Clr4^{SUV39H1} and Bdf2^{BRD4} ubiquitination mediate transcriptional silencing via heterochromatic phase transitions

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Abstract

Heterochromatin has crucial roles in genome stability by silencing repetitive DNA and by suppressing recombination^{1,2}. In the fission yeast *Schizosaccharomyces pombe*, Clr4 methylates histone H3 lysine 9 (H3K9) to maintain centromeric heterochromatin during the cell cycle, guided by RNA interference (RNAi)^{3,4}. Clr4 interacts with a specialized ubiquitination complex CLRC, which is recruited in part by small RNA and the RNAi transcriptional silencing complex (RITS) but otherwise, the role of CLRC is unknown⁵⁻⁸. Here we show that the E2 ubiquitin conjugating enzyme Ubc4 interacts with CLRC and targets Clr4 for mono-ubiquitination at an intrinsically disordered region. Mono-ubiquitination is essential for the transition from co-transcriptional (H3K9me2) to transcriptional (H3K9me3) gene silencing by transiently releasing Clr4 from centromeric heterochromatin, and is reversed by the deubiquitinating enzyme Ubp3. Addition of ubiquitin changes the liquid-liquid phase separation (LLPS) of Clr4 to enhance its dynamic activity. Ubc4-CLRC also poly-ubiquitinates Bdf2, the homologue of BET family double bromodomain protein BRD4 for degradation, which otherwise accumulates at centromeres to promote transcription and production of small RNAs.

Main text

Co-transcriptional gene silencing (CTGS) depends on the production of centromeric sRNAs by RNA polymerase II and RNAi, which guides H3K9me2 restoration during S phase of cell cycle. The subsequent transition from H3K9me2 to H3K9me3 and binding of HP1 proteins Swi6 and Chp2 to heterochromatin, suppresses RNA Polymerase II-dependent transcription by transcriptional gene silencing (TGS)^{2,9-11}. However, the

mechanism that promotes the transition from RNAi-dependent CTGS to TGS is unclear. Ubiquitination is a post-translational modification which requires ubiquitin, E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme and E3 ubiquitin ligase¹². We previously showed that a specific mutation in the E2 ubiquitin conjugating enzyme Ubc4 abolishes heterochromatic silencing at centromeres, mating type loci and telomeres¹³ (Extended Data Fig 1b, d). Overexpression of Ubc4 also causes chromosome mis-segregation¹⁴. We found that the *ubc4-G48D* (*ubc4-1*) mutation results in Ubc4 protein instability (Extended Data Fig. 1c, e). Replacement of *ubc4* with the mouse homologue UBE2D2 fully complemented *ubc4-1* and *UBE2D2-G48D* had similar silencing defects (Extended Data Fig. 1b). A spontaneous second-site suppressor, S137T (see Material & Methods), stabilizes Ubc4-1, restoring the silencing defects (Extended Data Fig. 1d-f).

Ubc4 and Cul4 regulate H3K9 methylation through RNAi

The E3 ubiquitin ligase, Cul4 is also involved in heterochromatin silencing and is part of the CLRC complex⁵⁻⁸. *ubc4-1* and the *cul4-GFP* fusion (*cul4-1*) mutants have very similar phenotypes, including loss of silencing of *ade6⁺* and *ura4⁺* reporter genes in centromeric heterochromatin (Extended Data Fig. 1b), as well as increased binding of Rpb1, the catalytic component of RNA polymerase II (Extended Data Fig. 2c). Interestingly, while H3K9me3 was lost from both *ubc4-1* and *cul4-1* mutants at all heterochromatic loci tested, H3K9me2 had increased occupancy at *dg* and *dh* repeats. Increased H3K9me2 was also observed at the centromeric *ade6⁺* reporter gene, which accumulates small RNA, but not at the *ura4⁺* gene, which does not (Fig. 1a and Extended Data Fig. 2a, 2b, 3a). Swi6^{HP1} protein binding was present at intermediate

levels, presumably because it recognizes both H3K9me2 and H3K9me3 (Extended Data Fig. 2e)^{9,15}. To determine whether RNAi is important for maintaining H3K9me2 in *ubc4-1* mutant cells, we introduced the *ubc4-1* mutation in *dcr1Δ* mutant cells and found that the *dcr1Δ ubc4-1* double mutant had greatly reduced H3K9me2, and was more sensitive to microtubule destabilizing agent thiabendazole (TBZ), showing that centromeric heterochromatin was completely lost (Extended Data Fig. 3b-e). The more severe alleles *ubc4-3xHA* and *cul4Δ* abolished H3K9me2 altogether (Extended Data Fig. 4a-c). Live imaging revealed that Ubc4 and Cul4 proteins localize to nuclear periphery (Extended Data Fig. 4d), and they form a stable complex by co-immunoprecipitation (Co-IP) (Extended Data Fig. 4e). Ubc4 also forms a complex with Raf1 and Raf2, two other components of CLRC (Extended Data Fig. 4f). These results clearly indicate that Ubc4 and Cul4 form the E2/E3 complex by which CLRC targets substrates important for heterochromatin assembly.

Ubc4-CLRC mono-ubiquitinates Clr4

Next, we set out to identify the substrates of this novel E2/E3 complex, Ubc4-CLRC. Interestingly, Clr4 co-purifies with the CLRC complex *in vivo*, but does not behave like a structural component of CLRC *in vitro*⁵⁻⁸. The Clr4 human homolog, SETDB1 is mono-ubiquitinated at its SET-Insertion domain and this modification is important for its H3K9 methyltransferase activity¹⁶. To test whether Clr4 is a substrate of CLRC, we purified 3xFlag-Clr4 protein from *S. pombe* cells (Methods) and detected a single higher molecular weight form that reacted with ubiquitin antibodies in a Ubc4-dependent fashion (Fig. 1c, Extended Data Fig. 5a, b). A pulldown of ubiquitinated proteins using

Ubiquitination Affinity Beads (UBA) revealed a similar higher molecular weight Clr4 which is also dependent on Ubc4 (Extended Data Fig. 5c). In addition to Ubc4, the presence of this higher molecular weight Clr4 depends on Cul4, Rik1, Raf1 and Raf2, the other components of CLRC but not on the proteasome inhibitor MG-132 (Extended Data Fig. 5d, e). These results strongly suggest that Ubc4 together with CLRC mono-ubiquitinates Clr4 *in vivo*. As the abundance of Clr4-Ub protein is too low to detect ubiquitinated residues by Mass Spectrometry, we ubiquitinated 6xHis-Clr4 recombinant protein purified from *E. coli* cells *in vitro*. This approach uncovered many possible ubiquitination sites, which mostly reside in an intrinsically disordered region (IDR) between the chromodomain (CD) and PreSET domain (Extended Data Fig. 5g-i) which is involved in nucleosome binding¹⁷. To identify which of these were functional *in vivo*, 36 lysines of Clr4 were arranged into 6 subgroups, and mutated into arginines (R). We also included a mutant of all 36 lysines (*AllKtoR*). The all-lysine mutant (*AllKtoR*) and the second block mutant (*2-8KtoR*), corresponding to the middle of the IDR, completely abolished ubiquitination (Fig. 1e and Extended Data Fig. 6b) consistent with results of Mass Spectrometry. Although no single lysine to arginine mutant lost ubiquitination (Extended Data Fig. 6c) we could narrow down the possible sites to 4 lysines (*clr4-2-4KtoR*; K109, K110, K113, and K114) (Fig. 1e and Extended Data Fig. 6d-f). ChIP-Seq revealed that *clr4-AllKtoR* lost both H3K9me2 and H3K9me3, resembling strong alleles of *ubc4* and *cul4*, while *clr4-2-4KtoR* had the same increase of H3K9me2 and decrease of H3K9me3 seen in *ubc4-1* and *cul4-1* hypomorphs (Fig. 1g and Extended Data Fig. 6g). Thus mono-ubiquitination of Clr4 by Ubc4-CLRC is required for H3K9 trimethylation at centromeric heterochromatin.

Clr4 ubiquitination induces its dissociation from chromatin

Clr4 ubiquitination does not change the histone methylation activity of Clr4 (Extended Data Fig. 7a) but binding to chromatin was increased in *ubc4-1*, *cul4-1*, *clr4-2-8KtoR* or *clr4-AllKtoR* mutants, especially at *dh* where sRNAs are produced (Fig. 2a and Extended Data Fig. 7b). To further explore the effect of Clr4 mono-ubiquitination on chromatin binding, we carried out nucleosome binding assays using *in vitro* ubiquitinated 6xHis-Clr4 recombinant protein or 3xFlag-Clr4 purified from *S. pombe* cells with tethered nucleosomes (Extended Data Fig. 7c). In contrast to unmodified Clr4 protein which had strong binding affinity to the nucleosome, especially when H3K9me3 was present, ubiquitinated Clr4 did not bind to the nucleosome (Fig. 2b Extended Data Fig. 7d). These results are consistent with the increased binding *in vivo* in the absence of mono-ubiquitination in *ubc4-1* or *cul4-1*. Binding in the absence of ubiquitin depended on the Clr4 chromodomain (Fig. 2c and Extended Data Fig. 7e)^{9,18}. These results indicate that Clr4 mono-ubiquitination reduces the binding activity of Clr4 to chromatin and that this is important for the transition from H3K9 di- to tri-methylation.

