Importance of polyadenylation in the selective elimination of meiotic mRNAs in growing *S. pombe* cells

Soichiro Yamanaka, Akira Yamashita, Yuriko Harigaya[1], Ryo Iwata and Masayuki Yamamoto*

Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Hongo, Tokyo, Japan

A number of meiosis-specific mRNAs are initially weakly transcribed, but then selectively removed during fission yeast mitotic growth. These mRNAs harbour a region termed DSR (determinant of selective removal), which is recognized by the YTH family RNA-binding protein Mmi1p. Mmi1p directs the destruction of these mRNAs in collaboration with nuclear exosomes. However, detailed molecular mechanisms underlying this process of selective mRNA elimination have remained elusive. In this study, we demonstrate the critical role of polyadenylation in this process. Two-hybrid and genetic screens revealed potential interactions between Mmi1p and proteins involved in polyadenylation. Additional investigations showed that destruction of DSR-containing mRNAs by exosomes required polyadenylation by a canonical poly(A) polymerase. The recruitment of Pab2p, a poly(A)-binding protein, to the poly(A) tail was also necessary for mRNA destruction. In cells undergoing vegetative growth, Mmi1p localized with exosomes, Pab2p, and components of the polyadenylation complex in several patchy structures in the nucleoplasm. These patches may represent the sites for degradation of meiosis-specific mRNAs with untimely expression.

Introduction

Most eukaryotic cells have the genetic potential to carry out meiosis, but in reality only a limited population of cells, such as germ cells, actually performs meiotic division. As the untimely expression of meiotic genes during vegetative proliferation could be destructive, it is presumable that cells strictly separate the

*Corresponding author. Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Hongo, Tokyo 113-0033, Japan. Tel.: +81 3 5841 4386; Fax: +81 3 5802 2042; E-mail: yamamoto@biochem.s.u-tokyo.ac.jp
[1]Present address: Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ 85721-0106, USA

mitotic and meiotic cell division programmes. Consistent with this hypothesis, our previous analyses revealed that the fission yeast, *Schizosaccharomyces pombe*, possesses a mechanism for the elimination of meiosis-specific mRNAs from vegetatively growing cells (Harigaya *et al*, 2006). These mRNAs harbour a unique region, termed DSR (determinant for selective removal), which serves as a marker for mRNA degradation. Mmi1p was identified as a new type of RNA-binding protein that binds to the DSR region and directs transcript degradation. Furthermore, we also observed that Mei2p, the master regulator of meiosis in fission yeast (Watanabe and Yamamoto, 1994; Watanabe *et al*, 1997), sequesters Mmi1p in a nuclear dot structure (Mei2 dot) during meiotic prophase to prevent Mmi1p from directing the degradation of meiosis-specific mRNAs during this stage of cell division (Harigaya *et al*, 2006). The Mei2 dot was observed to be composed of Mei2p and meiRNA encoded by the *sme2* gene, and to be attached to the *sme2* locus on chromosome II (Watanabe *et al*, 1997; Yamashita *et al*, 1998; Shimada *et al*, 2003).

The transcription of meiotic genes does not stop completely during vegetative growth in fission yeast, and elimination of unnecessary meiosis-specific messages by Mmi1p seems to be physiologically indispensable, as growth is severely impaired if cells lose Mmi1p expression (Harigaya *et al*, 2006). The mRNA elimination system involving DSR and Mmi1p is likely to be the first example of a mechanism to selectively remove unnecessary mRNA species to maintain a certain cellular status. Hence, it is of great interest to clarify of the detailed mechanisms responsible for this selective elimination. Mmi1p is a relatively small protein of 488 amino acids with no obvious known features, other than a putative YTH family RNA-binding domain. Mmi1p also seems to cooperate with the exosome, a multi-subunit protein complex with nuclease activity (Mitchell *et al*, 1997; Allmang *et al*, 1999), to degrade mRNAs within the nucleus (Harigaya *et al*, 2006).

To gain further insight into the molecular mechanisms that underlie selective elimination of DSR-containing mRNAs, we set out to identify and characterize new components of this targeted degradation system. The analysis of the factors identified in the search delineates polyadenylation of the target mRNAs and subsequent recruitment of a poly(A)-binding protein to them as crucial steps in selective mRNA degradation.

Results

Identification of factors involved in 3′-end processing of mRNA, which participate in selective mRNA elimination

To search for factors that might cooperate with Mmi1p in facilitating selective elimination of meiosis-specific mRNAs, we carried out a genome-wide, yeast two-hybrid screen

using Mmi1p as bait. Several candidates, including Rna15p and Pab2p, were identified as possible Mmi1p-interacting proteins in this analysis (Supplementary Figure S1). Rna15p (SPAC644.16) is an apparent orthologue of *Saccharomyces cerevisiae* RNA15, which is a subunit of the multi-subunit cleavage factor CF1A, a component of the polyadenylation complex (Minvielle-Sebastia *et al*, 1994; Kessler *et al*, 1996). Pab2p is a previously characterized nuclear poly(A)-binding protein (Perreault *et al*, 2007).

To identify factors that might be necessary to promote selective mRNA elimination, we also carried out a screen for mutations that could suppress meiotic arrest in the *sme2Δ* mutant, based on the observation that a reduction in Mmi1p activity suppressed this arrest (Harigaya *et al*, 2006). The *sme2Δ* mutant lacks meiRNA and cannot form the previously characterized Mei2 dot structures (Watanabe *et al*, 1997; Yamashita *et al*, 1998; Shimada *et al*, 2003), resulting in a failure to downregulate Mmi1p. In this screen, we observed that an insertion introduced in an intron of the *pla1* gene, which encodes a poly(A) polymerase composing the polyadenylation complex (Ohnacker *et al*, 1996), could suppress *sme2Δ* (Supplementary Figure S2). This insertion (*pla1-10*) resulted in the production of a truncated form of Pla1p (Supplementary Figure S2).

We confirmed the physical interaction of Mmi1p with Pla1p, Rna15p and Pab2p by immunoprecipitation and pull-down analyses, as shown in Supplementary Figure S3. Given these results, we tested whether the loss of function in *pla1*, *rna15* or *pab2* could affect expression of meiosis-specific transcripts in vegetative cells. As *pla1* and *rna15* are essential for cell growth, we isolated temperature-sensitive (ts) mutants of these two genes and used a viable *pab2* deletion mutant in subsequent experiments. As shown in Figure 1A, cells of each mutant at least partially accumulated meiosis-specific mRNAs when the respective gene function was eliminated. However, the levels of mRNA accumulation in these mutants were generally lower than those observed in the *mmi1-ts3* mutant. The pattern of affected mRNAs also seemed to vary to some extent according to the gene mutated, suggesting an underlying complexity of the whole system relevant to selective mRNA elimination.

