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Involvement of the Spliceosomal U4 Small Nuclear RNA in Heterochromatic Gene Silencing at Fission Yeast Centromeres

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prp13-1 is one of the mutants isolated in a screen for defective pre-mRNA splicing at a nonpermissive temperature in fission yeast Schizosaccharomyces pombe. We cloned the prp13+ gene and found that it encodes U4 small nuclear RNA (snRNA) involved in the assembly of the spliceosome. The prp13-1 mutant produced elongated cells, a phenotype similar to cell division cycle mutants, and displays a high incidence of lagging chromosomes on anaphase spindles. The mutant is hypersensitive to the microtubule-destabilizing drug thiabendazole, supporting that prp13-1 has a defect in chromosomal segregation. We found that the prp13-1 mutation resulted in expression of the ura4+ gene inserted in the pericentromeric heterochromatin region and reduced recruitment of the heterochromatin protein Swi6p to that region, indicating defects in the formation of pericentromeric heterochromatin, which is essential for the segregation of chromosomes, in prp13-1. The formation of centromeric heterochromatin is induced by the RNA interference (RNAi) system in S. pombe. In prp13-1, the processing of centromeric noncoding RNAs to siRNAs, which direct the heterochromatin formation, was impaired and unprocessed noncoding RNAs were accumulated. These results suggest that U4 snRNA is required for the RNAi-directed heterochromatic gene silencing at the centromeres. In relation to the linkage between the spliceosomal U4 snRNA and the RNAi-directed formation of heterochromatin, we identified a mRNA-type intron in the centromeric noncoding RNAs. We propose a model in which the assembly of the spliceosome or a sub-spliceosome complex on the intron-containing centromeric noncoding RNAs facilitates the RNAi-directed formation of heterochromatin at centromeres, through interaction with the RNA-directed RNA polymerase complex.

The recognition and removal of introns from pre-mRNAs are essential for gene expression in eukaryotic cells. Pre-mRNA splicing takes place in a large complex, the spliceosome, which assembles through ordered interactions of four small nuclear

riboinucleoprotein particles (snRNPs), U1, U2, U4/U6, and U5 snRNPs, and numerous non-snRNP proteins (for a review, see Ref. 1, 2). During assembly of the spliceosome and catalysis of the splicing reaction, the U snRNPs undergo ordered dynamic changes in composition and structure. The U1 and U2 snRNPs initially bind to the pre-mRNA and generate the pre-spliceosome, or complex A. The preformed U4/U6.U5 tri-snRNP is then recruited to complex A to form a pre-catalytic complex B containing all five snRNAs. Subsequently, a large conformational rearrangement occurs in the spliceosome in which U1 and U4 snRNPs are released, accompanied by base-pairing between U6 and U2, and generates complex C. After the first step of splicing, the spliceosome is converted into complex C. The second step generates the mature mRNA product and is followed by the release of the remaining snRNPs from the spliced-out intron (1, 2). In this way, pre-mRNA splicing is performed via RNA-RNA and RNA-protein interactions and each snRNA plays a role in the recognition of pre-mRNA sequences or functions as a catalytic element like a ribozyme (3). U6 snRNA base-paired with U4 snRNA has been believed to have a catalytic role in pre-mRNA splicing. It forms two intermolecular RNA helices (helix I and helix II) with U4 snRNA, both of which are disrupted during the activation of the spliceosome resulting in a subsequent release of U4 snRNA. U6 snRNA interacts with U2 snRNA and the 5′-end of the intron to form part of the catalytic center (3). Only U4 snRNA does not have a sequence for the recognition of pre-mRNA directly. U4 snRNA has been thought of as an RNA chaperone to keep U6 snRNA in a repressed state by masking its catalytic residues (4). Precise roles of U4 snRNA in the spliceosome, however, remain to be clarified.

We and others have isolated prp (pre-mRNA processing) mutants of the fission yeast Schizosaccharomyces pombe, which are defective in pre-mRNA splicing at a nonpermissive temperature (5–8). So far, 14 prp mutants (prp1–prp14) have been isolated, 8 of which produce elongated cells, like cell-division-cycle (cdc) mutants, suggesting a possible link between the pre-mRNA splicing and the cell cycle progression (7, 8).

The centromere is the chromosomal region for the assembly of the kinetochore, which ensures equal segregation of chromosomes at cell division through its interaction with the microtu-
splicing factors, such as Cwf10p and Prp10p, affect the generation of siRNAs from noncoding RNAs and consequently the formation of heterochromatin that is important for centromere function.

