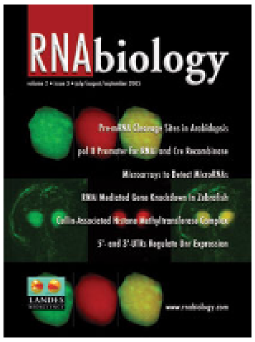

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Research Paper

A Cullin E3 Ubiquitin Ligase Complex Associates with Rik1 and the Clr4 Histone H3-K9 Methyltransferase and Is Required for RNAi-Mediated Heterochromatin Formation

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RNAi, heterochromatin, Clr4, Rik1, Cul4, histone H3-K9 methylation, epigenetics

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ABSTRACT

The assembly of heterochromatin in fission yeast and metazoans requires histone H3-lysine 9 (-K9) methylation by the conserved Clr4/Suv39h methyltransferase. In fission yeast, H3-K9 methylation requires components of the RNAi machinery and is initiated by the RNA-Induced Transcriptional Silencing (RITS) complex. Here we report the purification of a novel complex that associates with the Clr4 methyltransferase, termed the CLRC (CLr4-Rik1-Cul4) complex. By affinity purification of the Clr4-associated protein Rik1, we show that, in addition to Clr4, Rik1 is associated with the fission yeast E3 ubiquitin ligase Cullin4 (Cul4, encoded by *cul4+*), the ubiquitin-like protein, Ned8, and two previously uncharacterized proteins, designated Cmc1 and Cmc2. In addition, the complex contains substochiometric amounts of histones H2B and H4, and the 14-3-3 protein, Rad24. Deletion of *cul4+*, *cmc1+*, *cmc2+* and *rad24+* results in a complete loss of silencing of a *ura4+* reporter gene inserted within centromeric DNA repeats or the silent mating type locus. Each of the above deletions also results in accumulation of noncoding RNAs transcribed from centromeric repeats and telomeric DNA regions, and a corresponding loss of small RNAs that are homologous to centromeric repeats, suggesting a defect in the processing of noncoding RNA to small RNA. Based on these results, we propose that the components of the Clr4-Rik1-Cul4 complex act concertedly at an early step in heterochromatin formation.

INTRODUCTION

Heterochromatin is a conserved feature of eukaryotic chromosomes and plays important roles in centromere function and epigenetic gene regulation.[1,2] In the fission yeast *Schizosacchromyces pombe* and metazoans, heterochromatin assembly is associated with methylation of histone H3-lysine 9 (-K9) by Clr4/Suv39h methyltransferase.[1-3] H3-K9 methylation creates a binding site for the chromodomain protein Swi6/HP1 and sequential cycles of methylation and Swi6/HP1 binding have been proposed to mediate the spreading of heterochromatin.[4-7] At centromeres, heterochromatin assembly is initiated by components of the RNA interference (RNAi) machinery,[8] whereas at the silent mating type loci, *mat2/3*, RNAi and site-specific DNA binding protein play redundant roles in initiating heterochromatin assembly.[9,10]

Transcription from divergent promoters at centromeric repeats generates long double-stranded RNA, which is processed by Dcr1, the fission yeast Dicer homolog, into small interfering RNAs of ~22 nucleotides in size.[8,11] The RNAi effector complex, RITS, uses Dcr1-produced siRNAs as specificity factors to target a subset of chromosome regions for inactivation.[12] The generation of siRNAs also requires Rdp1, the fission yeast RNA-directed RNA polymerase, and Clr4.[13,14] Rdp1 is a component of the RNA-directed RNA polymerase complex (RDRC), which also contains the Hrr1 helicase and Cid12, a member of the polyA polymerase family of enzymes.[13] The RITS and RDRC complexes physically associate in a Dcr1 and Clr4 dependent manner, suggesting that both siRNAs and H3-K9 methylation are important for RITS-RDRC interaction.[13] Furthermore, the components of RITS and RDRC complexes can be cross linked to centromeric DNA as well as noncoding centromeric transcripts.[12,13,15] These observations have given rise to the hypothesis that RITS recruits RDRC and Clr4 to chromatin-associated nascent transcripts in order to initiate dsRNA synthesis and H3-K9 methylation.[13] In this model Clr4 plays a dual role in heterochromatin assembly. First, it tethers RITS to chromatin via the chromodomain of Chp1 and mediates dsRNA and siRNA synthesis by regulating RITS-dependent RDRC recruitment. Second, it creates a binding pocket for Swi6 and this mediates heterochromatin spreading.

