Supporting Information

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SI Materials and Methods

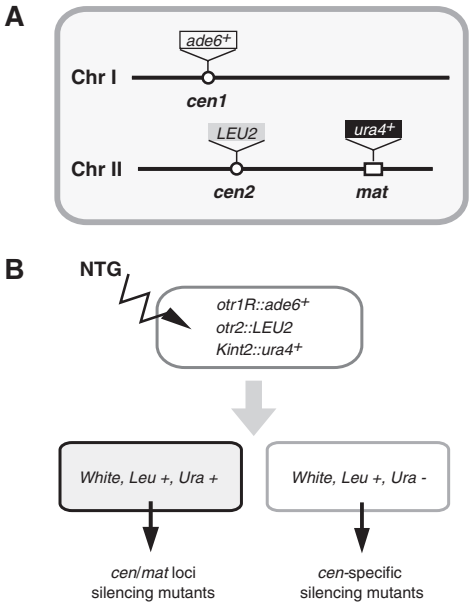
Screening of Heterochromatin Silencing Defect Mutants. Strain AHY420, which harbors three marker genes (*otr1R::ade6*$^+$, *otr2::LEU2*, and *Kint2::ura4*$^+$) was treated with 3 mg/mL *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine for 30 min at 30 °C. The mutagenized cells were incubated overnight at 26 °C and plated on yeast extract plates. After 3–5 d, 466 white/pink colonies were picked up and streaked on YEA plates to obtain single colonies (master plates). The cells were then replicated on minimal medium (EMM) lacking leucine or EMM lacking uracil to examine the silencing defect of the other marker genes. In this first screen, we obtained 26 mutants that showed a silencing defect for all the marker genes (Ade$^+$, Leu$^+$, and Ura$^+$) and 65 mutants that showed a centromeric-specific silencing defect (Ade$^+$, Leu$^+$, and Ura$^-$). These mutants were further subjected to three backcrosses, and mutants that displayed an uncertain phenotype were excluded from further analysis. Finally, we isolated 15 mutants that showed a global silencing defect and 31 mutants with a centromere-specific silencing defect. To identify the corresponding genes of these mutants, the mutant cells were transformed with a fission yeast genomic library pTN-L1 (National BioResource Project). 5FOA-resistant colonies were isolated by replica plating from ∼50,000 transformants. The plasmids were recovered from the isolated cells and introduced into *Escherichia coli* for DNA sequencing.

RNA Preparation and RT-PCR Analysis. RNA preparation and RT-PCR analyses were carried out as described previously (1). DNase I-treated total RNA prepared from each strain was subjected to quantitative RT-PCR analysis using the One Step SYBR PrimeScript RT-PCR Kit II (TaKaRa) and the 7300 Real-Time PCR system (ABI). The primers used in these analyses are listed in Table S3.

RNA Analysis. Total RNA was extracted and centromeric transcripts were detected as described previously (1). Small RNAs were detected as described previously (2), with some modifications. The total RNA prepared from cells growing at exponential phase in YEA medium was fractionated using a mirVana miRNA Isolation Kit (Ambion). The collected small RNAs (5–10 μg) were resolved on a 15% (wt/vol) denaturing PAGE gel and transferred to nylon membrane (Hybond-N+; GE Healthcare). A 367-bp region within centromeric *dh* repeats was amplified with primers SP6-dhII-Fw and T7-dhII-Rv, and the amplified products were used as a template for in vitro transcription utilizing SP6 or T7 RNA polymerase. ^{32}P-labeled riboprobes were incubated in hydrolysis buffer (60 mM $NaHCO_3$ and 90 mM $NaCO_3$) at 60 °C for 2–2.5 h and used for hybridization. Small RNA blots were also hybridized with an oligonucleotide complementary to the *S. pombe* U6 snRNA.

Fluorescence Microscopy. EGFP-fused Ers1 protein was expressed under control of the *nmt41* promoter. Live-cell imaging was carried out as described previously (3). Cells were cultured in EMM lacking leucine to early-log phase at 26 °C. The cells were suspended in EMM and mounted on a slide glass sealed with silicon for microscope observation. Zeiss AxioVision 3.1 was used for image acquisition and analysis. For categorization of EGFP-Ers1 nuclear dots, the value of fluorescent intensity (FI) of the brightest pixel within each spot was used and categorized as strong (FI ≥ 200), moderate (200 > FI > 180), or weak (180 > FI). The average background FI in the nucleus was around 90.

