Dicer is required for chromosome segregation and gene silencing in fission yeast cells

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RNA interference is a form of gene silencing in which the nuclease Dicer cleaves double-stranded RNA into small interfering RNAs. Here we report a role for Dicer in chromosome segregation of fission yeast. Deletion of the Dicer (*dcr1*$^+$) gene caused slow growth, sensitivity to thiabendazole, lagging chromosomes during anaphase, and abrogated silencing of centromeric repeats. As Dicer in other species, Dcr1p degraded double-stranded RNA into ≈23 nucleotide fragments *in vitro*, and *dcr1*Δ cells were partially rescued by expression of human Dicer, indicating evolutionarily conserved functions. Expression profiling demonstrated that *dcr1*$^+$ was required for silencing of two genes containing a conserved motif.

In RNA interference (RNAi), the nuclease Dicer cleaves double-stranded RNA (dsRNA) into 21- to 23-nt small interfering RNAs (1–4). These small interfering RNAs guide a multicomponent nuclease, the RNA-induced silencing complex (RISC), to destroy specific mRNAs (5). Dicer-like activities have been detected in *Drosophila* (4), *Caenorhabditis elegans* (6), and mouse embryonal carcinoma F9 and P19 (7) cell extracts or immunoprecipitates (IPs), and in Dicer-mediated suppression of gene expression induced by dsRNA in *Drosophila melanogaster* (4) and *C. elegans* (6, 8). Dicer is also involved in the synthesis of the endogenous small temporal RNAs *lin-4* and *let-7* in flies, humans, and worms, and has been implicated in developmental timing in *C. elegans* (6, 8, 9). The family of tiny RNAs has recently been expanded by the identification of several additional microRNAs in HeLa cells, *Drosophila* (10), and *C. elegans* (11, 12).

Before the identification of Dicer, the RNase III family of nucleases was suggested to play critical regulatory roles in eukaryotic cells (13) and to be involved in RNAi (14). Very recently, RNAi was shown to be important for formation of heterochromatin and centromere silencing by promoting H3 lysine 9 methylation in fission yeast (15). In addition, small RNAs corresponding to *Schizosaccharomyces pombe* centromeric heterochromatic repeats have been detected (16), and a role for small RNAs in genome rearrangement in *Tetrahymena* was suggested (17).

In this study, we further characterized the Dicer homologue in fission yeast. Several components of the RNAi machinery are conserved in *S. pombe*, indicating that this organism is an excellent model for studying the cellular function of RNAi. We report that deletion of the *S. pombe* Dicer (*dcr1*$^+$) gene caused slow growth and defects in chromosome segregation, which could be explained by a loss of centromeric heterochromatin structure. We used microarray expression profiling analysis to show that *dcr1*$^+$ is required for silencing of two genes containing a conserved motif. Importantly, we demonstrate that *S. pombe* Dcr1p degraded dsRNA into ≈23 nucleotide fragments *in vitro*, and that *dcr1*Δ cells were partially rescued by expression of human Dicer, indicating an evolutionarily conserved function.

Materials and Methods

***S. pombe* Strains and Media.** Supplemented yeast extract (rich) medium (YES) and minimal medium with glutamate as nitrogen source (PMG) have been described (18). Standard genetic techniques were used according to ref. 19. Comparative plating and serial dilution experiments were performed as described (18). Thiabendazole (TBZ; Sigma) was dissolved in DMSO as a stock solution at 20 mg/ml.

Preparation of Dicer Constructs. The *S. pombe dcr1*$^+$ gene was amplified from genomic DNA by PCR, whereas human Dicer cDNA was obtained by cDNA library screening (20). The *S. pombe dcr1*$^+$ gene was cloned as a *Bam*HI fragment into the *Bam*HI site of pREP41X vector, carrying a *Leu* selection marker, or pREP42X, carrying a *Ura* marker (21). The human Dicer gene was cloned as a *Sal*I fragment into the *Xho*I site of pREP41X or pREP42X. The correct orientation of the inserts was ascertained by restriction analysis. In these vectors, the Dicer genes are under the control of the thiamine-regulatable *nmt1* promoter (21); gene expression was induced in the absence of thiamine and repressed at 15 μM thiamine.

Homologous Recombination. The *dcr1*$^+$ gene was tagged with the HA epitope by using the pFA6a-3HA-kanMX6 vector, whereas the *dcr1*$^+$ gene was deleted using the pFA6a-kanMX6 vector, in a PCR-based approach (22). The PCR products were used to transform *S. pombe* cells by electroporation, and transformants were selected based on their resistance to kanamycin; homologous integration was confirmed by PCR.

