RNA-binding protein DUS16 plays an essential role in primary miRNA processing in the unicellular alga *Chlamydomonas reinhardtii*

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Canonical microRNAs (miRNAs) are embedded in duplexed stem–loops in long precursor transcripts and are excised by sequential cleavage by DICER nuclease(s). In this miRNA biogenesis pathway, dsRNA-binding proteins play important roles in animals and plants by assisting DICER. However, these RNA-binding proteins are poorly characterized in unicellular organisms. Here we report that a unique RNA-binding protein, Dull slicer-16 (DUS16), plays an essential role in processing of primary-miRNA (pri-miRNA) transcripts in the unicellular green alga *Chlamydomonas reinhardtii*. In animals and plants, dsRNA-binding proteins involved in miRNA biogenesis harbor two or three dsRNA-binding domains (dsRBDs), whereas DUS16 contains one dsRBD and also an ssRNA-binding domain (RRM). The null mutant of *DUS16* showed a drastic reduction in most miRNA species. Production of these miRNAs was complemented by expression of full-length *DUS16*, but the expression of RRM- or dsRBD-truncated *DUS16* did not restore miRNA production. Furthermore, DUS16 is predominantly localized to the nucleus and associated with nascent (unspliced form) pri-miRNAs and the DICER-LIKE 3 protein. These results suggest that DUS16 recognizes pri-miRNA transcripts cotranscriptionally and promotes their processing into mature miRNAs as a component of a microprocessor complex. We propose that DUS16 is an essential factor for miRNA production in *Chlamydomonas* and, because DUS16 is functionally similar to the dsRNA-binding proteins involved in miRNA biogenesis in animals and land plants, our report provides insight into this mechanism in unicellular eukaryotes.

Chlamydomonas | microRNA biogenesis | dsRNA-binding protein | small RNA-seq | mutagenesis

MicroRNAs (miRNAs) generally function as posttranscriptional repressors by promoting degradation and/or translation inhibition of their target mRNAs. Further, miRNAs are key regulators of diverse biological processes, such as developmental timing, cell proliferation, and cell death, in animals and plants. Thus, miRNA abundance and activities must be tightly regulated at the levels of transcription, processing, and turnover of these small RNAs (1–3). The miRNA biogenesis pathway begins with transcription of primary-miRNAs (pri-miRNAs), generally synthesized by RNA polymerase II. After transcription, pri-miRNAs fold into hairpins and are processed by RNase III enzymes into precursor-miRNAs (pre-miRNAs), and then are processed further into duplexes consisting of guide (miRNA) and passenger (miRNA*) strands with 2-nt 3′ overhangs. Guide-strand miRNAs are preferentially incorporated into Argonaute (AGO) proteins and form an effector ribonucleoprotein complex termed the "RNA-induced silencing complex" (RISC). RISC induces endonucleolytic cleavage and/or translation repression of target mRNAs (2, 4, 5).

Several RNA-binding proteins play important roles in producing subsets of miRNAs by assisting the accurate and efficient processing of pri-miRNA and pre-miRNAs in animals and plants (6–8). In humans, pri-miRNAs are cotranscriptionally recognized by the microprocessor complex consisting of the RNase III enzyme Drosha and the dsRNA-binding protein DGCR8 and then are processed to liberate pre-miRNA hairpins from pri-miRNA in the nucleus (9–12). DGCR8 interacts directly at the junction between ssRNA and the dsRNA region of the pri-miRNA stem and directs dicing by Drosha 11 bp from the junction (10, 13). Following export of pre-miRNAs by Exportin-5 to the cytoplasm, another RNase III enzyme, Dicer, cleaves them into miRNA/miRNA* duplexes (14–16). Human Dicer also associates with two different dsRNA-binding proteins, TRBP and PACT (17, 18). These dsRNA-binding proteins are not required for dicing activity but function as key regulatory factors in defining the dicing site and facilitating RISC formation (19–21).

The biogenesis and functions of plant miRNAs have been documented mainly in *Arabidopsis thaliana*. Processing of pri-miRNAs is completed in two steps by the same RNase III enzyme, DICER LIKE 1 (DCL1). As in animals, DCL1 cleaves the base of pri-miRNAs to yield pre-miRNAs and then cleaves pre-miRNA again to release miRNA/miRNA* duplexes (22–24). Plant miRNA duplexes are generated in the nucleus and then are exported to the cytoplasm by HASTY, a homolog of Exportin-5, for RISC loading (25). DCL1 interacts with the dsRNA-binding protein

Significance

MicroRNAs are important regulators of gene expression in unicellular and multicellular eukaryotes. They are generally embedded in stem–loops of precursor transcripts and are excised by the dsRNA-specific nuclease DICER with the assistance of dsRNA-binding proteins. In animals and plants, proteins harboring two or three dsRNA-binding domains (dsRBDs) are involved in microRNA (miRNA) biogenesis. In contrast, we found that the Dull slicer-16 (DUS16) protein, which contains a single dsRBD and also an ssRNA-binding domain, is involved in miRNA biogenesis in the unicellular green alga *Chlamydomonas*. This finding sheds light on a molecular mechanism of miRNA biogenesis in unicellular organisms that may be similar to that in a common ancestor of animals and plants.