1. Iida T, Kawaguchi R, Nakayama J (2006) Conserved ribonuclease, Eri1, negatively regulates heterochromatin assembly in fission yeast. *Curr Biol* 16:1459–1464.
2. Kato H, et al. (2005) RNA polymerase II is required for RNAi-dependent heterochromatin assembly. *Science* 309:467–469.
3. Hayashi A, et al. (2009) Localization of gene products using a chromosomally tagged GFP-fusion library in the fission yeast *Schizosaccharomyces pombe*. *Genes Cells* 14: 217–225.



C

Mutants obtained by screening

Phenotype	Gene	No. of alleles
Ade+/Leu+/Ura+	*clr4*$^{+}$	6
	rik1^{+}	3
	raf1^{+}	5
	raf2^{+}	2
Ade+/Leu+/Ura-	*hrr1*$^{+}$	10
	ago1^{+}	5
	dcr1^{+}	5
	rdp1^{+}	4
	SPBC582.04c	3
	arb1^{+}	2
	chp1^{+}	1
	ers1^{+}	1

Fig. S1. Screening of silencing defective mutants. (*A*) Schematic drawing of the parental strain used in the genetic screening. Three auxotrophic marker genes (*ade6*$^{+}$, *LEU2*, and *ura4*$^{+}$) are inserted at heterochromatic loci, *cen1*, *cen2*, and the mating type locus, respectively. Chr, chromosome. (*B*) Cells harboring marker genes were mutagenized, and silencing defect mutants were isolated as described in *Materials and Methods*. Obtained mutants were classified into two categories according to the pattern of marker gene expression. NTG, N-methyl-N′-nitro-N-nitorosoguanidine. (*C*) Summary of the genetic screening.

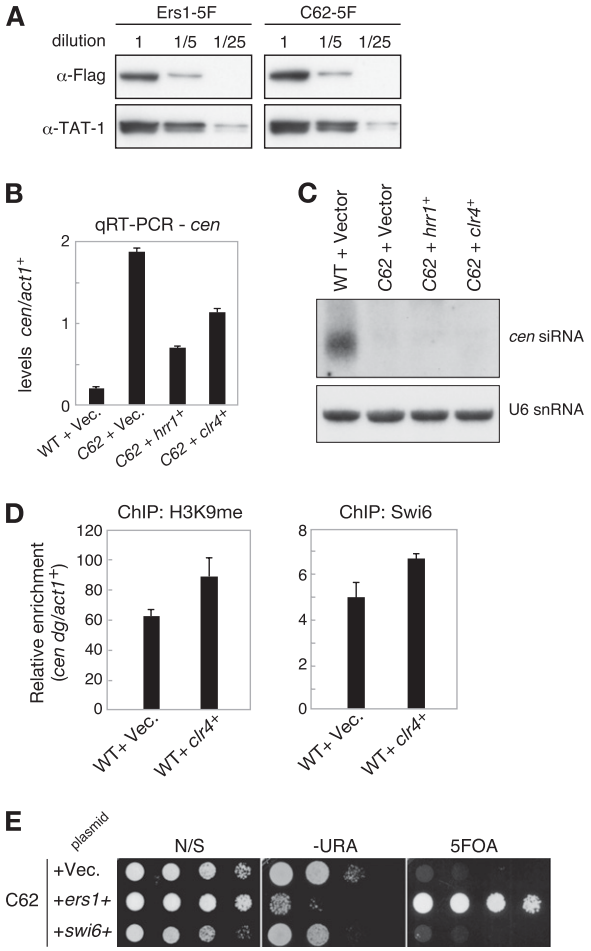


Fig. S2. Characterizing the suppressive effect of Hrr1 and Clr4 on the *C62* mutant phenotype. (*A*) Comparison of protein stability between WT and mutant Ers1. Whole-cell extracts prepared from cells expressing either Ers1-Flag or Ers1^{C62}-Flag were diluted (1-fold, 1/5-fold, or 1/25-fold) and subjected to Western blot analysis using anti-Flag M2 antibody. Anti-TAT1 antibody was used as a control. (*B*) Real-time quantitative RT (qRT)-PCR analysis of centromeric *dh* transcript levels relative to *act1*$^+$. *C62* cells expressing either *hrr1*$^+$ or *clr4*$^+$ were cultured in EMM containing 5FOA before preparing total RNA. *C62* harboring an empty vector (Vec.) was used as a control. (*C*) Northern blot analysis of centromeric siRNAs prepared from the indicated strain as in *B*. (*D*) ChIP analysis of H3K9me2 and Swi6 levels associated with *cen dg*, relative to control *act1*$^+$ locus. WT cells expressing *clr4*$^+$ were subjected to ChIP analysis by real-time PCR. Cells harboring an empty vector were used as a control. Results are the mean ± SD of at least three independent experiments. (*E*) Expression of *swi6*$^+$ does not suppress the *ers1-C62* silencing defect. *ers1*$^+$ or *swi6*$^+$ was introduced in *ers1-C62* mutant cells, and silencing states at the *otr1R::ura4*$^+$ locus were assayed. An empty vector was used as the control. N/S, nonselective medium; −URA, EMM lacking uracil.