Immunofluorescence Microscopy. Cell growth, fixation in 3.8% paraformaldehyde, staining, detection of α-tubulin by using mouse Tat1 monoclonal antibody (23), and collection of images and spindle length measurements have been described (24).

ORF Microarray. Array experiments were essentially done according to those of Xue *et al.* (Y. Xue, S. Haas, T. Driss, D. Marechal, M. Vingron, K.E., and A.W., unpublished work). Briefly, RNA was extracted from log phase yeast cells by using a standard acid phenol extraction. The RNA was then cleaned up using the RNeasy kit (Qiagen, Valencia, CA). Reverse transcription of the RNA was done with *S. pombe* array-specific and polydT primers, and Cy3- or Cy5-conjugated dCTP. Unincorporated dNTPs and primers were removed with the QIAquick PCR purification kit. Probes were hybridized to cDNA arrays from Eurogentec (Brussels) spotted with 5,029 annotated ORFs. The CHIPREADER V.1.8 (Virtek, Waterloo, ON, Canada) was used to scan the slides. Quantification of signals was done with IMAGENE V.4.01 (Biodiscovery, Los Angeles), and preliminary analysis was done with GENESIGHT LIGHT (Biodiscovery). The final analysis was done with GENESPRING (Silicon Genetics, Redwood City, CA). DNA matrix analysis of the up-regulated genes was performed using MACVECTOR 6.5 (Genetics Computer Group).

Abbreviations: dsRNA, double-stranded RNA; RNAi, RNA interference; TBZ, thiabendazole.

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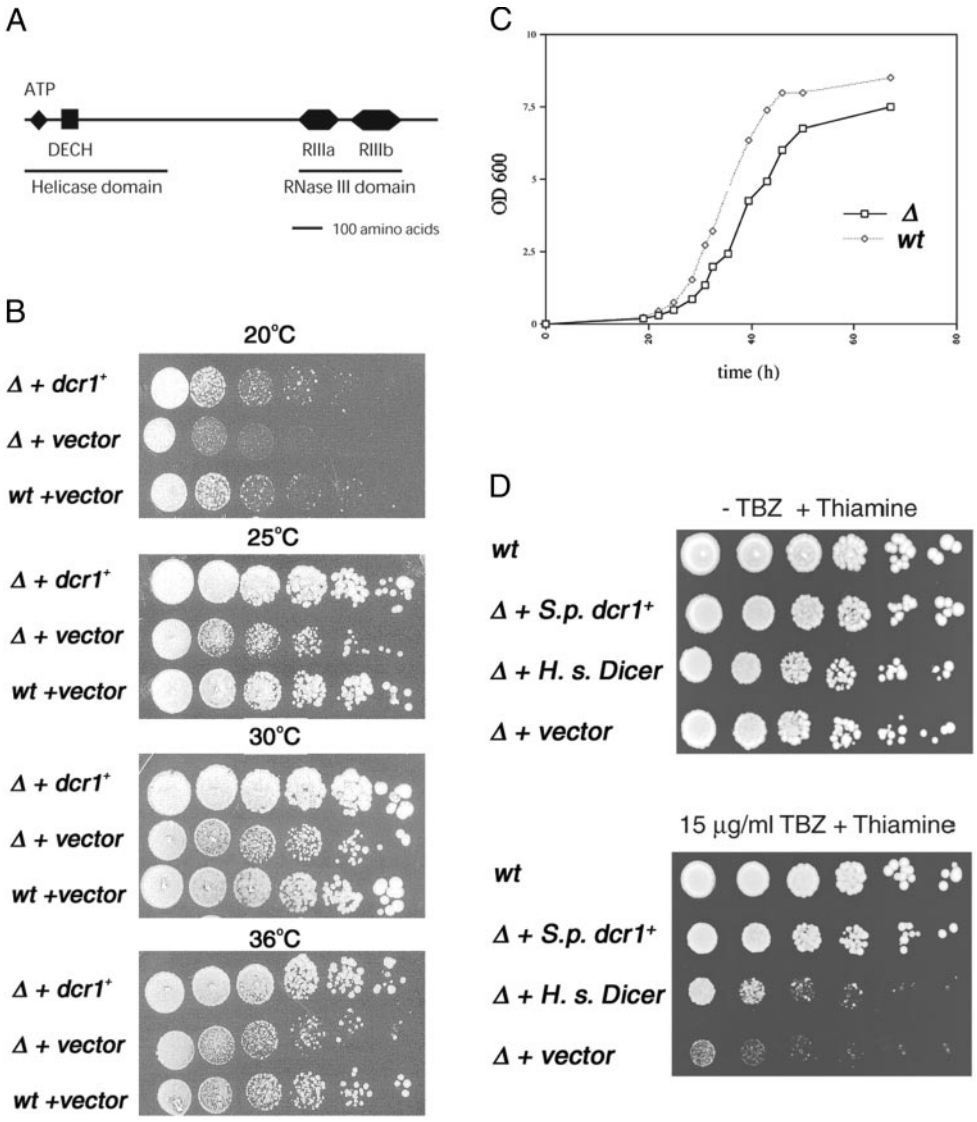