Despite their conserved roles in heterochromatin assembly, histone H3-K9 methyltransferases that are specific to heterochromatin assembly have not yet been purified. In order to gain insight into the mechanisms that regulate H3-K9 methylation and the potential roles of this modification in regulation of the RNAi pathway, we purified a Clr4 containing complex from fission yeast, using an affinity tag on the Clr4-associated protein Rik1. Our purifications reveal that Clr4 and Rik1 are associated with the Cullin 4 ubiquitin ligase in a complex. We show that Cul4, the fission yeast Cullin 4 homolog, as well as the other Rik1-associated proteins are required for gene silencing at several different heterochromatic regions. Cullin 4 complexes have previously been shown to be associated with DNA-damage binding proteins, Ddb1 and Ddb2.[16-20] Interestingly, Rik1 is homologous to Ddb1, suggesting possible parallels between mechanisms that recruit the Clr4-Rik1-Cul4 and DDB1-Cul4 complexes to specific nucleic acid targets, siRNA-dependent structure at heterochromatin or damaged DNA, respectively.

Table 1 **List of strains used in this study**

Strain	Genotype
SPY28	(SPG1011) *h⁺ leu1-32 ade6-216 ura4DS/E imrR(NcoI)::ura4⁺ oriI*
SPY44	*h⁺ leu1-32 ade6-216 ura4DS/E imrR(NcoI)::ura4⁺ oriI rik1::TAP-Kan^R*
SPY95	*h⁺ leu1-32 ade6-216 ura4DS/E imrR(NcoI)::ura4⁺ oriI chp1Δ::TAP- Kan^R*
SPY137	*h⁺ leu1-32 ade6-210 ura4DS/E otrR(SphI)::ura4⁺*
SPY139	*h^{90} leu1-32 ade6-210 ura4DS/E mat3::ura4⁺*
EYP19	*h⁺ leu1-32 ade6-210 ura4DS/E otrR(SphI)::ura4⁺ clr4Δ::Kan^R*
EYP23	*h^{90} leu1-32 ade6-210 ura4DS/E mat3::ura4⁺ clr4Δ::Kan^R*
EYP48	*h⁺ leu1-32 ade6-216 ura4DS/E imrR(NcoI)::ura4⁺ oriI cmc1Δ::Nat^R*
EYP51	*h⁺ leu1-32 ade6-210 ura4DS/E otrR(SphI)::ura4⁺ cmc1Δ::Nat^R*
EYP57	*h⁺ leu1-32 ade6-216 ura4DS/E imrR(NcoI)::ura4⁺ oriI cmc2Δ::Nat^R*
EYP59	*h⁺ leu1-32 ade6-210 ura4DS/E otrR(SphI)::ura4⁺ cmc2Δ::Nat^R*
EYP75	*h⁺ leu1-32 ade6-216 ura4DS/E imrR(NcoI)::ura4⁺ oriI cmc2Δ::TAP-Kan^R*
EYP108	*h⁺ leu1-32 ade6-216 ura4DS/E imrR(NcoI)::ura4⁺ oriI rik1Δ::Kan^R*
EYP109	*h⁺ leu1-32 ade6-210 ura4DS/E otrR(SphI)::ura4⁺ rik1Δ::Kan^R*
EYP111	*h⁺ leu1-32 ade6-216 ura4DS/E imrR(NcoI)::ura4⁺ oriI cul4Δ::Nat^R*
EYP122	*h⁺ leu1-32 ade6-216 ura4DS/E imrR(NcoI)::ura4⁺ oriI clr4Δ::Kan^R*
EYP130	*h⁺ leu1-32 ade6-216 ura4DS/E imrR(NcoI)::ura4⁺ oriI rad24Δ::Nat^R*
EYP132	*h⁺ leu1-32 ade6-210 ura4DS/E otrR(SphI)::ura4⁺ rad24Δ::Nat^R*
EYP181	*h^{90} leu1-32 ade6-210 ura4DS/E mat3::ura4⁺ cmc1Δ::Nat^R*
EYP203	*h^{90} leu1-32 ade6-210 ura4DS/E mat3::ura4⁺ rik1Δ::Kan^R*
EYP204	*h^{90} leu1-32 ade6-210 ura4DS/E mat3::ura4⁺ cmc2Δ::Nat*
EYP205	*h^{90} leu1-32 ade6-210 ura4DS/E mat3::ura4⁺ cul4Δ::Nat^R*

MATERIALS AND METHODS

Strain construction. Strains used in this study are listed in (Table 1.) Deletion and epitope-tagged strains were made using a single-step gene replacement strategy.[21,22]

Silencing assays. Silencing of a *ura4⁺* reporter inserted in various heterochromatic regions was performed as described.[23]

TAP purification and protein identification. Rik1-TAP and Cmc2-TAP were purified as described and associated proteins were identified by LC-MS/MS analysis of tryptic digests of excised silver-stained gel bands and total mixtures of proteins[23,24] using a hybrid linear ion trap -7 Tesla ion cyclotron resonance Fourier transform instrument (LTQ-FT, Thermo Finnigan, San Jose, CA).