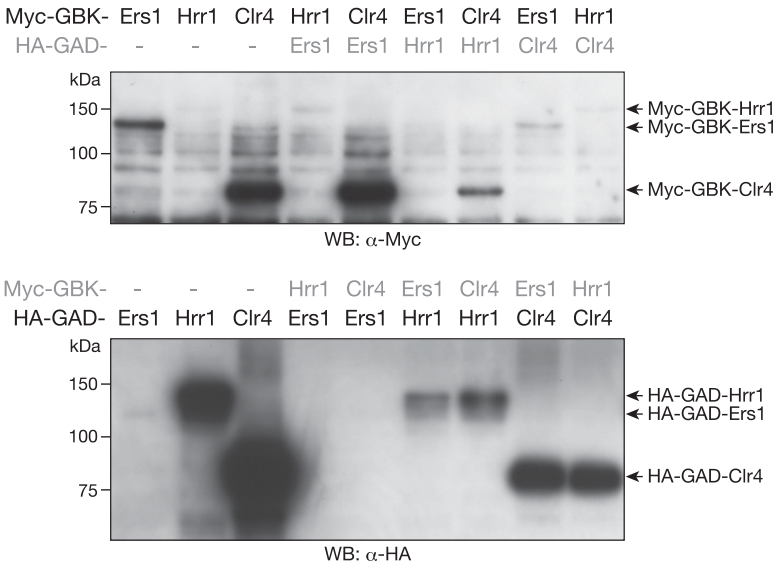


Fig. S3. Detection of fused proteins expressed in YTH analysis. Western blot (WB) analysis of activation domain (GAD)- or binding domain (GBD)-fused proteins. The GAD-fused and GBD-fused proteins were detected using anti-Myc (*Upper*) and anti-HA antibodies (*Lower*), respectively. Arrows indicate bands for each fusion protein.

