Supplemental Data

Conserved Ribonuclease, Eri1, Negatively Regulates Heterochromatin Assembly in Fission Yeast

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Supplemental Experimental Procedures

Strains

The strains used in this study are listed in Table S1. Deletion and tagging to produce 5xFLAG fusion proteins of endogenous *eri1*$^+$, *dcr1*$^+$, and *tas3*$^+$ were performed with a PCR-based gene-targeting protocol [S1].

Cloning of *eri1*$^+$ cDNA

To obtain the *eri1*$^+$-encoding cDNA, we purified total RNAs from yeast cells, and we performed RT-PCR by using a SuperScript II reverse transcriptase (Invitrogen) and oligo-dT primers. The resultant cDNA library was subjected to PCR amplification with BamHINdeIeri1F (5′-tggatcccatATGGAGTCGCCAGTGCAGATTTTAG-3′) and eri1-BamHISmaIR (5′-cccggggatccTTAGCTTGCGAAATATGGCG GG-3′) by KODplus DNA polymerase (TOYOBO). Purified PCR fragments were incubated with *Taq* DNA polymerase and cloned into the TA-vector, pCRII (Invitrogen).

Plasmid Construction

To obtain pGEX-eri1, we cloned the *eri1* cDNA into GST expression vector pGEX-6P-3 (GE Healthcare). The pGEX-eri1 was changed to pGEX-eri1AA via a primer, eri1AAf (5′-GAGGGAAGCGAGgcTCGCG GAATAGcTGATGCGAGG-3′), and a site-directed mutagenesis method [S2]. To construct pGEX-eri1-SAP, we amplified the DNA fragment of pGEX-eri1 by PCR with eri1SAPr (5′-ACTAGTAGAA AACTTTTGATAACGTTCTTG-3′) and pGEX6P3f (5′-TAAGGATCCcc gaattcccgggtcgactcg-3′), and the amplified fragment was ligated. The pGEX-eri1-EXO was constructed by ligation of the PCR fragment that was amplified from pGEX-eri1 by eri1EXOf (5′-AATGA AAACAAAACTTGTTTACGATACTTG-3′) and pGEX6P3r (5′-CATATG GGATCCcaggggcccctggaacag-3′).

Purification of Recombinant Proteins

The expression vector carrying the cDNA of *eri1*$^+$ or the *eri1* mutant (pGEX, pGEX-eri1, pGEX-eri1AA, pGEX-eri1-SAP, or pGEX-eri1-EXO) was introduced into *E. coli* strain BL21 (DE3). Protein expression was induced by the addition of IPTG to a final concentration of 0.1 mM. The culture was incubated for 30 min more at 25ºC before harvesting, and the cells were then lysed by sonication. GST and GST fusion proteins were purified via glutathione-Sepharose 4B resins, according to the manufacturer's instructions (GE Healthcare). The eluted materials were dialyzed against PBS with 10% glycerol and concentrated via spin columns. The concentrated proteins were stored at −80ºC before use.

In Vitro Ribonuclease Assay

Ribonuclease assays were performed as previously described [S3]. Each reaction consisted of 10 μl reaction buffer (135 mM KCl, 50 mM Tris-Cl [pH 8.0], 2.5 mM $MgCl_2$, 2.5% glycerol, 1 mg/ml bovine serum albumin) with 50 fmol ^{32}P-labeled oligo and either 3 or 6 pmol GST fusion proteins. Reaction mixtures were incubated at 37ºC for 30 min and then boiled with 40 μl of 7 M urea loading dye. Five microliters of each sample was separated on 15% polyacrylamide gels containing 8 M urea; the gels were dried, and the bands were visualized with a phosphoimager screen or X-ray film. In assays with excess proteins, each reaction contained 3 (1-fold) to 240 (80-fold) pmol proteins. Radioactive double-stranded oligo probes were prepared from 2 μM 5′ ^{32}P-labeled sense and 5′ phosphorylated antisense oligos in annealing buffer (30 mM HEPES-KOH [pH 7.4], 0.1 M KoAc, 1 mM $Mg(oAc)_2$). Equal amounts of sense and antisense oligos were mixed, boiled (95ºC), and gently cooled to room temperature (final concentration, 1 μM). The sense RNA oligo (5′-UCUAGCUUCGCCAUCAAUAAGUA-3′) was used to make ssRNA, dsRNA, and RNA/DNA. The sense DNA oligo (5′-TCTAGCTTCGCCA TCAATAAGTA-3′) was used to make ssDNA. The anti-sense RNA oligo (5′-CUUAUUGAUGGCGAAGCUAGAUC-3′) was used to make dsRNA. The anti-sense DNA oligo (5′-CTTATTGATGGCGAAGCTA GATC-3′) was used to make RNA/DNA.

