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SHORT COMMUNICATION

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Cooperative processing of primary miRNAs by DUS16 and DCL3 in the unicellular green alga *Chlamydomonas reinhardtii*

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ABSTRACT

We have previously reported that the RNA-binding protein Dull slicer 16 (DUS16) plays a key role in the processing of primary miRNAs (pri-miRNAs) in the unicellular green alga *Chlamydomonas reinhardtii*. In the present report, we elaborate on the interaction of DUS16 with Dicer-like 3 (DCL3) during pri-miRNA processing. Comprehensive analyses of small RNA libraries derived from mutant and wild-type algal strains allowed the de novo prediction of 35 pri-miRNA genes, including 9 previously unknown ones. The pri-miRNAs dependent on DUS16 for processing largely overlapped with those dependent on DCL3. Our findings suggest that DUS16 and DCL3 work cooperatively, presumably as components of a microprocessor complex, in the processing of the majority of pri-miRNAs in *C. reinhardtii*.

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Argonaute; *Chlamydomonas reinhardtii*; Dicer; miRNA; RNA-binding protein; small RNA-seq

MicroRNAs (miRNAs) are loaded into Argonaute (AGO) proteins during the formation of the RNA-induced silencing complex (RISC).[1] The main function of miRNAs in RNA silencing is guiding RISC to target transcripts for inducing endonucleolytic RNA cleavage and/or translational repression. In general, miRNAs are embedded in long primary miRNA (pri-miRNA) transcripts containing stem-loop structures and have to be processed to mature miRNAs with the assistance of RNase III Dicer and associated RNA-binding proteins.[2,3] We have recently reported that in the unicellular green alga *Chlamydomonas reinhardtii*, an RNA-binding protein, Dull slicer 16 (DUS16), is required for pri-miRNA processing and associates with Dicer-like 3 (DCL3), which in turn is involved in the biogenesis of the majority of miRNAs (Fig. 1).[4,5] We also reported that AGO3, which is one of the 3 AGOs encoded in the *C. reinhardtii* genome, predominantly binds to mature miRNAs and determines miRNA-mediated post-transcriptional gene silencing (Fig. 1).[6] The present report contains a comprehensive analysis of our previously published small RNA-seq (sRNA-seq) data [from the *AGO3* mutant (*ago3–1*); the *DUS16* mutant (*dus16–1*); the parental strain of these mutants Gluc(1×), which expresses a reporter luciferase transgene in the wild-type background; and the wild-type strain CC-124] to predict de novo pri-miRNAs and gain insight into the functional coupling between DUS16 and DCL3.

From the sRNA-seq raw data of CC-124, Gluc(1×), *ago3–1*, and *dus16–1*, adaptor sequences were removed and reads ranging from 17 to 25 nucleotides in length were selected for further analyses. The alignment of sorted sRNA reads from the Gluc(1×) sRNA library to the *C. reinhardtii* genome (Ch_genome_v5.0) using miRA,[7] an miRNA discovery tool for plants and algae, led to the identification of 1,062 inverted repeat loci encoding stem-loop RNAs. To stringently screen for genuine pri-miRNA genes, sRNA sequences with <10 read counts were excluded from the libraries, and the remaining redundant sRNA reads were aligned with *C. reinhardtii* gene models encompassing the inverted repeats using CLC genomic workbench (QIAGEN, https://www.qiagenbioinformatics.com/products/clc-genomics-workbench/). Gene models with <90 mapped-sRNA read counts in the sRNA libraries of CC-124 and Gluc(1×) and/or those without a predominant sRNA species on an arm of the predicted stem-loop structure were discarded.

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Supplemental data for this article can be accessed on the publisher's website.

