Splicing Factors Facilitate RNAi-Directed Silencing in Fission Yeast Elizabeth H. Bayne, Manuela Portoso, Alexander Kagansky, Isabelle C. Kos-Braun, Takeshi Urano, Karl Ekwall, Flavia Alves, Juri Rappsilber, Robin C. Allshire

SUPPORTING ONLINE MATERIAL
This supplement contains: Materials and Methods Figs. S1 to S5 Tables S1 and S2 References

Supporting online material - Materials and Methods:

Yeast strains and plasmids. S. pombe strains are listed in Table S1. Standard procedures were used for growth and genetic manipulations (*1*). All strains were grown at 25°C unless otherwise stated. Plasmid pH-cc2, and analysis of H3K9me2 establishment on it, have been described previously (*2*).

Establishment assays. The plasmid pH-CC2 contains a 2 kb region from the outer repeat *dg* element from centromere 1 (*2*). pH-CC2 DNA prepared from *E.coli* was transformed into wild-type, *cwf10-1*, *prp10-1* and *dcr1Δ S. pombe* cells. The establishment assay tests if heterochromatin, specifically H3K9me2, can be established on naked DNA templates after introduction into wild-type or mutant strains (*2*). ura^+ transformants were cultured under selection for the plasmid and processed for ChIP with anti-H3K9me2 antibody. In wild-type cells H3K9me2 is enriched on the plasmid *dg* element relative to the euchromatic *fbp1*$^+$ *gene*, but this is not detected in cells lacking Dcr1 (Fig. 3C). In *cwf10-1* and *prp10-1* cells H3 K9 methylation can be established on this dg element but at reduced amounts, consistent with the greatly reduced levels of centromeric siRNA detected in *cwf10-1* and *prp10-1* (Fig. 1D and 1E).

ChIP. ChIP was performed as described (*3*), with the following modifications. Cells were grown at 25°C. For H3K9me2 ChIPs, cells were fixed in 1% PFA for 15 min at room temperature. For Swi6 ChIPs, cells were incubated for 2hr at 18°C prior to fixing in 3% PFA for 30 min at room temperature. For ChIP of Cwf10, Prp8 and Cid12, cells were incubated for 2hr at 18°C prior to double fixation with 2.5mM DMA for 45 min followed by 3% PFA for 30 min at room temperature. Cells were lysed using a bead beater (Biospec products) and the chromatin was sheared using a Bioruptor (Diagenode) sonicator (15 min of 30s ON and 30s OFF on 'high' [200 W] power). 1ul of monoclonal H3K9me2 antibody (m5.1.1)(*4*), 10ul of polyclonal Swi6 antibody (*5*) or 1 ul of 12CA5 antibody (Kumiko Samejima) were used per ChIP. Multiplex PCR was performed using primers listed in table S2; the primers used for analysis of pH-cc2 were DF151 and DF169. PCR products were separated on 1.5%

agarose gels and post-stained with ethidium bromide. Quantitation of bands was performed using the Kodak EDAS 290 system and 1D Image Analysis Software (Eastman Kodak). Relative enrichments were calculated as the ratio of product of interest to control product in IP over input.

qPCR. Real-time PCR was performed in the presence of SYBR Green on a Bio-Rad iCycler. Primers used for real-time PCR are listed in table S2; the primers used for analysis of *fbp1*$^{+}$ were the same as those for ChIP/RT-PCR. Data were analyzed with iCycler iQ Optical System Software. Histograms represent the results of at least three independent experiments, and error bars represent one standard deviation.

RNA analysis. Northern analysis of centromeric siRNAs and transcripts was performed as described previously (*6*). Oligos used to make the probes are listed in table S2. For cen-dh siRNA analysis the probe was a PCR product produced using primers cen-dh-FOR and dhH-siRNA. For cen-dh/dg siRNA analysis the probe was a mixture of three labeled oligos complementary to dh and dg repeats (IK8, IK9 and IK10). For the loading control a labeled oligo complementary to snRNA58 was used. Quantification of siRNAs in Fig. 1E was performed using ImageQuant software (Amersham Biosciences). The dilution series of wild-type RNA was used to generate a linear calibration curve for the ratio of siRNAs:snoRNA detected in wild-type. This was then used to calculate the amount of siRNAs detected in the mutants as a percentage of the amount that would be expected in wild-type for the observed amount of snoRNA. For transcript analysis the probe was a PCR product produced using primers lng-dg-FOR and lng-dg-REV. For RT-PCR, RNA was prepared using the RNeasy kit (Qiagen) according to the manufacturers instructions. Contaminating DNA was removed by treating with Turbo DNase (Ambion) and RT-PCR was performed using Superscript III Reverse Transcriptase (Invitrogen). Primers used for RT-PCR are listed in table S2.