Electrophoretic Mobility-Shift Assay

Electrophoretic Mobility-Shift Assays (EMSAs) were performed in 10 μl RNA binding buffer (20 mM HEPES-KOH [pH 7.6], 100 mM KCl, 2 mM EDTA, and 0.01% NP40) with 10–50 pmol GST, GST-SAP, or GST-EXO proteins, as well as 10 pmol of the radioactive double-stranded riboprobes used in the ribonuclease assay. Reaction mixtures were incubated at 37ºC for 30 min prior to electrophoresis. After the addition of 2 μl glycerol, the samples were separated on a 5% polyacrylamide gel in 0.25× TBE. Gels were dried and visualized with a phosphoimager screen or X-ray film. In the competition assay, reaction mixtures contained 50 pmol GST-SAP, 10 pmol radioactive double-stranded riboprobes, and 10 pmol, 100 pmol, or 1 nmol of unlabeled single-stranded or double-stranded oligo probes.

Silencing Assay

Silencing assays were conducted with unsaturated cultures grown in YEA medium. Ten-fold serial dilutions were made ($1 \times 10^5 \sim 1 \times 10^3$ cells) and spotted on plates of AA, AA lacking uracil, or AA containing 5-FOA. The plates were then incubated at 30ºC for 2.5–4 days. For cells harboring pREP plasmids, AA medium lacking leucine (with 2 nM thiamine in Figure 6B in the main text) were used for the culture and spotting.

Chromatin Immunoprecipitation

Chromatin Immunopredcipitation (ChIP) was performed as described previously [S1] with primer sets for *ura4*$^+$, *act1*$^+$, and centromeric *dg223* alleles.

RNA Isolation and Northern Hybridization

Total RNA extraction and detection of long centromeric transcripts were carried out as described previously [S1]. To detect *cen* siRNAs, We followed a previous method for northern analyses but included some modifications [S4, S5]. In this study, 25 μg samples of total RNA were analyzed for each assay, and Rapid-Hyb Buffer (GE Healthcare) was used for northern analysis. An oligo complementary to U6 (5′-TCATCCTTGTGCAGGGGCCATGCTAATCTTCTCTGTAT CG-3′) was used as a loading control. Sense-oligos of *ura4SE* (5′-CAAAGAAGTTGGTTTACCTTTGGGACGTGG-3′, 5′-TCTCTTGCTTT TGGCTGAAATGTCTTCCA-3′, 5′-AAGGCTCTTTGGCTACTGGTTCC TACACAG-3′, 5′-AACTATGTCCCCTGGTATCGGCTTGGATGT-3′, 5′-TAAAGGAGACGGGCTGGGACAGCAATATCG-3′, 5′-TACTCCTGAA GAAGTGATTGTAAACTGCGG-3′, 5′-GAAAACCTTAGAATGGTTTGA GAAGCATAC-3′, 5′-CGATTTTTGCTTTGGCTTTATAGCTGGTCG-3′, and 5′-TCGATTTCCTAACCTTCAAAGCGACTACAT-3′) were used for detection of small RNAs from *otr1R::ura4*$^+$.