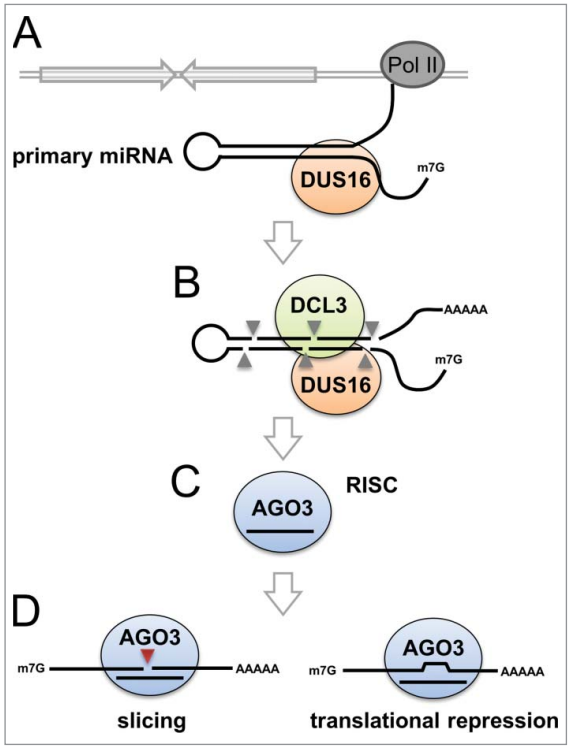

Figure 1. Model for miRNA biogenesis and action in *Chlamydomonas reinhardtii*. Dull slicer 16 (DUS16) recognizes nascent pri-miRNA transcripts (A). Dicer-like 3 (DCL3) mediates processing of most pri-miRNAs to miRNA duplexes with assistance of DUS16 (B). Argonaute 3 (AGO3) incorporates most *Chlamydomonas* mature miRNAs, having a U as their 5′ nucleotide, and forms the RISC (C). AGO3-RISC recognizes target transcripts and induces slicing and/or translational repression (D).

Based on the above workflow, 35 gene models were annotated as pri-miRNA genes, including 9 previously unknown ones (Table 1).

A comparison of total sRNA read counts, mapped on the predicted pri-miRNA genes, from *dus16–1* and Gluc (1×) revealed that the production of mature sRNAs from 33 of the 35 pri-miRNAs is significantly lower in *dus16–1*, suggesting that these pri-miRNAs are mainly processed in a DUS16-dependent manner (Table 1, Fig. S1). Twenty-four of the 35 identified miRNA genes were previously annotated as pri-miRNAs by Valli *et al.* and are predominantly processed by DCL3 (annotated as "high confidence," "medium confidence" and/or "upregulated" in Table 1, Fig. S1).[5] Furthermore, 22 of these 24 pri-miRNAs (91%) appear to require DUS16 for processing (Table 1; Fig. S1). This result suggests that, in addition to our previous finding of DUS16 physically interacting with DCL3,[4] DUS16 is functionally coupled to DCL3, presumably as part of a microprocessor complex involved in the processing of the majority of *C. reinhardtii* pri-miRNAs.

On the other hand, 2 pri-miRNA transcripts corresponding to *Cre04.g217925* and *Cre06.g274550*, which give rise to mature miR-1144 and miR-1162, respectively, are processed in a DCL3-dependent and DUS16-independent manner (Table 1). In the *ago3–1* mutant, the number of mature sRNAs generated from these pri-miRNAs is very low, indicating that most likely, they are authentic pri-miRNAs (Table 1, Fig. 2, Fig. S1). Some