Ubp3 deubiquitinates Clr4

Ubiquitination is a reversible, highly dynamic and transient modification, and its removal is catalyzed by deubiquitinating enzymes¹². A deletion mutant of deubiquitinating enzyme Ubp3 rescues heterochromatic silencing defects of *ago1Δ*, and *ubp3Δ* shows positive epistatic genetic interactions with CLRC component mutants^{19,20}. While deletion of Ubp3 did not increase Clr4 mono-ubiquitination (Extended Data Fig. 8a), overexpression had a silencing defect at the centromeric *ura4⁺* reporter gene and

reduced the mono-ubiquitination of Clr4 (Fig. 1f and Extended Data Fig. 8b). The overexpression of Ubp3 also resulted in an increase of H3K9me2 and decrease of H3K9me3 as well as increased RNA Pol II binding, noncoding RNA transcription and Clr4 binding at centromeric heterochromatin (Fig. 1g and Extended Data Fig. 8c-f). These results indicate that Ubp3 counteracts Clr4 mono-ubiquitination activity of Ubc4-CLRC and reverses Clr4 activity.

Clr4^{IDR} displays ubiquitin-dependent phase separation properties.

Swi6 forms 4 foci in the nucleus corresponding to the chromocenter and the three telomeric clusters, all of which depend on Clr4 (Fig. 3a and Extended Data Fig. 9a). In *ubc4-1* mutants, the telomeric foci disappear and the chromocenter is retained, while in *dcr1Δ* mutants the telomeric foci are retained and the chromocenter is reduced (Fig. 1b and 3a and Extended Data Fig. 3c, 3d and 9a). Along with HP1 proteins from human, Swi6 has been shown to undergo liquid-liquid phase separation (LLPS) which contributes to the highly dynamic features of heterochromatin^{21,22}. Clr4 protein has an intrinsically disordered region (IDR) or low-complexity domain (LCD) between its chromodomain (CD) and PreSET domain¹⁷, and IDR/LCD are domains typically responsible for phase separation (Extended Data Fig. 9b)²³. We found that recombinant GFP-Clr4^{Full} and GFP-Clr4^{IDR} (51-220 amino acids) proteins form liquid droplets *in vitro* at low salt conditions even without addition of crowding agents like PEG8000 (Fig. 3b and Extended Data Fig. 9c, d). Next, we treated the proteins with 1,6-Hexanediol which dissolves liquid droplets by disrupting hydrophobic interaction of protein-protein or protein-RNA²⁴. We found that the formation of liquid droplets of GFP-Clr4^{IDR} was greatly

reduced by 1,6-haxanediol treatment indicating that these liquid droplets are formed by LLPS (Extended Data Fig. 9d). Clr4^{IDR} binds ubiquitin²⁵ and we added concentrated ubiquitin to GFP-Clr4^{IDR} in order to mimic the effect of ubiquitination of Clr4. Strikingly, we found that adding ubiquitin greatly reduced the size of LLPS of GFP-Clr4^{IDR} (Fig. 3b, c). Furthermore, fluorescence recovery after photobleaching (FRAP) of the liquid droplets showed a faster recovery rate of GFP-Clr4^{IDR} with ubiquitin than GFP-Clr4^{IDR} alone (Fig. 3d, Extended Data Fig. 9e) indicating that increased dynamics of GFP-Clr4^{IDR} by addition of ubiquitin confer reduced size of liquid droplets and escape from the LLPS.

Ubc4 and Cul4 regulate transcription of sRNA through Bdf2^{BRD4}

In *ubc4-1* and *cul4-1* mutants, we observed an almost 2-fold increase of centromeric sRNA, as previously observed for *cul4* (Fig. 4a, Extended Data Fig. 10a)¹⁸, accompanied by increased chromatin binding of Ago1 and Chp1, two other components of the RNA-induced transcriptional gene silencing (RITS) complex (Extended Data Fig. 10c). The putative H3K9 demethylase, Epe1, localizes to centromeric heterochromatin in a Swi6-dependent manner and contributes to sRNA production^{26,27}. The BET family double bromodomain protein Bdf2 binds to the centromeric heterochromatin boundary in Epe1-dependent manner and interacts with the TFIID transcription factor complex^{28,29}. *bdf2Δ* suppressed the increased sRNA production in *ubc4-1* and *cul4-1* mutant cells (Fig. 4a). The chromatin binding of Bdf2 at centromeric heterochromatin was greatly increased in both *ubc4-1* and *cul4-1* (Fig. 4b) and at euchromatic genes in *ubc4-1* (Extended Data Figure 10d, e), while the protein level was increased in *ubc4-1*

only (Fig. 4c). These results suggest that Ubc4 is a general regulator for Bdf2, while Cul4 regulates Bdf2 specifically at centromeric heterochromatin. Bdf2 protein purified from cells treated with proteasome inhibitor MG-132 accumulated multiple ubiquitinated bands dependent on Ubc4, suggesting that Ubc4 poly-ubiquitinates Bdf2 for its degradation (Fig. 4d). These results indicate that Ubc4 and Cul4 regulates the protein level and chromatin binding of Bdf2 by poly-ubiquitination, which is important for sRNA production by recruiting TFIID and RNA Pol II to centromeric heterochromatin.

Discussion

We demonstrate that Ubc4-CLRC is essential for the switch between RNAi-dependent CTGS and RNAi-independent TGS (Fig. 4e). During S phase, centromeric heterochromatin is transcribed, and CLRC is recruited to centromeric heterochromatin by the RITS complex and small RNA^{10,11,30}. Clr4 di-methylates newly deposited H3K9, and has strong binding affinity to existing H3K9me3. Subsequent mono-ubiquitination of Clr4 by Ubc4-CLRC releases it from H3K9me3, allowing tri-methylation of adjacent H3K9me1 or -me2 nucleosomes independent of RNAi³¹. Mono-ubiquitination also triggers dissolution of subcellular phase separated droplets, allowing replacement by Swi6 condensates that also bind H3K9me3 and silence transcription²². Poly-ubiquitination of Bdf2 further reduces transcription and small RNA production completing the transition to TGS. De-ubiquitination by Ubp3 reverses the process and allows Clr4 recruitment in the next cell cycle (Fig. 4e).

In *ubc4-1* and *cul4-1* mutants, or in mutants of Clr4 that can no longer be ubiquitinated, CTGS cannot transition to TGS, leading to increased sRNA production, and failure to release Clr4. In contrast, strong *ubc4-3xHA* and *cul4Δ* mutants completely abolish centromeric heterochromatin resembling *clr4Δ*, *rik1Δ*, *raf1Δ* and *raf2Δ* (Extended Data Fig. 4a-c), because Clr4 is no longer recruited to heterochromatin by CLRC and RNAi in the first place (Extended Data Fig. 5f). Ubc4-CLRC may possess additional targets, such as H3K14, which was recently shown to be a prerequisite for H3K9 methylation *in vitro*²⁵. Bdf2, like its human homologue BRD4, has IDRs which may induce LLPS of acetylated chromatin (Extended Data Fig. 9f)³². Poly-ubiquitination of Bdf2 may similarly release Bdf2 from the heterochromatin phase for subsequent degradation³³. The ubiquitin-related protein modifier SUMO contributes to small RNA-dependent localization of the Clr4 homologue SETDB1 to chromatin in *Drosophila*³⁴, and is required for heterochromatic silencing in *S. pombe*³⁵, which we speculate may also be mediated by a similar mechanism as reported here³⁶. The existence of IDR/LCD between chromo and PreSET domains of mammalian SUV39H1 (Extended Data Fig. 9g) and the interaction of human BRD4 with CUL4B³⁷ suggest that parallel mechanisms might regulate transcriptional silencing in higher eukaryotes.

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Materials and Methods

Yeast strains and plasmids manipulation

Yeast strains, oligonucleotide sequences and plasmids are listed in Supplementary Information Table S1-3. All yeast strains were constructed by PCR-based targeting method³⁸ and lithium acetate transformation method³⁹. For N-terminus tagging of 3xFlag to *clr4*, *rik1* and *raf1*, endogenous promoters and terminators of each gene were used. For some 3xFlag-Clr4 lysine mutants, *adh1* terminator with Kan resistant (Kan^R) gene was inserted at the end of *clr4* open reading frames (ORF). For overexpression of *ubp3* and *ubp8*, 500 bp of *adh1* promoter was inserted between their promoter and ORF. To construct pET-14b-Clr4, the coding DNA sequence of full-length Clr4 (amino acids 1-490) was amplified from *S. pombe* genomic DNA, digested with *NdeI* and cloned into *NdeI*-digested pET-14b. For pHis-parallel-GFP-Clr4^{Full} or pHis-parallel-GFP-Clr4^{IDR} construction, the coding DNA sequence of full-length Clr4^{Full} (amino acids 1-490) or Clr4^{IDR} (amino acids 51-220) were amplified from *S. pombe* genomic DNA, digested with *BamHI*/*XhoI* and cloned into *BamHI*/*XhoI*-digested pHis-parallel-GFP.

