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Ubiquitin ligase component Cul4 associates with Clr4 histone methyltransferase to assemble heterochromatin

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In eukaryotes, heterochromatin mediates diverse processes including gene silencing and regulation of long-range chromatin interactions[1,2]. The formation of heterochromatin involves a conserved array of histone modifications; in particular, methylation of histone H3 at Lys 9 (H3K9me) is essential for recruiting HP1/Swi6 proteins[3]. In fission yeast, the Clr4 methyltransferase is responsible for H3K9me across all heterochromatic domains[4,5]. However, the mechanism of Clr4 recruitment to these loci is poorly understood. We show that Clr4 associates with Cul4, a cullin family protein that serves as a scaffold for assembling ubiquitin ligases. Mutations in Cul4 result in defective localization of Clr4 and loss of silencing at heterochromatic loci. This is accompanied by a severe reduction in H3K9me and Swi6 levels, and accumulation of transcripts corresponding to naturally silenced repeat elements within heterochromatic domains. Moreover, heterochromatin defects in Cul4 mutants could not be rescued by expression of Cul4 protein lacking Nedd8 modification, which is essential for its ubiquitin ligase activity. Rik1, a protein related to DNA damage binding protein DDB1 and required for H3K9me[4,6], also interacts with Cul4, the association of which might serve to target Clr4 to heterochromatic loci. These analyses uncover a role for Cul4-based protein ubiquitination in regulating H3K9me and heterochromatin formation.

Large regions of higher eukaryotic genomes are composed of repetitive DNA elements, which are preferential targets for heterochromatin assembly[1]. The genome of the fission yeast *Schizosaccharomyces pombe* contains sizeable blocks of heterochromatin associated with inverted arrays of *dg* and *dh* pericentromeric repeats, subtelomeric regions, ribosomal DNA (rDNA) and a 20-kilobase (kb) domain at the mating-type locus encompassing the *mat2* and *mat3* silent mating-type loci[5]. The assembly of heterochromatin is believed to globally inhibit transcription and recombination throughout these chromosomal domains, and the ability of heterochromatin to prohibit illegitimate recombination is thought to be critical for maintaining genomic integrity[1]. Heterochromatin is also implicated in recruiting the cohesion protein complex that is essential for sister chromatid cohesion in order to ensure proper segregation of chromosomes[7,8].

Heterochromatin can be nucleated directly by specific *cis*-acting DNA sequences, which are recognized by DNA-binding proteins capable of recruiting general silencing factors[9,10]. Additionally, the RNA interference (RNAi) machinery is believed to be critical for recognizing specific classes of repetitive DNA elements embedded within genomes, making them preferred targets for heterochromatin assembly[11–13]. Transcripts that originated from repetitive elements are processed by the RNAi machinery into small interfering RNAs (siRNAs), which are believed to provide specificity for targeting heterochromatin to cognate sequences.

Post-translational modifications of histones have crucial roles in the assembly of heterochromatin[3]. Genetic and biochemical analyses have implicated deacetylase and methyltransferase activities in establishing a specific histone modification pattern, which specifies the recruitment of downstream factors essential for heterochromatin assembly[3]. Specifically, H3K9me provides binding sites not only for the localization of Swi6/HP1 but also the RITS RNAi effector complex to heterochromatic loci[12,14]. Although multiple enzymes methylate H3 at Lys 9 in mammals, Clr4 is probably the sole enzyme responsible for H3K9me in *S. pombe*[4]. In the absence of Clr4, H3K9me is abolished across the entire chromatin landscape, which correlates with loss of heterochromatin and delocalization of RNAi factors from chromatin[5]. Whereas the role of Clr4-mediated H3K9me in heterochromatin formation is well-studied, it remains unclear how Clr4 is recruited to the target loci.

To gain insights into the mechanism of Clr4 recruitment, we performed affinity purification of the Clr4 complex. For this purpose, a strain expressing the Clr4 protein fused with three Flag epitopes at the amino terminus (Flag–Clr4) was constructed. The tagged *clr4*$^{+}$ is under the control of its natural promoter so as to achieve wild-type levels of expression, and cells expressing Flag–Clr4 did not show any obvious defect in silencing at centromeres (Fig. 1a). Immuno-affinity purification with Flag antibody revealed protein bands that were specific to the Flag–Clr4-purified fraction but were absent in the untagged control fraction (Fig. 1b). Mass spectrometry of the excised bands identified the

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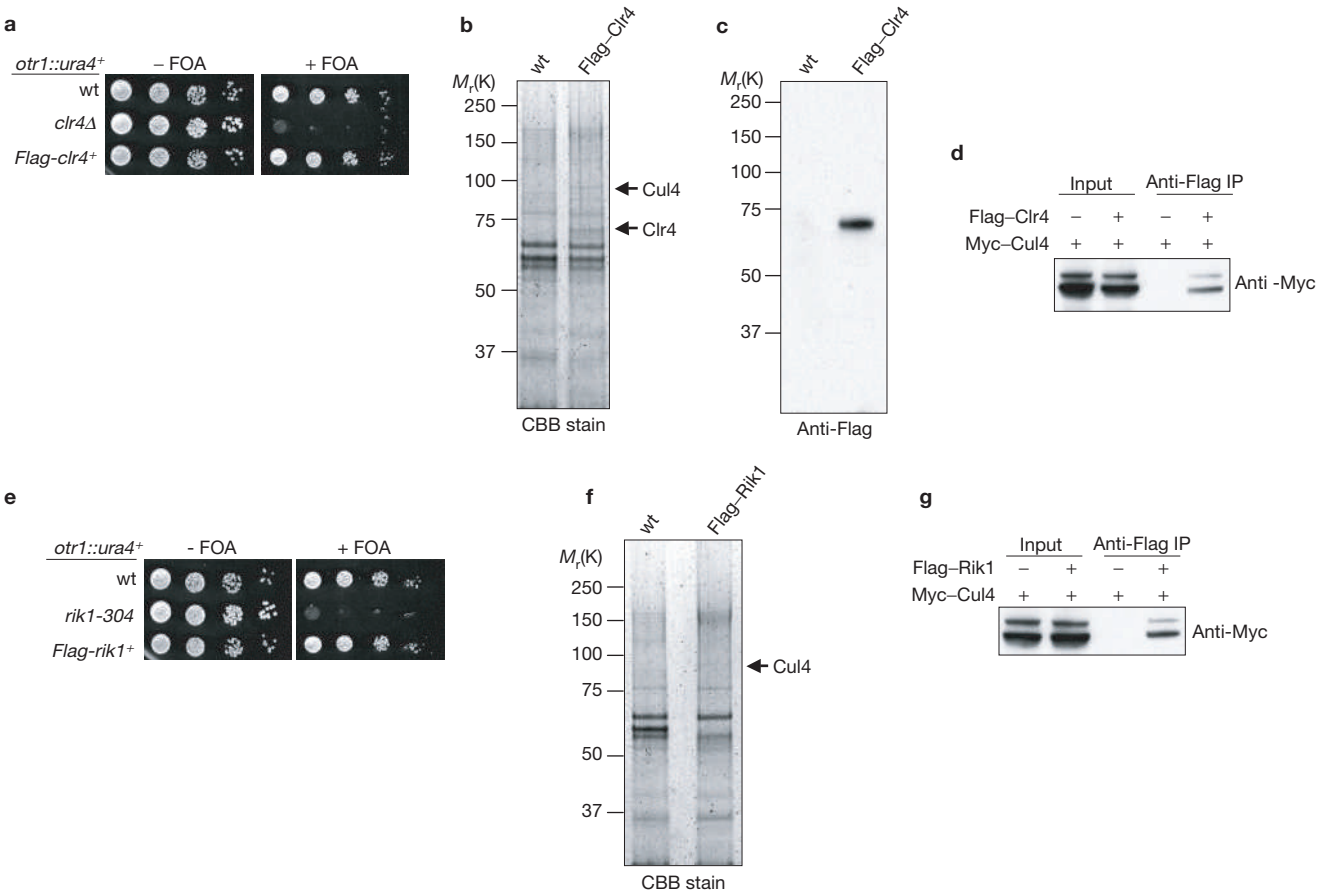

