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INVESTIGATION

Dependency of Heterochromatin Domains on Replication Factors

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ABSTRACT Chromatin structure regulates both genome expression and dynamics in eukaryotes, where large heterochromatic regions are epigenetically silenced through the methylation of histone H3K9, histone deacetylation, and the assembly of repressive complexes. Previous genetic screens with the fission yeast Schizosaccharomyces pombe have led to the identification of key enzymatic activities and structural constituents of heterochromatin. We report here on additional factors discovered by screening a library of deletion mutants for silencing defects at the edge of a heterochromatic domain bound by its natural boundary—the IR-R+ element—or by ectopic boundaries. We found that several components of the DNA replication progression complex (RPC), including Mrc1/Claspin, Mcl1/Ctf4, Swi1/Timeless, Swi3/Tipin, and the FACT subunit Pob3, are essential for robust heterochromatic silencing, as are the ubiquitin ligase components Pof3 and Def1, which have been implicated in the removal of stalled DNA and RNA polymerases from chromatin. Moreover, the search identified the cohesin release factor Wpl1 and the forkhead protein Fkh2, both likely to function through genome organization, the Ssz1 chaperone, the Fkbp39 proline cis-trans isomerase, which acts on histone H3P30 and P38 in Saccharomyces cerevisiae, and the chromatin remodeler Fft3. In addition to their effects in the mating-type region, to varying extents, these factors take part in heterochromatic silencing in pericentromeric regions and telomeres, revealing for many a general effect in heterochromatin. This list of factors provides precious new clues with which to study the spatio-temporal organization and dynamics of heterochromatic regions in connection with DNA replication.

KEYWORDS chromatin remodeling cohesins DNA replication fission yeast gene silencing genetic screen heterochromatin mating-type region nuclear organization proline isomerase

The various chromatin states found in eukaryotes fall into two histologically distinct types: euchromatin, which sustains gene expression, and heterochromatin, with repressive functions. While numerous forms of euchromatin can be distinguished based on histone modifications, heterochromatin is more homogenously characterized by the methylation of histone H3 at lysine 9 (H3K9me). H3K9me silences repeats and transposable elements that together constitute more than half of the human genome (Gregory 2005), and recent investigations of embryonic stem cells and cell reprogramming have further shown the importance of H3K9me in maintaining cell identity in differentiated cells through the formation of facultative heterochromatin (Becker et al. 2016). Beyond the control of gene expression, chromatin fulfills structural roles and participates in DNA recombination, and in the repair, replication, and condensation of chromosomes. How these various functions are coordinated to accommodate each other at the chromatin level is an important aspect of eukaryotic genome biology.

Much knowledge of chromatin structure and function stems from studies of the fission yeast Schizosaccharomyces pombe. Genetic screens in this organism produced mutants of the H3K9 methyltransferase Clr4 (Ekwall and Ruusala 1994; Thon et al. 1994), which together with its metazoan homolog SUVAR39 was the first identified histone methyltransferase (Rea et al. 2000). Clr4 and SUVAR39 establish large heterochromatic domains. The genetic screens that identified the clr4 locus relied on derepression of the silent mating-type loci and nearby reporters. Not only do clr4 mutants express these normally silent genes due to loss of H3K9me heterochromatin, but several aspects of mitotic
and meiotic recombination are also altered in the mutants (Thon et al. 1994; Ellermeier et al. 2010). Thus, the system provides an example where chromatin structure coordinately controls gene expression and recombination through H3K9me. In addition to Clr4, genetic screens have produced mutants in: the HP1-like protein Swi6 (Gutz and Schmidt 1983; Lorenz et al. 1994); the Clr7 (a.k.a. Raf2, Cmc2, and Dos2), Clr8 (a.k.a. Raf1, Cmc1, and Dos1), and Rik1 subunits of the Clr4-associated complex CLRC (Li et al. 2005; Thon et al. 2005); the Clr1, Clr2, and Clr3 subunits of the SHREC histone deacetylase complex (Thon and Klar 1992; Ekwall and Ruusala 1994; Thon et al. 1994; Sugiyama et al. 2007; Job et al. 2016); Clr5 (Hansen et al. 2011); the Clr6 histone deacetylase (Grewal et al. 1998); and the Ubc4 E2 ubiquitin ligase (Irvin et al. 2009). This mutant assortment mirrors mutants isolated in Drosophila (Elgin and Reuter 2013) and mouse (Blewitt et al. 2005), and emphasizes the evolutionarily conserved properties of heterochromatin, such as H3K9me and histone hypoacetylation.