It has been shown that factors related to RNA interference (RNAi) play essential roles in the formation of heterochromatin at the centromeres in fission yeast (for a review, see Ref. 10). The outermost dh and dg repeats are packaged into heterochromatin that is important for centromere functions.

The generation of siRNA is dependent on dsRNA synthesis by the RNA-directed RNA polymerase complex (RDRC) and processing by the Dicer ribonuclease, which physically associates with RITS (13, 14). The targeting of RITS by siRNAs then promotes the dimethylation of histone H3-lysine 9 (H3K9me2), the localization of the chromodomain protein Swi6, a heterochromatin protein 1 (HP1) homologue, to the centromeric outer regions (12). The generation of siRNA is dependent on dsRNA synthesis by the RNA-directed RNA polymerase complex (RDRC) and processing by the Dicer ribonuclease, which physically associates with RITS (13, 14). The targeting of RITS by siRNAs then promotes the dimethylation of histone H3-lysine 9 (H3K9me2), the localization of the chromodomain protein Swi6, a heterochromatin protein 1 (HP1) homologue, to the centromeric outer regions (12).

Recently, Bayne et al. (18) reported that defects in specific splicing factors, such as Cwf10p and Prp10p, affect the generation of siRNAs from noncoding RNAs and consequently the centromeric heterochromatin integrity, although the molecular mechanism involved remains unknown. They revealed that Cid12p, a component of RDRC, interacts physically with those splicing factors. In this study, we revealed that a mutation in the spliceosomal U4 snRNA (the prp13-1 mutation) affects the formation of centromeric heterochromatin in S. pombe. We also found, for the first time that the noncoding RNAs involved in the formation of heterochromatin have an intron typical of pre-mRNAs. A possible model for the involvement of splicing factors, such as Cwf10p and Prp10p, affect the generation of siRNAs from noncoding RNAs and consequently the formation of heterochromatin that is important for centromere function.

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and disrupted by glass beads in TELS buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 mM LiCl, and 1% SDS). PCI (phenol/chloroform/isoamyl alcohol, 25:24:1) was added, and the samples were vortexed for 20 s 4 times. After centrifugation, RNAs were precipitated by the addition of ethanol. Following treatment with DNase (Promega), RNA samples were treated with PCI, and precipitated with ethanol. RT-PCR was performed using a PrimeScript RT-PCR kit (TAKARA). The primers used are listed in Table 2.

Silencing Assays—Cell suspensions (2 × 10^5 cells/ml) were serially diluted 5-fold and spotted on N/S plates (nonselective YE medium supplemented with adenine, leucine, and uracil), or 5-FOA plates (N/S plates containing 1 mg/ml 5-fluoroorotic acid). The plates were then incubated at 26 °C for ts mutants or 33 °C for cs mutants for 3–4 days.

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Chromatin Immunoprecipitation—Immunoprecipitation of fixed chromatin with the Swi6 antibody (kindly provided by Dr. Jun-ichi Nakayama) was performed as described previously (15), except that protein A dynabeads (BERITAS) were used. Briefly, cells grown to mid-log phase were fixed with 3% formaldehyde for 30 min. The cells were then ruptured with a Multi beads shocker (Yasui, Co.), and the chromatin was shared using a Biorupter Sonicator (BM Equipment, Co.). The samples were next incubated with the anti-Swi6p antibody overnight, then mixed with protein A dynabeads with rotation at 4 °C for 1 h. After being washed, the samples were decross-linked at 65 °C and treated with protease K.

Competitive PCR of the ura4+ gene to quantify Swi6p enrichment was done using the ura4 DS/E Fw and Rv primers (Table 2). Real-time quantitative PCR was performed in the presence of SYBR Green on a Roche Diagnostics LightCycler with the primers listed in Table 2. Ura4-5 and ura4-6 primers can amplify the ura4+ gene inserted in the otr1R locus specifically. The fbp1+ gene was amplified as a control for the euchromatic region. Data were analyzed with Lightcycler Software version 3.5. ChIP experiments were done independently three times.

