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Candida albicans Dicer (CaDcr1) is required for efficient ribosomal and spliceosomal RNA maturation

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The generation of mature functional RNAs from nascent transcripts requires the precise and coordinated action of numerous RNAs and proteins. One such protein family, the ribonuclease III (RNase III) endonucleases, includes Rnt1, which functions in fungal ribosome and spliceosome biogenesis, and Dicer, which generates the siRNAs of the RNAi pathway. The recent discovery of small RNAs in Candida albicans led us to investigate the function of C. albicans Dicer (CaDcr1). CaDcr1 is capable of generating siRNAs in vitro and is required for siRNA generation in vivo. In addition, CaDcr1 complements a Dicer knockout in Saccharomyces castellii, restoring RNAi-mediated gene repression. Unexpectedly, deletion of the C. albicans CaDcr1 results in a severe slow-growth phenotype, whereas deletion of another core component of the RNAi pathway (CaAGO1) has little effect on growth, suggesting that CaDcr1 may have an essential function in addition to producing siRNAs. Indeed CaDcr1, the sole functional RNase III enzyme in C. albicans, has additional functions: it is required for cleavage of the 3′ external transcribed spacer from unprocessed pre-rRNA and for processing the 3′ tail of snRNA U4. Our results suggest two models whereby the RNAi enzymes of a fungal ancestor, containing both a canonical Dicer and Rnt1, evolved through a series of gene-duplication and gene-loss events to generate the variety of RNase III enzymes found in modern-day budding yeasts.

Argonaute | CD10 | bifunctional dicer

RNA processing plays pivotal roles in ribosome biogenesis (1), mRNA maturation (2), tRNA synthesis (3), and siRNA generation (4). Each requires the accurate and efficient maturation of precursor RNAs to generate specialized RNA molecules designed to perform distinct cellular tasks.

Dicer is the key ribonuclease important for the generation of siRNAs and related types of small RNAs, which in most eukaryotic organisms have key functions in viral defense, transposon silencing, and cellular gene repression (5–10). Dicer generates siRNA duplexes from long double-stranded RNA (dsRNA). One strand of the duplex is loaded into an effector complex containing Argonaute, where it base pairs to target RNAs, thereby directing gene repression during the process of RNAi. Both Dicer and Argonaute are absent in Saccharomyces cerevisiae (8) but present in some other budding-yeast species, including Saccharomyces castellii and Candida albicans, a fungal pathogen (11). The S. castellii Dicer (SceDcr1) and Argonaute (SceAgo1) are required for gene silencing but are not essential for viability (11). C. albicans has a Dicer-like activity that can process dsRNA into small RNAs in vitro, and in vivo produces small RNAs that bear the chemical signature of molecules generated by a ribonuclease III (RNase III) cleavage (11). However, C. albicans Argonaute (CaAgo1) and Dicer (CaDcr1) are reported to be insufficient to induce RNAi-mediated gene silencing in C. albicans (12). Thus, the in vivo roles of CaDcr1 and CaAgo1 remain a mystery.

Although the domain structure of budding-yeast Argonaute proteins is similar to that of Argonaute proteins found in other eukaryotes, the budding-yeast Dicers are quite dissimilar from their canonical counterparts found in other fungi and higher eukaryotes (11). These canonical fungal Dicers contain two RNase III domains as well as helicase and Piwi Argonaute Zwille (PZ) domains (13). By contrast, ScaDcr1 and CaDcr1 have only a single RNase III domain adjacent to a dsRNA-binding domain (dsRBD) and an additional C-terminal dsRBD (11) (Fig. 1). Despite significant differences in domain structure between the budding-yeast and canonical Dicers, the RNase III signature motif and active-site residues important for dsRNA cleavage are conserved in ScaDcr1 and CaDcr1, suggesting that the RNase III cleavage activity is conserved in ScaDcr1 and CaDcr1 (14–16).

