Schizosaccharomyces pombe RanGAP Homolog, SpRna1, Is Required for Centromeric Silencing and Chromosome Segregation[□]

Ayumi Kusano,* Tomoko Yoshioka, Hitoshi Nishijima, Hideo Nishitani, and Takeharu Nishimoto[†]

Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

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We isolated 11 independent temperature-sensitive (ts) mutants of *Schizosaccharomyces pombe* RanGAP, SpRna1 that have several amino acid changes in the conserved domains of RanGAP. Resulting *Sprna1^{ts}* showed a strong defect in mitotic chromosome segregation, but did not in nucleocytoplasmic transport and microtubule formation. In addition to *Sprna1⁺* and *Spksp1⁺*, the *clr4⁺* (histone H3-K9 methyltransferase), the *S. pombe* gene, *SPAC25A8.01c*, designated *snf2SR⁺* (a member of the chromatin remodeling factors, Snf2 family with DNA-dependent ATPase activity), but not the *spi1⁺* (*S. pombe* Ran homolog), rescued a lethality of *Sprna1^{ts}*. Both *Clr4* and *Snf2* were reported to be involved in heterochromatin formation essential for building the centromeres. Consistently, *Sprna1^{ts}* was defective in gene-silencing at the centromeres. But a silencing at the telomere, another heterochromatic region, was normal in all of *Sprna1^{ts}* strains, indicating SpRna1 in general did not function for a heterochromatin formation. *snf2SR⁺* rescued a centromeric silencing defect and Δ *clr4⁺* was synthetic lethal with *Sprna1^{ts}*. Taken together, SpRna1 was suggested to function for constructing the centromeres, by cooperating with *Clr4* and *Snf2SR*. Loss of SpRna1 activity, therefore, caused chromosome missegregation.

INTRODUCTION

Ran, a small GTPase, plays a key role in the nucleocytoplasmic transport of macromolecules, the mitotic spindle formation and the postmitotic nuclear envelope assembly (Moore, 2001; Dasso, 2002; Hetzer *et al.*, 2002; Weis, 2003). RanGAP, RanGTPase activating protein, is primarily cytosolic, whereas RCC1 (Kai *et al.*, 1986), mammalian RanGEF (RanGDP/GTP exchange factor; Bischoff and Ponstingl, 1991), is a chromosomal protein (Ohtsubo *et al.*, 1989). Ran-GTP concentration, thus, is high in the nucleus and low in the cytoplasm (Kalab *et al.*, 2002). The Ran-GTP/Ran-GDP gradient is currently thought to be critical for Ran-mediated cellular processes. Proteins carrying a nuclear export signal (NES), for instance, make a complex with the nuclear export receptors by the aid of Ran-GTP in the nucleus, and they enter the cytoplasm where Ran-GTP is hydrolyzed to Ran-GDP by the aid of RanGAP, releasing NES-cargo proteins into the cytoplasm (Weis, 2003). The high local concentration of Ran-GTP

in the vicinity of mitotic chromosomes promotes an assembly of mitotic spindle and a fusion of the nuclear membrane vesicles, by the aid of RanGAP (Hetzer *et al.*, 2001, 2002).

Although RanGAP is exclusively localized to the cytoplasm, *Saccharomyces cerevisiae* RanGAP, Rna1, possesses a novel type of nuclear localization signal (NLS) and two of the classical NES, and the endogenous Rna1 location is dependent on the nuclear export receptor, Xpo1/Crm1 (Feng *et al.*, 1999). In *Drosophila melanogaster*, a naturally occurring meiotic drive system of the *Segregation Distorter* (*SD*; Lyttle, 1991), which shows preferential transmission of the *SD* chromosome from *SD/SD⁺* heterozygous males, is caused by the mutated RanGAP, referred to as Sd-RanGAP that is an enzymatically active protein with a truncated NES (Kusano *et al.*, 2001, 2002). Sd-RanGAP, therefore, accumulates in the nucleus. The *SD* phenotype was thus suggested to be induced by abolishing the Ran-GTP/Ran-GDP gradient (Kusano *et al.*, 2001). Indeed, overexpression of Ran rescues the *SD* phenotype (Kusano *et al.*, 2001). Taken together, RanGAP obviously enters the nucleus. However, it is unknown whether nuclear RanGAP participates in a complete RanGTPase nuclear cycle or serves a novel nuclear function (Feng *et al.*, 1999). To address this issue, we chose RanGAP.

A series of temperature-sensitive (ts) mutants of *Schizosaccharomyces pombe* RanGAP homolog, *Sprna1⁺*, were isolated using the error-prone polymerase chain reaction (PCR) as described previously (Oki *et al.*, 1998). Resulting *Sprna1^{ts}* showed a defect in the chromosome segregation, rather than the mitotic spindle formation and the nucleocytoplasmic transport. Subsequently, we isolated the multicopy suppressors of *Sprna1^{ts}* that encoded *Clr4*, a methyltransferase spe-

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* Present address: Research Organization of Information and Systems, National Institute of Genetics, Genetics Strains Research Center, Laboratory for Frontier Research, 1111 Yata Mishima, 411-8540, Japan.

[†] Corresponding author. E-mail address: tnishi@mailserver.med.kyushu-u.ac.jp.

cific for histone H3-K9 (Rea *et al.*, 2000; Bannister *et al.*, 2001; Nakayama *et al.*, 2001), and Snf2SR, a member of chromatin remodeling factors, Snf2 family that have DNA-dependent ATPase activity (Havas *et al.*, 2001; Becker and Horz, 2002; Lusser and Kadonaga, 2003), in addition to SpRna1 and SpKsp1, *S. pombe* homolog of *S. cerevisiae* Ksp1 (Fleischmann *et al.*, 1996). Consistent with the previous reports that both Ctr4 and Snf2-homolog function for heterochromatin formation in *Arabidopsis thaliana* (Jeddeloh *et al.*, 1999; Gendrel *et al.*, 2002; Johnson *et al.*, 2002), most of *SpRna1^{ts}* strains showed a silencing defect at the innermost repeat domain and one mutant showed a clear silencing defect at the outer repeat domain of the centromeres where Swi6, *S. pombe* homolog of mammalian heterochromatin protein 1 (HP1)-dependent heterochromatin is formed (Bannister *et al.*, 2001). A silencing at the telomeres, another heterochromatic chromosomal region, was normal in all of *SpRna1^{ts}*. SpRna1, therefore, in general did not function for heterochromatin formation. A half of *SpRna1^{ts}* also showed a significant defect at the central core of the centromeres. Especially one mutant showed a slight, but significant, silencing defect only at the central core. Taken together with the report that mammalian RanGAP1 is localized on kinetochores in mitotic cells (Joseph *et al.*, 2004), SpRna1 was suggested to have a novel function that is required for constructing the centromeres.

