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RNA interference regulates the cell cycle checkpoint through the RNA export factor, Ptr1, in fission yeast

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ABSTRACT

Ago1, an effector protein of RNA interference (RNAi), regulates heterochromatin silencing and cell cycle arrest in fission yeast. However, the mechanism by which Ago1 controls cell cycle checkpoint following hydroxyurea (HU) treatment has not been elucidated. In this study, we show that Ago1 and other RNAi factors control cell cycle checkpoint following HU treatment via a mechanism independent of silencing. While silencing requires *dcr1*$^+$, the overexpression of *ago1*$^+$ alleviated the cell cycle defect in *dcr1*Δ. Ago1 interacted with the mRNA export factor, Ptr1. The *ptr1-1* mutation impaired cell cycle checkpoint but gene silencing was unaffected. Genetic analysis revealed that the regulation of cell cycle checkpoint by *ago1*$^+$ is dependent on *ptr1*$^+$. Nuclear accumulation of poly(A)$^+$ RNAs was detected in mutants of *ago1*$^+$ and *ptr1*$^+$, suggesting there is a functional link between the cell cycle checkpoint and RNAi-mediated RNA quality control.

1. Introduction

The RNA interference (RNAi) is a conserved silencing mechanism mediated by small interfering RNAs (siRNAs) [1]. In fission yeast (*Schizosaccharomyces pombe*), the RNAi mechanism is involved in assembly of centromeric heterochromatin, which depends on the processing of centromeric noncoding RNAs into siRNAs by Dcr1 [2,3]. Double-stranded siRNAs are processed to single-stranded siRNAs by two Ago1-containing complexes, the Argonaute siRNA chaperone complex (ARC) (consisting of Ago1, Arb1, and Arb2) and the RNA-induced transcriptional silencing complex (RITS) (consisting of Ago1, Chp1, and Tas3) [4,5]. RITS associates with nascent transcripts and chromatin through siRNAs and methylation of histone H3 at Lys 9 (H3K9) [6]. This RNA binding of RITS recruits the Rdp1-containing RNA-directed RNA polymerase complex (RDRC) and the Clr4-Rik1 complex (CLRC), a H3K9 methylase complex, to facilitate siRNA amplification and H3K9 methylation, respectively [6,7]. Swi6, a heterochromatin protein 1 (HP1) homolog expressed in fission yeast, is recruited following H3K9 methylation, causing the degradation of heterochromatin-associated transcripts [8,9].

RNA degradation at heterochromatin is mediated by RNA surveillance factors [10,11]. Recently, Clr4 and RITS were found to interact with Mlo3, a protein related to mRNA quality control and export, to suppress antisense RNA at heterochromatin and euchromatin regions [11]. In budding yeast, Tom1, a HECT-type ubiquitin ligase, ubiquitinates Yra1, a homolog of Mlo3/Aly/REF, to promote dissociation of mRNA ribonucleoprotein (mRNP) and mRNA degradation prior to mRNA export [12]. Ptr1, a homolog of Tom1 in fission yeast, is an RNA export factor [13]. Whether Ptr1 plays a role in RNA surveillance remains uncertain; however the *ptr1-1* mutant exhibits nuclear accumulation of poly(A)$^+$ RNAs similar to the phenotype observed in mutants with defects in RNA export and/or RNA surveillance [13].

Dcr1 and Ago1 are also involved in cell cycle arrest induced by hydroxyurea (HU) treatment [14], which inhibits DNA replication and triggers S-phase checkpoint [15–17]. In the previous study, cell cycle arrest was unaffected in the *rdp1* deletion mutant, suggesting that Ago1 and Dcr1 have functionally diverged from Rdp1 to

Abbreviations: RNAi, RNA interference; HU, hydroxyurea; siRNAs, small interfering RNAs; ARC, Argonaute siRNA chaperone; RDRC, RNA-directed RNA polymerase complex; RITS, RNA-induced transcriptional silencing complex; Cen, centromeric-noncoding; HP1, heterochromatin protein 1; LC–MS, liquid chromatography–mass spectrometry; RT-PCR, reverse transcriptase-polymerase chain reaction.

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control cell cycle events [14]. However, the mechanism by which RNAi contributes to the cell cycle checkpoint has not been elucidated. In this study, we characterized RNAi components and provide evidence of a functional linkage between RNAi, RNA quality control and the cell cycle checkpoint.

