Iron homeostasis regulates facultative heterochromatin assembly in adaptive genome control

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Iron metabolism is critical for sustaining life and maintaining human health. Here, we find that iron homeostasis is linked to facultative heterochromatin assembly and regulation of gene expression during adaptive genome control. We show that the fission yeast Clr4/Suv39h histone methyltransferase is part of a rheostat-like mechanism in which transcriptional upregulation of mRNAs in response to environmental change provides feedback to prevent their uncontrolled expression through heterochromatin assembly. Interestingly, proper iron homeostasis is required, as iron depletion or downregulation of iron transporters causes defects in heterochromatin assembly and unrestrained upregulation of gene expression. Remarkably, an unbiased genetic screen revealed that restoration of iron homeostasis is sufficient to re-establish facultative heterochromatin and proper gene control genome-wide. These results establish a role for iron homeostasis in facultative heterochromatin assembly and reveal a dynamic mechanism for reprogramming the genome in response to environmental changes.

The genome of eukaryotic cells can be reprogrammed to allow adaptation to changes in growth conditions or to respond to developmental signals1–4. Reprogramming requires intricate epigenetic mechanisms that orchestrate dynamic changes in gene expression patterns through factors involved in the assembly of ‘open’ or ‘closed’ chromatin domains known as euchromatin and heterochromatin, respectively5–8. Moreover, post-transcriptional mechanisms that regulate RNA stability also control gene expression9–12. However, how transcriptional and post-transcriptional factors coordinate to establish genome-wide expression patterns that are critical for growth under diverse conditions is poorly understood.

The fission yeast Schizosaccharomyces pombe is an excellent model system to explore adaptive control of the genome. Chromatin modification factors involved in gene regulation in higher eukaryotes, such as histone-modifying activities that assemble specialized chromatin domains, are highly conserved in fission yeast1. Clr4, a member of the mammalian SUV39 histone methyltransferase family, is the sole enzyme that methylates histone H3 lysine 9 (H3K9) to assemble constitutive heterochromatin domains and discrete facultative heterochromatin islands13,14. Additionally, histone deacetylases (HDACs) help to assemble repressive chromatin structures15. Among the HDACs, Cdr6 exists in at least two distinct protein complexes: complex I, which contains the essential Sin3 homolog Pst1 and targets gene promoters, and complex-II, which contains the nonessential Sin3 homolog Pst2 and targets open reading frames (ORFs)16.

Gene expression is also regulated by RNA processing and degradation factors that are conserved from S. pombe to mammals17. A network of nuclear RNA elimination factors, including the Mt1l–Red1 core (MTREC) protein complex, composed of the Mtr4-like RNA helicase Mt1l and the zinc-finger protein Red1, prevents untimely gene expression18,19. MTREC, which is functionally related to mammalian PAXT20, and its associated proteins promote RNA degradation by the 3′–5′ exosome exonuclease Rrp6 (refs 17,19). Additionally, the RNAi machinery, including Argonaute (Ago1), Dicer (Dcr1) and RNA-dependent RNA polymerase (Rdp1), as well as the CCR4–NOT complex that plays roles in RNA metabolism from synthesis to decay20, target various mRNAs for degradation21–23 (Supplementary Fig. 1).

In addition to RNA degradation, RNA processing factors mediate RNA-dependent targeting of heterochromatin24,25,26,27,28,29. The RNA interference (RNAi) machinery targets facultative heterochromatin assembly at regulated genes24,26,29 and constitutive heterochromatin assembly at centromeric repeats24,25. MTREC-associated proteins recruit Clr4 to assemble facultative heterochromatin islands at meiotic genes24,26,27,28. These facultative heterochromatin domains can be modulated in response to nutritional conditions24,25. However, whether these pathways directly regulate the fluctuating pool of transcripts as part of cellular adaptation to environmental changes remains to be determined.

In this study, we explore the roles of heterochromatin and RNA processing factors in the regulation of gene expression patterns at a low temperature. We find that transcriptional upregulation of mRNAs in response to an environmental change provides negative feedback to prevent their uncontrolled expression through a buffering mechanism that involves heterochromatin factor Clr4. Surprisingly, we find that iron homeostasis is critical for facultative heterochromatin assembly and regulation of gene expression at low-temperature conditions. We discuss the implications of these findings in understanding how cells fine-tune gene expression and show that Clr4 limits RNA polymerase II (RNAPII) transcription at target genes to optimize their expression.

Results

Cells grown at a low temperature show widespread gene expression changes. To explore mechanisms of gene control in response
to varying growth conditions, we first asked how the transcriptome of *S. pombe* is affected by growth at a suboptimal low temperature (18°C). We analyzed the transcriptome of wild-type cells cultured at 18°C using RNA-sequencing (RNA-seq) analysis and compared our results to those obtained from cells grown under standard laboratory conditions (30°C). We observed widespread transcriptome changes in cells grown at 18°C, including 279 transcripts that were upregulated and 129 transcripts that were downregulated at least two-fold compared to cells grown at 30°C (Fig. 1a). Among the loci with increased expression at 18°C, the most highly upregulated transcripts included stress-response genes, noncoding RNAs, and genes encoding transmembrane transporters (Supplementary Fig. 2). Notably, genes encoding iron transporters were significantly upregulated at 18°C (Fig. 1a). Thus, cells exposed to a low temperature undergo an adaptive response that involves substantial changes at the transcript level.

