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Ago1 and Dcr1, Two Core Components of the RNA Interference Pathway, Functionally Diverge from Rdp1 in Regulating Cell Cycle Events in *Schizosaccharomyces pombe*

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In the fission yeast *Schizosaccharomyces pombe*, three genes that function in the RNA interference (RNAi) pathway, *ago1*$^+$, *dcr1*$^+$, and *rdp1*$^+$, have recently been shown to be important for timely formation of heterochromatin and accurate chromosome segregation. In the present study, we present evidence that null mutants for *ago1*$^+$ and *dcr1*$^+$ but not *rdp1*$^+$, exhibit abnormal cytokinesis, cell cycle arrest deficiencies, and mating defects. Subsequent analyses showed that *ago1*$^+$ and *dcr1*$^+$ are required for regulated hyperphosphorylation of Cdc2 when encountering genotoxic insults. Because *rdp1*$^+$ is dispensable for this process, the functions of *ago1*$^+$ and *dcr1*$^+$ in this pathway are presumably independent of their roles in RNAi-mediated heterochromatin formation and chromosome segregation. This was further supported by the finding that *ago1*$^+$ is a multicopy suppressor of the S-M checkpoint deficiency and cytokinesis defects associated with loss of Dcr1 function, but not for the chromosome segregation defects of this mutant. Accordingly, we conclude that Dcr1-dependent production of small interfering RNAs is not required for enactment and/or maintenance of certain cell cycle checkpoints and that Ago1 and Dcr1 functionally diverge from Rdp1 to control cell cycle events in fission yeast. Finally, exogenous expression of hGERp95/EIF2C2/hAgo2, a human Ago1 homolog implicated in posttranscriptional gene silencing, compensated for the loss of *ago1*$^+$ function in *S. pombe*. This suggests that PPD proteins may also be important for regulation of cell cycle events in higher eukaryotes.

INTRODUCTION

*P*AZ *P*iwi *D*omain (PPD) proteins belong to a family of highly conserved proteins found in all multicellular organisms and in the fission yeast *Schizosaccharomyces pombe*. They are not present in the budding yeast *Saccharomyces cerevisiae* or bacteria. Family members are defined by the presence of two signature domains: a 100-amino acid PAZ domain and a 300 amino acid Piwi domain in the N- and C-terminal regions, respectively (Cerutti *et al*., 2000). The function of the PAZ domain is not known, but recent studies have shown that the Piwi domain (Doi *et al*., 2003; our unpublished data) is involved in binding to Dicer (Hammond *et al*., 2000), a ribonuclease that is required for RNA interference (RNAi) (Fire *et al*., 1998).

Genetic and biochemical studies first linked PPD proteins to signaling processes that affect cellular differentiation and development (Bohmert *et al*., 1998; Cox *et al*., 1998; Moussian *et al*., 1998; Cikaluk *et al*., 1999; Lynn *et al*., 1999; Cox *et al*., 2000; King *et al*., 2001; Tahbaz *et al*., 2001). In addition, numerous groups have shown that PPD proteins function as part of the RNAi machinery to mediate posttranscriptional gene and chromatin silencing (Tabara *et al*., 1999; Fagard *et al*., 2000; Hammond *et al*., 2001; Hall *et al*., 2002; Pal-Bhadra *et al*., 2002; Volpe *et al*., 2002). Although it is not known how they work, PPD proteins are hypothesized to serve as effector molecules that provide specificity in gene-silencing pathways (Baulcombe, 2001; Hannon, 2002). Dicer processed micro-RNAs or small interfering RNAs (siRNA) bind PPD proteins to form ribonucleoprotein complexes termed *R*NA *i*nduced *s*ilencing *c*omplexes (RISCs), which are thought to mediate mRNA degradation, translational repression, or chromatin silencing in a homology-dependent manner (Matzke *et al*., 2001; Hannon, 2002). Interestingly, mutations in PPD genes that affect either gene silencing or development (but not both processes) have been isolated, demonstrating that these two functions can be genetically uncoupled (Morel *et al*., 2002).

Because all metazoan genomes encode multiple family members (Carmell *et al*., 2002), the phenotype caused by a mutation in a given PPD gene may in fact be tempered by the overlapping functions of paralogous genes (Lynn *et al*., 1999; Grishok *et al*., 2001; Doi *et al*., 2003). In this respect, *S. pombe* is an ideal model organism with which to study PPD gene function because its genome contains a single member of this family, *ago1*$^+$. The *ago1*$^+$ gene encodes for Ago1, a protein that is 33% identical and 55% similar to human GERp95/EIF2C2/hAgo2 (Carmell *et al*., 2002). During the course of this work, a number of studies on the RNAi

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

Strain	Genotype	Source	Institution
FY254	*h⁻ can1-1 leu1-32 ade6-M210 ura4-D18*	Dr. S. Forsburg	Salk Institute
FY261	*h⁻ can1-1 leu1-32 ade6-M216 ura4-D18*	Dr. S. Forsburg	Salk Institute
JC254K	*h⁻ ago1⁺::G418ᴿ can1-1 leu1-32 ade6-M210 ura4-D18*	This study	
JC261K	*h⁺ ago1⁺::G418ᴿ can1-1 leu1-32 ade6-M216 ura4-D18*	This study	
FY326	*h⁺ wee1-50 leu1-32 ura4 ade6-M216*	Dr. S. Forsburg	Salk Institute
FY118	*h⁹⁰ ura4-D18 leu1-32 ade6-M216*	Dr. S. Forsburg	Salk Institute
JCD-1	*h⁻/h′ can1-1 leu1-32 ade6-M210/ade6-M216 ura4-D18*	This study	
JCDK	*h⁻/h⁺ can1-1 leu1-32 ade6-M210/ade6-M216 ura4-D18 ago1′::G418ᴿ*	This study	
JC90DK	*h⁹⁰/h⁺ ago1⁺::G418ᴿ ura4-D18 leu1-32 ade6-M216/ade6-M210 can1-1*	This study	
JC90K	*h⁹⁰ ago1⁺::G418ᴿ ura4-D18 leu1-32 ade6-M216*	This study	
TV292	*h⁻ ago1⁺::kanMX6 ura4-D18 DS/E*	Dr. T. Volpe	Cold Spring Harbor
TV293	*h⁺ dcr1⁺::kanMX6 ura4-D18 DS/E*	Dr. T. Volpe	Cold Spring Harbor
TV294	*h⁻ ura4-D18 DS/E*	Dr. T. Volpe	Cold Spring Harbor
TV296	*h⁺ rdp1⁺::kanMX6 ura4-D18 DS/E*	Dr. T. Volpe	Cold Spring Harbor
FY1106	*h⁺ rad3⁺::ura4⁺ ura4-D18 leu1-32 ade6-M210*	Dr. S. Forsburg	Salk Institute

machinery of *S. pombe* were reported. First, it was shown that *ago1⁺*, *dcr1⁺*, and *rdp1⁺* were required for timely formation of centromeric heterochromatin (Hall *et al.*, 2002; Volpe *et al.*, 2002). Moreover, deletion of any one of these genes results in missegregation of chromosomes due to loss of centromeric cohesion and mitotic spindle assembly defects (Provost *et al.*, 2002; Hall *et al.*, 2003; Volpe *et al.*, 2003). A model derived from this work entails that double-strand centromeric transcripts serve as substrates for the RNase Dcr1 (Allshire, 2002; Bailis and Forsburg, 2002; Volpe *et al.*, 2002). Dcr1 cleavage products are then subsequently incorporated into an Ago1-containing RISC that functions to mediate locus-specific chromatin silencing in concert with histone methylation complexes. Although Rdp1 is also required for RNAi-mediated chromatin silencing, it is not clear whether this enzyme is involved mainly in producing double-strand RNA species by using centromeric transcripts as templates and/or in amplification of siRNAs at a later step.