Figure 1 Cul4 associates with Clr4 and Rik1. (**a**, **e**) The Flag–Clr4 and Flag–Rik1 fusion proteins are fully functional. Serial dilution plating assays were performed to measure the expression of *otr1::ura4*$^+$. wt, wild type. FOA, 5-fluoroorotic acid. (**b**, **f**) Purification of Flag–Clr4 or Flag–Rik1. Extracts prepared from the indicated strains were used to perform affinity purification with anti-Flag agarose resin, resolved by SDS–PAGE and stained with Coomassie blue (CBB). The identity of protein bands, as determined by mass spectrometry, is indicated on the right. (**c**) Expression of Flag–Clr4. A portion of the samples, as in **b**, was subject to western blot analysis with Flag antibody. (**d**, **g**) Clr4 and Rik1 associate with Cul4 *in vivo*. Extracts prepared from the indicated strains were incubated with Flag antibody. The immunoprecipitated fractions were analysed by western blotting with Myc antibody. Two bands of Myc–Cul4 were observed, and the upper band represents the Nedd8-modified form of Cul4.

band with a relative molecular mass of 65,000 (M_r 65K) to be Clr4, which was also confirmed independently by western blot analysis (Fig. 1c). The band with M_r 90K was identified as Cul4 (see Supplementary Information, Fig. S1a), a member of the cullin family of proteins that are components of E3 ubiquitin ligases involved in protein ubiquitination[15]. We also observed a band corresponding to Cul4 in the tandem affinity-purified (TAP) fraction using extracts prepared from a strain expressing TAP-Clr4 (see Supplementary Information, Fig. S2). Mass spectrometry analysis of purified protein mixtures associated with Flag–Clr4 also identified Cul4 as a Clr4-interacting factor (see Supplementary Information, Fig. S1c). In addition, we identified several peptides corresponding to Rik1 protein, which is known to interact with Clr4 and is required for heterochromatin assembly[4,16]. Similarly, mass spectrometry of Flag–Rik1 affinity-purified proteins also identified Cul4 as a Rik1-interacting protein (Fig. 1f and see Supplementary Information, S1b). Taken together, these results suggested that Rik1, Cul4 and Clr4 form a complex.

To confirm the interaction between Clr4, Rik1 and Cul4, we expressed fully functional Myc–Cul4 from a plasmid under the control of the medium-strength *nmt1* promoter. When protein extracts prepared from cells expressing Myc–Cul4 in the presence of Flag–Clr4 or Flag–Rik1 were used to perform immunoprecipitation analysis with anti-Flag antibody, Myc–Cul4 co-immunoprecipitated with both Clr4 and Rik1 (Fig. 1d, g). These results confirmed that Clr4, Rik1 and Cul4 indeed form a complex *in vivo* and that these factors might be components of a larger ubiquitin ligase complex required for heterochromatin formation. Interestingly, Rik1 is structurally related to the DDB1 family of proteins, which have been reported to associate with cullin 4 and are believed to serve as receptor/adaptor to recruit ligase activity to the target proteins[6,15,17,18]. *S. pombe* contains a second Rik1 orthologue called Ddb1 that has been shown to function coordinately with Cul4 to regulate genome stability and cell-cycle progression[19,20]. However, Ddb1 is not required for heterochromatic silencing (O. Nielsen, personal communication). Moreover, we did not find peptides corresponding to Ddb1 in our affinity-purified fractions of Clr4 and Rik1, suggesting that Rik1, but not Ddb1, might coordinate with Cul4 to recruit Clr4 to heterochromatic loci.

The interaction of Cul4 with Clr4 and Rik1 suggests that Cul4 may be involved in heterochromatin formation. Indeed, we found that deletion of *cul4* causes loss of silencing of a *ura4*$^+$ marker gene inserted at the centromeric repeats (*otr1::ura4*$^+$) (see Supplementary Information, Fig. S3). However, *cul4Δ* cells were extremely slow-growing and showed severe defects in cell-cycle progression (data not shown). To separate the function of Cul4 in cell-cycle regulation from heterochromatin formation, we

