**a**

```
                                                                48
spUbc4   MALKRINRELADLGKDPPSSCSAGPVGDDLFHWQATIMGPADSPYAGGVFFLSIHFPTDY 60
mUBE2D2  MALKRIHKELNDLARDPPAQCSAGPVGDDMFHWQATIMGPNDSPYQGGVFFLTIHFPTDY 60
mUBE2D3  MALKRINKELSDLARDPPAQCSAGPVGDDMFHWQATIMGPNDSPYQGGVFFLTIHFPTDY 60
         ******::** **.:***:.*********:********** **** ******:*******

spUbc4   PFKPPKVNFTTRIYHPNINSNGSICLDILRDQWSPALTISKVLLSICSLLTDPNPDDPLV 120
mUBE2D2  PFKPPKVAFTTRIYHPNINSNGSICLDILRSQWSPALTISKVLLSICSLLCDPNPDDPLV 120
mUBE2D3  PFKPPKVAFTTRIYHPNINSNGSICLDILRSQWSPALTISKVLLSICSLLCDPNPDDPLV 120
         ******* **********************.****************** *********

                  137
spUbc4   PEIAHVYKTDRSRYELSAREWTRKYAI 147
mUBE2D2  PEIARIYKTDREKYNRIAREWTQKYAM 147
mUBE2D3  PEIARIYKTDRDKYNRISREWTQKYAM 147
         ****::*****.:*:  :****:***:
```

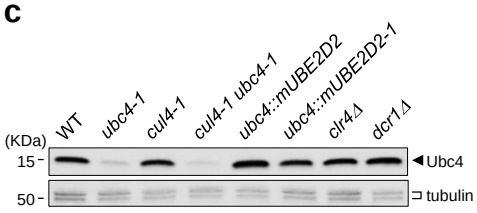
Red(*) : Identical
Blue(:) : Strongly similar
Green(.) : Weakly similar
Black( ) : None

**b**

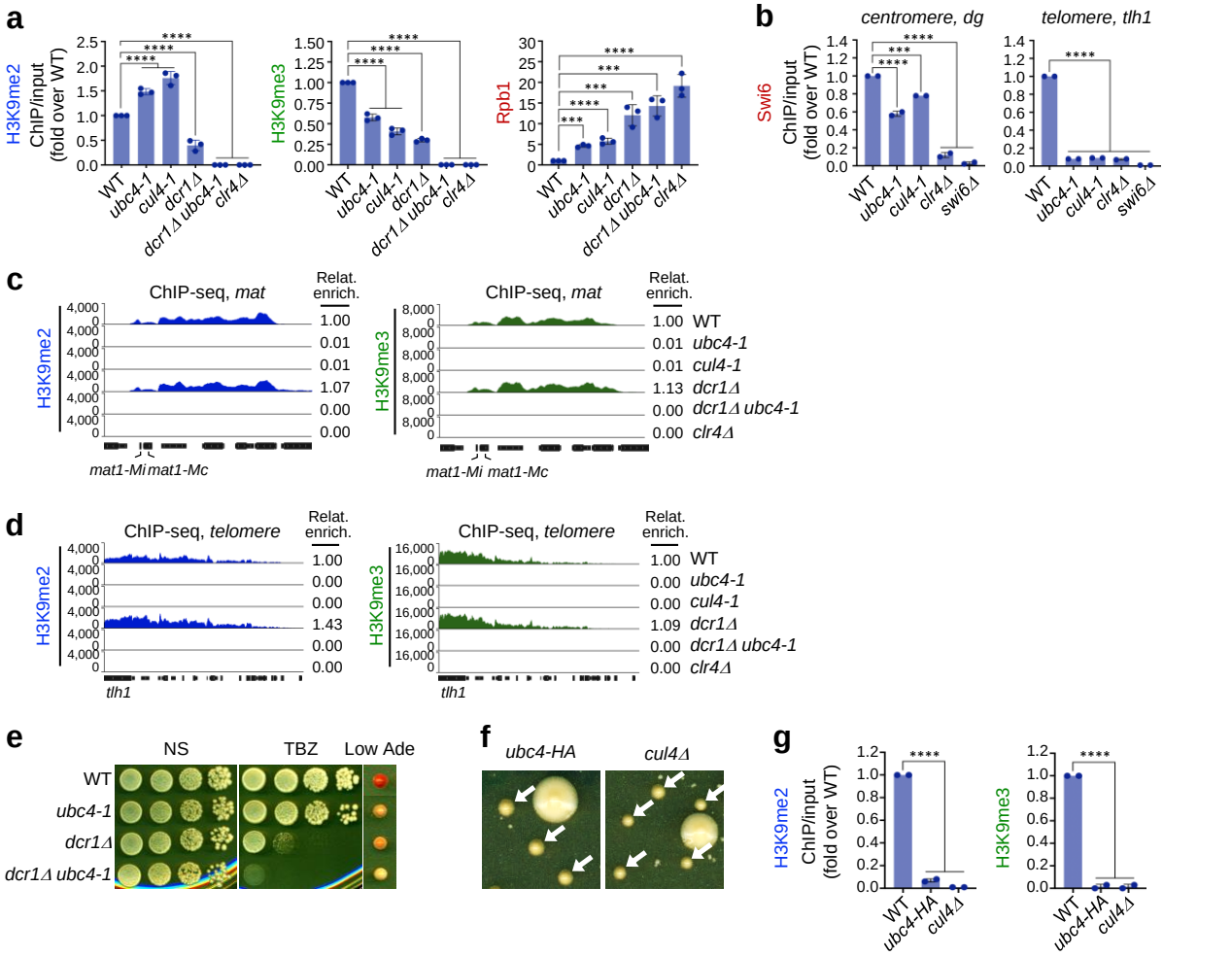
| | spUbc4 | mUBE2D2 | mUBE2D3 |
|---|---|---|---|
| spUbc4 | 100 (100) | 84.4 (95.2) | 84.4 (95.9) |
| mUBE2D2 | 84.4 (95.2) | 100 (100) | 97.3 (100) |
| mUBE2D3 | 84.4 (95.9) | 97.3 (100) | 100 (100) |

Identity (Similarity), %


**c**




**Supplementary Fig. 1 | Comparison of Ubc4 with mouse homologs.**
**a** Left, Amino acid sequence comparison between Ubc4 with its mouse homologs using Clustal Omega. Glycine 48 of Ubc4 is conserved but Serine 137, which is important for Ubc4 stability (see below), is not conserved.
**b** The summary of amino acid sequence comparison between *S. poombe* Ubc4 with mouse UBE2D2 and UBE2D3. The comparison of Ubc4 with mUBE2D3 is highlighted with red. Bottom, cladogram for the relationship of these proteins.
**c** Ubc4 protein was greatly reduced in *ubc4-G48D* (*ubc4-1)* mutant. For WB analyses of Ubc4 protein in the indicated strains, antibody for mUBE2D3 was used to detect Ubc4. WB of tubulin was used as loading control. Molecular weight markers are shown and uncropped images are provided in a Source Data file.




**Supplementary Fig. 2 | Ubc4 and Cul4 regulate heterochromatin silencing.**

**a** ChIP-qPCR assays showing enrichment of H3K9me2 (left), H3K9me3 (middle) and Rpb1 (right) at centromeric *dh* locus in the indicated strains. Error bars, S.D. (n = 3). Data are presented as mean ± SD (n = 3). *P* values are from one-way ANOVA test (Dunnett's multiple comparisons test). $P < 0.0001$ for all mutants (left and middle). $P = 0.0002$ for *ubc4-1*, $P < 0.0001$ for *cul4-1*, $P = 0.0008$ for *dcr1Δ*, $P = 0.0003$ for *dcr1Δ ubc4-1* and $P < 0.0001$ for *clr4Δ* (right).

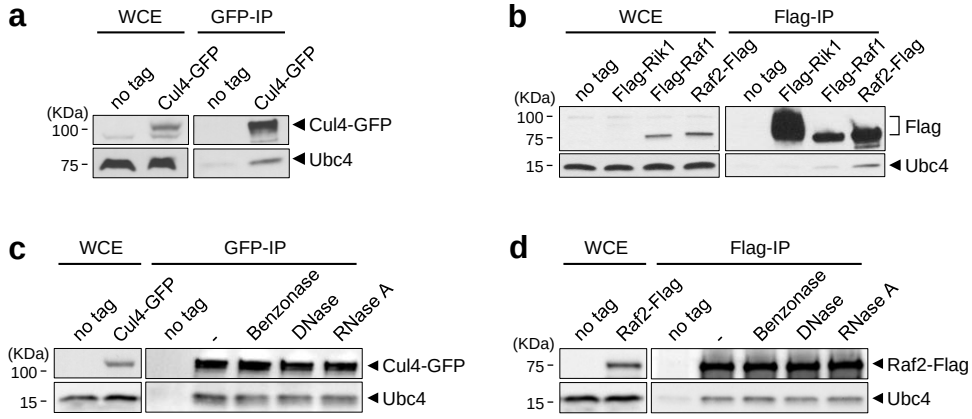
**b** ChIP-qPCR assays showing enrichment of HP1 protein, Swi6 at centromeric *dg* locus (left) and telomere 1 left region (right) in the indicated strains. Error bars, S.D. (n = 2). Data are presented as mean ± SD (n = 3). *P* values are from one-way ANOVA test (Dunnett's multiple comparisons test). $P < 0.0001$ for *ubc4-1*, $P = 0.0003$ for *cul4-1* and $P < 0.0001$ for *clr4Δ* and *swi6Δ* (left). $P < 0.0001$ for all mutants (right).