In addition to their well-known roles in RNAi, evidence from Lin and co-workers suggest that PPD proteins regulate cell cycle events. For example in *Drosophila*, altering the expression level of Piwi leads to changes in the proliferation rates of germline stem cells (Cox *et al.*, 2000). Furthermore, the Piwi signaling network exhibits cross talk with the Hedgehog signaling machinery (King *et al.*, 2001), a developmental patterning system that influences the cell cycle through the actions of Patched1 on cyclin B1 (Barnes *et al.*, 2001; Roy and Ingham, 2002). Presently, it is not clear whether Piwi's role in cell cycle progression is dependent upon siRNAs. In the present study, we demonstrate that *ago1⁺* and *dcr1⁺* are required for regulated hyperphosphorylation of the cyclin-dependent kinase Cdc2. Inhibitory phosphorylation of Cdc2 is required to prevent the onset of mitosis in situations where damaged or unreplicated DNA is present (Enoch and Nurse, 1990; Rhind *et al.*, 1997; Rhind and Russell, 1998). Conversely, dephosphorylation of Cdc2 is required for progression through the cell cycle (Russell and Nurse, 1986; Millar *et al.*, 1991). Because *rdp1⁺* does not seem to be required for regulation of Cdc2 phosphorylation, we suggest that in addition to their roles in chromatin silencing and chromosome segregation, Ago1 and Dcr1 function in a divergent pathway that is not dependent upon Dcr1-derived siRNAs.

MATERIALS AND METHODS

Strains and Media

A list of *S. pombe* strains used in this study and their corresponding genotypes is shown in Table 1. Unless otherwise indicated, yeast were cultured at 30°C in YES, YPD, YE, or EMM lacking nutritional supplements (Alfa *et al.*, 1993). To induce expression of genes under the control of the no message thiamine promoter, cultures were grown overnight in EMM supplemented with thiamine (5 μg/ml) to repress expression of the gene, washed twice in EMM to remove residual thiamine, and cultured for an additional 48 h to allow expression of the genes of interest. *Escherichia coli* strain DH5α was used for propagation of plasmids (Table 2).

Cloning of ago1⁺

Genomic and cDNA clones of *ago1⁺* (*SPCC736.11*) were obtained by polymerase chain reaction (PCR) amplification of genomic DNA from FY254 and an *S. pombe* cDNA library (BD Biosciences Clontech, Palo Alto, CA) with Expand High Fidelity PCR System (Roche Diagnostics, Indianapolis, IN). PCR products were purified from agarose gels by using ELU-QUIK DNA purification kit (Schleicher & Schuell, Keene, NH) and ligated into the pGEM-T vector (Promega, Madison, WI). Individual clones were sequenced using the Department of Cell Biology sequencing facility (University of Alberta, Edmonton, AB, Canada).

Gene Replacement

A G418-resistance cassette was amplified by PCR from pFA6a-kanMX6 (Bahler *et al.*, 1998) and then used to disrupt the *ago1⁺* open reading frame by electroporation (Moreno *et al.*, 1991) in a one-step gene replacement procedure. Replacement of *ago1⁺* was verified by PCR and Southern blot analyses on genomic DNA isolated (Hoffman and Winston, 1987) from transformants that were resistant to G418 (100 μg/ml).

Mating Assays

Heterothallic strains were grown to mid-log phase in liquid YE and equivalent numbers of opposite mating type cells were mixed together and spotted onto malt extract plates. Homothallic (h^{90}) strains were cultured in YE until mid-log phase, washed, and resuspended in media lacking nitrogen or in low glucose/low nitrogen media (Okazaki *et al.*, 1998). Mating assays were conducted over a range of 18–30 h. Mating frequencies were determined by dividing the number of zygotes formed by the total number of cells. Three

Table 2. List of plasmids

Vector	Source	Institution
pREP3X	Dr. S. Forsburg	Salk Institute
pGEM-T	Promega	
pFA6a-kanMX6	Dr. J. Pringle	University of North Carolina
pDS473a	Dr. S. Forsburg	Salk Institute
pSLF273	Dr. S. Forsburg	Salk Institute

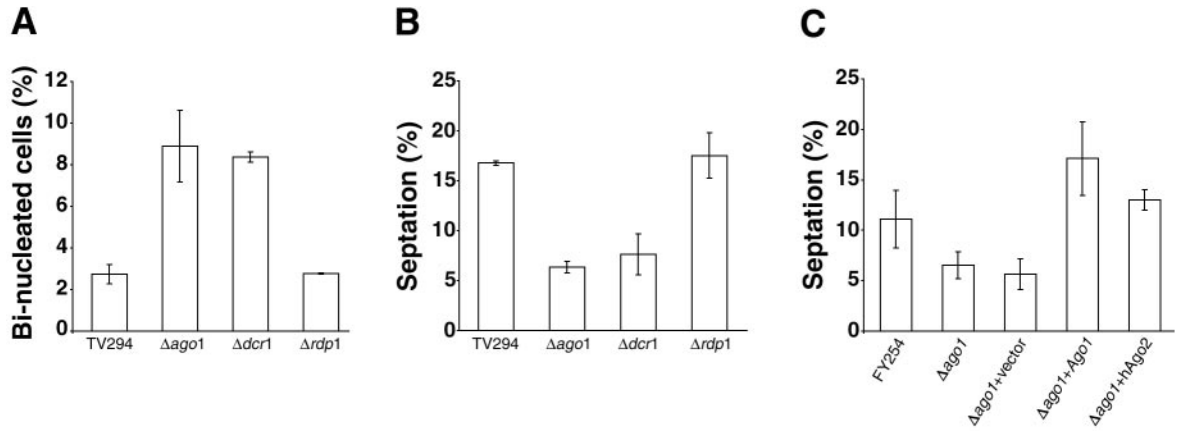


Figure 1. ago1$^+$ and *dcr1*$^+$ are both required for normal cytokinesis. All strains were cultured at 30°C in YE to mid-log phase (OD_{595} = 0.6–0.9), stained with 4,6-diamidino-2-phenylindole and placed onto microscope slides coated with poly L-lysine (5 mg/ml). Samples were examined by fluorescence and differential interference contrast microscopy. Representative fields were photographed and scored for nuclei or septation. For each sample, at least 500 cells were scored. (A) Quantitation of binucleated cells in parental strain TV294, Δ*ago1* (TV292), Δ*dcr1* (TV293), and Δ*rdp1* (TV296) strains. (B) Quantitation of septation indices of TV294, Δ*ago1* (TV292), Δ*dcr1* (TV293), and Δ*rdp1* (TV296). (C) Quantitation of septation indices in FY254 and Δ*ago1* (JC254K) strains transformed with vector (pREP3X), pREP3X +*ago1*$^+$, or pREP3X +hAgo2.

independent mating assays were performed for each data point with at least 300 cells scored per assay.

Flow Cytometry

Aliquots of 10^6 cells were collected from liquid cultures, pelleted, and fixed by resuspension in 1 ml of 70% ethanol. Cells were washed in 3 ml of 50 mM sodium citrate, resuspended in 0.5 ml of 50 mM sodium citrate containing 0.1 mg/ml RNaseA and incubated at 37°C for 2 h. Cells were stained by adding 0.5 ml sodium citrate solution containing 2 μM Sytox Green (Molecular Probes, Eugene, OR) and stored at 4°C until processing. Cells were sonicated for 45 s before analyses on an FACScan (BD Biosciences, San Jose, CA).