Immunoaffinity purification. Immunoaffinity purifications were performed essentially as described (*7*) with the following modifications: *S. pombe* cultures were grown to a cell density of 10^{8}cells/ml in 4x concentrated YES media. For each sample, 5g of cells, milled in solid phase, were used. Immunoprecipitations were performed using Dynabeads coupled to anti-Flag antibody (Sigma, F3165) for 90 minutes. After washes Dynabeads with immunoprecipitated material were subjected to on-bead Tryptic digestion. After digestion, samples were acidified by adding TFA to a final concentration of 0.1% and spun onto StageTips as described elsewhere (*8,*

9). Peptides were eluted in 20 µL of 80% acetonitrile and 0.5% acetic acid and were concentrated to 2 µL (Concentrator 5301, Eppendorf AG). They were then diluted to 5 µL in 0.1% TFA and injected for LC-MS/MS analysis. For co-immunoprecipitation, flag immunoprecipitations were performed as above, but following washes the dynabeads were resuspended in SDS sample buffer and analysed by standard SDS-PAGE using monoclonal anti-flag M2-HRP (Sigma) and monoclonal anti-HA 12CA5 antibody. Co-immunoprecipitation could not be performed with Prp10 because the tagged protein was non-functional.

Mass spectrometry analysis. An LTQ-Orbitrap mass spectrometer (ThermoElectron) was coupled online to an Agilent 1100 binary nanopump and an HTC PAL autosampler (CTC). To prepare an analytical column with a self-assembled particle frit (*10*), C18 material (ReproSil-Pur C18-AQ 3 mm; Dr. Maisch, GmbH) was packed into a spray emitter (75-µm ID, 8-µm opening, 70-mm length; New Objectives) using an air-pressure pump (Proxeon). Mobile phase A consisted of water, 5% acetonitrile, and 0.5% acetic acid; mobile phase B, consisted of acetonitrile and 0.5% acetic acid. The gradient went from 0% to 20% buffer B in 75 min and then to 80% B in 13 min at 300 nL/min flow. The six most intense peaks of the MS scan were selected in the ion trap for MS^2, (normal scan, wideband activation, filling $5x10^5$ ions for MS scan, 10^4 ions for MS^2, maximum fill time 100 msec, dynamic exclusion for 180 sec). Raw files were processed using DTAsupercharge 0.62 (a kind gift from Matthias Mann, Max Planck Institute of Biochemistry, Martinsried, Germany). The generated peak lists were searched against the SGD database (version 11.05.2007) using Mascot 2.0 with the parameters: monoisotopic masses, 8 ppm peptide tolerance and 0.6 Da MS/MS tolerance, ESI TRAP parameters, fully tryptic specificity, with two missed cleavage sites allowed. The results were passed through MSQuant (http://msquant.sourceforge. net/), and a cutoff 5-ppm peptide tolerance was applied to the recalibrated list. Peptides with scores of 25 or higher were reported and in individual cases manually validated. The list presented in Fig. 4C contains all proteins represented by two or more peptides in three independent purifications of Cid12-FLAG, less those represented by two or more peptides in a control purification from untagged cells.