Immunostaining—Cells grown to a mid-log phase in YE medium were fixed with 3% formaldehyde and 0.2% glutaraldehyde for 90 min. After washing with PEM buffer (100 mM PIPES, pH 6.9, 1 mM EGTA, and 1 mM MgSO4), the cells were treated with 0.5 mg/ml zymolyase at 37 °C for 8–10 min, then incubated for 2 min in PEMST (PEM containing 1M sorbitol, and 1% Triton X-100). After being washed with PEMS (PEM containing 1 M sorbitol), the cells were treated with the TAT1 monoclonal antibody against tubulin (kindly provided by Dr. Keith Gull) in PEMBAL (PEM containing 0.1M -lysine, 1% bovine serum albumin, and 0.1% sodium azide) at room temperature overnight, and then with anti-mouse IgG antibody conjugated with fluorescein isothiocyanate (FITC) (ICN Biomedicals). After counterstaining with DAPI, fluorescence images of the cells were obtained using an Olympus AX70 fluorescence microscope equipped with a Photometrics Quantix-cooled CCD camera.

RESULTS

Cloning of the prp13+ Gene—To clone the prp13+ gene, we transformed the prp13-1 mutant with the S. pombe genomic library constructed in the cosmid pSS10 (22). We isolated six cosmids that rescue the temperature-sensitive growth of prp13-1 (Fig. 1A). Four clones including cosmid 3 showed the same restriction enzyme-cutting patterns, suggesting that genomic DNA fragments contained in these cosmids are iden-
The prp13 mutation (data not shown). Cosmids 7 and 19 showed restriction enzyme-cutting patterns different from that of cosmid 3. Several DNA fragments from cosmid 7 were subcloned into pSP1, the S. pombe ars1 multicopy vector. We found that a 1.6-kb Spel fragment complemented the temperature-sensitive phenotype of prp13-1 at 36 °C (Fig. 1B). Partial sequencing of the fragment revealed that it contains the U4 snRNA gene (Fig. 1B). PCR analyses using primers specific for U4 snRNA showed that cosmid 3 also contains the U4 snRNA gene (data not shown).

A 7.8-kb BamHI fragment of cosmid 19 also complemented the temperature-sensitive phenotype of prp13-1 at 36 °C. After subcloning, a 3.6-kb SacI fragment was found to complement the prp13 mutation (data not shown). The fragment contained the prp31+ gene that encodes a component of U4/U6/U5 tri-snRNP (24).

To determine the site of mutation in prp13-1, we amplified the gene for U4 snRNA and the prp31+ gene from the prp13-1 genomic DNA and sequenced them directly. We identified one nucleotide change in the U4 snRNA gene in prp13-1; G at position +35 in the 5’ stem-loop structure was replaced with A (Fig. 2). There was no nucleotide change in the prp31+ gene in prp13-1. These results indicate that the U4 snRNA gene is the authentic prp31+ gene, and the prp31+ gene is a multicopy suppressor for prp13-1.

The 5’ stem-loop structure in the Y-shaped U4/U6 DNA domain is essential for the late stage of spliceosome assembly (25, 26) and is a binding site for the 15.5-kDa protein (Snu13p in yeasts) in the U4/U6/U5 tri-snRNPs (27). To elucidate effects of the prp13-1 mutation, we examined if the binding of Snu13p to U4 snRNA is impaired in prp13-1. To that end, we expressed Snu13p tagged with HA in prp13-1 and the wild-type strain HM123. The immunoprecipitation assay showed that U4 snRNA bound to HA-Snu13p significantly decreased in prp13-1, suggesting that the mutation in the 5’ stem-loop structure reduced the ability of U4 snRNA to bind Snu13p (supplemental Fig. S1).

The U4 snRNA Mutation Causes a High Incidence of Lagging Chromosomes—It has been shown that some S. pombe splicing mutants including prp13-1 produce elongated cells at a non-permissive temperature, a typical phenotype of cell division cycle mutants, cdc (7, 8, 28). To examine a cause of the cdc-like phenotype of prp13-1, we stained cells with DAPI to see a manner of the nuclear division in prp13-1. As a result, we found that prp13-1 yields lagging chromosomes on anaphase spindles at both permissive and nonpermissive temperatures (Fig. 3A and supplemental Fig. S2).