Chromatin Immunoprecipitation (ChIP)

Chromatin Immunoprecipitation (ChIP) experiments were performed as previously described²⁹. Briefly, freshly grown 40 ml cells in YES were fixed with 1% formaldehyde from 37% stock (Sigma F8775) for 20 min at room temperature for H3K9me2, H3K9me3, RNA Pol II, Ago1 and Chp1 ChIP. For Swi6 and 3xFlag-Clr4 ChIP, 40 ml cells were pre-incubated at 18 °C for 2 h and fixed with 1.45% Formaldehyde from 16% stock (ThermoFisher 28908) for 30 min at 18 °C. Cells were quenched with 360 mM

glycine and 2.4 mM Tris for 5 min. Whole-cell extracts (WCE) were prepared using FastPrep-24 and sonicated using Covaris. 1~2 µg of antibodies like anti-H3K9me2 (Abcam ab1220), anti-H3K9me3 (Absolute Antibody Ab00700-1.26), anti-RNA polymerase II (BioLegend 904001), Swi6 (Abcam ab188276), Ago1 (Abcam Ab18190), Chp1 (Abcam ab18191) and anti-Flag (Sigma F1804) were pre-incubated with 50 µl Protein A and Protein G sepharose (GE 17513801 and 17061801) mix for 3~4 h and then incubated with sheared chromatin overnight at 4 °C. After washing, bead-antibody-chromatin mixture were treated with Proteinase K (ThermoFisher 100005393) for 1 h at 42 °C and incubated 5~6 h at 65 °C to reverse cross-links. DNA was cleaned up using ChIP DNA Clean & Concentrator (Zymo Research D5205) and subsequently used for qPCR or ChIP-Seq.

ChIP-Seq

ChIP-Seq libraries were constructed using NEBNext Ultra II DNA Library Prep Kit for Illumina (NEB E7645) and barcoded multiplex libraries were sequenced using Illumina MiSeq platform, which generates paired-end 101 bp reads. Raw reads were trimmed using Trimmomatic, mapped to *S. pombe* genome using Bowtie2 and visualized in IGV genome browser. Total ChIP-seq read counts for defined region were analyzed using MultiBigwigSummary or MultiBamSummary.

Co-Immunoprecipitation (Co-IP) and Immunoprecipitation (IP) for ubiquitinated Clr4

Whole-cell extracts (WCE) were prepared from freshly grown yeast cells in lysis buffer

(40mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% Glycerol, 0.1% NP-40, 300 mM NaCl) with Protease inhibitor cocktail (Roche 11836170001). For Co-IP of Cul4-GFP with Ubc4, 2 µg of GFP antibody (Roche 11814460001) was pre-incubated with 50 µl Protein A and Protein G Sepharose (GE Healthcare 17513801 and 17061801) mix for 3~4 h and then incubated with WCE overnight at 4 °C. After washing, purified proteins were loaded on 4–20% Mini-PROTEAN TGX Gel (Bio-rad 456-1093) and Western blotting was performed with anti-GFP (Abcam 290) and anti-mUBE2D3 (Proteintech 11677-1-AP) antibodies to detect Cul4-GFP and Ubc4 using ECL (Thermo Fisher 32209) assay according to the manufacturer's instruction. For Co-IP of Flag-Rik1, Flag-Dos1 or Dos2-Flag with Ubc4, 50 µl of Anti-Flag M2 Affinity Gel (Sigma A2220) was incubated with WCE overnight at 4 °C. For detection of Flag tagged protein and Ubc4, anti-Flag (Sigma F7425) and anti-mUBE2D3 (Proteintech 11677-1-AP) antibodies were used. For Immunoprecipitation of ubiquitinated Clr4, WCE prepared from freshly grown cells in lysis buffer (50mM Hepes, pH 7.6, 150mM NaCl, 300mM KAc, 5mM MgAc, 10% Glycerol, 20mM β-GP, 1mM EDTA, 1mM EGTA, 0.1% NP-40, 1mM DTT, 250U/ml Dnase I (Roche 04716728001), 25~50 µg/ml Rnase A (Sigma R4642), 1mM PMSF, 0.3mM PR-619 (Sigma 662141)) with Protease inhibitor cocktail (Roche 11836170001) and De-ubiquitination/sumoylation Inhibitor (Cytoskeleton NEM09BB) was incubated with Anti-Flag M2 Affinity Gel or Ubiquitination Affinity Beads (UAB, Cytoskeleton UBA01- Beads) overnight at 4 °C. For detection of ubiquitinated Clr4 protein, anti-Flag (Sigma F7425) or anti-ubiquitin (Cell Signalling 3936T) antibodies were used. Cells were also treated with proteasome inhibitor 100 µM MG-132 (APEX BIO A2585) for 4 h before harvesting.

Quantitative reverse transcription PCR (RT-qPCR)

Total RNAs were purified from freshly grown yeast cells using *Quick-RNA* Fungal/Bacterial Miniprep Kit (Zymoresearch R2014). Genomic DNAs were removed using DNase I (Roche 04716728001) and RNAs were cleaned using RNA Clean & Concentrator (Zymoresearch R1013). Reverse transcription was carried out using SuperScript IV First-Strand Synthesis System (ThermoFisher 18091050) with random hexamer or oligo dT as primers. For quantitative PCR (qPCR), iQ SYBR Green Supermix (Bio-rad 1708882) was used with the primers listed in Supplementary Information Table 2 and samples were run on CFX96 Real-Time PCR Detection System (Bio-rad).

sRNA-Seq

Total RNAs were purified from freshly grown yeast cells using *Quick-RNA* Fungal/Bacterial Miniprep Kit (Zymoresearch R2014) and sRNA were enriched using RNA Clean & Concentrator (Zymoresearch R1013). sRNA libraries were constructed using NEBNext Small RNA Library Prep Set for Illumina (NEB E7330) and barcoded multiplex libraries were sequenced using Illumina MiSeq platform, which generates single-end 36 nt reads. Reads were adapter-trimmed using FASTX-Toolkit, keeping a minimum read size of 15 nt, and mapped to the *S. pombe* genome using Bowtie⁴⁰ allowing up to 1 mismatch. The strand-specific genomic coverage was calculated using Bedtools⁴¹ and represented in 20 nt windows.

Silencing spot assay

Cells grown freshly on YES plate were picked and resuspended in water to make a concentration of $\sim 5 \times 10^4$ cells per μl . 8 μl of 10-fold serial dilutions were spotted on appropriate plates like YES, YE, EMM-Ura, YES+FOA, YES+TBZ, and incubated 3 days at 30 °C for photography. *ura4⁺* reporter gene silencing was also tested on 5-fluoro-orotic acid (FOA) plates, which kills cells expressing Ura4.

Iodine staining

Freshly grown yeast cells were spotted on SPA plates to induce sporulation, incubated at room temperature for 3 days, and then stained with iodine vapor for 2-3 min. Staining of starch which is abundant in mature spore produces a dark brown color.

EMS mutagenesis and whole-genome sequencing for *ubc4-G48D* suppressor

EMS mutagenesis was carried out as previously described⁴². Briefly, freshly grown 1×10^9 yeast cells were washed with distilled water, resuspended in 1.5 ml of 0.1 M sodium phosphate buffer (pH 7.0) and treated with 50 μl EMS (Sigma M0880) for 1h at 30 °C. 0.2 ml of treated cells was moved to 8 ml of sterile 5% sodium thiosulfate to inactivate EMS. Cells were washed, resuspended in sterile water and plated on YES plate. Single colonies from plates were patched on SPA plates to induce sporulation, incubated at room temperature for 3 days, and then stained with iodine vapor for 2-3 min. For whole-genome sequencing for *ubc4-G48D* suppressors, genomic DNA was purified from freshly grown yeast cells using Genomic-tip 20/G column (QIAGEN 10223). DNA library was constructed using the TruSeq DNA PCR-Free Library Prep Kit (Illumina 20015962).