**c** and **d** ChIP-seq reads of H3K9me2 and H3K9me3 mapped to mating type locus (**b**) and telomere (**c**) in the indicated strains.

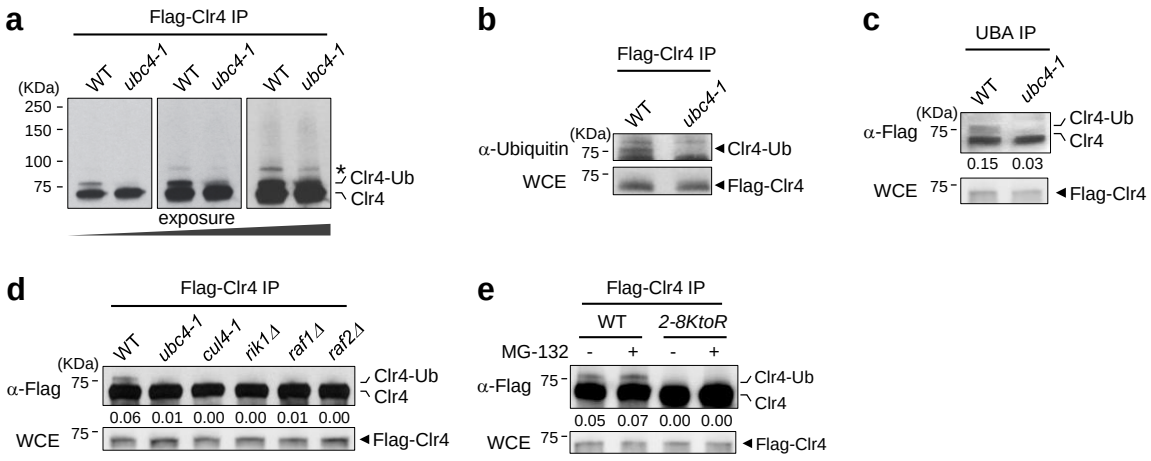
**e** Assays for silencing of centromeric *ade6*$^+$ on Low Ade medium and for chromosome segregation by viability on 12.5 μg/ml TBZ medium in the indicated strains.

**f** *ubc4* mutants tagged with triple HA at carboxyl-terminus (*ubc4-HA*) and *cul4* deletion mutants (*cul4Δ*) are viable but have a severe growth defect (arrows). Large colonies are WT controls.

**g** ChIP-qPCR assays showing enrichment of H3K9me2 and H3K9me3 at centromeric *dg* repeat in the indicated strains. Error bars, S.D. (n = 2). Data are presented as mean ± SD (n = 2). *P* values are from one-way ANOVA test (Dunnett's multiple comparisons test). $P < 0.0001$ for all mutants (left and right).




**Supplementary Fig. 3 | Ubc4 and Cul4 form Ubc4-CLRC complex independently of nucleases.**
**a** and **b** Co-immunoprecipitation (Co-IP) of Cul4-GFP (**a**) and Flag-Rik1, Flag-Raf1 and Raf2-Flag (**b**) with Ubc4. Molecular weight markers are shown and uncropped images are provided in a Source Data file.
**c** and **d** Co-IP of Cul4-GFP (**c**) and Raf2-Flag (**d**) with Ubc4 in the absence and presence of Benzonase (1.25 U/μl), TURBO DNase (0.1 U/μl) and RNase A (0.5 μg/μl). Molecular weight markers are shown and uncropped images are provided in a Source Data file.




**Supplementary Fig. 4 | Mono-ubiquitination of Clr4.**
**a** Extended exposure of WB analysis for Flag-Clr4 IP from WT and *ubc4-1* mutant cells (Fig. 1B). Asterisk indicates possible di- ubiquitination which is also largely dependent on Ubc4. Molecular weight markers are shown and uncropped images are provided in a Source Data file.
**b** Flag-Clr4 IP and WB analysis using ubiquitin antibody. Molecular weight markers are shown and uncropped images are provided in a Source Data file.
**c** WB analyses Flag-Clr4 enrichment by ubiquitination affinity beads (UBA IP). Molecular weight markers are shown and uncropped images are provided in a Source Data file.
**d** WB analyses of immunoprecipitated Flag-Clr4 (Flag-Clr4 IP) in the indicated strains. Molecular weight markers are shown and uncropped images are provided in a Source Data file.
**e** Proteasome-independent Clr4 ubiquitination. WB analyses of Flag-Clr4 IP with or without treatment of proteasome inhibitor MG-132 in the indicated strains. Molecular weight markers (KDa) are shown and uncropped images are provided in a Source Data file.

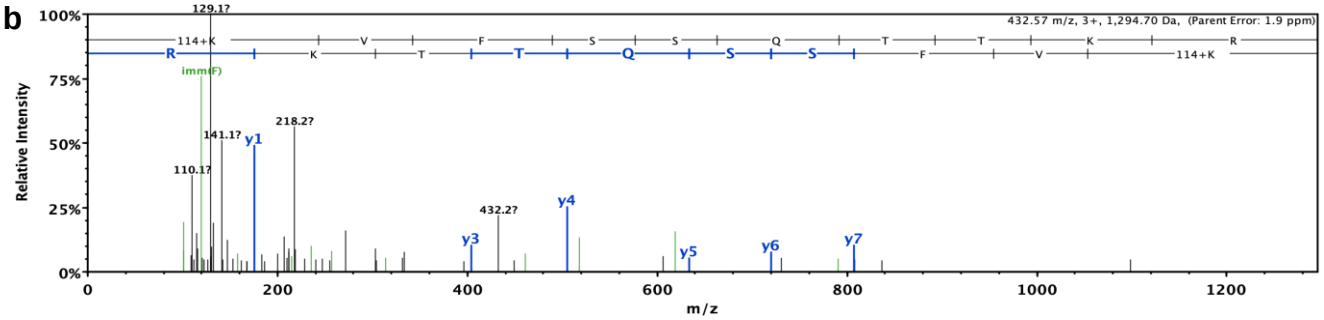
**a**

| Sequence | A.A. | Prob | Modifications |
|---|---|---|---|
| (R)NGAVKLYR(I) | K25 | **99%** | GG (+114) |
| (R)LKGSNSDSDSPHHASNPHPNSR(Q) | K65 | **99%** | GG (+114) |
| (K)KVFSSQTTKR(Q) | K114 | **96%** | GG (+114) |
| (R)KQSTALTTNDTSIILDDSLHTNSKK(L) | K151 | **96%** | GG (+114) |
| (K)TRNEVKEESQKR(E) | K160 | 85% | GG (+114) |
| (R)ELVSNSIKEATSPK(T) | K174 | 84% | GG (+114) |
| (R)ELVSNSIKEATSPKTSSILTKPR(N) | K180 | **100%** | GG (+114) |
| (K)TSSILTKPR(N) | K187 | **97%** | GG (+114) |
| (R)TLPLEIFKTK(E) | K336 | **99%** | GG (+114) |

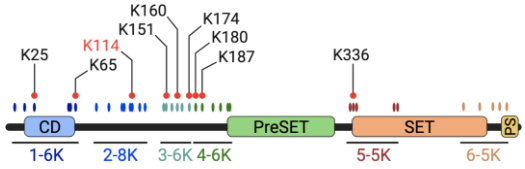
**c**

```
MGSSHHHHHH SSGLVPRGSH MSPKQEEYEV ERIVDEKLDR NGAVKLYRIR
WLNYSSRSDT WEPPENLSGC SAVLAEWKRR KRRLKGSNSD SDSPHHASNP
HPNSRQKHQH QTSKSVPRSQ RFSRELNVKK ENKKVFSSQT TKRQSRKQST
ALTTNDTSII LDDSLHTNSK KLGKTRNEVK EESQKRELVS NSIKEATSPK
TSSILTKPRN PSKLDSYTHL SFYEKRELFR KKLREIEGPE VTLVNEVDDE
PCPSLDFQFI SQYRLTQGVI PPDPNFQSGC NCSSLGGCDL NNPSRCECLD
DLDEPTHFAY DAQGRVRADT GAVIYECNSF CSCSMECPNR VVQRGRTLPL
EIFKTKEKGW GVRSLRFAPA GTFITCYLGE VITSAEAAKR DKNYDDDGIT
YLFDLDMFDD ASEYTVDAQN YGDVSRFFNH SCSPNIAIYS AVRNHGFRTI
YDLAFFAIKD IQPLEELTFD YAGAKDFSPV QSQKSQQNRI SKLRRQCKCG
SANCRGWLFG
```