Transcripts upregulated at 18°C are normally repressed by Clr6. To understand the mechanisms that control expression of transcripts upregulated in low-temperature conditions, we tested whether the loss of heterochromatin or RNA degradation factors affects the levels of these RNAs at 30°C. Specifically, we analyzed the transcriptomes of mutant *S. pombe* strains grown at 30°C, including cells lacking Clr4, RNAi factor Ago1, the CCR4–NOT subunit Ccr4, the MTREC component Red1, and the nuclear exosome subunit Rrp6, as well as a strain expressing a temperature-sensitive mutant allele of Clr6 HDAC. Expression profiling revealed the highest correlation between transcripts upregulated in wild-type cells grown at

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**Fig. 1** | Transcriptome analyses of cells grown at 18°C. **a**, Volcano plot showing statistical significance (−log10 P value) versus fold change (log2 fold change) of RNA-seq data from wild-type (WT) cells grown at 30°C or 18°C (n = 2 independent experiments per condition). Genes with increased expression (fold-change value ≥ 1 and P value < 0.05 by the Wald test, as implemented within the DESeq2 R library) are shown in red, and genes with decreased expression (fold-change value ≤ 1 and P value < 0.05 by the Wald test) are shown in blue. Iron-transporter genes are underlined. **b**, Heat map of fold-change values relative to WT cells grown at 30°C for the 279 transcripts upregulated in WT cells grown at 18°C, compared to mutant cells grown at 30°C (top) or 18°C (bottom). Data for *clr6-1* temperature-sensitive mutant are shown for 30°C only. **c**, Box plots representing fold-change values for the indicated strains (top, 30°C; bottom, 18°C) relative to WT cells at 30°C for the 279 transcripts upregulated in WT cells at 18°C. Whiskers for box plots are drawn at the 12.5 and 87.5 percentiles. Top and bottom edges of the box approximate the third and first quantiles, respectively, and the central line is drawn at the median. **d,e**, Area-proportional Venn diagrams representing the numbers of genes with increased expression in WT cells grown at 18°C compared with *ccr4Δ* and *clr4Δ* cells grown at 30°C (d) or 18°C (e). **f**, Heat map of fold-change values for upregulated transcripts relative to WT cells at 30°C (top) or 18°C (bottom). Clusters are grouped according to transcripts upregulated in WT only (1); WT, *ccr4Δ*, and *clr4Δ* (2); *ccr4Δ* and *clr4Δ* (3); *ccr4Δ* only (4); and *clr4Δ* only (5). Black lines on the right indicate stress-response genes. Source data for **a–f** are available with the paper online.
18 °C and those in the clr6 HDAC mutant grown at 30 °C (Fig. 1b and Supplementary Fig. 3a), suggesting that loci upregulated at low temperature are repressed by Clr6 under normal growth conditions.

Notably, we found iron-transporter genes among the Clr6-repressed loci (Supplementary Fig. 3b). To address whether Clr6 directly targets iron-transporter genes, we performed chromatin immunoprecipitation coupled to microarray analysis (ChIP-chip) of Clr6 complex subunits. Indeed, we found that Clr6 and Pst1 localized to iron-transporter genes (Supplementary Fig. 3c), suggesting that the Clr6 complex plays a direct role in their silencing.

We noted that the loss of Ago1, Red1, or Rrp6 affected a subset of upregulated transcripts in wild-type cells grown at 18 °C (Fig. 1b). These mutants formed a distinct cluster based on their correlation coefficients (Fig. 1b), which is consistent with our prior findings that RNAi, MTREC, and the exosome share targets across the genome. Surprisingly, loss of Clr4 or Ccr4 in cells cultured at 30 °C had no major impact on gene expression, suggesting that under standard growth conditions these factors are largely dispensable for the regulation of transcripts that are upregulated in wild-type cells grown at 18 °C (Fig. 1b–d).

Cells lacking Clr4 or Ccr4 show hyper-elevation of transcripts upregulated at 18 °C. We next tested whether loss of heterochromatin or RNA degradation factors affects transcript levels in cells grown at a low temperature. Interestingly, loss of Ago1 caused only minimal changes in levels of transcripts upregulated in wild-type cells grown at 18 °C (Fig. 1b,c), suggesting that RNAi is dispensable for their regulation in low-temperature conditions. By contrast, transcripts upregulated in wild-type cells grown at 18 °C were further derepressed in the absence of the RNA elimination factors Red1 or Rrp6 (Fig. 1b).