Bacteria and some budding yeasts encode a single protein with this RNase III domain, whereas higher eukaryotes often encode several RNase III enzymes that perform distinct tasks in RNA metabolism. Rnt1, the sole RNase III protein in S. cerevisiae, plays crucial roles in ribosomal and spliceosomal RNA maturation (17–20). For example, S. cerevisiae Rnt1 (SceRnt1) cleaves the 3′ external transcribed spacer (ETS) hairpin from primary 35S rRNA transcript, thereby permitting downstream rRNA processing events (20). Mutants lacking SceRnt1 function grow slowly because they have a severe defect in processing the nascent 35S rRNA transcript, resulting in decreased ribosome biogenesis (20, 21). S. castellii encodes two RNase III proteins, ScaRnt1 and ScaDcr1, each thought to perform specialized functions in RNA processing (11). ScaRnt1 has the same domain structure as SceRnt1 and presumably plays analogous roles in ribosomal and spliceosomal RNA maturation. ScaDcr1 contains a second dsRBD not found in SceRnt1 (Fig. 1B), suggesting that this domain functions in Dicer-specific activities as seen with Klaveromyces polysporus Dcr1 (16).

The in vivo roles for C. albicans RNase III homologs are unknown. To better understand the roles of Dicer and Argonaute in C. albicans, we analyzed the consequences of deleting the genes encoding each of these proteins. C. albicans mutants lacking AGO1 grow normally. We were only able to knock out both endogenous copies of DCR1 when DCR1 was expressed in trans from an inducible promoter. Analysis of strains defective in DCR1 show that this protein is unusual: DCR1 encodes a versatile RNase III enzyme that acts as both a Dicer, catalyzing the production of siRNAs, and as an RNA and snRNA processing enzyme (cleaving the 3′ ETS from unprocessed pre-rRNA and processing the 3′ tail of snRNA U4) like the S. cerevisiae Rnt1 enzyme. The Rnt1-like roles of CaDcr1 are likely responsible for the severe slow growth phenotype of mutant strains lacking DCR1. Our data suggest that the RNase III enzymes of fungi have evolved through a number of


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gene-duplication, neo-functionalization, sub-functionalization, and gene-loss events.

Results

C. albicans AGO1 and DCR1 Mutants. To characterize the in vivo roles of CaDcr1 and CaAgol, we attempted to construct homologous deletions of DCR1 and AGO1. The homologous deletion strains of AGO1 (ORF19.2903) were constructed from wild-type strains by successive transformations with the same deletion construct, with marker removal in between as described in Materials and Methods. Homologous AGO1 deletion in two different C. albicans strain backgrounds had no effect on growth rate (Fig. S1), germ tube formation, or colony morphology.

A search of the C. albicans genome for homologs of S. castellii Dcr1 revealed two ORFs predicted to encode RNase III domains. One previously identified as the Candida Dicer ortholog is encoded by ORF19.3796 (11), and another is encoded by ORF19.3773. Both of these genes are conserved throughout the Candida clade and have the same domain structure as ScDcr1 (Fig. 1A and B). Alignment of all of the RNase III domains from the Candida clade divided the RNase III-containing proteins into two families, ORF19.3796 homologs and ORF19.3773 homologs (Fig. 1A and C). Examination of the ORF19.3773 sequence revealed that amino acids corresponding to E278, D282, N315, K346, D350, and E353 of SceRnt1, which are highly conserved residues involved in metal-ion coordination at the active sites of RNase III enzymes, are altered to catalytically inactive residues in the Cdl1 (Candida dicer-like) protein family are labeled (*) and shaded red when inactive. The 9-aa RNase III signature motif is labeled. ClustalW 1.81 with default setting was used to generate both the phylogenetic tree and the sequence alignment.

Fig. 1. (A) Phylogenetic relationship between RNase III enzymes of indicated species. Bootstrap values for key nodes are shown, with central region (boxed) enlarged for clarity. Table indicates the presence (+) or absence (-) of the indicated gene. For Dicer, (+) designates budding-yeast Dicer, whereas - indicates canonical Dicer. (B) Domain structure of budding yeast RNase III enzymes. (C) Sequence alignment around the RNase III catalytic residues from the protein sequences from A. Sequence conservation is shown in gray scale, with the most highly conserved residues most darkly colored. The six residues (corresponding to SceRnt1 E278, D282, N315, K346, D350, and E353) altered to catalytically inactive residues in the Cdl1 (Candida dicer-like) protein family are labeled (*) and shaded red when inactive. The 9-aa RNase III signature motif is labeled. ClustalW 1.81 with default setting was used to generate both the phylogenetic tree and the sequence alignment.
growth of deletion strains in which the transgene was not induced, suggest that C. albicans DCR1 encodes an essential function. The slow growth of strains under repressed conditions might be attributable either to the incomplete repression of the ectopic copy of DCR1 or to residual Dcr1 protein.