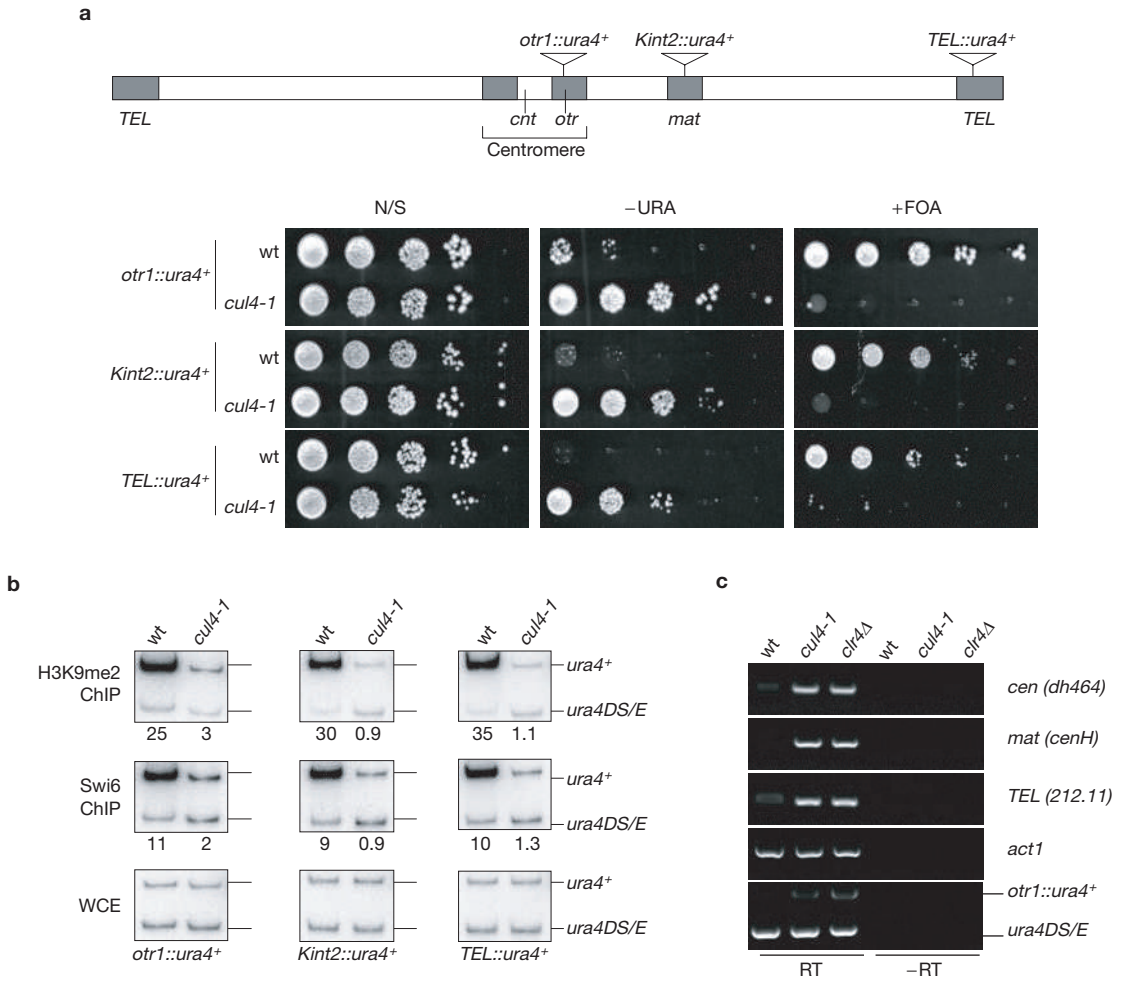

Figure 2 Cul4 is required for heterochromatin formation. (**a**) *cul4-1* affects silencing at all heterochromatin loci. Shown is a schematic diagram of a chromosome (top). Grey boxes represent heterochromatin at the centromeres, silent mating-type locus (*mat*) and telomeres (*TEL*). The positions of *ura4*$^+$ reporter genes are indicated. Serial dilution plating assays were performed to measure the expression of *ura4*$^+$ reporter genes (bottom panels). N/S, nonselective medium. (**b**) *cul4-1* affects H3K9me and Swi6 levels at all heterochromatic loci. Levels of dimethylated H3K9 (H3K9me2) and Swi6 at the indicated locations were determined by ChIP assay. Relative fold enrichment values, calculated as described in the Methods section, are shown below each lane. WCE, whole-cell extract. (**c**) *cul4-1* results in the accumulation of transcripts derived from heterochromatin-associated repeats. RT–PCR was performed with total RNA isolated from indicated strains to measure the amount of transcripts derived from repetitive sequences. Primers used were centromeric *dh* repeats (*dh464*), the *cenH* element at the silent mating-type region, and the telomere-associated SPAC212.11 locus (*212.11*). *act1* was used as a positive control. *otr1::ura4*$^+$ expression was also analysed and compared with euchromatic *ura4DS/E*. –RT, no reverse transcription.

isolated three mutant alleles of *cul4* that did not affect its growth-related function but caused severe defects in heterochromatic silencing (see Supplementary Information, Fig. S3). The *cul4-1* mutant showed the strongest silencing defect and was used for all subsequent analysis. The silencing phenotype observed in the *cul4-1* mutant background could be rescued by a plasmid expressing the *cul4*$^+$ gene, demonstrating that the silencing defect was specific to the loss of Cul4 function in heterochromatin formation (Fig. 4a).

We next explored whether Cul4 also affects silencing at other heterochromatic loci including the mating-type locus and telomeres, as well as investigating the role of Cul4 in Clr4-mediated H3K9me, and recruitment of Swi6 at heterochromatic loci. Mutation in *cul4* abolished silencing of the *ura4*$^+$ reporter gene inserted either within a centromere-homologous (*cenH*) repeat at the silent mating-type region (*Kint2::ura4*$^+$) or adjacent to the telomeres (*TEL::ura4*$^+$) (Fig. 2a). More importantly, chromatin immunoprecipitation (ChIP) analysis showed that the levels of H3K9me and Swi6 were severely reduced at the heterochromatic regions (Fig. 2b). The residual enrichment of H3K9me and Swi6 observed at centromeres presumably reflects low levels of Cul4 activity remaining in mutant cells (see below).

We previously showed that components of RNAi and heterochromatin machineries form a positive feedback loop, which is essential for maintenance of heterochromatic silencing and for the processing of aberrant transcripts produced by repetitive DNA elements[14,21]. Loss of H3K9me results in the delocalization of RNAi factors from heterochromatic loci, which correlates with accumulation of transcripts produced by the repetitive elements within these domains. Reverse transcriptase-polymerase chain reaction (RT–PCR) analyses of total RNA extracted from *cul4-1* cells showed that transcripts originating from the heterochromatic repeat elements accumulated to levels comparable to those of the *clr4Δ* strain (Fig. 2c), indicating defects in the heterochromatin assembly and RNAi pathways. These results suggested that Cul4 interaction with Clr4 is functionally important for the assembly of heterochromatin and the silencing of repeat elements at all major heterochromatic domains.