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Data deposition: Small RNA-seq raw data have been deposited in the DNA Data Bank of Japan (DDBJ) sequence read archive [accession nos. (CC-124 replicate #1, DRR045092; CC-124 replicate #2, DRR045093; Gluc1(×) replicate #1, DRR045094; Gluc1(×) replicate #2, DRR045095; dus16-1 replicate #1, DRR048495; and dus16-1 replicate #2, DRR048496)]. Plasmid pMO449 is available on the Chlamydomonas Resource Center, www.chlamycollection.org.

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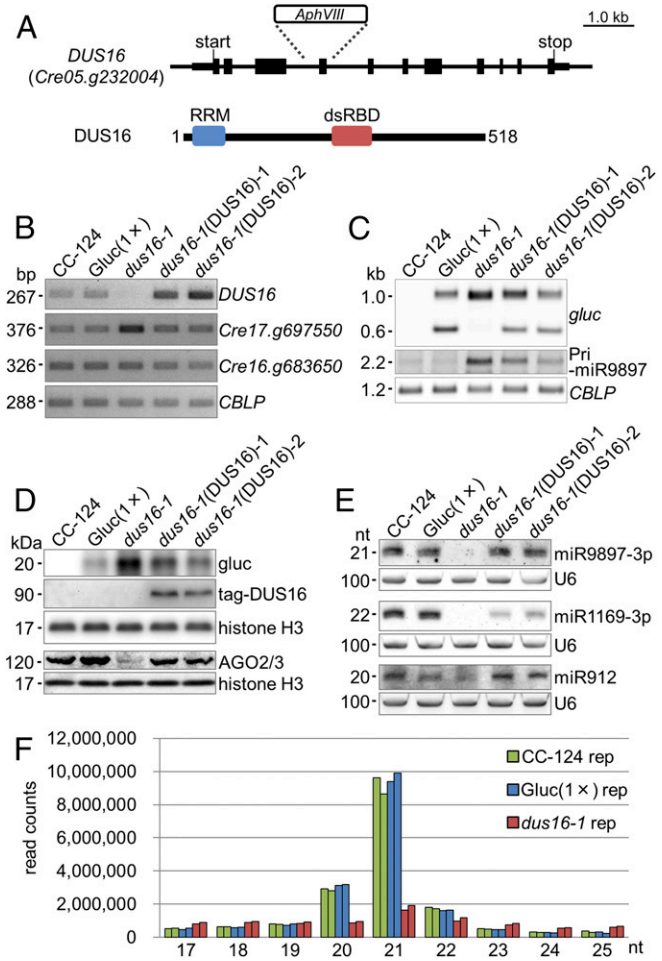

Fig. 1. Characterization of the *DUS16* mutant. CC-124, wild-type strain; Gluc(1×), CC-124 transgenic strain expressing *Gaussia* luciferase (*gluc*) bearing a sequence perfectly complementary to *Chlamydomonas* miR9897-3p; *dus16-1*, *DUS16*-defective mutant of Gluc(1×); *dus16-1*(DUS16)-1 and -2, strains expressing HA-FLAG-tagged *DUS16* in a *dus16-1* background. (*A*) Schematic diagram of the gene structure and transcript of *DUS16* (*Cre05.g232004*). dsRBD, dsRNA-binding domain; RRM, ssRNA-binding domain. (*B*–*E*) Each panel is representative of three independent experiments. (*B*) RT-PCR analysis in the indicated strains. *Cre17.g697550* is a target of miR B-mediated cleavage. *Cre16.g683650* is a target of miR C-mediated translation repression. A housekeeping gene, *CBLP*, was used as a control. (*C*) Northern blotting of *gluc* mRNA and the pri-miR9897 transcript in the indicated strains. Detection of *CBLP* was performed to check for equivalent loading of the RNA samples. (*D*) Immunoblotting of gluc, tagged DUS16, and AGO2/3 proteins in the indicated strains. The tagged DUS16 protein was detected with a monoclonal antibody against the HA tag. Both AGO2 and AGO3 polypeptides are recognized by a polyclonal antibody. Detection of histone H3 was performed to confirm similar loading of the protein samples. (*E*) Northern blotting of *Chlamydomonas* mature miRNAs in the indicated strains. U6 small nuclear RNA was used as a control for equal loading of the RNA samples. (*F*) Small RNA-seq analysis of CC-124 (green), Gluc(1×) (blue), and *dus16-1* (red). Biological duplicates of sRNA libraries were generated from the indicated strains. sRNA reads, 17–25 nt in length, were classified according to their size. Absolute read counts, without normalization, are indicated.