MATERIALS AND METHODS

Yeast Media and Strains

The *S. pombe* strain was grown in rich medium (YE5S) or Edinburgh minimal medium (EMM) with appropriate supplements. Δ *SpRna1*-A1 strain: 478 (Table 1), was provided by S. Sazer (Baylor College of Medicine; Matynia *et al.*, 1996). To isolate a haploid derivative that carries the *SpRna1::ura4⁺* allele, Δ *SpRna1*-A1 was transformed first with pREP81X-*SpRna1⁺* which carries the open reading frame (ORF) of wild-type *SpRna1⁺* and *LEU⁺* marker (Matynia *et al.*, 1996). Δ *SpRna1*-A1 [pREP81X-*SpRna1⁺*] sporulated on ME plates, and spores were treated with β -glucuronidase (Sigma, St. Louis, MO) and replated for germination. The strains possessing *Ura⁺* and *Leu⁺* markers were identified as *SpRna1::ura4⁺* cells carrying pREP81X-*SpRna1⁺*. The strains used in this experiment are listed in Table 1.

Isolation of *SpRna1^{ts}*

SpRna1^{ts} were generated through the error-prone PCR method as described previously (Oki *et al.*, 1998). Briefly, the *SpRna1⁺* genomic DNA fragment, which covers an entire ORF of *SpRna1⁺* along with about 1 kilobase pair (kbp) of both flanking regions derived from the upstream and the downstream of *SpRna1⁺* ORF, was amplified by PCR using the following primers: the 5' primer, 5'-CAC AGT AAT GAA TAA GAA ATG, and the 3' primer, 5'-AGC ACT TTC ATC CAA ACA CTC, in the presence of 0.2 mM Mn²⁺. Amplified DNA fragments were introduced into the haploid *SpRna1::ura4⁺* cells carrying pREP81X-*SpRna1⁺* through electroporation. Transfected cells were grown for four generations in EMM medium supplemented with leucine, uracil, and adenine and then incubated in the presence of 1.5 mg/ml 5FOA (5-fluoroorotic acid) at 26°C. Resulting cells were replica-plated at 26 and 35°C. Cell lines that grew at 26°C (permissive temperature), but not at 35°C (restrictive temperature), were identified as ts mutants.

Each *SpRna1^{ts}* strain was crossed to Δ *ctr4* strain, gifted from R. Allshire (University of Edinburgh; Bannister *et al.*, 2001), to generate the double mutants of *SpRna1^{ts} Δctr4*.

Isolation of Multicopy Suppressors for *SpRna1^{ts}*

S. pombe genomic library provided from M. Yanagida (Kyoto University), was introduced into *SpRna1^{ts}* with electroporation. Transformed cells were plated and incubated at either 34 or 35°C for 5 d. Cells that grew on plates at 35°C were selected for further analysis. Plasmids retained in *ts⁺* transformants of *SpRna1^{ts}* were recovered in *Escherichia coli*, and their nucleotide sequences of *S. pombe* genomic DNA carried on plasmids were determined using the ABI Genetic Analyzer 3100 (Applied Biosystems, Tokyo, Japan).

Construction of NLS-NES-GFP and NLS-NES^{P12}-GFP

pREP3X-NLS-NES-GFP and pREP3X-NLS-NES^{P12}-GFP constructs were created by introducing a DNA fragment that encodes NLS-NES-GFP or NLS-NES^{P12}-GFP, derived from pKW430 or pKW431 (Stade *et al.*, 1997), into the pREP3X vector.

Immunofluorescence Microscopy

DNA. Cells were spotted onto a glass slide, fixed on 70°C heat block, and then mounted in VECTASHIELD with DAPI (Vector Laboratories, Burlingame, CA) to visualize chromosomes.

Microtubules. Cells were fixed with 3.3% formaldehyde in phosphate-buffered saline, stained first with the mouse anti-Tat1 antibody, gifted from K. Gull (University of Manchester; Sherwin and Gull, 1989), followed by the fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (IgG, Jackson ImmunoResearch Laboratories, West Grove, PA).

Spindle Pole Body (SPB). Cells fixed as described above, were first stained with the rabbit anti-Sad1 antibody gifted from I. Hagan (University of Manchester; Hagan and Yanagida, 1995), followed by the Alexa Fluor 568-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR).

Images were visualized with the Zeiss Axioskop Fluorescence Microscope (Carl Zeiss, Thornwood, NY), and processed with Adobe Photoshop (Adobe Systems, San Jose, CA).

RESULTS

Isolation of *SpRna1^{ts}*

The *SpRna1⁺* genomic DNA fragment that contains the entire ORF of *SpRna1⁺* and 1 kb of flanking regions was amplified by PCR in the presence of 0.2 mM of Mn²⁺ as described previously (Oki *et al.*, 1998). Amplified DNA fragments were introduced into the haploid strain; *SpRna1::ura4⁺* carrying pREP81X-*SpRna1⁺* and then temperature-sensitive (*ts*) cells were isolated as described in *Materials and Methods*.

Initially, 57 of *ts* cells were independently isolated. The *SpRna1⁺*-ORF of the resulting *ts* cells was sequenced to confirm that the temperature-sensitive lethality of cells was caused by the mutation of SpRna1. In parallel, pREP81X-*SpRna1⁺* was introduced into resulting *ts* cells to examine whether a temperature-sensitive lethality of isolated strains could be rescued by this plasmid. According to the sequencing data, 11 lines of *SpRna1^{ts}* strains were finally chosen for further analyses (Table 2). Most of them had a single point mutation, which was mainly localized either in the conserved leucine-rich repeats (LRRs) domain (Hillig *et al.*, 1999) or in the Ran-interacting domain of SpRna1 (Seewald *et al.*, 2002; Figure 1).