It has been shown that fission yeast mutants defective in kinetochore-microtubule attachment essential for chromosomal segregation are generally hypersensitive to the microtubule destabilizing drug thiabendazole (TBZ). We examined the sensitivity to TBZ of prp13-1 and other prp mutants at a permissive temperature. Δago1 and Δdrcl1, the wild-type genes of which are required for the accurate segregation of chromosomes, were used as TBZ-sensitive controls. Serial dilutions of mutant cells were spotted onto YEALU plates without TBZ (N/S), or with 10 μg/ml of TBZ (+TBZ). The plates were incubated at 26 °C for ts- cells or 33 °C for cs- mutants (prp11-1, prp14-2). Δago1 and Δdrcl1 were spotted as controls for defective chromosomal segregation.

The U4 snRNA Mutation Results in Defective Centromeric Silencing—To clarify the causes of the abnormal chromosomal segregation and hypersensitivity to TBZ in prp13-1, we examined if the mutation in U4 snRNA affects the formation of centromeric heterochromatin. In S. pombe, the centromeric region consists of a central core, where the kinetochore is assembled, and some of the imr repeats, is packaged as heterochromatin that silence transcrip-
The formation of heterochromatin at the centromeric outer repeat domain in fission yeast is directed by the RNAi machinery (39). The central core domain is comprised of the inner part of the imr elements. The outer repeat domain encompasses the dg/dh elements and a small part of the imr elements. The ura4+ marker gene was inserted in the inner region or the outer region. Serial dilutions of the cells were spotted onto plates with or without 5-fluoroorotic acid (5-FOA). In contrast, the imr1R or otr1R region was sensitive to 5-FOA, indicating that the ura4+ marker gene was expressed in these strains. The swi6 mutant containing the ura4+ transgene, the wild type of which encodes a chromodomain protein, was used as a control for defects in the formation of centromeric heterochromatin.

Expression of the prp3-3, prp4-2, prp8-1, prp10-1, prp12-1, and prp14-2 mutations in U4 snRNA results in defective gene silencing at the centromere. (A) Schematic representation of the structure of the fission yeast centromere 1. Vertical lines in the imr1L and imr1R regions indicate clusters of (or single) tRNA genes that have been proposed to function as boundary elements (39). The central core domain is comprised of the inner part of the imr elements and the outer repeat domain encompasses the dg/dh elements and a small part of the imr elements. The ura4+ marker gene was inserted in the imr1R or otr1R region. Serial dilutions of the cells containing the inserted ura4+ gene were spotted on YEALU plates (N/S) or YEALU plates containing 1 mg/ml of 5-FOA and incubated at 26 °C. prp13-1 with the ura4+ gene inserted in the imr1R or otr1R region was sensitive to 5-FOA, indicating that the ura4+ marker gene was expressed in these strains. The swi6 mutant containing the ura4+ transgene, the wild type of which encodes a chromodomain protein, was used as a control for defects in the formation of centromeric heterochromatin. C. Expression of the ura4+ mRNAs from the genes inserted in the pericentromere region was confirmed by RT-PCR. Total RNA was isolated from the indicated strains and subjected to RT-PCR using primers corresponding to the dg noncoding RNA and act1 mRNA. No bands were detected in any samples without the reverse transcription reaction (−RT). Act1 mRNA was amplified by RT-PCR as an internal control. Upper and lower arrows denote bands derived from the authentic dg noncoding RNA and its spliced form, respectively.

FIGURE 4. The formation of heterochromatin is affected by the prp13-1 mutation. We carried out a chromatin immunoprecipitation (ChiP) assay using the anti-Swi6 antibody. As shown in Fig. 5, we found that the prp13-1 mutation results in a decreased level of Swi6p in the pericentromeric outer domain, which is consistent with the finding that the processing of noncoding RNAs into siRNAs is severely inhibited in this mutant. Introduction of the wild-type U4 snRNA gene into the prp13-1 mutant recovered reproductively.

The formation of heterochromatin at the centromeric outer repeat domain in fission yeast is directed by the RNAi machinery (11). Thus, we examined if the dg noncoding transcripts transcribed from the otr regions, which are processed into siRNAs that target the RITS complex to the centromere, is accumulated in prp13-1 and other prp mutants. Total RNAs isolated from the prp strains and the ∆dcr1 strain were subjected to an RT-PCR assay using primers for the dg transcript. Reverse transcription was done using the oligo(dT). As shown in Fig. 4D, the dg noncoding transcripts were accumulated in prp13-1 similar to ∆dcr1, suggesting the processing of the centromeric transcripts into siRNA by Dicer to be impaired. Accumulation of the dg transcripts was also observed in prp3-3, prp4-2, prp8-1, prp10-1, prp12-1, and prp14-2.