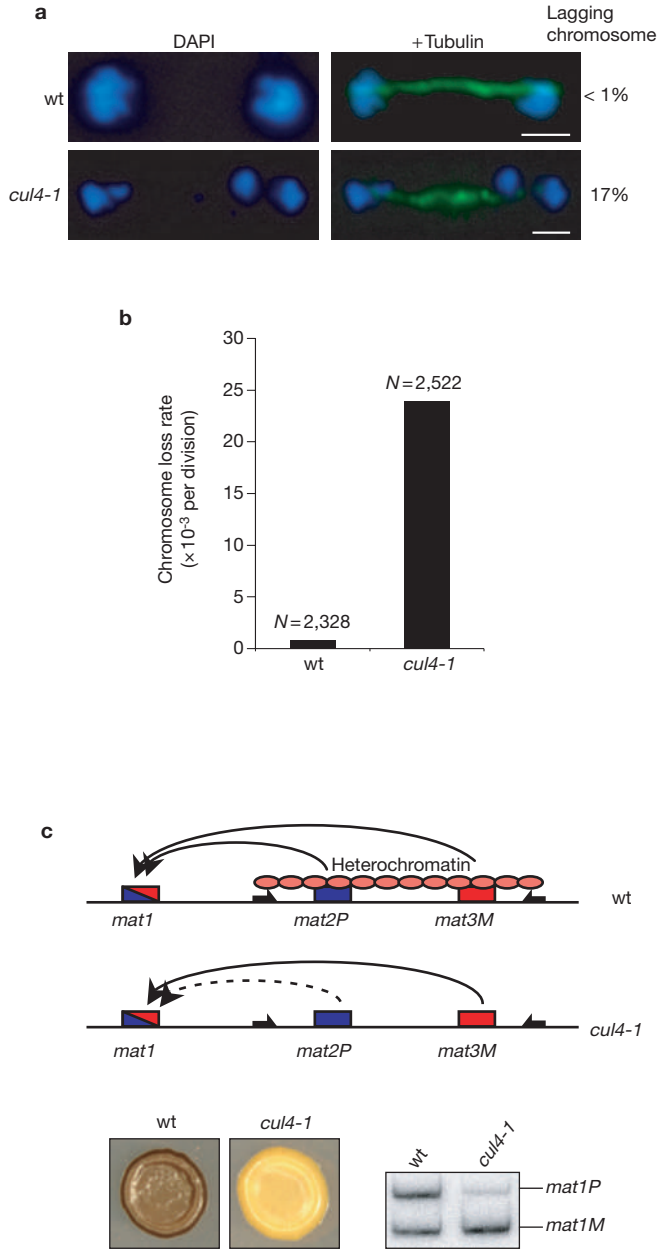

Figure 3 Mutation in Cul4 affects chromosome segregation and mating-type switching. (**a**) *cul4-1* affects mitotic chromosome segregation. Chromosome segregation in late anaphase cells was analysed by 4,6-diamidino-2-phenylindole (DAPI) staining and immunofluorescence with the anti-tubulin TAT1 antibody. The percentage of cells with lagging chromosomes was determined by microscopic examination of more than 100 cells with fully elongated mitotic spindles. Scale bars, 2 μm. (**b**) *cul4-1* cells showed an increased rate of mini-chromosome loss as compared with wild-type cells. (**c**) *cul4-1* affects mating-type switching. Top: schematic diagram indicating the mating-type switching pattern in wild-type and *cul4-1* mutant cells (not drawn to scale). Whereas both donors are used equally in wild-type cells (solid arrows), *cul4-1* cells are defective in utilization of *mat2P* (dotted arrow), owing to heterochromatin assembly defects. Bottom left: iodine staining phenotypes. Colonies were replicated onto sporulation medium and allowed to grow at 25 °C for 3 days before staining with iodine vapours. Bottom right: PCR-based assay was used to determine the *mat1* genetic content, indicating the utilization of *mat2P* or *mat3M* as donors during mating-type switching.

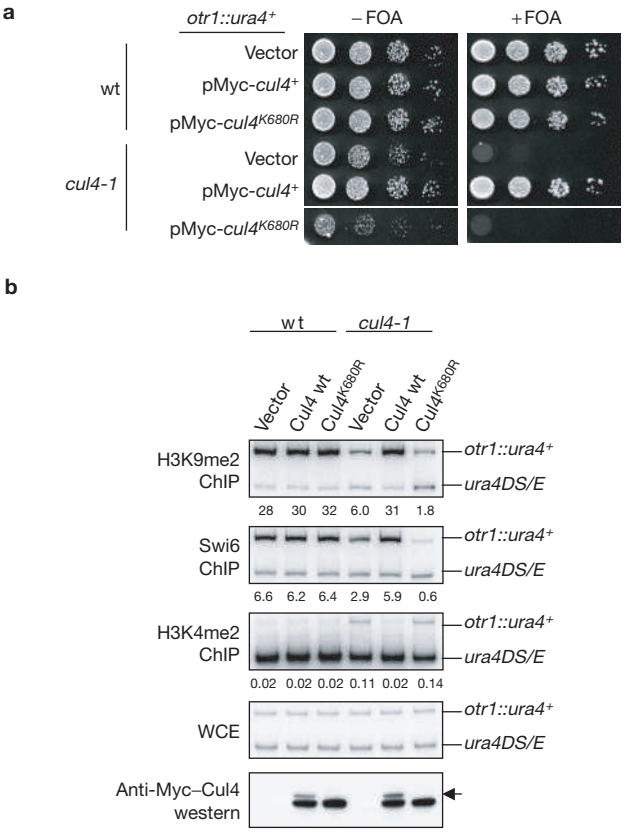

Figure 4 The ubiquitin ligase activity of Cul4 is required for heterochromatin formation. (**a**) The Cul4^{K680R} mutant protein that is defective in neddylation loses the ability to complement the silencing defect of *cul4-1* cells. Wild-type or *cul4-1* cells were transformed with a *LEU2*-based plasmid expressing either Myc–Cul4 or Myc–Cul4^{K680R} under the control of a medium-strength *nmt1* promoter. Cells were grown on EMM-Leu medium to induce Cul4 expression before a serial dilution plating assay was performed to measure the expression of *otr1::ura4*$^{+}$. Colonies formed by *cul4-1* cells expressing the Cul4^{K680R} allele were photographed separately due to their slow growth phenotype. (**b**) Neddylation of Cul4 is essential for H3K9me and Swi6 localization at centromeres. ChIP assays were performed to measure the levels of dimethylated H3K9 (H3K9me2), Swi6 and dimethylated H3K4 (H3K4me2) at centromeres. The expression of wild-type or Cul4^{K680R} proteins was confirmed by western blot analyses. Arrow indicates the Nedd8-modified form of Cul4, which is absent in strains expressing Cul4^{K680R}. Relative fold enrichment values are shown below each lane.