The cellular amount of mutated SpRna1 was not reduced in most of *SpRna1^{ts}*, except for *SpRna1-47^{ts}* and *SpRna1-82^{ts}*, after incubation for 5 h at 35°C, the restrictive temperature (unpublished data). Both *SpRna1-1^{ts}* and *SpRna1-86^{ts}* have a mutation at the amino acid in the NES region estimated from *S. cerevisiae* Rna1, but a corresponding *S. cerevisiae* Rna1 mutant has no effect on the nuclear export (Feng *et al.*, 1999). The other *SpRna1^{ts}* mutants have no mutation in the putative SpRna1 NES. Expectedly, all of mutated SpRna1 proteins were mainly localized in the cytoplasm, even after incubation at the restrictive temperature (unpublished data).

Mitotic Chromosome Segregation Was Defective in *SpRna1^{ts}*

RanGTPase is involved in several cellular processes (Moore, 2001; Dasso, 2002; Hetzer *et al.*, 2002; Weis, 2003), which were then examined in *SpRna1^{ts}* isolated.

First, the nucleocytoplasmic transport of proteins was examined. To address this issue, GFP-fused reporter proteins appended with NLS and NES (NLS-NES) were expressed in *SpRna1^{ts}*. The representative results are shown in Figure 2. Because the activity of the used NES is stronger than that of NLS, the expressed GFP was localized in both the cytoplasm and the nuclear rim of wild-type cells, both at 26 and at 37°C, as reported (Stade *et al.*, 1997; Figure 2, A and C). On the other hand, GFP-fused reporter proteins appended with NLS and an inactive NES (NLS-NES^{P12}), which are mainly

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

Strains ^a	Genotype
478	h ⁺ /h ⁻ , ura4-D18/ura4-D18, leu1-32/leu1-32, ade6m-210/ade6m-216, sprna1::ura4 ⁺
h ⁺ , 975	h ⁺ , 975
h ⁺ , ura4-D18	h ⁺ , ura4-D18
AK1-1	h ⁺ , ura4-D18, leu1-32, sprna1-1 ^{ts}
AK4-4	h ⁺ , ura4-D18, leu1-32, sprna1-8 ^{ts}
AK6-2	h ⁺ , ura4-D18, leu1-32, sprna1-11 ^{ts}
AK7-5	h ⁺ , ura4-D18, leu1-32, sprna1-15 ^{ts}
AK26-4	h ⁺ , ura4-D18, leu1-32, sprna1-47 ^{ts}
AK14-7	h ⁺ , ura4-D18, leu1-32, sprna1-48 ^{ts}
AK16-3	h ⁺ , ura4-D18, leu1-32, sprna1-50 ^{ts}
AK17-3	h ⁺ , ura4-D18, leu1-32, sprna1-55 ^{ts}
AK20-3	h ⁺ , ura4-D18, leu1-32, sprna1-82 ^{ts}
AK21-5	h ⁺ , ura4-D18, leu1-32, sprna1-86 ^{ts}
AK23-4	h ⁺ , ura4-D18, leu1-32, sprna1-86 ^{ts}
FY2267	h ⁺ , ura4-D18, leu1-32, ade6-m210, clr4 ⁺ ::ura4, otr1R(dg-glu)Sph::ade6 ⁺
AK936	h ⁺ , ura4-D18, clr4 ⁺ ::ura4, sprna1-1 ^{ts}
AK938	h ⁺ , ura4-D18, clr4 ⁺ ::ura4, sprna1-8 ^{ts}
AK940	h ⁺ , ura4-D18, clr4 ⁺ ::ura4, sprna1-15 ^{ts}
AK941	h ⁺ , ura4-D18, clr4 ⁺ ::ura4, sprna1-47 ^{ts}
AK943	h ⁺ , ura4-D18, clr4 ⁺ ::ura4, sprna1-48 ^{ts}
AK945	h ⁺ , ura4-D18, clr4 ⁺ ::ura4, sprna1-50 ^{ts}
AK947	h ⁺ , ura4-D18, clr4 ⁺ ::ura4, sprna1-55 ^{ts}
AK949	h ⁺ , ura4-D18, clr4 ⁺ ::ura4, sprna1-82 ^{ts}
AK950	h ⁺ , ura4-D18, clr4 ⁺ ::ura4, sprna1-86 ^{ts}
AK951	h ⁺ , ura4-D18, clr4 ⁺ ::ura4, sprna1-87 ^{ts}
FY498	h ⁺ , ura4-DS/E, leu1-32, ade6-m210, imr1R(NcoI)::ura4 ⁺
AK89	h ⁺ , ura4-DS/E, leu1-32, imr1R(NcoI)::ura4 ⁺
AK60	h ⁺ , ura4-DS/E, leu1-32, imr1R(NcoI)::ura4 ⁺ , sprna1-1 ^{ts}
AK64	h ⁺ , ura4-DS/E, leu1-32, imr1R(NcoI)::ura4 ⁺ , sprna1-11 ^{ts}
AK66	h ⁺ , ura4-DS/E, leu1-32, imr1R(NcoI)::ura4 ⁺ , sprna1-15 ^{ts}
AK68	h ⁺ , ura4-DS/E, leu1-32, imr1R(NcoI)::ura4 ⁺ , sprna1-47 ^{ts}
AK70	h ⁺ , ura4-D18, leu1-32, imr1R(NcoI)::ura4 ⁺ , sprna1-48 ^{ts}
AK91	h ⁺ , ura4-D18, leu1-32, imr1R(NcoI)::ura4 ⁺ , sprna1-82 ^{ts}
AK72	h ⁺ , ura4-D18, leu1-32, imr1R(NcoI)::ura4 ⁺ , sprna1-86 ^{ts}
AK74	h ⁺ , ura4-D18, leu1-32, imr1R(NcoI)::ura4 ⁺ , sprna1-87 ^{ts}
FY648	h ⁺ , ura4DS/E, leu1-32, ade6-m210, otr1R(NcoI)::ura4 ⁺
AK90	h ⁺ , ura4-D18, leu1-32, otr1R(NcoI)::ura4 ⁺
AK76	h ⁺ , ura4-D18, leu1-32, otr1R(NcoI)::ura4 ⁺ , sprna1-1 ^{ts}
AK79	h ⁺ , ura4-D18, leu1-32, otr1R(NcoI)::ura4 ⁺ , sprna1-11 ^{ts}
AK80	h ⁺ , ura4-D18, leu1-32, otr1R(NcoI)::ura4 ⁺ , sprna1-15 ^{ts}
AK82	h ⁺ , ura4-DS/E, leu1-32, otr1R(NcoI)::ura4 ⁺ , sprna1-47 ^{ts}
AK84	h ⁺ , ura4-DS/E, leu1-32, otr1R(NcoI)::ura4 ⁺ , sprna1-48 ^{ts}
AK92	h ⁺ , ura4-DS/E, leu1-32, otr1R(NcoI)::ura4 ⁺ , sprna1-82 ^{ts}
AK86	h ⁺ , ura4-D18, leu1-32, otr1R(NcoI)::ura4 ⁺ , sprna1-86 ^{ts}
AK88	h ⁺ , ura4-D18, leu1-32, otr1R(NcoI)::ura4 ⁺ , sprna1-87 ^{ts}
FY336	h ⁺ , leu1-32, ura4DS/E, ade6-m210, cnt1/TM(NcoI)-ura4 ⁺
AK906	h ⁺ , ura4-DS/E, leu1-32, cnt1/TM(NcoI)-ura4 ⁺
AK885	h ⁺ , ura4-DS/E, leu1-32, cnt1/TM(NcoI)-ura4 ⁺ , sprna1-1 ^{ts}
AK889	h ⁺ , ura4-DS/E, leu1-32, cnt1/TM(NcoI)-ura4 ⁺ , sprna1-11 ^{ts}
AK891	h ⁺ , ura4-DS/E, leu1-32, cnt1/TM(NcoI)-ura4 ⁺ , sprna1-15 ^{ts}
AK893	h ⁺ , ura4-DS/E, leu1-32, cnt1/TM(NcoI)-ura4 ⁺ , sprna1-47 ^{ts}
AK895	h ⁺ , ura4-DS/E, leu1-32, cnt1/TM(NcoI)-ura4 ⁺ , sprna1-48 ^{ts}
AK900	h ⁺ , ura4-DS/E, leu1-32, cnt1/TM(NcoI)-ura4 ⁺ , sprna1-82 ^{ts}
AK902	h ⁺ , ura4-DS/E, leu1-32, cnt1/TM(NcoI)-ura4 ⁺ , sprna1-86 ^{ts}
AK904	h ⁺ , ura4-DS/E, leu1-32, cnt1/TM(NcoI)-ura4 ⁺ , sprna1-87 ^{ts}
FY1862	h ⁹⁰ , his3-D1, leu1-3, ura4-D18, ade6-D1, otr1R::ade6 ⁺ , tel(1)-his3 ⁺ , tel(2)-ura4 ⁺
t1m+	h ⁻ , ura4-D18, tel(2)-ura4 ⁺ , tel(1)-his3 ⁺ , otr1R::ade6 ⁺
t1m1	h ⁺ , ura4-D18, tel(2)-ura4 ⁺ , sprna1-1 ^{ts} , tel(1)-his3 ⁺ , otr1R::ade6 ⁺
t1m11	h ⁻ , ura4-D18, tel(2)-ura4 ⁺ , sprna1-11 ^{ts} , tel(1)-his3 ⁺
t1m15	h ⁺ , ura4-D18, tel(2)-ura4 ⁺ , sprna1-15 ^{ts} , otr1R::ade6 ⁺
t1m48	h ⁺ , ura4-D18, tel(2)-ura4 ⁺ , sprna1-48 ^{ts} , otr1R::ade6 ⁺
t1m82	h ⁹⁰ , ura4-D18, tel(2)-ura4 ⁺ , sprna1-82 ^{ts} , tel(1)-his3 ⁺ , otr1R::ade6 ⁺
t1m86	h ⁹⁰ , ura4-D18, tel(2)-ura4 ⁺ , sprna1-86 ^{ts} , tel(1)-his3 ⁺
t1m87	h ⁹⁰ , ura4-D18, tel(2)-ura4 ⁺ , sprna1-87 ^{ts} , otr1R::ade6 ⁺