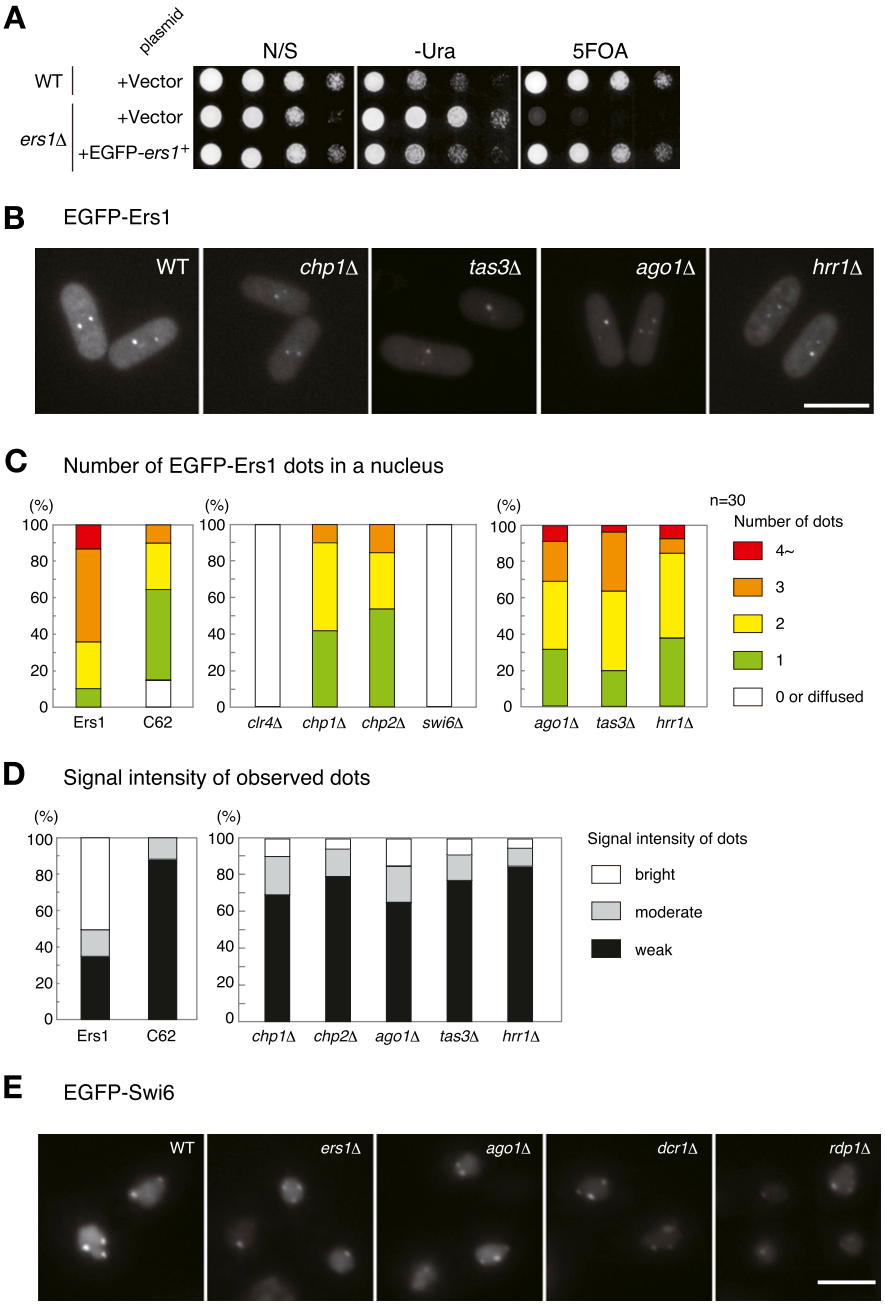


Fig. S4. Localization of EGFP-Ers1 and EGFP-Swi6 in RNAi mutant cells. (*A*) EGFP-Ers1 protein is functional. The *ers1*Δ cells expressing Ers1-EGFP are assayed in the silencing states at *otr1R::ura4*$^+$. An empty vector was used as the control. Plates are nonselective (N/S), lacking uracil (−URA), and supplemented with 5FOA. (*B*) Ers1-EGFP localization in *chp1*Δ, *tas3*Δ, *ago1*Δ, and *hrr1*Δ mutant cells. All images were taken with the same exposure time (3 s) at a wavelength of 495 nm. (Scale bar, 5 μm.) (*C*) Number of Ers1-EGFP (EGFP-Ers1) nuclear dots in WT and RNAi mutant cells was counted, and obtained percentages are represented by stacked bar graphs (white, 0 dot or diffused; green, 1 dot; yellow, 2 dots; orange, 3 dots; red, more than 4 dots). Thirty independent cells of indicated strains (n) were examined to obtain the percentages. (*D*) Comparison of EGFP-Ers1 signal intensity. Intensities of Ers1-EGFP nuclear dots in each strain were measured and divided into three categories according to relative level. The percentages of nuclear dots in each category are plotted on stacked bar graphs (white, bright dots; gray, moderate dots; black, weak dots). (*E*) EGFP-Swi6 localization in WT and RNAi mutant cells. EGFP-Swi6 was expressed under the control of *nmt41* promoter. All images were taken with the same exposure time (2 s). (Scale bar, 5 μm.)