Surprisingly, despite Clr4 and Ccr4’s having little effect on transcript levels under standard growth conditions (Fig. 1b–d), they play a critical role in limiting transcript levels at 18 °C. Notably, Clr4 and Ccr4 affected a common set of targets, including transcripts upregulated in wild-type cells cultured at a low temperature that were further derepressed in cells lacking Clr4 or Ccr4 (Fig. 1b,c,e,f). Several coding and noncoding RNAs were also derepressed in the absence of Ccr4 or Clr4 (Fig. 1e,f). Strikingly, a large fraction of loci that are upregulated in clr4Δ cells at 18 °C are normally repressed by Clr6 HDAC under standard growth conditions (Supplementary Fig. 3a), thus indicating a global shift in cellular strategy to control gene expression as cells respond to temperature change. These results suggest that loss of Clr4 or Ccr4 disables an important buffering mechanism that normally prevents unrestrained gene expression during adaptation to low-temperature growth.

Loss of Clr4 affects meiotic gene silencing at low temperature. Clr4 methylates H3K9 at several meiotic genes that are maintained in a silent state during vegetative growth14. However, deletion of clr4 alone had little or no effect on meiotic gene expression under standard growth conditions14 (Supplementary Fig. 4a). Given the shift in cellular strategy to control gene expression at 18 °C, we wondered whether Clr4 becomes required for repression of meiotic genes under suboptimal growth conditions. Indeed, cells lacking Clr4 exhibited untimely expression of several meiotic genes during vegetative growth at 18 °C (Supplementary Fig. 4a), suggesting a critical role for this conserved factor in preventing inappropriate gene expression. Notably, clr4Δ cells displayed a slow-growth phenotype at 18 °C compared to wild-type cells (Supplementary Fig. 4b).

Cells cultured at a low temperature form new heterochromatin islands. Our finding that loss of Clr4 causes further upregulation of various transcripts at a low temperature suggested that cells grown at 18 °C might exhibit a unique heterochromatin profile and display additional facultative heterochromatin domains14,26. To explore potential changes in heterochromatin distribution, we performed genome-wide analyses of H3K9 methylation (H3K9me) in wild-type cells grown at 18 °C. Remarkably, we identified 36 novel facultative heterochromatin islands (Fig. 2a,b). Our quantitative ChIP–PCR (ChIP–qPCR) time course analyses revealed a gradual increase in H3K9me at selected new facultative heterochromatin islands following the shift to low temperature (Fig. 2c). Importantly, these new islands mapped preferentially to stress-responsive genes (Supplementary Fig. 2) and coincided with loci that show upregulation of transcripts in wild-type cells grown at 18 °C (Fig. 2a–c). These transcripts were further upregulated in clr4Δ cells grown at 18 °C, which we also confirmed by RT–qPCR analysis of SPAPB1A11.02 (Fig. 3a). Together, these findings suggest that Clr4 is involved in the assembly of new facultative heterochromatin islands in cells grown at 18 °C and regulates expression of environmentally controlled transcripts.

An RNA-based mechanism forms new heterochromatin islands. We were intrigued by the finding that new H3K9me peaks form at loci showing upregulation of mRNAs at 18 °C and that these mRNAs are further derepressed upon loss of Clr4. Taken together, these results suggested that mRNAs might provide feedback to regulate their own expression through recruitment of Clr4. We tested this possibility by inserting the ura4 terminator sequence into the SPAPB1A11.02 ORF to impair mRNA production, which we confirmed using 3’ RACE (Fig. 3b). Disruption of SPAPB1A11.02 mRNA production resulted in defects in heterochromatin assembly specifically at this locus (Fig. 3c) and did not affect H3K9me enrichment at another locus, SPBC1289.14, in cells grown at 18 °C (Fig. 3d). These results suggest that gene transcripts upregulated at a low temperature are likely part of a self-regulatory feedback mechanism that recruits Clr4 in cis to buffer against their uncontrolled expression.

To confirm this self-regulatory feedback mechanism, we examined whether an increase in SPAPB1A11.02 mRNA could trigger heterochromatin formation under standard growth conditions. We tested this possibility using a clr6 mutant that showed an increase in SPAPB1A11.02 mRNA in cells cultured at 30 °C, similar to that observed in wild-type cells cultured at 18 °C (Fig. 3e). Remarkably, we found that an increase in SPAPB1A11.02 mRNA was linked to an increase in H3K9me enrichment in clr6 mutant cells grown at 30 °C (Fig. 3e,f). Moreover, disruption of transcription by the ura4 terminator sequence in the SPAPB1A11.02 ORF caused a strong reduction in H3K9me levels in clr6 mutant cells (Fig. 3g), further supporting our model in which mRNAs generated from the target genes themselves recruit Clr4 to modulate their expression.

RNA elimination and termination factors but not RNAi are required for new H3K9me islands at 18 °C. Transcription and RNA-mediated assembly of heterochromatin involves RNA processing factors. Whereas RNAi machinery is required to establish H3K9me at repetitive DNA elements and certain regulated genes20,21, RNAi-independent mechanisms involving MTREC and its associated factors assemble facultative heterochromatin at meiotic genes14,17,25. Our ChIP-chip and ChIP analyses showed that loss of Ago1 did not affect H3K9me peaks detected in cells grown at 18 °C (Supplementary Fig. 5a,b), indicating that RNAi is dispensable in the assembly of these heterochromatin islands.