Barcoded DNA library was sequenced using Illumina MiSeq platform, which generates paired-end 151 nt reads and analyzed as previously described⁴³. Briefly, reads were adapter-trimmed and quality-filtered using Sickle (paired-end mode), then mapped to the *S. pombe* genome using Bowtie2⁴⁴. Duplicate reads were removed using Samtools ($\leq 0.14\%$ per library). SNPs were called using FreeBayes to find SNPs present in suppressor strain but absent in the parental strain⁴⁵. This uncovered a G→C transversion at position chr2:716192, a second-site mutation in the *ubc4* gene. The mutation was verified by Sanger sequencing and confirmed by constructing fresh same mutation.

Live cell imaging

Images of freshly growing cells were collected using Zeiss Axio Imager M2 with DIC and EGFP channels and processed using Zeiss Zen software.

CLRC complex purification from *S. pombe*

Protein purifications were performed as described previously⁴. Briefly, Rik1-TAP were purified from 6 to 12 g of cells. Cells were lysed in 1 volume of lysis buffer (50 mM HEPES-KOH, pH 7.6, 300 mM KAc, 10% glycerol, 1 mM EGTA, 1 mM EDTA, 0.1% NP-40, 1 mM DTT, 5 mM MgAc, 1 mM NaF, 20 mM β -GP, 1 mM PMSF, 1 mM Benzamidine and 1 μ g/ml of Leupeptin, Aprotinin, Bestatin and Pepstatin). 1 volume lysis buffer was added and the lysate was centrifuged at 16,500 rpm for 25 min at 4 °C in a SA600 rotor. The first affinity purification was performed using Ig-G Sepharose beads at 4 °C for 2 h. The beads were washed with lysis buffer and TEV-C buffer (10 mM Tris-HCl, pH 8.0,

150 mM KAc, 0.1% NP-40, 0.5 mM EDTA, 1 mM DTT) and bound protein was eluted using a GST-TEV protease. The eluate was diluted in 2 volumes of CAM-B buffer (10 mM Tris-HCL, pH 8.0, 150 mM NaCl, 1 mM MgAc, 1 mM Imidazole, 2 mM CaCl₂, 10 mM βME) and the second affinity purification was performed by adding Calmodulin-Sepharose at 4 °C for 1 h. The beads lysate was washed with CAM-B buffer containing 0.1 % NP-40 and bound protein was eluted with CAM-E buffer (10 mM Tris-HCL, pH 8.0, 150 mM NaCl, 1 mM MgAc, 1 mM Imidazole, 10 mM EGTA, 10 mM βME).

Recombinant protein purification from *E. coli*, *in vitro* ubiquitination, Histone Methylation assay and nucleosome binding assay

BL21-CodonPlus (DE3)-RIL strains (Agilent Technologies 230245) that express 6xHis-Clr4, 6xHis-GFP-Clr4^{Full} or 6xHis-GFP-Clr4^{IDR} were grown with 0.4 mM IPTG for 20 h at 18 °C. Induced proteins were purified on HisPur Ni-NTA Superflow Agarose (ThermoFisher 25214) and eluted with elution buffer containing 150 mM Imidazole. For *in vitro* ubiquitination, purified 6xHis-Clr4 protein was mixed with human Ubiquitin-activating Enzyme (UBE1, R&D systems E-305), human UbcH5c/UBE2D3 (R&D systems E2-627), human Ubiquitin (R&D systems U-100H) or human HA-Ubiquitin (R&D systems U-110), Mg-ATP (R&D systems B-20) and 10x E3 Ligase Reaction Buffer (R&D systems B-71). Addition of Rik1-TAP complex purified from *S. pombe* also added to the reaction gave similar results. For Histone Methylation assay, ubiquitinated or unmodified Clr4 proteins were mixed with Recombinant Mononucleosomes (H3.1, Active Motif 31467) or Recombinant Mononucleosomes H3K9me3 (H3.2, Active Motif 31555) and S-(5'-Adenosyl)-L-methionine chloride dihydrochloride (SAM, Sigma 7007)

in HMT buffer (50mM Tris, pH 8.0, 10 mM MgCl₂, 1 mM DTT, 5% Glycerol and 1 mM PMSF). Samples were boiled and loaded on 4–20% Mini-PROTEAN TGX Gel (Bio-rad 456-1093) and Western blotting was performed with anti-His (ThermoFisher MA1-135), anti-HA (Roche 11583816001), anti-H3 (Abcam ab1791), and anti-H3K9me3 (Abcam ab8898) using ECL (Thermo Fisher 32209) assay according to the manufacturer's instruction. For nucleosome binding assay, biotin labeled Recombinant Mononucleosomes (H3.1, Active Motif 31467) or Recombinant Mononucleosomes H3K9me3 (H3.2, Active Motif 31555) was pre-incubated with Streptavidin beads (GE Healthcare 17-5113-01) and incubated in vitro ubiquitinated Ctr4 or Ctr4 proteins purified from *S. pombe* cells. Samples were boiled and loaded on 4–20% Mini-PROTEAN TGX Gel (Bio-rad 456-1093) and Western blotting was performed with anti-Flag (Abcam ab1791), anti-H3K9me3 (Abcam ab8898) and anti-H3 (Abcam ab1791), and using ECL (Thermo Fisher 32209) assay according to the manufacturer's instruction.

Mass Spectrometry

The gel band sample was reduced and alkylated with *N*-ethylmaleimide (NEM), digested with trypsin for 1.5 h at 37 °C and washed with ammonium bicarbonate/acetonitrile to remove stain, SDS and other reagents. Peptides were extracted from the gel pieces, dried down and re-dissolved in 2.5% acetonitrile, 0.1% formic acid. Each digest was run by nanoLC-MS/MS using a 2 h gradient on a 0.075 mm x 250 mm C18 column feeding into a Q-Exactive HF mass spectrometer. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version

2.6.1). Mascot was set up to search the cRAP_20150130.fasta; custom4_20190215.fasta; SwissProt_2019_02 database (selected for *Schizosaccharomyces pombe*, unknown version, 5269 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.060 Da and a parent ion tolerance of 10.0 PPM. Deamidated of asparagine and glutamine, oxidation of methionine, phospho of serine, threonine and tyrosine, GG of lysine, N-ethylmaleimide of cysteine were specified in Mascot as variable modifications. Scaffold (version Scaffold_4.8.9, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 80.0% probability by the Peptide Prophet algorithm⁴⁶ with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm⁴⁷. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters.

Liquid-Liquid Phase Separation (LLPS)

LLPS of GFP-Clr4^{Full} and GFP-Clr4^{IDR} proteins were induced by diluting protein to 12 μ M and 16 μ M respectively in 20 mM Tris, pH 7.5, 30 mM NaCl, 0.5 mM MgAc, 1% Glycerol and 15 mM Imidazole. To observe the effect of ubiquitin on GFP-Clr4^{IDR} LLPS, 0.9 μ g/ μ l of human ubiquitin (R&D systems U-100H) or buffer only (10 mM Hepes, pH 7.6) were added to the GFP-Clr4^{IDR}. To observe the effect of 1,6-Hexanediol (Sigma

H11807) on GFP-Clr4^{IDR} LLPS, GFP-Clr4^{IDR} protein was diluted to 44.8 μ M in 56 mM Tris, pH 7.5, 84 mM NaCl, 1.4 mM MgAc, 2.8% Glycerol, 42 mM Imidazole and 3.6% PEG8000 with or without 7.2% 1,6-Hexanediol. LLPS was imaged on Zeiss Axio Imager M2 with differential interference contrast (DIC) and EGFP channels and processed using Zeiss Zen software.

FRAP experiments

FRAP experiments were performed as previously described⁴⁸. Briefly, GFP-Clr4^{IDR} protein were diluted to 44.8 μ M in 56 mM Tris, pH 7.5, 84 mM NaCl, 1.4 mM MgAc, 2.8% Glycerol, 42 mM Imidazole and 3.6% PEG8000 with or without 0.65 μ g/ μ l ubiquitin. FRAP experiments were carried out using Zeiss LSM710 Confocal Laser Scanning Microscope with 63x objective. All Bleaching experiments were performed with 488 nm argon laser at 100% intensity. Images before and after photobleaching were taken at 5 s interval and analyzed using standard methods.

Data availability

Genome-wide datasets are deposited in the Gene Expression Omnibus (GEO) under the accession number GSE156069. The token for private access is **ojipouckhladzeh**.

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Author contributions

H.S.K. performed experiments and bioinformatics. B.R. contributed sRNA-seq experiments and bioinformatics. S.B. contributed to IP for ubiquitinated Clr4 and *in vitro* protein purification. A.Y.C. contributed to EMS mutagenesis, C.H. contributed to LLPS

562 and LT and AV contributed to Rik1-TAP purification. H.S.K. and R.A.M. designed the
563 experiments and wrote the manuscript.

564 **Competing interests**

565 The authors declare no competing interests.

Supplementary Information Table 1. *S. pombe* strains used in this study.