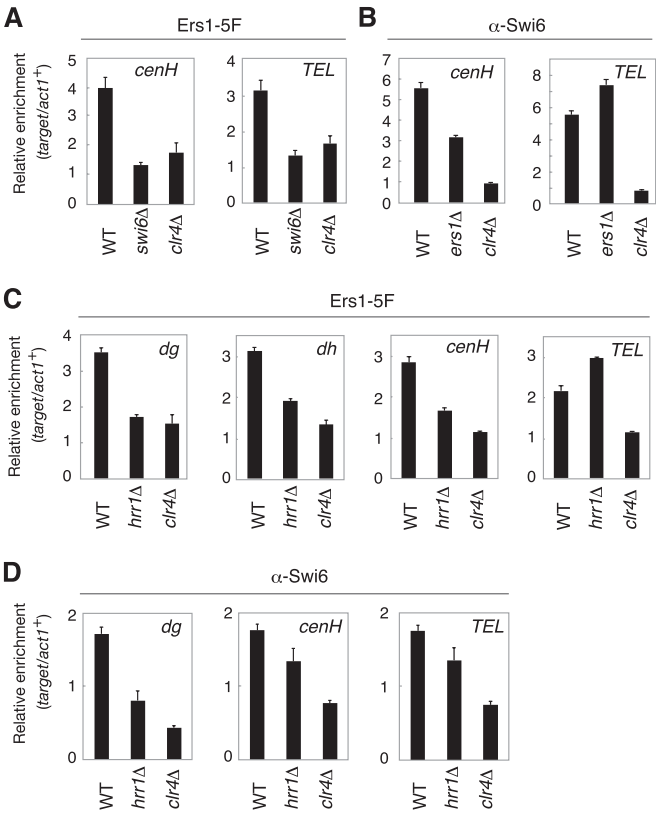


Fig. S5. Ers1 localization is dependent on Swi6. ChIP analysis of Ers1-Flag (*A* and *C*) and Swi6 (*B* and *D*) levels associated with centromeric *dg* and *dh*, the mating-type locus (*cenH*), and telomere (*TEL*) regions, relative to *act1*$^+$. Immunoprecipitated DNA was subjected to quantitative PCR analysis. Results are the mean ± SD of at least three independent experiments.

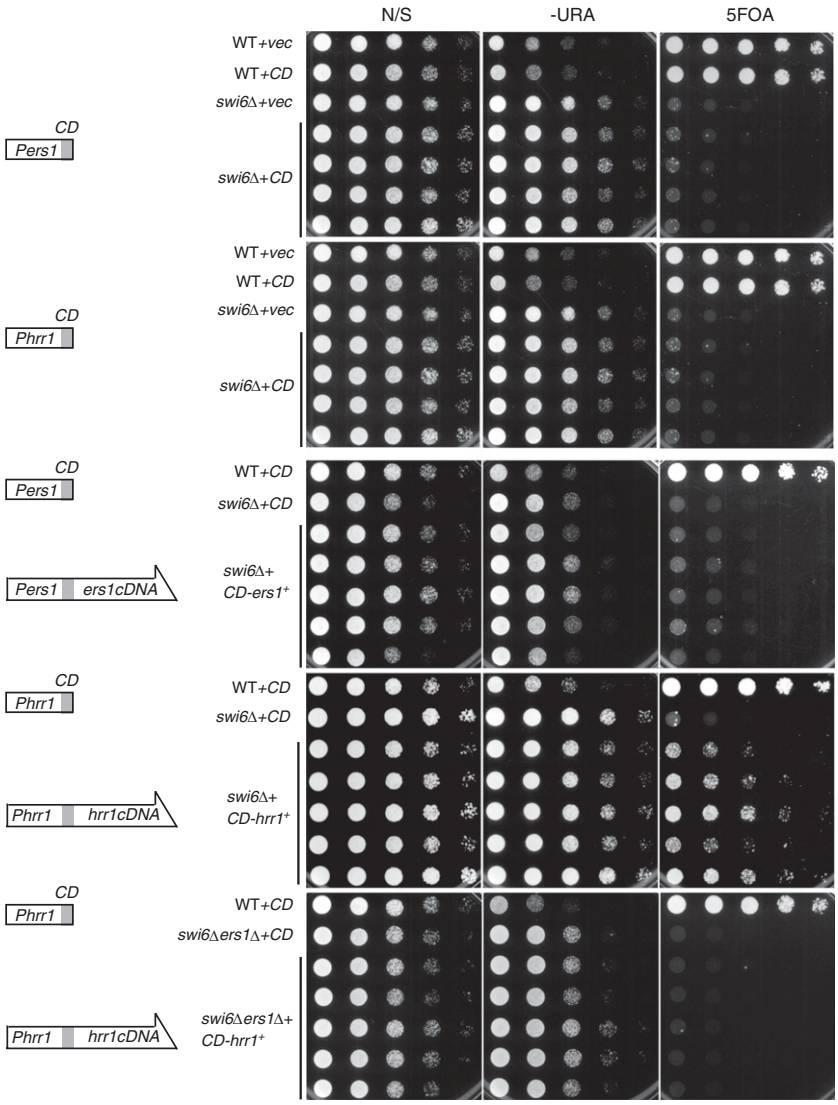


Fig. S6. Silencing assay of *swi6*Δ cells expressing CD-fused proteins. CD alone or CD-fused Ers1 or Hrr1 was expressed in *swi6*Δ cells under the control of each promoter. At least 10 transformants were isolated, and the silencing state of *otr1R::uar4*$^{+}$ was analyzed by a spot assay. Fivefold-diluted cultures of the indicated strains were plated onto minimal nonselective medium (N/S), EMM lacking uracil (−URA), and EMM containing 5FOA. Five representative clones for each transformant are shown with control strains.