We then evaluated whether the mutations that we created in *pla1*, *rna15* and *pab2* could suppress meiotic arrest in *sme2Δ* cells. As shown in Figure 1B, the mutants suppressed the *sme2Δ* phenotype. To our surprise, the *pla1-ts37* and *rna15-ts10* mutations could suppress the *sme2Δ* phenotype at 25°C (Figure 1B), although strains carrying these mutations exhibited no obvious growth retardation at this temperature (data not shown). As *pla1-10*, the original insertion mutation that suppressed *sme2Δ*, did not seem to retard vegetative growth, a defect in the polyadenylation complex might have a more significant impact on meiotic transcripts than on mitotic transcripts. Overall, the observed genetic interactions suggested that polyadenylation might contribute to the selective elimination of meiosis-specific mRNAs in mitotic cells.

Excessive polyadenylation of meiosis-specific mRNAs generated in an exosome mutant

We have previously shown that Mmi1p could physically interact with Rrp6p (Harigaya *et al*, 2006), which is a nucleus-specific subunit of the exosome (Briggs *et al*, 1998;
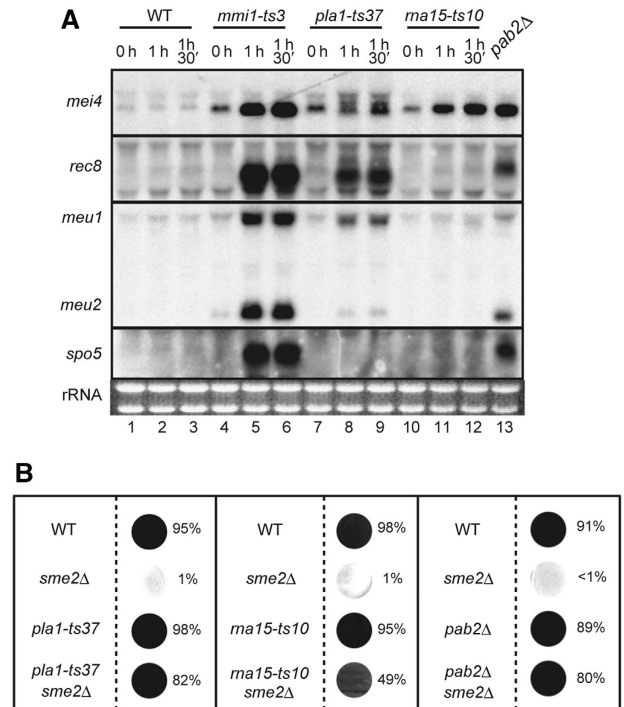

Figure 1 Components of the polyadenylation complex and the poly(A)-binding protein Pab2p contribute to the elimination of DSR-containing mRNAs. (**A**) JY450 (WT), JV564 (*mmi1-ts3*), JT452 (*pla1-ts37*) and JT453 (*rna15-ts10*) cells were cultured at 25°C in YE liquid medium and then shifted to 37°C. After the indicated times, total RNA was extracted from each sample and processed for northern blot analysis (lanes 1–12). For JT454 (*pab2Δ*), total RNA was extracted from cells cultured at 30°C (lane 13). A total of 5 µg of RNA was loaded in each lane. (**B**) Suppression of *sme2Δ* by *pla1-ts37*, *rna15-ts10* and *pab2Δ*. JY450 (WT), JZ464 (*sme2Δ*), JT452 (*pla1-ts37*), JT453 (*rna15-ts10*), JT455 (*pla1-ts37 sme2Δ*) and JT456 (*rna15-ts10 sme2Δ*) cells were cultured in YE medium at 25°C and spotted onto an SSA plate. Incubation was continued at 25°C for 4 days, and then cells were stained with iodine and the sporulation efficiency of each cell line was calculated. JT454 (*pab2Δ*) and JT457 (*pab2Δ sme2Δ*) cells and control JY450 and JZ464 cells were cultured at 30°C and after 2 days the sporulation efficiency was measured at 30°C.

Burkard and Butler 2000). Furthermore, a defect in Rrp6p was as effective in suppressing the *sme2Δ* phenotype as a reduction in Mmi1p activity, suggesting that exosomes were likely to eliminate meiosis-specific mRNAs within the cell nucleus (Harigaya *et al*, 2006). We next examined the expression of meiosis-specific transcripts in the ts exosome mutant cell lines, *rrp6* and *dis3*; the latter of which encodes a core subunit of the exosome (Ohkura *et al*, 1988; Dziembowski *et al*, 2007; Murakami *et al*, 2007). As shown in Figure 2A, the transcripts of five meiotic genes, namely *mei4*, *rec8*, *meu1*, *meu2* and *spo5*, were detected in both *rrp6-ts32* and *dis3-ts4* cells shifted to the restrictive temperature in growth medium (lanes 8, 9, 11 and 12) at levels that were comparable to those observed in *mmi1-ts3* cells (lanes 5 and 6). These transcripts were never induced by a temperature shift in wild-type cells, indicating that nuclear exosomes are required for the elimination of meiosis-specific mRNAs during vegetative growth (lanes 2 and 3; Harigaya *et al*, 2006).