Drug Treatment and Cdc2 Phosphorylation Assays

Strains were cultured (streaks or serial dilutions) for the indicated time periods at 30°C on YE or EMM plates with indicated hydroxyurea (HU) (3.5–10 mM) or thiabendazole (TBZ) (10 μg/ml). For Cdc2 phosphorylation assays, strains were cultured in YE to OD_{595} = 0.3 at which time HU (10 mM) was added and incubation continued for various times. For the experiments shown in Figure 3B, 4 h post-HU treatment, cultures were washed, resuspended in YE, and incubations continued at 30°C for the remainder of the experiment. Aliquots were removed at various times, and the proportions of septated cells were determined by microscopic analyses (n = 300).

Whole cell lysates for all Cdc2 phosphorylation assays were prepared by mechanically breaking cells in radioimmunoprecipitation buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with glass beads by using Beadbeater 8 device. After boiling for 3 min, lysates were cleared by centrifugation (1000 × *g*) for 15 min at 4°C, resolved by SDS-PAGE on 10% gels, and then transferred to Immobilon-P membranes. Total Cdc2 levels and Cdc2 (tyrosine 15) phosphorylation levels were measured by immunoblot analyses by using anti-PSTAIR (Sigma-Aldrich, St. Louis, MO) and anti-phospho-cdc2 (Tyr15) (Cell Signaling Technology, Beverly, MA), respectively, followed by enhanced chemiluminescence detection.

UV Irradiation Assay

Yeast were grown to OD_{595} = 0.8 in YE, counted, and spread on YE plates at a density of 100–300 cells per plate. The plates were irradiated (0, 75, 150, 225, or 300 J/M^2) by using a Hoefer UVC 500 apparatus, wrapped in aluminum foil, and incubated for 24 h at 30°C. The foil was removed and incubation was continued at 30°C for an additional 72 h. Surviving colonies were counted and presented as a percentage of the surviving nonirradiated cells.

Ago1 and Dcr1 Expression Constructs

The cDNAs encoding Ago1 and Dcr1 were subcloned into pREP3X and pREP41HA-N expression vectors (Forsburg and Sherman, 1997), respectively. Yeast were transformed and selected for growth on EMM lacking leucine. Overexpression of Ago1 and HA-Dcr1 were confirmed by immunoblot analysis of whole cell lysates by using rabbit anti-Ago1 or a monoclonal antibody to hemagglutinin (HA), respectively (our unpublished data).

RESULTS

ago1$^+$ *and dcr1*$^+$ *Null Mutants Are Delayed in Cytokinesis*

One-step targeted gene disruption was used to replace the entire *ago1*$^+$ open reading frame with a G418 resistance cassette in the haploid strain FY254. Disruption of the *ago1*$^+$ locus in the resulting strain JC254K was confirmed by PCR and Southern blot analyses (our unpublished data). In agreement with a recently published study (Volpe *et al*., 2002), we found that *ago1*$^+$ was not essential for viability at 25, 30, or 36°C, but it was also clear that deletion of this gene resulted in pronounced growth and morphological defects (see below). Uncondensed and/or mislocalized chromosomes as well as cut phenotypes were also evident in this strain. The latter phenotype is indicative of cytokinesis being uncoupled from DNA segregation. These results are in agreement with those reported in recent studies (Provost *et al*., 2002; Hall *et al*., 2003). Backcrossing of the Δ*ago*1 strains with FY261 was performed to rule out the possibility that the morphological defects were the result of second site mutations. As well, the morphological defects of *ago1* null mutants were suppressed by plasmid-driven expression of Ago1 (our unpublished data).

A striking feature of the *ago1* null mutants when examined by light microscopy was the relative abundance of binucleated cells. This apparent cytokinesis defect was first noticed in the Δ*ago1* strain JC254K (our unpublished data). Because *dcr1*$^+$ and *rdp1*$^+$ are known to function together with *ago1*$^+$ in a chromatin silencing pathway (Volpe *et al*., 2002), we investigated whether cytokinesis would also be affected in these null strains. The proportions of binucleated cells were significantly higher in a Δ*dcr1* strain and a second Δ*ago1* strain (TV292), compared with the parental (TV294) strain, whereas no change was observed in a Δ*rdp1* strain (Figure 1A). Binucleates, which comprised ~3% of the parental and Δ*rdp1* populations, increased by threefold to ~9% of cells in the Δ*ago1* and Δ*dcr1* strains under the same culture conditions.

Cytokinesis is preceded by septum formation in fission yeast and therefore the septation index can be used as an

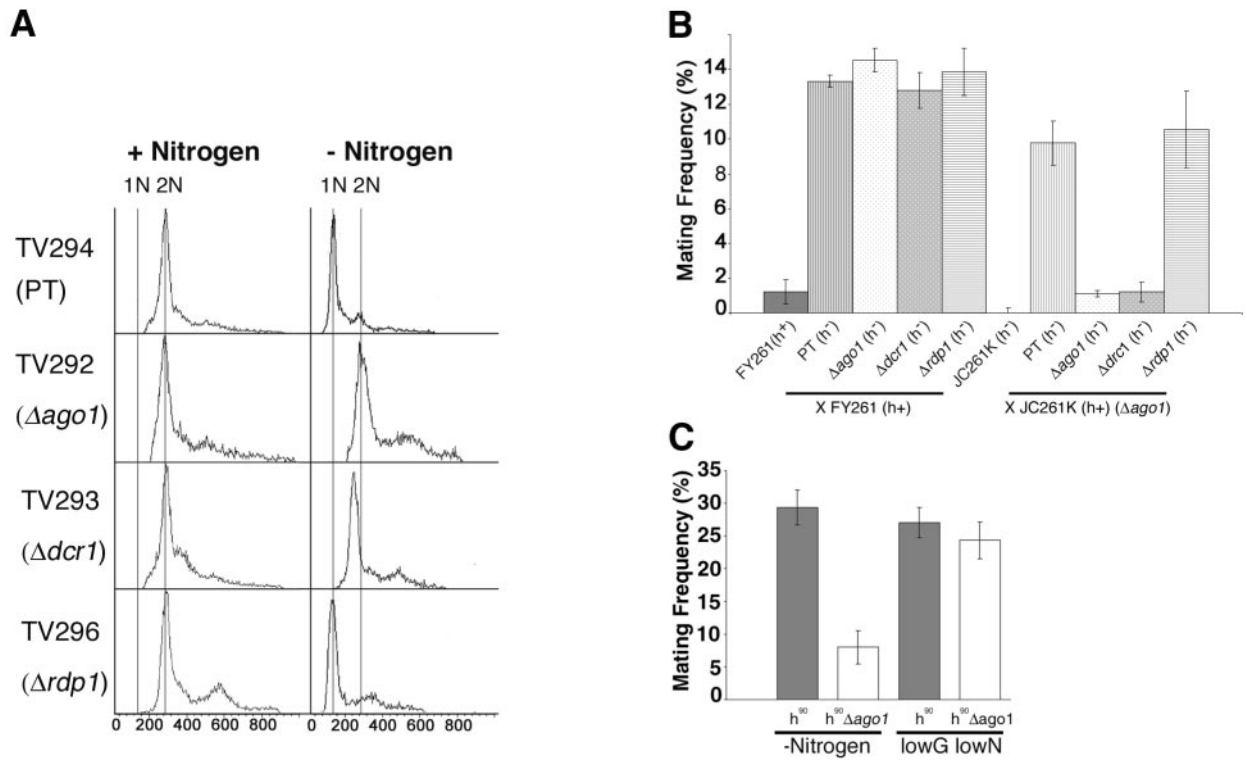


Figure 2 (continued on facing page).