We next tested the potential involvement of RNA elimination factors, including MTREC and CCR4–NOT, in heterochromatin island assembly. H3K9me peaks detected at 18 °C did not form in the absence of Ccr4 (see below and Supplementary Fig. 5a,c). Moreover, cells lacking Red1 were defective in the assembly of these heterochromatin islands (Supplementary Fig. 5c). These data are consistent with our transcriptome analyses showing that RNA elimination factors, but not RNAi machinery, are required for preventing inappropriate gene expression at low temperature (Fig. 1b,c).
RNA elimination factors cooperate with the conserved exonuclease Dhp1 (Xrn2 in higher eukaryotes) that, in addition to promoting transcription termination, facilitates Clr4 recruitment to assemble heterochromatin. We tested whether the formation of heterochromatin islands detected at 18 °C requires Dhp1. Because dhp1 is an essential gene, we used a temperature-sensitive dhp1 mutant allele in a clr6 mutant background and assayed H3K9me enrichment at the restrictive temperature (37 °C). Unlike the single clr6 mutant that showed significant H3K9me enrichment at the SPAPB1A11.02 locus, no enrichment was detected in the clr6 dhp1 double mutant (Fig. 3h), suggesting that Dhp1 is required for heterochromatin assembly.

Genetic screen connects iron homeostasis to cellular adaptation. Among the factors affecting facultative heterochromatin in cells grown at 18 °C, ccr4Δ cells showed the most severe cold sensitivity. We hypothesized that the cold sensitivity of ccr4Δ cells might be related to misregulated gene expression. To explore this possibility, we performed a genetic screen for suppressors of the ccr4Δ cold-sensitive phenotype (Fig. 4a and Methods) and isolated three suppressor mutants.
Tup11 or Tup12 causes upregulation of iron-transporter genes involved in various physiological processes (Fig. 4c,d). Moreover, colocalization of Fep1 and Ssn6, including at the promoters of genes involved in the repression of genes encoding iron-using proteins, did not occur preferentially at the promoters of the gene encoding iron-transporter genes (Fig. 4d and Supplementary Fig. 6b). We also observed preferential enrichment of these proteins at the promoters of wild-type cells (Fig. 4d and Supplementary Fig. 6b). Collectively, these results suggest an important connection between factors that regulate iron homeostasis and growth at 18°C.

Whole-genome sequencing analyses revealed that two suppressors contained mutations in a gene encoding the iron-sensing transcription factor fep1, which represses iron-transporter genes. Both mutations introduce premature stop codons into the fep1 ORF (Fig. 4a). Supporting these results, deletion of fep1 mimicked the suppression of the ccr4Δ cold sensitivity that we observed with the mutant alleles (Fig. 4b). This suppression was specific to fep1, as deletion of php4, which is involved in the repression of genes encoding iron-using proteins, did not suppress the growth defect of ccr4Δ cells cultured at 18°C.

Interestingly, the third suppressor mutation mapped to the gene encoding Ssn6 (Fig. 4a), a component of the conserved Snf6–Tup11/12 transcriptional corepressor that acts together with HDACs. Tup11 physically associates with Fep1 (ref. 43). Loss of Tup11 or Tup12 causes upregulation of iron-transporter genes, which we found are also repressed by the Ctr6 HDAC (Supplementary Fig. 3b). Our genome-wide ChIP-chip analysis revealed widespread colocalization of Fep1 and Ssn6, including at the promoters of genes involved in various physiological processes (Fig. 4c,d). Moreover, their localization coincided with the Pst1-containing Ctr6 complex at several gene promoters (Supplementary Fig. 6a). We also observed preferential enrichment of these proteins at the promoters of iron-transporter genes (Fig. 4d and Supplementary Fig. 6b). Collectively, these results suggest an important connection between factors that regulate iron homeostasis and growth at 18°C.

The remarkable identification of two distinct factors involved in iron homeostasis as suppressors of the ccr4Δ cold-sensitivity phenotype prompted us to test iron uptake in ccr4Δ cells and to explore the connection between Fep1 and Ssn6 in controlling this process. Strikingly, iron-transporter genes were downregulated in ccr4Δ cells at 18°C, as determined by our RNA-seq and RT–qPCR analyses (Fig. 5a). However, lack of Fep1, which abolished localization of the Ssn6 corepressor at iron-transporter gene promoters (Fig. 4d), fully restored expression of these loci in ccr4Δ cells (Fig. 5a and Supplementary Fig. 6c). Consistently, we found that intracellular iron levels were lower in ccr4Δ cells relative to those of wild-type cells cultured at 18°C and that loss Fep1 in these cells restored intracellular iron (Supplementary Fig. 6d). Together with our results showing that iron transporters are upregulated in wild-type cells grown at 18°C (Fig. 1a), these findings suggest that proper control of iron homeostasis is crucial for cellular adaptation to growth at a low temperature.

Iron homeostasis affects facultative heterochromatin and global gene control. We next investigated whether the suppression of cold sensitivity is coupled to the restoration of proper global gene expression in ccr4Δ fep1-1 double-mutant cells, as compared to the ccr4Δ single mutant. Our expression profile comparisons revealed widespread normalization of transcript levels in ccr4Δ fep1-1 cells.
A particularly striking finding was that expression levels of a wide array of transcripts that were upregulated in ccr4∆ cells compared to wild-type cells cultured at 18 °C were restored to near wild-type levels in the ccr4∆ fep1-1 double mutants (Fig. 5b,c). This remarkably broad restoring effect on gene expression included not only the highly upregulated transcripts in wild-type cells grown at 18 °C, but also other transcripts that were derepressed genome-wide in ccr4∆ and clr4∆ cells (Fig. 5b).