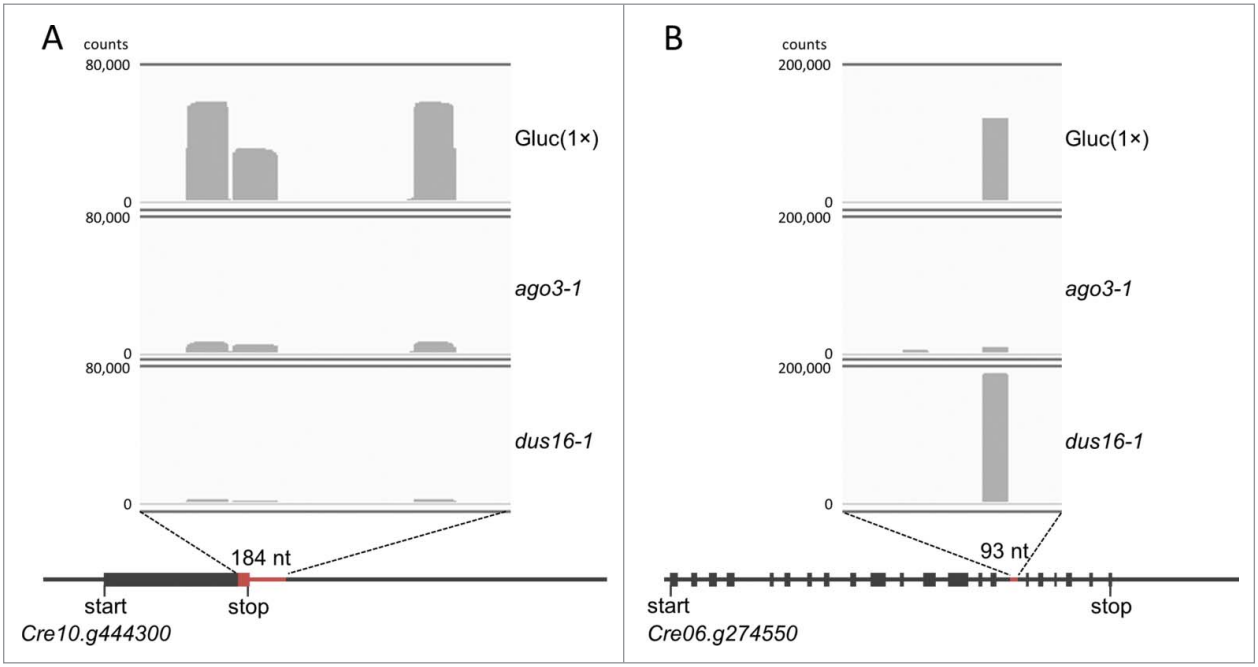

Figure 2. Frequency (counts) of small RNA (sRNA) reads matching the inverted repeat regions of *Cre10.g444300* (A) and *Cre06.g274550* (B) in the *AGO3* mutant (*ago3–1*), the *DUS16* mutant (*dus16–1*), and their parental strain Gluc(1×). Schematic diagrams of gene structures, indicating predicted start and stop codons, are shown at the bottom of each panel. Inverted repeat regions are indicated in red. Gray bars represent the coverage of sRNA read counts on the corresponding sequences.

Table 1. De novo prediction of primary and mature miRNAs.

