig. 1H, Table 1, and SI Appendix, Fig. S3 and Table S1). Approximately \(6\%\) of IR-R\(\Delta\) cells and \(1\%\) of B-box\(\Delta\) cells showed derepression in this experiment and similar values were obtained in subsequent experiments (SI Appendix, Table S1). The more widespread derepression by IR-R\(\Delta\) than by B-box\(\Delta\) reflects what had been observed with \((\text{EcoRV})::\text{ade}6^+\) strains (Fig. 1A–D). In the case of \((\text{EcoRV})::\text{ade}6^+\), inheritance of the Ade6 protein [estimated at \(\sim 80,000\) molecules per cell when expressed from the endogenous locus (24) with a half-life of \(18\) h (25)] is likely to contribute to adenine prototrophy during colony growth, masking the cell-to-cell heterogeneity seen with \((\text{EcoRV})::\text{mCherry}\).

The pattern of \((\text{EcoRV})::\text{mCherry}\) expression in IR-R mutants raised the questions of whether and how the populations of OFF and ON cells interconvert. Bimodal patterns of gene expression have been observed for other heterochromatin mutants such as for the \(S.\, \text{achromomycoses cerevisiae sir1}\) mutants, where defects in heterochromatin establishment and maintenance result in mixed populations of OFF and ON cells (26–28). Here we considered that heterochromatin might be labile in the absence of boundary, in which case ON cells would arise from heterochromatin loss in the OFF cells. It also appeared possible that the absence of boundary would reduce the efficiency of heterochromatin formation; reduced heterochromatin establishment would increase the proportion of ON cells in the populations. These possibilities were investigated in several ways.

**Participation of the IR-R Boundary in De Novo Heterochromatin Establishment.** Heterochromatin establishment was monitored by reintroducing \(\text{clr4}^+\) by genetic crosses into \(\text{clr4}\Delta\) strains harboring the \(\text{Kint2}:\text{YFP}\) and \((\text{EcoRV})::\text{mCherry}\) fluorescent reporters, using prototrophies to select for the desired recombinant progeny at spore germination as described in Materials and Methods, similar to ref. 21. \(\text{Kint2}:\text{YFP}\) \((\text{EcoRV})::\text{mCherry}\) cells could thus be examined from the first divisions after they acquired the \(\text{clr4}^+\) gene and subsequently at regular time intervals by sampling liquid cultures maintained in exponential phase. The rate of silencing of the fluorescent reporters was then used as a proxy for the rate of heterochromatin establishment. Rates were measured in the IR-R mutants to dissect the effect these have on heterochromatin establishment (Fig. 2 and SI Appendix, Fig. S5). In this assay, cells with no YFP signal have nucleated heterochromatin at cenH, while cells with no mCherry signal have established heterochromatin near \(\text{mat3}-M\).

Heterochromatin establishment of \(\text{cenH}\) was fast and not affected by IR-R mutations (SI Appendix, Fig. S5 and Table S2). \((\text{Kint2})::\text{YFP}\) was silenced in the vast majority of cells within 10 generations, reflecting what had been observed previously for the wild type (21) and consistent with our steady-state measurements where no expression of \((\text{Kint2})::\text{YFP}\) was detected (Fig. 1 G and H and SI Appendix, Fig. S4).

In contrast, silencing of \((\text{EcoRV})::\text{mCherry}\) occurred at different rates in the three strains (Fig. 2 B and C and SI Appendix, Table S2). For cells with wild-type IR-R, silencing was almost fully established at the population level 20 generations after spore germination. In the IR-R\(\Delta\) strain, even after \(\sim 40\) generations, 10% of cells still expressed the normally silent region (Fig. 2C). In a strain lacking the B boxes, heterochromatin establishment occurred at a rate intermediate between the wild type and IR-R\(\Delta\) (Fig. 2C). The mean lifetimes of the ON state (2.8 generations for IR-\(R^+\); 7.7 for IR-R\(\Delta\); 4.5 for B-box\(\Delta\)) again reflect the differences previously observed between the B-box\(\Delta\) and IR-R\(\Delta\) mutants and further support the notion that molecular differences exist between the two mutants, in this case resulting in different kinetics of heterochromatin establishment.