RT-PCR Analysis

Total RNA prepared from each strain was pre-incubated with RNase-free DNase I (0.4 U/μg RNA, TaKaRa) to digest contaminated genomic DNAs. cDNA samples were synthesized with Superscript III reverse transcriptase (Invitrogen) and an oligo dT primer and subjected to quantitative PCR analyses with qPCR MasterMix Plus for SYBR Green (Eurogentec) and a 7300 Real-Time PCR System (ABI). The primers used in these analyses were dhII-Fw (5′-CCCATGCTGTTGGATCAATG-3′) and dhII-Rv (5′-GCTCAAAAGTGT GGCGCTATATC-3′), for the endogenous *dh* transcript, and ura4-RT-Fw (5′-GGCCTCAAAGAAGTTGGTTTACC-3′) and ura4-RT-Rv (5′-GAAGACATTTCAGCCAAAAGCA-3′) for the *otr1R::ura4*$^+$ locus, respectively.

RITS Immunoprecipitation and RNA Detection

To purify the RITS complex, we collected 2×10^{10} cells expressing FLAG-tagged Tas3, and extracts were prepared as described [S6]. Tas3-F was immunoprecipitated with 250 μl anti-FLAG (M2) agarose beads (Sigma), and 1/50 of each immunoprecipitate sample was detected by HRP-conjugated anti-FLAG antibody (M2-HRP, Sigma) and anti-Chp1 rabbit polyclonal antibody in western blot analysis. To monitor RITS-associated RNAs, we purified the RNAs from the Tas3-F immunoprecipitates and labeled them at their 3′ end with [α-^{32}P] cordycepin (NEN Life Sciences) and polyA polymerase (USB), as described previously [S7].

Subcellular Fractionation

Subcellular fractionation experiments followed a previous method with some modifications [S8]. In this assay, all buffers except for the STOP buffer contained 1% (v/v) of Protease inhibitor cocktail for fungi (Sigma) and 1 mM PMSF. Exponentially growing 5×10^8 cells (2.5×10^9 cells = five tubes for RNA fractionation) in YEA were washed in ice-cold STOP buffer (50 mM NaF, 10 mM Tris-Cl 7.5, and 0.02% NaAzide) and resuspended in 600 μl S buffer (1.4 M Sorbitol, 40 mM HEPES-KOH [pH 7.0], 0.5 mM MgCl2). Spheroplasts were made by incubating the cell suspension at 30ºC for 40 min in the presence of 20 mg/ml zymolyase-100T (SEIKAGAKU Co.). Cells were gently lysed in 300 μl ice-cold F buffer (40 mM HEPES-KOH [pH 7.0], 0.5 mM MgCl2, 1 mM EGTA, and 18% w/v Ficoll 400) with a micro teflon-homogenizer (ISO Inc.) on ice. To remove unlysed cells, we gently spun the suspension (2000 rpm at 4ºC for 5 min, repeated multiple times). This suspension was used as whole-cell extract (WCE) sample. WCE (150 μl) was layered onto 100 μl of GF buffer (40 mM HEPES-KOH [pH 7.0], 0.5 mM $MgCl_2$, 1 mM EGTA, 7% w/v Ficoll 400, and 20% v/v glycerol) and fractionated by gentle centrifugation (6000 × g at 4ºC for 5 min). The resulting supernatant (100 μl of the upper layer) was recovered as the cytoplasmic (Cyt) fractions, and nuclei in the pellet were resuspended in 100 μl of F buffer as the nuclear (Nuc) fraction. All protein samples were precipitated by the addition of a 1/5 volume of 50% TCA, and the precipitates were boiled in 1× SDS-PAGE sample buffer prior to western blotting. Western analysis was carried out with anti-FLAG (M2-HRP: Sigma) for Eri1-F, anti-Chp1, anti-RNA polymerase II (H5; COVANCE) and TAT1 antibodies (gifted form Prof. K. Gull) for α-tublin.