Number	Name	Genotype	Reference
FY2002	WT	<i>h⁺ otrIR(SphI)::ade6 ImrIL(NcoI)::leu1-32 ade6-DN/N ura4-DS/E leu1-32</i>	R. Allshire
HSK29	<i>ubc4-G48D (ubc4-1)</i>	FY2002_ <i>ubc4-G48D-KanMX6</i>	This study
HSK335	<i>cul4-GFP (cul4-1)</i>	FY2002_ <i>cul4-GFP-KanMX6</i>	This study
HSK351	<i>clr4Δ</i>	FY2002_ <i>clr4Δ::KanMx6</i>	This study
HSK770	<i>dcr1Δ</i>	FY2002_ <i>dcr1Δ::NatMx6</i>	This study
HSK790	<i>dcr1Δ ubc4-1</i>	FY2002_ <i>dcr1Δ::NatMx6 ubc4-G48D-KanMX6</i>	This study
HSK606	<i>3xFlag-Clr4</i>	FY2002_ <i>HphMX6-3xFlag-Clr4</i>	This study
HSK618	<i>3xFlag-Clr4 ubc4-1</i>	FY2002_ <i>HphMX6-3xFlag-Clr4 ubc4-G48D-KanMX6</i>	This study
HSK1118	<i>3xFlag-Clr4-AllKtoR</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-AllKtoR</i>	This study
HSK971	<i>3xFlag-Clr4-2-8KtoR</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-2-8KtoR</i>	This study
HSK1121	<i>3xFlag-Clr4-2-4KtoR</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-2-4KtoR</i>	This study
HSK918	<i>3xFlag-Clr4 ubp3-OE</i>	FY2002_ <i>HphMX6-3xFlag-Clr4 NatMX6-adh1p-ubp3</i>	This study
HSK1049	<i>3xFlag-Clr4-W31G</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-W31G</i>	This study
HSK1054	<i>3xFlag-Clr4-W31G ubc4-1</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-W31G ubc4-G48D-KanMX6</i>	This study
HSK679	<i>GFP-Swi6</i>	FY2002_ <i>HphMX6-GFP-Swi6</i>	This study
HSK778	<i>GFP-Swi6 clr4Δ</i>	FY2002_ <i>HphMX6-GFP-Swi6 clr4Δ::KanMX6</i>	This study
HSK700	<i>GFP-Swi6 ubc4-1</i>	FY2002_ <i>HphMX6-GFP-Swi6 ubc4-G48D-KanMX6</i>	This study
HSK776	<i>GFP-Swi6 dcr1Δ</i>	FY2002_ <i>HphMX6-GFP-Swi6 dcr1Δ::NatMX6</i>	This study
HSK808	<i>GFP-Swi6 dcr1Δ ubc4-1</i>	FY2002_ <i>HphMX6-GFP-Swi6 dcr1Δ::NatMX6 ubc4-G48D-KanMX6</i>	This study
HSK518	<i>bdf2Δ</i>	FY2002_ <i>bdf2Δ::KanMX6</i>	This study
HSK543	<i>ubc4-1 bdf2Δ</i>	FY2002_ <i>ubc4-G48D-KanMX6 bdf2Δ::NatMX6</i>	This study
HSK545	<i>cul4-1 bdf2Δ</i>	FY2002_ <i>cul4-GFP-KanMX6 bdf2Δ::NatMX6</i>	This study
HSK524	<i>Bdf2-3xFlag</i>	FY2002_ <i>Bdf2-3xFlag-HphMX6</i>	This study
HSK571	<i>Bdf2-3xFlag ubc4-1</i>	FY2002_ <i>Bdf2-3xFlag-HphMX6 ubc4-G48D-KanMX6</i>	This study
HSK573	<i>Bdf2-3xFlag cul4-1</i>	FY2002_ <i>Bdf2-3xFlag-HphMX6 cul4-GFP-KanMX6</i>	This study
HSK583	<i>Bdf2-3xFlag ddb1Δ</i>	FY2002_ <i>Bdf2-3xFlag-HphMX6 ddb1Δ::KanMX6</i>	This study
HSK575	<i>Bdf2-3xFlag clr3Δ</i>	FY2002_ <i>Bdf2-3xFlag-HphMX6 clr3Δ::KanMX6</i>	This study
HSK458	<i>cul4-1 ubc4-1</i>	FY2002_ <i>cul4-GFP-KanMX6 ubc4-G48D-NatMX6</i>	This study
HSK608	<i>ubc4::mUBE2D2</i>	FY2002_ <i>KanMX6-ubc4::mUBE2D2</i>	This study
HSK610	<i>ubc4::mUBE2D2-G48D</i>	FY2002_ <i>KanMX6-ubc4::mUBE2D2-G48D</i>	This study
KFP26	<i>h⁻</i>	<i>h⁻ ura4-D18 leu1-32 ade6-M210 his3-D1</i>	Lab stock
SPG18	<i>h⁹⁰</i>	<i>h⁹⁰ ura4-D18 leu1-32 ade6 -M216 his2</i>	Lab stock
DI304	<i>h⁹⁰ ubc4-G48D</i>	<i>h⁹⁰ leu1-32 ubc4-G48D-KanMX6</i>	Irvine et al 2009
AY709	<i>h⁹⁰ubc4-G48D/S137T</i>	<i>h⁹⁰ leu1-32 ubc4-G48D-KanMX6 ubc4-S137T</i> (EMS mutagenesis)	This study
HSK33	<i>raf1Δ</i>	FY2002_ <i>raf1Δ::KanMx6</i>	This study
HSK307	<i>raf2Δ</i>	FY2002_ <i>raf2Δ::KanMx6</i>	This study
HSK355	<i>rik1Δ</i>	FY2002_ <i>rik1Δ::KanMx6</i>	This study
HSK351	<i>clr3Δ</i>	FY2002_ <i>clr3Δ::KanMx6</i>	This study
HSK644	<i>ubc4-3xHA</i>	FY2002_ <i>ubc4-3xHA-KanMX6</i>	This study
HSK646	<i>cul4Δ</i>	FY2002_ <i>cul4Δ::NatMx6</i>	This study
HSK716	<i>GFP-Swi6 ubc4-3xHA</i>	FY2002_ <i>HphMX6-GFP-Swi6 ubc4-3xHA-KanMX6</i>	This study
HSK717	<i>GFP-Swi6 cul4Δ</i>	FY2002_ <i>HphMX6-GFP-Swi6 cul4Δ::NatMX6</i>	This study
HSK642	<i>ubc4-GFP</i>	FY2002_ <i>ubc4-GFP-KanMX6</i>	This study
HSK393	<i>3xFlag-Rik1</i>	FY2002_ <i>HphMX6-3xFlag-Rik1</i>	This study
HSK389	<i>3xFlag-Raf1</i>	FY2002_ <i>HphMX6-3xFlag-Raf1</i>	This study
HSK274	<i>Raf2-3xFlag</i>	FY2002_ <i>Raf2-3xFlag-HphMX6</i>	This study
HSK620	<i>3xFlag-Clr4 cul4-1</i>	FY2002_ <i>HphMX6-3xFlag-Clr4 cul4-GFP-KanMX6</i>	This study
HSK920	<i>3xFlag-Clr4 rik1Δ</i>	FY2002_ <i>HphMX6-3xFlag-Clr4 rik1Δ::NatMx6</i>	This study
HSK921	<i>3xFlag-Clr4 raf1Δ</i>	FY2002_ <i>HphMX6-3xFlag-Clr4 raf1Δ::NatMx6</i>	This study