Supporting online material – Supplementary figures:

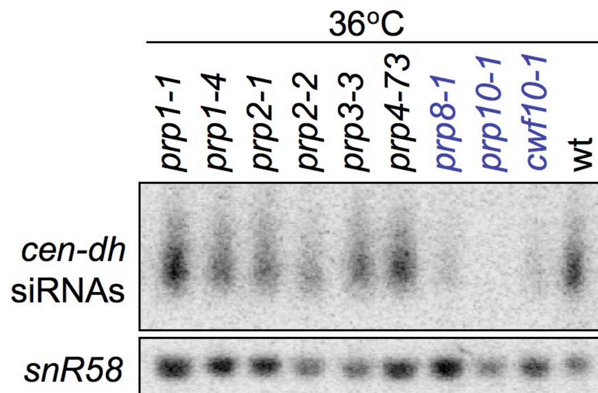

Figure. S1 Northern analysis of siRNAs corresponding to *cen-dh* in cells grown at permissive temperature (25°C) and then shifted to restrictive temperature (36°C) for 6 hours before harvesting. Mutants that disrupt silencing at 25°C are highlighted in blue; those in black do not. snoRNA58 was used as a loading control. The temperature shift is sufficient to inhibit splicing in all the mutants (Fig. 2A), but does not suppress siRNA accumulation in mutant alleles of *prp1*, *prp2*, *prp3* or *prp4*. This indicates that the reduction in siRNA accumulation seen in *prp8-1*, *prp10-1* and *cwf10-1* at both 25°C and 36°C is not explained by defective splicing.

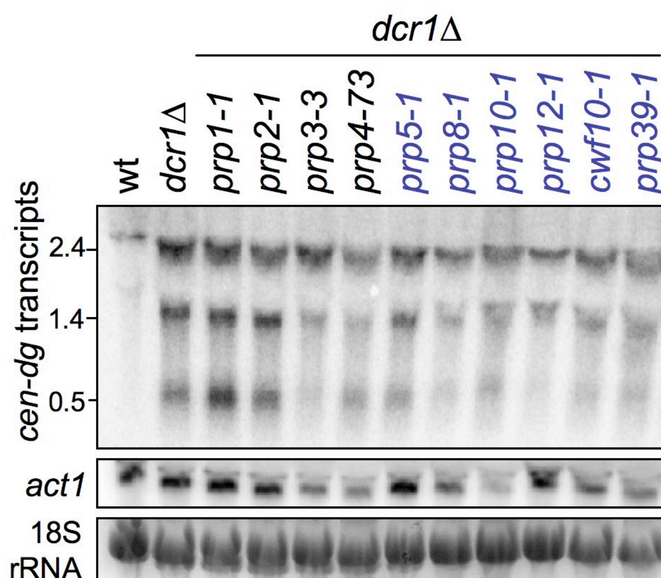

Figure. S2 Northern analysis detecting three *cen-dg* transcripts in splicing mutants in a *dcr1*Δ background at 25°C. Mutants that disrupt silencing at 25°C are highlighted in blue; those in black do not. *act1*$^{+}$ transcripts and EtBr-stained 18S rRNA are loading controls. No change in transcript abundance or length is seen in the splicing mutants as compared to *dcr1*Δ alone, indicating that splicing mutants do not affect transcription of centromere repeats.