Immunostaining

Immunostaining was carried out according to Shimanuki et al.'s method with slight modifications [S9]. Exponentially growing cells were collected and resuspended in PEM (100 mM PIPES, 1 mM EGTA, 1 mM MgSO4 at pH 6.9) containing 3% paraformaldehyde and 0.2% glutaraldehyde and then incubated with shaking at 26ºC for 1 hr. Cells were collected and washed with PEM, incubated in PEM containing 0.1 M glycine, and then washed with PEMS (PEM with 1.2 M sorbitol). Cells were suspended in PEMS containing 0.5 mg/ml Zymolyase 100T (Seikagakukogyo) and incubated at 37ºC for 90 min. Cells were collected and washed with PEMS. Cells were suspended in PEMS containing 0.1% Triton X-100 and incubated for 2 min, followed by washing with PEMS and PEM. Cells were resuspended in PEMBAL (PEM with 1% BSA, 0.1% sodium azide, and 1% lysine HCl) containing 1:1000 of anti-FLAG M2 antibody (Sigma) and incubated for 16 hr. Cells were washed with PEMBAL, resuspended in PEMBAL containing Alexa488-conjugated anti-mouse IgG antibody (Molecular Probes), and incubated for 16 hr. Cells were washed with PEMBAL and resuspended in PBS containing 0.2 μg/ml DAPI.

Supplemental References

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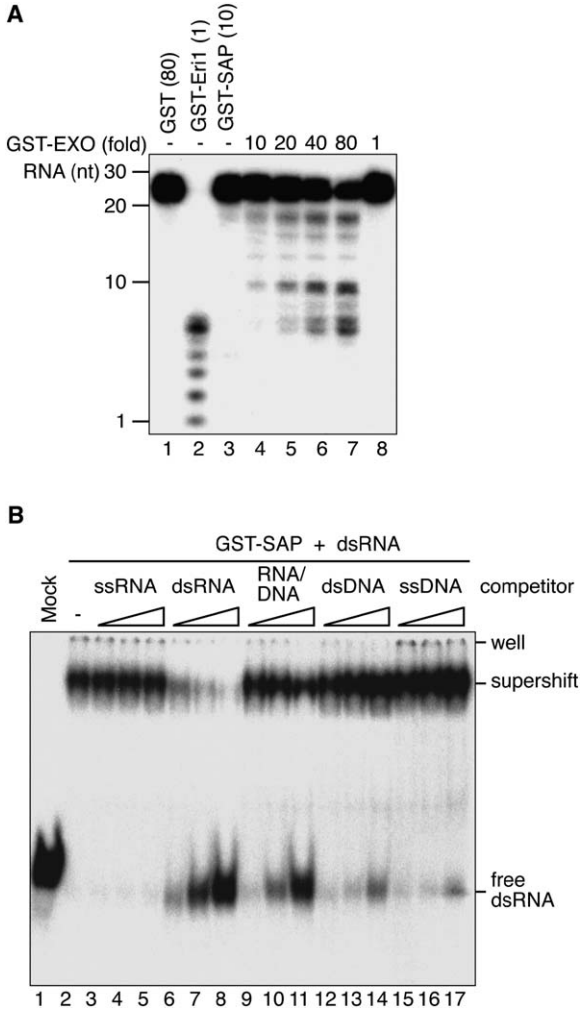

Figure S1. Functions of Conserved SAP and EXO Domains in the *Sp* Eri1 Protein

(A) An excess amount of GST-EXO (1-, 10-, 20-, 40-, and 80-fold) was incubated with dsRNA in the presence or absence of GST-SAP (10-fold excess) as shown in Figure 1C in the main text.

(B) dsRNA binding activity of the SAP domain. An electrophoretic mobility-shift assay (EMSA) was performed as in Figure 1E, with unlabeled competitors (1-, 10-, and 100-fold amount of labeled probe). The reaction lacking recombinant proteins was defined as "Mock."