In the exosome mutants, we noted that some ectopically expressed, meiosis-specific transcripts appeared to be smeary and larger than the natural transcripts produced during
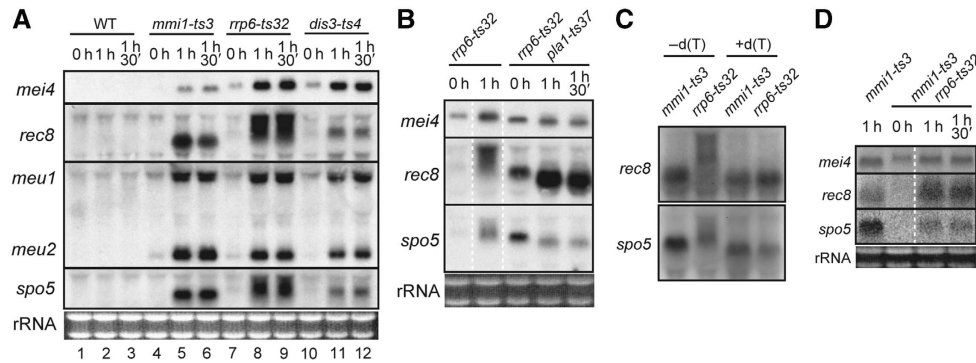
Figure 2 DSR-containing mRNAs suffer Pla1p-dependent excessive polyadenylation in the *rrp6-ts32* strain at the restrictive temperature. (**A**) Northern blot analysis of the expression of meiosis-specific genes *mei4*, *rec8*, *meu1*, *meu2* and *spo5* in exosome mutants. Cells were cultured in YE liquid medium at 25°C, shifted to the restrictive temperature 37°C and then sampled for RNA extraction after incubation for the indicated times. Lanes 1–3, JY450 (WT; wild-type); lanes 4–6, JV564 (*mmi1-ts3*); lanes 7–9, JT432 (*rrp6-ts32*); and lanes 10–12, JT449 (*dis3-ts4*). Total RNA (5 μg) was loaded in each lane. (**B**) JT432 (*rrp6-ts32*) and JT458 (*rrp6-ts32 pla1-ts37*) cells were cultured at 25°C, and then shifted to 37°C. At the indicated times, total RNA was extracted from each sample and processed for northern blot analysis as in (**A**). (**C**) Northern blots of RNase H-treated *rec8* and *spo5* transcripts derived from growing mutant strains as indicated. RNase H treatment was performed in the absence (lanes 1 and 2) and presence (lanes 3 and 4) of poly(dT). (**D**) JV564 (*mmi1-ts3*) and JT496 (*mmi1-ts3 rrp6-ts32*) cells were cultured at 25°C, and then shifted to 37°C. At the indicated times, total RNA was extracted from each sample and processed for northern blot analysis as in (**A**).

meiosis or those expressed in the *mmi1-ts3* mutant. For example, *rec8* transcripts accumulated in *rrp6-ts32* cells were extremely larger, *spo5* transcripts were substantially larger and *mei4* transcripts were slightly larger in comparison with natural transcripts (Figure 2A). In the experiments described below, we tested whether the increase in the size of these transcripts might result from excessive polyadenylation.

First, we analysed *rec8*, *spo5* and *mei4* transcripts produced in the absence of active canonical poly(A) polymerase. As shown in Figure 2B, the increase in transcript size observed in the *rrp6-ts32* mutant was almost completely lost in the *rrp6-ts32 pla1-ts37* double mutant, supporting the idea that the increase was caused by polyadenylation. Notably, the double mutant accumulated a considerable quantity of meiosis-specific transcripts at the permissive temperature 25°C, implying that the *rrp6-ts32* and the *pla1-ts37* alleles were both partially defective at this temperature, although each single mutant showed no obvious growth defect. The confirmation that the *rrp6-ts32* allele was not fully functional at 25°C was based on the observation that the *rrp6-ts32 pab2Δ* double mutant suffered severe growth retardation at this temperature (data not shown).

To estimate the length of poly(A) additions, poly(A) tracts were removed by RNase H digestion in the presence of oligo(dT) (Hausen and Stein, 1970). After digestion, both *rec8* and *spo5* transcripts yielded a single, non-smeary band in gel electrophoresis of a size that was close to that observed for fragments derived from *mmi1-ts3* cells (Figure 2C). These results strongly suggest that *rec8* and *spo5* transcripts that have escaped from destruction by exosomes are likely to exhibit various extents of excessive polyadenylation.

Interestingly, excessive polyadenylation of *rec8*, *spo5* and *mei4* transcripts, as was seen in the *rrp6-ts32* mutant, did not occur in the *mmi1-ts3 rrp6-ts32* double mutant (Figure 2D). This suggested that Mmi1p was responsible for the excessive polyadenylation observed in *rrp6-ts32* cells, and that the polyadenylation reaction involving Mmi1p might be unusual either quantitatively or qualitatively.

Meanwhile, slow migrating bands were also observed for *mei4* mRNA produced in *pla1-ts37* cells shifted to the restrictive temperature, though their intensity was moderate

(Figure 1, lanes 8 and 9). They turned out not to represent excessive polyadenylation. Transcripts in these bands were not homogeneous with regard to the 3′ end, but they generally carried a shorter poly(A) tail and a longer 3′ UTR stretch, which apparently resulted from a failure in cleavage at the proper polyadenylation site (data not shown).

Polyadenylation is required for the selective elimination of meiosis-specific mRNAs