2. Materials and methods

2.1. Media, yeast strains and plasmids

Fission yeast media and genetic methods have been described previously [18]. The *S. pombe* strains used in this study are listed in Supplementary Table 1. Genotyping was carried out by Southern analysis and PCR analysis of both wild-type and mutant alleles using genomic DNA as the template. The plasmids used in this study were the control vector, pREP1, and the *ago1*$^+$ plasmid, pREP1-3flag-ago1 [4].

2.2. Checkpoint assay

HU-induced cell cycle checkpoint in yeast was analyzed by calculating the septation index as described previously with minor modifications [19]. Briefly, exponentially growing cells (0.5–1.0 $\times$ 10^7 cells/ml) were divided into two aliquots, one aliquot was treated with HU (10 mM) and the other aliquot was left untreated. Cells were incubated for 3 h at 30 °C, and they were fixed with 70% ethanol. The fixed cells were resuspended in PBS and stained with Calcofluor. Cells were imaged with a microscope equipped with a UV lamp and septated cells were counted. For cultures in synthetic media, cells were treated with HU for 6 h. At least 300 cells were scored in each sample to calculate the septation index.

2.3. Affinity purification and immunoprecipitation assay

Cells (PIT2 and PIT280) were treated with HU (10 mM) for 4 h and 3Flag-Ago1 was affinity purified. Purification was performed according to Buker et al. [4], using 50 μl (bed volume) of anti-Flag antibody (M2)-conjugated agarose (Sigma) and lysis buffer (50 mM HEPES–KOH pH 7.5, 150 mM KOAc, 0.05% Tween-20, 0.005% NP-40, 10% glycerol, 1$\times$ complete protease inhibitor cocktail (Roche), 2 mM NaF, 0.4 mM Na_3VO_4, 0.5 mM Na-pyrophosphate). After washing with 50 ml of lysis buffer, beads were incubated in 1 ml of lysis buffer containing 10 μg/mL Flag peptides (Sigma) for 1 h. Immunocomplexes were eluted by incubating with 100 μg/mL 3X Flag peptide (Sigma) for 3 h. For immunoprecipitation, 5 μg of antibodies (M2 for Flag, anti-GST and anti-Ptr1 for Ptr1) were added to 500 μl of cell extracts in lysis buffer. After 3 h at 4 °C, 50 μl of protein-G magnetic beads (Dynal) were added and lysates were incubated for 1 h at 4 °C. Magnetic beads were washed three times with 1 ml of lysis buffer at 4 °C. Beads were boiled in 10 μl of 1$\times$ SDS–PAGE sample buffer and samples were analyzed by Western blotting with HRP-conjugated antibodies against Flag (M2) and Ptr1.

2.4. Liquid chromatography–mass spectrometry (LC–MS) analysis

In-gel digestion of proteins in silver-stained gels was performed as described previously [20], and Trypsin Gold (Promega) was used for tryptic digestion. Peptides were separated using EASY-nLC and a Capillary column (NTCC-360) (Nikkyo Technos), and analysis was performed using HCT-ultra (Bruker Daltonics) and the Mascot search engine (Matrix Science).

2.5. Antibodies

The M2-monoclonal antibody (Sigma) was used to detect Flag-tagged proteins. The GST-fusion protein containing amino acids 1826–2167 of Ptr1 (GST-Ptr1M) was purified from *Escherichia coli* (BL21). Rabbits were immunized with purified GST-Ptr1M and antiserum was collected. Anti-GST and anti-Ptr1 antibodies were purified from the antiserum using affinity columns coated with GST and GST-Ptr1M.

2.6. Silencing assay

The silencing assay was performed as described previously [18].

2.7. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Quantitative real-time RT-PCR was performed as previously described [18]. The following primers were used to amplify *act1*$^+$ transcripts: prIT207-5′ AACCCTCAGCTTTGGGTCTT and prIT208-5′ TTTGCATACGATCGGCAATA. The following primers were used to amplify *cen* transcripts: prIT183-5′ GAAAACACATCGTTGTCTTCAGAG and prIT184-5′ CGTCTTGTAGCTGCATGTGAA.

