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# The FHA domain proteins DAWDLE in *Arabidopsis* and SNIP1 in humans act in small RNA biogenesis

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Proteins containing the forkhead-associated domain (FHA) are known to act in biological processes such as DNA damage repair, protein degradation, and signal transduction. Here we report that DAWDLE (DDL), an FHA domain-containing protein in *Arabidopsis*, acts in the biogenesis of miRNAs and endogenous siRNAs. Unlike mutants of genes known to participate in the processing of miRNA precursors, such as *dcl1*, *hyponastic leaves1*, and *serrate*, *ddl* mutants show reduced levels of pri-miRNAs as well as mature miRNAs. Promoter activity of *MIR* genes, however, is not affected by *ddl* mutations. DDL is an RNA binding protein and is able to interact with DCL1. In addition, we found that SNIP1, the human homolog of DDL, is involved in miRNA biogenesis and interacts with Drosha. Therefore, we uncovered an evolutionarily conserved factor in miRNA biogenesis. We propose that DDL participates in miRNA biogenesis by facilitating DCL1 to access or recognize pri-miRNAs.

DCL1 | microRNA | siRNA | Drosha | SMAD

A class of sequence-specific repressors of gene expression in eukaryotes is 20- to 24-nt small RNAs, which include miRNAs and siRNAs. miRNAs are processed from stem-loop precursor RNAs, called pri-miRNAs. In animals, pri-miRNAs are processed in the nucleus by Drosha to form pre-miRNAs, which are exported to the cytoplasm by exportin 5 and further processed by Dicer to produce mature miRNAs (reviewed in ref. 1). In *Arabidopsis*, mature miRNAs are produced through two processing steps (pri-miRNAs to pre-miRNAs and pre-miRNAs to miRNAs) in the nucleus by DCL1 with the assistance of HYL1 and SERRATE (reviewed in ref. 2). After processing, miRNAs are 2'-O-methylated by HEN1 (3). siRNAs are produced from long, double-stranded RNAs. Plants contain several classes of endogenous siRNAs, such as transacting siRNAs (ta-siRNAs), natural antisense siRNAs (nat-siRNAs), and siRNAs from endogenous repeat sequences and transposons (reviewed in ref. 4).

The forkhead-associated (FHA) domain is an 80- to 100-aa module that is thought to recognize phosphothreonine-containing motifs and mediate protein-protein interactions in prokaryotes and eukaryotes (reviewed in ref. 5). DAWDLE (DDL) is a nuclear-localized FHA domain-containing protein in *Arabidopsis* (6). DDL appears to act in multiple developmental processes such as growth, fertility, and root, shoot, and floral morphogenesis (6).

Smad nuclear interacting protein 1 (SNIP1) is a human FHA domain-containing protein that functions as an inhibitor of TGF- $\beta$  and NF- $\kappa$ B signaling pathways by competing with the TGF- $\beta$  signaling protein Smad4 and the NF- $\kappa$ B transcription factor p65/RelA for binding to the transcriptional coactivator p300 (7, 8). Recently, Fujii *et al.* (9) reported that SNIP1 interacts with the transcription factor/oncoprotein c-Myc and enhances its activity by bridging its interaction with p300.

Here we report that DDL is required for the accumulation of miRNAs and endogenous siRNAs in *Arabidopsis*. Its affinity for RNA, its potential association with DCL1, and the reduction in

pri-miRNA levels in *ddl* loss-of-function mutants suggest that DDL is a candidate protein recruiting DCL1 to its substrates. In addition, we show that SNIP1 is a human ortholog of DDL and that it also acts in miRNA biogenesis.