microRNA precursor		Gluc(1X)			*dus16–1*								Valli *et al.*					
Gene ID[a]	encoded proteins/domains	rep#1[b]	rep#2[b]	mean[c]	rep#1[b]	rep#2[b]	mean[c]	*dus16–1* /Gluc1(1x)[d]	Position of stem-loop (strand)	Length (nt)[e]	Location of stem-loop	MIR gene[f]	Voshall *et al.*[g]	Predicted as miRNA precursor with[h]	Upregulated in the *DCL3* mutant[i]	Mature miRNA miRNA sequences	Length (nt)	*ago3–1* /Gluc (1×)[j]
Cre01.g011500	RNP11, 26S proteasome regulatory subunit	13,070	12,102	12,586	2,202	1,741	1,972	0.16	chromosome_1:2125423..2125712 (+)	290	intron	MIR906				CGGTTGGTGGGCGTGATCAGC	21	2.10
Cre01.g023913	no putative conserved proteins/ domains	2,050	1,944	1,997	117	61	89	0.04	chromosome_1:3724948..3725125 (-)	178	3′UTR			medium confidence		TGACACATGGAACAACACAACA	22	0.50
Cre01.g038350	no putative conserved proteins/ domains	3,881	3,515	3,698	84	54	69	0.02	chromosome_1:5449977..5451159 (+)	1,183	5′UTR-exon-intron				upregulated	TCATTGTCAGACGTTCGGAAG	21	0.27
Cre02.g143427	no putative conserved proteins/ domains	2,353	2,190	2,272	93	40	67	0.03	chromosome_2:9129472..9129630 (+)	159	3′UTR		Cluster14712	medium confidence	upregulated	TGCGTGCTTGCGCCCTCTAGC	21	1.70
Cre03.g195950	protein kinase domain	7,435	6,963	7,199	128	89	109	0.02	chromosome_3:6573882..6574002 (+)	121	3′UTR		Cluster16411	medium confidence		TGACATGCGGTGAATGTGAAT	21	0.51
Cre03.g206250	no putative conserved proteins/ domains	2,279	2,071	2,175	327	166	247	0.11	chromosome_3:7376018..7376645 (+)	728	exon-intron				upregulated	TACGGGCTCGTCTTCGGAGACA	22	0.20
																AGAGAAGCAGCTGGAATGATG	21	0.71
Cre04.g217925	KELCH repeat domain	2,154	2,159	2,157	4,616	3,668	4,142	1.92	chromosome_4:457731..458006 (+)	276	intron-exon	MIR1144		medium confidence		TTGGCACCGGGCACGCAGGAGG	22	0.14
Cre04.g220461	no putative conserved proteins/ domains	3,896	3,279	3,588	253	116	185	0.05	chromosome_4:2304022..2305586 (-)	1,565	3 different model				upregulated	TGACGGAGCTTCTGACCGAGC	21	0.21
Cre04.g225700	mediator of RNA polymerase II transcription subunit 8	55,579	52,177	53,878	2,535	2,010	2,273	0.04	chromosome_4:3100596..3100778 (+)	183	intron	MIR1153	Cluster17620	hight condidence		TGGGCCATCGTATTACTATCAG	22	0.16
Cre05.g238343	no putative conserved proteins/ domains	17,163	15,132	16,148	206	117	162	0.01	chromosome_5:2985422..2986713 (+)	1,293	exon-3′UTR-intron					TGCCATCCTTGGGACTCCTGG	21	0.09
Cre05.g239950	no putative conserved proteins/ domains	57,037	54,236	55,637	790	595	693	0.01	chromosome_5:3227648..3227768 (-)	121	exon			hight condidence	upregulated	AGGCGTGAAAAGTGTGGAATG	21	1.32
Cre05.g242180	no putative conserved proteins/ domains	6,071	5,467	5,769	0	0	0	0.00	chromosome_5:1813823..1814182 (-)	360	eoxn-3′UTR					TTCTGCAAAATGAGGAACTTGC	22	0.08
									chromosome_5:1814195..1814341 (-)	147	5′UTR					TCTTGGGACGCTGCTTAGACG	21	0.23
Cre05.g242301	no putative conserved proteins/ domains	6,514	5,970	6,242	14	0	7	0.00	chromosome_5:1790617..1790877 (+)	261	3′UTR	MIR913		medium confidence		ACGGACTCGCAGGTGTGCAAG	21	0.98
Cre05.g247100	CotH, spore coat protein	4,209	3,519	3,864	760	492	626	0.16	chromosome_5:935231..935515 (-)	285	intron	MIR918/ 919	Cluster18100			TCGGTCAGCATCTCGATTGGC	21	0.19
																TACCTGAAGCGGACATCTTGC	21	0.06
Cre06.g266052	WD40 repeat domain	19,750	18,386	19,068	349	202	276	0.01	chromosome_6:2201552..2201759 (-)	208	intron-exon		Cluster19166		upregulated	TTGGGCGGCGTTGTAAGATT	20	0.32
Cre06.g274550	protein kinase	123480	117542	120511	190817	141689	166253	1.38	chromosome_6:3067367..3067459 (+)	93	intron	MIR1162	Cluster19538	hight condidence		TGTTGTAGTAGTTTAGCCCTGC	22	0.08
Cre06.g278206	lipoprotein leucine-zipper	71,633	65,283	68,458	2,312	1,338	1,825	0.03	chromosome_6:4031321..4031518 (+)	198	5′UTR-exon	MIR907			upregulated	AAGACATCGCTGGCACCGTG	20	0.37
																TCTTCTGCGAGCGGTGCGAGC	21	0.25
Cre06.g295350	no putative conserved proteins/ domains	3,314	2,618	2,966	103	64	84	0.03	chromosome_6:6854015..6854278 (+)	264	exon-3′UTR					TACAGGAGCCTGATGAGGATG	21	0.22
Cre07.g312650	no putative conserved proteins/ domains	1,933	1,875	1,904	96	58	77	0.04	chromosome_7:77597..78113 (+)	517	exon-intron-3′UTR				upregulated	AGACTGTCTGGAGTGCCGACT	21	0.74
Cre08.g358535	no putative conserved proteins/ domains	12,763	12,226	12,495	412	269	341	0.03	chromosome_8:121841..121961 (+)	121	3′UTR		Cluster22587	high confidence	upregulated	TGGCTTTCGTCGGTCCTAGG	20	0.33
																TAGGACCGACGAAAGCCACT	20	0.41
Cre10.g444300	no putative conserved proteins/ domains	87,015	81,861	84,438	2,858	1,766	2,312	0.03	chromosome_10:3399862..3400009 (-)	148	exon-3′UTR	MIR9897	Cluster2675	medium confidence	upregulated	TACCGGGCGTGGGGAGGGCAGG	22	0.16
																TTACGGCTCCTTCTTATCGGC	21	0.13
Cre10.g452700	no putative conserved proteins/ domains	33,037	28,233	30,635	1,841	1,280	1,561	0.05	chromosome_10:4598637..4598830 (+)	194	intron-exon					AGCGCGATGATGGATGAGAAG	21	0.56
																CTTGGCGGGCTGAAGACATAG	21	0.52