**Requirement for the IR-R Boundary in Heterochromatin Maintenance.** The fact that a proportion of \((\text{EcoRV})::\text{mCherry}\) \(\text{clr4}^+\) cells expressed \(\text{mCherry}\) in cultures started from single cells (Fig. 1 F–H), together with the adenine prototrophy of \((\text{EcoRV})::\text{ade}6^+\) boundary mutants (Fig. 1 B–D), suggested that heterochromatin

### Table 1. Fraction of the cell population expressing the \(\text{Kint2}:\text{YFP}\) or \((\text{EcoRV})::\text{mCherry}\) reporter in wild-type and mutant cell cultures

<table>
<thead>
<tr>
<th>Genotype</th>
<th>YFP-ON cells, mean</th>
<th>mCherry-ON cells, mean</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR-(R^+)</td>
<td>(0.1 \times 10^{-3})</td>
<td>(0.1 \times 10^{-2})</td>
<td>16</td>
</tr>
<tr>
<td>IR-(R\Delta)</td>
<td>(0.2 \times 10^{-3})</td>
<td>(7.1 \times 10^{-2})</td>
<td>19</td>
</tr>
<tr>
<td>B-box(\Delta)</td>
<td>(0.2 \times 10^{-3})</td>
<td>(1.8 \times 10^{-2})</td>
<td>16</td>
</tr>
<tr>
<td>IR-L(\Delta) IR-(R^+)</td>
<td>(0.2 \times 10^{-3})</td>
<td>(0.4 \times 10^{-3})</td>
<td>12</td>
</tr>
<tr>
<td>IR-L(\Delta) IR-(R\Delta)</td>
<td>(0.4 \times 10^{-4})</td>
<td>(1.4 \times 10^{-2})</td>
<td>15</td>
</tr>
<tr>
<td>IR-L(\Delta) B-box(\Delta)</td>
<td>(0.3 \times 10^{-3})</td>
<td>(1.7 \times 10^{-2})</td>
<td>12</td>
</tr>
<tr>
<td>amo1(\Delta) IR-(R^+)</td>
<td>(0.5 \times 10^{-3})</td>
<td>(0.1 \times 10^{-2})</td>
<td>6</td>
</tr>
<tr>
<td>amo1(\Delta) IR-(R\Delta)</td>
<td>(0.5 \times 10^{-3})</td>
<td>(2.8 \times 10^{-2})</td>
<td>6</td>
</tr>
<tr>
<td>amo1(\Delta) B-box(\Delta)</td>
<td>(0.1 \times 10^{-3})</td>
<td>(9.3 \times 10^{-2})</td>
<td>6</td>
</tr>
<tr>
<td>amo1(\Delta) IR-L(\Delta) IR-(R^+)</td>
<td>(0.3 \times 10^{-3})</td>
<td>(0.1 \times 10^{-2})</td>
<td>6</td>
</tr>
<tr>
<td>amo1(\Delta) IR-L(\Delta) IR-(R\Delta)</td>
<td>(0.9 \times 10^{-3})</td>
<td>(5.2 \times 10^{-2})</td>
<td>6</td>
</tr>
<tr>
<td>amo1(\Delta) IR-L(\Delta) B-box(\Delta)</td>
<td>(0.2 \times 10^{-3})</td>
<td>(7.9 \times 10^{-2})</td>
<td>6</td>
</tr>
</tbody>
</table>

\(n\) represents the number of independent cultures examined in each case.
might be unstable in boundary mutants, in addition to being established at a slower rate than for wild-type cells. To measure stability, clr4Δ cells with the Kim2::YFP and (EcoRV)::mCherry reporters were placed under small agar patches in minimal medium and imaged by time-lapse fluorescence microscopy at each generation for six or seven generations. The formation of 22 microcolonies for each strain was observed in this way. Both generation for six or seven generations. The formation of 22 microcolonies for each strain was observed in this way. Both might be unstable in boundary mutants, in addition to being established at a slower rate than for wild-type cells. To measure stability, clr4Δ cells with the Kim2::YFP and (EcoRV)::mCherry reporters were placed under small agar patches in minimal medium and imaged by time-lapse fluorescence microscopy at each generation for six or seven generations. The formation of 22 microcolonies for each strain was observed in this way. Both gains and losses of the silenced state were observed as well as clonal inheritance of each state for multiple divisions (Movies S1–S9).