Fig. 1. Domain structure of Dcr1p, documentation of the temperature-sensitive, slow-growth sensitivity to TBZ phenotypes of the *dcr1*Δ strain, and functional complementation by human Dicer. (*A*) Predicted domain structure of Dcr1p deduced from the amino acid sequence of Dicer (SWISS-PROT accession no. Q09884). (*B*) Growth of *dcr1*Δ (Δ) and WT (*wt*) cells transformed with a vector expressing *S. pombe* Dcr1, or empty vector only, at 20, 25, 30, or 36°C. Cells were serially diluted at 5-fold dilutions and plated on minimal medium plates. Plates were photographed after 4 days of growth. Strains were Hu655 (Δ) and Hu111 (*wt*). (*C*) Growth of *dcr1*Δ or WT cells in liquid YES [supplemented yeast extract (rich)] medium at 25°C. Cultures were incubated with shaking at 25°C. Cell density (OD_{600}) was monitored. (*D*) Growth of WT (*wt*) and *dcr1*Δ (Δ) cells transformed with a vector expressing *S. pombe* Dcr1 (*S.p. dcr1*$^+$), human Dicer (*H.s. Dicer*), or empty vector only, plated on minimal medium plates containing 20 μM thiamine and 0 or 15 μg/ml TBZ as indicated. Strains were Hu655 (Δ) and Hu111 (*wt*). Cells were serially diluted at 5-fold dilutions. Plates were photographed after 4 days of growth at 30°C.

RT-PCR. cDNA was synthesized from *S. pombe* RNA preparations using Reverse Transcriptase (BRL) according to the manufacturer's instructions. cDNA samples were analyzed using *ade6*$^+$, *ura4*$^+$, *hsp16*$^+$, or SPBC19C7.04c gene primers in multiplex or competitive PCR strategies (see legend of Fig. 4).

Results and Discussion

Database searches with the human Dicer sequence, which we initially found in a yeast two-hybrid screening using 5-lipoxygenase as a bait (25), identified homologous sequences in a variety of organisms, except the budding yeast *Saccharomyces cerevisiae*. Notably, the recently sequenced genome of the fission yeast *S. pombe* (26) contains a homologous gene. The gene SPCC584.10c (SWISS-PROT accession no. Q09884), here designated *dcr1*$^+$, is 4,125 bp long and encodes a protein of 1,374 aa with an N-terminal helicase domain containing an ATP-binding site motif ^{32}MRTGAGKT^{39} and a ^{145}DECH^{148} box, and tandem C-terminal ribonuclease (RNase) III signatures [^{1120}QQLEFLGDA^{1128} (RIIIb) and ^{930}DRLEFYGDC^{938} (RIIIa)] (divergent residues are underlined). No other ORF sequences combining these putative functional domains could be identified in the *S. pombe* genome, unlike the situation in *Drosophila*, which has at least two Dicer genes (Dcr-1 and Dcr-2) (4). Alignment of the multiple Dicer sequences revealed a relatively high sequence similarity (see Fig. 5, which is published as supporting information on the PNAS web site, www.pnas.org). In this alignment, conservation of the ATP-binding site, the DECH box, and the second RNase III motif (RIIIb) is noteworthy, whereas the first RNase III motif (RIIIa) in *dcr1*$^+$ is slightly shifted toward the C terminus, as compared with the same motif in the mammalian proteins. The domain structure of *dcr1*$^+$ also differs from the other Dicer proteins in that it appears not to contain a Piwi/