^a The strain 478 is described in Matynia *et al.* (1996). FY498, FY648, and FY336 are described in Nakagawa *et al.* (2002). FY2267 and FY1862 are described in Bannister *et al.* (2001) and Nimmo *et al.* (1998), respectively. Strains starting with AK and t1m were generated in this study.

Table 2. Amino acid changes in the SpRna1 of *Sprna1^{ts}*

<i>Sprna1^{ts}</i>	Amino acid change
<i>Sprna1-1^{ts}</i>	L305R ^a
<i>Sprna1-8^{ts}</i>	N226I ^b
<i>Sprna1-11^{ts}</i>	G232D
<i>Sprna1-15^{ts}</i>	E148G, G260V
<i>Sprna1-47^{ts}</i>	K76R ^b , K181I, G232D
<i>Sprna1-48^{ts}</i>	Q277P
<i>Sprna1-50^{ts}</i>	H121R
<i>Sprna1-55^{ts}</i>	L161S ^a
<i>Sprna1-82^{ts}</i>	V38E, L249Q ^a
<i>Sprna1-86^{ts}</i>	L308P ^a
<i>Sprna1-87^{ts}</i>	L223P ^a

^a Conserved leucine (L) residues in the LRRs (Hillig *et al.*, 1999).

^b Residues shown to be required for interaction with RanGTPase (Seewald *et al.*, 2002).

^c Underlined residues are conserved from yeast to mammal (Hillig *et al.*, 1999).

localized in the nucleus (Figure 2, B and D), were used to estimate the nuclear-protein import activity of *Sprna1^{ts}*. Most of *Sprna1^{ts}* strains did not show obvious defects in the nucleocytoplasmic transport (Figure 2, E and G, F and H). Only both *Sprna1-82^{ts}* and *Sprna1-87^{ts}* showed a defect in the nuclear protein import at 37°C. In these mutants, there was no obvious nuclear accumulation of GFP-fused NLS-NES^{P12} proteins, compared with *Sprna1-86^{ts}* and wild-type cells (Figure 2, J and L, N and P).