**C. albicans Dcr1 Generates Small RNAs in Vivo and in Vitro.** The construction of a strain lacking Dcr1 activity enabled us to determine whether DCR1 function was required to generate siRNAs. Northern analysis of total RNA prepared from cells grown under repressive conditions revealed that two of the most abundant siRNAs in *C. albicans* (11) were significantly lower in *MAL2p-DCR1, Δdcr1/Δdcr1* than in strains expressing DCR1 (Fig. 3A and Fig. S3A and B). Growth of *MAL2p-DCR1, Δdcr1/Δdcr1* in media containing maltose restored siRNA production and actually increased the levels of these siRNAs (Fig. 3A and Fig. S3 A and B) over those found in wild-type cells. Furthermore, a lower abundance siRNA, previously identified by high-throughput sequencing, was only detectable by Northern blotting in *MAL2p-DCR1* containing strains grown on maltose (Fig. S3 C and D). The increase in siRNA production in the maltose-inducible strains when grown in maltose was likely attributable to increased Dcr1 levels as a result of increased promoter strength, suggesting that Dcr1 is normally limiting for siRNA production. Homozygous deletion of *AGO1 (Δago1/Δago1)* had no effect on siRNA accumulation as tested by Northern blotting (Fig. S3E).

Because Δdcr1/Δdcr1 mutants had diminished siRNA levels in vivo, we sought to determine whether purified Dcr1 had in vitro dicing activity. Recombinant Dcr1 was purified from *Escherichia coli* and incubated with a 500-bp radiolabeled dsRNA substrate. Purified Dcr1 cleaved the substrate to 22-nt products (Fig. 3B), a size matching that of small RNAs sequenced from *C. albicans* (11). These results show that purified CaDcr1 is capable of making siRNAs in vitro and are in agreement with previous work showing that *C. albicans* crude extracts can generate siRNAs from an exogenous dsRNA substrate (11).

These results raised the question of whether CaDcr1 can act in the RNAi pathway to silence a gene in vivo. Because a gene-silencing system does not yet exist for *C. albicans*, we tested whether CaDcr1 can mediate silencing in *S. castellii*. *SacDcr1* is required to generate the siRNAs that direct gene silencing (11). A recoded *CaDCR1* was transformed into an *S. castellii* Δdcr1 mutant that expresses green fluorescent protein (GFP) from the *URA3* promoter and a hairpin against GFP from the *GAL1* promoter. The *CaDCR1* complemented the *S. castellii* Δdcr1 mutant, restoring silencing to levels observed in an *S. castellii* strain with a functional *S. castellii DCR1* (Fig. 3C). Together, these in vitro and in vivo data demonstrate that *C. albicans DCR1* encodes a gene with Dicer function.

**C. albicans DCR1 Is Required for rRNA Processing.** The requirement of functional CaDcr1 but not CaAgo1 for normal growth suggests that CaDcr1 might have additional roles in *Candida*. In other fungi, mutants lacking either Dicer or Argonaute have minimal effects on growth rates (11, 22, 23). One possibility is that CaDcr1 functions like the SceRnt1 of *S. cerevisiae*, which plays a critical role in the initial steps of ribosome biosynthesis. Decreased expression of *SceRnt1* leads to a severe slow-growth phenotype resembling that observed in the *C. albicans Δdcr1/Δdcr1* mutant. Furthermore, the only additional gene in the *Candida* genome that contains an RNase III domain, *CDL1*, is unlikely to have catalytic activity, which leaves CaDcr1 as the only RNase III with resemblance to levels observed in an *S. castellii* strain with a functional *S. castellii DCR1* (Fig. 3C). Together, these in vitro and in vivo data demonstrate that *C. albicans DCR1* encodes a gene with Dicer function.