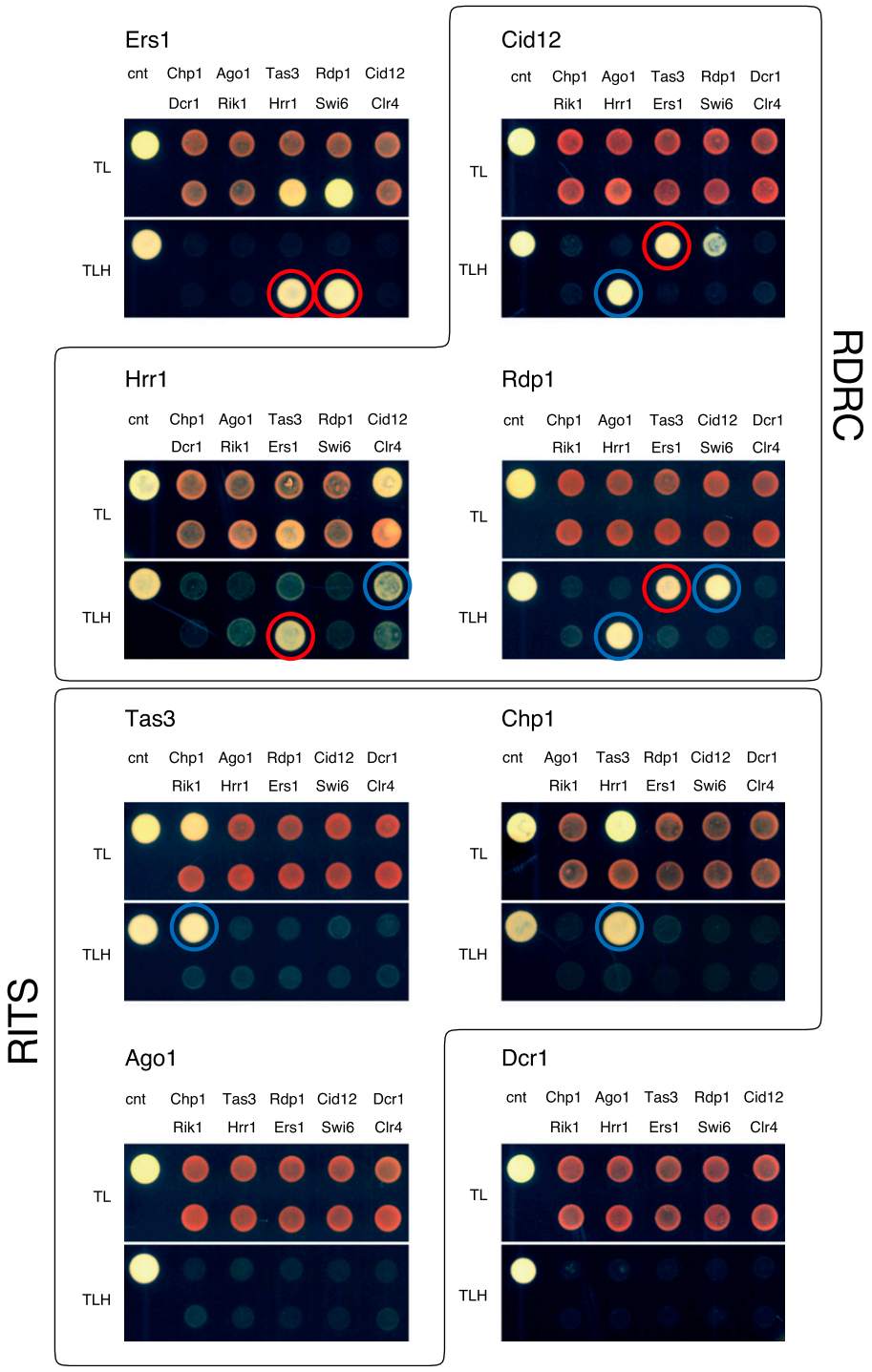


Fig. S7. Interaction map of factors involved in the RNAi pathway. YTH assay for Ers1, RDRC components (Rdp1, Hrr1, and Cid12), RITS complex components (Ago1, Tas3, and Chp1), and Dcr1. Blue and red circles indicate intra- and intercomplex interactions, respectively. TL, SD/−Trp/−Leu medium; TLH, SD/−Trp/−Leu/−His medium.