RT-PCR. RNA from exponentially growing cultures was isolated as follows. Four milliliters of overnight culture was refreshed in 25 ml of rich medium for four hours. Cells were lysed by adding 1.2 ml of lysis buffer (50 mM sodium acetate, 10 mM EDTA, 0.8% SDS) and 1.2 ml of acid phenol (pH 4.3), followed by repeated cycles of vortexing and incubation at 65°C. 1.3 ml of chloroform was added, and the aqueous phase containing RNA was separated from the cell debris/organic phase using the Phase-Lock Gel (Eppendorf). The recovered solution was supplemented with $MgCl_2$ and $CaCl_2$ to final concentrations of 25 and 5 mM, respectively, and was treated with 50 units of DNase I (Roche) for two hours at 32°C. DNase I was removed by phenol/chloroform extraction, and RNA was precipitated with isopropanol. One microgram of RNA was used for each RT-PCR reaction using the one-step RT-PCR kit (Qiagen) with primers listed in Table 2. For detection of RNA from heterochromatic regions and *act1⁺* after reverse transcription, 26 and 21 cycles of PCR were performed, respectively.

Northern blot analysis of small RNAs. RNA was prepared as described for RT-PCR. Small RNAs were enriched using the RNeasy midi kit according to the manufacturer's instruction (Qiagen). Twenty micrograms of small RNAs were separated on 18% polyacrylamide gels containing urea, which were subsequently electroblotted on nylon membranes using semi-dry transfer apparatus (BioRad) at 10 volts for one hour. Membranes were cross-linked at 1,200 μjoules, and baked at 80°C for 1 hour to immobilize RNA. Membranes were hybridized in Ultra Hyb-oligo buffer (Ambion) with radioactively labeled probes at 35°C overnight. Radioactive probes were prepared by labeling 40 pmoles of oligos that are complementary to centromere heterochromatic repeats[11] with 40 pmoles of [γ-32P] ATP (3000 Ci/mmol, NEN Life Science), using polynucleotide kinase (Roche). Similarly labeled oligos complementary to snoR69 were used as a loading control (EJP126 in Table 2). Probes were purified using microspin G-25 columns (Amersham)

Table 2 **List of oligonucleotides used in this study**

Name	Sequence
ACT1-1	5'GAGTCATCTTCTCACGGTTGG3'
ACT1-2	5'TCCTACGTTGGTGATGAAGC3'
RT-PCR-1	5'GAAAACACATCGTTGTCTTCAGAG3'
RT-PCR-2	5'CGTCTTGTAGCTGCATGTGAA3'
AV360	5'ACATACTAGTGATGCAAGCGTTC3'
AV361	5'TACTATTCTCAACTTAAGTAGTC3'
AV362	5'TTCAAGACTTGGAAATCATGCAG3'
AV363	5'CCGTTCGAATGATATCCCTAACG3'
EJP126	5'CAATGTAAATACTCCGAGTGAGCTGGGTTTAAC3'

Transcripts from *act1⁺*, *otr*, *imr* and SPAC212.11 (silent telomeric ORF) were detected by pairs of oligonucleotides ACT1-1/ACT1-2, RT-PCR-1/RT-PCR-2, AV360/AV361, and AV362/AV363, respectively. snoR69 was detected with probes generated from EJP126.

prior to hybridization. Membranes were washed in 2X SSC, 0.5% SDS, and small RNAs were visualized using a phosphoimager screen.

RESULTS

Purification of Rik1 and identification of Rik1-associated proteins. Rik1 has previously been shown to coimmunoprecipitate with Clr4 and like Clr4 is required for H3-K9 methylation and heterochromatin formation.[25,26] We created a strain that expressed a C-terminally TAP-tagged Rik1 protein, which was fully functional for centromeric silencing (Fig. 1A). Rik1-TAP was purified as previously described and its associated proteins were identified by LC-MS/MS analysis of excised gel bands and trichloroacetic acid (TCA) precipitated mixture of proteins[23,27] (Fig. 1B, Table 3). Analysis of the precipitated mixture identified Rik1, Cul4, Clr4, SPCC613.12c, SPCC970.07c, Ned8, histone H4, histone H2B and Rad24 as Rik1-associated proteins (Table 3). All proteins, except H2B and H4, were represented with two or more peptides. We designated the proteins encoded by SPCC613.12c