Number	Name	Genotype	Reference
HSK922	<i>3xFlag-Clr4 raf2Δ</i>	FY2002_ <i>HphMX6-3xFlag-Clr4 raf2Δ::NatMx6</i>	This study
HSK1125	<i>3xFlag-Clr4-1-6KtoR</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-1-6KtoR</i>	This study
HSK1127	<i>3xFlag-Clr4-3-6KtoR</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-3-6KtoR</i>	This study
HSK1129	<i>3xFlag-Clr4-4-6KtoR</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-4-6KtoR</i>	This study
HSK1131	<i>3xFlag-Clr4-5-5KtoR</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-5-5KtoR</i>	This study
HSK1132	<i>3xFlag-Clr4-6-5KtoR</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-6-5KtoR</i>	This study
HSK1140	<i>3xFlag-Clr4-K87R</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-K87R</i>	This study
HSK1142	<i>3xFlag-Clr4-K94R</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-K94R</i>	This study
HSK1144	<i>3xFlag-Clr4-K109R</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-K109R</i>	This study
HSK1146	<i>3xFlag-Clr4-K110R</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-K110R</i>	This study
HSK1148	<i>3xFlag-Clr4-K113R</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-K113R</i>	This study
HSK1150	<i>3xFlag-Clr4-K114R</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-K114R</i>	This study
HSK1152	<i>3xFlag-Clr4-K122R</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-K122R</i>	This study
HSK1154	<i>3xFlag-Clr4-K127R</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-K127R</i>	This study
HSK1013	<i>3xFlag-clr4-K109/110/113/114R</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-K109/110/113/114R-KanMX6</i>	This study
HSK999	<i>3xFlag-Clr4-K87/94/109/110/113/114R</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-K87/94/109/110/113/114R-KanMX6</i>	This study
HSK1000	<i>3xFlag-Clr4-K109/110/113/114/122/127R</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-K109/110/113/114/122/127R-KanMX6</i>	This study
HSK1015	<i>3xFlag-Clr4-K110/113/114R</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-110/113/114R-KanMX6</i>	This study
HSK1017	<i>3xFlag-Clr4-K109/113/114R</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-109/113/114R-KanMX6</i>	This study
HSK1019	<i>3xFlag-Clr4-K109/110/114R</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-109/110/114R-KanMX6</i>	This study
HSK1021	<i>3xFlag-Clr4-K109/110/113R</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-109/110/113R-KanMX6</i>	This study
HSK1082	<i>3xFlag-Clr4-K109/110R</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-109/110R-KanMX6</i>	This study
HSK1062	<i>3xFlag-Clr4-K109/113R</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-109/113R-KanMX6</i>	This study
HSK1064	<i>3xFlag-Clr4-K109/114R</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-109/114R-KanMX6</i>	This study
HSK1086	<i>3xFlag-Clr4-K110/113R</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-110/113R-KanMX6</i>	This study
HSK1065	<i>3xFlag-Clr4-K110/114R</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-110/114R-KanMX6</i>	This study
HSK1084	<i>3xFlag-Clr4-K113/114R</i>	FY2002_ <i>HphMX6-3xFlag-Clr4-113/114R-KanMX6</i>	This study
HSK919	<i>3xFlag-Clr4 ubp3Δ</i>	FY2002_ <i>HphMX6-3xFlag-Clr4 ubp3Δ::NatMx6</i>	This study
HSK395	<i>ubp3-OE**</i>	FY2002_ <i>HphMX6-adh1p-ubp3 (ubp3 overexpression)</i>	This study
HSK401	<i>ubp8-OE**</i>	FY2002_ <i>HphMX6-adh1p-ubp8 (ubp8 overexpression)</i>	This study

Supplementary Information Table 2. Oligonucleotides used in this study.

Name	Description	Purpose	Sequence
p30-qPCR-F	<i>dg</i>	ChIP-qPCR, RT-qPCR	CCATATCAATTTCCTATGTTCC
p30-qPCR-R	<i>dg</i>	ChIP-qPCR, RT-qPCR	CATCAAGCGAGTCGAGATGA
p33-qPCR-F	<i>dh</i>	ChIP-qPCR, RT-qPCR	TATCCTGCGTCTCGGTATCC
p33-qPCR-R	<i>dh</i>	ChIP-qPCR, RT-qPCR	CTGTTCTGTAATGCTGAGAAAG
act1-qPCR-F	<i>act1</i>	ChIP-qPCR, RT-qPCR	TGCACCTGCCTTTTATGTTG
act1-qPCR-R	<i>act1</i>	ChIP-qPCR, RT-qPCR	TGGGAACAGTGTGGGTAACA
ade6-qPCR-F	<i>ade6</i>	ChIP-qPCR	TTCCTACTGCCATCAAAGCA
ade6-qPCR-R	<i>ade6</i>	ChIP-qPCR	GCACGCTGTTGAATTGAGAA
ura4-qPCR-F	<i>ura4</i>	ChIP-qPCR	TACCTTTGGGACGTGGTCTC
ura4-qPCR-R	<i>ura4</i>	ChIP-qPCR	CCCGTCTCCTTTAACATCCA
irc3-R-qPCR-F	<i>irc-3R</i>	ChIP-qPCR	GGCAACCTTTCAAGTTCTCG
irc3-R-qPCR-R	<i>irc-3R</i>	ChIP-qPCR	ATTCTTCCCATCTGCGTTTG
tlh1-R-qPCR-F	<i>tlh1</i>	ChIP-qPCR	GTTCTCCATGCTCCACTGGT
tlh1-R-qPCR-R	<i>tlh1</i>	ChIP-qPCR	CCAACAGTGGTCTTCGGTTT
ubp3-qPCR-F	<i>ubp3</i>	RT-qPCR	GCCAAATTTTTGGTGGTCAG
ubp3-qPCR-R	<i>ubp3</i>	RT-qPCR	TGAATGTCGAGTTGCAGAGG
Clr4(His)-NdeI-F	6xHis-Clr4	Plasmid construction	CCGCGCGGCAGCCATATGTCGCCTAAACAAGAGGAGTATG
Clr4(His)-NdeI-R	6xHis-Clr4	Plasmid construction	CGGATCCTCGAGCATATGTTAACCAGAAAAGCCAGCCACGACAA
Clr4-BamHI-F	6xHis-GFP-Clr4	Plasmid construction	CGGGTACCATGGATCCGATGTCGCCTAAACAAGAGGAGTATG
Clr4-XhoI-R	6xHis-GFP-Clr4	Plasmid construction	GGTGGTGGTGCTCGAGTTAACCAGAAAAGCCAGCCACGACAA
Clr4-IDR-BamHI-F	6xHis-GFP-IDR	Plasmid construction	CGGGTACCATGGATCCGCTCTGCCGTTTTAGCAGAATGGAAAA
Clr4-IDR-XhoI-R	6xHis-GFP-IDR	Plasmid construction	GGTGGTGGTGCTCGAGCTATTCCGGTCCTTCAATTTCCCGC

Supplementary Information Table 3. Plasmids used in this study.

Name	Description	Application
pET-14b-Clr4	6xHis-Clr4	Recombinant 6xHis-Clr4 protein purification from <i>E. coli</i>
pHis-parallel-GFP-Clr4 ^{Full}	6xHis-GFP-Clr4 ^{Full}	Recombinant 6xHis-GFP-Clr4 ^{Full} protein purification from <i>E. coli</i>
pHis-parallel-GFP-Clr4 ^{IDR}	6xHis-GFP-Clr4 ^{IDR}	Recombinant 6xHis-GFP-Clr4 ^{IDR} protein purification from <i>E. coli</i>

Figure 1

Figure 1. Mono-ubiquitination of Clr4 by Ubc4-CLRC is required for H3K9 trimethylation.

a, ChIP-qPCR assays showing enrichment of H3K9me2 (left) and H3K9me3 (right) at centromeric *dg* locus in the indicated strains. *clr4* deletion mutant (*clr4Δ*) shows complete loss of H3K9me2 and H3K9me3. Error bars, standard deviation (S.D., 3 biological replicates).

b, ChIP-qPCR assays showing enrichment of H3K9me2 at centromeric *dg* locus in the indicated strains. *dcr1Δ ubc4-1* double mutant shows complete abolishment of H3K9me2. Error bars, S.D. (3 biological replicates).

c, Western blot (WB) analyses of immunoprecipitated 3xFlag-Clr4 (3xFlag IP) from WT and *ubc4-1* mutant cells treated with proteasome inhibitor MG-132 (100μM) for 4 h before harvest. Bottom, WB analysis of 3xFlag-Clr4 from whole cell extract (WCE).

d, Schematic diagram of domain structure of Clr4 protein and the position of lysine residues. CD: chromodomain, PS: PostSET. Ovals show the position of all 36 lysines of Clr4, which are subgrouped to 6 groups. Second group (2-8K) is highlighted and amino acid sequences are shown. 2-4K region covers K109-K114.

e, WB analyses same as **c**, but showing WT, all-lysine mutant (*AllKtoR*), second block mutant (*2-8KtoR*) and mutant of 4 lysines (K109, K110, K113 and K114) of second block (*2-4KtoR*).

f, Western blot (WB) analysis of immunoprecipitated 3xFlag-Clr4 from WT and *ubp3*-overexpression (*ubp3-OE*) strains.

g, ChIP-seq reads of H3K9me2 and H3K9me3 mapped to chromosome 3 centromere right arm in the indicated strains. Relat. enrich., relative enrichment. Total count of ChIP-seq reads from different strains were divided by the total count of WT to show relative enrichment. Bottom, schematic diagram of chromosome 3 centromere right arm showing innermost repeat (*imr*), outermost repeats (*dg*, *dh*) and boundary region (*irc*) between heterochromatin and euchromatin. Vertical red lines and pink boxes represent tRNA and euchromatic genes respectively.