Table 1. List of the strains used in this study

Strain	Genotype	Source or reference
Hu111	*h*$^+$ *leu1-32 ade6-DN/N ura4-DS/E*	This study
Hu303	*h*$^-$	Standard strain 972 *h*$^-$
Hu642	*h*$^-$ *leu1-32*	This study
Hu643	*h*$^+$ *leu1-32*	This study
Hu647	*leu1-32 ura4-DS/E*	This study
Hu648	*leu1-32 ura4-DS/E*	This study
Hu652	*h*$^-$ *ura4-DS/E*	This study
Hu653	*h*$^+$	This study
Hu655	*h*$^+$ *leu1-32 ade6-DN/N ura4-DS/E dcr1ΔkanMX6*	This study
Hu656	*h*$^-$ *leu1-32 ade6-DN/N ura4-DS/E dcr1ΔkanMX6*	This study
Hu657	*h*$^-$ *leu1-32 ura4-DS/E dcr1ΔkanMX6*	This study
Hu658	*leu1-32 ura4-DS/E dcr1ΔkanMX6*	This study
Hu659	*leu1-32 ura4-DS/E dcr1ΔkanMX6*	This study
Hu676	*h*$^+$ *ura4-DS/E dcr1ΔkanMX6*	This study
Hu677	*h*$^-$ *ura4-DS/E dcr1ΔkanMX6*	This study
Hu678	*h*$^+$ *dcr1Δ kanMX6*	This study
Hu679	*h*$^-$ *dcr1Δ kanMX6*	This study
Hu906	*dcr1-HA::kanMX6*	This study
Hu913	*dcr1-HA::kanMX6*$^+$ *leu1-32 ade6-DN/N ura4-DS/E cen1-otr1R(Sph1)::ade6*$^+$	This study
Fy2002	*h*$^+$ *leu1-32 ade6-DN/N ura4-DS/E cen1-imrL(Nco1)::ura4*$^+$ *otr1R(Sph1)::ade6*$^+$	35

Argonaute/Zwille (PAZ) domain (27). This domain, whose exact function remains unknown, is also present in *Drosophila* Argonaute (28).

To characterize the function of *dcr1*$^+$, a *dcr1*Δ strain was created (see Table 1). To document the effect of Dicer deletion on growth, colony growth assays were performed at 20, 25, 30, or 36°C (Fig. 1*B*). Growth assays in liquid cultures were also performed at 25°C (Fig. 1*C*). The *dcr1*Δ strain exhibited a temperature-sensitive phenotype, as compared with the WT strain; we observed a slight impairment of cell growth at 30 and 36°C, but a more severe growth defect at lower temperatures (20 and 25°C). The temperature-sensitive slow growth phenotype of *dcr1*Δ cells is suggestive of a possible defect in mitosis or other cell division-related phenomena. To investigate whether chromosome segregation was affected in *dcr1*Δ cells, their sensitivity to TBZ was investigated. *dcr1*Δ cells displayed a marked supersensitivity to TBZ, not growing at low concentrations of the drug that were well tolerated by WT cells (Fig. 1*D*, rows 1 and 4). Next, functional complementation of the slow growth and TBZ sensitivity phenotypes of *dcr1*Δ cells was investigated. We used plasmid constructs, in which the *S. pombe dcr1*$^+$ or human Dicer genes were inserted downstream of the *nmt1* promoter (see *Materials and Methods*). The *dcr1*Δ strain was transformed, and the transformants were grown on selective media at 25, 30, or 36°C. As shown in Fig. 1 *B* and *D*, plasmid expression of *dcr1*$^+$ rescued the growth defect and TBZ sensitivity of the *dcr1*Δ cells. Importantly, a partial functional rescue of *dcr1*Δ cells was achieved by episomal expression of human Dicer (see Fig. 1*D*, row 3), indicating that the function of this new class of proteins has been conserved during evolution.