The assembly of heterochromatic structures at centromeres is crucial for faithful segregation of chromosomes during mitosis[7,8]. Because Cul4 is required for heterochromatin assembly, we investigated its role in proper segregation of chromosomes. Our analyses revealed that *cul4-1* cells had a significantly higher percentage of cells with lagging chromosomes during late anaphase than wild-type cells (Fig. 3a). Moreover, the fidelity of segregation of a mini-chromosome Ch16, a 530-kb derivative of chromosome III[22], was severely affected in *cul4* mutant cells (Fig. 3b). Heterochromatin also regulates mating-type switching in *S. pombe* by promoting long-range chromatin interactions that are essential for non-random choice of *mat2* or *mat3* loci during *mat1* switching[2]. This directionality of the switching process is lost in *clr4* or *swi6* mutants, resulting in poor iodine staining of colonies composed mainly of *mat1M* cells (owing to predominant utilization of *mat3M* as a donor), in contrast to the dark staining of wild-type colonies with equal numbers of *mat1P* and *mat1M* cells[2,6,23,24]. We found that *cul4-1* mutant cells are defective

in directionality of switching, as indicated by light iodine staining of colonies and the predominant utilization of *mat3M* as a donor (Fig. 3c), as was also observed in *clr4* mutant background cells[24]. Collectively, these results demonstrate that Cul4 is required for biological functioning of heterochromatin in *S. pombe*.

Cullins are modified by the ubiquitin-like protein Nedd8, which is required for their ubiquitin ligase activity[25]. Cullin mutants that are defective in neddylation show severe growth defects and an elongated cell phenotype[26]. To determine whether the ubiquitin ligase activity of Cul4 might be required for H3K9me and heterochromatin formation, we created a mutant Cul4 allele in which Lys 680, the site of Nedd8 conjugation, is mutated to arginine ($Cul4^{K680R}$). Western blot analysis of extracts prepared from cells expressing mutant Cul4 revealed that the K680R mutation specifically abolished a slower migrating band corresponding to the Nedd8-modified form of Cul4 (Fig. 4b)[26]. We next examined whether expression of the wild-type or $Cul4^{K680R}$ alleles can rescue the silencing and heterochromatin assembly defects observed in the *cul4-1* mutant. Whereas the expression of wild-type Cul4 could complement the silencing defect of the *cul4-1* mutant, the expression of $Cul4^{K680R}$ failed to restore silencing at centromeres (Fig. 4a). More notably, ChIP analyses revealed that the expression of wild-type Cul4, but not the $Cul4^{K680R}$ mutant, restored H3K9me and Swi6 localization at centromeres, concomitant with a reduction in H3K4me (Fig. 4b). Interestingly, we found that expression of the $Cul4^{K680R}$ mutant protein resulted in a further reduction in the residual H3K9me and Swi6 levels found at the centromeres in *cul4-1* mutant background cells, whereas no such change was observed in wild-type background cells, suggesting that $Cul4^{K680R}$ might interfere with or negate the effect of residual Cul4 activity remaining in *cul4-1* cells. More importantly, the failure of neddylation-defective Cul4 to support H3K9me and Swi6 localization at centromeres suggests that the ubiquitin ligase activity of Cul4 might be required for the assembly of heterochromatic structures. While this work was under review, another independent study reported the identification of Cul4 as a Rik1-interacting protein and demonstrated that Rik1-containing complexes possess ubiquitin ligase activity *in vitro*[27]. The authors concluded that expression of the $Cul4^{K680R}$ allele has no effect on H3K9me at centromeres. However, these analyses relied on overexpression of the $Cul4^{K680R}$ allele to create a dominant-negative effect in otherwise wild-type background cells, rather than assessing the ability of the K680R allele to restore heterochromatin in the *cul4* mutant that is specifically defective in silencing. Thus it is possible that the activity associated with wild-type Cul4 present in cells prevented the detection of H3K9me defects associated with the neddylation-defective Cul4.

We also addressed whether Cul4 is required for the localization of Clr4 and Rik1 to heterochromatic regions. ChIP analysis showed that whereas Clr4 was enriched at all major heterochromatic regions in wild-type cells, its levels at these loci were significantly reduced in *cul4-1* cells (Fig. 5a), even though the protein levels of Clr4 and Swi6 remained unchanged in the *cul4* mutant background (Fig. 5c). This result suggests that the targeting of Clr4 to heterochromatic loci is dependent on Cul4, which may function at a step prior to Clr4 localization. Consistent with this notion, overexpression of Clr4 could not rescue the loss of silencing in the *cul4-1* mutant (Fig. 5b). ChIP analysis of a functional Rik1–Myc strain showed that Rik1 also localized to all heterochromatic loci, in a manner similar to Clr4 (Fig. 5d and see Supplementary Information, Fig. S4). However, in contrast to the severe defects of Clr4 localization,

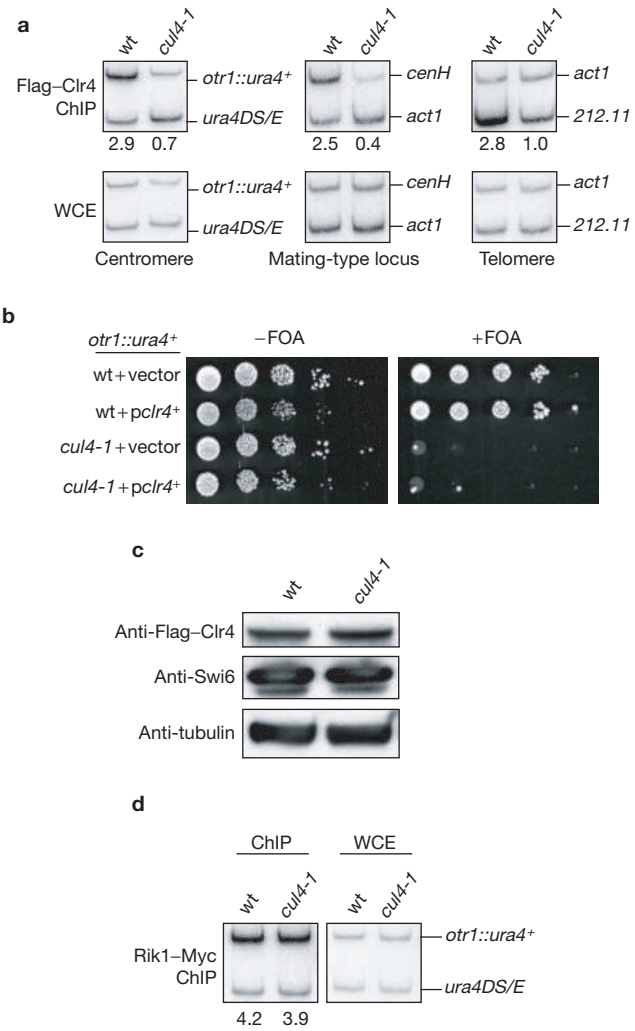

Figure 5 Cul4 recruits Clr4 to heterochromatic loci. (**a**) Clr4 is delocalized from all heterochromatic loci in the *cul4-1* mutant. Flag–Clr4 relative enrichment at the indicated locations was determined by ChIP assay. (**b**) Overexpression of Clr4 could not rescue the silencing defect of *cul4-1*. A serial dilution plating assay was performed to measure the expression of *otr1::ura4*$^+$. (**c**) *cul4-1* does not affect Clr4 or Swi6 protein levels. A western blot of whole-cell extract prepared from indicated strains was performed with antibodies against Flag, Swi6 or tubulin. Both strains expressed Flag–Clr4. (**d**) Rik1 localization to heterochromatin is not affected in *cul4-1* mutant cells. Levels of Rik1–Myc at the *otr1::ura4*$^+$ were determined by ChIP with Myc antibody. Relative fold enrichment values are shown below each lane.