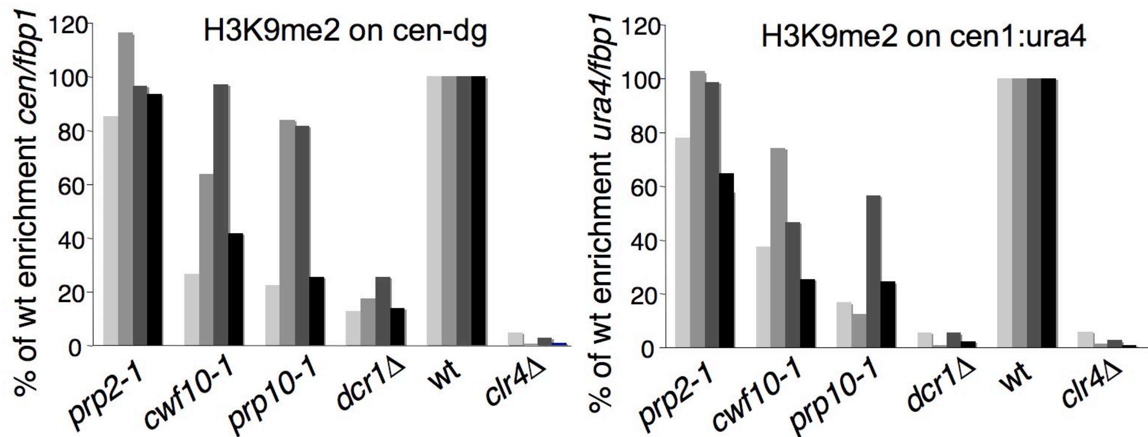

Figure. S3 ChIP analysis of H3K9me2 enrichment at *cen-dg* or *cen1:ura4*$^{+}$ relative to a euchromatic control locus (*fbp1*$^{+}$). The histograms represent an expanded version of the qPCR analysis of four ChIP experiments shown in the histograms in Fig. 3, with each of the four experiments now represented by a separate shaded bar. Relative enrichments were calculated as the ratio of *cen-dg* or *cen1:ura4*$^{+}$ to *fbp1*$^{+}$ DNA, in IP relative to input (in), and are shown as a percentage of wild-type enrichment.

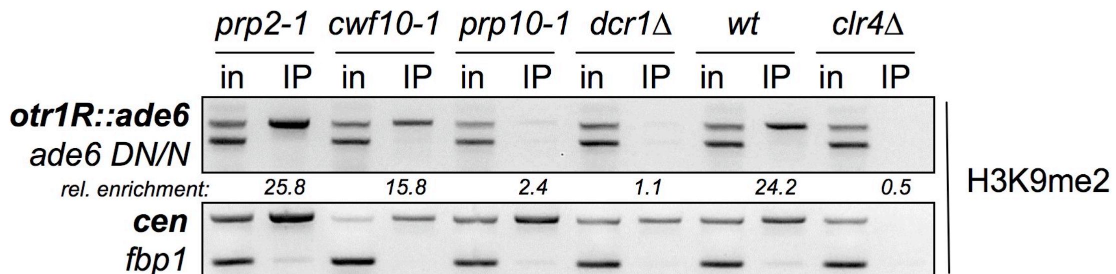

Figure. S4 ChIP analysis of H3K9me2 enrichment at *cen1:ade6*$^{+}$ relative to the euchromatic control locus *ade6-DN/N* (truncated ade6^{+}), and at *cen-dh* relative to *fbp1*$^{+}$. Representative gels are shown. Relative enrichments at *cen1:ade6*$^{+}$ were calculated as the ratio of *cen1:ade6*$^{+}$ to *ade6-DN/N* DNA in IP relative to input (in).

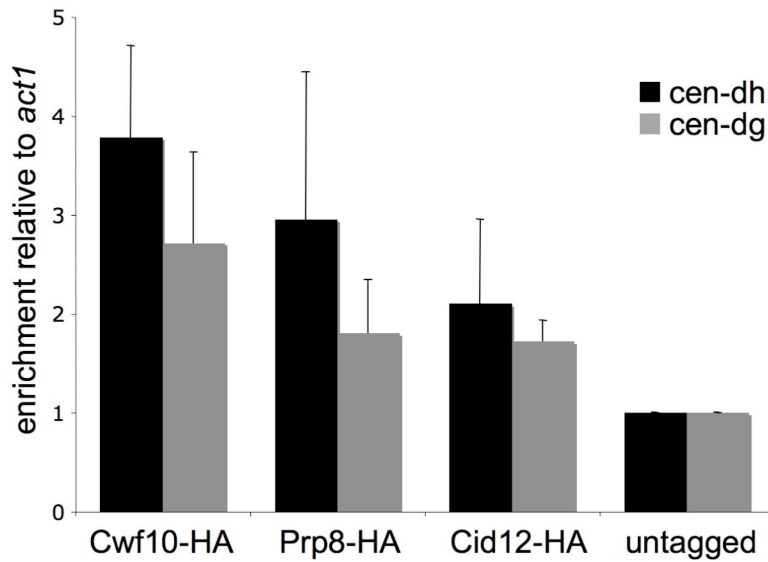

Figure. S5 ChIP analysis of Cwf10, Prp8 and Cid12 enrichment at *cen-dh* or *cen-dg* relative to a euchromatic, unspliced control locus *act1*$^{+}$. Relative enrichments were calculated as the ratio of *cen-dh* or *cen-dg* to *act1*$^{+}$ DNA, in IP relative to input (in), and are shown normalised to enrichment in an untagged control strain.