Figure 2. Effects of DCR1 on *C. albicans* growth. (A) WT [BWP17 (■)], Δdcr1/Δdcr1 [ΔAB151 (▲)], Δdcr1/Δdcr1 ENO1/eno1::MAL2p-DCR1 [ΔAB225 (●)], and Δdcr1/Δdcr1 ENO1/eno1::MAL2p-DCR1 [ΔAB225 (×)] grown on a roller drum in synthetic complete (SC) maltose. (B) Strains from A grown in SC glucose. OD600 was used to measure growth. Points plotted are the average ODs of three replicates, and error bars represent 1 SD of the mean.

Figure 3. Functions of *DCR1* in vivo and in vitro. (A) Small RNA Northern blot analysis of RNA purified from cells grown overnight in SC and resolved on a denaturing gel. Strains are of indicated genotype, and probe was Northern Primer 1. Left: BWP17 background; Right: CAI4 background. Below each blot, ribosomal RNA has been visualized with ethidium bromide to show loading. (B) In vitro processing of radiolabeled dsRNA by recombinant *C. albicans Dcr1*. Products were resolved on a denaturing gel. *SacDcr1* had no detectable levels of unprocessed RNA transcripts, whereas the Δdcr1/Δdcr1 strains grown in restrictive conditions accumulated unprocessed RNA.
transcripts (Fig. 4 A and Fig. S4 A, C, and E). By contrast, all four strains grown in permissive media had undetectable levels of unprocessed rRNA transcripts (Fig. 4 B and Fig. S4 B, D, and F). Homozygous deletion of AGO1 had no effect on rRNA processing (Fig. S4 J). The rRNA processing defect was unlikely to be an indirect consequence of the loss of an essential gene function, because strains lacking another essential gene (PES1 under control of MAL2p) had severely reduced growth on glucose but did not display the same rRNA processing defect we observed in Δdcr1/Δdcr1 MAL2p-DCR1 (Fig. 4 J).

Because S. cerevisiae Rnt1 plays an additional role in the proper maturation of some snRNA transcripts, we determined whether CaDcr1 also plays an analogous role in snRNA processing. In S. cerevisiae, mtl1 cells accumulate unprocessed U4 transcript (24). On a growth medium containing glucose, we observed an accumulation of unprocessed U4 snRNA transcript in Δdcr1/Δdcr1 MAL2p-DCR1 (Fig. 4 C) but not in DCR1/DCR1, Δdcr1/DCR1, or Δdcr1/DCR1 MAL2p-DCR1 (Fig. 4 D), indicating that CaDcr1 participates in U4 snRNA processing in vivo. Correctly processed U4 snRNA also accumulated in nonpermissive conditions, a result similar to that obtained in S. cerevisiae (24). This result suggests that C. albicans encodes other proteins able to act in a functionally redundant manner, or the small amount of expression in glucose is sufficient for detectable processing.

Discussion

Our findings suggest that CaDcr1, which seems to be the only active RNase III enzyme in C. albicans, is versatile, possessing a dicin function that generates siRNAs and additional functions involved in ribosome and spliceosome biogenesis. We attribute the slow-growth phenotype of Δdcr1/Δdcr1 to its ribosome-maturation defects because loss of CaAGO1 did not affect the growth rate. Knockouts of S. cerevisiae RNT1 and its Schizosaccharomyces pombe homolog PAC1 also result in severe growth defects that are likely attributable to ribosome-maturation defects similar to those observed in Δdcr1/Δdcr1 (21, 25, 26).

The dual function of the C. albicans Dicer is unique among the fungal Dicers that have been analyzed. An S. castellii strain lacking Dcr1 function resulted in loss of siRNAs but no obvious loss in viability or discernible phenotype related to growth or morphology (11). The only phenotype that has been noted is the loss of RNAi leads to a compatibility with dsRNA killer viruses (27). Previous work identified small RNAs from C. albicans Zorro L1 elements, suggesting a role in genome defense. Because the Δdcr1/Δdcr1 strain has poor viability, the biological roles of siRNAs and other components of the RNAi pathway were best queried using the Δago1/Δago1 strain. Although the siRNAs derived from Zorro L1-like elements imply that these transposable elements are silenced by the RNAi pathway (11), we did not observe any obvious difference in Zorro transposition between AGO1/AGO1 and Δago1/Δago1 strains. However, the Zorro transposition frequency is very low in Candida, and slight differences would not have been detected (28).