HYPONASTIC LEAVES1 (HYL1/DRB1) and the ssRNA-binding protein SERRATE (SE) and forms a plant microprocessor complex in the nucleus (26–28). HYL1 assists in the efficient and precise cleavage of pri-miRNA by interaction with DCL1 (26). SE enhances the accuracy of DCL1-dependent pri-miRNA processing together with HYL1 (28). TOUGH (TGH), another ssRNA-binding protein, is also a component of the plant microprocessor. TGH contributes to the interaction between pri-miRNA and HYL1 and may regulate DCL1 cleavage efficiency and/or recruitment of pri-miRNAs (29). *A. thaliana* encodes four additional dsRNA-binding proteins, DRB2–5, which are closely related to HYL1. These DRBs also function in canonical and noncanonical miRNA biogenesis pathways (30, 31).

Although many studies have shed light on the mechanism(s) of RNA-mediated gene silencing, it remains poorly understood in the unicellular green alga *Chlamydomonas reinhardtii* (32–41), particularly with respect to miRNA biogenesis. Here we show that the *Chlamydomonas* RNA-binding protein Dull slicer-16 (DUS16), which has an ssRNA-binding domain (RRM) and a dsRNA-binding domain (dsRBD), associates with nascent pri-miRNA transcripts cotranscriptionally and assists in processing pri-miRNAs into mature miRNAs.

Results

DUS16 Is Involved in the Processing of pri-miRNAs. We have generated an insertional mutant library from the *Chlamydomonas* transgenic strain Gluc(1×), which expresses *Gaussia* luciferase (*gluc*) bearing a sequence perfectly complementary to miR9897-3p (38, 40). Measurement of gluc activity as an index of silencing allowed us to identify silencing-defective mutants in the library. One of the mutants, *dus16-1*, was defective in silencing by disruption of *Cre05.g232004* (renamed "*DUS16*") because of a single-tag insertion. It is noteworthy that *DUS16* contains two different RNA-binding domains, an RRM and a dsRBD (Fig. 1 *A* and *B* and Fig. S1) (42–45). To verify the impairment of miR9897-3p–mediated silencing in *dus16-1*, we measured the abundance of the intact *gluc*

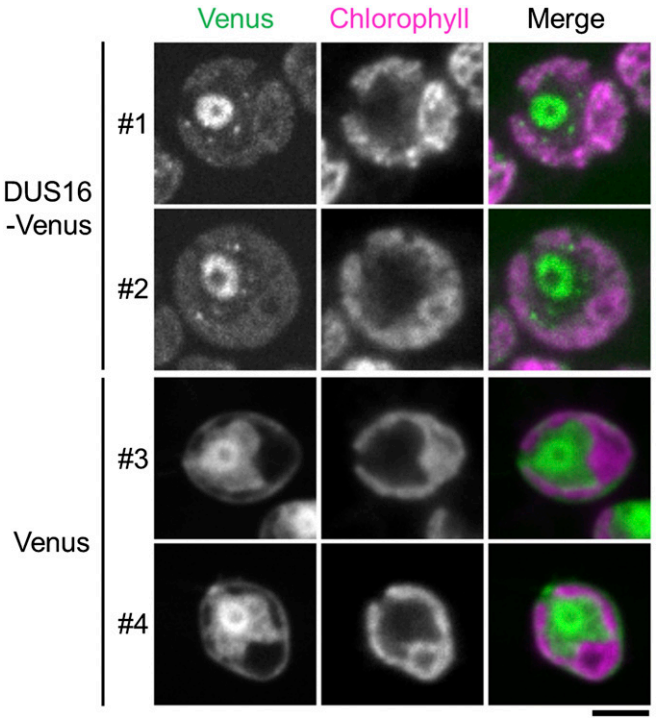

Fig. 2. Subcellular localization of the DUS16-Venus fusion protein. Cells of transgenic *Chlamydomonas* expressing DUS16-Venus in a *dus16-1* background (DUS16-Venus, strain #1 and #2) and wild-type cells expressing Venus (Venus, strain #3 and #4) were examined by confocal microscopy. Average-projection images of six z-stacks, each around the midplane, are shown. Note that, because of the low expression of the protein, a long exposure and high laser intensity were used for imaging DUS16-Venus; this imaging technique also increased chlorophyll and eyespot autofluorescence in the Venus channel (also see Fig. S5). (Scale bar, 5 μm.)