To establish the significance of polyadenylation for the elimination of meiosis-specific transcripts, we devised a system for transcribing a DSR-containing reporter gene with or without the addition of a poly(A) tail. The reporter constructs used are shown schematically in Figure 3A. The parental construct (Construct 1) was comprised of the constitutive *adh1* promoter, the jellyfish green fluorescent protein (GFP) open reading frame (ORF), the DSR region derived from *spo5* and the terminator region of *adh1*. The transcripts produced from Construct 1 were barely detectable in mitotic cells (Figure 3B, lane 1). To confirm that DSR-dependent selective elimination was functional for Construct 1, we substituted the DSR sequence with a non-functional sequence termed DSR-M10 that had six nucleotide substitutions (data not shown). The resultant construct (Construct 2) escaped degradation, resulting in transcript accumulation within mitotic cells (Figure 3B, lane 2). We then proceeded to examine the effect of a poly(A) tail in this system. To terminate transcripts with no poly(A) addition, we replaced the *adh1* terminator in Construct 1 with the terminator of the *snu2* gene, which encodes a small nuclear RNA (Brennwald *et al*, 1988; Zhou *et al*, 1999). Interestingly, this construct (Construct 3) produced detectable amounts of transcripts in mitotic cells, which were nearly as much as its counterpart with DSR-M10 instead of a functional DSR (Construct 4; Figure 3B, lanes 3 and 4). Data were quantified by measuring the intensity of each band in Figure 3B and then calculating the ratio between the counterparts bearing DSR and DSR(M10) (Figure 3C). These results suggest that a poly(A) tail is necessary for targeting DSR-containing mRNAs for selective elimination.
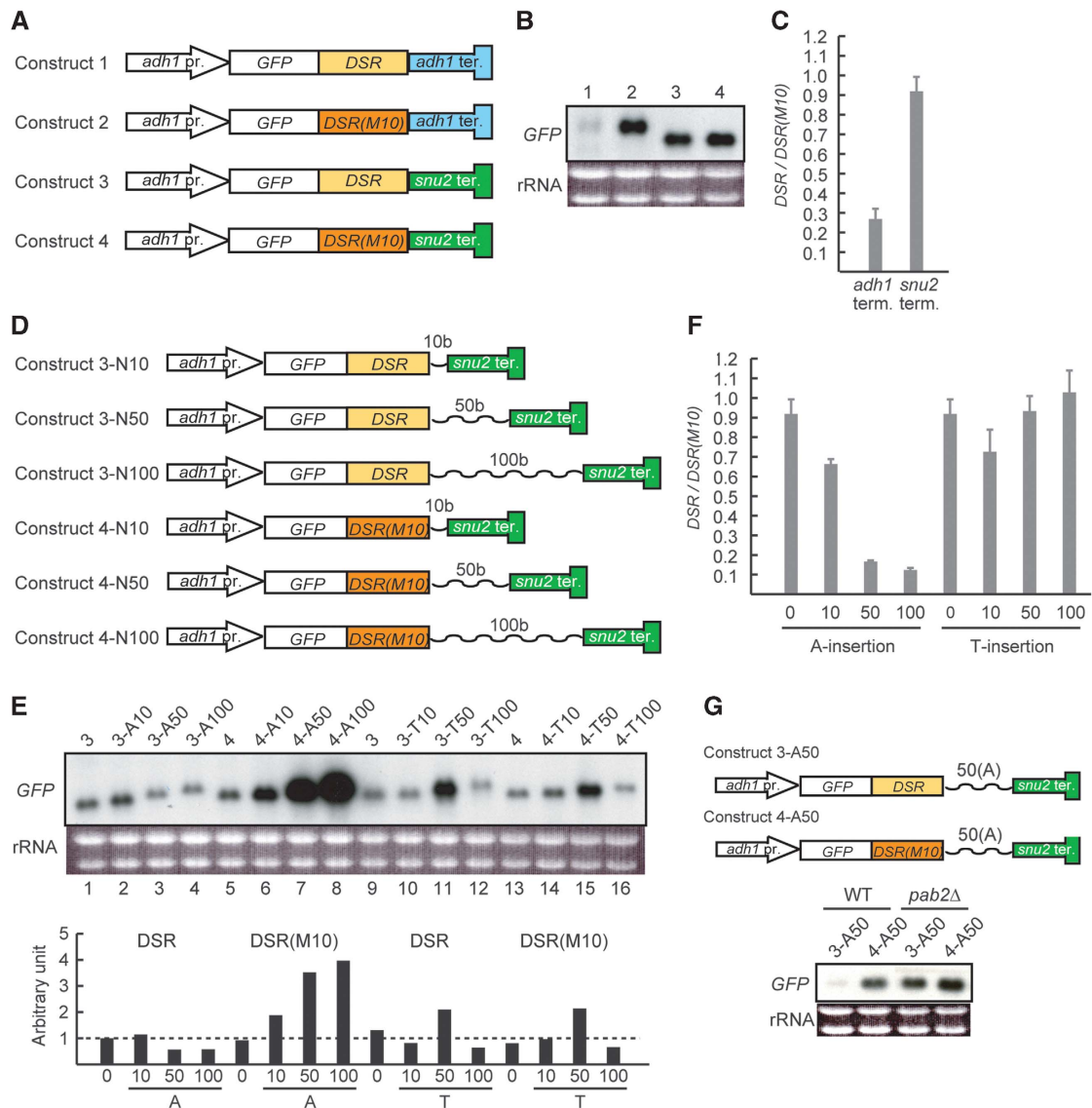
Figure 3 A poly(A) tail is required for the elimination of DSR-containing RNA. (**A**) Schematic illustration of the reporter constructs used in (**B**). A detailed explanation of each construct is provided in the text. *adh1* pr., the promoter for the *adh1* gene; GFP, the ORF for jellyfish green fluorescent protein; DSR, the DSR sequence of the *spo5* gene; DSR(M10), a defective form of DSR; *adh1* ter., the terminator for the *adh1* gene; and *snu2* ter., the terminator for the *snu2* gene. (**B**) Constructs 1–4, as shown in (**A**), were integrated at the *lys1* locus of the parental strain JY333. The resulting four strains were cultured in YE at 30°C. Total RNA was extracted from each culture and analysed by northern blot analysis. RNA (5 µg) was loaded in each lane and the *GFP* sequence was used as the probe. (**C**) Comparison of the band intensity in (**B**) between lanes 1 and 2 (*adh1* term.) and lanes 3 and 4 (*snu2* term.). (**D**) Schematic illustration of the reporter constructs used in (**E**). Either a poly(dA) tract or a poly(dT) tract was inserted after the DSR sequence in Construct 3 and Construct 4. The tract length was 10, 50 or 100 bases. (**E**) Constructs carrying a poly(dA) or poly(dT) tract, as shown in (**D**), were integrated at the *lys1* locus of JY333. The resulting strains were examined for *GFP* expression by northern blot analysis as described in (**B**). The intensity of each band was measured and normalized by the amount of rRNA as the loading control, which is graphically shown under the northern blot panel. (**F**) Comparison of the band intensity in (**E**) between lanes 1 and 5 (A-0), lanes 2 and 6 (A-10), lanes 3 and 7 (A-50), lanes 4 and 8 (A-100), lanes 9 and 13 (T-0), lanes 10 and 14 (T-10), lanes 11 and 15 (T-50) and lanes 12 and 16 (T-100). (**G**) Evaluation of the necessity of Pab2p for the elimination of DSR-containing RNA with an artificial poly(A) tract. The expression of *GFP* was monitored by northern blot analysis in wild-type (JY450) and *pab2Δ* (JT454) cells carrying either Construct 3-A50 or 4-A50.

To determine whether artificial addition of a poly(A) tract could stimulate the elimination of DSR-containing transcripts, we inserted poly(dA) sequences of various lengths between the DSR and the *snu2* terminator on Construct 3, as is schematically shown in Figure 3D (Construct 3-A10, 10 nucleotides; Construct 3-A50, 50 nucleotides; and Construct 3-A100, 100 nucleotides). Corresponding constructs were also prepared from Construct 4, which carried DSR-M10 (Constructs 4-A10, 4-A50 and 4-A100; Figure 3D). Although the