Second, the mitotic spindle formation and the localization of SPB stained with the anti-Tat1 and the anti-Sad1 antibodies, respectively, were examined in all of *Sprna1^{ts}*. The representative results are shown in Figure 3. Both the mitotic spindle formation and the localization of SPB were indistinguishable from those of wild-type cells, even after incubation at 37°C, in all of *Sprna1^{ts}*. When DNA of the same samples was stained with DAPI, however, chromosomal DNA was dislocated from SPB on elongated mitotic spindles in most of *Sprna1^{ts}*, and the images were similar to the *sim* mutants (Pidoux *et al.*, 2003), indicating the uneven segregation of mitotic chromosomes occurred in *Sprna1^{ts}* at 37°C. To further confirm this issue, *Sprna1^{ts}* was grown at 35°C for 4 h in liquid medium and then fixed to stain with DAPI.

Apparent uneven chromosome segregation was detected in all of *Sprna1^{ts}*. The representative results are shown in Figure 4. Cells marked with arrows and arrowheads showed an aberrant chromosome segregation as indicated in Figure 4D. Compared with wild-type cells, uneven chromosome segregation was apparent in *Sprna1^{ts}* at 35°C.

Clr4 and *Snf2SR* Rescued a Temperature-sensitive Lethality of *Sprna1^{ts}*

To elucidate the molecular mechanism of uneven chromosome segregation, *S. pombe* genomic library was screened for the multicopy suppressor genes of *Sprna1^{ts}* that rescue a temperature-sensitive lethality of *Sprna1^{ts}*. In addition to *Sprna1⁺* and *Spksp1⁺*, both *clr4⁺* and the *S. pombe* gene, SPAC25A8.01c, encoding a member of Snf2 family proteins (Lusser and Kadonaga, 2003), designated as *snf2SR⁺*-like Suppressor of *Sprna1^{ts}*, were frequently obtained from *ts⁺* transformants of several *Sprna1^{ts}* that grew on plates at 35°C (Table 3).

The ability of *clr4⁺* and *snf2SR⁺* to rescue a temperature-sensitive lethality of *Sprna1^{ts}* was well correlated (Figure 5). On overexpression of *clr4⁺* or *snf2SR⁺* genes, *Sprna1^{ts}* such as *Sprna1-1^{ts}*, *Sprna1-8^{ts}*, *Sprna1-15^{ts}*, *Sprna1-48^{ts}*, and *Sprna1-86^{ts}*, grew at the restrictive temperature (34 and 35°C), but not the other *Sprna1^{ts}* such as *Sprna1-11^{ts}*, *Sprna1-47^{ts}*, *Sprna1-50^{ts}*, *Sprna1-55^{ts}*, *Sprna1-82^{ts}*, and *Sprna1-87^{ts}*. The frequency of uneven chromosome segregation was also significantly reduced in *Sprna1-1^{ts}*, *Sprna1-8^{ts}*, *Sprna1-15^{ts}*, *Sprna1-48^{ts}*, and *Sprna1-86^{ts}*, but not in *Sprna1-11^{ts}*, *Sprna1-47^{ts}*, *Sprna1-50^{ts}*, *Sprna1-55^{ts}*, *Sprna1-82^{ts}*, and *Sprna1-87^{ts}* by overexpression of *clr4⁺* or *snf2SR⁺* (representative results are shown in Figure 6). The ability to rescue a temperature-sensitive lethality of *Sprna1^{ts}* by *clr4⁺* or *snf2SR⁺* was well correlated with the ability to achieve proper chromosome segregation, suggesting that *Sprna1^{ts}* became lethal by uneven chromosome segregation at the restrictive temperature.

Sprna1^{ts} Was Defective in Centromeric, but not in Telomeric, Silencing

Proper chromosome segregation is carried out by interaction between mitotic spindle and kinetochores. Because mitotic spindle formation and SPB distribution seemed to be normal (Figure 3), the uneven chromosome segregation suggested that the kinetochore-centromere function that ensures an interaction between mitotic spindle and kinetochores be-

Figure 1. Mutation sites in *Sprna1^{ts}* mutants. Mutation sites of *Sprna1^{ts}* were localized on a ribbon presentation of the structure of SpRna1 (modified from Hillig *et al.*, 1999). Red ribbons and green arrows represent α -helices and β -sheet structures, respectively. Yellow lines show hinge region between α -helix and β -sheet. Mutations at the Ran interaction site are indicated in green, and the mutations at the conserved leucine residues of the LRRs domain are indicated in red. Mutations at other amino acid residues are indicated in blue. The number in parentheses indicates the number of times that the same mutation was independently isolated. As for *Sprna1-15^{ts}*, *Sprna1-47^{ts}*, and *Sprna1-82^{ts}*, which have two to three amino acid changes (see Table 2), the mutation site in the Ran-interaction or the LRRs domains was shown.

Figure 2. Nucleocytoplasmic transport in *Sprna1^{ts}* mutants. Cultures of indicated cells were incubated at 26°C, and then half of cultures were incubated at 37°C for 4 h. Images of wild-type, *Sprna1-86^{ts}*, *Sprna1-82^{ts}*, and *Sprna1-87^{ts}* cells expressing the indicated GFP-fused NLS-NES or NLS-NES^{p12} proteins were shown as representatives. Left columns of the indicated NLS-NES and NLS-NES^{p12} show GFP localization, and right columns show merged images with DAPI-staining. Scale bar, 5 μ m.

came defective at the restrictive temperature. Ctr4, which methylates specifically lysine 9 of histone H3 (Rea *et al.*, 2000; Bannister *et al.*, 2001; Nakayama *et al.*, 2001), is essential for constructing heterochromatin. Snf2SR is a member of the Snf2 family (Havas *et al.*, 2001; Becker and Horz, 2002; Lusser and Kadonaga, 2003). The Snf2-like protein is required for heterochromatin formation and maintenance in *A. thaliana* (Gendrel *et al.*, 2002; Johnson *et al.*, 2002). The fact that both suppressors of *Sprna1^{ts}* are involved in constructing heterochromatin, which is important for the centromeric function (Jeddeloh *et al.*, 1999; Pidoux *et al.*, 2003; Appलगren *et al.*, 2003), indicated that the centromeres might become defective in *Sprna1^{ts}* at the restrictive temperature. To ad-

dress this issue, *Sprna1^{ts}* was examined for a gene-silencing activity at the centromere, based on previous reports that the expression of the euchromatic gene inserted into the centromere is repressed (Nakagawa *et al.*, 2002; Pidoux *et al.*, 2003).