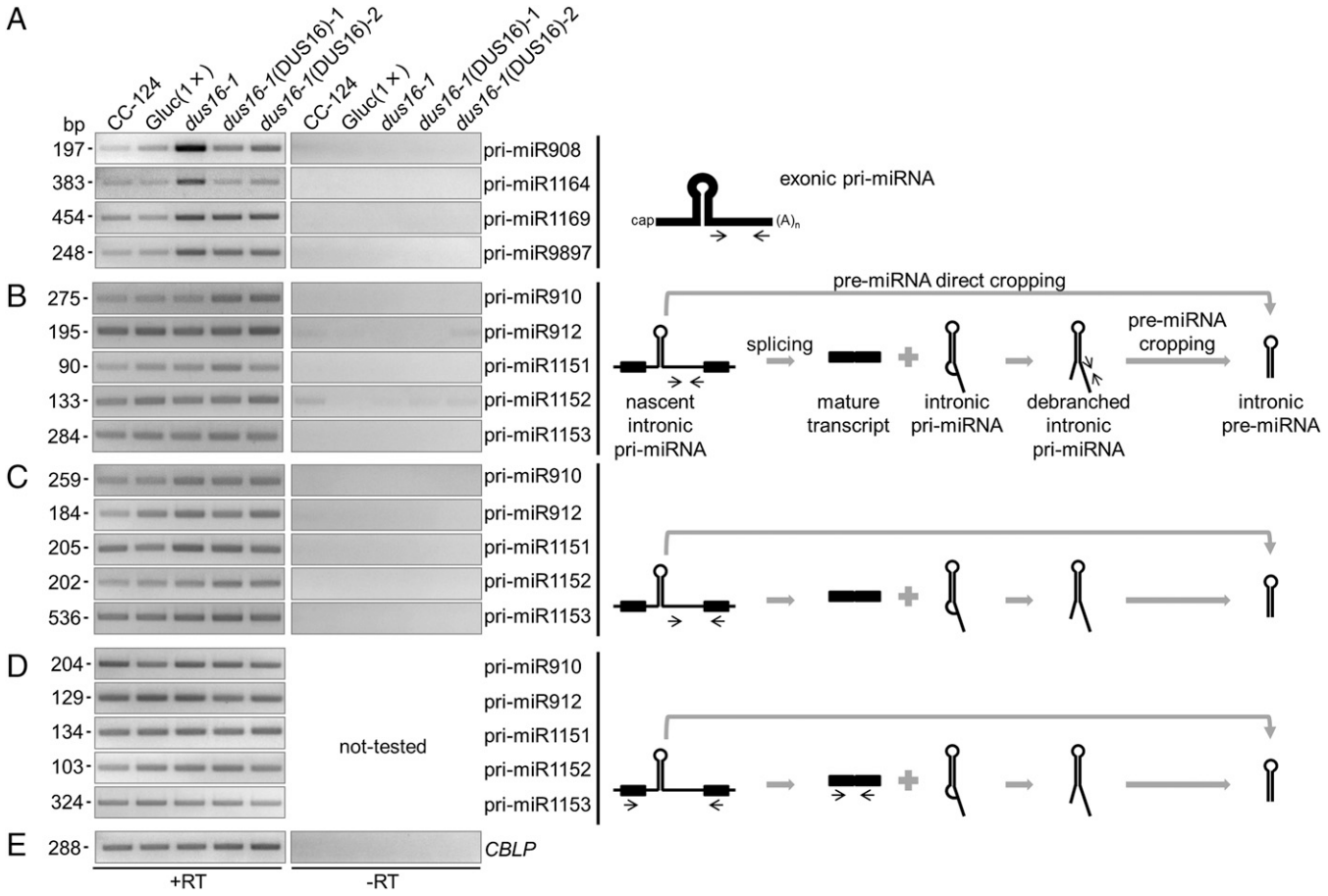

Fig. 3. RT-PCR analysis of exonic and intronic pri-miRNAs. (*A–E*, *Left*) Each panel is representative of three independent experiments. (*A*) RT-PCR analysis of exonic pri-miRNAs in the indicated strains. The stem–loops are located in the 3′ UTRs, and primers were designed to amplify downstream of the stem–loops. (*B*) RT-PCR analysis of intronic pri-miRNAs in the indicated strains. The primers were designed to amplify downstream of the stem–loops. (*C*) RT-PCR analysis of nascent (unspliced form) intronic pri-miRNAs in the indicated strains. (*D*) RT-PCR analysis of mature transcripts resulting from proper splicing out of intronic pri-miRNAs. (*E*) RT-PCR analysis of mature *CBLP* mRNA as a control. (*Right*) Schematic diagrams of the secondary structure of exonic and intronic pri-miRNAs. Thick black lines in the diagrams indicate exons, and narrow black lines indicate introns. Narrow black arrows indicate the location and direction of primers for PCR amplification. Gray arrows and symbols indicate the direction of processing.