the *cul4-1* allele had no significant effect on the localization of Rik1 at centromeric repeats (Fig. 5d). Taken together, these analyses support a model in which Rik1 targets Cul4-dependent ubiquitination machinery and Clr4 methyltransferase to heterochromatic loci, and that these factors act together to promote H3K9me, which is essential for heterochromatin formation. It is possible that the Rik1 protein, which possesses the WD β-propeller domain found in chromatin assembly and RNA processing factors[17,28,29], might be involved in RNAi-dependent targeting of Clr4 to heterochromatic loci, as also proposed earlier[30]. Rik1 along with its associated factors might serve as an adaptor connecting Clr4 to siRNA-bound factors such as the RITS complex, which is involved in RNAi-mediated heterochromatin assembly. However, another possibility is that the Rik1–Cul4–Clr4 complex is directly recruited to sites of transcription at repeat loci in an RNAi-dependent manner.

Accumulating evidence suggests a broader role for protein ubiquitination machinery in epigenetic regulation of chromatin structure. Silencing of the *Hox* gene cluster in mammalian cells is associated with the ubiquitination of H2A at Lys 119 by the PRC1 complex[31]. Moreover, there seems to be crosstalk between ubiquitination and methylation of histones. In particular, ubiquitination of histone H2BK123 by the Rad6–Bre1 complex is required for H3K4me[32,33] and H3K79me[34,35], markers commonly associated with euchromatin. Our analysis suggests that cells carrying a mutation corresponding to H2BK123 (K120 in *S. pombe*) are not defective in H3K9me and Swi6 localization at centromeres, suggesting that ubiquitination of H2BK123 might not be the target of the Cul4 ligase complex (M. Zofall and S.I.S.G., unpublished observations). However, it is possible that ubiquitination of additional sites on histones and/or non-histone proteins is important for the methylation of H3K9 by Clr4, and the assembly of heterochromatin. Alternatively, ubiquitination of silencing factor(s) could prime its heterochromatin formation activity.

Cul4 is a highly conserved protein that performs a variety of functions in several different organisms including *S. pombe*, *Caenorhabditis elegans* and mammals[15]. However, Cul4 is absent in the budding yeast *Saccharomyces cerevisiae*, which also lacks Clr4, Swi6 and RNAi machinery. This suggests that Cul4-dependent protein ubiquitination and the H3K9me-related process probably evolved together to regulate heterochromatin assembly, which might be a conserved mechanism in higher eukaryotes.

METHODS

Fission yeast strains and plasmids. *Flag-clr4* and *Flag-rik1* strains were generated by inserting the *ura4*$^+$ reporter gene at the promoter region of the corresponding gene, and then replacing *ura4*$^+$ with the 3 × Flag-tagged sequence. *cul4-1*, *cul4-2*, *cul4-3* and *rik1-Myc* strains were constructed using a PCR-based module method. *cul4-1* contains a green fluorescent protein (GFP) moiety at the carboxyl terminus, and *cul4-2* and *cul4-3* contain frame-shift mutations at the C terminus of the protein, which results in the replacement of the last 5 or 8 amino acids by 11 random amino acids, respectively. Genetic crosses were used to construct all other strains. Yeast strains were grown under standard conditions. For serial dilution plating assays, tenfold dilutions of a log phase culture were plated on indicated medium and grown for 3 days at 33 °C. For dilution analysis involving *TEL::ura4*$^+$, all strains were grown on medium without adenine to select for the non-essential Ch16 mini-chromosome, which harbours the *TEL::ura4*$^+$ reporter gene. pREP41-Myc-*cul4*$^+$ was generated by inserting a PCR fragment encoding the full-length *cul4*$^+$ gene between the *Sal*I and *Sma*I sites of pREP-41-MHN. The K680R mutation was generated with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Yeast cells harbouring these plasmids were grown on EMM-Leu medium for at least 24 h to induce Cul4 expression before serial dilution plating assays or ChIP assays were performed.

Protein purification and immunoprecipitation. For affinity purification of Flag–Clr4- or Flag–Rik1-associated proteins, 12 litres of exponentially growing cells were harvested, washed with 2 × HC buffer (300 mM HEPES-KOH pH 7.6, 2 mM EDTA, 100 mM KCl, 40% glycerol, 2 mM DTT, protease inhibitor cocktail (Roche, Indianapolis, IN)) and frozen in liquid nitrogen. Crude cell extracts were prepared by vigorously blending frozen yeast cells with dry ice using a household blender, followed by incubation with 30 ml 1 × HC buffer containing 250 mM KCl for 30 min. The lysates were cleared by centrifugation at 82,700*g* for 2 h and pre-cleared with 250 µl of protein A-agarose for 1 h. The supernatant was incubated with 250 µl of anti-Flag–agarose resin for 4 h and washed eight times with 1 × HC containing 250 mM KCl. Bound proteins were eluted with 200 mg ml^{-1} 3 × Flag peptide, precipitated by TCA, resolved by SDS–PAGE and stained with Coomassie blue. Immunoprecipitation was performed with Flag or Myc antibody using an IMMUNOcatcher kit (CytoSignal, Irvine, CA) according to the manufacturer's protocols.

Chromatin immunoprecipitation (ChIP) and immunofluorescence. ChIP analysis was performed as described previously[4]. Immunoprecipitation was performed with antibodies against Swi6, H3K9me2 or the Flag epitope. DNA isolated from immunoprecipitated chromatin fractions (ChIP) or from whole-cell extract (WCE) was quantitatively analysed by competitive PCR in which one primer pair amplifies heterochromatin-embedded sequences such as *ura4*$^+$ or DNA repeat elements, while the other primer pair amplifies control *ura4DS/E* minigene or *act1* at euchromatic locations. The ratios of intensities of heterochromatin to euchromatin signals in the ChIP and WCE lanes were used to calculate the relative fold enrichment. Values less than 1 indicate no enrichment. Immunofluorescence analysis was performed as described previously[14]. All samples were analysed with a Zeiss Axioplan2 fluorescent microscope. OPENLAB software (Improvision, Lexington, MA) was used for image analysis.