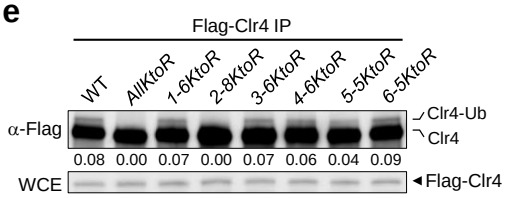
**b**



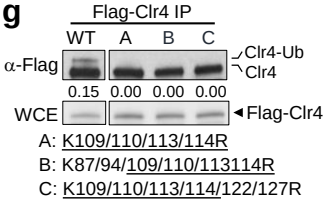

**d**



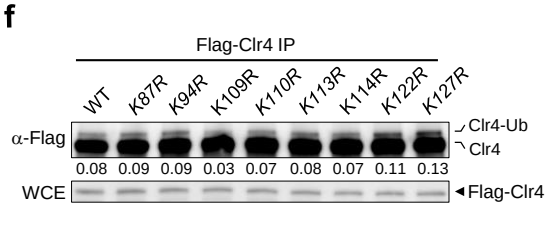

**e**



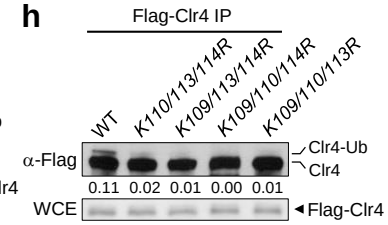

**g**



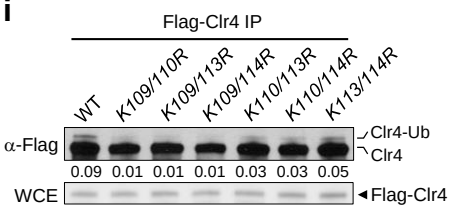

**f**



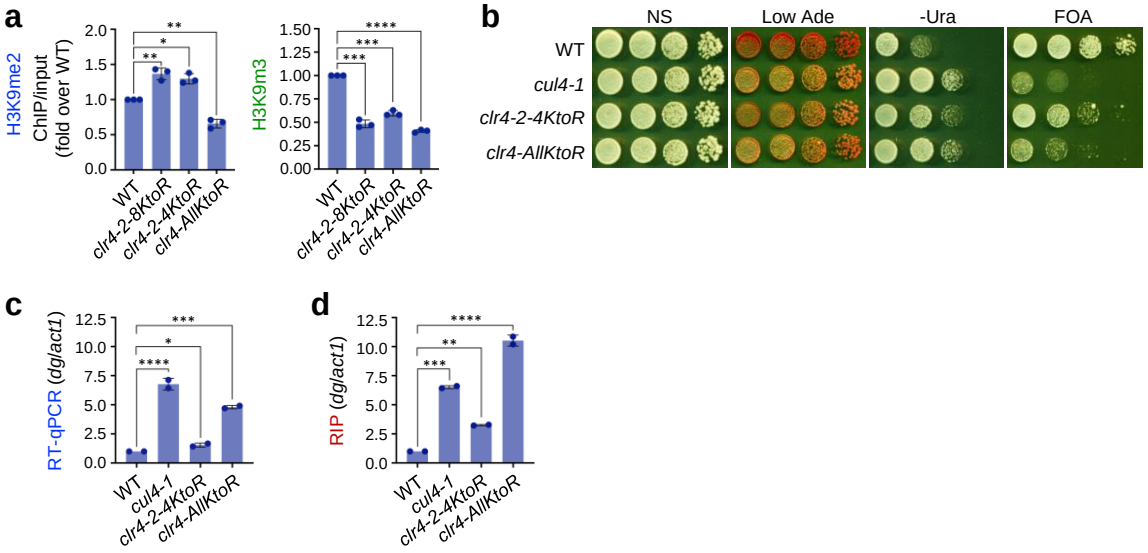

**h**




**i**




**Supplementary Fig. 5 | Characterization of ubiquitination sites of Clr4 by Mass Spec and genetic analyses.**
**a** 6xHis-Clr4 purified from *E. coli* cells were *in vitro* ubiquitinated and analyzed by Mass Spectrometry. Di-glycine (GG) remnants after trypsin digestion indicate possible ubiquitination sites. Prob., probability. K114 is highlighted.
**b** MS/MS spectrum of the peptide containing ubiquitinated K114. The observed y ions are shown. Predicted amino acids sequences are across the top.
**c** Amino acid sequence of 6xHis-Clr4 protein. Sequence coverage is highlighted in yellow. 6xHis (green) and translation start site (blue) are shown. Possible ubiquitination sites (red) are highlighted with black boxes and K114 is highlighted with red box.
**d** Schematic diagram of domain structure of Clr4 and ubiquitination sites characterized by Mass Spectrometry analysis. K114 is highlighted with red.
**e-i** WB analyses of Flag-Clr4 IP from indicated strains. Molecular weight markers and uncropped images are provided in a Source Data file.



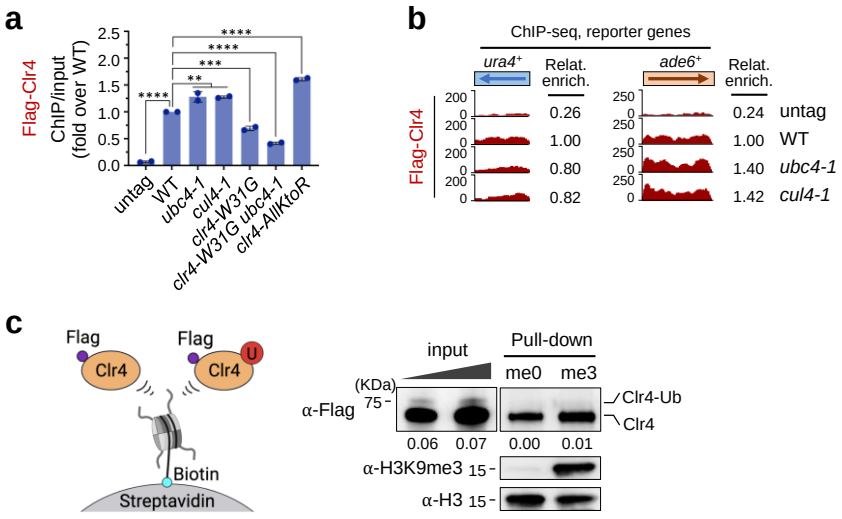

**Supplementary Fig. 6 | Heterochromatin silencing defects in *clr4-2-8KtoR*, *clr4-2-4KtoR* and *clr4-AllKtoR* mutants.**

**a** ChIP-qPCR assays showing enrichment of H3K9me2 and H3K9me3 at centromeric *dh* repeat in the indicated strains. Data are presented as mean ± SD (n = 3). *P* values are from one-way ANOVA test (Dunnett's multiple comparisons test). *P* = 0.0025 for *clr4-2-8KtoR*, *P* = 0.0233 for *clr4-2-4KtoR* and *P* = 0.0039 for *clr4-AllKtoR* (left). *P* = 0.0002 for *clr4-2-8KtoR*, *P* = 0.0003 for *clr4-2-4KtoR* and *P* < 0.0001 for *clr4-AllKtoR* (right).

**b** Assays for silencing of centromeric *ade6*$^{+}$ and *ura4*$^{+}$ on Low Ade, -Ura and FOA media in the indicated strains.

**c** RT-qPCR analysis for centromeric *dg* repeat (normalized to *act1*). Data are presented as mean ± SD (n = 2). *P* values are from one-way ANOVA test (Dunnett's multiple comparisons test). *P* < 0.0001 for *cul4-1*, *P* = 0.0433 for *clr4-2-4KtoR* and *P* = 0.0004 for *clr4-AllKtoR.*

**d** RNA immunoprecipitation (RNA IP) and qPCR of Flag-Clr4 to centromeric *dg* and *act1* transcript in the indicated strains. Data are presented as mean ± SD (n = 2). *P* values are from one-way ANOVA test (Dunnett's multiple comparisons test). *P* = 0.0001 for *cul4-1*, *P* = 0.0063 for *clr4-2-4KtoR* and *P* < 0.0001 for *clr4-AllKtoR.*

