

## Genes come and go

### The evolutionarily plastic path of budding yeast RNase III enzymes

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**O**ur recent finding that the *Candida albicans* RNase III enzyme CaDcr1 is an unusual, multifunctional RNase III coupled with data on the RNase III enzymes from other fungal species prompted us to seek a model that explained the evolution of RNase III's in modern budding yeast species. CaDcr1 has both dicer function (generates small RNA molecules from dsRNA precursors) and Rnt1 function, (catalyzes the maturation of 35S rRNA and U4 snRNA). Some budding yeast species have two distinct genes that encode these functions, a *Dicer* and *RNT1*, whereas others have only an *RNT1* and no *Dicer*. As none of the budding yeast species has the canonical *Dicer* found in many other fungal lineages and most eukaryotes, the extant species must have evolved from an ancestor that lost the canonical *Dicer*, and evolved a novel *Dicer* from the essential *RNT1* gene. No single, simple model could explain the evolution of RNase III enzymes from this ancestor because existing sequence data are consistent with two equally plausible models. The models share an architecture for RNase III evolution that involves gene duplication, loss, subfunctionalization, and neofunctionalization. This commentary explains our reasoning, and offers the prospect that further genomic data could further resolve the dilemma surrounding the budding yeast RNase III's evolution.

#### The RNase III Family of Proteins

RNase III enzymes are a family of double-strand RNases conserved from bacteria to higher eukaryotes.<sup>1,2</sup> RNase III family

members participate in a wide variety of RNA-processing reactions including the generation of small interfering RNAs (siRNAs)<sup>3</sup> capable of eliciting gene silencing and the processing of rRNA, snoRNA, and snRNA precursor molecules.<sup>4-7</sup> The hallmark of the RNase III family of proteins is the RNase III domain. This domain is distinguished by the presence of both a dsRNA-binding domain (dsRBD); and an RNase III signature motif. The RNase III signature motif contains a number of highly conserved amino acids important for coordination of 2 Mg<sup>2+</sup> ions and RNase III catalytic activity<sup>8-11</sup> while the dsRBD binds 2'-OH of the ribose moieties and the phosphate backbone of dsRNA. Furthermore, some RNase IIIs contain multiple dsRBDs that can act collectively to identify and bind specific substrates.<sup>12</sup> As their diverse biological functions suggest, RNase III enzymes may contain accessory domains such as PAZ, helicase, or additional dsRBD domains, which play roles in targeting the enzyme to a specific RNA substrate or facilitating specific biochemical tasks once present at the target RNA.<sup>13</sup>

In *S. cerevisiae*, the only protein predicted to contain an RNase III domain is Rnt1 (ScRnt1). The *RNT1* gene encodes a protein that is important for the initial steps of 35S rRNA processing, and for processing snoRNA and snRNA.<sup>4-7</sup> However, the ScRnt1 lacks dicer function, and *S. cerevisiae* does not encode an Argonaute, another key component of RNAi silencing systems. By contrast the *Saccharomyces castellii* genome encodes not only a *RNT1* ortholog, but a second RNase III gene. This second RNase III, ScaDcr1, is a

**Keywords:** RNase III, bifunctional dicer, DCR1, RNT1, *Candida*, yeast, evolution

Submitted: 05/16/12

Revised: 06/29/12

Accepted: 07/04/12

<http://dx.doi.org/10.4161/rna.21360>

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**Table 1.** RNase III domain containing proteins mentioned throughout this commentary

species	<i>S. cerevisiae</i>	<i>C. glabrata</i>	<i>C. albicans</i>	<i>S. castellii</i>	<i>S. bayanus</i>	<i>K. polysporus</i>	<i>S. pombe</i>
RNase III	<i>RNT1</i>	<i>RNT1</i> <i>RNT2</i>	<i>DCR1</i> <i>CDL1</i>	<i>RNT1</i> <i>DCR1</i>	<i>RNT1</i> <i>DCR1</i>	<i>RNT1</i> <i>DCR1</i>	<i>PAC1</i> <i>Dicer</i>

noncanonical Dicer enzyme. Although ScaDcr1 generates siRNA capable of mediating gene silencing, it encodes only a single RNase III domain, and lacks PAZ and helicase domains typically encoded by canonical Dicers.<sup>14</sup> Furthermore, ScaDcr1 is distinguished from Rnt1 enzymes by the presence of a second dsRBD at its C-terminus.