Table S1. List of yeast strains.

Strain	Genotype	Fig.
FY7095	*h+ otr1Rsph1::ade6 lys1::Nat ade6Δ::kan ura4-D18 leu1-32*	1,2,S1
FY7466	*h+ prp1-1 otr1Rsph1::ade6 lys1::Nat ade6Δ::kan*	1,2,S1[α]
FY7467	*h- prp1-4 otr1Rsph1::ade6 lys1::Nat ade6Δ::kan*	S1[α]
FY7468	*h- prp2-1 otr1Rsph1::ade6 lys1::Nat ade6Δ::kan*	1,2,S1[α]
FY7469	*h+ prp2-2 otr1Rsph1::ade6 lys1::Nat ade6Δ::kan*	S1[α]
FY7470	*h- prp3-3 otr1Rsph1::ade6 lys1::Nat ade6Δ::kan*	1,2,S1[α]
FY7471	*h- prp4-73 otr1Rsph1::ade6 lys1::Nat ade6Δ::kan*	1,2,S1[β]
FY7337	*h- prp5-1 otr1Rsph1::ade6 lys1::Nat ade6Δ::kan*	1,2[β]
FY7338	*h- prp8-1 otr1Rsph1::ade6 lys1::Nat ade6Δ::kan*	1,2,S1[α]
FY7472	*h- prp10-1 otr1Rsph1::ade6 lys1::Nat ade6Δ::kan*	1,2,S1[α]
FY7339	*h- prp12-1 otr1Rsph1::ade6 lys1::Nat ade6Δ::kan*	1,2[α]
FY7403	*h+ cwf10-1 otr1Rsph1::ade6 lys1::Nat ade6Δ::kan ura4-D18 leu1-32*	1,2,S1
FY7402	*h- prp39-1 otr1Rsph1::ade6 lys1::Nat ade6Δ::kan ura4-D18 leu1-32*	1,2
FY7341	*clr4Δ::ura4 otr1Rsph1::ade6 lys1::Nat ade6Δ::kan*	1,2
FY7343	*swi6Δ::ura4 otr1Rsph1::ade6 lys1::Nat ade6Δ::kan*	1
FY7005	*h+ dcr1Δ::Nat otr1Rsph1::ade6 ade6-210 leu1-32 ura4-D18*	1,S2
FY12195	*ago1::ago1cDNA otr1Rsph1::ade6 lys1::Nat ade6Δ::kan leu1-32*	2
FY12196	*ago1::ago1cDNA prp10-1 otr1Rsph1::ade6 lys1::Nat ade6Δ::kan leu1-32*	2
FY13111	*hrr1::hrr1cDNA otr1Rsph1::ade6 lys1::Nat ade6Δ::kan leu1-32 ura4-D18*	2
FY13112	*hrr1::hrr1cDNA prp10-1 otr1Rsph1::ade6 lys1::Nat ade6Δ::kan leu1-32*	2
FY11924	*prp1-1 dcr1Δ::kan ade6-210 leu1-32*	S2
FY11926	*prp2-1 dcr1Δ::kan ade6-210 leu1-32*	S2
FY11928	*prp3-3 dcr1Δ::kan ade6-210 leu1-32*	S2
FY11930	*prp4-73 dcr1Δ::kan ade6-210 leu1-32*	S2
FY11932	*prp5-1 dcr1Δ::kan ade6-210 leu1-32*	S2
FY11934	*prp8-1 dcr1Δ::kan ade6-210*	S2
FY11936	*prp10-1 dcr1Δ::kan ade6-210 leu1-32*	S2
FY11938	*prp12-1 dcr1Δ::kan ade6-210 leu1-32*	S2
FY11940	*cwf10-1 dcr1Δ::kan*	S2
FY11942	*prp39-1 dcr1Δ::kan ade6-210 leu1-32 ura4-DS/E*	S2
FY7591	*h+ ade6-704 arg3-D4 his3-D1 leu1-32 ura4-DSE cc2:his3*	3
FY9477	*h- dcr1Δ::nat ade6-704 arg3-D4 his3-D1 leu1-32 ura4-D18 cc2:his3*	3
FY11039	*h- prp10-1 ade6-704 arg3-D4 his3-D1 leu1-32 ura4-D18 cc2:his3*	3
FY11041	*h- cwf10-1 ade6-704 arg3-D4 his3-D1 leu1-32 ura4-D18 cc2:his3*	3
FY4841	*h- otr1Rsph1::ura4 ura4-DS/E leu1-32 ade6-210 his3-D1 arg3-D4*	3,S3