transcript (~1.0 kb) and the cleavage product of the *gluc* transcript (~0.6 kb) (38). Gluc(1×) accumulated the sliced *gluc* transcript, but *dus16-1* showed no detectable sliced *gluc* transcript (Fig. 1*C*). In agreement with the increase of intact *gluc* transcript, the gluc protein was increased significantly in *dus16-1* (Fig. 1 *C* and *D*). By small RNA (sRNA) blotting, we confirmed that miR9897-3p and other miRNAs (miR1169-3p and miR912) were nearly absent in *dus16-1* (Fig. 1*E*) (40). Moreover, the primary transcript of miR9897-3p (pri-miR9897), which is almost undetectable in the wild-type strain, was observed as an ~2.2-kb RNA in *dus16-1* (Fig. 1*C*). All these defects in *dus16-1* were largely restored by the expression of tagged-*DUS16*, even though the fusion of the epitope tag might modestly affect the function of DUS16. (Fig. 1 *B–E*). These results clearly indicate that *DUS16* is critically involved in the biogenesis of miR9897-3p, miR1169-3p, and miR912.

To investigate global changes in miRNA and sRNA levels, we performed sRNA-sequencing (sRNA-seq) analyses. The study revealed that the large majority of 20- and 21-nt sRNAs were absent in *dus16-1* (Fig. 1*F* and Table S1). To determine the number of sRNAs generated from individual pre-miRNAs, we counted redundant sRNA reads mapped to individual pre-miRNA sequences. Although 50 stem–loop sequences corresponding to putative pre-miRNAs in *Chlamydomonas* have been registered in the miRBase database (www.mirbase.org/), few or no reads from Gluc(1×) were mapped to four of the 50 sequences. Accordingly, these four sequences were excluded from further analyses. In comparison with Gluc(1×), *dus16-1* showed fewer than half the number of sRNA reads for 38 of the remaining 46 stem–loops; for eight stem–loops comparable or greater numbers of sRNA reads were observed (Fig. S2). These results suggest that *DUS16* is involved in the production of most miRNA species. Marked decreases in miRNA (miR) B and miR C levels were also found in *dus16-1* (Fig. S3). In agreement with this finding, the *Cre17.g697550* transcript, a slicing target of miR B, was noticeably increased, whereas *Cre16.g683650* mRNA, a translation repression target of miR C, was virtually unchanged in *dus16-1* (Fig. 1*B*) (39, 40). Moreover, the AGO2/3 proteins were almost absent in *dus16-1*, probably because of their destabilization resulting from the global decrease in AGO2/3-associated sRNAs (Fig. 1*D*).

RRM and dsRBD Are Essential for DUS16 Function. To evaluate the necessity of RRM and dsRBD for DUS16 function, we performed truncation analyses of *DUS16*. After removal of the RRM (Δ1–124), the dsRBD (Δ125–335), or the C-terminal region (Δ336–518) from the intact *DUS16* expression cassette, individual truncated-*DUS16* transgenes were transformed into the wild type and then were transferred to the *dus16-1* background by a genetic cross (Fig. S4). As described above, production of miR9897-3p was clearly restored by the expression of intact, full-length *DUS16* (Fig. S4). In contrast, no truncated