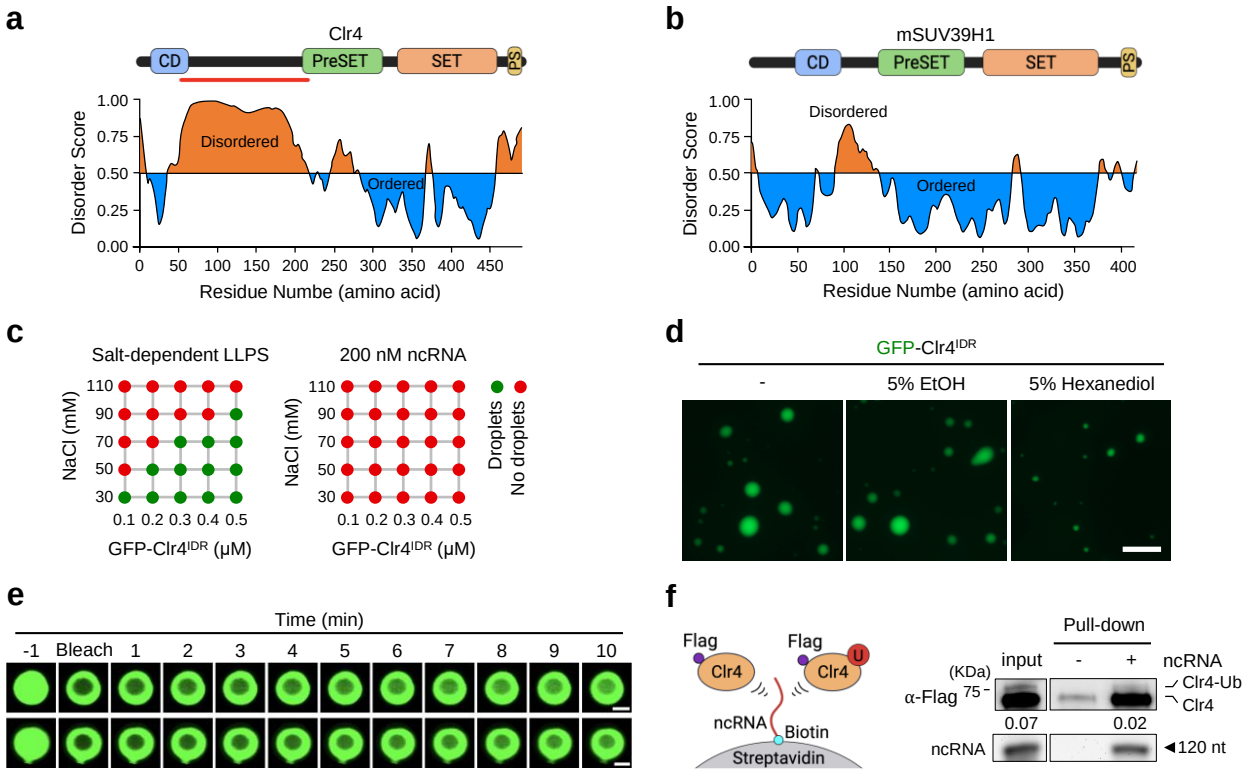



**Supplementary Fig. 7 | Clr4 mono-ubiquitination induces its dissociation from chromatin and from centromeric ncRNA.**

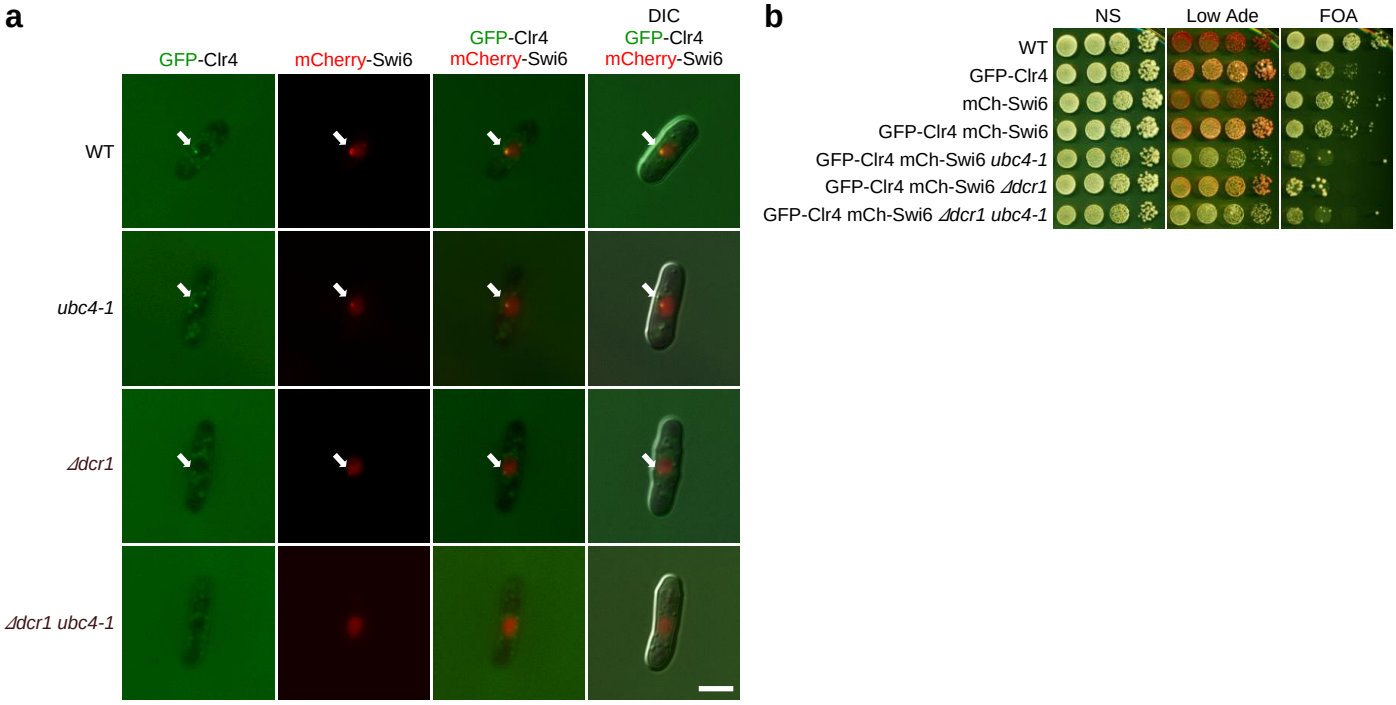
**a** ChIP-qPCR assays showing enrichment of Flag-Clr4 at centromeric *dh* repeat in the indicated strains. Data are presented as mean ± SD (n = 2). *P* values are from one-way ANOVA test (Dunnett's multiple comparisons test). $P < 0.0001$ for untag, $P = 0.0017$ for *ubc4-1*, $P = 0.0018$ for *cul4-1*, $P = 0.0009$ for *clr4-W31G* and $P < 0.0001$ for *clr4-W31G ubc4-1* and *clr4Δ*.

**b** ChIP-seq reads of Flag-Clr4 mapped to *ura4*$^+$ and *ade6*$^+$ reporter genes in indicated strains.

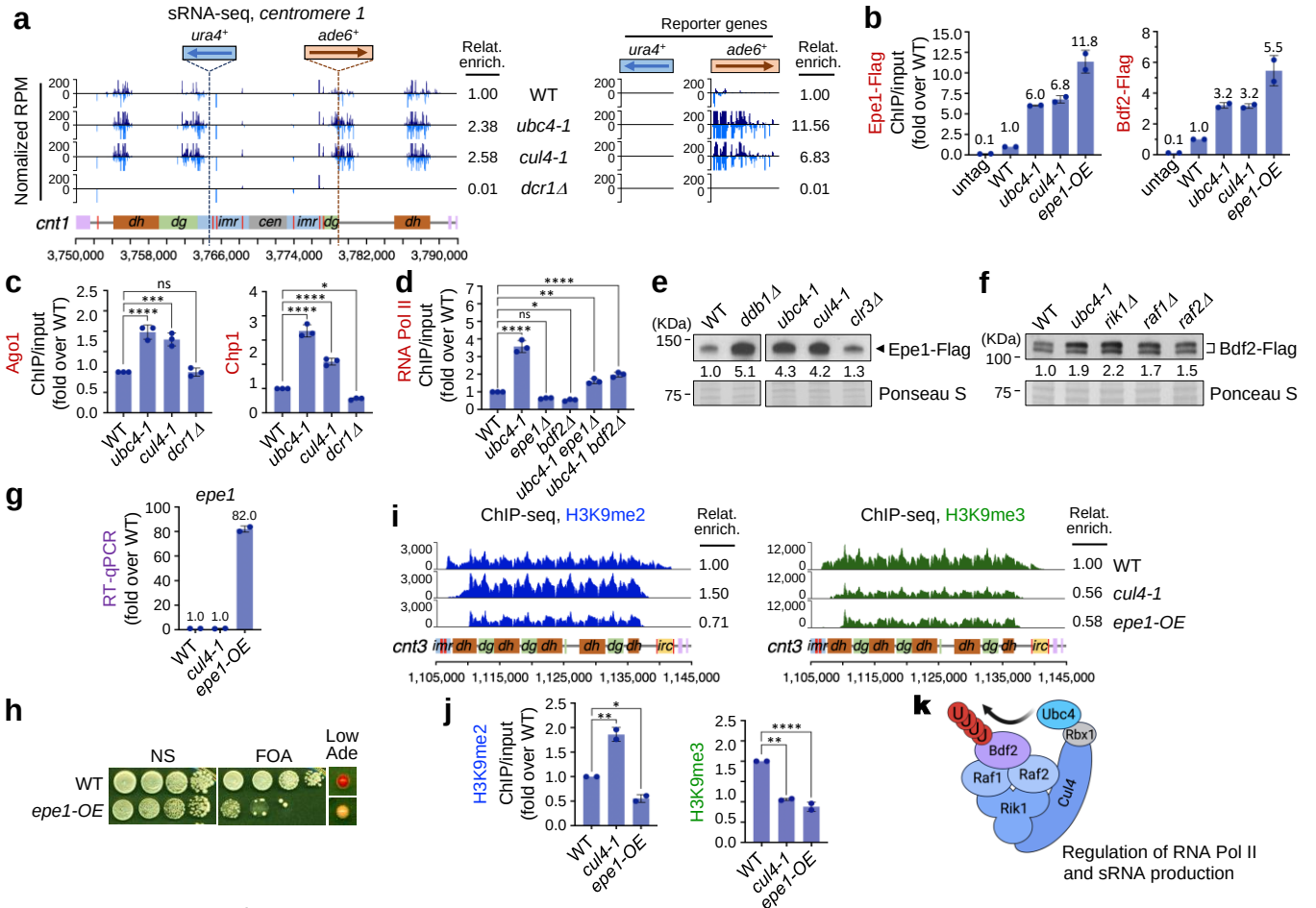
**c** Binding assay of Flag-Clr4 protein purified from *S. pombe* cells with nucleosomes with unmodified histone H3 (me0) or H3K9me3 histone (me3) anchored to streptavidin beads. Input and Flag-Clr4 proteins from pull-down were analyzed by WB using antibodies as shown. Schematic diagram of binding assay (left). Molecular weight markers are shown and uncropped images are provided in a Source Data file.