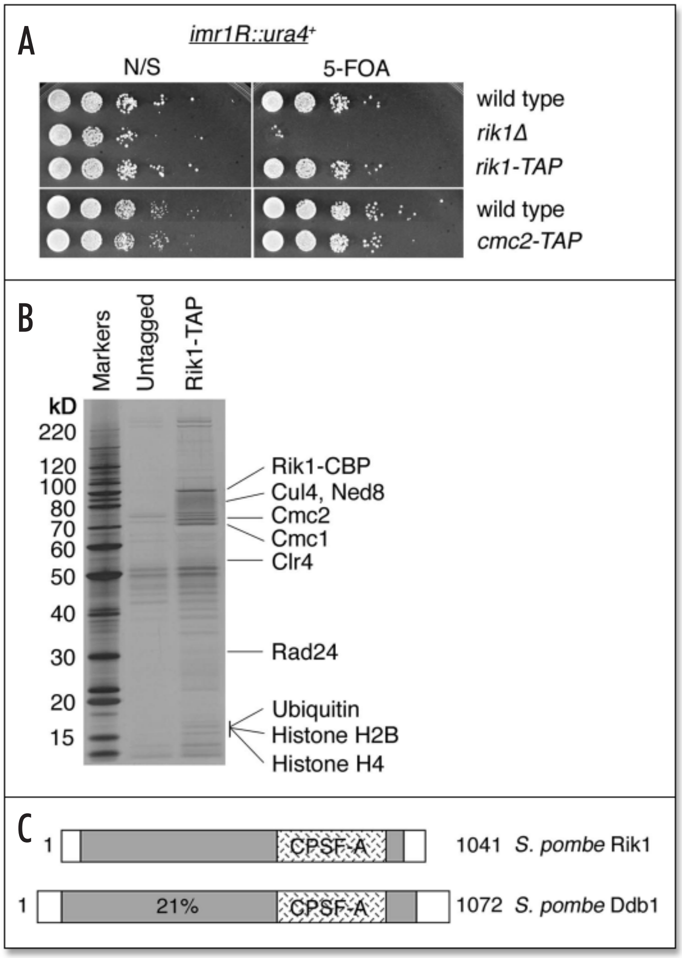

Figure 1. Purification of the *S. pombe* CLRC Complex and identification of its subunits. (A) Epitope-tagged Rik1 and Cmc2 are fully functional. A *ura4*$^+$ reporter inserted at the innermost centromeric repeats (*imr1R::ura4*$^+$) in strains containing *rik1-TAP* or *cmc2-TAP* shows the wild-type level of silencing. N/S indicates no selection, while 5-FOA indicates selection against *ura4*$^+$. (B) Silver-stained gel shows the purification of Rik1-TAP. A control purification from an untagged strain and molecular markers are also shown. Protein identities are based on LC-MS/MS analysis of excised gel bands. (C) *S. pombe* Rik1 and Ddb1 share sequence similarity. Schematic diagram represents the Rik1 and Ddb1 proteins. The shaded area (gray) denotes the region of amino acid sequence similarity. Rik1 and Ddb1 share 21% of sequence identity and 43% of sequence similarity. The dotted area indicates the region of sequence similarity with the cleavage and polyadenylation specificity factor-A (CPSF-A).

and SPCC970.07c, Cmc1 and Cmc2, respectively (Cullin-associated methyltransferase complex protein). Cmc1 is a WD-repeat protein, but Cmc2 shares no obvious sequence similarity with other proteins in databases. Analysis of silver-stained bands verified the presence of Rik1, Cul4, Ned8, Clr4, Cmc1, Cmc2, histone H2B, as well as three ubiquitin peptides in Rik-TAP purifications (Fig. 1B). The comigration of Ned8 with Cul4 is consistent with previous findings showing that activated Cullin subunits are neddylated (reviewed in ref. 28). The identification of histone in the Clr4-Rik1-Cul4 complex strongly suggests that the complex is chromatin associated. Furthermore, the presence of ubiquitin peptides in the 15–20 kD region of the gel suggests that one of the core histones, or an unidentified small protein, associated with Rik1 may be ubiquitylated.

We next generated strains that expressed C-terminally TAP-tagged Cmc1 and Cmc2, of which only the Cmc2-TAP strain was fully functional in a silencing assay (Fig. 1A). LC-MS/MS analysis of two independent Cmc2-TAP purifications identified nearly all of the same proteins that were identified in Rik1-TAP purifications (Table 3). In addition, Rbx1, the fission yeast homolog of the human and budding yeast ring domain protein, previously shown to be associated with and required for the activation of Cullin ubiquitin ligases,[29] was present in one of our Cmc2-TAP purifications.