## Results

**DDL Acts in miRNA Biogenesis in *Arabidopsis*.** *ddl-1* and *ddl-2* are two recessive, potentially null alleles in the *DDL* gene in the *Was-silewskija* genetic background (6). The *ddl-1* and *ddl-2* mutants show delayed growth and reduced fertility, and have defects in root, shoot, and floral morphology. These pleiotropic developmental defects resemble those of mutants deficient in miRNA biogenesis and prompted us to test whether the *ddl* mutants are compromised in miRNA accumulation. We examined the abundance of various miRNAs in *ddl-1* and *ddl-2* by RNA filter hybridization. Indeed, the levels of 9 of 10 tested DCL1-dependent miRNAs were reduced by 2- to 3.3-fold in *ddl-1* and *ddl-2*, relative to WT [Fig. 1 and supporting information (SI) Fig. S1]. The levels of an antisense miRNA, miR172\*, were reduced by 3.3 times in *ddl* mutants (Fig. 1). Introduction of a *DDL* transgene into *ddl-1* rescued the morphological defects of the mutants (6) and fully recovered the levels of miRNAs and miR172\* (Fig. 1 and Fig. S1), demonstrating that the defects in miRNA accumulation in the two mutants were due to *DDL* loss of function. To determine whether *DDL* plays a role in the methylation of miRNAs, we evaluated the methylation status of miR161 in *ddl* mutants by treating total RNAs with sodium periodate followed by  $\beta$ -elimination (3) and analyzing miR161 by filter hybridization. Loss of methylation would result in faster migration of the RNA in this assay (3). We found that the *ddl* mutations had no detectable effects on the methylation of miR161 (Fig. S1).

**DDL Is Required for the Biogenesis of ta-siRNAs and Repeated DNA-Associated siRNAs.** We next tested whether *DDL* is involved in the biogenesis of endogenous siRNAs. We found that two DCL4-dependent siRNAs—siRNA1511, a ta-siRNA from the *TAS2* locus (10), and siRNA255, a ta-siRNA from the *TAS1* locus (10)—were reduced in abundance in *ddl* mutants (Fig. 24). The reduced accumulation of these ta-siRNAs was rescued by the *DDL* trans-

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The authors declare no conflict of interest.

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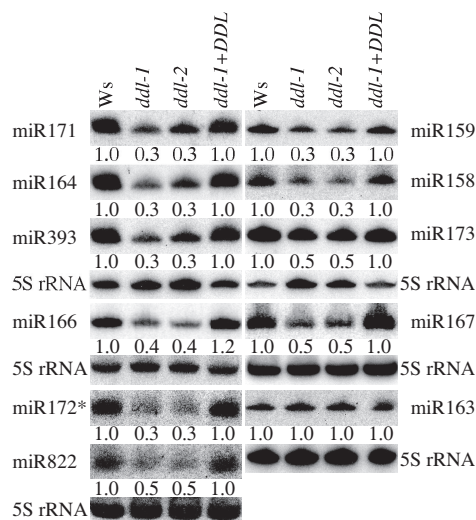
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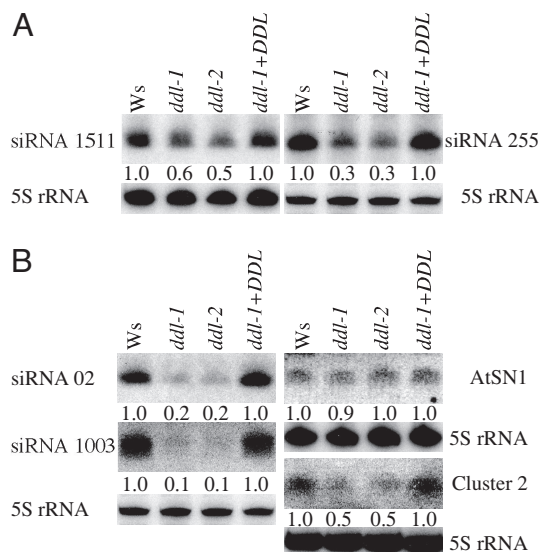
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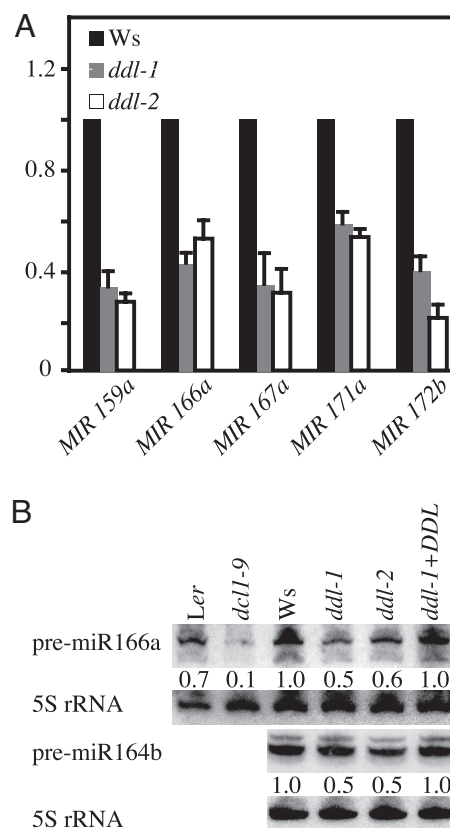