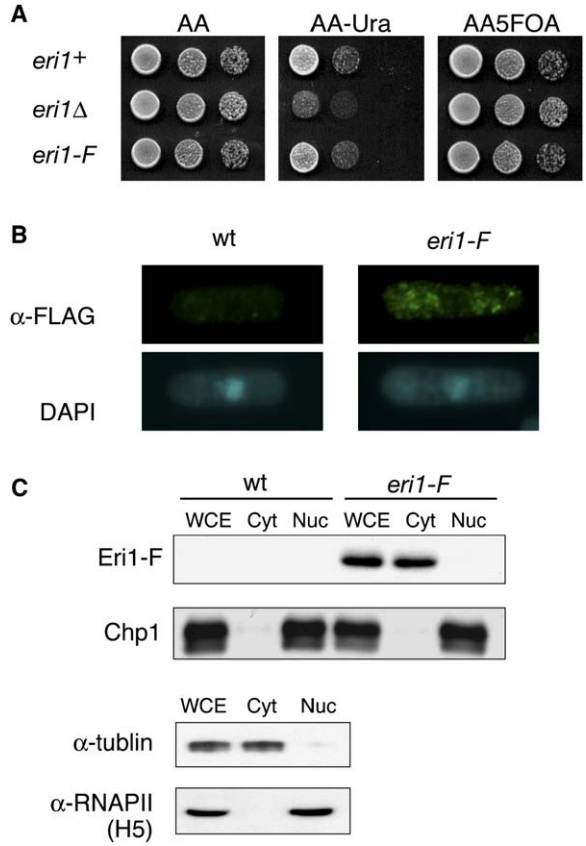

Figure S2. Eri1 Localizes Predominantly in the Cytoplasm

(A) Silencing assay of the *eri1-F*, *eri1Δ*, and wild-type cells. A 10-fold serial dilution of cultures of the indicated strains carrying *otr1R::ura4*$^{+}$ were spotted onto AA, AA-Ura, or AA5FOA medium. This assay indicates that the Eri1-F protein was fully functional.

(B) Immunostaining assays were carried out with wild-type and the *eri1-F* strains used in (A). FLAG-tagged proteins in fixed cells were visualized by an indirect immunofluorescence method with anti-FLAG primary antibody and Alexa488-conjugated anti-mouse IgG antibody (Green: upper panels). Nuclei in each cell were stained with DAPI (Blue: lower panels).

(C) Cells expressing Eri1-F from the native promoter were lysed and separated into cytoplasmic (Cyt) and nuclear (Nuc) fractions (upper panels). The Eri1-F and a nuclear protein, Chp1, were detected by western analysis with anti-FLAG and anti-Chp1 antibodies, respectively. Subcellular fractionation of wild-type cells successfully separates tublin and RNA polymerase II (lower panels). The cytoplasmic abundant protein, tublin, and the nuclear abundant protein, RNA polymerase II (RNAPII), were detected by western analysis with anti-TAT1 and anti-RNAPII (H5) antibodies, respectively.