2.8. Fluorescence in situ hybridization and immunofluorescence

Cells were cultured at 30 °C, fixed with 4% formaldehyde, and in situ hybridization with an Alexa488-oligo $(dT)_{50}$ probe was performed as described previously [13]. Samples were stained with DAPI and observed with a Ti-eclipse fluorescence microscope (Nikon).

3. Results and discussion

3.1. Involvement of RNAi components in the regulation of cell cycle checkpoint following HU treatment

In response to the ribonucleotide reductase inhibitor, HU, checkpoint kinases prevent passage through the S phase checkpoint by inhibiting Cdc2, a cyclin-dependent kinase important for cell cycle control [15–17]. In fission yeast, activation of the S phase checkpoint by HU treatment delays passage through S phase into mitosis and results in the accumulation of elongated cells without septum. Since a previous study proposed that cell cycle arrest can be controlled independently of *rdp1*$^+$ [14], we tested whether RNAi components are involved in the checkpoint control. We deleted *dcr1*$^+$, *ago1*$^+$ or *rdp1*$^+$ in a haploid strain with a silencing marker gene at a pericentromeric repeat (*otr1R::ura4*$^+$) [21]. All these mutants showed defect in silencing at centromere and had a higher septation index than the wild-type strain following HU treatment (data not shown). The reason why the phenotype of *rdp1*Δ observed here differs from that previously reported is unclear [14]. However, similar phenotypes found here were also observed in other background strains (Fig. 1A). We therefore conclude that *dcr1*$^+$, *ago1*$^+$ and *rdp1*$^+$ are involved in regulation of the cell cycle arrest by the cell cycle checkpoint.

The involvement of RNAi factors in the cell cycle checkpoint indicates that there may be a link between heterochromatin silencing and the checkpoint. To test this possibility, we constructed mutants of RITS (*chp1*Δ and *tas3*Δ), ARC (*arb1*Δ and *arb2*Δ), CLRC (*clr4*Δ) and HP1 (*swi6*Δ). Mutants of *tas3*$^+$, *arb1*$^+$ and *arb2*$^+$ exhibited defects in cell cycle checkpoint following HU treatment, similar to *ago1*Δ (Fig. 1A). Milder defects in cell cycle checkpoint were also observed in the *chp1*Δ and *clr4*Δ mutants (Fig. 1A). The septation index of the *swi6*Δ mutant was similar to the wild-type strain (Fig. 1A). CLRC plays a key role in the maintenance and establish-