RNA extraction and RT–PCR. Total cellular RNA was isolated from log phase cells using the MasterPure yeast RNA purification kit (Epicentre, Madison, WI) according to the manufacturer's protocol. Quantitative RT–PCR was performed with 250 ng of RNA as a template with One-step RT–PCR kit (Qiagen, Valencia, CA). Reactions in which the reverse transcription step was omitted were performed in parallel.

Chromosome segregation assay and mating-type switching assays. To assay the rate of chromosome loss, cells from Ade$^+$ colonies were plated on adenine-limited medium (YE) and incubated at 30 °C for 4 days. If chromosome loss occurs in the first division of a cell plated on YE, half of the resultant colony carrying Ch16 will be white, whereas the other half without Ch16 will be red. The number of half-sectored red/white colonies was determined and the rate of chromosome loss per cell division was calculated by dividing the number of half-sectored colonies by the total number of white colonies plus half-sectored colonies. Iodine staining and PCR-based assays for determining *mat1* genetic content have been described previously[2].

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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Figure S1

a

MPPEAKRIVV KGFDPRKSRQ RQETYYVTMI DRLNMALQVV MAGLGLK**TGY QELYSGVENL TR**ADQASRCF 70
NILQHHMSSG IQLLKDSAES FIQLEGTETD TNACTVVVGC WNKWLER**VEI VQNIFYYMDK** TFLSHHPDYP 140
TIEELSLSLF REKLMAVKNI QIPFLNSLLQ SFENLHSSK**S TDHAYLQDAM LMLHR**TEMYS SVFVPMYLVM 210
LSRFYDTESS QKIQELPLEE YLEYAMSSLE R**EDAYVEKFD IVR**DKKSIRE TVQRCLITSH LDTLTK**GISQ** 280
FIEKRDAHSC KLLYALLQFN HETEYLIQPW SDCLVDVGFK **LVNDESKDDT LVQELLSFHK** FLQVVVDESF 350
LHDETLSYAM RKAFETFING AKGSQREAPA RLIAK**YIDYL LR**VGEQASGG KPLKEVFSEI LDLFRYIASK 420
DIFEAYYKLD IAKRLLLNK**S ASAQNELMLL DMLKK**TCGSQ FTHSLEGMFR DVNISKEFTS SFRHSKAAHN 490
LHRDLYVNVL SQAYWPSYPE SHIRLPDDMQ QDLDCFEK**FY LSKQVGKK**IS WYASLGHCIV KARFPLGNKE 560
LSISLFQACV LLQFNNCLGG EGISYQDLKK **STELSDIDLT R**TLQSLSCAR **IRPLVMVPKS KKPSPDTMFY** 630
VNEKFTDKLY RVKINQIYLK EERQENSDVQ EQVVRDRQFE LQASIVRVMK QKEK**MKHDDL VQYVINNVKD** 700
RGIPLVSDVK TAIEKLLEKE YLEREDNDIY TYVT

b

MPPEAKRIVV KGFDPRKSRQ R**QETYYVTMI DRL**NMALQVV MAGLGLKTGY QELYSGVENL TRADQASRCF 70
NILQHHMSSG IQLLKDSAES FIQLEGTETD TNACTVVVGC WNKWLER**VEI VQNIFYYMDK** TFLSHHPDYP 140
TIEELSLSLF REKLMAVKNI QIPFLNSLLQ SFENLHSSKS TDHAYLQDAM LMLHR**TEMYS SVFVPMYLVM** 210
LSRFYDTESS QKIQELPLEE YLEYAMSSLE REDAYVEKFD IVRDKKSIRE TVQRCLITSH LDTLTK**GISQ** 280
FIEKRDAHSC KLLYALLQFN HETEYLIQPW SDCLVDVGFK **LVNDESKDDT LVQELLSFHK FLQVVVDESF** 350
LHDETLSYAM RKAFETFING AKGSQREAPA RLIAK**YIDYL LR**VGEQASGG KPLKEVFSEI LDLFR**YIAS**K 420
DIFEAYYKLD IAKRLLLNKS ASAQNELMLL DMLKKTCGSQ FTHSLEGMFR DVNISKEFTS SFRHSKAAHN 490
LHRDLYVNVL SQAYWPSYPE SHIRLPDDMQ QDLDCFEK**FY LSKQVGKK**IS WYASLGHCIV KARFPLGNKE 560
LSISLFQACV LLQFNNCLGG EGISYQDLKK **STELSDIDLT R**TLQSLSCAR IRPLVMVPKS K**KPSPDTMFY** 630
VNEKFTDKLY RVKINQIYLK EERQENSDVQ EQVVRDRQFE LQASIVRVMK QKEK**MKHDDL VQYVINNVKD** 700
RGIPLVSDVK TAIEKLLEKE YLEREDNDIY TYVT

c

Clr4	Cul4	Rik1
GWLFG	STELSDIDLTR	FLQLNPLPELTPR
TIYDLAFFAIK	STDHAYLQDAMLMLHR	FQNFPITNTNSFLEPK
LDSYTHLSFYEK	IQELPLEEYLEYAMSSLER	KVLLEHVPLQAIIFQNK
DIQPLEELTFDYAGAK	LVNDESKDDTLVQELLSFHK	MLFATEIGAIGSIVSLKDKELELEELTR
NPSKLDSYTHLSFYEK		
LKGSNSDSDSPHHASNPHPNSR		
QSTALTTNDTSIILDSLHTNSK		
KQSTALTTNDTSIILDDSLHTNSK		
DKNYDDDGITYLFDLDMFDDASEY TVDAQNYGDVSR		

Figure S1 LC-MS/MS analysis of proteins associated with FLAG-Clr4 and FLAG-Rik1. (a and b) Cul4 was identified by mass spectrometry of excised gel bands from FLAG-Clr4 (a) and FLAG-Rik1 (b) purifications. The amino acid sequence of Cul4 is shown. Peptides obtained by LC MS/MS are indicated in bold. (c) LC-MS/MS analysis of protein mixtures from FLAG-Clr4 purification identified Cul4 and Rik1. Identified peptides corresponding to each protein are shown.