In fission yeast, histone H3 in the heterochromatin is methylated at lysine 9 (H3K9me2), and this modification becomes a target of the chromodomain protein Swi6 to form silent heterochromatin (29, 30). To examine whether the heterochromatin structure is affected by the prp13-1 mutation, we carried out a chromatin immunoprecipitation (ChiP) assay using the anti-Swi6 antibody. As shown in Fig. 5, we found that the prp13-1 mutation results in a decreased level of Swi6p in the pericentromeric outer domain, which is consistent with the finding that the processing of noncoding RNAs into siRNAs is severely inhibited in this mutant. Introduction of the wild-type U4 snRNA gene into the prp13-1 mutant recovered reproductively.
U4 snRNA Is Involved in Centromeric Silencing

The Centromeric dg Noncoding RNA Has an mRNA-type Intron—The results presented above suggest that spliceosomal U4 snRNA is involved in the processing of centromeric noncoding RNAs into siRNAs and formation of centromeric heterochromatin. In the course of our analysis of the relationship between U4 snRNA and centromeric gene silencing, Bayne et al. (18) also reported that specific splicing factors such as Cwf10p and Prp10p play a role in the generation of siRNA in RNAi-directed centromere silencing. The molecular basis for that phenomenon is, however, unknown.

In relation to the cross-talk between the spliceosomal components and RNAi-directed heterochromatic silencing, we speculated that the centromeric noncoding RNAs have signals to allow the spliceosomal components to assemble on them. We thus searched for sequences matching the consensus sequences of the splice and branch sites in the pericentromeric noncoding RNAs. Interestingly, we found intron-like sequences that contain sequences suitable for the 5' and 3' splice sites and the branch site in the centromeric dg (Fig. 7A) and dh (supplemental Fig. S3) noncoding RNAs.

To examine whether these intron-like sequences are actually spliced, we performed RT-PCR using primers to amplify the dg and dh genomic regions containing the intron-like sequences. As a result, we detected smaller bands corresponding to the spliced form of the dg centromeric noncoding RNAs, in addition to the band derived from the primary transcripts (Fig. 4D). We then sequenced DNAs purified from the shorter band and confirmed that the intron-like sequence is actually spliced (Fig. 7B). We almost the same efficiency as in the wild-type cells (Fig. 6B). This result suggests that the defect in centromeric silencing in prp13-1 did not result from impaired splicing of these RNAi components.

We also confirmed that expression of the wild-type U4 snRNA in prp13-1 could complement the defective gene silencing at centromeres (Fig. 6C). Transformants with the plasmid containing the wild-type U4 snRNA gene grew on the plates with 5-FOA (Fig. 6C, rows 5 and 6), like wild-type cells, whereas the swi6 otr1R::ura4+ and prp13-1 otr1R::ura4+ strains did not (Fig. 6C, rows 2 and 4), indicating that the expression of the wild-type U4 snRNA rescues the defective centromeric silencing. In addition, introduction of the prp31” gene, a multicopy suppressor for prp13-1, complemented the defective heterochromatin silencing at the pericentromeric outer repeat domain in prp13-1 (Fig. 6C, rows 7 and 8).

**FIGURE 5.** The prp13-1 mutation abolished heterochromatin modification at the otr1R::ura4+ locus. A, ChIP analyses of Swi6p at otr1R::ura4+ relative to a euchromatic control locus (ura4 DS/E) were performed with the indicated strains. Relative enrichment was calculated as the ratio of the relative to that of the wild-type U4 snRNA gene (lanes in prp13+/psP1U4). A representative gel is shown. B, real-time quantitative PCR analyses were carried out with the samples analyzed in A to assess the Swi6p enrichment at the otr1R::ura4+ locus relative to that of the tbp1 euchromatic control locus. Error bars represent S.D.