Our results together with previous work provide insight into the evolution of the budding-yeast RNase III enzymes. Four types of RNase III-like proteins exist in budding yeast (Fig. 1 B). The S. cerevisiae genome encodes a single RNase III enzyme, Rnt1, that is essential for ribosome biosynthesis and also functions in generating spliceosomal RNAs (17, 19, 20), but SceRnt1 does not process dsRNA into siRNAs, consistent with the lack of RNAi in this species (11). S. castellii contains two functional RNase III enzymes, one of which likely plays a similar role to SceRnt1 in ribosome and snRNA processing and another, ScaDcr1, which generates siRNAs that guide RNA silencing (11). K. polysporus and Saccharomyces bayanus both contain syntenic homologs of the S. castellii DCR1 and RNT1. C. albicans encodes two proteins with an RNase III domain: Dcr1, which is multifunctional and plays roles in siRNA, snRNA, and rRNA maturation; and Cdl1, which is unlikely to have cleavage activity and whose role in vivo remains a mystery. These two genes are not syntenic with either DCR1 or RNT1 from non-Candida budding yeasts.

The domain structure of Cdl1 is identical to that of CaDcr1 (Fig. 1 B), suggesting that it could have originated from a recent gene-duplication event. However, Cdl1 is unlikely to have canonical RNase III activity, because six residues important for RNA cleavage and conserved in other eukaryotic RNase III enzymes are not conserved in Cdl1 homologs. Retention of Cdl1 in the Candida clade and conservation of other amino acids in the RNase III domain suggest that it has a function. Catalytically inactive enzyme homologs are common in signaling pathways, and these inactive proteins play roles regulating those pathways (29). Because RNase III proteins act as obligate dimers (14), the potential formation of a heterodimeric complex between CaDcr1 and CaCdl1 is intriguing.

Uncertainties in the branching order within the budding yeast RNase III phylogeny, combined with the absence of informative syntenic relationships, make it difficult to posit a single model that best explains the evolutionary paths of these enzymes. Therefore, we offer two models consistent with our data (Fig. 5). Both assume an ancestral species that had a canonical Dicer (DICER) and RNT1, as currently observed in the fission yeast S. pombe. Model 1 suggests RNT1 duplication with neofunctionalization to generate a non-canonical Dicer gene (DCR1) in a transitional species. The loss of DICER left both RNT1 and DCR1, as in present-day S. castellii. Loss of DCR1 and the rest of the RNAi pathway in many budding-yeast lineages (16) left these lineages with only RNT1, as observed in present-day S. cerevisiae and other members of the Saccharomyces complex that lack Argonaute and Dicer homologs. Meanwhile, neofunctionalization of DCR1 in the S. castellii-like ancestor of the Candida clade led to the multifunctional enzyme RNT1/DCR1. RNT1 loss (and Cdl1 gain through RNT1/DCR1 duplication with neofunctionalization) then generated the genes of C. albicans. Model 2 posits early neofunctionalization of an ancestral RNT1 to generate a transitional species with the multifunctional RNT1/DCR1. Subsequent DICER loss then generated the Candida-like budding yeast ancestor, which gained Cdl1 through RNT1/DCR1 duplication with neofunctionalization in the Candida lineage. Meanwhile, DCR1/RNT1 duplication with subfunctionalization generated the two RNase III enzymes present in S. castellii. This duplication probably did not occur during the whole-genome duplication (WGD) because the synten typical of paralogs created
at the WGD, such as that observed between Candida glabrata RNT1 paralogs, is not observed for DCR1 and RNT1. The ScaDcr1 RNase III domain is not significantly more similar to the RNase III domains of ScaRnt1 or CaDcr1 (Rnt1/Dcr1), thus we cannot distinguish between these two models with confidence. We favor model 1, because model 2 posits gain and then loss of the second dsRBD during the transitions from RNT1 to RNT1/DCR1 and back to RNT1. This objection would be mitigated in a hybrid of models 1 and 2, in which Fig. 5. Two models to explain the evolution of budding yeast DCR1 and RNT1. DICER, canonical Dicer as found in S. pombe; DCR1, budding yeast Dicer as found in S. castellii, K. polysporus, and S. bayanus; RNT1, Ribonuclease III as in S. cerevisiae; RNT1/DCR1, a multifunctional Dicer found in C. albicans; CDL1, Candida Dicer-like from the Candida clade.