Table S1. Result of YTH assay using Ers1 as bait

Gene	Product	Residues/full length, aa	Frequency
swi6^{+}	HP1	987/987	1
		684–987/987 (containing CSD)	1
taf111^{+}	TFIID subunit	2103–2940/2940	4
		2139–2940/2940	1
rpl3202^{+}	60S ribosomal	16–384/384	1

CSD, chromoshadow domain.

Table S2. List of strains used in this study

Strain	Genotype
AHY420	*h90 ade6-M210 his2 leu1-32 ura4-D18 otr1R::ade6*$^{+}$ *otr2::LEU2 Kint2::ura4*$^{+}$
FY648	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$
SPIT27	*h*$^{+}$ *ade6-210 ura4DS/E leu1-32 otr1::ura4*$^{+}$ *dcr1Δ::kanR*
SPIT31	*h*$^{+}$ *ade6-210 ura4DS/E leu1-32 otr1::ura4*$^{+}$ *ago1Δ::kanR*
SPIT34	*h*$^{+}$ *ade6-210 ura4DS/E leu1-32 otr1::ura4*$^{+}$ *rdp1Δ::kanR*
SPIT38	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *swi6Δ::kanR*
AHY488	*h90 ade6-M216 leu1-32 ura4-D18 ers1*$^{+}$*::ers1-13Myc-kanR*
AHY490	*h90 ade6-M210 leu1-32 ura4-D18 hrr1*$^{+}$*::hrr1-5Flag-kanR*
AHY491	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *ers1Δ::nat*
C62	*h*$^{+}$ *ade6-M210 leu1-32 ura4-D18 otr1R::ura4*$^{+}$ *ers1(C62) his7-366*
AHY498	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *ers1*$^{+}$*::ers1-5Flag-kanR*
AHY504	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *ers1*$^{+}$*::ers1-13Myc-kanR hrr1*$^{+}$*::hrr1-5Flag-kanR*
AHY511	*h*$^{+}$ *ade6-M210 leu1-32 ura4-D18 otr1R::ura4*$^{+}$ *hrr1*$^{+}$*::hrr1-5Flag-kanR*
AHY513	*h90 ade6-M216 leu1-32 ura4-D18 otr1R::ura4*$^{+}$ *ers1(C62)::C62-13Myc-kanR*
AHY514	*h*$^{+}$ *ade6-M210 leu1-32 ura4-D18 otr1R::ura4*$^{+}$ *ers1(C62)::C62-13Myc-kanR hrr1*$^{+}$*::hrr1-5Flag-kanR*
AHY526	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *chp1*$^{+}$*::chp1-5Flag-kanR*
AHY527	*h*$^{+}$ *ade6-M216 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *hrr1*$^{+}$*::hrr1-5Flag-kanR*
AHY528	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *rdp1*$^{+}$*::rdp1-5Flag-kanR*
AHY530	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *chp1*$^{+}$*::chp1-5Flag-kanR ers1Δ::nat*
AHY531	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *rdp1*$^{+}$*::rdp1-5Flag-kanR ers1Δ::nat*
AHY533	*h*$^{+}$ *ade6-M216 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *hrr1*$^{+}$*::hrr1-5Flag-kanR ers1Δ::nat*
AHY536	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4+ chp1*$^{+}$*::chp1-5Flag-kanR clr4Δ::nat*
AHY537	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *rdp1*$^{+}$*::rdp1-5Flag-kanR clr4Δ::nat*
AHY539	*h*$^{+}$ *ade6-M216 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *hrr1*$^{+}$*::hrr1-5Flag-kanR clr4Δ::nat*
AHY546	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *chp1*$^{+}$*::chp1-5Flag-kanR swi6Δ::nat*
AHY547	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *rdp1*$^{+}$*::rdp1-5Flag-kanR swi6Δ::nat*
AHY549	*h*$^{+}$ *ade6-M216 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *hrr1*$^{+}$*::hrr1-5Flag-kanR swi6Δ::nat*
AHY552	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *ers1*$^{+}$*::ers1-13Myc-kanR swi6Δ::nat*
AHY554	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *ers1*$^{+}$*::ers1-5Flag-kanR swi6Δ::nat*
AHY555	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *ers1*$^{+}$*::ers1-5Flag-kanR clr4Δ::nat*
AHY558	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *tas3*$^{+}$*::tas3-5F10H-hygR hrr1*$^{+}$*::hrr1-13Myc-kanR*
AHY563	*h*$^{+}$ *ade6-M216 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *hrr1*$^{+}$*::hrr1-13Myc-kanR*
AHY565	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *tas3*$^{+}$*::tas3-5F10H-hygR hrr1*$^{+}$*::hrr1-13Myc-kanR clr4Δ::nat*
SPIT270	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *tas3*$^{+}$*::tas3-5F10H-hygR*
AHY570	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *tas3::tas3-5F10H-hygR hrr1::hrr1-13Myc-kanR ers1Δ::nat*
AHY571	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *tas3::tas3-5F10H-hygR hrr1::hrr1-13Myc-kanR dcr1Δ::nat*
AHY580	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *swi6Δ::kanMX6 ers1Δ::nat*
AHY587	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *hrr1Δ::hygR*
AHY613	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *ers1*$^{+}$*::ers1-13Myc-kanR swi6::pAL2HBP-3Flag*
AHY617	*h*$^{+}$ *ade6-M216 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *hrr1*$^{+}$*::hrr1-13Myc-kanR swi6::pAL2HBP-3Flag*
AHY618	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *hrr1*$^{+}$*::hrr1-13Myc-kanR*
AHY619	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *ers1*$^{+}$*::ers1-13Myc-kanR*
AHY622	*h90 ade6-M216 leu1-32 ura4-D18 otr1R::ura4*$^{+}$ *ers1(C62)::C62-13Myc-kanR swi6::pAL2HBP-3Flag*
AHY623	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *tas3*$^{+}$*::tas3-5F10H-hygR hrr1*$^{+}$*::hrr1-13Myc-kanR swi6Δ::nat*
AHY624	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E otr1R::ura4*$^{+}$ *ers1*$^{+}$*::ers1-13Myc-kanR hrr1*$^{+}$*::hrr1-5Flag-kanR swi6Δ::nat*
SPM1626	*h*$^{+}$ *ade6-M210 leu1-32 ura4-D18 otr1R::ade6*$^{+}$ *clr4Δ::kanR*
SPM1675	*h*$^{+}$ *ade6-M210 leu1-32 ura4-D18 chp2Δ::ura4*$^{+}$
SMP1004	*h90 ade6-M210 leu1-32 ura4-D18 chp1Δ::kanR*
SPIT241	*h*$^{+}$ *ade6-M210 leu1-32 ura4DS/E cnt1/TM-ura4*$^{+}$ *tas3Δ::kanR*