We wondered whether restoration of iron homeostasis upon loss of Fep1 in ccr4∆ cells could also restore facultative heterochromatin assembly. Remarkably, our ChIP-chip analyses showed that H3K9me peaks lost in ccr4∆ cells were restored in ccr4∆ fep1-1 cells, to levels comparable to those in wild-type cells cultured at 18 °C (Fig. 6a,b). Similarly, a mutation in ssn6 restored H3K9me at loci that showed defects in facultative heterochromatin assembly in ccr4∆ cells grown at 18 °C (Supplementary Fig. 6e).
Iron depletion disrupts facultative heterochromatin assembly and gene regulation. Our genetic analyses suggested that iron homeostasis is critical for regulating gene expression and facultative heterochromatin formation at low temperature. To directly explore the role of iron in this process, we depleted intracellular iron using the membrane-permeable iron-chelating agent 2,2'-bipyridyl (Dip). Wild-type cells cultured at 18°C in the presence of Dip showed upregulation of transcripts that are regulated by Clr4 at low temperature (Fig. 7a,b). In addition to the further derepression of transcripts that are upregulated in wild-type cells grown at 18°C, iron chelation in wild-type cells affected additional gene clusters that were derepressed in clr4Δ cells grown at 18°C (Fig. 7a). Transcripts upregulated in iron-depleted cells were enriched in stress-response and catabolic genes, as well as ncRNAs, as we observed in clr4Δ cells cultured at 18°C. We obtained similar results when ccr4Δ fep1-1 double-mutant cells were treated with Dip (Supplementary Fig. 7a), suggesting that the normalization of gene expression observed in ccr4Δ fep1-1 requires iron and, therefore, is linked to restoration of iron homeostasis.

The re-establishment of facultative heterochromatin in the ccr4Δ fep1-1 double mutant led us to examine whether Clr4 is required by the factors that restore iron homeostasis and suppress the cold sensitivity of ccr4Δ cells. Indeed, we found that ccr4Δ fep1Δ clr4Δ cells failed to grow at 18°C (Fig. 6c), suggesting that Clr4 is indeed functionally important. Collectively, our analyses of cells lacking Ccr4, which show downregulation of iron-transporter genes, reveal an intimate connection between iron homeostasis and proper gene control via a mechanism involving Clr4, RNA processing factors, and facultative heterochromatin assembly.

We next investigated the effect of iron depletion on facultative heterochromatin assembly. Dip treatment abolished H3K9me peaks corresponding to heterochromatin islands in wild-type cells cultured at 18°C (Fig. 7c,d and Supplementary Fig. 7b), and similar results were observed in ccr4Δ fep1-1 cells treated with Dip (Supplementary Fig. 7c). Importantly, Dip treatment had no major impact on H3K9me levels at constitutive heterochromatic loci, including centromeres and telomeres (Fig. 7c), suggesting that iron depletion selectively affects the assembly of facultative heterochromatin islands at 18°C. Collectively, our findings underscore the importance of iron homeostasis in RNA-mediated targeting of Clr4 in assembling facultative heterochromatin and define gene expression patterns in response to environmental change.

Loss of Clr4 enhances Rnapii transcription of target loci at 18°C. We next addressed how Clr4 affects transcript levels at a low temperature. At constitutive heterochromatin regions, Clr4 influences expression at both transcriptional and post-transcriptional levels44. Because the assembly of heterochromatin islands in cells grown at 18°C involves RNA-mediated targeting of Clr4, we postulated that the changes in transcript levels observed in clr4Δ cells might be linked to defects in RNA degradation. However, as it was also possible that Clr4 affects Rnapii transcription, we performed native elongating transcript sequencing (NET-seq)45 to monitor Rnapii transcription in wild-type and clr4Δ cells grown at 18°C. clr4Δ cells showed an increase in Rnapii transcription at constitutive heterochromatin domains, supporting results from our previous ChIP analyses46, and an increase in Rnapii transcription was also observed at telomere-linked helicase (tlh) genes and several other loci within subtelomeric domains (Fig. 8a), which are
coated with H3K9me in wild-type cells. However, a surprising finding was that clr4Δ cells cultured at a low temperature showed a marked increase in RNAPII transcription at genes whose expression is elevated at 18 °C upon loss of Clr4 (Fig. 8b). At some locations, clr4Δ cells showed increased transcription of genes proximal to the heterochromatin islands detected in wild-type cells (for example, SPAC23H3.15) (Fig. 8b).