Figure S2

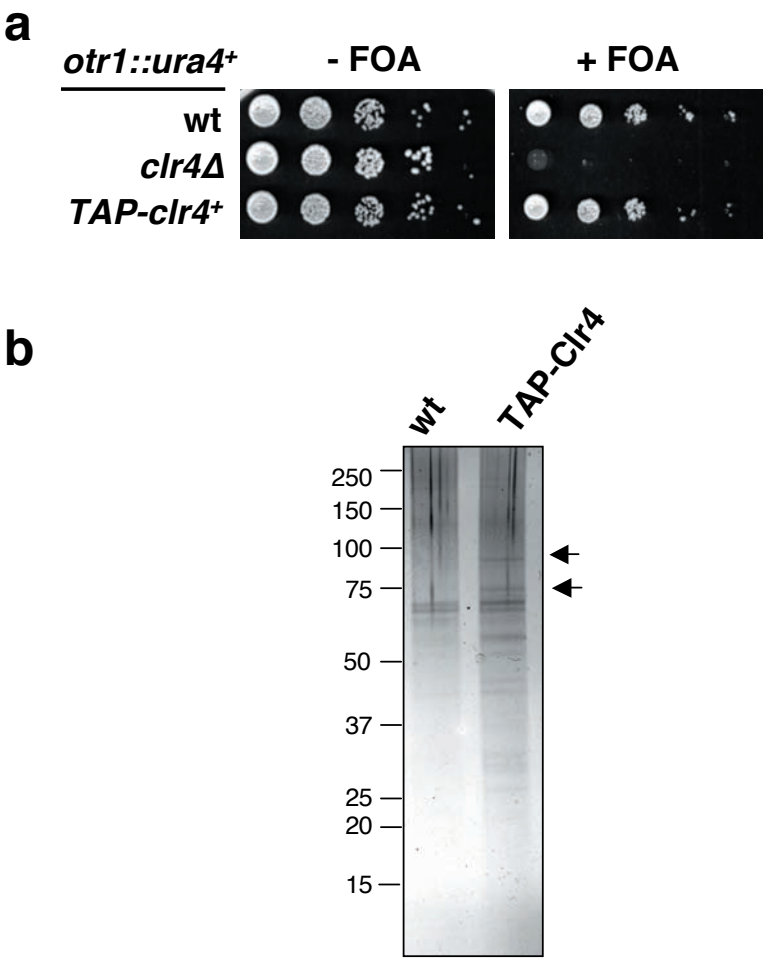

Figure S2 Tandem affinity purification of TAP-Clr4 identified a specific band corresponding to the size of Cul4. (a) TAP-Clr4 protein is fully functional. Serial dilution plating assays in the presence and absence of FOA were performed to measure the expression of *otr1::ura4+* reporter gene. (b) Tandem affinity purification of TAP-Clr4. Extracts prepared from indicated strains were purified by tandem affinity purification, resolved by SDS-PAGE and subjected to silver-staining. Arrows indicate protein bands specifically present in the TAP-Clr4 purification.

Figure S3

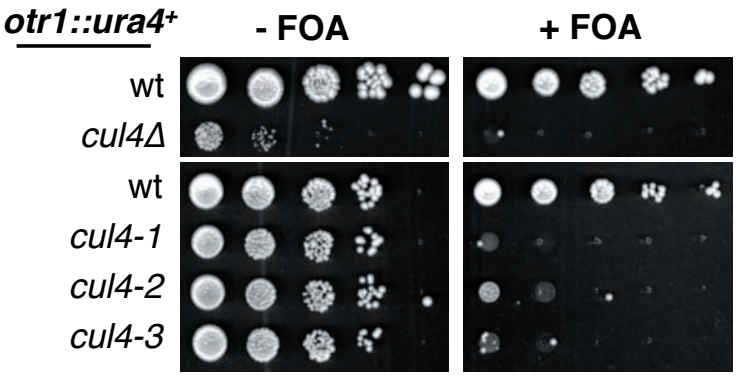

Figure S3 All *cul4* mutants show silencing defects. Serial dilution plating assays in the presence and absence of FOA were performed to measure the expression of *otr1::ura4*$^{+}$ reporter gene.

Figure S4

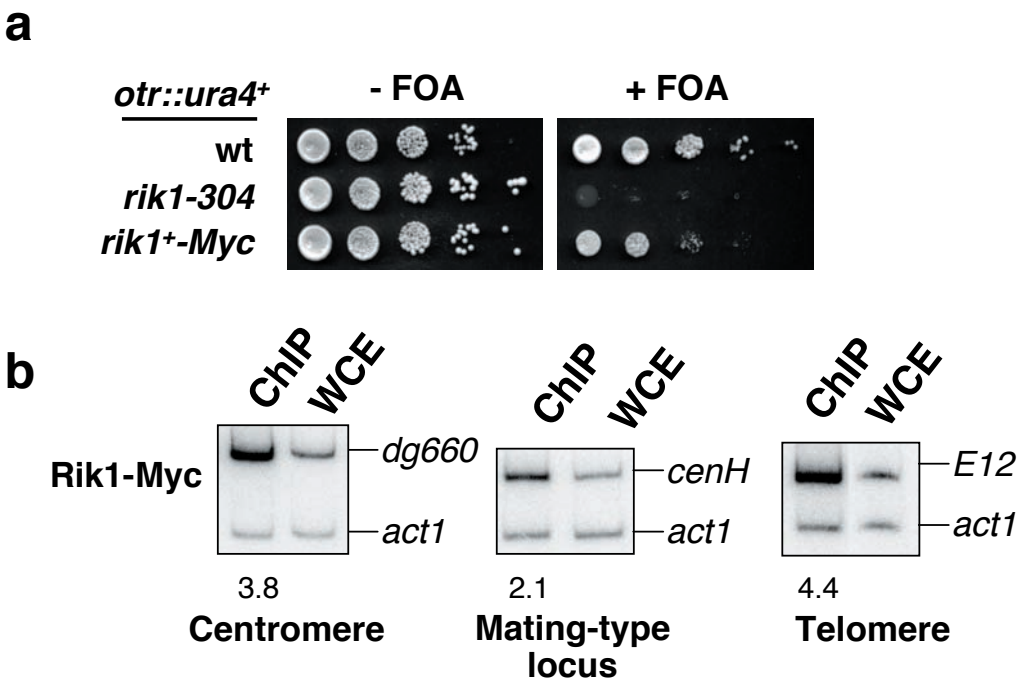

Figure S4 Rik1 is localized to repetitive DNA sequences at major heterochromatic loci. (a) Rik1-Myc protein is functional. Serial dilution plating assays in the presence and absence of FOA were performed to measure the expression of *otr1::ura4+* reporter gene. (b) Rik1 is localizes to major heterochromatic domains. Levels of Rik1-Myc at the indicated locations were determined by ChIP. Primers used were *dg660* (centromere), *cenH* (silent mating-type region), and *E12* (telomere).