Major players in S. pombe heterochromatin formation were identified by the screens mentioned above, although not all players. This is partly because some requirements for heterochromatin formation are location-specific. At centromeres, heterochromatin is strongly driven by RNA interference (RNAi), whereas this is less the case in the mating-type region (Ekwall and Ruusala 1994; Thon et al. 1994; Sugiyama et al. 2007; Job et al. 2016); Clr5 (Hansen et al. 2011); the Clr6 histone deacetylase (Grewal et al. 1998); and the Ubc4 E2 ubiquitin ligase (Irvin et al. 2009). This mutant assortment mirrors mutants isolated in Drosophila (Elgin and Reuter 2013) and mouse (Blewitt et al. 2005), and emphasizes the evolutionarily conserved properties of heterochromatin, such as H3K9me and histone hypoacetylation.

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**MATERIALS AND METHODS**

**Strains, genetic manipulations, media, and oligonucleotides**

The S. pombe strains used in this study are listed in Supplemental Material, Table S3 in File S1. Oligonucleotide sequences are provided in Table S4 in File S1. Media and yeast handling were conducted according to Ekwall and Thon (2017). NBA medium (Yeast Nitrogen Base) is identical to Saccharomyces cerevisiae SC medium.

**Screens of Bioneer deletion library**

An S. pombe deletion library of haploid strains (Bioneer, version 3) was screened in an arrayed format with the help of a ROTOR HDA robot (Singer Instruments), as described in Toteva et al. (2017) and based on Baryshnikova et al. (2010). Compared to Toteva et al. (2017), where the B6 and B7 strains were used as query strains, the following additional strains were mated here to the library: B14, B15, B16, B17, B18, and B19. In all cases, recombinant progeny of the crosses were selected for G418 resistance, conferred by the ORF deletions, and uracil prototrophy, conferred by the mating-type region of the query strains. (EcoRV): ade6 derepression was tested by growth in the absence of adenine, first in two rounds of high-throughput screening and subsequently by spot tests of a ten-fold dilution series for potential hits. Isolated colonies for the best-looking hits were obtained by streaking Bioneer library strains. The purified strains were then checked by PCR to confirm the expected ORF deletions, using Taq polymerase and primer Cn-P10 or Cp-C3, which anneal within the kanR cassette (Kim et al. 2010), in combination with loci-specific primers. Cp-N10 was used with Cp5 loci-specific primers and Cp-C3 with Cp3 loci-specific primers. Once confirmed, the purified strains were crossed again with the query strains to test the resulting Ade phenotype in ade6-DN/N progeny. ade6-DN/N was identified by PCR on genomic DNA with primers GTO-232 and GTO-233.

**Construction of mcl1, wpl1, and dpb4 deletion strains**

To retest the phenotypes of mcl1Δ::kanR (SPAPB1E7.02cΔ), wpl1Δ:: kanR (SPBC428.17cΔ), and dpb4Δ::kanR (SPBC3D6.09Δ), deletion cassettes were amplified from the corresponding Bioneer deletion library strains, respectively: V3-P19-76, V3-P02-77, and V3-P10-69, with the oligonucleotide pairs GTO-1332 and GTO-1333, GTO-1365 and GTO-1366, and GTO-1363 and GTO-1364, and Phusion HF DNA polymerase (Thermo Fisher Scientific). The mcl1Δ::kanR amplicon

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**Genes | Genomes | Genetics**
Table 1 List of silencing factors identified in this study, with references to prior identification in genetic screens or candidate and biochemical approaches