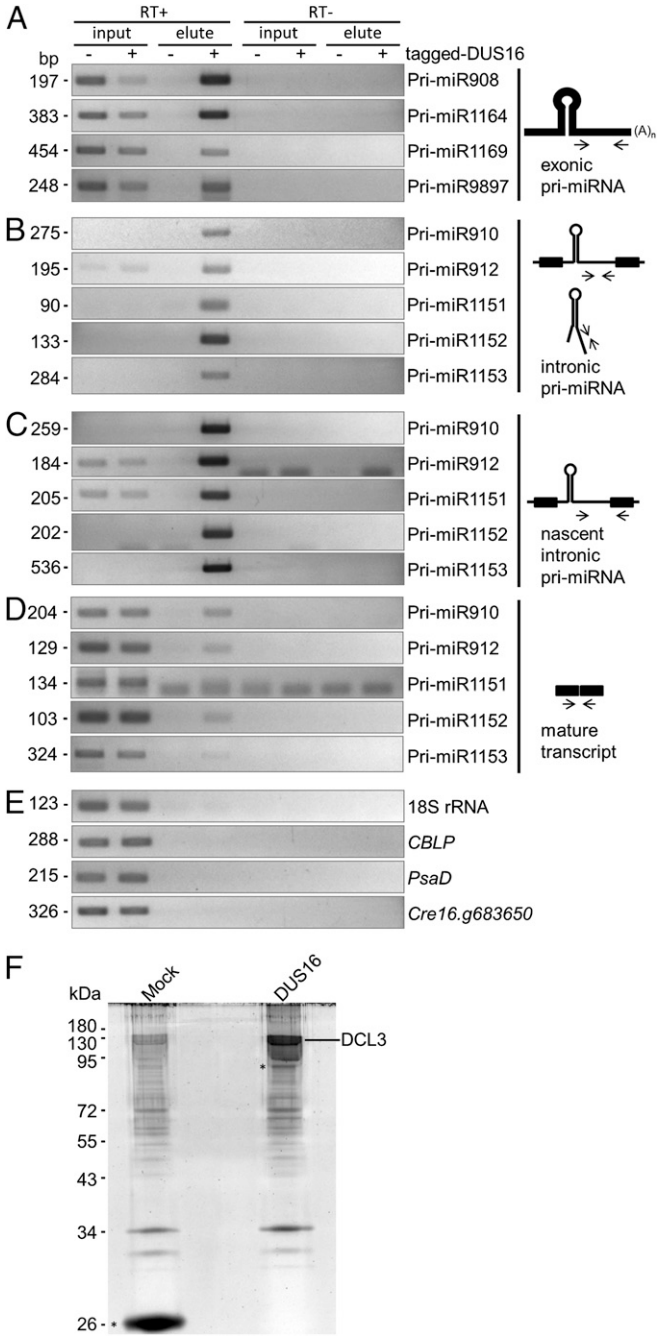

Fig. 4. RIP RT-PCR analysis and affinity purification of tagged-DUS16–associated transcripts and proteins. RIP with FLAG beads was performed with lysates from the *dus16-1* (indicated as tagged-DUS16 –) and *dus16-1*(DUS16)-1 (indicated as tagged-DUS16 +) strains. Input RNA corresponded to 0.05% of the total input amount. Reactions were stopped after any of the PCRs using the same primer set reached the midlogarithmic phase of amplification. Each panel is representative of three independent experiments. Affinity purification of proteins associated with epitope-tagged DUS16 was also carried out with FLAG beads. Subsequent mass spectrometry analysis was performed with lysate of the tagged ble-expressing strain and *dus16-1*(DUS16)-1. (*A*) Exonic pri-miRNAs. (*B*) Intronic pri-miRNAs. (*C*) Nascent (unspliced form) intronic pri-miRNAs. (*D*) Mature transcript, the product of proper splicing out of intronic pri-miRNA. (*E*) Analysis of 18S rRNA, *CBLP*, *PsaD*, and *Cre16.g683650* as negative controls. (*F*) Affinity purification of proteins associated with tagged DUS16 from the *dus16-1*(DUS16)-1 strain. Isolated tagged DUS16 and interacting proteins ware resolved by SDS/PAGE, stained with SYPRO Ruby, digested in the gel with trypsin, and identified by mass spectrometry. Mock purification with a tagged ble protein expressed in the CC-124 background served as a negative control. Tagged proteins are indicated by asterisks.

DUS16 transgene could restore the production of miR9897-3p (Fig. S4). These results suggest that the RRM, the dsRBD, and also the C-terminal region are functionally and/or structurally important for DUS16 function in the processing of pri-miR9897.

DUS16 Is Localized Mainly to the Nucleus, and the Remaining DUS16 Forms Speckles in the Cytoplasm. In animals and plants, initial processing of pri-miRNAs occurs exclusively in the nucleus. In agreement with this observation, HYL1, TGH, SE, and DCL1 are localized in specific subnuclear loci named "Dicer bodies" in *A. thaliana* (46–49). To observe the localization of DUS16 by confocal microscopy, we expressed Venus-fused DUS16 (DUS16-Venus) in *dus16-1* and restored the production of miR9897-3p (Fig. S5 *A* and *B*). Intriguingly, most DUS16-Venus localized to the nucleus, but a fraction of the protein appeared to form speckles in the cytoplasm (Fig. 2 and Fig. S5). In *A. thaliana*, HYL1 functions in the precise cleavage of pri-miRNAs through interaction with DCL1 (26). Because the results described above suggest that DUS16 could be a substitute for HYL1 in *Chlamydomonas*, we compared the precision of dicing sites on stem–loop sequences in Gluc(1×) and *dus16-1*. By comparing the mapped sRNA reads in the two strains, we found no obvious increased inaccuracy of processing in *dus16-1*, even though the number of sRNA reads was quite small (Fig. S6). These results suggest that DUS16 localizes predominantly to the nucleus and contributes to the initial processing of pri-miRNA but does not assist strongly in adjusting dicing sites.