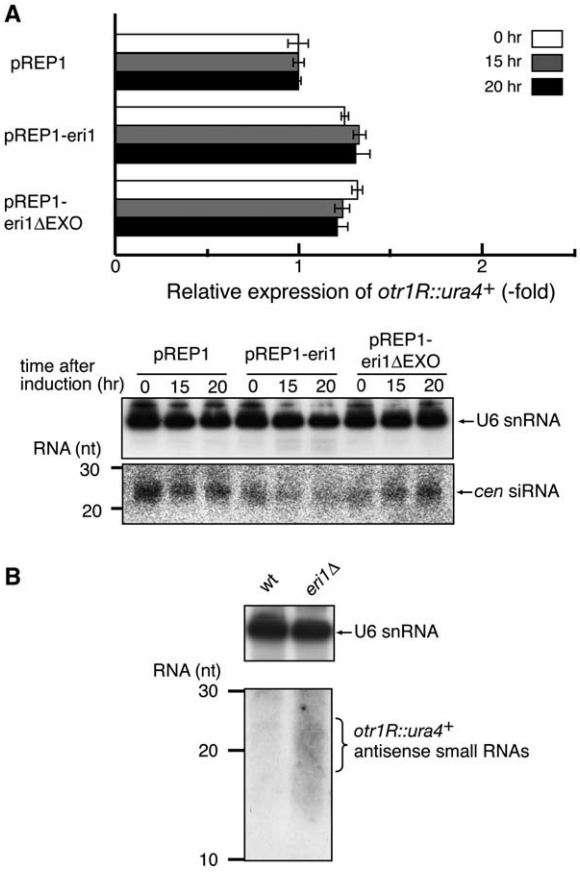

Figure S3. Effects on *otr1R::ura4*$^+$ by Overexpression or Deletion of Eri1

(A) Overexpression of the *eri1*$^+$ gene and its effect on the *otr1R::ura4*$^+$ silencing, centromeric (*cen*) siRNAs, and U6 snRNA. Cells harboring a control vector pREP1, pREP1-*eri1*, or pREP1-*eri1ΔEXO* (exonuclease domain-deleted *eri1*$^+$) plasmid were cultured in AA-Leu medium with thiamine and then were shifted into AA-Leu medium lacking thiamine (0 hr: white bar). After induction of the Eri1 or Eri1ΔEXO protein under the *nmt1* promoter, RNA samples were prepared from exponentially growing cells after 15 (gray bar) and 20 hr (black bar). Real-time RT-PCR analysis was performed, and the expression levels of *otr1R::ura4*$^+$ transcripts of each strain were compared. The levels of *otr1R::ura4*$^+$ expression were normalized by *act1*$^+$ expression, and relative expression levels to control strain (pREP1) were shown in the graph (top). Centromeric siRNAs and U6 snRNA in the prepared total RNA samples were detected by northern blot analyses with probes for siRNAs and U6 snRNA, respectively (bottom).

(B) Small RNAs derived from the *otr1R::ura4*$^+$ locus accumulated in the *eri1Δ* mutant cells. RNA samples were prepared from wild-type and *eri1Δ* cells harboring *otr1R::ura4*$^+$. U6 snRNA and small RNAs derived from the anti-sense strand of *otr1R::ura4*$^+$ locus were detected by northern blot analysis with probes for U6 snRNA and *ura4*Stu*I*-Eco*RV* (*ura4SE*), respectively.

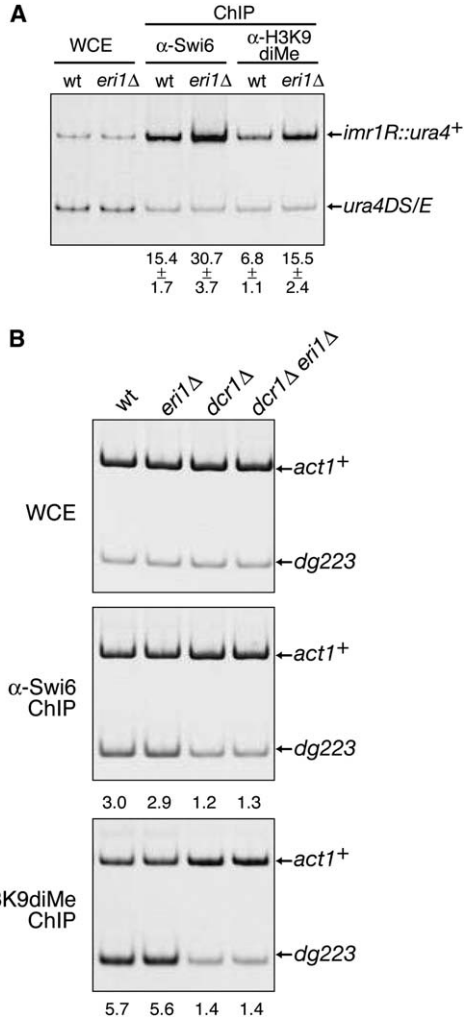

Figure S4. Effect of *eri1Δ* Mutation on Heterochromatin Formation at *imr1R::ura4*$^+$ and Centromeric *dg* Repeats

Chromatin-immunoprecipitation assays were performed with DNA isolated from anti-Swi6 or anti-H3K9diMe immunoprecipitates as in Figure 5A.

(A) Purified DNAs from the indicated strains were used as a template for the PCR amplification of the *ura4*$^+$ gene at the centromeric *imr* repeat locus on chromosome I.

(B) PCR amplification at the centromeric *dg* repeat regions (*dg223*) and the euchromatic control *act1* region. Fragment position of *dg223* is indicated in Figure 3A. The signal enrichments relative to the control signal (*ura4DS/E* and *act1*) in the ChIP results were normalized to the WCE signals and are shown beneath each lane.