The *Candida albicans* Dicer, CaDcr1, is a multifunctional RNase III. Inspection of budding yeast genome sequences revealed that non-canonical Dicer enzymes could be found in a number of species including the human pathogen *C. albicans*.<sup>14</sup> Since small RNAs with the structure similar to those of Argonaute-bound guide RNAs had previously been identified in *C. albicans*, we hypothesized that this *C. albicans* RNase III (*CaDCR1*) was responsible for siRNA generation. *C. albicans* strains homozygous for a deletion of the resident *CaDCR1*, but encoding an inducible copy at an ectopic site were very slow growing when the exogenous copy was not expressed, suggesting that in contrast to *ScaDCR1*, *CaDCR1* was important for WT growth.<sup>14</sup> However, knockout of a *C. albicans* Argonaute (*CaAGO1*) had no obvious growth defect, which suggested that the *Cadcr1/Cadcr1* growth defect was unlikely to be a consequence of diminished silencing.<sup>15</sup> These results raised the question: does CaDcr1 function as a Dicer? Subsequent experiments showed that the small-RNA levels observed in wild type were decreased in the *C. albicans* *CaDCR1* knockout and that *Candida* *DCR1* complemented a *S. castellii* *dcr1* in a GFP silencing assay. Moreover, in vitro CaDcr1 could process a dsRNA molecule into sRNA fragments, and the size of these fragments was consistent with the sRNA fragments identified in *C. albicans* cell extracts. These data suggested CaDcr1 was acting as a Dicer in vivo, but failed to explain why CaDcr1 was essential.<sup>15</sup>

Further inspection of the *Candida* genome revealed that the only other RNase III enzyme, *Candida* Dicer Like1 (*CDL1*), was likely to be catalytically inactive since a number of residues known to coordinate Mg<sup>2+</sup> in RNase III active sites had been altered.<sup>15</sup> As CaDcr1 was the only active RNase III enzyme in *C. albicans*, we hypothesized that *CaDCR1* might carry out functions similar to those of *S. cerevisiae* *RNT1*. Loss of Rnt1 function in *S. cerevisiae* leads to defects in rRNA and snRNA processing, and indeed, Northern analysis showed that knock out of *CaDCR1* similarly lead to an increased accumulation of rRNA and U4 precursor molecules.<sup>4,15,16</sup>

Our analysis showed that members of the budding-yeast lineage contain at least three distinct types of RNase III enzymes. *S. cerevisiae* contains only a single RNase III enzyme, SceRnt1, which carries out rRNA, snoRNA, and snRNA processing reactions. *S. castellii* contains two functional RNase III enzymes, one an ortholog of SceRnt1 that is likely to function similarly, and a noncanonical Dicer, that generates siRNA capable of gene silencing. Members of the *Candida* clade appear to encode two proteins with RNase III motifs: an active enzyme, CaDcr1, that functions both as a non-canonical Dicer and Rnt1 in vivo (Table 1), and *Candida* Dicer Like 1 (*Cdl1*), that lacks essential residues for RNase III activity.

### Evolution of the Budding Yeast RNase III

The discovery that extant members of the budding-yeast lineage encode such a wide variety of RNase III enzymes prompted our attempt to reconstruct an evolutionary history that could engender such diversity. Our aim was to identify a linear progression from a presumptive ancestral RNase III complement to that found in the current species of budding yeasts.