To visualize the mitotic defect of *dcr1*Δ cells, anti-tubulin immunofluorescence (IF) microscopy was performed. Clearly, *dcr1*Δ cells displayed a high incidence of lagging chromosomes on anaphase spindles (Fig. 2*A*), and this defect was more pronounced at 25°C (33%, $n = 100$) than at 30°C (16% lagging chromosomes, $n = 100$). For WT cells, no lagging chromosomes were observed. This phenotype is generally indicative of a defective centromeric heterochromatin function, such as in *swi6*Δ cells (24), and has been shown to be due to loss of centromeric cohesion (29). To test whether formation of centromeric heterochromatin depends on *dcr1*$^+$, the transcription of an inserted *ade6*$^+$ marker gene [*cen1-otrR*(*Sph*I)::ade6$^+$] was measured by RT-PCR (Fig. 2*B*). We observed that *ade6*$^+$ was transcribed from the centromere in *dcr1*Δ cells, but not in WT control cells, indicating that heterochromatin was disrupted in *dcr1*Δ cells. In *Drosophila*, multiple integrated copies of transgenes produce transcripts that are silenced posttranscriptionally by RNAi (30). To test whether the *S. pombe* centromeric multiple copy repeats themselves also produce transcripts that may be degraded by Dcr1p, we carried out RT-PCR analysis of the noncoding centromere *otr-dg* repeats in WT and *dcr1*Δ cells. Our RT-PCR analysis showed that *otr-dg* transcripts were not detectable in RNA preparations from WT cells, but accumulated to high levels in *dcr1*Δ cells (Fig. 2*C*). Our RT-PCR analysis of the integrated *ade6*$^+$ marker gene centromere repeats are in agreement with the recently reported Northern and RT-PCR analysis of integrated *ura4*$^+$ marker gene and centromeric repeats (15). Also recently, small RNAs corresponding to *S. pombe* centromeric heterochromatic repeats were detected (16). The accumulation of long RNA molecules corresponding to heterochromatic repeats that we observed in *dcr1*Δ cells indicates that Dcr1p is normally required for posttranscriptional degradation of these long RNAs via dsRNA substrates and, thus, Dcr1p should be capable of degrading such dsRNA substrates *in vitro*.

To test the enzymatic activity of the Dcr1 protein *in vitro*, it was tagged with the HA epitope (see *Materials and Methods*). The Dcr1-HA-tagged protein was fully functional, as judged by growth and TBZ assays (unpublished data). Dcr1-HA could be immunoprecipitated from *S. pombe* cell homogenates by using anti-HA antibody, as shown by immunoblot analysis (Fig. 3*A*), and these immunoprecipitates exhibited dsRNase activity (Fig. 3 *B* and *C*). In this assay, an ≈23-nt band was produced by Dcr1-HA immunoprecipitates when incubated with the human let-7 microRNA precursor (Fig. 3*B*, lane 3), but not by immunoprecipitates prepared from *dcr1*Δ cells (Fig. 3*B*, lane 4). Similar results were obtained when using a 500-bp (Fig. 3*C*) dsRNA substrate. Thus, the *S. pombe* Dicer enzyme is capable of processing dsRNA *in vitro*.

To investigate whether Dcr1p is also required for silencing of genes in euchromatin, we have performed an initial exploration of the genes whose expression may be regulated, directly or indirectly, by Dcr1p, using a microarray expression profiling approach (Y. Xue, S. Haas, T. Driss, D. Marechal, M. Vingron,