FY8536	*prp2-1 otr1Rsph1::ura4 ura4-DS/E leu1-32 ade6-210 arg3-D4*	3,S3
FY8537	*cwf10-1 otr1Rsph1::ura4 ura4-DS/E leu1-32 ade6-210 his3-D1*	3,S3
FY8111	*prp10-1 otr1Rsph1::ura4 ura4-DS/E leu1-32 ade6-210 his3-D1*	3,S3
FY8538	*h+ dcr1Δ::kan otr1Rsph1::ura4 ura4-DS/E leu1-32 ade6-210 his3-D1*	3,S3
FY707	*hA clr4-s5 otr1R sph1::ura4 ura4-DS/E leu1-32 ade6-210*	3,S3
FY511	*h90 mat3-M::ura4 ura4-D18 leu1-32 ade6-216*	4
FY9603	*h90 prp2-1 mat3-M::ura4 ura4-D18 leu1-32 ade6-210*	4
FY9602	*h90 cwf10-1 mat3-M::ura4 ura4-D18 leu1-32 ade6-210*	4
FY9745	*h90 prp10-1 mat3-M::ura4 ura4-D18 leu1-32 ade6-210*	4
FY9296	*h90 swi6Δ::kan mat3-M::ura4 ura4-DS/E leu1-32 ade6-210*	4
FY9299	*h90 dcr1Δ::nat mat3-M::ura4 ura4-DS/E*	4
SPY440	*ura4^{+}:5BoxB-hph*	4[χ]
SPY452	*ura4^{+}:5BoxB/hph tas3:λN-kan*	4[χ]
FY9180	*prp2-1 ura4^{+}:5BoxB/hph tas3:λN-kan ade6-210*	4
FY9181	*cwf10-1 ura4^{+}:5BoxB/hph tas3:λN-kan ade6-210*	4
FY9182	*prp10-1 ura4^{+}:5BoxB/hph tas3:λN-kan ade6-210*	4
FY9183	*dcr1Δ::nat ura4^{+}:5BoxB/hph tas3:λN-kan ade6-210*	4
FY9768	*h+ cid12-3xFLAG-nat otr1Rsph1::ade6 ade6-210 leu1-32 ura4-DS/E*	4
FY10350	*h+ cwf10-HA-nat otr1Rsph1::ade6 ade6-210 leu1-32 ura4-D18*	4,S5
FY12527	*cwf10-HA-nat cid12-3xFLAG-nat otr1Rsph1::ade6 ade6-210 leu1-32 ura4-D18*	4
FY12476	*cwf10-HA-nat prp10-1 otr1Rsph1::ade6 ade6-210 leu1-32 ura4-D18*	4
FY12535	*cwf10-HA-nat dcr1Δ::kan otr1Rsph1::ade6 ade6-210 leu1-32 ura4-D18*	4
FY9643	*h- otr1Rsph1::ade6 lys1::Nat ade6-DN/N leu1-32 ura4-D18*	S4
FY9631	*h- prp2-1 otr1Rsph1::ade6 lys1::Nat ade6-DN/N leu1-32 ura4-D18/DS/E*	S4
FY9633	*h- cwf10-1 otr1Rsph1::ade6 lys1::Nat ade6-DN/N leu1-32 ura4-D18*	S4
FY9637	*h- prp10-1 otr1Rsph1::ade6 lys1::Nat ade6-DN/N leu1-32 ura4-D18/DS/E*	S4
FY11387	*h- otr1Rsph1::ade6 lys1::Nat ade6-DN/N leu1-32 ura4-D18*	S4
FY9641	*h- clr4::ura4 otr1Rsph1::ade6 lys1::Nat ade6-DN/N leu1-32 ura4-D18/DS/E*	S4
FY10353	*h+ prp8-HA-nat otr1Rsph1::ade6 ade6-210 leu1-32 ura4-D18*	S5
FY7097	*h- cid12-HA-nat leu1-32*	S5

[α]derivative of gift from T. Tani; [β]derivative of gift from N. Kaufer; [χ]gift from D. Moazed;

Table S2. List of primers used in this study.