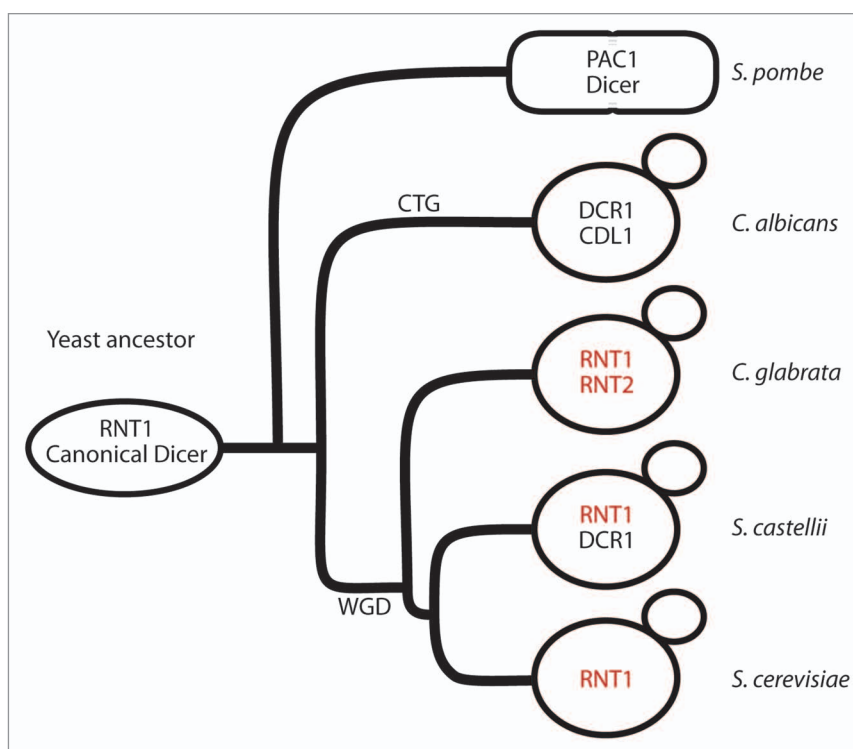
This route should be compatible with the known genome sequence relationships, assume no lateral transfer of genes, or multiple independent and simultaneous evolution of the noncanonical Dicer (*DCR*) and *RNT1* genes. Based on these considerations, we made the underlying assumption (suggested previously)<sup>15</sup> that both the budding yeast and non-budding yeast (e.g., *S. pombe*) lineages emerged from an ancestor that had both a canonical Dicer enzyme (*DICER*) as well as an *RNT1* ortholog (Fig. 1) (Table 1), and that *DICER* was lost from the budding-yeast lineage.

**Simple models require an unlikely sequence of events.** A simple model that could account for the diversity of RNase IIIs in budding yeast is that this lineage traces its origin back to an ancestor that had lost *DICER* and had only a single *RNT1* ortholog. After the whole-genome duplication event (WGD), there were two copies of this ancestral *RNT1*. In *S. castellii*, this duplication was followed by neofunctionalization of one copy to create *ScaRNT1* and *ScaDCR1*. In the *Candida* clade one of the *RNT1* ohnologs evolved to gain an additional Dicer activity and the second, *CDL1*, was inactivated. This model is unsatisfactory because it requires that the WGD occur prior to the formation of the *Candida* clade. However, evidence suggests the *Candida* clade split from the *Saccharomyces* clade prior to the WGD, making the WGD an improbable explanation for the duplication of an ancestral RNase III gene in the *Candida* clade.<sup>17</sup> Furthermore, regions of the *Candida* genome surrounding *CDL1* and *DCR1* are not syntenic, thus the two *Candida albicans* RNase III paralogs are likely to be a product of a more restricted duplication event. A comparison of the genes neighboring *ScaDCR1* with those adjacent to *ScaRNT1* also failed to reveal synteny consistent with a WGD derivation. By contrast, *C. glabrata* *RNT1*

paralogs show synteny in the genes flanking each copy suggesting that the *C. glabrata* RNT1's are possible products of the WGD (Fig. 1). So, while there is support for duplication of *RNT1* by WGD in the budding yeast lineage, the WGD event is unlikely to be responsible for the RNase III genes in *S. castellii* (*ScaDCR1* and *ScaRNT1*) or the *Candida* clade (*CaDCR1* and *CDL1*).

A second model posits that the evolution of the second RNase III protein present in *S. castellii* and the *Candida* clade involved rare independent events caused by the duplication of *RNT1* and its subsequent evolution to obtain Dicer function. This sequence of events seems an unlikely scenario as antecedent events for *S. castellii* for several reasons. First, if the *ScaDCR1* RNase III were the result of a recent autonomous duplication event of *ScaRNT1*, then ScaDcr1 should be more related to the ScaRnt1 than to other budding yeast Dcr1s. However, the RNase III domain sequences of ScaDcr1 are more closely related to the RNase III domains of *Saccharomyces bayanus* and *Kluyveromyces polysporus*, than they are to that of ScaRnt1 (Table 2).<sup>18</sup> *S. bayanus* and *K. polysporus*, are two yeasts from disparate branches of the *Saccharomycete* tree that like *S. castellii* encode two RNase IIIs, one homologous to *ScaRNT1* and one homologous to *ScaDCR1*. Not only are the RNase III domains of these DCR1's more closely related to each other than to the RNT1 gene family, they possess a distinct domain structure containing an additional C-terminal double stranded binding domain not found in Rnt1s. Although, the sequence of CaDcr1 RNase III domain is slightly more similar to the SceRnt1 and ScaRnt1 than the budding yeast Dcr1s RNase III domains, *Candida* Dcr1 possesses the distinct domain structure of *S. bayanus*, *K. polysporus*, and *S. castellii* Dcr1s (Table 2). These data suggest *S. bayanus*, *K. polysporus*, *S. castellii*, and *C. albicans* DCR1 did not independently evolve via recent duplications of *RNT1*. Instead, it is likely they originated from a common ancestral enzyme present before these lineages split.