Rik1-associated proteins are required for gene silencing. In order to test the possible role of Rik1-associated proteins in gene silencing within heterochromatic regions, we replaced the open reading frame for each *cmc1*$^+$, *cmc2*$^+$, *cul4*$^+$ and *rad24*$^+$ with an antibiotic resistance marker in strains that carried the *ura4*$^+$ reporter gene at the centromeric innermost (*imr1R::ura4*$^+$) or outermost (*otr1R::ura4*$^+$) repeat regions. As previously reported for *chp1*Δ, *rik1*Δ, and *clr4*Δ cells, we observed a complete loss of silencing of the *imr1R::ura4*$^+$ reporter gene in *cmc1*Δ, *cmc2*Δ, *cul4*Δ and *rad24*Δ cells (Fig. 2A). Deletion of *cmc1*$^+$, *cmc2*$^+$, and *rad24*$^+$ also abolished silencing of the *otr1R::ura4*$^+$ reporter gene (Fig. 2B). The newly identified Rik1-associated proteins were therefore required for silencing within the centromeric repeats. Rbx1 and Ned8 are essential for growth and their expected role in silencing could not be assessed.

Unlike components of the RNAi pathway, Rik1 and Clr4 are required for silencing at the silent *mat2/3* region. To determine whether the newly identified Rik1-associated proteins were similarly required for silencing at the *mat2/3* region, each of the above deletions was also tested for silencing of the *ura4*$^+$ gene inserted within the mating type region heterochromatin (*mat3::ura4*$^+$). As is the case with the deletion of *rik1*$^+$ and *clr4*$^+$, deletion of *cmc1*$^+$, *cmc2*$^+$ and *cul4*$^+$ resulted in a complete loss of silencing of the *mat3::ura4*$^+$ reporter (Fig. 3), indicating that each of the new gene products was required for mating type silencing.

Defects in components of the RNAi pathway and other genes that act upstream of H3-K9 methylation result in accumulation of noncoding RNAs that are transcribed from centromeric repeats.[8,13] We therefore tested whether deletion of each *cmc1*$^+$, *cmc2*$^+$, *cul4*$^+$ and *rad24*$^+$ affected the accumulation of noncoding heterochromatic RNAs in an RT-PCR assay. As shown in Figure 4, noncoding RNAs corresponding to the otr-dg repeats accumulate in *rik1*Δ, *cmc1*Δ, *cmc2*Δ, *cul4*Δ and *rad24*Δ cells, similar to what has been previously reported for *clr4*Δ cells.[13] Noncoding transcripts at the *imr* region of the centromere have not yet been mapped, but we detected the accumulation of RNAs corresponding to *imr*, as well as the subtelomeric SPAC212.11 open reading frame previously reported to be targeted by the RNAi pathway in each of the above mutant strains[30] (Fig. 4). In contrast, none of the deletions affected the level of actin mRNA (Fig. 4).

Small RNAs corresponding to the centromere heterochromatic repeats, called centromeric siRNAs, have

Table 3 **Identification of Rik1-associated proteins by mass spectrometry**

LC-MS/MS of Total Mixtures (# Unique Peptides, % Sequence Coverage by Mass)			
Rik1-TAP 1st	**Rik1-TAP 2nd**	**Cmc2-TAP 1st**	**Cmc2-TAP 2nd**
Rik1 (28, 29%)	Rik1 (33, 34%)	Rik1 (17, 20%)	Rik1 (13, 15%)
Cul4 (15, 22%)	Cul4 (9, 14%)	Cul4 (14, 24%)	Cul4 (8, 12%)
Cmc1 (10, 25%)	Cmc1 (15, 42%)	Cmc1 (15, 40%)	Cmc1 (7, 18%)
Cmc2 (11, 18%)	Cmc2 (9, 17%)	Cmc2 (12, 23%)	Cmc2 (15, 22%)
Clr4 (4, 14%)	Clr4 (6, 19%)	Clr4 (2, 6%)	Clr4 (4, 12%)
Ned8 (3, 25%)	Ned8 (1, 15%)	Ned8 (2, 25%)	-
histone H4 (2, 22%)	histone H4 (2, 22%)	-	-
histone H2B (1, 11%)	-	-	-
Rad24 (1, 5%)	-	Rad24 (1, 5%)	Rad24 (1, 5%)
-	-	-	Rbx1 (1, 10%)