transcripts produced in this system did not carry a poly(A) tail at their very 3′ end, the presence of a poly(A) tract seemed to perform a stabilizing function for the transcripts of derivatives of Construct 4; more transcripts accumulated within the cell as the poly(A) tract became longer (4-A10 through 4-A100; Figure 3E, lanes 5–8). However, this effect was not observed for derivatives of Construct 3, which carried the DSR. In these cells, the amount of transcripts did not increase, but rather decreased, as the poly(A) tract became longer (3-A10 through
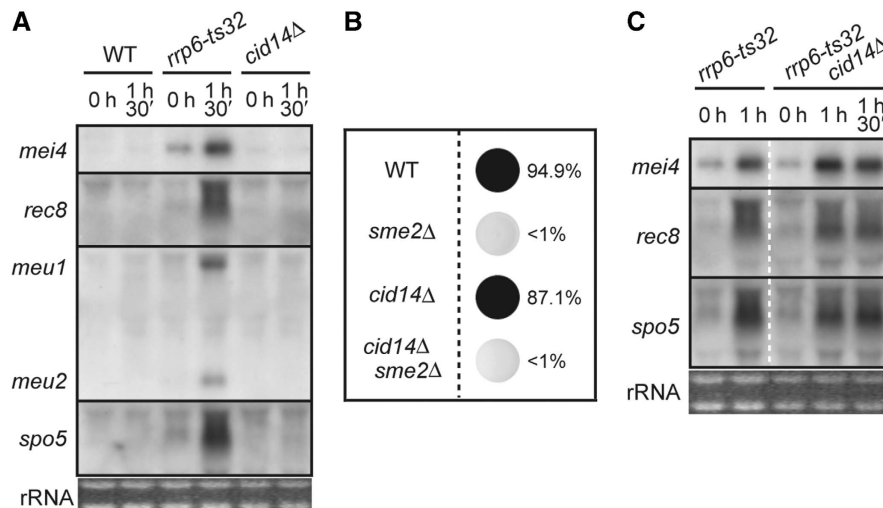
Figure 4 Cid14p is most likely not involved in the Mmi1-dependent selective elimination of the meiotic mRNAs (**A**) JY450 (WT), JT432 (*rrp6-ts32*) and JT654 (*cid14Δ*) cells were cultured at 25°C in YE medium, and then shifted to 37°C. At the indicated times, total RNA was extracted from each sample and processed for northern blot analysis. (**B**) JY450 (WT), JZ464 (*sme2Δ*), JT654 (*cid14Δ*) and JT655 (*cid14Δ sme2Δ*) cells were cultured in YE medium at 30°C and spotted onto an SPA plate. Incubation was continued at 30°C for 1 day, and then cells were stained with iodine and the sporulation efficiency was calculated. (**C**) JT432 (*rrp6-ts32*) and JT656 (*rrp6-ts32 cid14Δ*) cells were cultured at 25°C in YE medium, and then shifted to 37°C. At the indicated times, total RNA was extracted from each sample and processed for northern blot analysis.

3-A100; Figure 3E, lanes 1–4). This difference was obvious when the ratio between the counterparts with and without the functional DSR was plotted (Figure 3F).

To confirm the specificity of poly(A), we prepared analogues of the above constructs by substituting poly(dA) with poly(dT) to produce Constructs 3-T10, 3-T50, 3-T100, 4-T10, 4-T50 and 4-T100 (Figure 3D). Transcript abundance was comparable between 3-T10 and 4-T10, between 3-T50 and 4-T50 and between 3-T100 and 4-T100 (Figure 3E and F), although the T50 constructs exhibited abundance nearly twice as much as the others for reasons unknown. These data indicate that DSR-dependent, selective mRNA elimination does not operate using a poly(U) tract. Thus, it seemed that a poly(A) tract normally stabilizes the transcripts in this system, but the presence of a DSR sequence antagonizes this stabilization.

As shown in Figure 1A, Pab2p function was required for the degradation of endogenous DSR-containing mRNAs. This necessity for degradation was confirmed using the artificial reporter constructs Construct 3-A50 and Construct 4-A50. The former, which harboured a functional DSR, underwent transcript elimination in growing wild-type cells. However, both constructs accumulated transcripts in growing *pab2Δ* cells, indicating that Pab2p was necessary for the elimination of transcripts derived from Construct 3-A50 (Figure 3G). Thus, an important function of a poly(A) tract in selective mRNA elimination may be facilitating the recruitment of Pab2p to the transcript targeted for degradation.

The TRAMP complex is unlikely to be involved in selective elimination of meiotic mRNAs

There have been reports of polyadenylation activating the degradation of certain RNA species by exosomes. The best-studied case in this regard may be the system involving the TRAMP protein complex (LaCava *et al*, 2005; Vanacova *et al*, 2005; Wyers *et al*, 2005). The TRAMP complex has poly(A) polymerase activity and polyadenylates various substrates,

including rRNA, tRNA, snRNA and snoRNA, before substrate degradation. We therefore addressed whether the TRAMP complex was relevant to the selective elimination of meiosis-specific mRNAs. In fission yeast, Cid14p is most probably the catalytic subunit of TRAMP responsible for poly(A) polymerization (Win *et al*, 2006; Bühler *et al*, 2007). We examined the expression of meiosis-specific transcripts in viable, vegetatively growing *cid14Δ* cells. As shown in Figure 4A, the accumulation of meiosis-specific transcripts was not observed in these cells, which was in contrast to the results for *rrp6-ts32* cells, which were used as a control. Furthermore, *cid14Δ* did not suppress meiotic arrest in *sme2Δ* cells, unlike what occurred with a defect in *mmi1* or *rrp6* (Figure 4B). These observations suggest that the TRAMP complex is unlikely to have a major role in DSR-dependent, selective mRNA elimination.

We also tested whether Cid14p was relevant to the excessive polyadenylation of meiosis-specific transcripts in *rrp6-ts32* cells. The *rrp6-ts32* mutant and *rrp6-ts32 cid14Δ* double mutant exhibited quite similar transcript patterns (Figure 4C), indicating that the contribution of Cid14p to excessive polyadenylation, if any, is probably much smaller than the contributions of Pla1p. However, as the size of the transcripts seemed to be slightly reduced in the double mutant, we cannot completely dismiss the possibility that Cid14p may have a minor influence on this selective elimination system.