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Product</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histone deacetylase complexes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPBC2D10.17</td>
<td>Clr1</td>
<td>SHREC complex cryptic loci regulator 1 subunit</td>
<td>Thon and Klar (1992), Sugiyama et al. (2007)</td>
</tr>
<tr>
<td>SPAC1B3.17</td>
<td>Clr2</td>
<td>SHREC complex cryptic loci regulator 2 subunit</td>
<td>Ekwall and Ruusala (1994), Thon et al. (1994), Sugiyama et al. (2007)</td>
</tr>
<tr>
<td>SPBC800.03</td>
<td>Clr3</td>
<td>SHREC complex cryptic loci regulator 3 subunit (histone deacetylase)</td>
<td>Ekwall and Ruusala (1994), Thon et al. (1994), Sugiyama et al. (2007)</td>
</tr>
<tr>
<td>SPBP3SG2.10</td>
<td>Mit1</td>
<td>SHREC complex ATP-dependent DNA helicase subunit Mit1</td>
<td>Sugiyama et al. (2007)</td>
</tr>
<tr>
<td>SPAC16C9.05</td>
<td>Cph1</td>
<td>Ctr6 histone deacetylase-associated PHD protein-1</td>
<td>Nicolas et al. (2007)</td>
</tr>
<tr>
<td><strong>Histone H3K9 methyltransferase complex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPBC428.08c</td>
<td>Clr4</td>
<td>Cryptic loci regulator 4 (histone H3K9 methyltransferase)</td>
<td>Ekwall and Ruusala (1994), Thon et al. (1994), Horn et al. (2005)</td>
</tr>
<tr>
<td>SPCC970.07c</td>
<td>Raf2/</td>
<td>Rik1-associated factor 2/delocalization of Swi6 factor 2/cullin-associated</td>
<td>Hong et al. (2005), Horn et al. (2005), Li et al. (2005), Thon et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>Dos2/</td>
<td>Cmc2/</td>
<td></td>
</tr>
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<td>Clr7</td>
<td>2/cryptic loci regulator 7</td>
<td></td>
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<tr>
<td>SPCC613.12c</td>
<td>Raf1/</td>
<td>Rik1-associated factor 1/delocalization of Swi6 factor 2/cullin-associated</td>
<td>Hong et al. (2005), Horn et al. (2005), Li et al. (2005), Thon et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>Dos1/</td>
<td>Cmc1/</td>
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<td></td>
<td>Clr8</td>
<td>1/cryptic loci regulator 8</td>
<td></td>
</tr>
<tr>
<td>SPCC11E10.08</td>
<td>Rik1</td>
<td>Silencing protein Rik1</td>
<td>Ekwall and Ruusala (1994), Hong et al. (2005), Thon et al. (2005)</td>
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<tr>
<td><strong>HP1 family and associated factors</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SPAC1071.02</td>
<td>Mms19</td>
<td>Dos2 silencing complex subunit</td>
<td>Li et al. (2011)</td>
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<tr>
<td><strong>CRL4</strong></td>
<td></td>
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<td></td>
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<td>SPAC664.01c</td>
<td>Swi6</td>
<td>Chromodomain protein Swi6</td>
<td>Gutz and Schmidt (1985)</td>
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<tr>
<td>SPAC1851.03</td>
<td>Ckb1</td>
<td>CK2 family regulatory subunit</td>
<td>Shimada et al. (2009), Braun et al. (2011)</td>
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<td><strong>CSN</strong></td>
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<td></td>
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<td>SPBC215.03c</td>
<td>Csn1</td>
<td>COP9 signalosome complex subunit Csn1</td>
<td>Bayne et al. (2014)</td>
</tr>
<tr>
<td><strong>RPC</strong></td>
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<tr>
<td>SPAC694.06c</td>
<td>Mrc1</td>
<td>Mediator of replication checkpoint 1</td>
<td>Mamnun et al. (2006), Natsume et al. (2008)</td>
</tr>
<tr>
<td>SPAPB1E7.02c</td>
<td>Mcl1</td>
<td>DNA polymerase α accessory factor</td>
<td></td>
</tr>
<tr>
<td>SPBC216.06c</td>
<td>Swi1</td>
<td>Replication fork protection complex subunit Swi1</td>
<td></td>
</tr>
<tr>
<td>SPBC30D10.04</td>
<td>Swi3</td>
<td>Replication fork protection complex subunit Swi3</td>
<td></td>
</tr>
<tr>
<td><strong>Ubiquitin-SUMO-related</strong></td>
<td></td>
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<tr>
<td>SPBC338.16</td>
<td>Pof3</td>
<td>F-box protein</td>
<td>Katayama et al. (2002), Mamnun et al. (2006)</td>
</tr>
<tr>
<td><strong>Gene organization/replication timing</strong></td>
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<td></td>
<td></td>
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<tr>
<td>SPBC16G5.15c</td>
<td>Fkh2</td>
<td>Forkhead transcription factor Fkh2</td>
<td></td>
</tr>
<tr>
<td>SPBC428.17c</td>
<td>Wpl1</td>
<td>Cohesin loading/unloading factor (WAPL) Wpl1</td>
<td></td>
</tr>
<tr>
<td>SPAC6F6.17</td>
<td>Rif1</td>
<td>Telomere length regulator protein Rif1</td>
<td>Toteva et al. (2017)</td>
</tr>
<tr>
<td>SPBC1198.11c</td>
<td>Reb1</td>
<td>RNA polymerase 1 transcription termination factor /replication block</td>
<td>Jakociunas et al. (2013)</td>
</tr>
<tr>
<td><strong>Chromatin remodelers/chaperones</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPAC25A8.01c</td>
<td>Fft3</td>
<td>Fission yeast fun 30 related protein</td>
<td>Straifors et al. (2011)</td>
</tr>
<tr>
<td>SPAC31G5.19</td>
<td>Abo1</td>
<td>ATPase with bromodomain protein</td>
<td>Gal et al. (2016)</td>
</tr>
</tbody>
</table>