Table 1. Yeast strains

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RNT1 is duplicated before RNT1/DCR1 neo-functionalization and then retained in all ancestors of the Saccharomyces lineage. Regardless of the model, the evolution of the budding-yeast RNase III enzymes was not a simple linear path but instead required several instances of gene duplication/loss, often with neo-functionalization/sub-functionalization, to arrive at the diversity currently observed.

**Materials and Methods**

**Dicing Assay.** Radiolabeled dsRNA (140 pM) was incubated with purified Ca∆1 protein for 30 min at 37 °C, as described previously (11).

**Phylogenetic Analysis.** RNase III domains were identified using the Conserved Domain Domain search of the National Center for Biotechnology Information (30). Alignments of RNase III domains from selected fungi were created with ClustalW on the Biology Workbench [workbench.sdsc.edu (31)], using default parameters. A tree with bootstrap values was created with Clustaltree on Biology Workbench, and Treeview (32). Synteny analysis was performed using the Yeast Gene Order Browser (33) and Candida Gene Order Browser (34).

**FACS.** Cells were grown overnight in appropriate media, diluted in PBS, and subjected to flow cytometry on a BD FACS Calibur with data acquisition using BD CellQuest Pro. Flow cytometry data analysis was done using FlowJo (Treestar).

**Strain Construction.** All Candida strain genotypes in Table 1 were verified by Southern analysis. The KpnI/SacI fragment from plasmid DAB124 was transformed into C. albicans strains BW17 and CAI4 and selected on yeast extract, peptone dextrose media with Nourseothricin (YPD + Nat). Correct integrants were grown in Difco yeast carbon base with bovine serum albumin (YCB + BSA) to induce expression of the flip recombinase and subsequent removal of the NAT gene from the genome generating DAB151 and DAB157. DAB151 and DAB157 were transformed with pV397 integrating Teto–DCR1 at the ADH1 locus. Correct integration in strains DAB151 and DAB157 generated DAB184 and DAB196, respectively. We also transformed DAB151 and DAB157 with pV434 integrating MAL2p–DCR1 at the ENO1 locus. Correct integration in strains DAB151 and DAB157 generated DAB223 and DAB224, respectively. A KpnI/SacI fragment of DAB126 was transformed to disrupt the second endogenous copy of DCR1, generating dcr1-1. The transformation of DAB196 and DAB184 generated DAB204 and DAB232, respectively. An analogous strategy was used to generate strains DAB225 and DAB228 from DAB223 and DAB224, using plasmid DAB177. Although the construction of DAB225 from DAB223 resulted in the subsequent loss of the URA3, the construction of the Dox-driven expression system resulted in auxotrophically matched strains. Because we see a similar slow-growth phenotype in both constructions, the slow growth of the maltose-inducible strains is not due to the rescue of the uracl1 auxotrophy. Strains BW17 and SC5314 were transformed with either pV308 or pV307 (respectively) digested with either pV308 or pV307 digested with KpnI/SacI. Correct integrants were grown on YCB + BSA medium to induce expression of FLP, leaving only FRT sites, resulting in strains YVS523 and YVS519. These strains were subjected to an additional round of transformation and flipping out, resulting in strains YVS537 and YVS561. S. castelli strains DPB333 and DPB339 were transformed using the lithium acetate method, with either pV401 or pV406 for complementation analysis, and selected on YPD + Nat.

**Additional methods included in SI Materials and Methods.**

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