**Supplementary Fig. 8 | Phase separation of Clr4.**
**a** Schematic diagram of domain structure of Clr4 protein. Disorder prediction shows that the hinge region between the chromodomain (CD) and PreSET domain of Clr4 is highly disordered. Red bar shows the location of amino acids which is used for GFP-Clr4$^{IDR}$ fusion (51-220 amino acids).
**b** Schematic diagram of domain structure and disorder prediction (PONDR) of mSUV39H1 protein.
**c** Summary of LLPS behaviors of Clr4$^{IDR}$ with different concentrations of salt (NaCl, left). Summary of LLPS behaviors of Clr4$^{IDR}$ with different concentrations of salt in the presence of 200 nM centromeric ncRNA (right).
**d** Representative images of phase-separated liquid droplets of GFP-Clr4$^{IDR}$ after treatment with 5% Ethanol (EtOH) or 5% Hexanediol. Scale bar, 5 μm.
**e** FRAP of GFP-Clr4$^{Full}$. Representative phase-separated liquid droplests were monitored as shown. Photo bleach was applied around time 0 min. Scale bar, 2 μm.
**f** Flag-Clr4 protein purified from *S. pombe* cells were incubated with centromeric ncRNA anchored to streptavidin beads. Input and bound Flag-Clr4 proteins from pull-down were analyzed by WB. ncRNA from input and pull-down was stained with SYBR Gold. Schematic diagram (left). Molecular weight marker is shown and uncropped images are provided in a Source Data file.




**Supplementary Fig. 9 | Co-localization of GFP-Clr4 and mCherry-Swi6.**
**a** Live cell imaging showing condensed GFP-Clr4 and mCherry-Swi6 heterochromatic foci in indicated strains. Co-localization of GFP-Clr4 and mCherry-Swi6 condensates were seen clearly in WT and with reduced intensity in *ubc4-1* and *dcr1⊿* mutants. Both GFP-Clr4 and mCherry-Swi6 condensates are completely lost in *dcr1⊿ ubc4-1* mutant. Scale bar, 2.5 μm.
**b** Assays for silencing of centromeric *ade6*$^{+}$ and *ura4*$^{+}$ on Low Ade and FOA media in the indicated strains.




**Supplementary Fig. 10 | Ubiquitination by Ubc4 and Cul4 regulates centromeric sRNA transcription through Epe1 and Bdf2$^{BRD4}$.**

**a** sRNA-seq reads mapped to chromosome 1 centromere. The *ura4*$^+$ reporter gene is inserted in *imr* with no sRNA production whereas *ade6*$^+$ is located in *otr* where sRNA are produced. Increased sRNA production was observed at the *ade6*$^+$ reporter gene, but not at the *ura4*$^+$ reporter gene, in *ubc4-1* and *cul4-1* mutants.

**b** ChIP-qPCR assays for enrichment of Epe1-Flag and Bdf2-Flag at centromeric *dh* repeat in the indicated strains. Data are presented as mean ± SD (n = 2).

**c** ChIP-qPCR assays for enrichment of Ago1 and Chp1 at centromeric *dh* repeat in the indicated strains. Data are presented as mean ± SD (n = 3). *P* values are from one-way ANOVA test (Dunnett's multiple comparisons test). *P* < 0.0001 for *ubc4-1* and *P* = 0.0002 for *cul4-1* (left). *P* < 0.0001 for *ubc4-1* and *cul4-1* and *P* = 0.0206 for *dcr1Δ*. (right).

**d** ChIP-qPCR assays for enrichment of RNA Pol II (Rpb1) at centromeric *dg* repeat in the indicated strains. Data are presented as mean ± SD (n = 3). *P* values are from one-way ANOVA test (Dunnett's multiple comparisons test). *P* < 0.0001 for *ubc4-1*, *P* = 0.0172 for *bdf2Δ*, *P* = 0.0028 for *ubc4-1 epe1Δ* and *P* < 0.0001 for *ubc4-1 bdf2Δ*.

**e** and **f** WB analyses for Epe1-Flag and Bdf2-Flag in the indicated strains. Ponceau S staining is used as control. Molecular weight markers are shown and uncropped images are provided in a Source Data file.

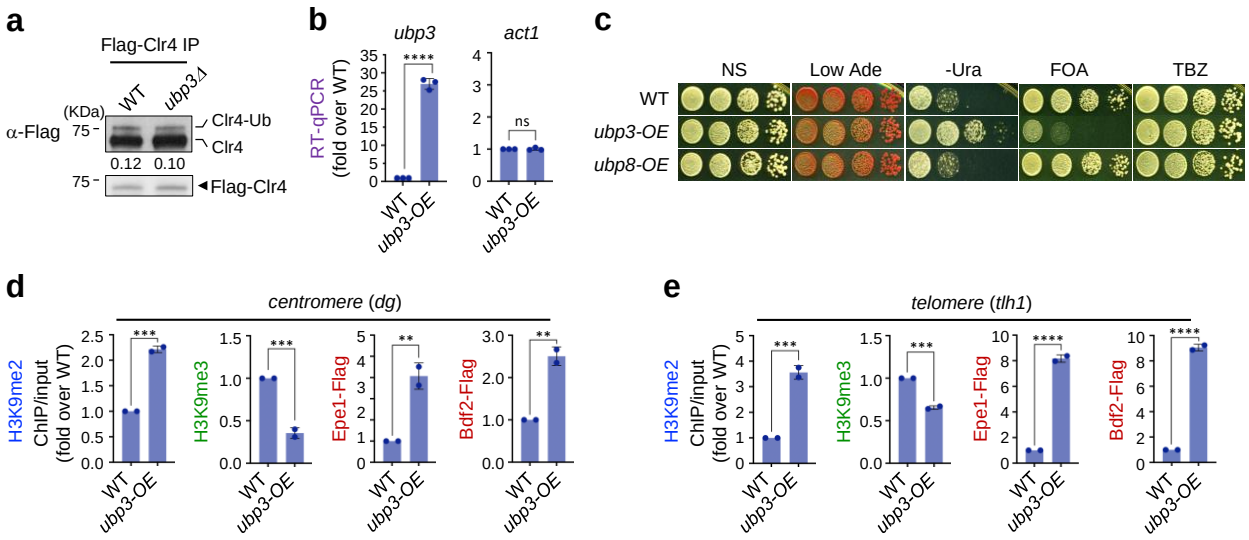
**g** RT-qPCR analysis for *epe1* transcripts in the indicated strains. Data are presented as mean ± SD (n = 2).

**h** Assays for silencing of centromeric *ura4*$^+$ and *ade6*$^+$ reporter genes on FOA and Low Ade media in the indicated strains.

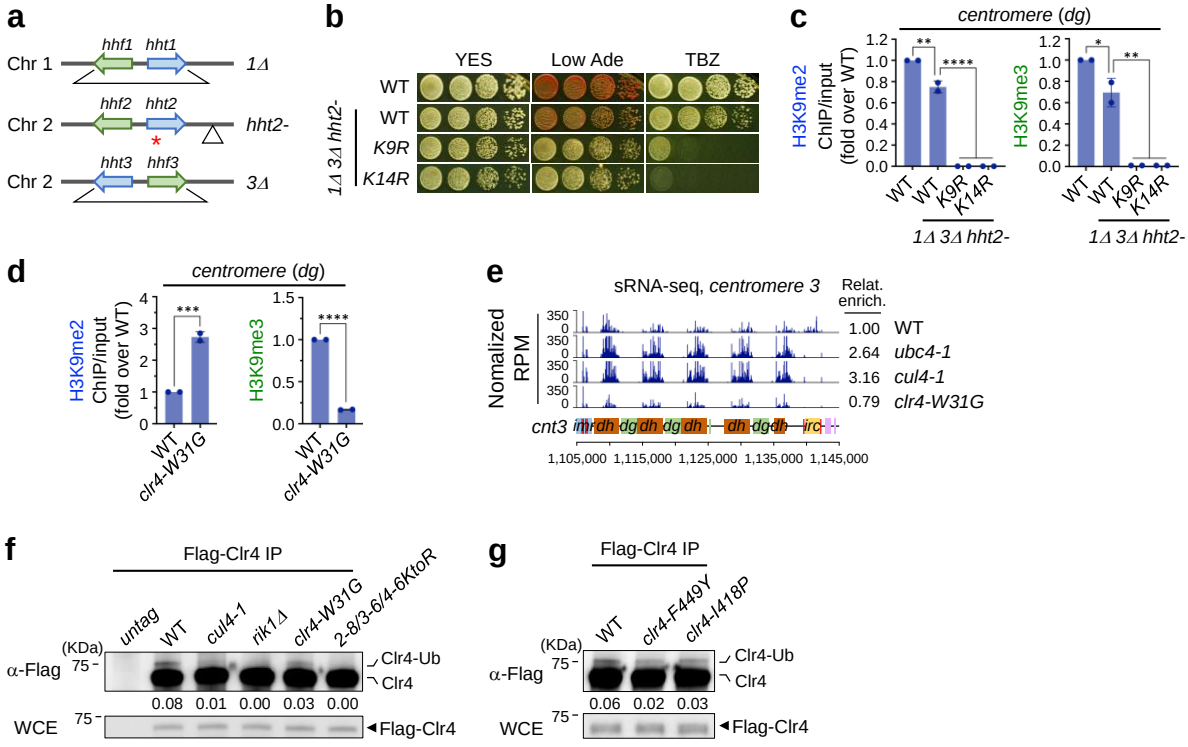
**i** ChIP-seq reads of H3K9me2 (blue) and H3K9me3 (green) mapped to centromere 3 (*cnt3*) right arm in the indicated strains.