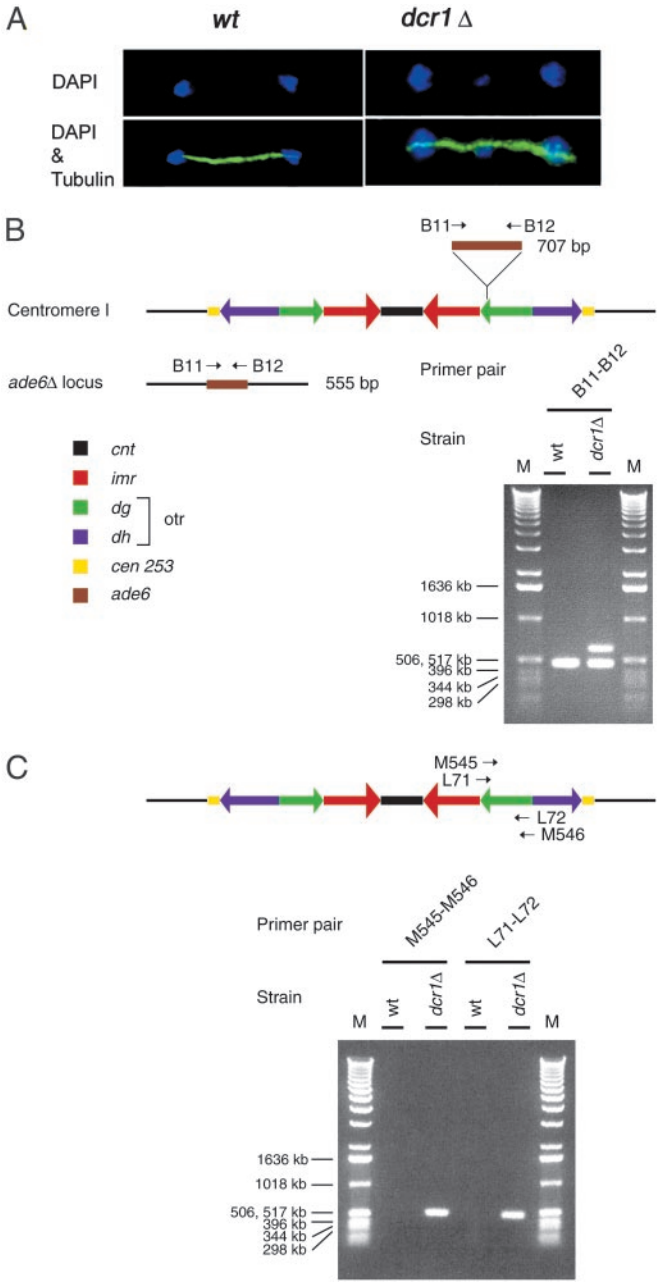

Fig. 2. Lagging chromosomes, centromere silencing defects, and transcription of noncoding *otr-dg* centromeric repeats in *dcr1*Δ cells. (*A*) Cells grown at 25°C were stained with TAT1 (α-tubulin, green) and 4′,6-diamidino-2-phenylindole (DAPI; DNA, blue). (*Left*) A normal anaphase *wt* cell. (*Right*) A lagging chromosome in a *dcr1*Δ cell. Strains were Hu655 (Δ) and Hu111 (*wt*). (*B*) Silencing defects of *dcr1*Δ. (*Upper*) Strategy for competitive RT-PCR. The top band (707 bp) represents the *ade6*$^+$ gene inserted in centromere [*cen1-otrR*(*SphI*):: ade6$^+$] and the bottom band (555 bp) represents *ade6*$^+$-*DN/N* allele at the endogenous *ade6*$^+$ locus amplified using the same set of primers. Strains were Hu913 (Δ) and Fy2002 (*wt*). (*C*) Accumulation of transcribed centromeric repeats in *dcr1*Δ cells. (*Upper*) Strategy for RT-PCR. The position of the primers are indicated on the schematic representation of *cen1*. Strains were Hu679 (Δ) and Hu303 (*wt*).