(*Continued on next page*)

Table 1. (*Continued*)

microRNA precursor		Gluc(1X)			*dus16–1*								Valli *et al*.					
Gene ID[a]	encoded proteins/domains	rep#1[b]	rep#2[b]	mean[c]	rep#1[b]	rep#2[b]	mean[c]	*dus16–1* /Gluc1(1x)[d]	Position of stem-loop (strand)	Length (nt)[e]	Location of stem-loop	MIR gene[f]	Voshall *et al*.[g]	Predicted as miRNA precursor with[h]	Upregulated in the *DCL3* mutant[i]	Mature miRNA miRNA sequences	Length (nt)	*ago3–1* /Gluc (1×)[j]
Cre10.g464300	no putative conserved proteins/ domains	35,962	33,490	34,726	2,437	1,685	2,061	0.06	chromosome_10:6199729..6199816 (+)	88	intron				upregulated	ATCTCGTCGTCGTCAGGCTTG	21	0.61
Cre11.g467650	conserbed hypothetical protein	12,488	10,717	11,603	2,461	1,773	2,117	0.18	chromosome_11:1824675..1824782 (+)	108	3′UTR				upregulated	AAGGACGCTCCTCGTACTGACG	22	0.63
Cre12.g536301	no putative conserved proteins/ domains	28,770	24,661	26,716	272	134	203	0.01	chromosome_12:6166877..6167231 (+)	355	intron-3′UTR					TGGCAAAGAGGAAAGCGGAGC	21	0.29
Cre13.g576700	conserbed hypothetical protein	17,130	15,285	16,208	152	95	124	0.01	chromosome_13:2001062..2001207 (-)	146	3′UTR		Cluster7085	medium confidence	upregulated	AAGCAGTCAGGTAGAAGCGC	20	0.66
																TGACTCTCACTCCTACTCGGC	21	0.23
Cre13.g579050	anaphase promoting complex subunit 1	105061	90,219	97,640	3,332	2,426	2,879	0.03	chromosome_13:2301400..2301727 (-)	328	3′UTR				upregulated	TGTTTGTGTGACGTGGTTCTT	21	0.25
Cre14.g615950	translation elongation factor 3	40,709	36,425	38,567	1,913	1,337	1,625	0.04	chromosome_14:1191293..1192047 (-)	755	intron	MIR1159				ATGACGAGTGGCTAGGCAGCG	21	0.33
																GCGGCAGTCGGGCACTGTGGC	21	0.57
Cre16.g686203	no putative conserved proteins/ domains	2,430	2,412	2,421	0	0	0	0.00	chromosome_16:4838378..4838521 (+)	144	intron-exon					TCTTTCGTGCCTAGGGCCTTG	21	0.07
Cre16.g686398	no putative conserved proteins/ domains	1,133	1,008	1,071	21	0	11	0.01	chromosome_16:7434870..7435158 (+)	289	5′UTR-exon-intron					TGCACGCTGTGACTGTCTAGC	21	1.12
Cre17.g697550	no putative conserved proteins/ domains	32,592	28,227	30,410	6,170	4,240	5,205	0.17	chromosome_17:194516..194869 (+)	354	exon-intron-exon					ATGCACGGCACGGGCGACGGT	21	0.62
Cre17.g697800	chromosome segregation protein	1,527	1,308	1,418	458	330	394	0.28	chromosome_17:228757..228889 (+)	133	intron				upregulated	CGGTCCTGTAAGCATCAAAACG	22	0.95
Cre17.g735375	FAP164, flagellar Associated Protein 164	60,726	54,267	57,497	28,321	21,351	24,836	0.43	chromosome_17:5152751..5152951 (-)	201	5′UTR		Cluster12364	medium confidence		TCGGAGAAGCGGGTAGCTGAGG	22	0.41
																ATGTCGCACAGCCAGTGTCCG	21	0.27
Cre17.g741601	no putative conserved proteins/ domains	12,965	11,544	12,255	369	223	296	0.02	chromosome_17:6144100..6144226 (-)	127	3′UTR		Cluster12551	hight condidence	upregulated	TCGCCTTGTCTGTTTATGTGG	21	0.18
																TAAACAGACAAGGCGACCGACA	22	0.42
Cre24.g755697	conserbed hypothetical protein	3,094	2,846	2,970	276	211	244	0.08	scaffold_24:82169..82327 (+)	159	3′UTR	MIR1172		medium confidence	upregulated	AGGATTGCAGCAGCAACGGGGC	22	0.44