(continued)
was cloned into the EcoRV site of plasmid pJET1.2 (Thermo Fisher Scientific), generating plasmid pPA7. This plasmid was digested with BglII to release the mcl1Δ:kanR insert for *S. pombe* transformation, while the purified amplicons for wpl1Δ:kanR and dpb4Δ:kanR were used directly. Each deletion was introduced into the *S. pombe* strains PG9350, PC152, PT600, and PG3947, and transformants were selected on YES plates with G418. Gene replacements were confirmed by PCR with primers Cp-N10 or Cp-C3 (Kim et al. 2010) employed as above with locus-specific primers: Cpg-mcl1D, Cpg-wpl1D, and Cpg-dpb4D were used with Cp-N10, and Cpg-mcl1D, Cpg-wpl1D, and Cpg-dpb4D were used with Cp-C3. The PG9350, PC152, PT600, and PG3947 transformants with mcl1Δ:kanR were named PA71, PA72, PA73, and PA74; those with dpb4Δ:kanR were named PA77, PA78, PA79, PA80, and PA81; and those with wpl1Δ:kanR were named PA82, PA83, PA84, and PA85. During the construction of these strains, transformants were replica- plated onto medium with limited adenine (YE) and medium lacking adenine (AA-ade) at several steps, including following the original G418 selection of transformants, during the colony purification of multiple candidates to gauge effects on (EcoRV):ade6* expression, and to ensure that the phenotypes of the strains saved in the collection were representative.

**RNA preparation and RT-QPCR**

(EcoRV)::ade6* RNA levels were monitored as described previously (Hansen et al. 2005; Jacobiunas et al. 2013). Briefly, for each strain to be examined, three isolated colonies were first patched on YES and subsequently propagated overnight in 50 ml EMM2 cultures supplemented with adenine, leucine, and uracil, to an OD600 of 0.4. Cell pellets were flash frozen in liquid nitrogen and stored at −80°C. Following RNA extraction, 2 μg RNA were treated with RQ1 DNase (Promega), diluted with DEPC-treated H2O to a final volume of 200 μl, and stored at −20°C. QPCRs were set up with 7.5 μl DNase-treated RNA samples in 25 μl reactions using a QuantiFast Multiplex RT-PCR kit (QIAGEN) and two primers at 1 μM each. Three technical replicates were set up for each reaction. The reactions were performed as suggested by the manufacturer: 10 min at 50°C, followed by 5 min at 95°C and 39 cycles of 10 sec at 95°C, and 30 sec at 60°C, with data capture at each cycle, in a Bio-Rad CFX96 Thermal Cycler. Melting curves were produced on the final products and no RT controls were included. Reactions were set up with the primer pairs GTO-218 and GTO-219 (ade6*), and TJO-55 and TJO-58 (act1*). Data were analyzed using Bio-Rad CFX Manager and Microsoft Excel, normalizing ade6* expression to act1* in all samples.

Mean normalized expression (MNE) values were calculated according to the following equation: MNE = (Eact1CTact1, mean) / (Eade6CTade6, mean), where Eact1 is the efficiency of act1* RNA amplification determined on 10-fold serial dilutions of genomic DNA, CTact1,mean is the mean cycle threshold for act1* RNA amplification, Eade6 is the efficiency of amplification of the target of interest (ade6*) determined on 10-fold serial dilutions of genomic DNA, and CTade6,mean is the mean cycle threshold for ade6* RNA amplification.

**Data availability**

Strains are available upon request. All Supplemental Tables and Figures are regrouped in File S1. Table S1 in File S1 presents a list of Bioneer deletion strains selected for the second and third mutant screens and Table S2 in File S1 gives a list of potential false positives. Figures S1–S8 in File S1 show the phenotypes of mutants examined in the second screen, while Figures S9–S11 in File S1 show the phenotypes of mutants examined in the third screen. Table S3 in File S1 contains a list of strains and their genotypes, while Table S4 in File S1 contains oligonucleotide sequences.