Derepression of YFP was never observed under these conditions, confirming that heterochromatin at cenH is unaffected by IR-R. As expected, in wild-type cells, heterochromatin was also stable at EcoRV. No case of (EcoRV)::mCherry derepression was observed among the imaged cells (n = 685 divisions). In contrast, derepression of (EcoRV)::mCherry occurred readily in the boundary mutants. Each event was scored by visual inspection, allowing an estimate of the rates of silencing loss. This showed that the derepression rate was increased to a similar extent in the B-boxΔ and IR-RΔ mutants, respectively, 3.5 × 10⁻² (n = 482 divisions) and 3.1 × 10⁻² (n = 655 divisions) loss of silencing events per cell division.

Role of IR-L in Setting Up the Right Domain Boundary. The distribution profiles of (EcoRV)::mCherry expression gave us the opportunity to investigate a potential interplay between the left and right boundaries by examining the expression of (EcoRV)::mCherry in strains lacking IR-L (Fig. 3A–D, Table 1, and SI Appendix, Figs. S6 and S7 and Table S1). It had previously been observed that deletion of IR-L derepressed a reporter gene near the mat2-P mating-type cassette [(XbaI)::ura4⁺] but did not detectably affect (EcoRV)::ura4⁺ expression near mat3-M (1). Consistently, here (EcoRV)::mCherry remained silent when IR-L but not IR-R was deleted. When both IR-L and IR-R were deleted, a synergistic derepression of (EcoRV)::mCherry occurred. Fluorescence intensity did not increase in individual mCherry-positive cells, but higher proportions of mCherry-positive cells were present in the double mutant compared with the mutant lacking only IR-R. The effect was seen in all cultures propagated in parallel for this specific experiment and corroborated by subsequent measurements, for a total of 16 cultures of IR-RΔ cells and 12 cultures of IR-LΔ IR-RΔ cells (SI Appendix, Table S1). On average, compared with ∼7% derepressed cells in the IR-RΔ mutant, ∼15% were derepressed by the double-boundary deletion. In contrast, no synergistic effect was detected when only the B boxes were deleted together with IR-L (Fig. 3B–D, Table 1, and SI Appendix, Figs. S3–S7 and Table S1). Thus, IR-L participates in the repression of (EcoRV)::mCherry, located 20 kb away, close to the other edge of the heterochromatic domain.

The Boundaries Are Required for RNAi-Independent Heterochromatin in cenH Mutants. Mutants lacking the cenH element (∆K mutants) display a bistable state where cells are either similar to wild type or lack heterochromatin over the mat2-P mat3-M region (29, 30). Each state has a mean half-life greater than 1,000 generations (30), and thus the two phenotypes are clearly distinguishable not
only at the colony level but also upon restreaking of colonies. The bistability is believed to reflect a de novo heterochromatin establishment defect in ΔK cells due to loss of the RNAI-dependent heterochromatin nucleation center cenH (20); establishment would still occur, but insufficiently. Here, we wondered whether deletion of the domain boundaries would affect the bistable phenotype of ΔK mutants. We created a strain identical to one of the original ΔK mutants (30) but lacking both boundaries (Fig. 3E).