Table S1. Yeast Strains Used in This Study

Strain	Genotype	Reference
SPYB106	*h^{90} ade6-M216 leu1-32 his2 ura4DS/E Kint2::ura4$^+$*	[S1]
SPIT22	*h^{90} ade6-M216 leu1-32 his2 ura4DS/E Kint2::ura4$^+$ eri1Δ::hphMX4*	This study
FY336	*h$^-$ ade6-M210 leu1-32 ura4DS/E cnt1/TM (NcoI)-ura4$^+$*	[S10]
SPIT116	*h$^-$ ade6-M210 leu1-32 ura4DS/E cnt1/TM (NcoI)-ura4$^+$ eri1Δ::hphMX4*	This study
FY498	*h$^+$ ade6-M210 leu1-32 ura4DS/E imr1R (NcoI)::ura4$^+$*	[S10]
SPIT120	*h$^+$ ade6-M210 leu1-32 ura4DS/E imr1R (NcoI)::ura4$^+$ eri1Δ::hphMX4*	This study
FY648	*h$^+$ ade6-M210 leu1-32 ura4DS/E otr1R (SphI)::ura4$^+$*	[S10]
SPIT24	*h$^+$ ade6-M210 leu1-32 ura4DS/E otr1R (SphI)::ura4$^+$ eri1Δ::hphMX4*	This study
SPIT26	*h$^+$ ade6-M210 leu1-32 ura4DS/E otr1R (SphI)::ura4$^+$ dcr1Δ::kanMX6*	This study
SPIT28	*h$^+$ ade6-M210 leu1-32 ura4DS/E otr1R (SphI)::ura4$^+$ eri1Δ::hphMX4 dcr1Δ::kanMX6*	This study
SPIT30	*h$^+$ ade6-M210 leu1-32 ura4DS/E otr1R (SphI)::ura4$^+$ ago1Δ::kanMX6*	This study
SPIT32	*h$^+$ ade6-M210 leu1-32 ura4DS/E otr1R (SphI)::ura4$^+$ eri1Δ::hphMX4 ago1Δ::kanMX6*	This study
SPIT34	*h$^+$ ade6-M210 leu1-32 ura4DS/E otr1R (SphI)::ura4$^+$ rdp1Δ::kanMX6*	This study
SPIT36	*h$^+$ ade6-M210 leu1-32 ura4DS/E otr1R (SphI)::ura4$^+$ eri1Δ::hphMX4 rdp1Δ::kanMX6*	This study
SPIT39	*h$^+$ ade6-M210 leu1-32 ura4DS/E otr1R (SphI)::ura4$^+$ swi6Δ::kanMX6*	This study
SPIT40	*h$^+$ ade6-M210 leu1-32 ura4DS/E otr1R (SphI)::ura4$^+$ eri1Δ::hphMX4 swi6Δ::kanMX6*	This study
SPIT58	*h$^+$ ade6-M210 leu1-32 ura4DS/E otr1R (SphI)::ura4$^+$ tas3::tas3-F/kanMX6*	This study
SPIT160	*h$^+$ ade6-M210 leu1-32 ura4DS/E otr1R (SphI)::ura4$^+$ tas3::tas3-F/kanMX6 eri1Δ::hphMX4*	This study
SPIT162	*h$^+$ ade6-M210 leu1-32 ura4DS/E otr1R (SphI)::ura4$^+$ tas3::tas3-F/kanMX6 dcr1Δ:: hphMX4*	This study
SPIT20	*h$^+$ ade6-M210 leu1-32 ura4DS/E otr1R (SphI)::ura4$^+$ eri1::eri1-F/kanMX6*	This study
SPIT173	*h$^+$ ade6-M210 leu1-32 ura4DS/E otr1R (SphI)::ura4$^+$ eri1::eri1AA-F/kanMX6*	This study
SPIT174	*h$^+$ ade6-M210 leu1-32 ura4DS/E otr1R (SphI)::ura4$^+$ dcr1::dcr1-F/kanMX6*	This study