**j** ChIP-qPCR assays for enrichment of H3K9me2 and H3K9me3 at centromeric *dh* repeat in the indicated strains. Data are presented as mean ± SD (n = 2). *P* values are from one-way ANOVA test (Dunnett's multiple comparisons test). *P* = 0.0045 for *cul4-1* and *P* = 0.0287 for *epe1-OE* (left). *P* = 0.0013 for *cul4-1* and *P* = 0.0008 for *epe1-OE* (right).

**k** Schematic diagram showing the presumptive subunit arrangement in the complex of Ubc4-CLRC and its substrate Bdf2. Poly-ubiquitination activity of Ubc4-CLRC toward Bdf2 promotes degradation of Bdf2, reduced RNA Pol II recruitment to centromeric heterochromatin and subsequently reduced centromeric sRNA production. Red circle (U), ubiquitin.




**Supplementary Fig. 11 | Ubp3 counteracts Ubc4-CLRC activities.**
**a** WB analyses of Flag-Clr4 IP reveals that *ubp3* deletion does not cause significant change of Clr4 ubiquitination. Molecular weight markers are shown and uncropped images are provided in a Source Data file.
**b** RT-qPCR analysis for *ubp3*, centromeric *dg* and *act1* transcripts in the indicated strains. Data are presented as mean ± SD (n = 3). *P* values are from one-way ANOVA test (Dunnett's multiple comparisons test). $P < 0.0001$ for *ubp3-OE* (left).
**c** Assays for silencing of centromeric *ade6*$^+$ and *ura4*$^+$ reporter genes on Low Ade, -Ura and FOA media and for chromosome segregation by viability on 15 μg/ml TBZ medium in *ubp3* or *ubp8* overexpression (-OE) cells.
**d** ChIP-qPCR assays for enrichment of H3K9me2, H3K9me3, Epe1-Flag and Bdf2-Flag at centromeric *dg* locus. Data are presented as mean ± SD (n = 2). *P* values are from one-way ANOVA test (Dunnett's multiple comparisons test). $P = 0.0001$ for *ubp3-OE* (H3K9me2), $P = 0.0007$ for *ubp3-OE* (H3K9me2), $P = 0.0056$ for *ubp3-OE* (Epe1-Flag) and $P = 0.0021$ for *ubp3-OE* (Bdf2-Flag)
**e** ChIP-qPCR assays for enrichment of H3K9me2, H3K9me3, Epe1-Flag and Bdf2-Flag at telomeric *tlh1* locus. Data are presented as mean ± SD (n = 2). *P* values are from one-way ANOVA test (Dunnett's multiple comparisons test). $P = 0.0008$ for *ubp3-OE* (H3K9me2), $P = 0.0002$ for *ubp3-OE* (H3K9me2) and $P < 0.0001$ for *ubp3-OE* (Epe1-Flag) and *ubp3-OE* (Bdf2-Flag).




**Supplementary Fig. 12 | Mutation of histone H3 lysines 9 and 14 abolishes both H3K9me2 and H3K9me3, while *clr4* mutants with H3K9me2-to-H3K9me3 transition defect show reduced mono-ubiquitination.**
**a** Schematic diagram of histone H3 and H4 genes. After the *hhf1-hht1* and *hht3-hhf3* gene pairs were deleted (open trapezoid, *1Δ 3Δ*), the *hht2* gene was mutagenized by inserting a marker gene (triangle) and simultaneous homologous recombination (*hht2-*). Asterisk indicates mutation site in *hht2*.
**b** Assays for silencing of centromeric *ade6*$^{+}$ on Low Ade medium and for chromosome segregation by viability on 15 μg/ml TBZ medium in the indicated strains. Both H3K14R and H3K9R mutants have a strong silencing defect with white colonies on Low Ade medium, and high sensitivity to TBZ.
**c** ChIP-qPCR assays for enrichment of H3K9me2 and H3K9me3 at centromeric *dg* repeats in the indicated strains. Both H3K14R and H3K9R mutants completely lost H3K9me2 and H3K9me3 at the centromere. Data are presented as mean ± SD (n = 2). *P* values are from one-way ANOVA test (Dunnett's multiple comparisons test). *P* = 0.0021 for *1Δ 3Δ hht2*-WT and *P* < 0.0001 for *1Δ 3Δ hht2-K9R and 1Δ 3Δ hht2-K14R* (left). *P* = 0.0238 for *1Δ 3Δ hht2*-WT and *P* = 0.0012 for *1Δ 3Δ hht2-K9R and 1Δ 3Δ hht2-K14R* (right).
**d** ChIP-qPCR assays showing enrichment of H3K9me2 (left), H3K9me3 (right) at centromeric *dg* repeat in the indicated strains. Data are presented as mean ± SD (n = 2). *P* values are from one-way ANOVA test (Dunnett's multiple comparisons test). *P* = 0.0006 for *clr4-W31G* (left) and *P* < 0.0001 for *clr4-W31G* (right).
**e** sRNA-seq reads mapped to centromere 3 right arm in indicated strains.
**f** and **g** WB analyses of Flag-Clr4 IP from indicated strains. Molecular weight markers are shown and uncropped images are provided in a Source Data file.