**FIGURE 6.** Impaired pre-mRNA splicing is not a cause of defective centromeric silencing. A, prp13-1 shows weak or no defects in pre-mRNA splicing. Total RNA was isolated from strains cultured at 37 °C for the periods indicated and analyzed by RT-PCR using primers for tub1 or cdc2. White and black arrowheads indicate bands for pre-mRNAs and mature mRNAs, respectively. The cdc2 gene has two introns. B, no severe defects were observed in the splicing of hrr1”, ago1”, and sir2” pre-mRNAs, the products of which are essential for the RNAi pathway. Total RNA extracted from the wild-type 972 cells or prp13-1 cells cultured at 26 °C or 37 °C for 2 h was subjected to a RT-PCR assay using primers for the indicated genes. Ago1 pre-mRNA is spliced with a low efficiency in both the wild-type and prp13-1 cells. C, introduction of the genes for U4 snRNA, Prp31p, and Snu13p recovered the centromeric gene silencing in prp13-1. Each strain or transformant was spotted on YEALU plates with 5-FOA (+ FOA) or without 5-FOA (N/S), and incubated at 26 °C for 5 days. Two independent clones (1 and 2) were spotted for each transformant.
U4 snRNA Is Involved in Centromeric Silencing

(A) SPNCRNA232

| CGTCTTTTTTCCACACAACACGATTTAAATCGATAAAGAATTTTCCA |
| TCCGCAAGTGGGAGTACATCATTCTACTTCGATATTACCTTTTGAAGA |
| AAAGTGAACGGTAATGAAATTTCCAGACACAGAATTATCTGAGCA |
| CATATTATATGCAGAAGCCACAAACAAATATTTAGTTGAGCA |
| GTACAATGCTAATCGACCTCTTTTGGAGTTAATAGGATCCTGGAACAAAC |
| TAGAAAGAAATCCGAAAGGATTTATAGGATCCTGGAACAAAC |
| CATGGTACAGCTTATCGTGTTGTATTATAATCATCAGGCTTCCTCTCTA |
| TATCTCATATCTCCTATATAGATATAAAGATGGAGTTTTGAAGTAG |

(B) FIGURE 7. The centromeric dg noncoding RNA contains an mRNA-type intron. A, nucleotide sequence of the region containing the mRNA-type intron in the centromeric dg element. A part of the genomic region, where the dg noncoding RNA is transcribed from, is shown. The intron region is written in red lowercase letters. The sequences that matched the consensus sequence of the 5' and 3' splice sites in S. pombe are boxed. The putative branch site is underlined. Arrows indicate primers used for the RT-PCR analysis. B, intron-like region in the dg noncoding RNA is precisely removed in the cDNA corresponding to the lower band observed in Fig. 4D. Sequence data determined using the ABI 310 sequencer are shown. A vertical arrow indicates the position of the intron-like sequence in the dg noncoding RNA.

could not detect the splicing of the intron-like sequence in the dh noncoding centromeric transcript (supplementary Fig. S3).

DISCUSSION

In this study, we revealed that a mutation in the spliceosomal U4 snRNA causes a defect in the formation of heterochromatin at the centromere, suggesting a linkage between the splicing machinery and the RNAi-directed formation of centromeric heterochromatin. As the present study was being conducted, Bayne et al. (18) also reported that defects in specific splicing factors affect the generation of centromeric siRNAs and integrity of centromeric heterochromatin. They showed that csp4 and csp5 (centromere: suppressor of position effect), which alleviated silencing of marker genes inserted in the otf of centromere 1, are alleles of the splicing factors Cwf10p and Prp39p, respectively. Interestingly, Cwf10p is a homologue of the S. cerevisiae Snu114p that is required for the unwinding of U4/U6 snRNA (31). Prp39p in S. cerevisiae is known to be a factor associated with U1 snRNA and involved in the early step of the splicing reaction; that is, commitment to the splicing of pre-mRNA (32).

To exclude the possibility that the defect in heterochromatin silencing is a secondary effect of the impaired splicing in the prp mutants, we showed that splicing of the transcripts for major factors related to the RNAi pathway, whose genes have introns, is not blocked significantly at the permissive temperature of 26 °C, the temperature at which chromatin silencing was disrupted (Figs. 4 and 6). To demonstrate that defective splicing in the prp mutants is not a cause of the impaired RNAi-directed gene silencing, Bayne et al. (18) replaced the endogenous intron-containing ago1+ and hrr1+ genes with corresponding intron-less cDNAs and showed that the prp10-1 mutation still alleviated heterochromatin silencing in S. pombe, although we cannot exclude the minor possibility that splicing of unknown essential genes involved in the RNAi system is specifically impaired even at the permissive temperature, resulting in defective gene silencing.