indicator of cell division (Alfa *et al*., 1993). As shown in Figure 1B, the proportions of septated cells were significantly reduced when *ago1*$^+$ or *dcr1*$^+$ gene function was ablated. However, deletion of *rdp1*$^+$ had no effect on this process. Plasmid-driven overexpression of Ago1 or the human PPD protein hAgo2 corrected the cytokinesis defect in the *ago1*$^+$ mutant (Figure 1C). Although it cannot be ruled out that loss of *ago1*$^+$ or *dcr1*$^+$ results in accelerated mitosis, these results suggest that Ago1 and Dcr1 may function together to regulate septum formation and subsequent cytokinesis in *S. pombe*.

ago1$^+$ *and dcr1*$^+$*, but Not rdp1*$^+$ *Are Required for* G_1 *Arrest and Mating in Response to Nitrogen Starvation*

To enable survival when starved of nitrogen, *S. pombe* cells normally arrest in G_1 and if a partner of opposite mating type is in proximity such that pheromones are sensed, commit to sexual differentiation and subsequent meiosis (Egel and Egel-Mitani, 1974; Crandall *et al*., 1977; Breeding *et al*., 1998). Alternatively, cells enter a quiescent state (G_0) when mating partners are absent. These processes are mediated by the Wis1 MAPK pathway (Shiozaki and Russell, 1996). Fluorescence-activated cell sorting (FACS) analyses of JC254K demonstrated that *ago1*$^+$ was required for G_1 arrest during nitrogen depletion (our unpublished data). The inability to arrest in G_1 was also observed in another Δ*ago1* strain (TV292) as well as in a Δ*dcr1* (TV293) strain (Figure 2A). Moreover, the characteristic shortening and rounding of cells (Alfa *et al*., 1993; Su *et al*., 1996) in response to nitrogen depletion was not evident in the nitrogen-starved Δ*ago1* and Δ*dcr1* strains (Figure 2D). In contrast, the control parental strain (TV294) and the Δ*rdp1* strain (TV296) both arrested in G_1 in response to nitrogen limitation.

Because they cannot effectively block in G_1 under nitrogen limiting condition, we anticipated that the Δ*ago1* and Δ*dcr1* strains would be compromised in their abilities to undergo sexual differentiation. Indeed, heterothallic Δ*ago1* (TV292) and Δ*dcr1* (TV293) strains were unable to mate efficiently with a Δ*ago1* (JC261K) strain of opposite mating type (Figure 2B). In contrast, crossing null *ago1* and *dcr1* strains with FY261 resulted in formation of zygotes at the same level as TV294/FY261 crosses or with the *rdp1* null strain. Because mating is dependent upon sexual partners being in proximity to each other, any mating defect associated with the *ago1* null phenotype would be exaggerated by using two different mating strains. To circumvent this problem, we constructed an h^{90} strain deleted for the *ago1*$^+$ gene and tested the ability of this strain to form zygotes. The h^{90} *ago1* null strain (h^{90}Δ*ago1*) mated at a greatly reduced frequency compared with the parental strain (h^{90}) under nitrogen-deficient conditions (Figure 2C). Interestingly, when subjected to low glucose/low nitrogen conditions, the h^{90}Δ*ago1* strain showed the characteristic morphological change indicative of a G_1 arrest (Figure 2D). Moreover, under the same conditions, FACS analyses (our unpublished data) were used to confirm that this strain had efficiently blocked in G_1. As expected, the h^{90}Δ*ago1* strain mated with the same efficiency as the parental h^{90} strain under low glucose/low nitrogen conditions (Figure 3C). These results indicate that *ago1*$^+$ and *dcr1*$^+$ but not *rdp1*$^+$, are required for mating through the facilitation of a nitrogen-specific block in G_1.

ago1 and dcr1 Null Mutants Fail to Enact and/or Maintain the S-M Checkpoint

The above-mentioned results demonstrate that yeast lacking Ago1 function share a number of similarities to Spc1/StyI mutants (Warbrick and Fantes, 1991; Stettler *et al*., 1996). Specifically, cells are elongated, do not block in G_1 in response to nitrogen deprivation, and mating ability is com-