We assayed the silencing of the *ura4⁺* gene inserted at three different domains of Chromosome I centromere: the outer repeat (*otr1R::ura4⁺*), the innermost repeat (*imr1R::ura4⁺*), and the central core (*cnt1::ura4⁺*), as reported previously (Nakagawa *et al.*, 2002). Expression of the inserted *ura4⁺* gene was monitored by plating serially diluted cells of indicated each strain on the nonselective (N/S), the selective (Ura⁻), or the counterselective (5FOA) plates (5FOA is a toxic substrate for the Ura4 protein). As

Figure 3. Mitotic spindle formation in *Sprna1^{ts}* mutants. Cultures of indicated cells were incubated at 26°C, and then half of cultures were incubated at 37°C for 4 h. Images of wild-type, *Sprna1-86^{ts}*, and *Sprna1-87^{ts}* are shown as representatives. Mitotic spindle and SPB were detected with anti-Tat1 (green) and anti-Sad1 (red) antibodies, respectively. DNA was stained with DAPI (blue). Left columns show an anti-Tat1 staining pattern and right columns show an anti-Tat1 staining image merged with anti-Sad1 for SPB (red) and DAPI (blue), respectively, at the indicated temperature. Scale bar, 5 μ m.

Figure 4. *Sprna1^{ts}* mutants showing aberrant mitotic chromosome segregation. Cultures of indicated cells were grown in liquid medium at 26°C, and then half of cultures were incubated at 35°C for 4 h. Images of wild-type (A), *Sprna1-86^{ts}* (B), and *Sprna1-87^{ts}* (C) cells are shown as representatives. (D) The frequency of the indicated patterns of aberrant chromosome segregation that are shown by black and white arrowheads and an arrow. Chromosomal DNA was stained with DAPI. Scale bar, 5 μ m.

expected, *ura4⁺* cells grew on Ura⁻ plates, but not on 5FOA plates. In contrast, *ura4⁻* cells grew on 5FOA plates, but did not on Ura⁻ plates (Figure 7, D and E, *h⁺ 975* and *h⁺ ura4-D18*). In *Sprna1⁺* (wt) cells, the *ura4⁺* gene expression was repressed at the outer repeat (*otr1R::ura4⁺*) and the innermost repeat (*imr1R::ura4⁺*), but it was partially repressed at the central core (*cnt1::ura4⁺*; Figure 7, A–C, wt, compare Ura⁻ and 5FOA plates), as previously reported (Pidoux *et al.*, 2003). After incubation at 30°C for 5 d, a silencing defect at the centromeres was observed, as shown in Figure 7. *Sprna1-1^{ts}*, *Sprna1-11^{ts}*, *Sprna1-47^{ts}*, and *Sprna1-86^{ts}* showed a significant silencing defect at the central core (Figure 7A, 5FOA plate). Except *Sprna1-86^{ts}* that has a silencing defect only at the central core, all of the other *Sprna1^{ts}* strains also showed a silencing defect at the innermost repeat. In addition to the innermost repeat, a clear silencing defect at the outer repeat domain where

Swi6-mediated heterochromatin is formed (Bannister *et al.*, 2001) was observed only in *Sprna1-87^{ts}*. Consistently, the distribution of Swi6, which binds to the outer repeat domain (Bannister *et al.*, 2001), was slightly changed in this mutant, whereas it seemed to be normal in other *Sprna1^{ts}* strains, after incubation at the restrictive temperature (Supplementary Figure 1).

To address the question whether a defect of SpRna1 affects gene silencing at the heterochromatic domains other than the centromeres, the expression of the *ura4⁺* gene inserted into the telomere was examined. It was clearly repressed in all of *Sprna1^{ts}* strains examined (Figure 7E), indicating that SpRna1 in general did not function for heterochromatin formation.

The *snf2SR⁺* rescued a centromeric silencing defect of *Sprna1^{ts}* mutants. Representative results at the innermost repeat domain (*imr1R::ura4⁺*) were shown (Figure 7D). When Clr4 was overexpressed, the gene silencing ability at three domains of the centromeres became defective in *Sprna1⁺* (wt) cells. So, we could not determine whether Clr4 rescues the centromeric silencing defect of *Sprna1^{ts}* or not. In the supplemental figure, we showed the representative results at the innermost repeat domain of the centromeres. Curiously, Clr4 rescued a silencing defect of some *Sprna1^{ts}* strains at this domain, although it abolished a silencing at the same domain of *Sprna1⁺* (wt) cells (Supplementary Figure 2, 5FOA).

Sprna1^{ts} Was Synthetically Lethal with Δ clr4

To confirm a functional relationship between SpRna1 and Clr4, the *clr4⁺* gene of each *Sprna1^{ts}* was disrupted. The temperature-sensitive lethality was enhanced by a disruption of the *clr4⁺* gene, in most of *Sprna1^{ts}* strains (Figure 8). Among them, *Sprna1-11^{ts}*, *Sprna1-47^{ts}*, and *Sprna1-87^{ts}*,

Table 3. Multicopy suppressors of *Sprna1^{ts}*

Suppressor genes	Known function	Frequency identified as multicopy suppressor ^a
<i>Spksp1⁺</i>	Ser/Thr protein kinase	3.6×10^{-5}
<i>clr4⁺</i>	Histone H3 methyltransferase	2.6×10^{-5}
<i>snf2SR⁺b</i>	Chromatin remodeling factor	3.0×10^{-5}
<i>Sprna1⁺</i>	Ran GTPase activating protein	2.5×10^{-4}

^a Frequency of multicopy suppression was calculated as follows: the number of *Leu⁺* transformant carrying the indicated gene at the restrictive temperature divided by the total *Leu⁺* transformant at the permissive temperature.

^b *snf2SR⁺* (*snf2*-like Suppressor of *Sprna1^{ts}*): *S. pombe* gene number: SPAC25A8.01C.