Subcellular localization of the factors involved in the selective elimination

We have previously reported that Mmi1p forms several patchy structures in the nucleus of growing cells (Harigaya *et al*, 2006). To determine whether the factors cooperating with Mmi1p might also localize to these patches, Rrp6p, Dis3p, Pla1p and Pab2p were tagged with a fluorescent marker using expression constructs. The majority of Rrp6p and Dis3p proteins, which compose the exosome, was
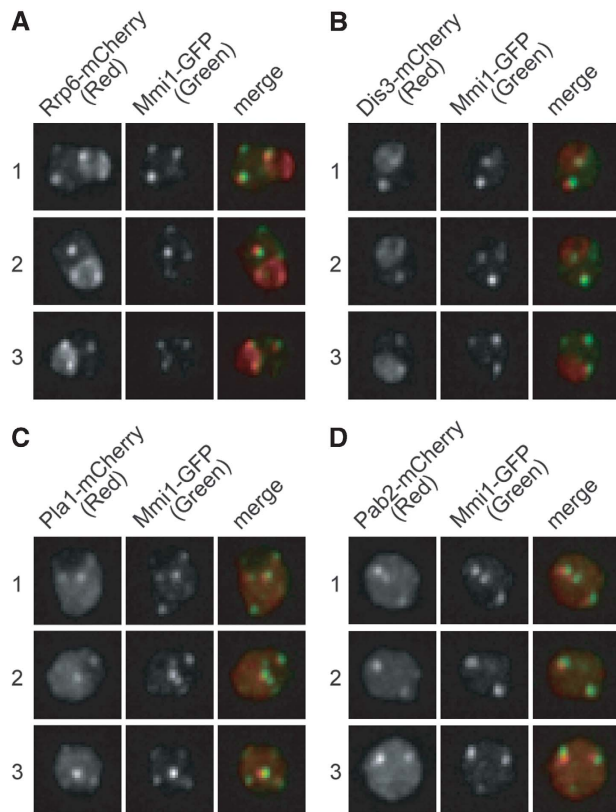
Figure 5 Factors involved in selective elimination of DSR-containing mRNA form patchy structures in the nucleoplasm. (A–D) GFP-tagged Mmi1p (Mmi1–GFP) was co-expressed with either (**A**) Rrp6–mCherry, (**B**) Dis3–mCherry, (**C**) Pla1–mCherry or (**D**) Pab2–mCherry from the respective endogenous promoters. Three independent cells (numbered 1–3) were examined for each combination. An image of the nuclear region, stacked along the *z*-axis, is shown.

localized rather evenly in the nucleolus and also formed bright patches in the nucleoplasm. These patches showed co-localization with Mmi1p (Figure 5A and B).

Pla1p was localized evenly in the nucleoplasm, occasionally forming patchy foci (Supplementary Figure S4). Most of these foci seemed to coincide with the Mmi1p patches (Figure 5C). Pab2p localized to the nucleolus and the nucleoplasm and formed patchy foci in the latter. Again, these foci seemed to coincide with the Mmi1p patches (Figure 5D). On the basis of these observations, we propose that the machinery for the selective meiotic mRNA elimination is localized to several patchy multi-protein structures in the nucleoplasm, which include the following components: Mmi1p, exosomes, canonical poly(A) polymerase, a poly(A)-binding protein and possibly some additional factors. Similar analyses have shown that Rna15p also co-localizes with Mmi1p (data not shown). Cid14p is reported to be enriched in the nucleolus, but it does not seem to form patchy structures in the nucleus (Win *et al*, 2006). These data may provide additional evidence that the TRAMP complex is unlikely to participate in this Mmi1p-dependent mRNA elimination system.

Discussion

In this study, we have demonstrated that the acquisition of a poly(A) tail is crucial for mRNA degradation by exosomes in the DSR/Mmi1p-dependent selective elimination system.

An important role for the poly(A)-binding protein, Pab2p, in the system has been also demonstrated.

A polyadenylation-mediated RNA degradation system has been well established in bacteria and was the subject of a recent review (Richards *et al*, 2008). In *Escherichia coli*, mRNA stability is established through a stem-loop structure at the 3′ end of the mRNA. When an mRNA is to be degraded, it is cleaved by endoribonuclease RNase E, the major component of the degradosome. This cleavage provides a signal for the polyadenylation of the resulting fragments involving the recruitment of poly(A) polymerase I (PAP I) to the fragments, most likely as a result of its interaction with the degradosome. Poly(A) polymerase I adds a poly(A) tail to the fragments, which in turn promotes the binding of polynucleotide phosphorylase (PNPase), another component of the degradosome. On binding to the poly(A) tail, PNPase degrades the RNA fragment in cooperation with the helicase RhlB, which is also a component of the degradosome. It should be noted that polyadenylation is now established as having a much more crucial role in bacterial RNA quality control than was previously anticipated. Thus, the basic scheme for selective elimination of meiosis-specific mRNAs in fission yeast seems to have many similarities to this bacterial system, except that the bacterial system represents a general degradation mechanism involving most mRNAs with no apparent strong sequence specificity for the mRNA target.

By contrast, polyadenylation is thought to facilitate the function of mRNAs in eukaryotic cells through mRNA stabilization, promotion of cytoplasmic export and stimulation of translation. Thus, contrary to prokaryotes, eukaryotes use mRNA polyadenylation for the prevention of transcript degradation. However, recent studies have provided examples whereby polyadenylation activates the degradation of certain eukaryotic RNA species by exosomes. In this regard, of particular interest is the TRAMP complex (LaCava *et al*, 2005; Vanacova *et al*, 2005; Wyers *et al*, 2005), which has poly(A) polymerase activity and polyadenylates various substrates, including rRNA, tRNA, snRNA and snoRNA before their destruction. For example, TRAMP polyadenylates tRNAs that are not methylated properly and converts them to degradation substrates for exosomes, thereby contributing to the quality control of a cell's tRNA population (Kadaba *et al*, 2004). Recent studies have also indicated that polyadenylation of yeast cryptic unstable transcripts (CUTs) by TRAMP directs them to degradation by nuclear exosomes (Wyers *et al*, 2005). Our analyses, however, suggest that the TRAMP complex is not directly relevant to the selective elimination of meiosis-specific mRNAs. Rather, this selective elimination system apparently uses the general polyadenylation complex, which includes Pla1p and Rna15p, to polyadenylate transcripts targeted for degradation.

We also describe the unexpected observation that mutation of *pla1-ts37* or *rna15-ts10* could suppress *sme2Δ* at the permissive temperature for vegetative growth. In our original screening, we isolated an insertion mutation in *pla1*, which permitted vegetative growth but suppressed meiotic arrest in the *sme2Δ* strain. These observations indicate that a small flaw in the polyadenylation system that does not severely affect mRNA production can effectively block the Mmi1p-dependent selective elimination mechanism.

A recent study has shown that the fission yeast *pab2Δ* strain accumulates excessively polyadenylated snoRNAs,

although it does not seem to affect mRNAs (Lemay *et al*, 2010). The authors proposed that Pab2p promotes poly(A) tail trimming from pre-snoRNAs through nuclear exosome recruitment. Whether this observation is directly relevant to the function of Pab2p in selective elimination remains unclear, but it is interesting to note that Pab2p apparently activates exosomes in both systems.