Notes. [a]Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias = Org_Creinhardtii).
[b]Absolute sRNA read counts from the individual sRNA libraries that align to each gene model.
[c]Mean values of 2 replicates.
[d]Ratio of the means of abundant mature miRNAs in *dus16–1* over Gluc(1×).
[e]Length of the sequences corresponding to a stem-loop RNA.
[f]miRBASE (http://www.mirbase.org/).
[g]Previously annotated pri-miRNA genes published by Voshall *et al*.[12]
[h]Previously annotated pri-miRNA genes with high or medium confidence interval pubslihed by Valli *et al*.[5]
[i]Putative pri-miRNA genes with abundant upregulated transcripts in the *DCL3* mutant (Valli *et al*.).[5]
[j]Ratio of the means of abundant mature miRNAs in *ago3–1* over Gluc(1×).

sRNAs are also produced from the transcripts of inverted repeats in a DCL3-independent manner.[5] These results imply the presence of minor DUS16- and/or DCL3-independent pri-miRNA-processing pathways in *C. reinhardtii*.

C. reinhardtii appears to possess canonical miRNA biogenesis pathways and miRNA-mediated post-transcriptional gene regulation with certain similarities to those in animals and plants[8,9,10,11,12] Mutant analyses revealed that the initial processing of the majority of pri-miRNAs relies on a putative microprocessor complex comprising both DUS16 and DCL3.[4,5] In addition, our analyses also uncovered a minor set of pri-miRNAs that are likely processed in a DUS16 and/or DCL3-independent manner.

Accession numbers

Small RNA-seq raw data has been deposited in the DDBJ sequence read archive (DRA) under accession numbers DRA003930 and DRA004107 (CC-124 replicate #1, DRX040414; CC-124 replicate #2, CCDRX040415; Gluc1(×) replicate #1, DRX040416; Gluc1(×) replicate #2, DRX040417; *ago3–1* replicate#1, DRR045098; *ago3–1* replicate#2 DRR045099; *dus16–1* replicate #1, DRX043778; and *dus16–1* replicate #2, DRX043779).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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