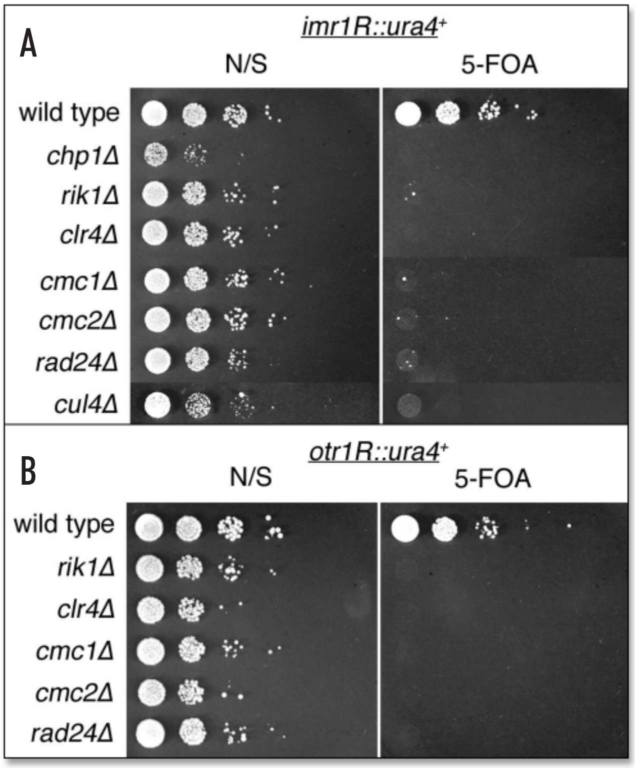

Figure 2. Rik1-associated proteins are required for silencing at centromeric DNA regions. Silencing of *ura4*+ inserted at (A) the innermost centromeric repeats (*imr1R::ura4*+) and (B) outermost centromeric repeats (*otr1R:: ura4*+) is lost in *cmc1*Δ, *cmc2*Δ, *rad24*Δ, and *cul4*Δ cells . Silencing in *chp1*Δ, *rik1*Δ and *clr4*Δ cells is shown for comparison. N/S indicates no selection, while 5-FOA indicates selection against *ura4*+.

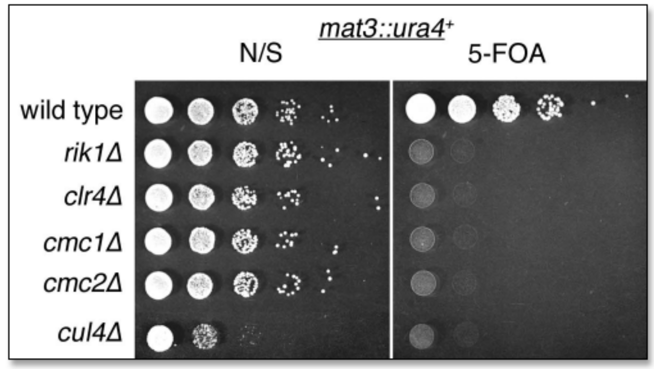

Figure 3. Rik1-associated proteins are required for silencing at the mating type region. Deletion of *cmc1*+, *cmc2*+, and *cul4*+ results in loss of silencing of a *mat3::ura4*+ reporter gene.

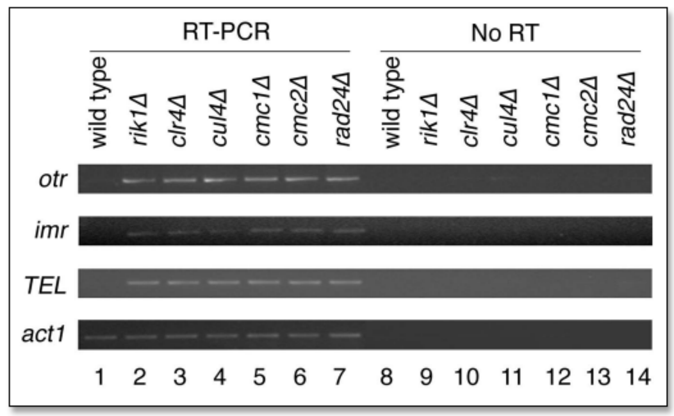

Figure 4. Noncoding heterochromatic transcripts accumulate in cells lacking Rik1-associated proteins. RT-PCR shows that the transcripts from silent loci, but not actin mRNA (*act1*+), become more abundant in cells lacking the CLRC subunits (compare lanes 2-7 with lane 1). *otr*, outer centromeric repeats; *imr*, innermost centromeric repeats; *TEL*, subtelomeric open reading frame, SPAC212.11.

been proposed to be important for recruitment of RITS and RDRC, the RNAi machineries required for initiation of heterochromatin formation. To test whether siRNA production is affected by the loss of Rik1-associated proteins, we used Northern blot analysis to examine changes in siRNA levels that might result from deleting genes that encode the Rik1-associoeted proteins. In contrast to wild-type cells, in *rik1*Δ, *clr4*Δ, *cmc1*Δ, *cmc2*Δ, *cul4*Δ and *rad24*Δ cells, siRNAs homologous to centromeric repeats were absent, as was the case in *dcr1*Δ cells (Fig. 5). These results indicate a failure to process noncoding RNA to siRNAs, and strongly suggests that Cmc1, Cmc2, Cul4, and Rad24 act together with Rik1 and Clr4 in an early step in heterochromatin formation.