In addition, our second model does not satisfactorily explain the origin of the two RNase IIIs found in *C. albicans*. The



**Figure 1.** Cartoon depicting where WGD and CTG codon usage changes are estimated to have occurred in the budding yeast lineage. Genes colored in red are syntenic with one another.

*Candida* clade is distinct from other budding yeast because they do not encode a Rnt1 homolog and one of their RNase III homologs, Cdl1, appears to have been inactivated through precise alteration of several key catalytic residues.<sup>11,15</sup> Since multiple highly conserved residues in RNase III domains have been altered in Cdl1, it is not surprising that Cdl1's RNase III domain is less homologous to active RNase III domains.<sup>18</sup> Nonetheless, Cdl1's RNase III domain is most highly homologous to CaDcr1's RNase III domain, and the Cdl1 domain structure is consistent with it being more closely related to a noncanonical dicer than a Rnt1 enzyme<sup>14</sup> (Table 2). In view of these considerations, it is likely that Cdl1 is a product of gene duplication and subsequent evolution of CaDcr1. This duplication occurred after the noncanonical Dicer ancestor had been established in the budding-yeast lineage. We cannot rule out that the duplication creating Cdl1 occurred prior to the split of the *C. albicans*, *S. bayanus*, *K. polysporus*, and *S. castellii* lineages, and the duplicated gene was subsequently lost from all except

the *Candida* clade, where it was instead inactivated.

We considered a third model in which duplication of an ancestral canonical Dicer followed by subfunctionalization and neofunctionalization of one of the copies resulted in the generation of a noncanonical dicer-like enzyme. Loss of canonical dicer in *S. castellii* would then account for the present day allocation of its RNase III enzymes. According to this model, loss of canonical Dicer as well as the noncanonical Dicer would leave only *RNT1* as is presently observed in *S. cerevisiae*. Duplication of noncanonical Dicer as well as apparent inactivation of one copy accompanied by *RNT1* and loss of the canonical Dicer would result in the complement observed in the *Candida* clade. We found these scenarios to be unlikely because the RNase III domain sequence from CaDcr1 and ScaDcr1 are more similar to one another, and to SceRnt1 or ScaRnt1 than they are to modern-day canonical Dicer RNase III domains<sup>18</sup> (Table 2). This suggests that the noncanonical Dicers likely evolved from an ancestral Rnt1-like enzyme and not from a canonical Dicer.

**Table 2.** Pairwise comparison of RNase III domains.

RNaseIII	CaDcr1	ScaDcr1	ScaRnt1	SceRnt1	SpPac1	SpDcr1	SpDcr2	KpDcr1	SbDcr1	CgRnt1	CgRnt2	CaCdl1
CaDcr1	705	273	303	359	277	191	179	270	263	338	302	202
	ScaDcr1	709	255	241	224	162	207	484	581	255	244	108
		ScaRnt1	685	415	260	139	171	255	233	393	360	143
			SceRnt1	732	249	156	167	253	233	451	390	177
				SpPac1	611	167	149	218	225	258	233	162
					SpDcr1	726	163	185	148	144	165	128
						SpDcr2	819	175	199	172	195	124
							KpDcr1	762	469	298	226	120
								SbDcr1	818	232	246	117
									CgRnt1	695	358	167
										CgRnt2	716	132
											CaCdl1	705

Higher scores indicate greater homology.

### Two Models that Explain the Evolution of Budding Yeast *DCR1* and *RNT1*

The failure of these models to explain the diversity of contemporary fungal RNase IIIs led us to propose that both the budding-yeast *DCR1*s evolved from an ancestral *Rnt1* (Fig. 2).