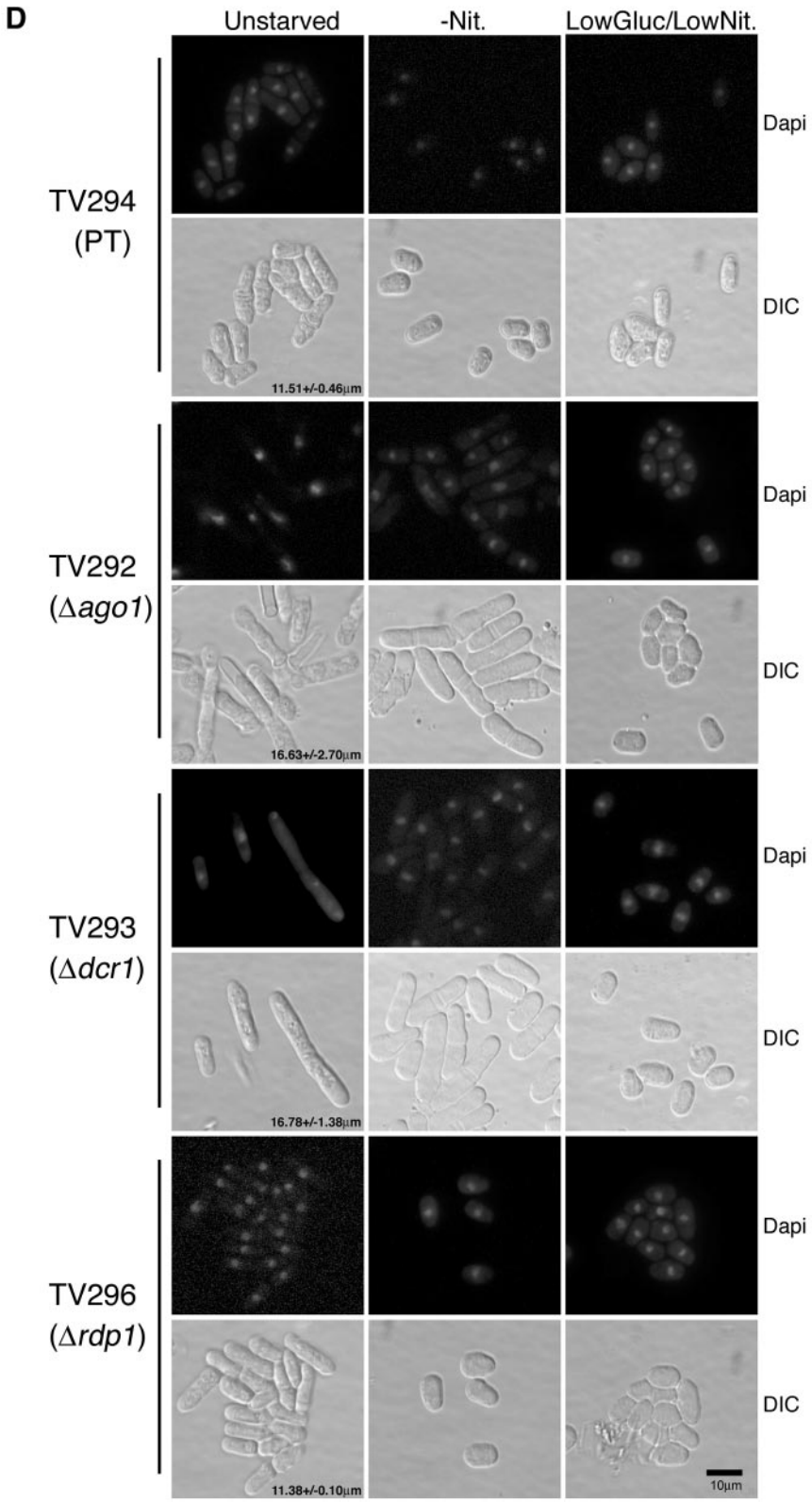


Figure 2. ago1 and *dcr1* mutants are defective for G_1 arrest and mating after nitrogen limitation. (A) FACS analyses of log phase (+Nitrogen) and nitrogen starved (−Nitrogen) TV294, Δ*ago1* (TV292), Δ*dcr1* (TV293), and Δ*rdp1* (TV296) cultures. Insets at the right show the morphologies of yeast cells under nitrogen-deficient conditions. (B and C) Mating mixtures were incubated at 30°C for 18–30 h on malt extract plates and scored for zygote production microscopically. (B) Mating frequencies of TV294, Δ*ago1* (TV292), Δ*dcr1* (TV293), and Δ*rdp1* (TV296) crossed with FY261 (parental type) or JC261K (Δ*ago1*). The low level of self-mating in strains FY261 and JC261K presumably occurs due to mating type switching. (C) Mating frequencies for homothallic wild-type (h^{90}) or homothallic *ago1* null (h^{90}Δ*ago1*) strains under nitrogen-deficient or low glucose/low nitrogen condition. (D) 4,6-diamidino-2-phenylindole-stained and corresponding differential interference contrast (DIC) images of TV294, Δ*ago1*, Δ*dcr1*, and Δ*rdp1* strains cultured under normal growth (unstarved), nitrogen starvation (−Nit.) and low glucose/low nitrogen (LowGluc./LowNit.) conditions. Average cell lengths for each strain (unstarved condition) are shown at the bottoms of the DIC panels.

promised. It has also been shown that strains deficient in Spc1/Sty1 are highly sensitive to deoxyribonucleotide depletion (Taricani *et al*., 2001) and radiation-induced DNA damage (Degols and Russell, 1997). In response to the ribonucleotide reductase inhibitor HU, checkpoint kinases ultimately mediate enactment of the S-M checkpoint through inhibitory phosphorylation of tyrosine 15 on Cdc2 (al-Khodairy and Carr, 1992; Enoch *et al*., 1992; Furnari *et al*., 1997; Rhind and Russell, 1998). Cdc2 is a cyclin-dependent kinase and is the central regulator of the cell cycle that controls

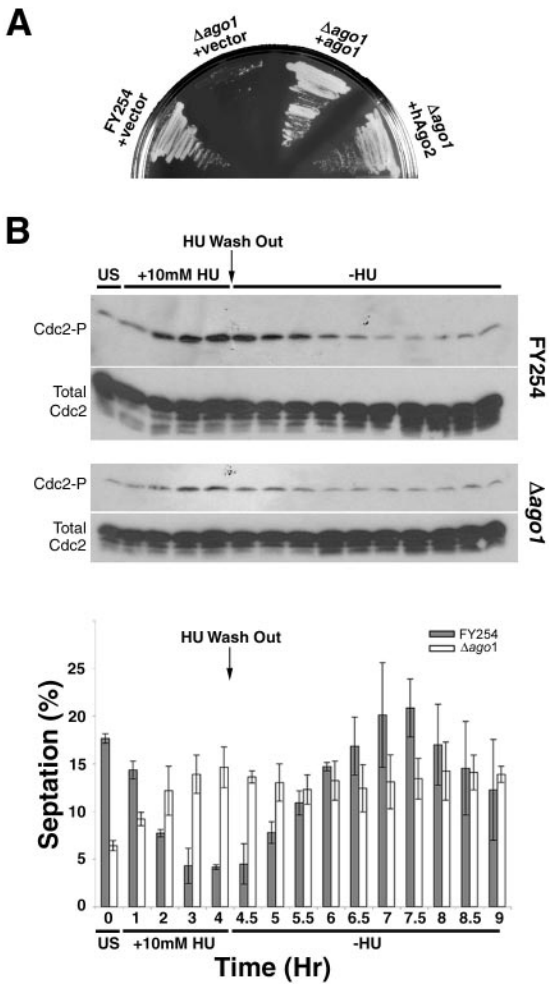


Figure 3. ago1 null mutants fail to activate the S-M DNA replication checkpoint. (A) FY254 and Δ*ago1* strains transformed with vector (pREP3X), pREP3X +*ago1*$^+$, or pREP3X +hAgo2 were streaked onto leucine-deficient EMM plates containing 10 mM HU and incubated for 3 d at 30°C. Viability of the Δ*ago1* strain containing pREP3X was confirmed by growth on EMM lacking both leucine and HU (our unpublished data). (B) Immunoblot analyses of total and phosphorylated Cdc2 (Cdc2-P) from FY254 and Δ*ago1* strains in unsynchronized cultures (US), during HU treatment (+10 mM HU) and after removal of HU (Wash Out). Mean septation indices (determined from four independent experiments) are shown for each time point (bar graph).

passage through S-phase and into mitosis (Nurse, 1990; Fisher and Nurse, 1996). Experimental results shown in Figure 3A offered the first clue that *ago1*$^+$ may be important for enactment of the S-M checkpoint. JC254K (Δ*ago1*) and the parent strain FY254 transformed with vector pREP3X, pREP3X +*ago1*$^+$, or pREP3X +hAgo2 were streaked onto plates containing 10 mM HU to test their abilities to activate the DNA replication checkpoint. After 3 d, Δ*ago1* cells transformed with vector alone were not able to grow under these conditions, indicating that they were unable to activate, maintain or recover from activation of this checkpoint (Figure 3A). In comparison, the Δ*ago1* strain ectopically expressing Ago1 or hAgo2 grew well on HU-containing media.

Because hyperphosphorylation of Cdc2 is required for initiation of the S-M checkpoint, we predicted that phosphorylation of this kinase would be decreased in the Δ*ago1* strain. As expected in response to HU treatment, the levels of phosphorylated Cdc2 in the parent strain FY254 increased, coinciding with a decrease in the septation index (Figure 3B). After removal of HU, the levels of phosphorylated Cdc2 decreased and were accompanied by a concomitant increase in the number of septated cells. In contrast, Cdc2 hyperphosphorylation was delayed in Δ*ago1* cultures, and peak phosphorylation levels were substantially lower than those seen in FY254 (Figure 3B). Consequently, the septation index of Δ*ago1* cultures continued to increase rather than decrease in the presence of HU (Figure 3B). The Δ*dcr1* strain was also hypersensitive to HU, whereas the Δ*rdp1* mutant was significantly less sensitive to this drug (Figure 4A). Acting on the premise that the HU sensitivity of the Δ*dcr1* strain resulted from an inability to activate/maintain the S-M checkpoint, we verified that this mutant was indeed unable to hyperphosphorylate Cdc2 in response to HU treatment (Figure 4B). On the contrary, HU-induced hyperphosphorylation of Cdc2 was not affected by loss of Rdp1 function. Overexpression of Ago1 or Dcr1 in their cognate null mutants, restored the ability of these strains to hyperphosphorylate Cdc2 in response to HU treatment (Figure 4C).