To address the specific effect on RNAPII transcription at loci showing hyperelevation (> 5-fold) of transcripts in clr4Δ cells grown at 18 °C, we compared the NET-seq profiles of wild-type and clr4Δ cells. Remarkably,
Fig. 7 | Depletion of iron from growth medium abolishes heterochromatin assembly at a low temperature. 

a, Heat map of fold-change values for upregulated transcripts relative to WT 30 °C cells in the indicated strains grown at 18 °C. Clusters are grouped according to the analysis presented in Fig. 1f. 

b, RNA-seq analysis of the expression levels of two representative heterochromatic loci are shown for the indicated strains grown at 18 °C. 

c, ChIP-chip analysis of genome-wide H3K9me relative enrichment in WT cells at 18 °C, either untreated or treated with 250 µM Dip. New facultative heterochromatin peaks at 18 °C are indicated on the WT 18 °C plot. Note that H3K9me at constitutive heterochromatin domains (cen, mat and tel) and meiotic islands (ssm4 and mei4) is largely maintained in Dip-treated cells. WT (untreated) ChIP-chip data are also presented in Fig. 2a. 

d, H3K9me relative enrichment at individual loci in WT cells, either untreated or treated with 250 µM Dip, at 18 °C. WT (untreated) ChIP-chip data are also presented in Fig. 2b. Source data for a are available with the paper online.
the NET-seq average gene profile clearly showed higher levels of RNAPII transcription at Clr4 target loci in clr4Δ cells compared to wild-type cells cultured at 18 °C (Fig. 8c). This difference was not observed at loci that showed no expression change in clr4Δ cells cultured at 18 °C (Fig. 8c).

Thus, in addition to constitutive heterochromatic loci, Clr4 specifically limits RNAPII activity at loci that are upregulated in cells cultured at a low temperature.

Discussion
In this study, we provide evidence that heterochromatin factor Clr4/Suv39h and nuclear RNA processing and termination factors are components of a dynamically regulated mechanism that controls gene expression during growth at a low temperature. Furthermore, we show that iron homeostasis is critical for facultative heterochromatin assembly and for the normalization of upregulated gene expression that occurs during adaptation to this suboptimal condition.

Our analyses suggest that a rheostat-like mechanism prevents upregulated transcripts from reaching hyperelevated levels. Many genes that are repressed by Clr6 HDAC under standard growth conditions are controlled by a Clr4-based mechanism that fine-tunes their expression at low temperature. Specifically, we find that transcriptional upregulation of the mRNAs provides feedback...
that limits gene expression through Clr4 recruitment. Consistently, transcripts that are upregulated in wild-type cells are considerably further elevated in cells lacking Clr4. Moreover, we show that Clr4 inhibits RNAPII transcription at its target loci.

How cells recruit Clr4 to suppress RNAPII activity remains a key question. Clr4-mediated suppression of RNAPII activity may involve RNAi machinery implicated in transcription-coupled heterochromatin formation. However, we find that RNAi is dispensable for both facultative heterochromatin assembly and gene regulation in cells grown at 18°C. Instead, this process requires RNA elimination machinery and Dhp1/Xnr2, an RNAPII termination factor linked to heterochromatin modifications. Clr4 might recruit silencing effectors by methylating H3K9, as it does at constitutive heterochromatic loci. Nevertheless, we note that Clr4 exists in a cullin 4 (Cul4)-containing complex, which contains E3 ubiquitin ligase activity that might also contribute to limiting RNAPII transcription, particularly at loci showing little or no H3K9me. Regardless of the mechanism, these findings will help to elucidate the functions of heterochromatin factors at transcribed genes in higher eukaryotes. Interestingly, the localization of heterochromatin factors at heat-shock puffs in Drosophila is contemporaneous with the wave of increased expression, and in mammals, H3K9me is detected in the coding regions of activated genes. Whether, or even how, heterochromatin machinery regulates gene activity at these loci is unclear. In light of this study, these observations are remarkably consistent with the view that the transcription-coupled recruitment of heterochromatin factors buffers against uncontrolled gene expression in response to developmental and environmental signals.

Another surprising finding from our current study was that iron is required for adaptive gene control at a low temperature. Notably, cells depleted of iron, as well as ccr4Δ cells that are defective in iron homeostasis owing to the downregulation of iron-transporter genes, show defects in facultative heterochromatin assembly and hyperelongation of Clr4 target genes. Suppressors mutations that restore iron homeostasis in ccr4Δ cells re-establish facultative heterochromatin and proper gene control genome-wide, thus supporting a critical role for iron in these adaptive processes. In particular, mutations in the iron-sensing factor fep1 derepress iron transporters silenced by Clr6 HDAC and the Ssn6–Tup11/12 corepressor. This study, which in turn restores intracellular iron levels required for adaptive genome control. Interestingly, wild-type cells cultured at 18°C show elevated transcription of iron transporters that correlates with the formation of facultative heterochromatin islands across the genome. It is likely that intracellular iron concentrations are carefully regulated as part of adaptive gene control in response to changes in growth conditions.