Model 1 proposes duplication of *RNT1* with neofunctionalization of one copy to generate a non-canonical Dicer gene (*DCR*) 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 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 (CaDcr1). *RNT1* loss (and *CDL1* gain through *RNT1/DCR1* duplication with neofunctionalization/inactivation) generated the RNase III genes of *C. albicans*.<sup>15</sup>

Model 2 posits early neofunctionalization of an ancestral *RNT1* to generate a transitional species with the multifunctional *RNT1/DCR1*. Subsequent canonical Dicer loss then generated the *Candida*-like budding yeast ancestor, which gained *CDL1* through *RNT1/DCR1* duplication with neofunctionalization of *CDL1* in the *Candida* lineage. Meanwhile, *RNT1/DCR1* duplication with subfunctionalization of each copy generated the two RNase III enzymes present in *S. castellii*.<sup>15</sup>

Model 1 predicts that the *S. castellii* Dcr1 RNase III domain should be more similar to the RNase III domain of CaDcr1 (Rnt1/Dcr1) than that of *S. castellii* Rnt1 whereas Model 2 predicts that the *S. castellii* Dcr1 RNase III domain should be more similar to the RNase III domain of *S. castellii* Rnt1 than that of CaDcr1, however, the *S. castellii* Dcr1 RNase III domain is not significantly more similar to the RNase III domains of ScaRnt1 or CaDcr1 (Rnt1/Dcr1) (Table 2). We are thus unable to distinguish between the two models.

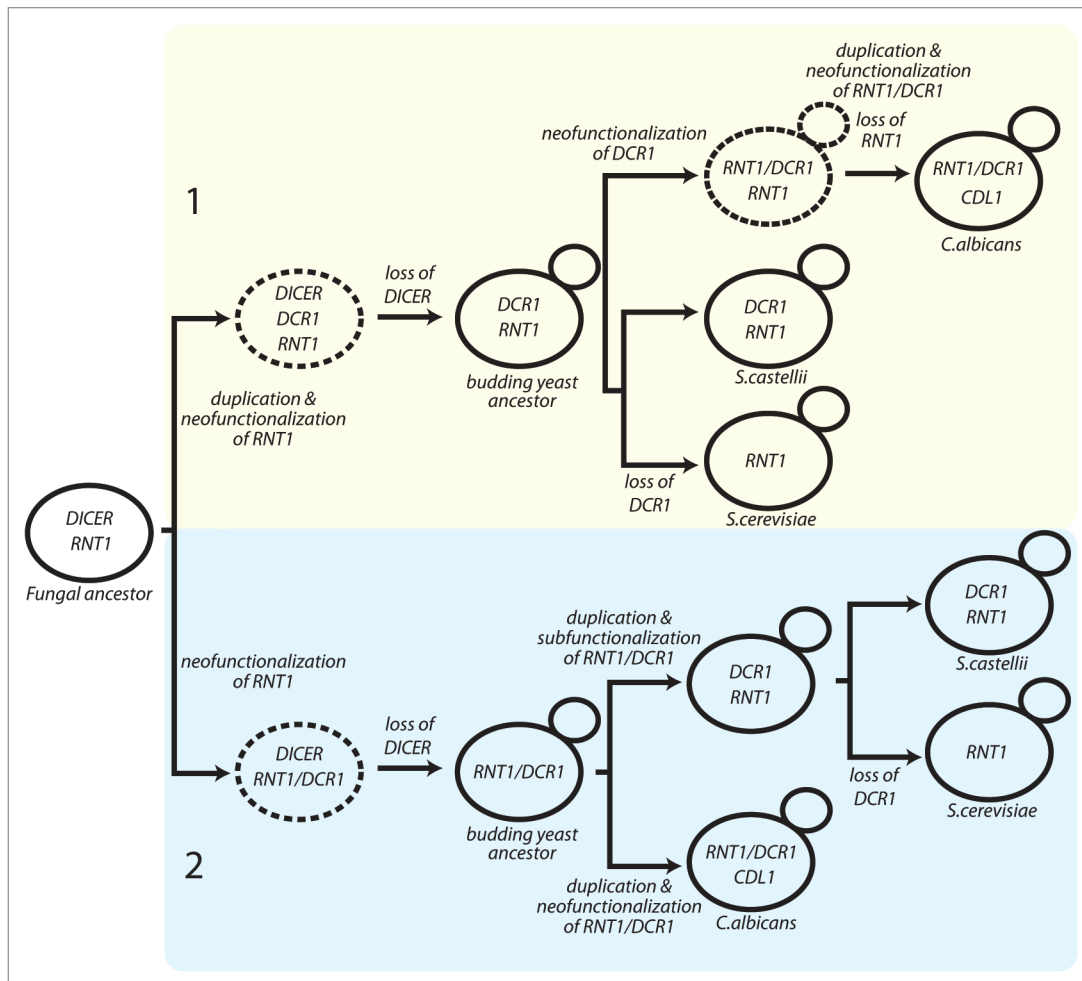
**Substrates affect RNase III evolution.** What are the selective pressures that shaped the evolution of these RNase IIIs, their loss, and the emergence of novel functions over time? The answers to these questions require consideration of not only the RNases, but also the interplay between these proteins and their substrates.