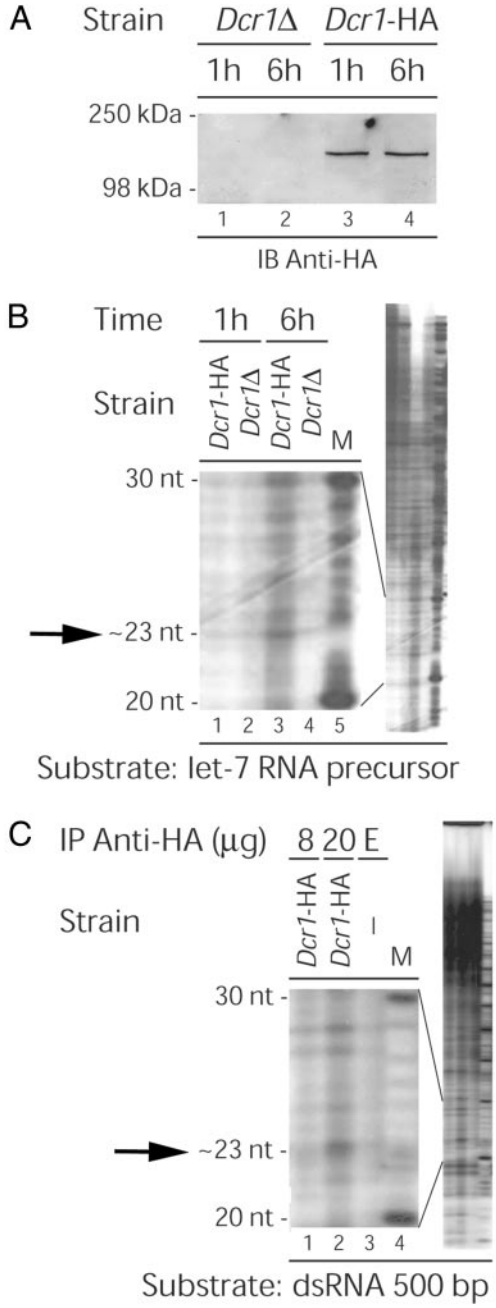

Fig. 3. Enzyme activity of the Dcr1 protein. The *dcr1*Δ and Dcr1-HA-tagged strains were grown and harvested, and the epitope-tagged Dcr1-HA protein was immunoprecipitated with anti-HA antibody (20 μg; clone 12CA5, Roche) and Protein A-Agarose (Roche). (*A*) Dcr1-HA protein expression. After several washes, the protein was eluted from the beads by boiling in loading buffer, and analyzed by immunoblotting using anti-HA antibody. (*B–C*) Detection of dsRNase activity. Twenty-three-nucleotide degradation products are indicated by arrows. ^{32}P-labeled dsRNA substrates were prepared, as described in refs. 20 and 36. After several washes, the beads were incubated with ^{32}P-labeled (*B*) 103-nt human let-7 microRNA precursor substrate (37) or (*C*) 500-bp Coactosin-like Protein (CLP) (nucleotides 150–649; accession no. L54057) dsRNA, in 10 μl of assay buffer (20 mM Tris·HCl/5 mM $MgCl_2$/1 mM DTT/1 mM ATP/5% Superase·In, pH 7.5) for 1 h (*A* and *B*) or 6 h (*A–C*) at 30°C. E, empty Protein A-Agarose beads; M indicates a 10-nt RNA size marker (Ambion, Austin, TX) prepared with [γ-^{32}P]ATP. Reactions were stopped on ice, and an equal volume of gel loading buffer was added. After heating at 85°C for 5 min, the samples were analyzed by electrophoresis on a 10% polyacrylamide/7M urea gel run in Tris-borate-EDTA (TBE) buffer. The gel was fixed and dried, and the ^{32}P-labeled RNA products were detected by autoradiography. The 20- to 30-nt region is shown, with the full-length gel on the right.

M., K.E., and A.W., unpublished work). Gene expression in the *dcr1*Δ cells was assessed and compared with that of the WT strain (Fig. 4*A*). Two genes, *hsp16*$^+$ and SPBC19C7.04c, were found to be consistently up-regulated by >3-fold in *dcr1*Δ cells as compared with WT cells at 25°C ($P < 0.05$, *t* test). To