How does iron regulate facultative heterochromatin assembly and gene expression? We can speculate that iron may affect chromatin-modifying activities. However, given that iron depletion has no impact on constitutive heterochromatin formation, it is unlikely that iron affects Clr4 activity, but rather, iron may promote specific RNA-dependent targeting of H3K9me by RNA processing and termination factors. Supporting this notion, iron deprivation causes the accumulation of aberrantly spliced RNAs, which are normally degraded by RNA processing factors. Furthermore, our hierarchical clustering analysis of expression profiles showed that iron-depleted cells have the highest correlation with mtl1 mutant cells. Indeed, RNA-modifying enzymes containing iron-sulfur clusters and radical SAM domains catalyze a variety of RNA modifications that affect RNA stability or function. Determining whether Clr4 recruitment may be facilitated through iron-dependent RNA-modification activities will be an important topic for future studies.

The conservation of RNA processing and heterochromatin pathways from S. pombe to mammals underscores the importance of these findings for understanding epigenetic reprogramming of higher eukaryotic genomes and for discovering the molecular underpinnings of human diseases linked to iron deficiencies.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41594-018-0056-2.

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Author contributions

S.I.S.G. and P.S.G. conceived and supervised the project. S.P.S.G., M.L.I.D., V.B., H.X., and C.W. performed experiments and analyzed data. R.C., G.T., and D.W. performed bioinformatics analyses of genomic datasets. S.I.S.G. and P.S.G. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Methods

Strains and media. Yeast cell culture and genetic manipulations were performed using standard methods\(^1\). *S. pombe* strains used in this study are listed in Supplementary Table 1. Yeast extract rich medium supplemented with adenine (YEA) was used to grow cultures. Cells cultured at 18 °C were grown to mid-log phase (OD\(_{600}\)=0.4–0.8) at 30 °C and then shifted to 18 °C for 3 d. Cultures were maintained in early- to mid-log phase (OD\(_{600}\)=0.1–0.8) during 18 °C growth. Cells cultured at 37 °C were first grown at 26 °C and then shifted to 37 °C for 5 h. For iron chelation experiments, cultured cells were treated with 250 μM DpIm (Sigma) immediately before the shift to 18 °C.

RNA analyses. RNA-seq libraries were constructed as previously described\(^2\). Briefly, total RNA was used for the MasterPure Yeast RNA Purification Kit (Epicentre) according to the manufacturer's instructions. rRNA was removed using the Ribozero® Gold rRNA Removal Magnetic Kit (Yeasen) before library construction using the TruSeq V2 RNA Library Preparation Kit (Illumina). Sequencing was performed on the Illumina MiSeq platform. Adapter trimmed reads were aligned to the *S. pombe* ASM294v2.30 reference genome using the BWA\(^1\) short read aligner with default parameters. Duplicate reads were marked in the resulting BAM files using picard-tools\(^2\) to produce a single VCF file containing mutations called in each of the six genomes. Mutations found in the mutant segregants but not in any of the controls were identified from the VCF file using an in-house Perl script\(^2\). Mutation impact was assessed using Snpeff\(^3\).

Iron measurement. Iron-55 labeling of *S. pombe* cells was performed as previously described\(^4\), with some modifications. Yeast cells were inoculated in 50 ml of YE A rich medium in 125 ml flasks and grown overnight in an orbital platform at 30 °C. Cultures were diluted to an OD\(_{600}\) of 0.2 in 100 ml of fresh YEA in 250 ml flasks and grown for 48 h at 18 °C. Cells were transferred to 50 ml Falcon tubes and spun down at 2,000 × g for 5 min. Cell pellets were washed with water and resuspended in synthetic iron-free medium at 0.5 g of cell pellet per 10 ml of medium supplemented with iron-55 (Perkin Elmer, cat # NE043002M) at 30 μCi/ml of cell suspension. Resuspended cells were incubated at 150 r.p.m. for 3 h at 18 °C. Cells were then collected and washed with 50 mM sodium citrate (50 mM citrate, 1 mM EDTA, pH 7.0), then with 20 mM HEPES (20 mM HEPES/KOH, pH 7.4) buffer to remove residual iron-55 from the medium and the outside of cells. Washed cells were resuspended at 1 g/mL in TENTG (10 mM Tris/HCl, pH 7.4, 2.5 mM EDTA, 150 mM NaCl), 10% (vol/vol) glycerol, 0.5% (vol/vol) Triton X-100, 0.1% (wt/vol) Protease Inhibitors EDTA-free complete (Roche), 50 μM 1,4-Butanediol, 2 μg/ml of cell suspension. Cell lysates were prepared, and radioactivity was determined by scintillation counting as described\(^4\).