Figure 2. Ctr4 mono-ubiquitination induces its dissociation from chromatin.

a, ChIP-seq reads of 3xFlag-Clr4 mapped to chromosome 3 centromere (*cnt3*) right arm in indicated strains. Relat. enrich., relative enrichment. Bottom, schematic diagram of chromosome 3 centromere (*cnt3*) right arm.

b, *In vitro* protein binding assay of *in vitro* ubiquitinated Clr4 protein with nucleosomes. His-Clr4 purified from *E. coli* cells was ubiquitinated with human E1, E3 (UBE2D3) and HA-ubiquitin and incubated with nucleosomes with unmodified histone H3 (me0) or H3K9me3 histone (me3) anchored to streptavidin beads. Input and precipitated proteins were analyzed by Western blot (WB) using antibodies as shown.

c, ChIP-seq reads of 3xFlag-Clr4 mapped to chromosome 3 centromere (*cnt3*) right arm in indicated strains. Relat. enrich., relative enrichment. Bottom, schematic diagram of chromosome 3 centromere (*cnt3*) right arm.

d, Schematic diagram showing the presumptive subunit arrangement in the complex of Ubc4-CLRC and its substrate Clr4. Red circle (U), ubiquitin. The arrow shows the mono-ubiquitination activity of Ubc4-CLRC toward Clr4.

Figure 3. Ubiquitin reduces GFP-Clr4^{IDR} LLPS activity.

a, Live cell imaging showing GFP-Swi6 heterochromatic foci in indicated strains. In WT 4 foci represent three telomeres and the centromeric chromocenter, which disappears in *dcr1Δ*. Only the chromocenter focus is retained in *ubc4-1* which loses H3K9me from telomeres. The foci are completely abolished in *clr4Δ* and *dcr1Δ ubc4-1* mutants. Scale bar, 2.5μm. Dashed lines show the outline of each *S. pombe* cells.

b, Reduced size of LLPS of GFP-Clr4^{IDR} (amino acid 51-220) by addition of ubiquitin. Buffer or ubiquitin was added to GFP-Clr4^{IDR} protein and the formation of LLPS was monitored. Scale bar, 10μm.

c, Reduced size of LLPS of GFP-Clr4^{IDR} by addition of ubiquitin (**b**). Top 30 LLPS droplets are measured and size distribution is shown. Black bar, average. ****P* value=1.1X10⁻⁵.

d, FRAP of GFP-Clr4^{IDR} by addition of ubiquitin before and after bleaching. The change of fluorescence was analyzed and indicated in mean and S.D. (n=7 and n=9 for Buffer and Ubiquitin, respectively). Arrow shows the time of bleaching (~40s).

Figure 4. Ubc4-CLRC poly-ubiquitinates Bdf2 and regulates sRNA production.

a, sRNA-seq reads mapped to chromosome 3 centromere right arm. The increased sRNA production in *ubc4-1* and *cul4-1* mutants is suppressed by the deletion mutation of *bdf2*. sRNA at boundary region (*irc*) were abolished in all mutants. Remaining sRNA peaks in *dcr1Δ* are tRNA-related products. Relat. enrich., relative enrichment of sRNA without tRNA. Bottom, schematic diagram of chromosome 3 centromere right arm.

b, ChIP-seq reads of Bdf2-3xFlag mapped to chromosome 3 centromere (*cnt3*) right arm in indicated strains. Relat. enrich., relative enrichment. Bottom, schematic diagram of chromosome 3 centromere right arm.

c, Western blot (WB) analyses for Bdf2-3xFlag in indicated strains. Ponceau S staining of membrane is shown for equal loading of protein for WB.

d, Western blot (WB) analyses of immunoprecipitated Bdf2-3xFlag (Flag IP) from WT and *ubc4-1* mutant cells treated with proteasome inhibitor MG-132 (100μM) for 4 h before harvest (left). Bdf2-3xFlag enrichment by ubiquitination affinity beads (UBA IP) are shown on right.

e, Model for the function of Ubc4-CLRC-mediated Clr4 mono-ubiquitination and Bdf2 poly-ubiquitination. Left, Bdf2 recruits RNA Pol II complex to produce centromeric ncRNAs, which are converted to sRNAs by the sequential action of RNAi machineries and these sRNAs reinforce the Clr4 activity for H3K9me2. Ubc4-CLRC poly-ubiquitinates Bdf2 for its degradation and reduces recruitment of RNA Pol II. Right, Clr4 converts H3K9me2 to H3K9me3 but because of its strong binding affinity to H3K9me3, it remains to bind nucleosome with H3K9me3. Clr4 mono-ubiquitination by Ubc4-CLRC dissociates Clr4 from chromatin and Ubp3 removes the mono-ubiquitin from Clr4. Unmodified Clr4 binds other nucleosomes with H3K9me3 and converts RNAi-independent H3K9me1 or -2 nucleosomes nearby to H3K9me3. Both reduction of sRNA and conversion of H3K9me2 to H3K9me3 promote the transition from CTGS to TGS.

c, Ubc4 protein is greatly reduced in *ubc4-G48D* mutant. For Western blot analysis of Ubc4 protein in the indicated strains, antibody for mUBE2D3 was used to detect Ubc4. Tubulin was used as loading control.

d, Mating-type switching defect in *ubc4-G48D* mutant was suppressed by the secondary mutation, *S137T*. Non-switchable *h⁻* and WT and *ubc4-G48D* mutants in switchable strain (*h⁹⁰*) were plated and incubated on sporulation medium and spores were stained with iodine vapors. The enrichment of starch in spore enables staining with iodine and dark staining indicates the strain is switch competent and can make spores but light staining indicates the strain is switching defective and cannot make spores efficiently. *ubc4-S137T* mutation which was identified by EMS mutagenesis of *ubc4-G48D*, iodine staining and whole genome sequencing suppresses the mating switching defect of *ubc4-G48D* mutant (*ubc4-G48D/S137T*).

e, *ubc4-S137T* mutation restores decreased protein level of *ubc4-G48D* mutant. Western blot analysis of Ubc4 protein and tubulin as control. Middle panel shows the longer exposure of upper panel for Ubc4 protein.

f, *ubc4-S137T* mutation suppresses the silencing defect of *ubc4-G48D* mutant. Silencing assay is same with **b**, but FOA containing (FOA) media, which kills *ura4⁺* expressing cells, was also used to assess *ura4⁺* expression.

Extended Data Figure 2. Ubc4 and Cul4 regulate H3K9me status.

a-c, ChIP-qPCR assays showing enrichment of H3K9me2 (**a**), H3K9me3 (**b**) and RNA Pol II (Rpb1, **c**) at centromeric *dh*, *ade6⁺* and *ura4⁺* reporter genes at centromere, boundary region between heterochromatin and euchromatin of centromere 3 (*irc3-R*) and telomeric *tlh1* locus. Error bars, S.D. (3 biological replicates).

d, ChIP-seq reads of H3K9me2 (left) and H3K9me3 (right) mapped to telomere 1 left region. Relat. enrich., relative enrichment. Bottom, schematic diagrams of *tlh1* gene locus.

e, ChIP-qPCR assays showing slightly decreased enrichment of HP1 protein, Swi6 at centromeric *dg* locus in *ubc4-1* and *cul4-1* strains. Error bars, S.D. (2 biological replicates).

e, ChIP-qPCR assays showing enrichment of RNA Pol II (Rpb1) at centromeric *dg* (left) and telomeric *tlh1* (right) loci in the indicated strains. Error bars, S.D. (2 biological replicates).

Extended Data Figure 4. Ubc4 and Cul4 localize to nuclear periphery and Ubc4 forms complex with CLRC.

a, *ubc4* mutant with triple HA tag at carboxyl-terminus (*ubc4-3xHA*) and *cul4* deletion mutant (*cul4Δ*) are viable but show severe growth defect. Big cells in each picture show WT cells. *ubc4-3xHA* cells were grown on YES media and *cul4Δ* cells were grown on low-adenine media.

b, ChIP-qPCR assays showing enrichment of H3K9me2 and H3K9me3 at centromeric *dg* locus in the indicated strains. Error bars, S.D. (2 biological replicates).

c, Live cell imaging showing the localization of GFP-Swi6 in indicated strains. The localization of GFP-Swi6 is completely abolished in *ubc4-3xHA* and *cul4Δ* mutants, which is consistent with H3K9me2 and H3K9me3 ChIP-qPCR results (**b**). Scale bar, 2.5μm.

d, Live cell imaging showing that Cul4-GFP and Ubc4-GFP localize to nuclear periphery. The strain without GFP tag (untag) was used as negative control. Scale bar, 2.5μm.

e, Co-immunoprecipitation (Co-IP) of Cul4-GFP with Ubc4. Whole-cell extracts (WCE) were immunoprecipitated with GFP antibody and Western blot analyses were carried out with GFP and mUBE2D3 antibodies.

f, Co-immunoprecipitation (Co-IP) of 3xFlag-Rik1, 3xFlag-Raf1 and Raf2-3xFlag with Ubc4. Whole-cell extracts (WCE) were immunoprecipitated with Flag antibody and Western blot analyses were carried out with Flag and mUBE2D3 antibodies.