ago1 and dcr1 Null Mutants Are Defective for the DNA Damage Checkpoint

The degree of overlap between components of the DNA replication and DNA damage checkpoints is considerable (al-Khodairy and Carr, 1992; Rowley *et al*., 1992; Furnari *et al*., 1997; Rhind *et al*., 1997; Rhind and Russell, 1998; Raleigh and O'Connell, 2000). In both cases, activation of the checkpoints after exposure to genotoxic insults requires hyperphosphorylation of Cdc2. Accordingly, we predicted that Δ*ago*1 and Δ*dcr*1 strains would be hypersensitive to UV irradiation, a commonly used inducer of the DNA damage checkpoint. Indeed, Δ*ago1* and Δ*dcr1* strains showed increased sensitivity to UV radiation compared with the *rdp1* null and the parental strain TV294, but were less sensitive than the control checkpoint mutant Δ*rad3* (Jimenez *et al*., 1992) (Figure 4D). To confirm that the sensitivity of *ago1* and *dcr1* null mutants to UV light was the result of a failure to activate the DNA damage checkpoint, strains were irradiated and the septation indices were determined over a 6-h period (Figure 4E). As expected the parental and Δ*rdp1* strains showed decreased septation after exposure to UV light (Francesconi *et al*., 2002), an indication that the DNA damage checkpoint was functioning in these strains. In contrast, *ago1* and *dcr1* null mutants did not exhibit decreased septation after exposure to UV radiation. These results suggest that *ago1*$^+$ and *dcr1*$^+$ are both required for survival when encountering DNA-damaging conditions, possibly through Cdc2-dependent activation of the DNA damage checkpoint.

Ago1 May Function Downstream of Dcr1 to Regulate Cell Cycle Events

To gain insight into how Ago1 and Dcr1 interact genetically to enact the S-phase replication checkpoint, we determined whether overexpression of Ago1 could compensate for lack of Dcr1 function and vice versa. We noticed that Δ*dcr1* strains overexpressing Ago1 exhibited normal morphology compared with Δ*dcr1* strains expressing vector only (our unpublished data). To investigate this finding further, we assayed whether Ago1 overexpression could correct the cytokinesis defect associated with loss of Dcr1 function. Data shown in Figure 5A clearly show that high level expression of Ago1 restores the septation index of Δ*dcr1* cells to normal

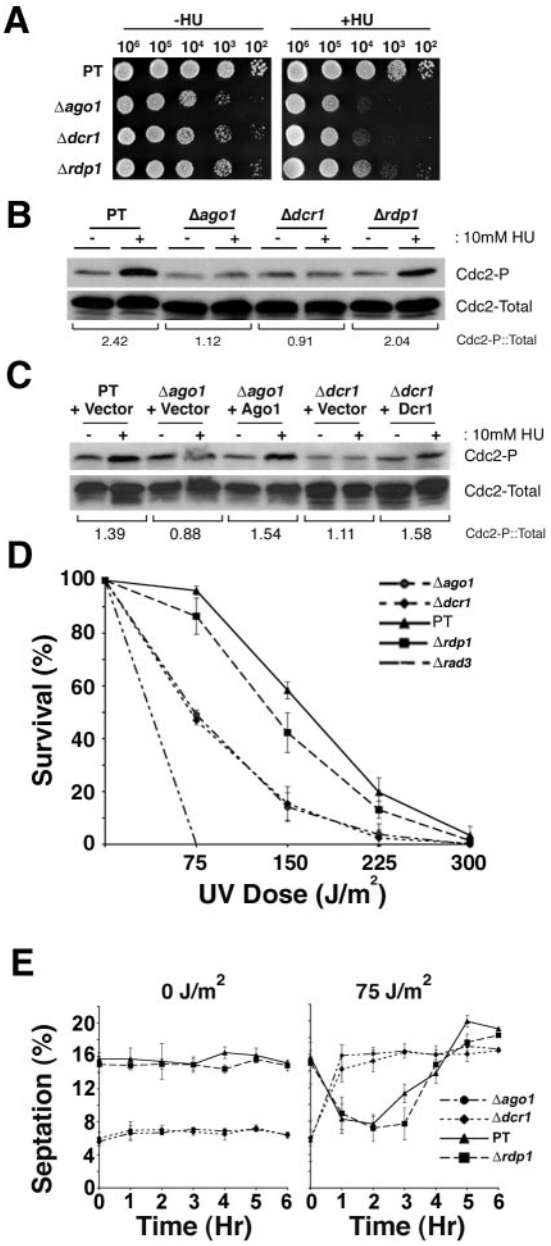


Figure 4. *ago1*$^+$ and *dcr1*$^+$ are both required for hyperphosphorylation of Cdc2 and enactment of the DNA damage and replication checkpoints. (A) Serial dilutions of TV294 and corresponding Δ*ago1*, Δ*dcr1*, and Δ*rdp1* strains were spotted on YE agar or YE agar containing 3.5 mM HU and were cultured for 3 and 5 d at 30°C, respectively. (B) TV294, Δ*ago1*, Δ*dcr1*, and Δ*rdp1* liquid cultures were grown at 30°C to an OD_{595} = 0.5, and aliquots were removed before (−) or after (+) a 4-h HU treatment. Whole cell lysates were prepared and separated by SDS-PAGE before immunoblot analyses of total and phosphorylated Cdc2. The normalized levels of HU-induced Cdc2 phosphorylation are shown below the immunoblots in B and C. (C) Levels of total and phosphorylated Cdc2 were analyzed in strains transformed with vector alone or vector encoding Ago1 or Dcr1. (D) TV294, Δ*ago1*, Δ*dcr1* Δ*rdp1*, and Δ*rad3* liquid cultures were grown at 30°C to an OD_{595} = 0.8, spread at a density of 100–300 cells per plate and exposed to UV radiation (0, 75, 150, 225, or 300 J/M^2). Cell survival is shown as a percentage of the nonirradiated control cell survival. (E) TV294, Δ*ago1*, Δ*dcr1*, and Δ*rdp1* were subjected to mock (0 J/m^2) treatment and UV irradiation (75 J/m^2), and the septation index for each strain was determined every hour postirradiation (n = 3, 300 cells counted/time point). Similar results were obtained when these strains were subjected to a 100 J/m^2 dose (our unpublished data).

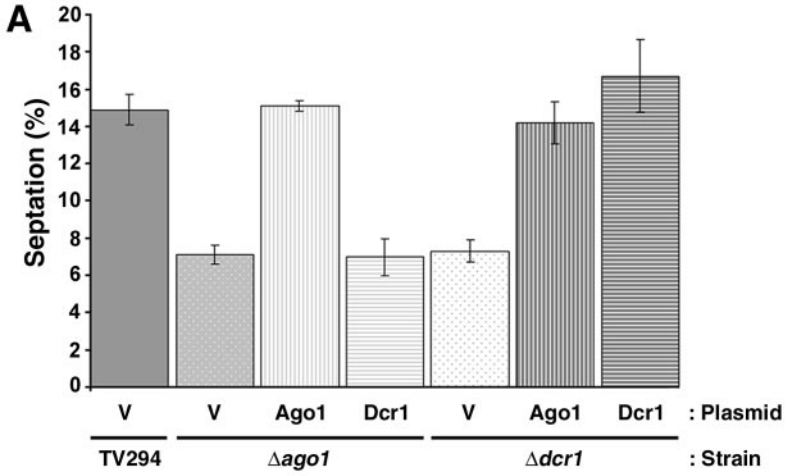
levels. In contrast, overexpression of Dcr1 did not compliment the cytokinesis defect of Δ*ago1* strains. Moreover, ectopic expression of Ago1 complemented that S-M checkpoint deficiency of both Δ*ago1* and Δ*dcr1* strains, whereas Dcr1 overexpression corrected the checkpoint deficiency in Δ*dcr1* strains only (Figure 5B). These results indicate that the requirement for Ago1 in regulation of cytokinesis and enactment of cell cycle checkpoints is downstream of Dcr1 and independent of Dcr1-derived siRNAs.