**RESULTS AND DISCUSSION**

**Screen for factors required for heterochromatin integrity close to domain boundaries**

An arrayed library of 3308 *S. pombe* ORF deletion strains (Kim et al. 2010) was used to determine the requirements for heterochromatic gene silencing at the edge of the mating-type region in eight strains: a strain with the wild-type IR-R+ boundary and strains with, respectively: STAR1-4, BTH1, an rDNA repeat, or the edge of cent1 in the place of IR-R+ (Figure 1A). The eight query strains contained the (EcoRV)::ade6* reporter gene close to the edge of the heterochromatic domain to provide a readout for gene silencing (Thon et al. 1999), and a euchromatic ura4* gene tightly linked to the mating-type region to allow for its selection (Xnm1::ura4*). The query strains were crossed with the deletion library in parallel large-scale experiments. Progeny combining the mating-type region of interest with each ORF deletion were selected according to the scheme in Figure 1B and tested for (EcoRV)::ade6* expression. Candidate factors whose deletions appeared to permit the expression of (EcoRV)::ade6* were identified. At this stage, even deletions with a dubious phenotype were chosen for further examination alongside deletions with a potentially strong effect. The corresponding 196 strains from the Bioneer library were rearrayed on a single plate.
(191 strains) or processed individually (five strains) and retested by genetic crosses against all query strains, leading to a shorter list of 48 factors potentially required for (EcoRV)::ade6+ repression (Figures S1–S8 and Table S1). Factors with extensively documented effects on heterochromatin formation were not tested further, and we focused instead on factors whose effects on heterochromatin had not been documented before, or not tested in the mating-type region. Recombinant Ura+ G418-resistant progeny were colony-purified and spotted on indicator and selective plates to better evaluate (EcoRV)::ade6+ expression, leading to a short list of 33 candidate genes with a heterochromatic silencing defect in at least one of the queried mating-type regions. For these candidates, single colonies were isolated from the Bioneer collection, the ORF deletions were verified by PCR using locus-specific primers, and we obtained recombinant progeny combining the mating-type regions of interest with each ORF deletion in an ade6-DN/N background. The ade6-DN/N background is well suited to the monitoring of ade6+ expression by colony color on a limiting concentration of adenine or by RT-QPCR with primers that specifically recognize ade6+. In addition to Bioneer strains, three deletion strains that were not present in the library were added to the analysis (abo2Δ is shown for comparison with the deletion of its paralog abo1Δ).

Comprehensive spot-test analyses assessing silencing by factors that made it through the entire screen are shown in Figures S9–S11. A subset of factors are presented in Figure 2 and Figure 3 for their effects on all query strains, and in Figure 4 and Figure 5 for their effects with the wild-type boundary IR-R+. Derepression of (EcoRV)::ade6+ occurs in IR-RΔ and clr4Δ cells, resulting in white or light pink colonies on medium with limiting adenine and improved growth in the absence of adenine. While not as pronounced as for clr4Δ, the phenotypes of the other mutations tested were distinctive and were confirmed by quantification of the (EcoRV)::ade6+ transcript (Figure 5). Increases ranging from 5- to 40-fold over wild-type were measured and normalized to (EcoRV)::ade6+ expression in the IR-RΔ strain in Figure 5.
amplified by PCR, together with 500 bp–1 kb flanking DNA from genomic DNA of the Bioneer deletion strain, and used to transform tester strains to confirm effects suggested by the genetic screens. Little effect was seen in dpb4Δ cells, suggesting that the originally observed phenotype was artifactual, while derepression by mcl1Δ and wpl1Δ was confirmed (Figure 7).

The silencing factors that were retested throughout the whole scheme are listed in Table 1, together with references to prior literature for those that had been described previously. In addition to known components of heterochromatin, the screens identified factors that had eluded all previous screens or that had not been examined for effects in the mating-type region. A few were element-specific, such as Rif1 acting with the STAR elements (Toteva et al. 2017), while others were required for (EcoRV):ade6+ silencing in all or nearly all query strains.

### Requirement for histone deacetylases and methyltransferases

The sole proteins required for (EcoRV):ade6+ silencing in all configurations of the mating-type region tested were Clr1, Clr2, and Clr3; that is, the deacetylase module of the SHREC complex (Figure 2). Most boundary elements also relied on the chromatin remodeler module of SHREC, Mit1, with the exception of BTH1. Some independence of the deacetylase and remodeler modules of SHREC is predicted by a structure–function analysis of the complex (Job et al. 2016). The histone H3K9 methyltransferase Ctr4, the Clr7, Clr8, and Rik1 subunits of the CLRC complex, and the chromodomain protein Swi6 were also broadly identified, in all screens except for the full-length rDNA repeat (Figure 2A). The rDNA repeat is exceptional in that it silences (EcoRV):ade6+ tightly through
antisense transcription and histone deacetylation, even in the absence of Clr4 (Jakociunas et al. 2013). The Clr6 histone deacetylase-associated PHD protein-1 Cph1 was also identified in several screens, albeit with relatively weak phenotypes (Figure 2). Moreover, deletion of the mec46 gene adjacent to clr4 resulted in the same strong derepression as clr4Δ (Figure 2), possibly due to impaired clr4 expression. Altogether, this first set of mutants emphasizes the central roles of deacetylation and methylation in heterochromatic gene silencing, and validates the screening strategy.