**Fig. 1.** DDL is required for the accumulation of miRNAs. The accumulation of various miRNAs and miR172\* as detected by Northern blotting in Ws (WT), *ddl-1*, *ddl-2*, and a *ddl-1* transgenic line harboring *DDL* genomic DNA. Note that except for miR822, which is DCL4-dependent, all miRNAs are DCL1-dependent. Total RNAs were extracted from inflorescences. The control 5S rRNA blots were below the corresponding miRNA blots. In cases where a membrane was used for several miRNAs, there would be one 5S rRNA blot for several miRNA blots. The numbers indicate the relative abundance of miRNAs or miR172\* among the four genotypes.

gene (Fig. 2*A*). Because the ta-siRNAs require a DCL1-dependent miRNA in their biogenesis, the reduction in ta-siRNA accumulation in *ddl* mutants could not support a direct role of DDL in the biogenesis of DCL4-dependent small RNAs. We tested the effect of *ddl* mutations on the accumulation of a DCL4-dependent miRNA, miR822 (11). We found that the *ddl* mutations led to



**Fig. 2.** DDL is required for the accumulation of endogenous siRNAs. (A) ta-siRNAs were detected in various genotypes by Northern blotting. (B) Repeated DNA-associated siRNAs were detected by Northern blotting in various genotypes. The same membrane was used to probe for siRNA 255, siRNA 02, and siRNA 1003, and the corresponding 5S rRNA control is shown twice to aid visual comparison. The numbers below the hybridization images indicate the relative abundance of siRNAs among the four genotypes. Ws, the WT control for the *ddl* mutants; *ddl-1+DDL*, a *ddl-1* mutant rescued by *DDL* genomic DNA.



**Fig. 3.** The accumulation of both pri- and pre-miRNAs in inflorescences is reduced in *ddl* mutants. (A) RT-PCR analysis of the levels of pri-miRNAs in WT (Ws), *ddl-1*, and *ddl-2* inflorescences. The levels of pri-miRNAs in *ddl* mutants were normalized to those of *UBIQUITIN 5* and compared with WT. (B) Northern blot analysis of pre-miRNAs in various genotypes. Ler, the WT control for *dcl1-9*; *dcl1-9*, a *ddl-1* mutant rescued by *DDL* genomic DNA. The numbers below the hybridization images indicate the relative abundance of pre-miRNAs among the four genotypes.

reduced levels of this miRNA, and that DDL genomic DNA rescued the molecular defect in *ddl-1* (Fig. 1).

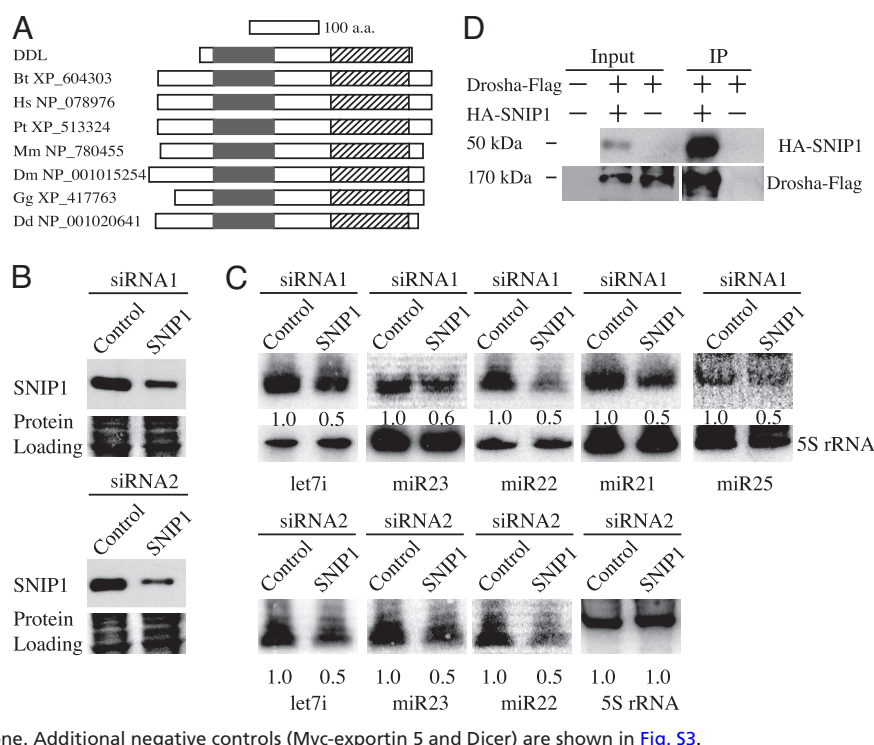
We also examined the levels of DCL3-dependent siRNAs from repeated DNA or transposons, such as siRNA02, siRNA1003, AtSN1 siRNAs, and cluster 2 siRNAs. Three of the four siRNAs were reduced by 2- to 10-fold in the *ddl* mutants, and this reduction was rescued by the *DDL* transgene (Fig. 2*B*).