ChIP/RT-PCR	
cen-dh-FOR	GAAAACACATCGTTGTCTTCAGAG
cen-dh-REV	CGTCTTGTAGCTGCATGTGAA
cen-dg-FOR	CACATCATCGTCGTACTACAT
cen-dg-REV	GATATCATCTATATTTAATGACTACT
ade6-FOR	TGC AAC TCT GCG ATG CAT TC
ade6-REV	CTT CAA TGG TGT AGT GAC CTG
ura4-FOR	GAGGGGATGAAAAATCCCAT
ura4-REV	TTCGACAACAGGATTACGACC
tbp1-FOR	CGCTTTACCCACCACGGCCTCGCAAG
tbp1-REV	TTCTGCATTACGTGCATGTAGCGC
fbp1-FOR	GGTTGCTGCTGGCTATACTATG
fbp1-REV	TGGATAAGCAAACAACCCACC
act1-FOR	GGCATCACACTTTCTACAACG
act1-REV	GAGTCCAAGACGATACCAGTG
DF151	GACTGTTGTTGAGTGCTGTG
DF169	CGCAATTAATGTGAGTTAGC
qPCR	
q_dg_FOR	AATTGTGGTGGTGTGGTAATAC
q_dg_REV	GGGTTCATCGTTTCCATTCAG
q_dh_FOR	CTACGCTTGATTTGAGGAAGG
q_dh_REV	AAAGTATGAGTCGCAGAAGTG
q_ade6_FOR	ATGCTTATCCTACAACTGAGACC
q_ade6_REV	TGAATTGAGAAGGGAAGACGAG
q_ura4_FOR	CGTGGTCTCTTGCT TTGG
q_ura4_REV	GTAGTCGCTTTGAAGGTTAGG
q_act_FOR	GGTTTCGCTGGAGATGATG
q_act_REV	ATACCACGCTTGCTTTGAG
q_tbp1_FOR	GCGTCTGGTAAAATGGTTG
q_tbp1_REV	GAAACAACTCAGGCTCATAAGATG
Northern probes	
lng-dg-FOR	CTACTCTTCTCGATGATCCTG
lng-dg-REV	GTAGTACGACGATGATGTGTTTTC
IK8	ATTCCTTTCTGAACCTCTCTGTTAT
IK9	TTTGATGCCCATGTTCATTCCACTTG
IK10	GGGAGTACATCATTCCTACTTCGATA
dhH-siRNA	TACTGTCATTAGGATATGCTCA
snR58	GATGAAATTCAGAAGTCTAGCATC

References

1. S. Moreno, A. Klar, P. Nurse, *Method Enzymol.* **194**, 795 (1991).
2. H. D. Folco, A. L. Pidoux, T. Urano, R. C. Allshire, *Science* **319**, 94 (2008).
3. A. Pidoux, B. Mellone, R. Allshire, *Methods* **33**, 252 (2004).
4. T. Nakagawachi *et al.*, *Oncogene* **22**, 8835 (2003).
5. K. Ekwall *et al.*, *Science* **269**, 1429 (1995).
6. S. C. Trewick, E. Minc, R. Antonelli, T. Urano, R. C. Allshire, *EMBO J.* **26**, 4670 (2007).
7. M. Oeffinger *et al.*, *Nat. Methods* **4**, 951 (2007).
8. J. Rappsilber, M. Mann, Y. Ishihama, *Nat. Protocols* **2**, 1896 (2007).
9. J. Rappsilber, Y. Ishihama, M. Mann, *Analytical Chemistry* **75**, 663 (2003).
10. Y. Ishihama, J. Rappsilber, J. S. Andersen, M. Mann, *Journal of Chromatography A* **979**, 233 (2002).