Requirement for Ddb1-Cdt2 and Csn1

Nearly all screens also identified the Ddb1, Cdt2, and Csn1 proteins (Figure 2 and Figure S9 in File S1 for ddb1Δ). Ddb1 and Cdt2 are part of the Cullin 4 ubiquitin ligase CRL4Cdt2 that targets specific proteins for degradation by the proteasome. In Cullin complexes, an adaptor protein, Cdt2 in this case, interacts with cognate substrates to display them to the ubiquitin ligase activity. Three CRL4Cdt2 substrates are known in S. pombe: the putative histone H3K9 demethylase Epe1 (Braun et al. 2011), the licensing factor Cdt1 that is required for the firing of replication origins (Ralph et al. 2006); and the inhibitor of ribonucleotide reductase Spd1 (Liu et al. 2003). According to previous work, stabilization of Epe1 in the cdt2Δ and ddb1Δ mutants and its accumulation in heterochromatin would suffice to derepress (EcoRV)::ade6+ (Braun et al. 2011). Deletion of the COP9 signalosome (CSN) subunit Csn1 also resulted in reduced silencing (Figure 2). Reduced silencing in the csn1Δ mutant might result from CRL4Cdt2 deregulation (Liu et al. 2003), or from the destabilization of other cullin ligases such as SCFPof3, whose F-box component Pof3 is proteolyzed in CSN mutants (Schmidt et al. 2009). Indicative of a general requirement in heterochromatin, Csn1 was also identified in a recent genetic screen for heterochromatic silencing defects at centromeres (Bayne et al. 2014).

Requirement for Def1, Pof3, and Pli1

Further links to ubiquitylation were revealed with the identification of the proposed homolog of S. cerevisiae Def1 and the F-box protein Pof3 (Figure 3, Figure 4, Figure 5, Figure 6, and Figures S9 and S10 in File S1). To our knowledge, this is the first evidence for an effect of Def1 in heterochromatin. In S. cerevisiae, Def1 mediates the ubiquitylation and degradation of the Rpb1 subunit of RNA polymerase II (RNAPII) during transcriptional stress (Wilson et al. 2013), and of Pol3, the catalytic subunit of the replicative DNA polymerase δ, in postreplicative repair (Daraba et al. 2014). In S. pombe, Def1 might control polymerase usage in heterochromatin, remove RNAPII from heterochromatin to promote
transcriptional silencing, or prevent collisions between DNA and RNA polymerases, a proposed cause of heterochromatin instability (Zaratiegui et al. 2011). The functions of Po3, homologous to Dia2 in S. cerevisiae, are understood in much greater detail than those of Def1. In S. pombe, chromosomes are lost in the absence of Po3, telomeres are shortened, telomere and centromere silencing is alleviated, and the DNA damage checkpoint is continuously activated (Katayama et al. 2002; Mamnun et al. 2006). Po3 associates with the replisome. Its targets, inferred from the increased stability in po3Δ cells, include the mediator of replication checkpoint Mcr1, Pol e, and subunits of the CMG helicase, each of which could be relevant to the observed effects (see below; Roseaulin et al. 2013a,b; Maculins et al. 2015). Other targets of Po3 potentially relevant to heterochromatic silencing would be Ams2, a regulator of histone gene transcription (Takayama et al. 2010), or other DNA-binding proteins that might be removed by Po3 as Po3 travels with the replication fork.

The screens also detected a small contribution of the SUMO E3 ligase Pli1 to (EcoRV)::ade6+ silencing (Figure 3), compatible with previously observed limited derepression of centromeric reporters in pli1Δ cells (Xhemalce et al. 2004).

**Requirement for the RPC**

Mcr1/Claspin, Mc1 (Cif4 in S. cerevisiae), Sw1 (Timeless in animals or Tof1 in S. cerevisiae), Swi3 (Tipin or Csm3), and the histone chaperone FACT are part of the RPC essential to normal replication fork progression (Gambus et al. 2006; Foltman et al. 2013; Yeeles et al. 2017). Here, Mcr1, Mc1, Swi1, Swi3, and the Pob3 subunit of FACT were all necessary for tight (EcoRV)::ade6+ silencing (Figure 4, Figure 7, and Figure S9 in File S1). Mcr1, Mc1, Swi1, and Swi3 also ensure replication fork protection at replication blocks (Noguchi et al. 2004; Shimmoto et al. 2009) and telomeric repeats (Gadaleta et al. 2016). Structurally, Mc1/Cif4 forms a trimer that connects the CMG helicase to the lagging-strand primase Polα, and various accessory factors to the replisome (Williams and McIntosh 2002, 2005; Tsutsumi et al. 2005; Simon et al. 2014; Samora et al. 2016; Villa et al. 2016). Po3/Dia2 in particular, also identified in our screen, is associated with the replisome in an Mc1- and Mcr1-dependent manner (Mamnun et al. 2006; Morohashi et al. 2009). Hence, the silencing defect in RPC mutants might be due to a failed association of Po3 with the fork in these mutants. Since Po3 is responsible for the short half-life of Mcr1 in wild-type S. pombe cells, and mediates the degradation of DNA polymerases and MCM helicases in the absence of the RPC (Roseaulin et al. 2013a,b), the silencing defect in po3Δ cells might also result from a misregulated RPC.