1 **Table S1. *S. pombe* strains used in this study.**

2

| Number | Name | Genotype | Reference |
|---|---|---|---|
| FY2002 | WT | *h⁺ otrIR(SphI)::ade6 lmrIL(NcoI)::leul-32 ade6-DN/N ura4-DS/E leu1* | R. Allshire |
| HSK29 | *ubc4-1* | FY2002_*ubc4-G48D-KanMX6* | This study |
| HSK335 | *cul4-1* | FY2002_*cul4-GFP-KanMX6* | This study |
| HSK458 | *cul4-1 ubc4-1* | FY2002_*cul4-GFP-KanMX6 ubc4-G48D-NatMX6* | This study |
| HSK608 | *ubc4::mUBE2D2* | FY2002_*KanMX6-ubc4::mUBE2D2* | This study |
| HSK610 | *ubc4::mUBE2D2-1* | FY2002_*KanMX6-ubc4::mUBE2D2-G48D* | This study |
| HSK351 | *clr4Δ* | FY2002_*clr4Δ::KanMx6* | This study |
| HSK770 | *dcr1Δ* | *FY2002_dcr1Δ::NatMx6* | This study |
| HSK790 | *dcr1Δ ubc4-1* | FY2002_*dcr1Δ::NatMx6 ubc4-G48D-KanMX6* | This study |
| HSK644 | *ubc4-3xHA* | FY2002_*ubc4-3xHA-KanMX6* | This study |
| HSK646 | *cul4Δ* | FY2002_*cul4Δ::NatMx6* | This study |
| HSK679 | *GFP-Swi6* | FY2002_*HphMX6-GFP-Swi6* | This study |
| HSK700 | *GFP-Swi6 ubc4-1* | FY2002_*HphMX6-GFP-Swi6 ubc4-G48D-KanMX6* | This study |
| HSK776 | *GFP-Swi6 dcr1Δ* | FY2002_*HphMX6-GFP-Swi6 dcr1Δ::NatMX6* | This study |
| HSK808 | *GFP-Swi6 dcr1Δ ubc4-1* | FY2002_*HphMX6-GFP-Swi6 dcr1Δ::NatMX6 ubc4-G48D-KanMX6* | This study |
| HSK778 | *GFP-Swi6 clr4Δ* | FY2002_*HphMX6-GFP-Swi6 clr4Δ::KanMX6* | This study |
| HSK716 | *GFP-Swi6 ubc4-3xHA* | FY2002_*HphMX6-GFP-Swi6 ubc4-3xHA-KanMX6* | This study |
| HSK717 | *GFP-Swi6 cul4Δ* | FY2002_*HphMX6-GFP-Swi6 cul4Δ::NatMX6* | This study |
| KFP26 | *h⁻* | *h⁻ ura4-D18 leu1-32 ade6-M210 his3-D1* | Lab stock |
| SPG18 | *h⁹⁰* | *h⁹⁰ ura4-D18 leul-32 ade6 -M216 his2* | Lab stock |
| DI304 | *h⁹⁰ ubc4-1* | *h⁹⁰ leul-32 ubc4-G48D-KanMX6* | Irvine, 2009 |
| AY709 | *h⁹⁰ubc4-1/S137T* | *h⁹⁰ leul-32 ubc4-G48D-KanMX6 ubc4-S137T* (EMS mutagenesis) | This study |
| HSK438 | *ubc4-1* | FY2002_*KanMX6-ubc4-G48D* | This study |
| HSK440 | *ubc4-S137T* | FY2002_*ubc4-S137T-HphMX6* | This study |
| HSK442 | *ubc4-1/S137T* | FY2002_*KanMX6-ubc4-G48D/S137T-HphMX6* | This study |
| HSK603 | *swi6Δ* | FY2002_*swi6Δ::KanMx6* | This study |
| HSK393 | *3xFlag-Rik1* | FY2002_*HphMX6-3xFlag-Rik1* | This study |
| HSK389 | *3xFlag-Raf1* | FY2002_*HphMX6-3xFlag-Raf1* | This study |
| HSK274 | *Raf2-3xFlag* | FY2002_*Raf2-3xFlag-HphMX6* | This study |
| HSK513 | *epe1Δ* | FY2002_*epe1Δ::NatMX6* | This study |
| HSK525 | *ubc4-1 epe1Δ* | FY2002_*ubc4-G48D-KanMX6 epe1Δ::NatMX6* | This study |
| HSK527 | *cul4-1 epe1Δ* | FY2002_*cul4-GFP-KanMX6 epe1Δ::NatMX6* | This study |
| HSK518 | *bdf2Δ* | FY2002_*bdf2Δ::KanMX6* | This study |
| HSK543 | *ubc4-1 bdf2Δ* | FY2002_*ubc4-G48D-KanMX6 bdf2Δ::NatMX6* | This study |
| HSK545 | *cul4-1 bdf2Δ* | FY2002_*cul4-GFP-KanMX6 bdf2Δ::NatMX6* | This study |
| HSK626 | *epe1-OE* | FY2002_*KanMX6-adh1p-epe1* | This study |
| HSK522 | *Epe1-3xFlag* | FY2002_ *Epe1-3xFlag-HphMX6* | This study |
| HSK561 | *Epe1-3xFlag ubc4-1* | FY2002_ *Epe1-3xFlag-HphMX6 ubc4-G48D-KanMX6* | This study |
| HSK563 | *Epe1-3xFlag cul4-1* | FY2002_ *Epe1-3xFlag-HphMX6 cul4-GFP-KanMX6* | This study |
| HSK634 | *epe1-OE-3xFlag* | FY2002_*kanMX6-adh1p-epe1-3xFlag-HphMX6* | This study |
| HSK524 | *Bdf2-3xFlag* | FY2002_ *Bdf2-3xFlag-HphMX6* | This study |
| HSK571 | *Bdf2-3xFlag ubc4-1* | FY2002_ *Bdf2-3xFlag-HphMX6 ubc4-G48D-KanMX6* | This study |
| HSK573 | *Bdf2-3xFlag cul4-1* | FY2002_ *Bdf2-3xFlag-HphMX6 cul4-GFP-KanMX6* | This study |
| HSK1259 | *Bdf2-3xFlag epe1-OE* | FY2002_ *Bdf2-3xFlag-HphMX6 KanMX6-adh1p-epe1* | This study |
| HSK355 | *rik1Δ* | FY2002_*rik1Δ::KanMx6* | This study |
| HSK585 | *Epe1-3xFlag ddb1Δ* | FY2002_ *Epe1-3xFlag-HphMX6 ddb1Δ::KanMx6* | This study |
| HSK565 | *Epe1-3xFlag clr3Δ* | FY2002_ *Epe1-3xFlag-HphMX6 clr3Δ::KanMX6* | This study |
| HSK1097 | *Bdf2-3xFlag rik1Δ* | FY2002_ *Bdf2-3xFlag-HphMX6 rik1Δ::KanMx6* | This study |
| HSK1099 | *Bdf2-3xFlag raf1Δ* | FY2002_ *Bdf2-3xFlag-HphMX6 raf1Δ::KanMx6* | This study |
| HSK1101 | *Bdf2-3xFlag raf2Δ* | FY2002_ *Bdf2-3xFlag-HphMX6 raf2Δ::KanMx6* | This study |
| HSK606 | *3xFlag-Clr4* | FY2002_*HphMX6-3xFlag-Clr4* | This study |

| Number | Name | Genotype | Reference |
|---|---|---|---|
| HSK618 | *3xFlag-Clr4 ubc4-1* | FY2002_*HphMX6-3xFlag-Clr4 ubc4-G48D-KanMX6* | This study |
| HSK620 | *3xFlag-Clr4 cul4-1* | FY2002_*HphMX6-3xFlag-Clr4 cul4-GFP-KanMX6* | This study |
| HSK920 | *3xFlag-Clr4 rik1Δ* | FY2002_*HphMX6-3xFlag-Clr4 rik1Δ::NatMx6* | This study |
| HSK921 | *3xFlag-Clr4 raf1Δ* | FY2002_*HphMX6-3xFlag-Clr4 raf1Δ::NatMx6* | This study |
| HSK922 | *3xFlag-Clr4 raf2Δ* | FY2002_*HphMX6-3xFlag-Clr4 raf2Δ::NatMx6* | This study |
| HSK1118 | *3xFlag-Clr4-AllKtoR* | FY2002_*HphMX6-3xFlag-Clr4-AllKtoR* | This study |
| HSK1125 | *3xFlag-Clr4-1-6KtoR* | FY2002_*HphMX6-3xFlag-Clr4-1-6KtoR* | This study |
| HSK971 | *3xFlag-Clr4-2-8KtoR* | FY2002_*HphMX6-3xFlag-Clr4-2-8KtoR* | This study |
| HSK1127 | *3xFlag-Clr4-3-6KtoR* | FY2002_*HphMX6-3xFlag-Clr4-3-6KtoR* | This study |
| HSK1129 | *3xFlag-Clr4-4-6KtoR* | FY2002_*HphMX6-3xFlag-Clr4-4-6KtoR* | This study |
| HSK1131 | *3xFlag-Clr4-5-5KtoR* | FY2002_*HphMX6-3xFlag-Clr4-5-5KtoR* | This study |
| HSK1132 | *3xFlag-Clr4-6-5KtoR* | FY2002_*HphMX6-3xFlag-Clr4-6-5KtoR* | This study |
| HSK1121 | *3xFlag-Clr4-2-4KtoR* | FY2002_*HphMX6-3xFlag-Clr4-2-4KtoR* | This study |
| HSK1140 | *3xFlag-Clr4-K87R* | FY2002_*HphMX6-3xFlag-Clr4-K87R* | This study |
| HSK1142 | *3xFlag-Clr4-K94R* | FY2002_*HphMX6-3xFlag-Clr4-K94R* | This study |
| HSK1144 | *3xFlag-Clr4-K109R* | FY2002_*HphMX6-3xFlag-Clr4-K109R* | This study |
| HSK1146 | *3xFlag-Clr4-K110R* | FY2002_*HphMX6-3xFlag-Clr4-K110R* | This study |
| HSK1148 | *3xFlag-Clr4-K113R* | FY2002_*HphMX6-3xFlag-Clr4-K113R* | This study |
| HSK1150 | *3xFlag-Clr4-K114R* | FY2002_*HphMX6-3xFlag-Clr4-K114R* | This study |
| HSK1152 | *3xFlag-Clr4-K122R* | FY2002_*HphMX6-3xFlag-Clr4-K122R* | This study |
| HSK1154 | *3xFlag-Clr4-K127R* | FY2002_*HphMX6-3xFlag-Clr4-K127R* | This study |
| HSK1013 | *3xFlag-clr4-K109/110/113/114R* | FY2002_*HphMX6-3xFlag-Clr4-K109/110/113/114R-KanMX6* | This study |
| HSK999 | *3xFlag-Clr4-K87/94/109/110/113/114R* | FY2002_*HphMX6-3xFlag-Clr4-K87/94/109/110/113/114R-KanMX6* | This study |
| HSK1000 | *3xFlag-Clr4-K109/110/113/114/122/127R* | FY2002_*HphMX6-3xFlag-Clr4-K109/110/113/114/122/127R-KanMX6* | This study |
| HSK1015 | *3xFlag-Clr4-K110/113/114R* | FY2002_*HphMX6-3xFlag-Clr4-110/113/114R-KanMX6* | This study |
| HSK1017 | *3xFlag-Clr4-K109/113/114R* | FY2002_*HphMX6-3xFlag-Clr4-109/113/114R-KanMX6* | This study |
| HSK1019 | *3xFlag-Clr4-K109/110/114R* | FY2002_*HphMX6-3xFlag-Clr4-109/110/114R-KanMX6* | This study |
| HSK1021 | *3xFlag-Clr4-K109/110/113R* | FY2002_*HphMX6-3xFlag-Clr4-109/110/113R-KanMX6* | This study |
| HSK1082 | *3xFlag-Clr4-K109/110R* | FY2002_*HphMX6-3xFlag-Clr4-109/110R-KanMX6* | This study |
| HSK1062 | *3xFlag-Clr4-K109/113R* | FY2002_*HphMX6-3xFlag-Clr4-109/113R-KanMX6* | This study |
| HSK1064 | *3xFlag-Clr4-K109/114R* | FY2002_*HphMX6-3xFlag-Clr4-109/114R-KanMX6* | This study |
| HSK1086 | *3xFlag-Clr4-K110/113R* | FY2002_*HphMX6-3xFlag-Clr4-110/113R-KanMX6* | This study |
| HSK1065 | *3xFlag-Clr4-K110/114R* | FY2002_*HphMX6-3xFlag-Clr4-110/114R-KanMX6* | This study |
| HSK1084 | *3xFlag-Clr4-K113/114R* | FY2002_*HphMX6-3xFlag-Clr4-113/114R-KanMX6* | This study |
| HSK967 | *clr4-2-8KtoR* | FY2002_*HphMX6-clr4-2-8KtoR* | This study |
| HSK1124 | *clr4-2-4KtoR* | FY2002_*HphMX6-clr4-2-4KtoR* | This study |
| HSK1080 | *clr4-W31G* | FY2002_*HphMX6-clr4-W31G* | This study |
| HSK1049 | *3xFlag-Clr4-W31G* | FY2002_*HphMX6-3xFlag-Clr4-W31G* | This study |
| HSK1054 | *3xFlag-Clr4-W31G ubc4-1* | FY2002_*HphMX6-3xFlag-Clr4-W31G ubc4-G48D-KanMX6* | This study |
| HSK927 | *3xFlag-Clr4-I418P* | FY2002_*HphMX6-3xFlag-Clr4-I418P* | This study |
| HSK928 | *3xFlag-Clr4-F449Y* | FY2002_*HphMX6-3xFlag-Clr4-F449Y* | This study |
| HSK1299 | *5xUAS-Ade6* | HSK1290 (*h$^{?}$ ade6-DN/N ura4$^{+}$*)_*ura4::5xUAS-ade6* | This study |
| HSK1313 | *5xUAS-Ade6 GBD-Clr4-ΔCD Clr4* | HSK1299_*GBD-Clr4-ΔCD leu1::Clr4* | This study |
| HSK1319 | *5xUAS-Ade6 GBD-Clr4-ΔCD Clr4 ubc4-1* | HSK1313_*ubc4-G48D-KanMX6* | This study |
| HSK1321 | *5xUAS-Ade6 GBD-Clr4-ΔCD Clr4 cul4-1* | HSK1313_*cul4-GFP-KanMX6* | This study |
| HSK395 | *ubp3-OE* | FY2002_*HphMX6-adh1p-ubp3* | This study |
| HSK401 | *ubp8-OE* | FY2002_*HphMX6-adh1p-ubp8* | This study |
| HSK918 | *3xFlag-Clr4 ubp3-OE* | FY2002_*HphMX6-3xFlag-Clr4 NatMX6-adh1p-ubp3* | This study |
| HSK1277 | *Epe1-3xFlag ubp3-OE* | FY2002_*Epe1-3xFlag-HphMX6 NatMX6-adh1p-ubp3* | This study |
| HSK1279 | *Bdf2-3xFlag ubp3-OE* | FY2002_*Bdf2-3xFlag-HphMX6 NatMX6-adh1p-ubp3* | This study |
| HSK919 | *3xFlag-Clr4 ubp3Δ* | FY2002_*HphMX6-3xFlag-Clr4 ubp3Δ::NatMx6* | This study |
| HSK388 | *1Δ 3Δ WT* | FY2002_*hhf1-hht1Δ::HphMX6 hht3-hhf3Δ::NatMX6* | This study |
| HSK480 | *1Δ 3Δ hht2-K9R* | HSK388_*hht2-K9R-KanMX6* | This study |
| HSK482 | *1Δ 3Δ hht2-K14R* | HSK388_*hht2-K14R-KanMX6* | This study |