Figure 5. Expression of *clr4*⁺ or *snf2SR*⁺ rescued the temperature-sensitive lethality of *Sprna1*^{ts} mutants. (A) Multicopy suppression of *Sprna1-8*^{ts}, *Sprna1-15*^{ts}, and *Sprna1-87*^{ts} by *clr4*⁺ or *snf2SR*⁺ are shown as representatives. Left and right columns show cells grown at 26 and 34°C, respectively. Clones are shown in the following clockwise order: wild-type, *Sprna1*^{ts} cells possessing a vector alone, *clr4*⁺ or *snf2SR*⁺ as indicated. (B) Allele specificity of multicopy suppression by *clr4*⁺ or *snf2SR*⁺. (+), suppressed; (-), not suppressed. The number of (+) indicated the efficiency of suppression.

which temperature-sensitive lethality could not be rescued by *clr4*⁺ or *snf2SR*⁺ (Figure 5), showed severe synthetic lethality with Δ *clr4*. In contrast, *Sprna1-1*^{ts}, *Sprna1-8*^{ts}, *Sprna1-15*^{ts}, *Sprna1-48*^{ts}, and *Sprna1-86*^{ts} in which temperature-sensitive lethality was well rescued by over-

expression of *clr4*⁺ or *snf2SR*⁺ (Figure 5), showed less severe synthetic lethality with Δ *clr4*. Thus, SpRna1 was suggested to have some functional relationship with Clr4. *Sprna1-50*^{ts}, *Sprna1-55*^{ts}, and *Sprna1-82*^{ts} did not show the synthetically lethality at the temperature examined.

Figure 6. Aberrant mitotic chromosome segregation of *Sprna1*^{ts} was rescued by *clr4*⁺ or *snf2SR*⁺. Cultures of *Sprna1*^{ts} mutants expressing no suppressors (left), *clr4*⁺ (middle), or *snf2SR*⁺ (right) grown in liquid medium at 26°C, were incubated at 35°C for 4 h and then stained with DAPI. Images of *Sprna1-86*^{ts} (A) and *Sprna1-87*^{ts} (B) are shown as representatives. Scale bar, 5 μ m. Black and white arrowheads indicate aberrant chromosome segregation as described in Figure 4. (C) Summary of A and B. The vertical line indicates a frequency of cells showing an aberrant mitosis (%). At least 300 cells were counted for each strain.

Figure 7. Gene silencing activity of *Sprna1^{ts}* mutants. Top panel, a schematic representation of the *cen1* and the *ura4⁺* insertion sites within the *cen1*. (A–D) Centromeric silencing. *Sprna1^{ts}* strains possessing the *ura4⁺* gene at the indicated domain are spotted (A–C). wt: AK906 (A); AK89 (B); AK90 (C). *Sprna1^{ts}* [*imr1R::ura4⁺*] strains expressing *snf2SR⁺* (D). (E) Telomeric silencing. *Sprna1^{ts}* strains possessing the *ura4⁺* gene at the telomere (Nimmo *et al.*, 1998) are spotted as indicated. wt: tlm⁺. The h⁺975 and h⁺*ura4-D18* were used as the control strains of either Ura⁺ or Ura⁻ cells, respectively. The dotted marks of these strains in D indicate that they have no plasmids. Each strain was grown to 1.0×10^7 cells/ml in YE5S. Serial dilution (1:5) of the indicated cultures were spotted onto nonselective (N/S), selective (Ura⁻), or countersselective (5FOA) plates and incubated at 30°C for 5 d. The highest density spots contained 1×10^4 cells.

DISCUSSION

We have isolated 11 *Sprna1^{ts}* strains that have one to three amino acid alterations in SpRna1. Except for *Sprna1-48^{ts}* and *Sprna1-50^{ts}*, all of the other *Sprna1^{ts}* have a mutation in the amino acid residues that are conserved from yeast to mammalian RanGAP (Hillig *et al.*, 1999). Specifically 5 of 11 mutants have a mutation in leucine residues of the RanGAP

LRR domains, suggesting that these regions have a key role for the RanGAP activity.

Sprna1^{ts} showed a little defect in the nucleocytoplasmic transport, but not in the mitotic spindle formation and the SPB localization. They showed a strong defect in the chromosome segregation, after incubation at the restrictive temperature. For proper chromosome segregation, both the kinetochores and the centromeres that underlie kinetochores are important, in addition to mitotic spindle and SPB. Although both mitotic spindle and SPB localization were normal after incubation at the restrictive temperature, chromosomal DNA was dislocated from SPB on elongated mitotic spindle, similar to the *sim4⁻*, which has a defect in the centromeres (Pidoux *et al.*, 2003). Indeed, a gene-silencing defect was observed at the centromeres of *Sprna1^{ts}*. The uneven chromosome segregation of *Sprna1^{ts}*, therefore, could be caused by a defect of centromere-kinetochore function that disrupts an interaction between mitotic spindles and kinetochores. The fact that Clr4 and Snf2SR, a member of Snf2 family, were isolated as suppressors of *Sprna1^{ts}* suggested that SpRna1 is required for building the centromeres, by the unknown mechanism, in which Clr4, Snf2SR, and probably another suppressor, SpKsp1, might be involved.

Clr4 performs histone H3 methylation at the 9th lysine (Nakayama *et al.*, 2001), which is essential for constructing heterochromatin. Snf2SR, another multicopy suppressor of *Sprna1^{ts}*, is a member of Snf2 family with DNA-dependent ATPase, comprising the chromatin-remodeling complex (Lusser and Kadonaga, 2003). SpKsp1 is an *S. pombe* homolog of *S. cerevisiae* Ksp1, which has been isolated as a multicopy suppressor of *prp20* (*S. cerevisiae* ts mutant of RCC1 homolog; Fleischmann *et al.*, 1996). It encodes Ser/Thr protein kinase. The *A. thaliana* Snf2-family protein, DDM1, has been reported to function for histone H3-K9 methylation and maintenance by cooperating with *A. thaliana* Clr4 homolog, KRYPTONITE (Gendrel *et al.*, 2002; Johnson *et al.*, 2002; Soppe *et al.*, 2002). The fact that both Clr4 and Snf2SR were frequently obtained as the multicopy suppressor of *Sprna1^{ts}* mutants, therefore, indicated that SpRna1 might be required for heterochromatin formation. However, all of *Sprna1^{ts}* strains did not show a silencing defect at the telomere, which is another heterochromatic domain of the chromosomes (Nimmo *et al.*, 1998). Thus, SpRna1 in general was not required for constructing heterochromatin.

SpRna1 seemed to have a tight functional relationship with Clr4, because Clr4 suppressed *Sprna1^{ts}* and Δ *clr4* is synthetic lethal with *Sprna1^{ts}*. Preliminarily, we found that recombinant SpRna1 binds histone H3 and activates the methylase activity of recombinant Clr4 without *S. pombe* Ran, Sp1, although SpRna1 was not coimmunoprecipitated with Clr4 (Nishijima *et al.*, unpublished results), indicating that there is a functional interaction between SpRna1 and Clr4. It is currently being investigated how SpRna1 interacts with Snf2SR and SpKsp1.