**Further requirements for DNA replication/genome organization factors**

Other proteins implicated in the control of DNA replication or genome organization identified in the screens were the forkhead protein Fkh2, whose S. cerevisiae homologs Fkh1 and Fkh2 organize the long-range clustering and activation of early-firing origins (Knott et al. 2012) and long-range chromosomal interactions occurring during mating-type switching (Li et al. 2012), and the cohesin release factor Wings apart-like Wpl1 (Figure 3, Figure 7, and Figure S9 in File S1). In the absence of Wpl1, (EcoRV)::ade6+ showed a strongly variegated phenotype (Figure 7). In mammals, ΔWAPL cells display altered chromatin loop structure, reduced long-range chromosomal interactions in cis, and decreased nuclear compartmentalization that correlates with gene derepression in normally silent compartments (Haarhuis et al. 2017). These effects might result from the aberrant distribution or accumulation of cohesin, and might be evolutionarily conserved since cohesins participate in S. pombe genome architecture (Mizuguchi et al. 2014) and a rad21 mutation causes heterochromatin loss in subtelomeric regions (Dheur et al. 2011). Furthermore, components of the RPC (including Swi1, Swi3, Mcr1, and Mc1) are required for sister chromatid cohesion (e.g., Williams and McIntosh 2002; Xu et al. 2004; Lengronne et al. 2006; Ansbach et al. 2008), hence the effects of the RPC, and Wpl1 might overlap mechanistically.

The screens also confirmed the importance of the iron–sulfur cluster assembly protein Mms19 to heterochromatin (Figure 3). Mms19 co-urifies with the silencing factor Clr7/Dos2 (Li et al. 2011).
Requirement for chromatin remodelers and chaperones

In addition to the SHREC subunit Mit1 and the FACT subunit Pob3, the SMARCAD1 family ATP-dependent DNA helicase Fft3 and the ATPase with bromodomain Abo1 were also broadly required for silencing by the various boundary elements used in the screens (Figure 3, Figure 4, Figure 5, Figure 6, and Figure S10 in File S1). In previous studies, Fft3 was found to affect heterochromatic boundaries at centromeres and telomeres (Stralfors et al. 2011), and functions of Abo1 in heterochromatin were recently documented as well (Gal et al. 2016). In our study, deletion of Abo1 conferred relatively modest, albeit distinguishable, phenotypes (Figure 3, Figure 4, Figure 5, Figure 6, and Figure S10 in File S1). The Abo1 paralogue Abo2 was not isolated in the initial screens, but was examined for comparison with Abo1 and displayed more minor effects, if any.

Newly identified factors whose deletion caused a pronounced derepression of (EcoRV)::ade6+ included the predicted peptidyl-prolyl cis-trans isomerase Fkbp39 and the Ssz1 chaperone, an HSP70 family

Figure 7 Effects of Mcl1 and Wpl1 on heterochromatic silencing. (A) The mating-type regions used in this experiment featured two ectopic boundaries, STAR2 and the 85 bp STAR2-S element (Toteva et al. 2017), in addition to wild-type IR-R. (B) mcl1Δ and wpl1Δ mutants with the indicated boundaries were spotted on low adenine to compare (EcoRV)::ade6+ expression with the wild-type background. mcl1Δ confers a pronounced growth defect; however, the formed (EcoRV)::ade6+ mcl1Δ colonies are Ade+. wpl1Δ induces strong variegation. The strains were wild-type: PG3947, PG3950, PC152, and PT600; mcl1Δ: PA71, PA72, PA73, and PA74; and wpl1Δ: PA82, PA83, PA84, and PA85. YE, medium with limited adenine.

Figure 8 Effects of selected factors on telomeric and centromeric silencing. Serial dilutions (10-fold) of cell suspensions were spotted. (A) Effects on the m23::ura4+-Tel[72] telomeric reporter gene. Derepression of the reporter improves plating efficiency on AA-ura and decreases white colony formation on FOA medium with low adenine concentration (where only cells with a repressed reporter can form white colonies). Strains used: LJ171, FY520, LJ163, LJ172, LJ173, LJ162, LJ174, LJ165, LJ170, LJ168, LJ161, and LJ166. (B) Effects on centromeric otr1(SphI)::ura4+ reporter gene. Strains used: LJ185, FY648, LJ180, LJ186, LJ190, LJ192, LJ187, LJ183, LJ181, LJ191, and LJ189. AA-ura, medium lacking uracil; ade, adenine; Chr, chromosome; FOA, fluoroorotic acid; WT, wild-type.
Effects at telomeres and centromeres