**dsRNA viruses shaped the fungal genome.** Drinnenberg et al. observed that loss of dicing activity in yeast correlates with the acquisition of killer virus. While dicing activity is hypothesized to play a protective role limiting the expression of transposons,<sup>19</sup> acquisition of killer virus provides a competitive advantage over yeast lacking the virus as these killer virus strains secrete a toxin that kills neighbors not infected with the virus.<sup>20</sup> Consequently, the budding yeast lineage has been faced with opposing selections: Retain the advantage of Dicer and RNAi at the risk of elimination by a strain lacking Dicer but harboring the killer virus. The advantage gained from the killer virus could explain why many budding yeast

lack either a canonical or noncanonical Dicer. The dichotomy between species with RNAi and no killer and those with killer and no RNAi is a striking example of how the fungal genome has been molded by a dsRNA substrate.<sup>20</sup>

**rRNA evolution could affect RNase III evolution.** Slight changes in the sequence of the ribosome substrate of Rnt1 could have led to the loss of the canonical *RNT1*, and the emergence of the multifunctional *Candida* CaDcr1. For example, mutation of a substrate in an essential reaction, such as the rRNA 3'ETS, might have permitted the eventual loss of canonical *RNT1* if Dcr1 acquired or possessed the ability to make this cleavage. Both in vitro and in vivo analysis have affirmed that the dsRBD binding of the AGNN tetra loop is required for SceRnt1 to cleave the double stranded 35S rRNA 3'ETS,<sup>21-23</sup> whereas preliminary analysis of 35S rRNA 3'ETS from the *Candida* clade suggests that a conserved AGNN tetra loop is not required for efficient cleavage.<sup>24</sup> Such an adaptation could explain our observation that *S. cerevisiae* *RNT1* is unable to complement a *CaDCR1* knockout. In the ancestor of modern *C. albicans*, mutation in the rRNA 3'ETS could have allowed CaDcr1 to process this altered substrate more efficiently than Rnt1. This change coupled with an ensuing period of Rnt1 decay could have permitted changes to additional Rnt1 substrates to be cemented in the *Candida* lineage and CaDcr1 to take over the role/s of an ancestral Rnt1.

Of course, we cannot rule out a dramatic change in the *Candida albicans*



**Figure 2.** Two potential 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, multifunctional Dicer found in the *Candida* clade; CDL1 *Candida* Dicer-like from the *Candida* clade. Proposed transitional species are shown in dashed lines. (This figure is nearly identical to the original model figure published in ref. 15).

*DCR1*, such as the addition of a C-terminal dsRBD, which allowed it to compete more efficiently for substrates previously cleaved by Rnt1. Such a change could have paved the way for the eventual loss of Rnt1 from the *Candida* lineage, and the subsequent drift of 35S rRNA 3'ETS sequence. Alternatively, evolution through loss of a dsRBD could have provided an equally significant functional adaptation as is posited in Model 2. Eukaryotic proteins have been found to encode up to five dsRBDs, and even seemingly minor changes in the number of dsRBDs can have profound effects on their biological function.<sup>25,26</sup> Furthermore, additional dsRNases could have played compensatory roles during the postulated transitional stages. Although some bacterial RNase III enzymes are

essential,<sup>27</sup> in other species they are dispensable presumably because other RNases perform essential RNase III functions in their absence.<sup>28,29</sup> Similar overlap of functions by ribonucleases with broad specificity could have supported budding yeast RNase III evolution.

### Summary and Future Directions

The discovery of the multifunctional CaDcr1 suggested that the RNase IIIs of extant species evolved from a common ancestor by gene duplication events coupled with subfunctionalization, neofunctionalization, and gene loss. The relationship among extant species is consistent with either of two models, but does not permit the choice between them.

The ambiguities could be resolved by genome sequences of additional yeast species. For example, the identification of an extant budding yeast with a complement of RNase IIIs labeled as transitional in Figure 2 could pave the way to a single model that satisfactorily traces the route from the ancestral species to modern budding yeast.

### Acknowledgments

The authors would like to acknowledge David Bartel and David Weinberg for helpful discussions. This work was funded by A.C.S. Grant PF-09-072-01-MBC to D.A.B., N.I.H. NRSA F32 AI729353 to V.K.V., Herman Sokol Fellowship to V.K.V., and N.I.H. Grant GM040266 (to G.R.F.).

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