The two phenotypes of the ΔK mutant are easily observed on sporulation plates by iodine staining of colonies. In this procedure, spores, but not vegetative cells, are stained dark by iodine vapors (31). Because heterochromatin is required for efficient sporulation (22, 32, 33), the ΔK mutant epitype with established heterochromatin forms brown colonies similar to wild-type (h<sup>+/+</sup>) strains, while the ΔK mutant epitype lacking heterochromatin forms light mottled colonies like <i>clr4Δ</i> strains (29, 30). We found that in a ΔK mutant lacking both boundaries the dark epitype could not be readily propagated (Fig. 3E). Colonies were not as uniformly stained as when the boundaries were present and, upon restreaking, light colonies and sectors appeared at high frequency, which was not the case when the boundaries were present (Fig. 3E). Thus, a stable heterochromatic state could not be attained in the absence of boundaries. Similarly, Wang and Moazed found that the boundaries stabilized heterochromatin induced by artificially tethering Cen4 (34).

**Protein Factors: Amo1, Bqt4, Lem2, and Man1.** Recently, two studies have suggested that the localization of the mating-type region at the nuclear periphery might be part of its silencing mechanism. First, it was reported that H3K9me is considerably reduced in the mating-type region in mutants lacking the nuclear membrane protein Bqt4 (35). Second, mutants in the nuclear rim protein Amo1 were also reported to reduce H3K9me in the <i>mat2-mat3</i> region, in particular outside cenH, attributed to defective maintenance (17). In the case of Amo1, reduced H3K9me was accompanied by partial deolocalization from the nuclear periphery (17). In the case of Bqt4, reduced H3K9me was accompanied by partial deolocalization from the nuclear periphery specifically during replication (35). Because anchoring at the nuclear periphery by the boundary elements could be part of their mechanism of action, we conducted an epistasis analysis to investigate the effects of combining mutations at IR-R with mutations in potential anchors at the nuclear envelope. We included a <i>lem2Δ</i> mutant (36), for which Barrales et al. previously detected a 5- to 25-fold increase of (EcoRV)::<i>ura4</i>+ transcript (37), a reporter at the same location as (EcoRV)::<i>ade6</i>+<sup>+</sup>, as well as a <i>man1Δ</i> mutant for which no effect on heterochromatin has been reported, to our knowledge.

Silencing of the sensitive (EcoRV)::<i>ade6</i>+ reporter appeared unaltered in the <i>bqt4Δ, lem2Δ, and man1Δ</i> mutants (Fig. 4A). While somewhat surprising in the cases of <i>bqt4Δ</i> and <i>lem2Δ</i> silencing near mat2-P is also maintained in these mutants (17). We thus focused on <i>amo1Δ</i>, which showed reduced silencing of (EcoRV)::<i>ade6</i>+ (Fig. 4A and B), consistent with its effect near mat2-P (17). (EcoRV)::<i>ade6</i>+ derepression by <i>amo1Δ</i> was not as pronounced as (EcoRV)::<i>ade6</i>+ derepression by IR-RΔ according to the light pink colony color of <i>amo1Δ</i> colonies, and there appeared to be cumulative defects in all cases when combining partial deletions of IR-R and <i>amo1Δ</i>, as shown for B-boxΔ in Fig. 4B. Quantitative analysis with (EcoRV)::<i>mCherry</i> confirmed these conclusions as fluorescent cells were more abundant in the <i>amo1Δ IR-RΔ and amo1Δ B-boxΔ</i> mutants than in each single mutant (Fig. 4E, Table 1, and SI Appendix, Figs. S3 and S4 and Table S1), with <i>amo1Δ</i> having a very small effect on its own [on the order of 0.1% of <i>amo1Δ</i> cells showed (EcoRV)::<i>mCherry</i> expression by fluorescence microscopy]. Furthermore, (EcoRV)::<i>mCherry</i> expression was also detected in a greater proportion of cells in the <i>amo1Δ IR-RΔ IR-RΔ</i> mutant than in the <i>IR-RΔ IR-RΔ</i> mutant (Fig. 4E, Table 1, and SI Appendix, Figs. S6 and S7 and Table S1). Altogether, these phenotypes indicate Amo1-independent functions of the boundaries in heterochromatin silencing, without ruling out some form of combined action. In addition, the great proportion of derepressed cells in the <i>amo1Δ IR-RΔ IR-RΔ</i> mutant (53% on average), greater than in the <i>amo1Δ IR-RΔ</i> mutant (29% on average), underscores the effect of IR-R at a distance.