Loss of DUS16 Results in Accumulation of Exonic pri-miRNAs, but Levels of Intronic pri-miRNAs Are Unchanged. Mammalian miRNAs are embedded mainly (~80%) into introns of protein-coding or noncoding genes, whereas plant miRNAs are encoded mostly in exonic regions of independent *miRNA* genes (50–52). In *C. reinhardtii*, 19 of the 46 stem–loop precursors discussed above are embedded into introns of gene models, whereas other 19 stem–loops are encoded in exons of gene models (three in 5′ UTRs, six in coding exons, and 10 in 3′ UTRs). The remaining eight stem–loops are found in intergenic regions (Fig. S2). In *dus16-1*, production of most sRNA species was impaired irrespective of the location of stem–loops in gene models (Fig. S2). We measured the accumulation levels of selected pri-miRNAs (pri-miR908, pri-miR1164, pri-miR1169, and pri-miR9897) that contain stem–loops in their 3′ UTRs. RT-PCR analyses revealed that these exonic pri-miRNAs were clearly increased in *dus16-1*, suggesting that they are processed by DCL protein(s) associated with DUS16 and that 5′- and 3′-cleavage fragments are likely destabilized and quickly degraded in the wild type (Fig. 3*A* and Fig. S7). We then measured the accumulation levels of other pri-miRNAs (pri-miR910, pri-miR912, pri-miR1151, pri-miR1152, and pri-miR1153) located in introns. In contrast to exonic pri-miRNAs, accumulation levels of these intronic pri-miRNAs appeared unchanged in *dus16-1* (Fig. 3*B*). Nascent (unspliced form) intronic-miRNAs and the mature transcripts also appeared unchanged in *dus16-1* (Fig. 3 *C*–*E*). These results suggest that splicing of intronic pri-miRNAs occurs efficiently and that the introns containing a stem–loop are degraded by the intron degradation pathway in *dus16-1*.

DUS16 Associates with Nascent pri-miRNAs and the DCL3 Protein. To obtain evidence that DUS16 associates with pri-miRNAs and promotes the production of miRNAs, we performed RNA immunoprecipitation (RIP) and subsequent RT-PCR (RIP RT-PCR). RNAs associated with tagged DUS16 were reverse-transcribed, and specific pri-miRNA regions were amplified by PCR with the primer sets used in Fig. 3. Interestingly, the exonic pri-miRNAs were recovered from the eluate of the *DUS16*-complemented strain, indicating that DUS16 associates with exonic pri-miRNAs (Fig. 4*A*). Furthermore, intronic pri-miRNAs (Fig. 4*B*) and nascent intronic pri-miRNAs (Fig. 4*C*) were recovered efficiently also. In contrast, DUS16 did not

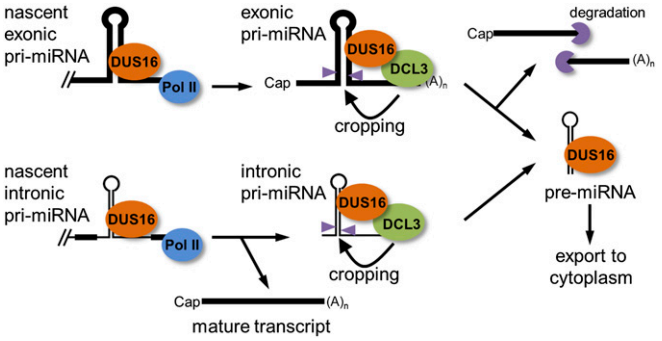

Fig. 5. Proposed model for the role of DUS16 in miRNA biogenesis in *Chlamydomonas*. DUS16 associates, likely cotranscriptionally, with nascent pri-miRNAs. (*Upper*) In exonic pri-miRNA processing, DCL3-mediated cropping of a stem–loop generates pre-miRNA and cleavage fragments, which are quickly degraded in the wild type. Lack of processing results in exonic pri-miRNA accumulation in *dus16-1*. (*Lower*) In intronic pri-miRNA processing, because splicing appears to be unaffected by the DCL3-mediated processing of intronic pri- and/or pre-miRNA, the mature transcript is stably produced irrespective of the presence of functional DUS16. Generated pre-miRNAs may be exported to the cytoplasm and subjected to further processing.

appear to interact efficiently with the mature transcripts (Fig. 4 *D* and *E*). Consistent with DUS16's being part of a microprocessor complex, affinity purification of tagged DUS16 and subsequent mass spectrometry analysis revealed that the protein associates with DICER-LIKE 3 (DCL3), which is the predominant DICER function in *Chlamydomonas* (Fig. 4*F*) (41). These results suggest that DUS16 recognizes and associates with nascent pri-miRNAs cotranscriptionally and assists DCL3-mediated processing of pri-miRNAs.

Discussion

Based on our results, we propose that DUS16 is involved in processing pri-miRNAs (Fig. 5). In animals, stem–loop structures in pri-miRNAs are recognized cotranscriptionally by the microprocessor complex, which consists of two core components: the RNase III enzyme Drosha and DGCR8 (53–55). In *A. thaliana*, DCL1 interacts with HYL1 and SE to form the plant microprocessor complex, and this complex positively regulates the efficiency and accuracy of pri-miRNA processing in the nucleus (26–28). In *C. reinhardtii*, DUS16 is localized mostly in the nucleus, associates with DCL3 and nascent pri-miRNAs, and is required for the processing of pri-miRNAs into mature miRNAs. Therefore DUS16 is very likely a component of the *Chlamydomonas* nuclear microprocessor complex. Further, given that DUS16 also forms several speckles in the cytosol, this protein may accompany pre-miRNAs to the cytoplasm and may form distinct bodies with DCL3 for further processing of precursor miRNAs into mature miRNAs.