**The Amount of pri- and pre-miRNAs Is Reduced in the *ddl* Mutants.** To determine the step at which a defect in miRNA biogenesis occurred in *ddl* mutants, we examined the levels of pri-miRNAs and pre-miRNAs in WT and *ddl* mutants. We determined the levels of pri-miRNAs at five *MIR* loci (*MIR159a*, *MIR166a*, *MIR167a*, *MIR171a*, and *MIR172b*) by RT-PCR. The levels of the five tested pri-miRNAs were reduced by 1.7- to 3.0-fold in *ddl-1* and 1.9- to 4.8-fold in *ddl-2* relative to WT (Fig. 3*A*). Next we examined the levels of pre-miR166a and pre-miR164b, which were shown to be detectable by RNA filter hybridization (12). We also included *dcl1-9*, which has reduced levels of pre-miRNAs (12), and its WT control (Ler) in the analysis. The levels of the two pre-miRNAs were reduced in *ddl* mutants (Fig. 3*B*). Furthermore, in the *ddl* mutants, the levels of pri-miRNAs, pre-miRNAs, and miRNAs appeared to be reduced to a similar extent (Figs. 1 and 3).

**DDL Does Not Control the Transcription of *MIR* Genes.** The reduction in pri-miRNA levels in *ddl* mutants raised the possibility that DDL is a general transcription factor for *MIR* genes or that DDL is a







(amino acids 1–833), but not a C-terminal portion (amino acids 814–1909), of DCL1 in the yeast two-hybrid assay (Fig. 5A).

To confirm the interaction between DDL and DCL1 with an independent assay, we tested coimmunoprecipitation (coIP) of the two proteins transiently expressed in *Nicotiana benthamiana*. The presence of the viral-silencing suppressor p19 of tomato bushy stunt virus was reported to result in at least a 50-fold increase in the expression of a target protein in a transient expression system in *N. benthamiana* (15). We used this system to separately express the DCL1 protein fused to a yellow fluorescent protein (DCL1-YFP) and the DDL protein fused to an HA epitope (HA-DDL). The HA-DDL extract was then mixed with the DCL1-YFP extract at a 1:1 ratio. We then used anti-GFP (and GFP variants) polyclonal antibodies conjugated to agarose beads to pull down DCL1-YFP from mixed DCL1-YFP/HA-DDL extracts or from HA-DDL extracts alone (control). As shown in Fig. 5B, an anti-HA antibody detected the enrichment of HA-DDL in the DCL1-YFP immunoprecipitate relative to the control immunoprecipitate, suggesting the association between DDL and DCL1. As another negative control, we expressed HA-HEN1 and performed coIP on DCL1-YFP and HA-HEN1 with the same procedure. We did not find any association between DCL1-YFP and HA-HEN1 (Fig. 5B).

It should be noted that only a small portion of the input HA-DDL was associated with DCL1-YFP in this assay. In fact, HA-DDL was not detected in the DCL1-YFP immunoprecipitate when the levels of DCL1-YFP were low (i.e., in the absence of p19). These observations suggested that only a portion of DCL1 molecules could interact with DDL or that DDL interacted transiently with DCL1.

**DDL Is an RNA Binding Protein.** BLAST analysis showed that an N-terminal domain of DDL was conserved in several proteins with roles in RNA metabolism (Fig. 6A) (data not shown). The presence of this domain prompted us to test whether DDL was an RNA binding protein. We performed a GST pull-down assay to test whether DDL could bind to pri-miR162b, which was transcribed *in vitro* in the presence of [ $\alpha$ - $^{32}$ P]UTP. We chose to test pri-miR162b because its 5' and 3' ends were determined experimentally (16).