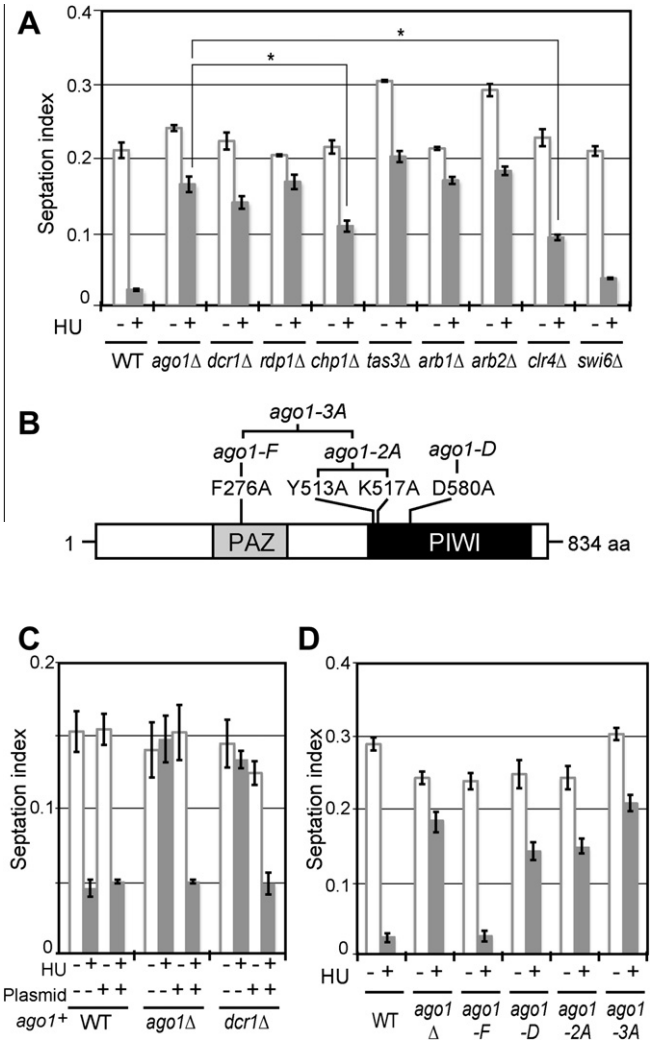

Fig. 1. Cell cycle arrest in response to HU treatment following mutation of various RNAi components. (A) Septation indices of wild-type cells and various mutants (*ago1*Δ, *dcr1*Δ, *rdp1*Δ, *chp1*Δ, *tas3*Δ, *arb1*Δ, *arb2*Δ, *clr4*Δ and *swi6*Δ). Septation indices are the proportion of septated cells following culture in rich media (YES) with or without HU. At least three replicates of each experiment were performed, and error bars indicate the standard deviation. Statistical significance was determined using a student's *t*-test (* indicates $P < 0.05$). (B) Functional domains of Ago1 and the locations of point mutations in the PAZ and PIWI domains. The F276A mutant, the Y513A K517A double mutant and the D580A mutant are referred to as *ago1-F*, *ago1-2A* and *ago1-D*, respectively. The triple mutation of F276A, Y513A and K517A is referred to as *ago1-3A*. (C) Strains expressing the control vector (−) or the *ago1*$^+$-containing vector (+) were tested in the checkpoint assay. Septation indices were calculated following culture of cells for 6 h in synthetic media (EMM-Leu) with or without HU. (D) Septation indices of various *ago1* mutants were determined as described in (A).

ment of heterochromatin through H3K9 methylation, which recruits chromodomain proteins such as Chp1 and Swi6 [7,9,22]. However, a previous study proposed that H3K9 and methylation of histone H3 at Lys 36 (H3K36) have redundant functions in the S phase checkpoint [19]. Taken together, these observations suggest the cell cycle checkpoint is regulated by RNAi components.

To determine whether *ago1*$^+$ overexpression rescues the checkpoint defect of *dcr1*Δ, we introduced expression vectors with or without the *ago1*$^+$ gene downstream of the *nmt1* promoter into the *dcr1*Δ strain (Fig. 1C). Interestingly, overexpression of *ago1*$^+$ rescued the checkpoint defect of *dcr1*Δ, suggesting that regulation of the checkpoint by RNAi requires Ago1. Since overexpression of *ago1*$^+$ does not rescue heterochromatin regulation in the *dcr1*Δ strain [23], these results suggest that RNAi regulates cell cycle checkpoint independently of the silencing mechanism.

Ago1, a single argonaute protein with conserved PAZ and PIWI domains in *S. pombe*, plays a crucial role in the RNAi mechanism [4,23,24]. The PAZ domain is important for siRNA binding [25–27], and the PIWI domain is responsible for the endonucleolytic cleavage of a target RNA, a process known as 'slicing' [28,29]. The substitution of single or multiple conserved residues within the PAZ and PIWI domains with alanine residues caused a defect in centromeric silencing (Fig. 1B) [4,23]. The cell cycle arrest of the PAZ domain F276A mutant (*ago1-F*) following HU treatment was similar to that of the wild-type strain. However, PIWI domain mutants (*ago1-D* or *ago1-2A*) and the PAZ-PIWI domain mutant (*ago1-3A*) exhibited defects in cell cycle checkpoint similar to those of the *ago1*Δ mutant (Fig. 1B and C). These results suggest that the slicer activity of the PIWI domain of Ago1 is required for checkpoint regulation.