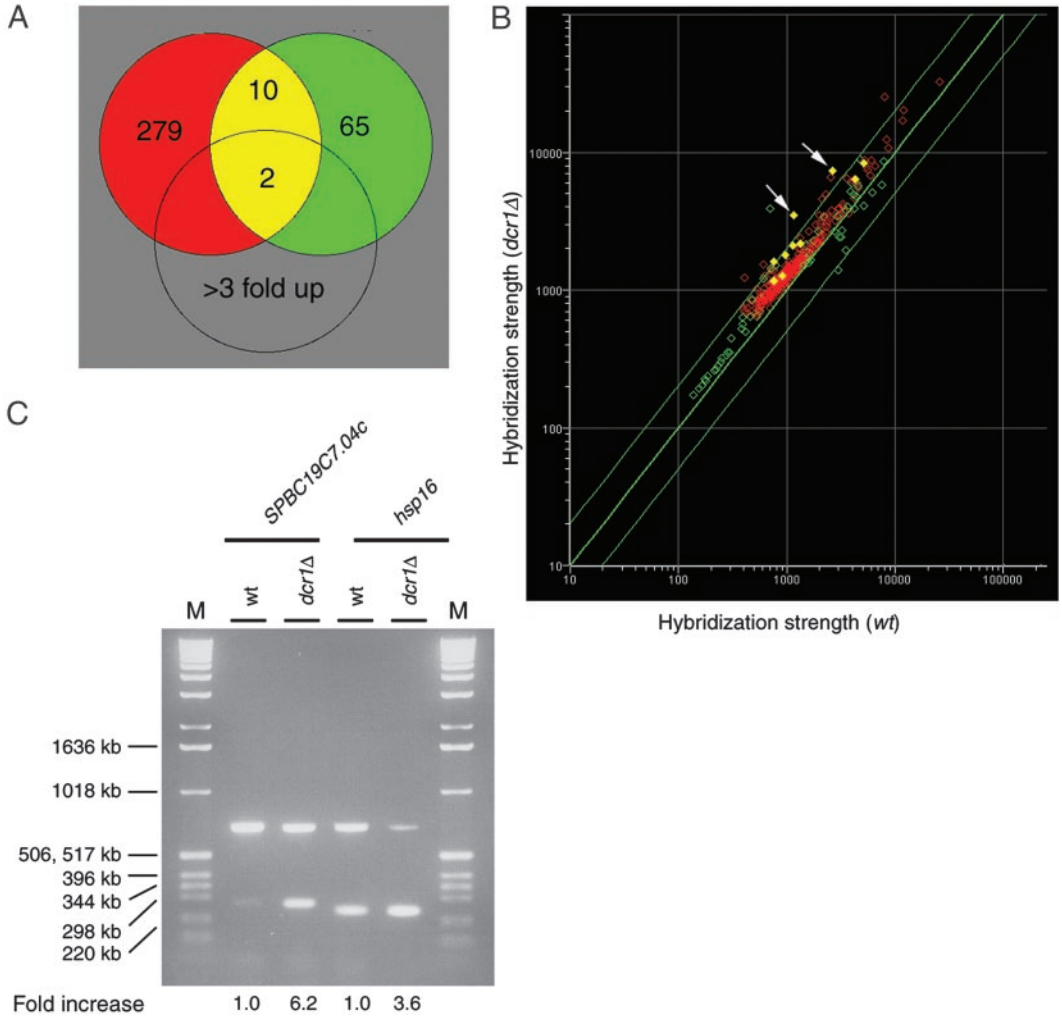

Fig. 4. ORF microarray expression profiling analysis of *dcr1*Δ cells. (*A*) The Venn diagram shows the number of genes whose expression was increased >1.5-fold in two independent experiments (red and green circles; see *Materials and Methods*). The yellow intersection shows the set of 10 reproducibly identified genes of which two (*hsp16*$^+$ and SPBC19C7.04c) were increased >3 fold. The complete data sets from the ORF microarray expression analyses are available at www.ki.se/biovetenskap/karl. The green circle corresponds to microarray ratios 1, and the red circle corresponds to microarray ratios 2. (*B*) The scatter plot shows nonprocessed hybridization intensity data from *dcr1*Δ and WT samples plotted against each other for the gene sets identified in *A*. The filled yellow points show the reproducibly identified genes, and points representing *hsp16*$^+$ and SPBC19C7.04c are shown by the white arrows. The diagonal green lines represent 2-fold changes. (*C*) RT-PCR analysis of the up-regulated genes, *hsp16*$^+$ and SPBC19C7.04c. The top band in each PCR reaction represents the *ura4*$^+$ control gene and the bottom band represents *hsp16*$^+$ or SPBC19C7.04c genes, as indicated. The fold increase of gene expression is indicated below each lane. For *A–C*, the strains used were Hu679 (Δ) and Hu303 (*wt*).

confirm the up-regulation of these two genes, an RT-PCR experiment was carried out (Fig. 4*C*). This experiment showed that *hsp16*$^+$ and SPBC19C7.04c genes were up-regulated 3.6- and 6.2-fold, respectively, in *dcr1*Δ cells. Interestingly, DNA matrix comparisons identified a common denominator to these two genes. Thus, an 11-nt DNA sequence (5′-GAGGACGT-TCA-3′) is present in four locations in the entire *S. pombe* genome: in opposite directions in the ORFs of *hsp16*$^+$ and SPBC19C7.04c, in one noncoding region (SPAC26H5), and in the gene encoding a putative U3 snoRNA component, which, in other organisms, is known to be required for rRNA processing (31). The presence of this conserved motif in ORFs and the fact that both genes containing this motif were strongly up-regulated in *dcr1*Δ cells are suggestive of a mechanism in which Dcr1p acts posttranscriptionally to degrade mRNA recognized by this motif.

We have shown that *dcr1*Δ cells display lagging chromosomes during anaphase, supporting a new role for Dicer in chromosome segregation and centromere silencing to maintain centromeric heterochromatin. Very recently, Dcr1, Ago1 (Argonaute), and Rdp1 (RNA-dependent RNA polymerase) were shown to be important for heterochromatin formation and centromere silencing by promoting H3 lysine 9 methylation in fission yeast (15). In addition, small RNA corresponding to *S. pombe* centromeric heterochromatin have been detected (16). These findings indicate that *S. pombe* is a suitable model for understanding biological roles for RNAi. Interestingly, an Argonaute homologue was isolated in a genetic screen as *csp* mutants (*c*entromere *s*uppressor of *p*osition effect) that alleviate centromeric silencing and cause defects in chromosome segregation very similar to those we report for *dcr1*Δ in this study (ref. 32; R. Allshire, personal communication). Thus, both Dicer and components of the RNA-induced silencing complex (RISC) are implicated in *S. pombe* centromere silencing, chromosome segregation, and centromere integrity. Examples of phenotypic abnormalities after inactivation of Dicer genes in other species are the unregulated cell division in *Arabidopsis* floral meristems (33) and sterility in *C. elegans* (6, 8, 34). We speculate that, as in other species,

aberrant small RNA formation underlies the effects of *dcr1*$^+$ inactivation in *S. pombe*, and that a functional Dicer is required for normal chromosome segregation and centromere integrity in other eukaryotes.

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