In animals, spliceosome assembly of intronic pri-miRNAs promotes cropping of pre-miRNA hairpins from pri-miRNAs, but the splicing itself occurs more slowly than that of the adjacent introns (53, 55). In the *A. thaliana* HYL1 mutant background, some pri-miRNAs of unusual length, containing introns, accumulate (56). In *Chlamydomonas*, the efficiency of splicing of intronic pri-miRNAs was apparently unchanged in the *dus16-1* background. In the analysis of the mature transcripts by RT-PCR, no additional longer amplicons that contained the intronic pri-miRNA were detected, indicating that the splicing efficiency of intronic pri-miRNAs was not strongly reduced. Thus, interactions between the DUS16 complex and the spliceosome in *Chlamydomonas* appear to be weak or nonexistent.

In the genome of *C. reinhardtii*, no multiple-dsRBD–containing gene, such as *HYL1* or *DGCR8*, was found. Likewise, no *DUS16* paralog was found. However, 8 of 46 examined pri-miRNAs were processed efficiently to mature miRNAs in *dus16-1*; indeed, some of these mature miRNAs were increased in the mutant. We hypothesize that *Chlamydomonas* likely harbors a DUS16-independent miRNA biogenesis pathway that is potentially activated in the *dus16-1* background. Stem–loops in *Chlamydomonas* pri-miRNAs are greatly heterogeneous in length and structure, with variable positioning of the miRNA/miRNA* duplex (57). Given that the stem–loops of the eight pri-miRNAs appear to vary in secondary structure and size, structural signatures do not appear to be key determinants of the DUS16-independent miRNA biogenesis pathway. Production of sRNAs from miR1144a stem–loop and miR1144b stem–loop, which are embedded in the same gene (*Cre04.g217937*), was not inhibited in *dus16-1*, possibly implying that the processing pathway of pri-miRNAs is determined for each primary transcript.

A putative homolog of *Chlamydomonas* DUS16 was found only in the closely related alga *Volvox carteri*. Although many RNA-binding proteins have been well characterized as essential factors for the production of miRNAs in animals and plants, the composition of RNA-binding domains in DUS16 is unique among them (6–8, 49). Dead End 1 (Dnd1) does consist of a single RRM and a single dsRBD, but it counteracts the function of miRNAs by binding to transcripts at miRNA target sites in humans and zebrafish and is not involved in miRNA biogenesis (58). By functional analogy to other RNA-binding proteins, we propose that DUS16 plays a role mostly in pri-miRNA processing efficiency (but not accuracy) and functions as a partial substitute of plant HYL1. DUS16 may partly retain the character and function of ancestral RNA-binding proteins involved in miRNA/sRNA production. Further analysis of DUS16 may provide insight into the mechanism of miRNA biogenesis not only in the unicellular eukaryotes but also in the last common ancestor of eukaryotes in which an miRNA/sRNA-processing system evolved.

Materials and Methods

Culture Conditions and Genetic Crosses. Unless otherwise noted, *C. reinhardtii* cells were grown photoheterotrophically in Tris/acetate/phosphate (TAP) medium (59). Genetic crosses were performed as described by Harris (60).

Plasmid Construction and *Chlamydomonas* Transformation. Transformation of *Chlamydomonas* was performed as described previously (38, 61, 62). Mutant screening has been performed previously (40). The detailed construction procedures of the plasmids are described in *SI Materials and Methods*.

Fluorescence Microscopy. Cells expressing Venus-tagged DUS16 were observed under a Leica DMI6000B microscope. The images were postprocessed using ImageJ version 1.50b (NIH) and Adobe Photoshop software. Detailed methods are provided in *SI Materials and Methods*.

DNA Analyses. Isolation of genomic DNA, Southern blotting to determine the copy number of the insertion tag, and determination of the tag-insertion site were performed as described previously (59, 63, 64).

RNA Analyses. Northern blotting and RT-PCR were performed as described previously (38, 65). The primers listed in Table S2 were used for RT-PCR and quantitative RT-PCR (qRT-PCR). Detailed methods are provided in *SI Materials and Methods*.

RIP. RIP was performed as described previously (40). Detailed methods are provided in *SI Materials and Methods*.

sRNA-Seq. sRNA-seq was performed as described previously (40). A summary of each sRNA library analyzed is presented in Table S1. Detailed methods are provided in *SI Materials and Methods*.

Immunoblotting. Immunodetection was performed as described previously (38). For details of antibodies, see *SI Materials and Methods*.

Affinity Purification. Affinity purification of tagged DUS16 and associated proteins was carried out in a similar way to RIP (66). Detailed methods are provided in *SI Materials and Methods*.

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