**Discussion**

Our experiments provide evidence that the IR-L and IR-R boundary elements in the fission yeast mating-type region facilitate heterochromatin propagation and maintenance over the silenced domain. They also reveal subelements that can be functionally separated, indicating B box-dependent and -independent mechanisms of boundary activity. How might boundaries facilitate the propagation of heterochromatin? The tethering of IR-L and IR-R to the nuclear envelope, physical interactions between them,
Fig. 4. Independent effects of the nuclear envelope protein Amo1 and boundary elements on heterochromatin integrity. (A) Four nuclear envelope proteins were tested for their requirement in the silencing of (EcoRV)::ade6+ by plating assay. As in Fig. 1, red colony color denotes repression, and pink or white denotes expression. A clr4Δ mutant is shown for comparison. (B) Epistasis analysis of amo1Δ and mutations at IR-R conducted with (EcoRV)::ade6+. (C–E) Epistasis analysis of amo1Δ and mutations at IR-R conducted with (EcoRV)::mCherry as in Fig. 1 G and H but examining six independent cultures for each genotype (nine for IR-RΔ and IR-LΔ IR-RΔ) [Kruskal–Wallis for (EcoRV)::mCherry, H = 73.13, P = 3.1 × 10−11, **P < 0.01]. The proportion of cells expressing (EcoRV)::mCherry was significantly higher in the IR-RΔ amo1Δ mutant than in the IR-R+ amo1Δ mutant (P = 0.003) and IR-RΔ (P = 0.002) mutants. It was also significantly higher in the IR-RΔ IR-LΔ amo1Δ mutant than in the IR-LΔ IR-RΔ amo1Δ (P = 0.003) and IR-LΔ IR-RΔ (P = 0.002) strains. Finally, the proportion was significantly higher in the IR-LΔ IR-RΔ amo1Δ mutant than the IR-RΔ amo1Δ strain (P = 0.003).

or the binding of protein complexes may affect chromatin dynamics in various ways that we discuss below. A mechanistic clue comes from the fact that the left and right repeats functionally interact to form the heterochromatin domain.

All our experiments point to gene silencing being alleviated in the absence of amo1Δ and full boundary-deletion mutants as well as increased derepression when combining amo1Δ with IR-RΔ or IR-LΔ IR-RΔ (Figs. 4 and 5 and Table 1). Thus, not just the B boxes but the whole boundaries as well retain their functionality in the absence of Amo1. Conversely, repression by Amo1 can occur in the absence of boundaries, this last effect plausibly due to tethering of the mating-type region to the nuclear periphery in a subnuclear compartment where reduced histone turnover stabilizes heterochromatin (17). The tethering would rely on the association of RIXC with heterochromatin throughout the silent domain and with the B boxes independent of heterochromatin (17).

In both RIXC and Amo1 mutants the mating-type region is displaced from the nuclear periphery and silencing defects are observed (17). Here, we observed a small derepression of (EcoRV)::ade6+ in the amo1Δ mutant (Fig. 4), consistent with the originally detected defect for amo1Δ in a sensitized background lacking the REII silencer near mat2-P (17). Derepression by amo1Δ was less pronounced than for the B-box mutant and cumulative effects were observed when combining the amo1Δ and B-boxΔ mutants (Fig. 4 and Table 1). Thus, while confirming a requirement for Amo1 for tight heterochromatic silencing, these phenotypes also suggest different mechanisms of action for Amo1 and the B-box elements that would account for the B-box-dependent silencing of (EcoRV)::ade6+ and (EcoRV)::mCherry in amo1Δ cells. One possibility is that other factors such as TFIIFC or Sap1 still exert a repression through the B boxes in the absence of Amo1.

Even larger phenotypic differences were observed between amo1Δ and full boundary-deletion mutants as well as increased derepression when combining amo1Δ with IR-RΔ or IR-LΔ IR-RΔ (Figs. 4 and 5 and Table 1). Thus, not just the B boxes but the whole boundaries as well retain their functionality in the absence of Amo1. Conversely, repression by Amo1 can occur in the absence of boundaries, this last effect plausibly due to tethering of the mating-type region to the nuclear periphery in a subnuclear compartment where reduced histone turnover stabilizes heterochromatin (17).