DISCUSSION

A Cullin-associated histone methyltransferase complex. In this study, we have used affinity purification to identify several proteins that associate with the fission yeast silencing protein Rik1. In addition to the Clr4 histone H3-K9 methyltransferase, Rik1 associated proteins include Cul4, the fission yeast Cul4 homolog, as well as two previously uncharacterized proteins, Cmc1 and Cmc2, the 14-3-3 protein Rad24, and the histones H2B and H4. Deletion of *cul4*+, *cmc1*+, *cmc2*+ and *rad24*+ abolishes the silencing of a *ura4*+ reporter gene at centromeric DNA repeats and the silent mating type loci and is accompanied by accumulation of noncoding centromeric RNAs. Our results demonstrate that an E3 ubiquitin ligase is associated with Rik1/Clr4 and is required for heterochromatin formation.

The purification of the Rik1 complex has been reported independently by Horn et al.[31] but their Rik1 purifications did not contain Ned8 or Rad24. In addition to identifying the protein subunits of the Rik1 complex, we have found that (1) the Rik1-associated Cul4 is neddylated, suggesting that it is an active ubiquitin ligase, (2) ubiquitin peptides comigrate with histones in our Rik1 purifications, suggesting that histones are the likely targets of Cul4-mediated ubiquitylation in the complex, (3) noncoding RNAs accumulate in cells with deletions of the genes that code for each one of the Rik1-associated proteins, and (4) these noncoding RNAs do not get processed to siRNAs that are normally loaded on RITS. We had previously shown that the RITS complex purified from *clr4*Δ cells is devoid of siRNAs.[13] The absence of siRNAs in total RNA preparations from cells carrying deletions of Rik1-associated proteins, assayed by Northern blotting (Fig. 5), suggests that Clr4, Rik1, and their associated proteins are required for siRNA generation, rather than siRNA loading onto RITS.

Cul4 E3 ubiquitin ligase complexes and heterochromatin assembly. Attachment of ubiquitin to target proteins involves activation by an E1 enzyme and subsequent transfer by an E2 ubiquitin-conjugating enzyme (reviewed in ref. 32). E2 enzymes are typically associated with an E3 ubiquitin ligase that recognizes the substrate. Eukaryotic

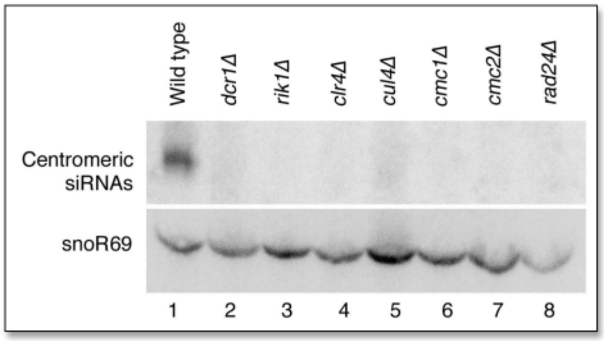

Figure 5. Production of centromeric siRNAs is impaired in cells lacking the CLRC subunits. Northern blot shows that siRNAs homologous to centromeric repeats are absent in cells lacking the CLRC subunits (compare lanes 2–7 with lane 1). snoR69 is shown as a loading control.