The effects of selected ORF deletions on telomeric silencing was assayed with the m23::ura4+ Tel[72] reporter close to the end of a truncated minichromosome Ch16 (Nimmo et al. 1994; Figure 8A). In this assay, cells containing the minichromosome are Ade+ due to intragenic complementation between the ade6-210 allele on chromosome 3 and ade6-216 on the minichromosome. Several deletions (mrc1Δ, def1Δ, szs1Δ, Fkbp39Δ, ril1Δ, and rif1Δ) increased growth on medium lacking uracil, indicative of m23::ura4+ Tel[72] derepression. For some strains (mrc1Δ, szs1Δ, and ft3Δ in Figure 8A), FOA strongly selected for cells that had lost their minichromosome, resulting in red colonies on FOA with limiting adenine. This is expected for mutants in which ura4+ cannot be repressed effectively; only cells that have lost the mini-chromosome are phenotypically Ura-. The ort1R(SphI)::ura4+ insertion in centromere 1 (Allshire et al. 1995) was used to test effects on pericentromeric silencing. These were less pronounced than for the edge of the mating-type region or sub-telomere (Figure 8B), perhaps due to the location of the ort1R(SphI)::ura4+ reporter, embedded in RNAi-responsive heterochromatin.

Interplays between replication and heterochromatin

Physical and functional links between DNA replication and heterochromatin formation have been discovered in numerous organisms, and are proposed to participate in the epigenetic maintenance of heterochromatin during cell division. In S. pombe, the Clr7/Dos2 subunit of the H3K9 methyltransferase complex CLRC binds Polε, and Polε mutants with reduced affinity for Dos2/Clr7 exhibit heterochromatin defects (Li et al. 2011). Mutations in the primase Polα (Nakayama et al. 2001; Natsume et al. 2008) or in the Mcm4 helicase (Toteva et al. 2017) also cause heterochromatin defects, as well as mutations in nonessential replication factors (Natsume et al. 2008). Our present results emphasize the tight links between replication and heterochromatin integrity by identifying multiple RPC components and associated chromatin modifiers (Figure 9).

A first mechanism through which RPC components might facilitate the maintenance of heterochromatin is through the stabilization of ongoing replication forks. It has been observed that replisome components are, under some circumstances, degraded upon replication stress (Roseaulin et al. 2013a,b; Daraba et al. 2014); in particular, both Polε and replicative helicases become unstable in S. pombe in the absence of the RPC component Swi1 (Roseaulin et al. 2013a,b). The breakdown of replisomes at replication forks that encounter an impediment in heterochromatin might lead to the dissociation of CLRC from the fork, or to a fork restart using different DNA polymerases that do not interact with CLRC, thereby weakening heterochromatin (Zaratiegui et al. 2011; Lee et al. 2013; Lee and Russell 2013). Similarly, control of homologous recombination, both during and outside S phase, might favor heterochromatin maintenance by preventing the use of polymerases other than Polε (Zaratiegui et al. 2011; Miyabe et al. 2015). A requirement
for the stabilization of replication forks in heterochromatic regions would be compounded by the fact that heterochromatin appears to be difficult to replicate and prone to fork stalling (Zaratiegui et al. 2011; Lee et al. 2013; Lee and Russell 2013).

A second mechanism through which RPC components might reinforce heterochromatin is through the control of replication origin firing. Mcr1/Claspin has dual roles in replication fork progression and in the DNA replication timing program. Other factors isolated in our screen also affect replication origin usage. Thus Rif1, necessary for boundary activity and heterochromatin protection by the STAR boundaries (Toteva et al. 2017), controls the replication timing program across species (Hayano et al. 2012; Yamazaki et al. 2012; Peace et al. 2014). In S. pombe, both Mcr1 and Rif1 mutations alter DNA replication genome-wide and bypass the requirement for the DNAK kinase Hsk1 (Matsumoto et al. 2011; Hayano et al. 2012). Consistent with coordinated control of origin firing and heterochromatin maintenance, a mutation in the N-terminus of the Mcm4 helicase, which removes a part of the protein that is predicted to confer DNAK control on origin firing, weakens heterochrominal silencing close to IR-R+ or artificial boundaries (Toteva et al. 2017). In addition, the forkhead protein Fkh2 has well-documented roles in replication origin usage in S. cerevisiae (Knot et al. 2012). Altogether, these observations indicate that the separation of replication domains, possibly facilitated by boundary elements, is of importance to heterochromatin integrity, for instance by controlling the type of replisomes that operate in particular domains.

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