3.2. Interaction between Ago1 and Ptr1

To investigate whether Ago1 requires other factors to regulate the cell cycle checkpoint, we attempted to identify binding partners of Ago1 using affinity purification of Flag-tagged Ago1 (3Flag-Ago1) from HU-treated cells (Fig. 2A). In a previous study, affinity purification of 3Flag-Ago1 from cell extracts containing 300 mM potassium acetate showed that Ago1 was a component of the ARC and RITS complexes [4]. To identify factors that interact weakly or transiently with Ago1, 3Flag-Ago1 was purified in low salt conditions (150 mM potassium acetate) using anti-Flag beads. When these samples were analyzed by SDS–PAGE and silver staining, bands corresponding to each of the components of the ARC complex, Ago1, Arb1 and Arb2, were observed (Fig. 2A). In addition, a minor band with a molecular weight over 300,000 Da was specifically detected in the sample containing 3Flag-Ago1 and was not observed in the negative control (Fig. 2A). LC–MS analysis of the band revealed that 53 peptides matched the 367,428 Da protein, Ptr1, with 15% coverage (Fig. 2A). Ptr1 is a homolog of the *Saccharomyces cerevisiae* HECT-type ubiquitin ligase, Tom1 [13]. The *ptr1*$^+$ gene is essential for growth and has been proposed to regulate poly(A)$^+$ RNA export [13]. Since Ptr1 could not be tagged with an epitope at either terminus (data not shown), we raised antibodies against the fusion protein GST-Ptr1M to enable detection of the Ptr1 protein. Ptr1 was specifically immunoprecipitated with 3Flag-Ago1 regardless of HU treatment (Fig. 2B). A similar result was observed when a reciprocal immunoprecipitation with the anti-Ptr1 antibody was performed (Fig. 2C). The co-immunoprecipitation of 3Flag-Ago1 and Ptr1 was inefficient, since less than 1% of the input was detected in the immunoprecipitate (Fig. 2B and C). These results suggest that Ago1 interacts with Ptr1 either weakly or transiently.

3.3. Heterochromatin silencing in the ptr1-1 mutant

Several factors that bind Ago1 regulate centromeric silencing [4,30], and so we investigated whether *ptr1*$^+$ is involved in heterochromatin silencing. The *ago1*Δ mutant exhibited defective silencing of a pericentromeric marker (*otr1R::ura4*$^+$) (Fig. 3A). However, *ptr1-1*, a temperature-sensitive mutant of the *ptr1*$^+$ gene, displayed silencing at semi-permissive temperatures (30 °C and 32 °C) similar to the wild-type strain (Fig. 3A and data not shown). Quantitative RT-PCR also revealed that centromeric-noncoding (*cen*) transcripts were efficiently silenced in the *ptr1-1* mutant at 30 °C (Fig. 3B). The heterochromatin defect in the *ago1* mutant is suppressed by RNAi-independent mechanisms when factors related to RNA quality control are mutated [31]. We constructed a double mutant of *ago1-3A* and *ptr1-1*, and examined whether the *ptr1-1*