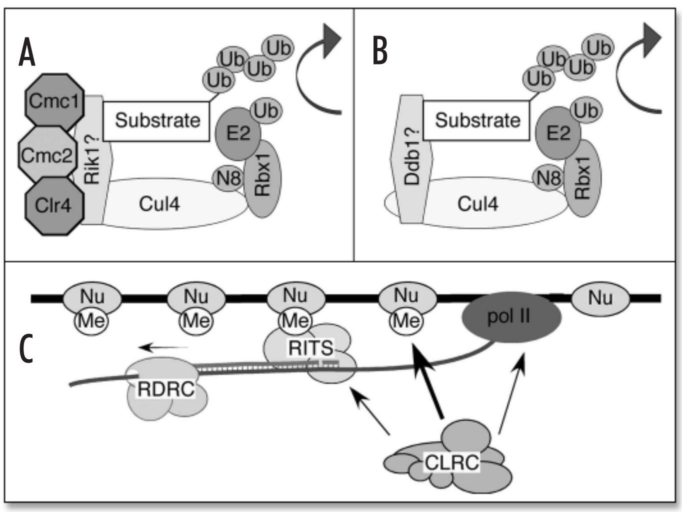

Figure 6. Parallels between the Clr4-Rik1-Cul4 and Ddb1-Cul4 complexes and model for recruitment of RNAi complexes and Clr4-Rik1-Cul4 to chromatin. Schematic diagrams of (A) Clr4-Rik1-Cul4 (CLRC) and (B) Ddb1-Cul4 (DDB) complexes, showing a similarity in composition. Arrow indicates the ubiquitylation of unknown substrate(s). In addition to the indicated subunits, Rad24 associates with CLRC and may regulate its activity. Ub, ubiquitin; N8, Ned8. (C) Model for the recruitment or Clr4-Rik1-Cul4 complex to chromatin via interactions with a RITS-associated nascent transcript. RITS, RNA-Induced Transcriptional Silencing complex; RDRC, RNA-Directed RNA polymerase Complex. Me, histone H3 methylated at lysine 9. See text for details.

cells contain numerous E3 ubiquitin ligases that are the primary determinants of substrate specificity. Cullins act as scaffold proteins for a subset of multisubunit E3 ubiquitin ligases that are involved in diverse biological processes. The founding member of these complexes is the SCF (Skp1-Cullin-F-box) complex in which the Cul1 subunit associates with Skp1 and an F-box protein that mediate substrate recognition.[33-35] In the Cul2 and Cul3 complex, other proteins replace the Skp1/F-box module and mediate substrate ubiquitylation.

Cul4 ubiquitin ligase complexes have been previously purified from fission yeast and mammalian cells and are associated with DNA damage repair and genome stability.[36] In human cells, Cul4 is associated with the DNA damage-binding proteins, Ddb1 and Ddb2, which form a complex together that recognizes cyclobutane pyrimidine dimers. Upon induction of DNA damage, the Ddb1/Cul4 complex binds damaged DNA and targets Cdt1, a replication licensing factor, for ubiquitylation and subsequent degradation in order to prevent rereplication of DNA.[37,38] As Ddb1 bridges the interaction between Cul4 and its substrate Cdt1, it has been proposed that Ddb1 is the specificity factor that recognizes target substrates[19] (Fig. 5B).

Rik1 shares sequence similarity with fission yeast Ddb1 over its entire length (see Fig. 1C). Based on its sequence similarity to Ddb1, we propose that Rik1, alone or in combination with Cmc1 (a WD- repeat protein) and Cmc2 proteins, constitutes the substrate specificity module in the Cul4-Clr4 complex (Fig. 5A). Whereas Ddb1 targets Cul4 to damaged DNA and other substrates such as Cdt1, we propose that Rik1 targets this E3 ubiquitin ligase, together with the Clr4 histone H3 methyltransferase, to a chromatin structure that is created during heterochromatin assembly (Fig. 5C). We have previously noted that the C-terminus of Rik1 shares sequence similarity to the CPSF-A subunit of the polyA polymerase complex[13] (see Fig. 1C). Sequence similarity between Rik1 and two nucleic acid binding proteins, CPSF-A and Ddb1, suggests that Rik1 may be a nucleic acid binding protein that recognizes some feature of noncoding nascent transcripts that are targeted by the RITS complex during heterochromatin assembly. This targeting would then trigger the ubiquitylation of an unknown chromatin substrate(s) and histone H3-K9 methylation. Previous studies have established a link between histone H2B ubiquitylation and histone H3-K4 and -K79 methylation in active chromatin.[39-42] An intriguing possibility suggested by our results is that ubiquitylation of a histone or another protein also mediates H3-K9 methylation in heterochromatin.

The 14-3-3 protein, Rad24, is required for heterochromatin formation. The 14-3-3 proteins bind to phosphoserine containing proteins and regulate numerous functional interactions.[43,44] In addition to its role in the DNA damage checkpoint, the fission yeast Rad24 has been found to associate with two RNA-binding proteins. The association of the RNA-binding protein Mei2 with MeiRNA is a required step for entry into meiosis; Rad24 disrupts Mei2-MeiRNA association and inhibits meiosis by binding to phosphorylated Mei2.[45] Rad24 also copurifies with the Cid13 polyA polymerase but the functional significance of this interaction is unknown.[46] The association of Rad24 with Rik1 and Cmc2, and its requirement for gene silencing, suggest that phosphorylation and Rad24 binding regulate CLRC function in heterochromatin assembly.

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