A clue for how the boundaries might function is provided by the synergy between IR-L and IR-R (Figs. 3 and 4, Table 1, and SI Appendix, Figs. S3, S4, S6, and S7 and Table S1). Derepression of (EcoRV)::mCherry at a distance by IR-LΔ, in a situation where heterochromatic silencing remains at Kin2::YFP in the intervening region, argues against the boundaries functioning as individual roadblocks against a linear spread of chromatic modifications, here euchromatic modifications spreading inward in the absence of boundaries. The synergy rather suggests the existence of a loop domain between the two boundaries, similar to models of boundary activity in Drosophila and other organisms (3). The mating-type region is delocalized from the nuclear...
loop domain model for the mating-type region. The mating-type region is anchored at the nuclear periphery by the Amo1 protein (in green, Left; Right, amo1Δ strains). (Top) The two boundaries (in blue, with TFIIIC binding sites in yellow) pair to define a heterochromatic domain in which the mCherry reporter (in red) is silenced independent of Amo1. A hypothetical condensin ring is represented in gray. (Middle) In the absence of the IR-R boundary, the mCherry gene occasionally slides out of the loop domain, resulting in heterochromatin loss and mCherry expression in some cells. Heterochromatin loss is compounded by delocalization from the nuclear envelope and higher histone turnover in the absence of Amo1. (Bottom) When both boundaries are deleted, the loop domain falls apart. Heterochromatin nucleated at cenH (purple) maintains nuclear envelope association through Amo1 but heterochromatin propagation and maintenance at mCherry are inefficient. Loss of silencing is again compounded by the absence of Amo1.

periphery in Amo1 mutants (17), yet we find that silencing is not affected nearly to the same extent by amo1Δ as by boundary deletions, indicating attachment to the nuclear envelope is not how the domain would be constrained. Instead, homologous pairing between the two elements together with TFIIIC binding might define the bottom of the loop (Fig. 5). In S. pombe, as in other organisms, condensin localizes to TFIIIC binding sites (12, 41–43). A loop constrained at its base by a condensin ring, or possibly by the long coil-coiled protein Sap1 detected at that location (21, 44), could create a domain of increased interactions, boosting positive feedbacks for nucleosomal modifications and heterochromatin propagation from the cenH nucleation center present in the loop (44) (Fig. 5). As it forms, heterochromatin would be further compacted by cohesin (45, 46) and altered nucleosomal contacts (47). Deletion of a single boundary would not preclude formation of the proposed looped domain, as a loop could be initiated and reeled in from the intact side (47) but the edge of the domain on the side lacking the boundary would not be precisely positioned, resulting in the observed variegation of the silencing phenotype on that side only, as observed (1). Only when both boundaries are deleted would silencing be affected at a distance due to loop disruption. Even then, silencing would remain in a proportion of cells as heterochromatin formation would still be initiated at cenH which, among other effects, would maintain the mating-type region at the nuclear envelope (Fig. 5).

In the budding yeast S. cerevisiae, a transfer RNA (tRNA) gene near the HMR silent mating-type cassette at one end of chromosome III facilitates long-range interactions with the HML locus at the other end of chromosome III and stabilizes gene silencing at both locations (48). Moreover, nucleoporins recruit HMR to the nuclear periphery through this tRNA gene and thereby contribute to HMR silencing (49). While the proposed loop domain for the S. pombe mating-type region (Fig. 5) helps understand observations specific to the fission yeast system, it thus appears possible that the two organisms rely on partially shared mechanisms to facilitate heterochromatin formation.

Materials and Methods

Growth Conditions and Genetic and Molecular Manipulations of S. pombe. Media (YES, EMM2, MSA) and genetic manipulations including transformations, genetic crosses, and chromosomal DNA preparations were according to published fission yeast protocols (50–53). YE (5 g/L yeast extract and 30 g/L glucose, a rich medium) and AA-ade [dropout medium lacking adenine (54)] were used to assay [EcoRⅤ]::ade6+ expression. Plasmid and strain constructions are detailed in SI Appendix. Strain genotypes are presented in SI Appendix, Table S3 and oligonucleotides are listed in SI Appendix, Table S4.