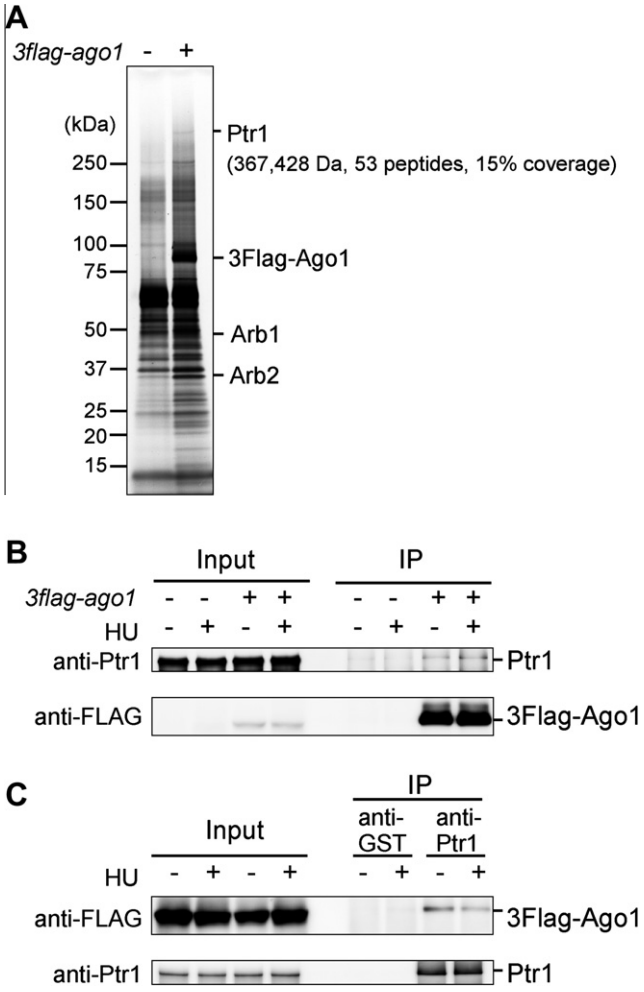

Fig. 2. Interaction between Ago1 and Ptr1. (A) Immunoprecipitation with the anti-Flag (M2) antibody was performed using extracts prepared from 3Flag-Ago1 expressing cells or control cells following HU treatment. A 4–12% gradient SDS–PAGE gel was silver stained. Results of the LC–MS analysis of the Ptr1 band are shown, and indicate the molecular weight, the number of unique peptides and sequence coverage. (B) Immunoprecipitation of 3Flag-Ago1 with an anti-Flag antibody. Strains expressing Flag-tagged or untagged *ago1*$^+$ were cultured in YES in the absence or presence of HU, lysates were prepared and immunoprecipitation with an anti-Flag antibody was performed. Precipitates were immunoblotted with anti-Flag and anti-Ptr1 antibodies. The input loaded represents 2% of the total lysate used. (C) Immunoprecipitation of Ptr1 with anti-Ptr1 antibodies. Immunoprecipitations with anti-GST or anti-Ptr1 antibodies was performed using a strain expressing Flag-tagged *ago1*$^+$ as described in (C). Precipitates were immunoblotted as in (C).

mutation suppressed the silencing defect of the *ago1-3A* mutant. *cen* transcripts accumulated to a similar level in *ago1*Δ, *ago1-3A* and *ago1-3A ptr1-1* mutants at 30 °C (Fig. 3B). These results suggest that the *ptr1-1* mutation is unable to rescue the silencing defect of the *ago1-3A* mutant and that *ptr1-1* does not affect silencing at centromeric heterochromatin at semi-permissive temperatures.

3.4. Checkpoint regulation by ago1$^+$ and ptr1$^+$

To examine whether *ptr1*$^+$ plays a role in the cell cycle checkpoint, we analyzed whether the checkpoint operated correctly in the *ptr1-1* mutant following HU treatment at 30 °C (Fig. 4A). In contrast to wild-type cells, the septation indices of *ptr1-1*, *ago1-3A* and *ago1-3A ptr1-1* remained high following HU treatment (Fig. 4A). These results indicate that *ago1*$^+$ and *ptr1*$^+$ regulate the cell cycle checkpoint via the same pathway, which is distinct from the pathway responsible for heterochromatin regulation.

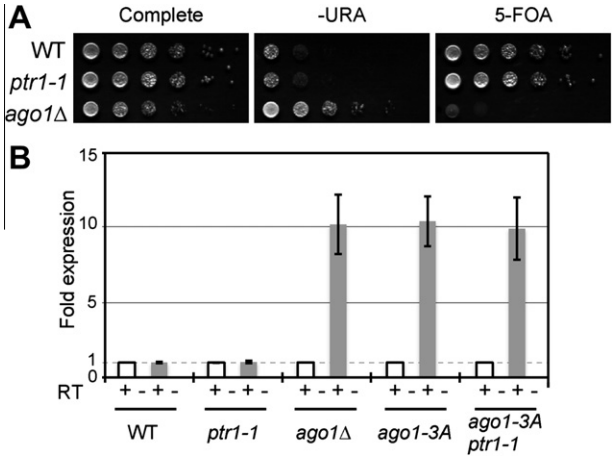

Fig. 3. *ptr1*$^+$ is not involved in centromeric silencing. (A) A silencing assay using a *ura4*$^+$ reporter inserted at the outmost centromeric repeat (*otr1R::ura4*$^+$) was performed with a wild-type strain, or *ptr1-1* or *ago1*Δ mutants at 30 °C. Complete, EMM medium; -URA, EMM medium lacking uracil; 5-FOA, EMM medium containing 5-fluoritic acid. (B) Quantitative RT-PCR analysis of *cen* and *act1*$^+$ in mutants of *ptr1*$^+$ and *ago1*$^+$. '+' and '–' indicate reactions with and without reverse transcriptase, respectively. qRT-PCR was repeated four times and the mean expression level relative to the wild-type strain is shown. Bars indicate standard deviations. Grey bars represent *cen*, and white bars represent *act1*$^+$.