We revealed that centromeric noncoding RNAs contain introns typical of pre-mRNAs (Fig. 7). This is the first report that centromeric noncoding RNAs have introns. The presence of the mRNA-type intron results in the assembly of the splicing factors including U4 snRNP play roles directly in the formation of pericentromeric noncoding RNAs by RNA polymerase II, a group of splicing factors and snRNPs recognize the splice sites and the noncoding RNAs have introns. The presence of the mRNA-type intron results in the assembly of the splicing factors on nascent centromeric noncoding RNAs. Interestingly, it has been shown that spliceosome subunits are co-purified with affinity-selected Cid12p, a component of RDRC involved in dsRNA synthesis, in cells lacking Rdpl (13). Cid12-FLAG was also co-immunoprecipitated with specific splicing factors, such as Cwf10p, in wild-type cells (18), indicating the physical interaction between the splicing factors and the RNAi component Cid12p. It is noteworthy that not all the spliceosomal factors were co-purified with the tagged Cid12p (18).

Fig. 8 shows a hypothetical model for the involvement of splicing factors and snRNPs in the formation of pericentromeric heterochromatin. During transcription of the centromeric noncoding RNAs by RNA polymerase II, a group of splicing factors and snRNPs recognize the splice sites and the branch site in the intron of the nascent transcripts from the centromeric region to form a spliceosome or a sub-spliceosome complex consisting of specific factors that affect processing of centromeric noncoding RNAs to siRNAs. The complex assembled on the noncoding RNAs then facilitates recruitment of...
RDRC through interaction with Cid12p. The assembled spliceosome or the sub-spliceosome complex might function as a platform to facilitate the processing of centromeric noncoding RNAs by RDRC, which synthesizes double-stranded RNAs used for the production of siRNA (18).

High-throughput sequencing of Argonaute-associated siRNAs revealed that siRNAs are generated from the intron of the dg centromeric noncoding RNA, as well as its exons (33). This means that splicing of the noncoding RNAs itself is not necessary for production of the centromeric siRNAs. Recognition of the intron sequences and assembly of the pre-spliceosome seem to be important for the recruitment of RDRC. Further analysis of the roles of the intron sequence in the RNAi pathway for heterochromatic gene silencing is now underway.

We and Bayne et al. (18) showed that some specific prp mutants are defective in heterochromatic gene silencing, thereby suggesting that subsets of splicing factors and snRNPs are involved in the silencing process. We cannot exclude the possibility that exhibition of the defective chromatin silencing phenotype might be dependent on the site of mutation in each prp mutant, as we found allele-specific sensitivity to TBZ in the prp2 mutants, the wild-type gene of which encodes U2AF59 (34) (Fig. 3). prp2-1 was moderately sensitive to TBZ, whereas prp2-2 was highly sensitive to the drug. Both alleles cause severe splicing defects at a nonpermissive temperature (34, 35). However, we could not detect the accumulation of noncoding RNAs in the prp2-1 or prp2-2 mutant, suggesting that sensitivity to TBZ in prp2-2 is not caused by the defect in the RNAi-directed formation of heterochromatin (supplemental Fig. S4). The TBZ sensitivity in prp2-2 might be due to the abnormal nuclear structure in this mutant (36). Thus, it is unlikely that Prp2p is involved in the RNAi-directed centromeric gene silencing.

We revealed that the prp13 mutation, the mutation in the U4 snRNA gene, caused weak splicing defects at the permissive temperature, although it caused severe defects in heterochromatic gene silencing at the same temperature. We demonstrated that the prp13-1 mutation in the 5’ stem-loop structure resulted in decreased binding of U4 snRNA to Snu13p even at the permissive temperature (supplemental Fig. S1). Unstable binding of Snu13p to U4 snRNA might affect the structure of U4/U6 snRNP and the sub-spliceosome complex assembled on the centromeric noncoding RNAs, leading to the decreased recruitment of RDRC and impairment of siRNA amplification. Analyses of the RDRC recruitment to the centromeric noncoding RNAs in the presence of the prp13 mutation or in the absence of the intron are now underway.

Recently, it was reported that the first cleavage reaction by the spliceosome generates the mature 3’-end of telomerase RNA in S. pombe (37). The results of the present study provide another line of evidence that the spliceosome is potentially multifunctional and has evolved to play essential roles not only in pre-mRNA splicing, but also in other biological processes, such as the maturation of telomere RNA and heterochromatic gene silencing, during evolution.

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U4 snRNA Is Involved in Centromeric Silencing

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