The centromeres of *S. pombe* are composed of two domains: the central core and the outer repeat domains (Appelgren *et al.*, 2003), both of which are required for a full function of the centromeres (Nakagawa *et al.*, 2002; Pidoux *et al.*, 2003). The central core, which has a chromatin structure distinct from the outer repeat, possesses the histone H3-variant Cnp1 (*S. pombe* CENP-A homolog). It actually underlies the kinetochores. The gene silencing activity at the central core is maintained by several centromere-associated proteins. For instance, there are two proteins: the centromere protein Sim4 (Pidoux *et al.*, 2003) and the kinetochore protein Mis6 (Takahashi *et al.*, 2000), both of which colocalize at

Figure 8. Synthetic lethality of *Sprna1^{ts} Δclr4* double mutants. (A) Cells with indicated genotypes were grown on YE5S plates at 26°C (left columns), 30°C (middle columns), or 33°C (right columns). Cell lines are shown in the following clockwise order: wild-type, *Δclr4*, *Sprna1^{ts}*, and *Sprna1^{ts} Δclr4*. Images of *Sprna1-47^{ts}* and *Sprna1-86^{ts}* are shown as representatives. (B) Lethality of each *Sprna1^{ts} Δclr4* double mutant. The temperature, at which each *sprna1^{ts}* mutant showed synthetic lethality with *Δclr4* is indicated as asterisks. Temperature-sensitive (ts) lethality is shown as ts lethal.

the centromeres (Pidoux *et al.*, 2003). The inactivation of either Sim4 or Mis6 causes a silencing defect at the central core but not at the outer repeat domains (Pidoux *et al.*, 2003), in contrast to loss of Swi6, which causes a silencing defect at the outer repeat but not at the central core domains (Nakagawa *et al.*, 2002). Swi6 is supposed to maintain heterochromatin structure by binding to histone H3 methylated at Lys 9 (Bannister *et al.*, 2001). Six of eight *Sprna1^{ts}* strains showed a silencing defect at the innermost repeat and only one

mutant, *Sprna1-87^{ts}*, showed a clear silencing defect at the outer repeat domains of the centromeres where Swi6-mediated heterochromatin is formed (Bannister *et al.*, 2001). Consistently, the Swi6-staining pattern was slightly changed in *Sprna1-87^{ts}* compared with that of *Sprna1-86^{ts}*, which showed a silencing defect only at the central core. The level of methylated histone H3-K9 might not be reduced enough for Swi6 to maintain heterochromatin structure, resulting in a gene silencing defect. In contrast, 4 *Sprna1^{ts}* strains showed

a silencing defect at the central core. *Sprrna1-86^{ts}* especially showed a silencing defect only at the central core domain where the histone H3 variant Cnp1 is present. Whether SpRna1 interacts with Cnp1, Sim4, or Mis6 remains to be investigated. Preliminary, we found that SpRna1 binds histone H3 (Nishijima *et al.*, unpublished results). It will be an intriguing question whether SpRna1 also binds to Cnp1 in order to construct the central core. Taken together, SpRna1 might act with Clr4, Snf2SR, histone H3, and perhaps other centromere-associated proteins throughout the centromere, not just at the Swi6-mediated heterochromatic region of the centromeres.

In mammalian cells, RanGAP is reported to be concentrated at the kinetochores and to function for microtubule-kinetochore interaction (Joseph *et al.*, 2004). This report is consistent with our present observation of *Sprrna1^{ts}*, although we do not know whether SpRna1 is localized at *S. pombe* kinetochore. A defect of centromere function observed in *Sprrna1^{ts}* might impair the spindle checkpoint, similar to *sim* mutants (Pidoux *et al.*, 2003), resulting in uneven chromosome segregation, with few lagging chromosomes (Figure 4D). Indeed, *Sprrna1^{ts}* did not exhibit a strong arrest at metaphase.

There are two formal possibilities regarding how SpRna1 functions to form the centromeres. First, SpRna1 participates in nucleocytoplasmic transport, so that proteins required for building centromeres, such as Snf2SR and Clr4, could be assembled onto the centromeres, by the aid of SpRna1. The second possibility is that SpRna1 is required to construct the centromeres, as a novel function of SpRna1. In the first case, overexpression of Spi1 (*S. pombe* Ran), should suppress *Sprrna1^{ts}*, as has been shown in the *SD* system (Kusano *et al.*, 2001). Because *Sprrna1-87^{ts}* has a defect in the nuclear protein import, a clear silencing defect of *Sprrna1-87^{ts}* at the outer repeat domain of *Chromosome 1* centromere could be caused by a defect in the nuclear protein import. However, the *spi1⁺* gene was not isolated as the multicopy suppressor of *Sprrna1^{ts}* strains including *Sprrna1-87^{ts}*. We obtained a lot of the *spi1⁺* gene as a multicopy suppressor of the *pim1^{ts}* mutants (Hirose *et al.*, unpublished results), using the same *S. pombe* genomic library as the presently used one. Furthermore, the *spi1⁺* gene carried on the same vector as the *clr4⁺* gene could not rescue a temperature-sensitive lethality of *Sprrna1^{ts}* (unpublished data). Taking together with the fact that the nucleocytoplasmic transport and the mitotic spindle formation seemed to be normal in most of *Sprrna1^{ts}* strains, the second possibility is currently reasonable. SpRna1, therefore, may have a novel function in the nucleus in order to properly segregate chromosome. Currently, it is unknown how SpRna1 functions to construct the centromeres. Our present finding will pave the way to addressing this question.

Because independently isolated *Sprrna1^{ts}* showed a similar silencing defect at the centromere, it could be concluded that our findings reflect a nuclear function of SpRna1. Because the concentration of Ran-GTP is high in the nucleus for nucleocytoplasmic transport, we reason that there must be some inhibitor(s) or regulator(s) of RanGAP activity for SpRna1 properly functioning in the nucleus, otherwise the nuclear SpRna1 may abolish the Ran-GTP concentration gradient from the nucleus to the cytoplasm. Furthermore, the mechanism of how SpRna1 enters the nucleus and is exported from the nucleus, is as yet unknown.

In conclusion, our present findings suggest that SpRna1 has a novel nuclear function that is required for constructing the centromeres.

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