Table S3. List of primers used in this study

Primer	Sequence	Experiment
dhII-RT-Fw2	5′-AACAAGTCCTGATTCTTGGCAAA-3′	qRT-PCR
dhII-RT-Rv2	5′-GGTGCGATTGGACTTGAGTGA-3′	qRT-PCR
act1-RT-Fw	5′-CGTGCCCCTGAAGCTCTTT-3′	qRT-PCR
act1-RT-Rv	5′-CTCATGAATACCGGCGTTTTC-3′	qRT-PCR
dg233in-Fw	5′-GAAACGATGAACCCATCATGAA-3′	qChIP
dg233in–Rv	5′-CCATTGTTTCATTTCATAAAG-3′	qChIP
dgF-Fw	5′-CTGCGGTTCACCCTTAACAT	qChIP
dgF-Rv	5′-CAACTGCGGATGGAAAAAGT	qChIP
dh260in-Fw	5′-CGTTCGGTAGGTATGAGTGAAATCT	qChIP
dh260in-Rv	5′-CGCTCGCCTTGCCAATGGCCTAA	qChIP
cenH-Fw	5′-TCAATAAGTATGAGACAAAGGAGTCCAT	qChIP
cenH-Rv	5′-TCCGCACAAAGTCTAGTACACCA	qChIP
Tel-Fw	5′-2GTCGTGGTCATAAACGCACATAC	qChIP
Tel-Rv	5′-CCAACACTACCATGAACACGATTC	qChIP
SP6-dhII-Fw	5′-ATTTAGGTGACACTATAGCCTCAAGTGACTGC-ATTAAAGCAT-3′	siRNA
T7-dhII-Rv	5′-TAATACGACTCACTATAGGGCTACTCTGAAGA-CAACGATGTGT-3′	siRNA
U6-antisense	5′-TCATCCTTGTGCAGGGGCCATGCTAATCTTCTCTGTATCG-3′	siRNA
ura4DS/E#1	5′-GAGGGGATGAAAAATCCCAT-3′	ChIP
ura4DS/E#2	5′-TTCGACAACAGGATTACGACC-3′	ChIP

Fw; forward; q, quantitative; Rv, reverse.