Recent studies have shown that *ago1*$^+$, *dcr1*$^+$, and *rdp1*$^+$ are required for accurate segregation of chromosomes and consequently, null mutations in any one of these genes results in hypersensitivity to the microtubule-destabilizing drug TBZ (Provost *et al*., 2002; Hall *et al*., 2003; Volpe *et al*., 2003). Because Rdp1 and Dcr1 are both required for efficient chromosome segregation, we assume that production and targeting of siRNAs are also required for this process. Accordingly, we predicted that ectopic expression of Ago1 would not complement the TBZ sensitivity of the Δ*dcr1* strains. Overexpression of Ago1 or Dcr1 in the *ago1* or *dcr1* mutants, respectively, resulted in decreased sensitivity to TBZ (Figure 6). However, overexpression of Ago1 in Δ*dcr1* or Dcr1 in Δago1 strains did not alleviate sensitivity to TBZ. Together, these results suggest that the roles of Ago1 and Dcr1 in centromere function and chromosome segregation are distinct from their functions in cytokinesis and cell cycle checkpoints.

DISCUSSION

Depending upon the organism, metazoan genomes encode between five and 24 PPD genes (Carmell *et al*., 2002). Not surprisingly, some paralogs have overlapping functions (Lynn *et al*., 1999; Doi *et al*., 2003), a situation that complicates the genetic and biochemical analyses of this family of proteins. In contrast, the *S. pombe* genome encodes for a single PPD gene *ago1*$^+$. This organism also encodes homologues of dicer and RNA-dependent RNA polymerase that together with *ago1*$^+$ function in chromatin silencing/segregation (Provost *et al*., 2002; Volpe *et al*., 2002; Hall *et al*., 2003). Accordingly, the fission yeast is an excellent experimental system with which to study the molecular functions of PPD proteins.

Targeted gene deletion demonstrated that *ago1*$^+$ was not essential for growth in haploid strains under a variety of conditions. Our results are consistent with recent studies that showed none of the genes that encode core RNAi components, *ago1*$^+$, *dcr1*$^+$, or *rdp1*$^+$, are essential for viability in *S. pombe* (Provost *et al*., 2002; Volpe *et al*., 2002). However, these genes were shown to be necessary for timely formation of centromeric heterochromatin (Hall *et al*., 2002; Volpe *et al*., 2002), a process that is not only important for epigenetic silencing in *S. pombe* but also for sister chromosome segregation (reviewed in Karpen and Allshire, 1997; Lee and Orr-Weaver, 2001). Heterochromatin formation at centromeric sites, results in enrichment of cohesion molecules that mediate sister chromosome attachment and kinetochore orientation, which in turn facilitates microtubule binding and proper chromosome segregation (Bernard *et al*., 2001; Nonaka *et al*., 2002). In keeping with recent work by others (Hall *et al*., 2003), we also found that assembly and or localization of mitotic spindles was abnormal in the Δ*ago1* strain JC254K (our unpublished data). Similar phenotypes were reported for *S. pombe* strains devoid of Dcr1 function (Provost *et al*., 2002; Hall *et al*., 2003). However, a finding not previously reported for Δ*ago1* and Δ*dcr1* strains is the fact that these mutants are impaired for cytokinesis. Interest-



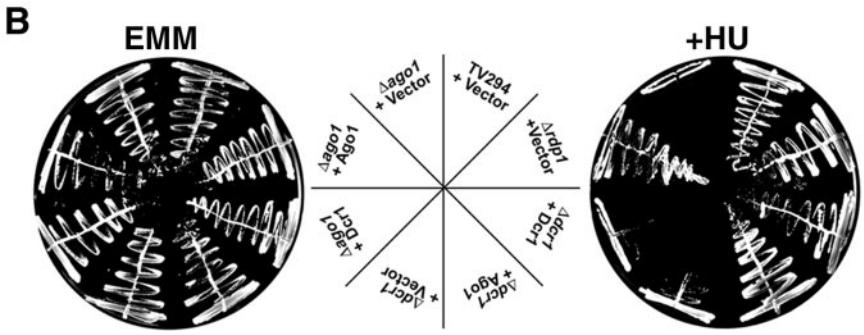


Figure 5. *ago1*$^+$ functions downstream of *dcr1*$^+$ to regulate cell cycle events. Yeast strains were transformed with plasmids encoding Ago1, HA-Dcr1, or vector alone. (A) Yeast strains grown in EMM-leu to log phase were examined by phase contrast microscopy, and septated cells were counted and expressed as a percentage of total cells. (B) Yeast strains were streaked onto EMM lacking leucine (EMM) in the presence or absence of 7.5 mM HU (+HU) and were then cultured at 30°C for 3 to 5 d.

ingly, the *rdp1* null mutant did not exhibit this phenotype, providing the first indication that Ago1 and Dcr1 may function independently of Rdp1 in a pathway distinct from that required for efficient heterochromatin formation and chromosome segregation.

PPD Proteins and Dicer Function to Regulate Cell Cycle Events

In *S. pombe*, cell division is preceded by the establishment of a septum in the medial portion of a dividing cell (Le Goff *et al*., 1999; Balasubramanian *et al*., 2000). This process is regulated by a signaling network known as the septation initiation network (McCollum and Gould, 2001). The absence of Ago1 or Dcr1 activity was associated with a dramatic reduction in septation and subsequent cytokinesis. However, these cell cycle defects are apparently not related to the inability to efficiently form heterochromatin or segregate chromomsomes because septation and cytokinesis were normal in *rdp1* mutants.

Of relevance to the present study is the previous finding that the Piwi signaling network exhibits cross talk with the hedgehog signaling pathway (King *et al*., 2001). The latter pathway is known to influence the regulation of various cell cycle events (reviewed in Barnes *et al*., 2001). With respect to Piwi, ectopic overexpression of this protein increases the division rate of germline stem cells, whereas mutations that compromise Piwi function seem to have the opposite effect (Cox *et al*., 2000). We also found that plasmid-driven overexpression of Ago1 increased the growth rate of parental yeast strains such as FY254, whereas growth is impaired in *ago1* null strains (our unpublished data). At this point, it is unknown whether siRNAs are required for Piwi function in signaling pathways that regulate the cell cycle.

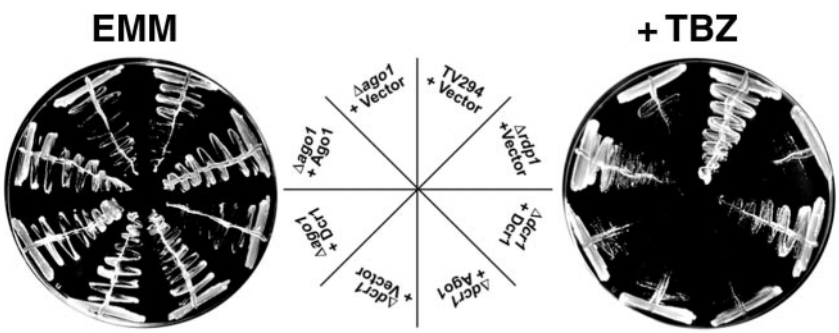


Figure 6. Ectopic overexpression of Ago1 does not correct the chromosome segregation defect of *dcr1* nulls. Yeast strains were transformed with plasmids encoding Ago1, HA-Dcr1 or vector alone. Strains were streaked onto EMM lacking leucine in the presence (+TBZ) or absence (EMM) of 10 μg/ml TBZ and were then cultured at 30°C for 3 to 5 d.