## Table S2. Plasmids used in this study.

| Name | Description | Application |
|---|---|---|
| pET-14b-Clr4 | 6xHis-Clr4 | Recombinant 6xHis-Clr4 protein purification from *E. coli* |
| pHis-parellel-GFP-Clr4$^{Full}$ | 6xHis-GFP-Clr4$^{Full}$ | Recombinant 6xHis-GFP-Clr4$^{Full}$ protein purification from *E. coli* |
| pHis-parellel-mCherry-Clr4$^{Full}$ | 6xHis-mCherry-Clr4$^{Full}$ | Recombinant 6xHis-mCherry-Clr4$^{Full}$ protein purification from *E. coli* |
| pHis-parellel-GFP-Clr4$^{IDR}$ | 6xHis-GFP-Clr4$^{IDR}$ | Recombinant 6xHis-GFP-Clr4$^{IDR}$ protein purification from *E. coli* |
| pET-14b-Flag-Clr4 | 6xHis-1xFlag-Clr4 | Recombinant 6xHis-1xFlag-Clr4 protein purification from *E. coli* |
| pET-14b-Myc-Clr4 | 6xHis-1xMyc-Clr4 | Recombinant 6xHis-1xMyc-Clr4 protein purification from *E. coli* |
| pET-14b-H3N-GST | H3N-GST-6xHis | Recombinant H3N-GST-6xHis protein purification from *E. coli* |

## Table S3. Oligonucleotides used in this study.

| Name | Description | Purpose | Sequence |
|---|---|---|---|
| p30-qPCR-F | *dg* | ChIP-qPCR, RT-qPCR | CCATATCAATTTCCCATGTTCC |
| p30-qPCR-R | *dg* | ChIP-qPCR, RT-qPCR | CATCAAGCGAGTCGAGATGA |
| p33-qPCR-F | *dh* | ChIP-qPCR, RT-qPCR | TATCCTGCGTCTCGGTATCC |
| p33-qPCR-R | *dh* | ChIP-qPCR, RT-qPCR | CTGTTCGTGAATGCTGAGAAAG |
| act1-qPCR-F | *act1* | ChIP-qPCR, RT-qPCR | TGCACCTGCCTTTTATGTTG |
| act1-qPCR-F | *act1* | ChIP-qPCR, RT-qPCR | TGGGAACAGTGTGGGTAACA |
| ade6-qPCR-F | *ade6* | ChIP-qPCR | TTCCTACTGCCATCAAAGCA |
| ade6-qPCR-R | *ade6* | ChIP-qPCR | GCACGCTGTTGAATTGAGAA |
| ubp3-qPCR-F | *ubp3* | RT-qPCR | GCCAAATTTTTGGTGGTCAG |
| ubp3-qPCR-R | *ubp3* | RT-qPCR | TGAATGTCGAGTTGCAGAGG |
| epe1-qPCR-F | *epe1* | RT-qPCR | GCAATTCTTCAAATCCATCCA |
| epe1-qPCR-R | *epe1* | RT-qPCR | TCGAGTCGTGATGGAATTGA |

## Table S4. Antibodies used in this study.

| Name | Company & Catalogue Numer | Description | Application |
|---|---|---|---|
| α-UBE2D3 | Proteintech 11677-1-AP | Rabbit polyclonal | WB (Western Blot) |
| α-RNA Pol II | BioLegend 904001 | Mouse monoclonal | ChIP (Chromatin immunoprecipitation) |
| α-H3K9me2 | Abcam, ab1220 | Mouse monoclonal | ChIP and WB |
| α-H3K9me3 | Absolute Antibody Ab00700-1.26 | Mouse scFv-Fc (synthetic antibody) | ChIP and WB |
| α-H3K9me3 | Abcam ab8898 | Rabbit polyclonal | WB |
| α-H3 | Abcam ab1791 | Rabbit polyclonal | WB |
| α-GFP | Sigma 11814460001 (Roche) | mouse monoclonal | IP (immunoprecipitation) |
| α-GFP | Abcam ab290 | Rabbit polyclonal | WB |
| α-Flag Agarose | Sigma A2220 | Mouse monoclonal | IP |
| α-Flag | Sigma F7425 | Rabbit polyclonal | WB |
| α-Flag | Sigma F1804 | Mouse monoclonal | ChIP and RNA IP |
| α-Myc | Abcam ab9106 | Rabbit polyclonal | WB |
| α-His | ThermoFisher MA1-135 | Mouse monoclonal | WB |
| α-HA | Roche 11583816001 | Mouse monoclonal | WB |
| α-Swi6 | Abcam ab188276 | Rabbit polyclonal | ChIP |
| α-Ago1 | Abcam Ab18190 | Rabbit polyclonal | ChIP |
| α-Chp1 | Abcam ab18191 | Rabbit polyclonal | ChIP |
| α-Tubulin | Abcam ab6160 | Rabbit monoclonal | WB |
| α-Ubiquitin | Enzo Life Sciences #ADI-SPA-200-F | Rabbit Polyclonal | WB |
| UBA TUBE | Cytoskeleton # UBA01-beads | Signal-Seeker Ubiquitination Affinity Beads | UBA TUBE IP |