The important question of how eukaryotic cells determine whether polyadenylated RNAs associated with nearly the same protein complex are targeted to the exosome for degradation or to the cytoplasm for function remains unanswered (Libri, 2010). Although the data obtained in this study are not yet sufficient to establish a clear mechanistic scheme for the selective elimination of mRNA, we would like to remark the followings, with a sketchy diagram comprising three steps (Figure 6).

We speculate that the physical affinity of Mmi1p for the polyadenylation complex, on top of its affinity for the nuclear exosome, may contribute significantly to gather exosomes to DSR-containing mRNAs. As mentioned in the Results section, Mmi1p seems to direct polyadenylation that is unusual either quantitatively or qualitatively, which might represent the major function of Mmi1p in the selective elimination (Figure 6, Step 1). The produced poly(A) tail, together with Mmi1p, probably recruits Pab2p (Step 2). Then, Pab2p, together with Mmi1p, may recruit a nuclear exosome to the transcript (Step 3).

We speculate further that Mmi1p and Pab2p may alter the fate of mRNAs through kinetic changes, slowing down their export from the nucleus and targeting them to a default exosome pathway, for instance. This possibility was previously hypothesized with regard to the putative role of Pab2p in the processing of pre-snoRNAs, under the naming of a 'take the money and run' strategy (Libri, 2010). This type of mechanism may be compatible with the observation that a modest flaw in the polyadenylation complex led to the suppression of selective elimination.

Intracellular localization analyses have shown that the factors necessary for selective mRNA elimination, including Mmi1p, Pla1p, Pab2p and exosomes, are assembled together into several patchy structures in the nucleoplasm of growing cells. One simple speculation is that these patches are the sites for selective degradation of meiosis-specific mRNAs. However, the number and position of these patches do not seem to be constant. Our preliminary analyses suggest that they are associated with neither major meiotic genes nor heterochromatin regions, although some of them do seem to show preferential localization on the periphery of the nucleolus. Thus, the nature of these patchy structures remains to be established. In this regard, it is noteworthy that several foci enriched with poly(A) RNA have been observed at the periphery of the nucleolar region in fission yeast mutants defective in nuclear mRNA export (Mizuki *et al*, 2007). Whether the patchy structures with Mmi1p localization have any relevance to the general control of mRNA quality, transport and metabolism remains an intriguing question.

Materials and methods

Yeast strains and growth media
Table I provides a summary of the yeast strains used in this study. The general genetic procedures for *S. pombe* have been described
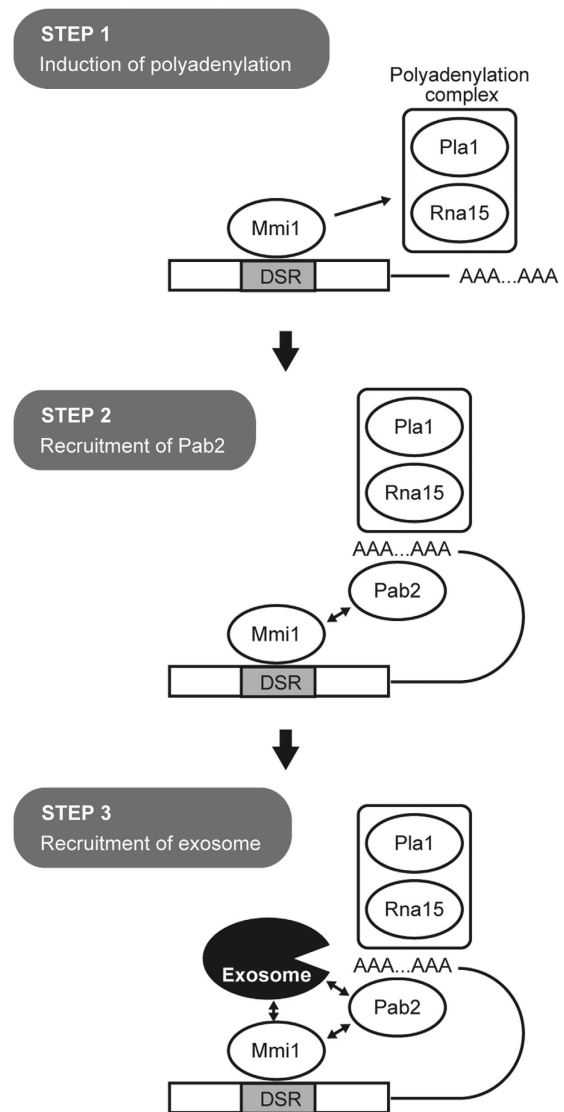

Figure 6 A putative model for the cooperation of Mmi1p, the polyadenylation complex, Pab2p and the exosome in the induction of selective elimination of a DSR-containing mRNA. In Step 1, Mmi1p bound to the DSR sequence on the transcript interacts with a conventional polyadenylation complex and promotes polyadenylation of apparently unusual nature. In Step 2, the poly(A)-binding protein Pab2p is recruited to the produced poly(A) tail. The affinity of Mmi1p for Pab2p may facilitate this recruitment. In Step 3, Pab2p, likely in collaboration with Mmi1p, recruits a nuclear exosome to the target transcript, which then digests it from the 3′ end.

previously (Gutz 1974; Moreno *et al*, 1991). The *pab2*Δ mutant was produced by the direct chromosomal integration method (Bähler *et al*, 1998). The production of various proteins tagged with a fluorescent marker was also performed using this method. The temperature-sensitive alleles of *rrp6*, *dis3*, *pla1* and *rna15* used in this study were produced essentially as described previously for the *mmi1* ts alleles (Harigaya *et al*, 2006 and Supplementary data therein). Briefly, we first constructed strains carrying a kanamycin cassette downstream of the respective gene on the chromosome. Using genomic DNA extracted from each strain as a template, we amplified DNA fragments that covered from roughly 0.5 kb upstream of the respective ORF to roughly 0.5 kb downstream of the kanamycin cassette by an error-prone PCR method (Zhou *et al*, 1991). Wild-type cells (JY450) were transformed with the resulting DNA fragments, which carried no annotated neighbouring gene, and transformants resistant to G418 (indicating the gain of the kanamycin cassette) were isolated at