Extended Data Figure 5. Characterization of ubiquitination sites of Clr4 by Mass Spectrometry analysis.

a, same as Fig. 1c, but shows extended exposure of WB analysis for immunoprecipitated 3xFlag-Clr4 from WT and *ubc4-1* mutant cells. Asterisk indicates possible di- or multi-ubiquitin chain which is also largely dependent on Ubc4.

b, WB analysis for 3xFlag-Clr4 enriched by α-Flag affinity beads and WB analysis using ubiquitin antibody.

c, WB analyses same as Fig. 1c, but showing 3xFlag-Clr4 enrichment by ubiquitination affinity beads (UAB IP).

d, Proteasome pathway-independent Clr4 ubiquitination. Western blot (WB) analysis of immunoprecipitated 3xFlag-Clr4 from wild type (WT) and second block mutant (*2-8KtoR*) with or without treatment of proteasome inhibitor MG-132 for 4h before harvest.

e, WB analyses same as Fig. 1c, but showing also CLRC mutants like *cul4-1*, *rik1Δ*, *raf1Δ* and *raf2Δ*.

f, ChIP-qPCR assay showing enrichment of 3xFlag-Clr4 at centromeric *dh* locus in the indicated strains. Error bars, S.D. (2 biological replicates).

g, 6xHis-Clr4 purified from *E. coli* cells were *in vitro* ubiquitinated with human E1, E3 (UBE2D3) and ubiquitin and analyzed by Mass Spectrometry. The peptides with possible ubiquitination sites were shown. Di-glycine-Lysine (GG) remnants after trypsin digestion indicate possible ubiquitination sites. Prob., probability. Mass Spectrometry results were analyzed using Scaffold program.

h, Amino acid sequence of 6xHis-Clr4 protein. Sequence coverage by Mass Spectrometry analysis is highlighted in yellow and amino acids with post-translational modifications (PTM) are shown in green. Possible ubiquitination sites are highlighted with a red box.

i, Schematic diagram of domain structure of Clr4 protein and the position of lysine residues same as Fig. 1d but also shows possible ubiquitination sites characterized by Mass Spectrometry analysis on top.

Extended Data Figure 6. Characterization of ubiquitination sites of Clr4 by genetic analysis.

a, Schematic diagram of domain structure of Clr4 protein and position of lysine residues, same as Fig. 1d.

b, Western blot (WB) analysis of immunoprecipitated 3xFlag-Clr4 from all-lysine mutant (*AllKtoR*) and 6 block mutants.

c-f, Western blot (WB) analysis of immunoprecipitated 3xFlag-Clr4 from 8 individual lysine to arginine mutants (**c**), 4 or 6 lysines to arginines mutants (**d**), triple combination of K109, K110, K113 and K114 to arginines mutants (**e**) or double combination of K109, K110, K113 and K114 to arginines mutants (**f**) of second block (2-8K) of Clr4.

g, ChIP-qPCR assays showing enrichment of H3K9me2 (left), H3K9me3 (right) at centromeric *dh* locus in the indicated strains. Error bars, S.D. (3 biological replicates).

Extended Data Figure 7. Increased enrichment of Clr4 protein at centromeric heterochromatin in *ubc4-1*, *cul4-1*, second block mutant (*2-8KtoR*) and all-lysine mutant (*AllKtoR*).

a, Histone methylation assay. Nucleosomes with unmodified histone H3 (H3K9me0) or H3K9me3 histone (H3K9me3) were incubated with unmodified (ubiquitin, -) or *in vitro* ubiquitinated Clr4 proteins (ubiquitin, +) for the indicated times. Western blot (WB) analyses were carried out using H3K9me2 and H3K9me3 antibodies.

b, ChIP-qPCR assay showing enrichment of 3xFlag-Clr4 at centromeric *dh* locus in the indicated strains. Error bars, S.D. (3 biological replicates).

c, Schematic diagram for *in vitro* protein binding assay with nucleosomes. First, nucleosomes were anchored to Streptavidin beads using Biotin moiety at the end of DNA. Unmodified or ubiquitinated Clr4 proteins are added to reaction mixture and bound proteins were analyzed further by WB analyses.

d, *In vitro* protein binding assay of Clr4 protein from *S. pombe* cells with nucleosomes. 3xFlag-Clr4 purified from *S. pombe* cells were incubated with nucleosomes with unmodified histone H3 (me0) or H3K9me3 histone (me3) anchored to streptavidin beads. Input and precipitated proteins were analyzed by WB using antibodies as shown. Similar to the results of Fig. 2b, ubiquitinated Clr4 did not bind to the nucleosome.

e, ChIP-qPCR assay showing enrichment of 3xFlag-Clr4 at centromeric *dh* locus in the indicated strains. Error bars, S.D. (3 biological replicates).

Extended Data Figure 8. The role of Ubp3 for de-ubiquitination of Clr4

- a**, Western blot (WB) analysis of immunoprecipitated 3xFlag-Clr4 shows that *ubp3* deletion does not cause clear change of Clr4 ubiquitination.
- b**, Silencing assay of centromeric *ade6⁺* gene (*otr1R::ade6⁺*) on low-adenine (Low ade) and *ura4⁺* gene (*imr1L::ura4⁺*) on no Uracil (-Ura) and 5'-fluroorotic acid (FOA) media for count selection of *ura4⁺* expression in Ubp3- or Ubp8-overexpression (-OE) cells. TBZ-sensitivity assay on media containing 15μg/ml thiabendazole (TBZ) media.
- c**, RT-qPCR analysis for *ubp3*, centromeric *dh* and *act1* transcripts in the indicated strains. Error bars, S.D. (3 biological replicates).
- d**, ChIP-qPCR assays show enrichment of H3K9me2 (left) and H3K9me3 (right) at centromeric *dg* locus in the indicated strains. Error bars, S.D. (3 biological replicates).
- e**, ChIP-qPCR assays showing enrichment of RNA Pol II (Rpb1) at centromeric *dg* locus in the indicated strains. Error bars, S.D. (3 biological replicates).
- f**, ChIP-qPCR assays show enrichment of 3xFlag-Clr4 at centromeric *dg* locus in the indicated strains. Error bars, S.D. (3 biological replicates).

Extended Data Figure 9. LLPS of GFP-Clr4 proteins and disorder prediction

a. Live cell imaging showing the localization of GFP-Swi6 in indicated strains, same as Fig. 3a but showing GFP and DIC together. Scale bar, 2.5μm.

b, Schematic diagram of domain structure of Clr4 protein. Disorder prediction (PONDR) shows that the hinge region between chromodomain (CD) and PreSET of Clr4 is highly disordered. Red bar below the Clr4 schematic diagram shows the location of amino acids which is used for GFP-Clr4^{IDR} construct (51-220 amino acids).

c, LLPS of GFP-Clr4^{Full} protein. DIC; Differential interference contrast. Scale bar, 10µm.

d, The effect of 7.2% (w/v) 1,6-Hexanediol on LLPS of GFP-Clr4^{IDR} protein. Scale bar, 5µm.

e, FRAP of GFP-Clr4^{IDR} by addition of ubiquitin before and after bleaching. Representative droplets from Buffer and Ubiquitin with or without bleach were shown. Arrow shows the time of bleaching (~40s). Scale bar, 2µm

f, Schematic diagram of domain structure of Bdf2 protein (top). Disorder prediction (PONDR) shows that two Bromo and BET domains are ordered but the other regions are highly disordered.

g, Schematic diagram of domain structure of mouse SUV39H1 protein (top). Disorder prediction (PONDR) shows that the hinge region between chromodomain (CD) and PreSET of mSUV39H1 is highly disordered.

Extended Data Figure 10. Increased centromeric sRNA in *ubc4-1* and *cul4-1* mutants.

a, sRNA-seq reads mapped to chromosome 1 centromere. *ura4⁺* reporter gene is inserted in left *imr* (innermost repeats) just next to where sRNAs are produced. *ade6⁺* is located in right *otr* (outer repeats) where sRNA is hugely produced especially in *ubc4-1* and *cul4-1* mutants. Relat. enrich., relative enrichment of sRNA without tRNA.

b, Increased sRNA production at *ade6⁺* reporter gene in *ubc4-1* and *cul4-1* mutants but not at *ura4⁺*. sRNA-seq reads of *ura4⁺* and *ade6⁺* reporter genes inserted at centromere 1 heterochromatin in the indicated strains. Relat. enrich., relative enrichment of sRNA.

c, ChIP-qPCR assays showing enrichment of Ago1 and Chp1 at centromeric *dh* locus in the indicated strains. Error bars, S.D. (3 biological replicates).

d, ChIP-qPCR assays showing enrichment of Bdf2-3xFlag at centromeric *dg* (left) and euchromatic *act1* (right) loci in the indicated strains. Error bars, S.D. (2 biological replicates).

e, ChIP-seq reads of Bdf2-3xFlag mapped to euchromatic loci on chromosome 1 right arm and rDNA loci on chromosome 3 right end in indicated strains. Relat. enrich., relative enrichment. Bottom, schematic diagram of chromosomal loci.