Heterochromatin Establishment Experiments. Reintroduction of the clr4+ gene was conducted essentially as in ref. 21. Strains with the mating-type regions of interest in a clr4Δ arg12Δ background, SC32, SC36, and SC37, were crossed with the clr4Δ arg12Δ strain PA90. In the progeny, arg12Δ was used to select for the tightly linked clr4+ gene and (XmnI)::ade6+ on the euchromatic side of IR-L+ was used to select for the mating-type region originating from SC32, SC36, or SC37. Practically, isolated colonies of all strains were patched onto rich medium (YES) at the beginning of the experiment and the patches were used, the following medium with low adenine concentration) and AA-ade [MSA supplemented with adenine, leucine, and uracil; MSA uses arginine as nitrogen source]. Two to three days later, free spores were prepared by resuspending each mating mix in 1 mL p-glucuronidase solution (Roche Diagnostics) diluted 1:100 in H2O, resuspended in 1 mL EMM2 minimal medium supplemented with uridine but lacking arginine and adenine, and placed at 30 °C to induce germination of Ade+ Arg+ spores. Liquid cultures inoculated with the germinated spores were kept in a shaking incubator at 30 °C and maintained in exponential phase for several days by diluting them in EMM2 containing uridine, keeping a culture volume of 1 mL throughout the experiment. Cell counting and imaging were performed at regular intervals to produce growth curves and to measure fluorescence. Crosses were monitored by plating the original random spore preparations on nonselective YES plates and by scoring all markers for 100 spore colonies, and subsequently by plating and genotyping cells from the growing cultures.

Acquisition and Analysis of Fluorescence Images. Liquid cultures were set up in 1 to 2 mL EMM2 minimum medium supplemented as needed and incubated with vigorous agitation at 30 °C. The cultures were inoculated with free spore preparations for heterochromatin establishment experiments or with single colonies to measure the proportion of cells expressing Kin12::YFP or [EcoRⅤ]::mCherry in strains of interest, in which case on the order of 6 to
10 cultures were inoculated in parallel for each strain. Fluorescence images were acquired with an Xcyto 10 Quantitative Cell Imager run with XcytoView 1.0. A glass coverslip was placed on top of 50 μl culture onto a 24-well chamber glass slide (ChemoMetec) and imaging 10,000 cells or 50 fields. Exposure times of 1 s were used for both YFP and mCherry. To exclude spots present at the early time points of heterochromatin establishment experiments and occasional cell aggregates, gating was imposed based on cell perimeter. Images were analyzed with XcytoView 1.0.109.0 software and visually inspected to introduce corrections when necessary. Files retrieved in .fcs format were further analyzed on a Nikon Ti Eclipse microscope two-color used for time-lapse microscopy and other fluorescence images were acquired with a Zeiss Axioscope microscope, in both cases using 100x objectives.

**Heterochromatin Maintenance Experiments.** Time-lapse microscopy was performed by imaging isolated cells and their descendants every 3 h. For each strain, SC50, SC51, and SC52, ~2 μl cell suspension was spotted onto a small agarose patch in supplemented EMM2 medium, left to dry for a few minutes, and inverted onto a 24 × 50-mm no. 1.0 microscope coverslip that could accommodate the three patches. The coverslip was used to close a small homemade imaging chamber and positioned on the stage of the Nikon Ti Eclipse microscope enclosed in a temperature-controlled chamber kept at 30°C. Approximately 10 positions were chosen under each patch using NIS-Elements software and images were collected every 3 h for 20 h in the bright-field, YFP, and mCherry channels, using a combination of autofocus and the Perfect Focus System to maintain the focus. The resulting time-lapse series were hand-counted to determine the number of total cell divisions and the number of cell divisions in which the fluorescent reporters had been turned on.

**Data Availability.** All study data are included in the article, SI and appendices.