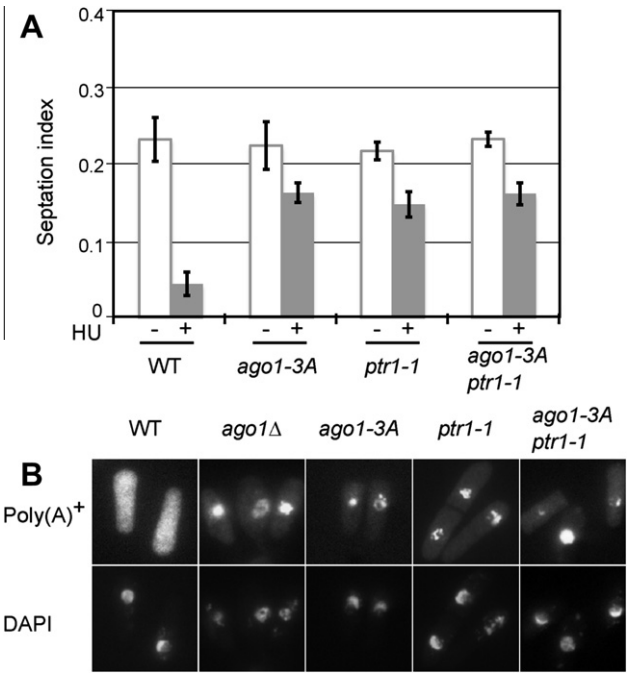

Fig. 4. Cell cycle arrest and poly(A)$^+$ RNA accumulation in *ago1*$^+$ and *ptr1*$^+$ mutants. (A) Septation indices of *ago1-3A*, *ptr1-1* and *ago1-3A ptr1-1*. Experimental conditions were identical to those described in Fig. 1 except that cells was cultured at 28 °C and shifted to the non-permissive temperature of 30 °C during HU treatment. (B) In situ hybridization with an Alexa488-oligo $(dT)_{50}$ probe revealed nuclear Poly(A)$^+$ RNA accumulation in *ago1*Δ, *ago1-3A*, *ptr1-1* and *ago1-3A ptr1-1* strains. Poly(A)$^+$ RNA and DNA were stained with Alexa488 and DAPI, respectively.

3.5. Poly(A)$^+$ RNA accumulation in mutants of ago1$^+$ and ptr1$^+$

In budding yeast, Tom1, a homolog of Ptr1, regulates the quality control of histone proteins and RNA by ubiquitinating target proteins [12,32]. However, Western blotting suggested that Ago1 and its associated factors, such as Tas3, Chp1, Arb1 and Arb2, are not ubiquitinated by Ptr1 (data not shown). In the future, the

identification of proteins that are ubiquitinated by Ptr1 should help reveal the mechanism and importance of Ptr1 ubiquitination.

To investigate whether *ago1*$^+$ and *ptr1*$^+$ play a role in RNA metabolism, we observed the distribution of poly(A)$^+$ RNA in the various mutants using fluorescence in situ hybridization with a oligo $(dT)_{50}$ probe. All mutants exhibited a similar level of nuclear accumulated poly(A)$^+$ RNAs at 30 °C, the semi-permissive temperature of the *ptr1-1* mutant (Fig. 4B). These results suggest that *ago1*$^+$ and *ptr1*$^+$ repress the nuclear accumulation of poly(A)$^+$ RNA via the same pathway.

Poly(A)$^+$ RNA accumulation is related to defects in various aspects of RNA quality control, such as mRNA export and the degradation of aberrant RNAs in the nucleus [11]. Therefore, the results of this study indicate that *ago1*$^+$ and *ptr1*$^+$ mediate a link between the cell cycle checkpoint and RNA quality control. It has recently been shown that *clr4*$^+$ and *mlo3*$^+$ mutants accumulate poly(A)$^+$ RNA in their nuclei, and that RNA quality control by *clr4*$^+$ and *mlo3*$^+$ suppresses antisense transcription at heterochromatin and euchromatin regions [11]. While *mlo3*Δ accumulates *cen* transcripts [11], we observed that *cen* transcripts were efficiently silenced in *ptr1-1* in this study (Fig. 3B). Despite these differing phenotypes in centromeric silencing, *mlo3*Δ exhibited a checkpoint defect similar to *ptr1-1* (data not shown). In conclusion, the quality control of RNA by RNAi prevents nuclear accumulation of aberrant RNA, and if this process is disrupted, the cell cycle checkpoint can be bypassed in fission yeast. Further analysis of the molecular mechanism that links RNA quality control and the cell cycle checkpoint is underway.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.027.

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