Our analyses showed that *ago1*$^+$ and *dcr1*$^+$ are both required for establishment and/or maintenance of G_1 arrest in response to nitrogen-limiting conditions. Accordingly, Δ*ago1* and Δ*dcr1* strains are severely impaired for mating, a hallmark phenotype for PPD and Dicer mutations in metazoans (Cox *et al*., 2000; Smardon *et al*., 2000; Grishok *et al*., 2001; Knight and Bass, 2001; Parrish and Fire, 2001). The results in this study are in apparent contrast to those of a previous study (using the same strains) where no obvious mating defects were reported (Volpe *et al*., 2002). However, the discrepancy in findings could potentially be explained if different mating assays were used in the two studies. For example, *ago1*$^+$ and *dcr1*$^+$-dependent mating defects were only observed in our hands when assays were conducted under nitrogen-deficient conditions. In contrast, when mating assays were performed under low glucose/low nitrogen conditions, these mutants mated with the same frequencies as parental and Δ*rdp1* strains. Importantly, these data suggest that the mating defects associated with the *ago1* and *dcr1* null mutants are not due to global defects stemming from lack of a functioning RNAi pathway or missegregation of chromosomes, but rather a failure to block in G_1 when nitrogen is absent.

Additional evidence for the functioning of *ago1*$^+$ and *dcr1*$^+$ in an *rdp1*$^+$-independent pathway came from the finding that the former two genes are essential for initiation and/or maintenance of the DNA replication and damage checkpoints. Significantly, enactment of these two checkpoints requires a common set of genes (al-Khodairy and Carr, 1992; Rowley *et al*., 1992; Furnari *et al*., 1997; Rhind *et al*., 1997; Rhind and Russell, 1998; Raleigh and O'Connell, 2000) whose collective functions ultimately determine the activity level of the master cell cycle regulator Cdc2. Inhibitory phosphorylation of tyrosine 15 in Cdc2 leads to a block in the cell cycle until replication inhibition or DNA damage is relieved. With respect to the *ago1* and *dcr1* null mutants, failure to enact these checkpoints likely results from their inability to hyperphosphorylate Cdc2 when exposed to genotoxic insults. Moreover, the inability to inactivate Cdc2 by inhibitory phosphorylation could also be the underlying cause of the cytokinesis and mating defects in the *ago1* and *dcr1* nulls. Activation of the septation initiation network and subsequent septation as well as sexual mating in yeast, requires a reduction in Cdc2 activity before these processes can occur (He *et al*., 1997; Wu and Russell, 1997; Stern and Nurse, 1998; Guertin *et al*., 2000; Chang *et al*., 2001). Intriguingly, a human Ago1 homolog, hAgo2, was able to fully complement the cytokinesis defect and DNA replication checkpoint deficiencies of *ago1* mutants, suggesting that PPD proteins may regulate cell cycle events in mammalian cells, a function not reported previously. In contrast, human dicer was found to only partially complement the chromosomal segregation defects in a *dcr1* knockout strain of *S. pombe* (Provost *et al*., 2002).

The phosphorylation status and activity of Cdc2 is controlled primarily by the opposing actions of the phosphatase Cdc25 and two kinases, Wee1 and to a lesser extent, Mik1 (Russell and Nurse, 1986, 1987; Gould and Nurse, 1989; Lundgren *et al*., 1991; McGowan and Russell, 1993; Lee *et al*., 1994). Accordingly, the decreased Cdc2 phosphorylation in *ago1* and *dcr1* mutants may result from inhibition of kinases that target Cdc2 and/or through increased Cdc25 phosphatase activity. Our repeated attempts to cross Δ*ago1* strains with a *wee1-50* mutant strain (Nurse, 1975) at the permissive temperature were unsuccessful. However, given the mating defects associated with *wee1* (Wu and Russell, 1997) and *ago1* mutants, successful conjugation between these two strain types may indeed be a rare event.

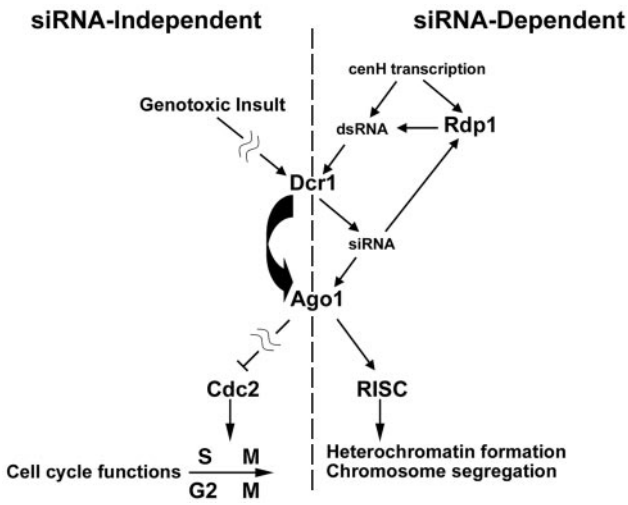


Figure 7. Model for RNAi-independent functions of Ago1 and Dcr1. The right side of the model depicts the classical RNAi pathway in which Rdp1 is required for production/amplification of centromere-derived double-strand transcripts (cenH) that serve as substrates for Dcr1. The resulting siRNAs are incorporated into Ago1-containing RISCs that facilitate heterochromatin formation, an event that is required for attachment of mitotic spindles to kinetochores and subsequent orderly chromosome segregation. The left side of the model depicts a pathway in which Dcr1 and Ago1 in response to genotoxic insults such as DNA damage or HU, mediate downstream events that lead to inhibitory phosphorylation of Cdc2. This process is proposed to be independent of siRNAs and Rdp1.

Ago1 and Dcr1 Function Independently of the Classical RNAi Pathway to Regulate Cell Cycle Events

Of course, it must be considered that the cell cycle defects associated with *ago1* and *dcr1* mutants are the result of an inability to enact transcriptional and/or posttranscriptional silencing of genes that modulate the cell cycle. However, if this were the case, it is difficult to explain why *rdp1*$^+$ is not required for these processes. Furthermore, expression profile analyses indicated that only two *S. pombe* genes require *dcr1*$^+$ for silencing (Provost *et al*., 2002) and neither of these genes has recognized functions in cell cycle regulation.

Rather, we propose that Ago1 and Dcr1 function independently of Rdp1 and the classical RNAi pathway to regulate Cdc2-dependent cell cycle events (Figure 7). This model, which also proposes that Ago1 acts downstream of Dcr1, is based upon the following experimental observations: 1) Rdp1 function is dispensable for regulated hyperphosphorylation of Cdc2. 2) Overexpression of Ago1 results in complementation of the S-phase check-point deficiency in a *dcr1* null strain. In contrast, *dcr1*$^+$ does not act as a multicopy suppressor to correct the S-phase checkpoint deficiency of Δ*ago1* mutants. 3) Overexpression of Ago1 in a *dcr1* null background does not complement the chromosome segregation defects associated with this mutant. Furthermore, a search of the *S. pombe* genome revealed that no other Dcr1-like or Rdp1-like sequences are present, and therefore it seems likely that Dcr1-dependent production of siRNAs is not required for Ago1-mediated regulation of cell cycle checkpoints. Together, these findings suggest that Ago1 and Dcr1 function in at least two pathways, one of which may be independent of siRNAs.

Presently, it is unknown how Dcr1 and Ago1 function to regulate Cdc2 phosphorylation, but given that neither of these proteins is predicted to have kinase or phosphatase activity, it seems likely that their actions are indirect. Consequently, important next steps are to understand how *ago1*$^+$ and *dcr1*$^+$ interact genetically with genes that regulate the phosphorylation status of Cdc2 and to determine whether PPD proteins in higher eukaryotes regulate cell cycle events by affecting the activity of this kinase.

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