NET-seq. NET-seq libraries were constructed as previously described\(^5\), with a few modifications. Briefly, 2.1 of cells expressing FLAG tagged Rpb3 subunit of RNPAP1 (Rpb3-FLAG) were cultured at 18°C for 3 d. Cells were harvested at early- to mid-log phase (OD\(_{600}\)=0.3–0.5) by vacuum filtration and flash frozen in liquid nitrogen. Frozen cells were lysed using a CryoMill (Retsch) via six cycles of pulverization for 3 min at 15 Hz. Nascent RNA was purified by immunoprecipitating RNPAP1 using anti-FLAG M2 affinity gel (Sigma) in lysis buffer (150 mM HEPES, pH 7.4, 110 mM KAc, 10 mM MgCl\(_2\), 0.5% Triton X-100, 0.1% Tween 20, 1× Protease Inhibitors EDTA-free complete (Roche), 50 μM 1,4-Butanediol, 2 μg/ml of cell suspension). NET-seq libraries were constructed as previously described\(^7\), with some modifications. The protocol for constructing NET-seq libraries is described\(^74\), with some modifications. Yeast cells were inoculated in 50 ml of synthetic iron-free medium at 0.5 g of cell pellet per 10 ml of medium supplemented with iron-55 (Perkin Elmer, cat # NE043002M) at 30 μCi/ml of cell suspension. Resuspended cells were incubated at 150 r.p.m. for 3 h at 18 °C. Cells were then collected and washed with 50 mM sodium citrate (50 mM citrate, 1 mM EDTA, pH 7.0), then with 20 mM HEPES (20 mM HEPES/KOH, pH 7.4) buffer to remove residual iron-55 from the medium and the outside of cells. Washed cells were resuspended at 1 g/mL in TENTG (10 mM Tris/HCl, pH 7.4, 2.5 mM EDTA, 150 mM NaCl), 10% (vol/vol) glycerol, 0.5% (vol/vol) Triton X-100, 0.1% (wt/vol) Protease Inhibitors EDTA-free complete (Roche), 50 μM 1,4-Butanediol, 2 μg/ml of cell suspension. Cell lysates were prepared, and radioactivity was determined by scintillation counting as described\(^4\).

ChIP and ChIP-chip. ChIP and ChIP-chip experiments were performed as previously described\(^6\). In ChIP experiments performed at 18 °C, cells were fixed at 18 °C with 3% paraformaldehyde for 10 min. In ChIP experiments performed at 30 °C or 37 °C, cells were fixed at room temperature with 3% paraformaldehyde for 30 min. Anti-H3K9me2 (ab151539, Abcam), anti-HA (12CA5, Roche), anti-GFP (ab290, Abcam) or anti-Myr (9E10, Santa Cruz) antibodies were used for immunoprecipitation. Antibodies were recovered using a Protein A/Protein G (1:1) bead slurry (Invitrogen). Immunoprecipitated DNA and input DNA were analyzed by performing ChIP-chip analysis or qPCR using iTaq Universal SYBR Green Supermix (BioRad). In ChIP-chip experiments, immunoprecipitated DNA and input DNA were processed with Cy3/Cy5 for microarray analyses using a custom 44 K oligonucleotide array (Agilent). Oligonucleotides used for ChIP-qPCR analysis are listed in Supplementary Table 1.

RT-qPCR. Total RNA was used for the MasterPure Yeast RNA Purification Kit (Epicentre) according to the manufacturer’s instructions. Gene-specific cDNA was synthesized with random hexamers (Thermo Scientific), and strand-specific cDNA was synthesized with custom DNA oligos using SuperScript III Reverse Transcriptase (Invitrogen). Expression was analyzed by performing qPCR using iTaq Universal SYBR Green Supermix (BioRad). Oligonucleotides used for cDNA synthesis and RT-qPCR are listed in Supplementary Table 1.

3’ RACE. Total RNA was used for the MasterPure Yeast RNA Purification Kit (Epicentre) according to the manufacturer’s instructions. cDNA was synthesized using the 3’ RACE CDS Primer A oligonucleotide using SuperScript III Reverse Transcriptase (Invitrogen). cDNA was then amplified by touch-down PCR with a gene-specific oligonucleotide and the universal primer mix using the Advantage 2 Polymerase mix (Clontech). Gene-specific oligonucleotides are listed in Supplementary Table 1.

Genetic screen. Exponentially growing cells were either plated to identify spontaneous suppressors of the *crrΔ* cold-sensitive phenotype or mutagenized from UV (150 mJ/m²) using a UV cross-linker (Thermo Scientific). Colonies were screened for growth on rich media (YE A) at 18 °C. Mutants were isolated and backcrossed to a non-mutagenized strain. Gene-specific oligonucleotides were designed from cold-sensitive (control) and three mutant segregants were subjected to whole-genome sequencing. DNA-sequencing libraries were constructed using the Nextera XT Kit (Illumina) and sequenced on the NextSeq 500 platform to an average depth of 40–50X. Mutations were identified as follows: adaptor trimmed reads were aligned to the *S. pombe* ASM294v2.30 reference genome using the BWA\(^1\) short read aligner with default parameters. Duplicate reads were marked in the resulting BAM files using picard-tools\(^2\) to produce a single VCF file containing mutations called in each of the six genomes. Mutations found in the mutant segregants but not in any of the controls were identified from the VCF file using an in-house Perl script\(^2\). Mutation impact was assessed using Snpeff\(^3\).

Data availability. Genomic datasets are deposited in the Gene Expression Omnibus with accession number GSE104547. Source data for Figs. 1a–f, 2a, 5a,b and 7a and Supplementary Fig. 8 are available online. Other primary data are available from the corresponding author upon reasonable request.
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ChIP experiments were performed using anti-H3K9me2 (ab115159, Abcam), anti-HA (12CA5, Roche), anti-GFP (ab290, Abcam), or anti-Myc (9E10, Santa Cruz) antibodies. NET-seq IP experiments were performed with anti-FLAG M2 affinity gel (Sigma).

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