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

Strain	Genotype	Source
JY333	h^- ade6-M216 leu1	Laboratory stock
JY450	h^{90} ade6-M216 leu1	Laboratory stock
JZ464	h^{90} sme2::ura4 ade6-M216 leu1 ura4-D18	Laboratory stock
JV564	h^- mmi1-ts3-(3HA)≪kan ade6-M216 leu1	Laboratory stock
JV838	h^{90} pab2-GFP≪kan^r ade6-M216 leu1	This study
JT3	h^{90} pla1-GFP≪kan^r ade6-M216 leu1	This study
JT432	h^{90} rrp6-32-GFP≪kan ade6-M216 leu1	This study
JT449	h^{90} dis3-4≪kan ade6-M216 leu1	This study
JT451	h^{90} sme2::ura4 dis3-4≪kan ade6-M216 leu1	This study
JT452	h^{90} pla1-37-GFP≪kan ade6-M216 leu1	This study
JT453	h^{90} rna15-10-GFP≪kan ade6-M216 leu1	This study
JT454	h^{90} pab2::kan ade6-M216 leu1	This study
JT455	h^{90} sme2::ura4 pla1-37-GFP≪kan ade6-M216 leu1	This study
JT456	h^{90} sme2::ura4 rna15-10-GFP≪kan ade6-M216 leu1	This study
JT457	h^{90} sme2::ura4 pab2::kan ade6-M216 leu1	This study
JT458	h^- rrp6-32-GFP≪kan pla1-37-GFP≪kan ade6-M216 leu1	This study
JT459	h^- lys1::adh1-GFP-DSR-adh ter.≪kan ade6-M216 leu1	This study
JT460	h^- lys1::adh1-GFP-DSR(M10)-adh ter.≪kan ade6-M216 leu1	This study
JT461	h^- lys1::adh1-GFP-DSR-snu2 ter.≪kan ade6-M216 leu1	This study
JT462	h^- lys1::adh1-GFP-DSR(M10)-snu2 ter.≪kan ade6-M216 leu1	This study
JT463	h^- lys1::adh1-GFP-DSR-10(A)-snu2 ter.≪kan ade6-M216 leu1	This study
JT464	h^- lys1::adh1-GFP-DSR-50(A)-snu2 ter.≪kan ade6-M216 leu1	This study
JT465	h^- lys1::adh1-GFP-DSR-100(A)-snu2 ter.≪kan ade6-M216 leu1	This study
JT466	h^- lys1::adh1-GFP-DSR(M10)-10(A)-snu2 ter.≪kan ade6-M216 leu1	This study
JT467	h^- lys1::adh1-GFP-DSR(M10)-50(A)-snu2 ter.≪kan ade6-M216 leu1	This study
JT468	h^- lys1::adh1-GFP-DSR(M10)-100(A)-snu2 ter.≪kan ade6-M216 leu1	This study
JT469	h^- lys1::adh1-GFP-DSR-10(T)-snu2 ter.≪kan ade6-M216 leu1	This study
JT470	h^- lys1::adh1-GFP-DSR-50(T)-snu2 ter.≪kan ade6-M216 leu1	This study
JT471	h^- lys1::adh1-GFP-DSR-100(T)-snu2 ter.≪kan ade6-M216 leu1	This study
JT472	h^- lys1::adh1-GFP-DSR(M10)-10(T)-snu2 ter.≪kan ade6-M216 leu1	This study
JT473	h^- lys1::adh1-GFP-DSR(M10)-50(T)-snu2 ter.≪kan ade6-M216 leu1	This study
JT474	h^- lys1::adh1-GFP-DSR(M10)-100(T)-snu2 ter.≪kan ade6-M216 leu1	This study
JT475	h^{90or-} lys1::adh1-GFP-DSR-50(A)-snu2 ter.≪kan pab2::hyg ade6-M216 leu1	This study
JT476	h^{90or-} lys1::adh1-GFP-DSR(M10)-50(A)-snu2 ter.≪kan pab2::hyg ade6-M216 leu1	This study
JT485	h^{90} LEU2>>mmi1(pro)-GFP-mmi1 rrp6-cherry≪hyg ade6-M216 leu1	This study
JT486	h^{90} LEU2>>mmi1(pro)-GFP-mmi1 dis3-cherry≪hyg ade6-M216 leu1	This study
JT487	h^{90} LEU2>>mmi1(pro)-GFP-mmi1 pla1-cherry≪hyg ade6-M216 leu1	This study
JT488	h^{90} LEU2>>mmi1(pro)-GFP-mmi1 pab2-cherry≪hyg ade6-M216 leu1	This study
JT496	h^{90or-} mmi1-ts3-(3HA)≪kan^r rrp6-ts32-GFP≪kan^r ade6-M216 leu1	This study
JT654	h^{90} cid14::nat^r ade6-M216 leu1	Danesh Moazed
JT655	h^{90} cid14::nat^r sme2::ura4 ade6-M216 leu1	This study
JT656	h^{90} rrp6-ts32-GFP≪kan^r cid14::nat^r ade6-M216 leu1	This study
JT657	h^{90} grn1-CFP≪kan^r rrp6-mCherry≪hyg^r ade6-M210 leu1	This study
JT658	h^{90} grn1-CFP≪kan^r pla1-mCherry≪hyg^r ade6-M216 leu1	This study
JT682	h^{90} rna15-GFP≪kan^r ade6-M216 leu1	This study

25°C. They were replica-plated at 37°C, and strains showing temperature-sensitive growth were picked. The introduction of each ts mutation to the proper gene was confirmed by complementation tests.

Northern blot analysis
S. pombe cells growing exponentially in YE medium were sampled and total RNA was prepared from each sample. Northern blot analysis was performed essentially according to a standard protocol (Thomas, 1980), with modifications as described previously (Watanabe *et al*, 1988). A PCR-amplified fragment covering the entire ORF of a specific gene was used as the DNA probe to detect each transcript.

RNase H treatment
A volume of 8 μl of a RNA solution containing 20 μg total RNA was mixed with 2 μl of a specific oligonucleotide solution (100 pmol/μl), which contained an oligonucleotide complementary to the transcript of either *mei4*, or *rec8* or *spo5*. A volume of 4 μl of oligo(dT) solution (100 pmol/μl) or DDW was then added to this mix. The final mixture was heated at 85°C for 5 min, incubated at 42°C for 10 min and then left at room temperature for 10 min. A volume of 20 μl of 10× buffer (200 mM Tris–HCl (pH7.5), 20 mM KCl, 1 mM EDTA and 1 mM DTT), 20 μl of 100 mM MgCl$_2$ and 1 μl of RNase H (TAKARA) were added to each mixture and digestion was carried out at 37°C for 30 min.

Microscopy
An Axioplan II microscope (Zeiss) and the Deltavision/SoftwoRx system (Olympus and Applied Precision) were used for fluorescence microscopy. For Figure 5, images consist of 10 optical sections along the *z*-axis taken at 0.4-μm intervals. The images were then deconvolved and merged into a single projection.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Conflict of interest

The authors declare that they have no conflict of interest.

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