GST-DDL or the control GST was expressed in *E. coli* and purified with glutathione beads. Labeled pri-miR162b was added to the beads containing GST-DDL or GST. After washes and elution, the RNA was resolved on a polyacrylamide gel. Pri-miR162b was retained by GST-DDL but not GST alone (Fig. 7A and B). Under the same conditions, double-stranded DNA corresponding to pri-miR162b was not retained by GST-DDL (Fig. 7B). We found that GST-DDL could also bind to an *in vitro* transcribed RNA corresponding to part of the *APETALA1* mRNA (data not shown). These observations indicate that DDL is an RNA binding protein, but it does not bind specifically to pri-miRNAs *in vitro*.

**The Human Homolog of DDL Is Involved in miRNA Biogenesis.** By BLAST searches of the nonredundant protein databases of the National Center for Biotechnology Information, we found that the SNIP1 proteins from human, mouse, and other organisms were obvious homologs of DDL (Fig. 6A). The human SNIP1 protein was reported to function in TGF- $\beta$  and NF- $\kappa$ B signaling pathways, but a potential role in miRNA metabolism was not evaluated.

We tested whether the human SNIP1 protein was involved in miRNA biogenesis. We first used an siRNA (siRNA1) targeting SNIP1 to knock down SNIP1 expression in HeLa cells. Indeed, siRNA1 targeting SNIP1 reduced the levels of SNIP1 protein by  $\approx 50\%$  relative to a nontargeting control siRNA (Fig. 6B). The accumulation of five tested miRNAs (let-7i, miR21, miR22, miR23, and miR25) showed 1.7- to 2-fold reduction after SNIP1 siRNA1 treatment (Fig. 6C). We further tested the effect of a different SNIP1 targeting siRNA (siRNA2). Transfection of HeLa cells with siRNA2 resulted in  $>50\%$  reduction in SNIP1 protein levels relative to a nontargeting control siRNA (Fig. 6B). A 2-fold reduction in three tested miRNAs was observed in siRNA2-treated cells (Fig. 6C). These data demonstrated that human SNIP1 functions in miRNA metabolism and is therefore likely an ortholog of DDL.

Both SNIP1 and Drosha, which processes pri-miRNAs in humans, are localized in the nucleus. We reasoned that, like the association between DDL and DCL1, which processes pri-miRNAs in plants, SNIP1 might be associated with Drosha. We tested the





factors as well as with the transcriptional coactivator p300 (7–9). At present, it is not clear whether SNIP1 is a multifunctional protein involved in several molecular processes or whether the observed molecular functions of SNIP1 (interactions with p300 and promotion of miRNA accumulation) represent aspects of a unified function.

## Materials and Methods

**Plant Materials.** The *ddl-1* and *ddl-2* mutants and the *ddl-1* mutant expressing a *DDL* genomic construct were reported by Morris *et al.* (6). To generate *DDL*<sup>+</sup> (*DDL/DDL* or *DDL/ddl-1*) or *ddl-1* plants harboring a single-locus GUS transgene, transgenic lines (in *Ler* background) containing a single locus of *pMIR172a::GUS*, *pMIR172b::GUS*, or *pPSTILLATA::GUS* (13) were crossed to *ddl-1*. In the F<sub>2</sub> population, plants showing the WT (of the *DDL/DDL* or *DDL/ddl-1* genotypes) and *ddl-1* phenotypes were screened for the GUS transgene by GUS staining. To generate *DDL*<sup>+</sup> (*DDL/DDL* or *DDL/ddl-1*) or *ddl-1* plants expressing *35S::MIR172a*, we crossed a transgenic line (in *Ler* background) containing a single locus of *35S::MIR172a* (14) to *ddl-1*. F<sub>2</sub> seeds were selected on Kanamycin medium for the presence of the transgene, and *DDL*<sup>+</sup> (*DDL/DDL* or *DDL/ddl-1*) or *ddl-1* plants were identified by their phenotypes.

**Plasmid Construction.** The construction of GST-DDL, *pMIR172a::GUS*, and *pMIR172b::GUS* plasmids is described in *SI Materials and Methods*.

**RT-PCR Analysis of pri-miRNAs.** cDNA was synthesized from 5 μg of total RNA by using reverse transcriptase (Invitrogen) and oligo(dT). Quantitative PCR was performed in triplicate on a Bio-Rad IQcycler apparatus with the Quantitech SYBR green kit (Bio-Rad). The primers used are listed in Table S1.

**RNA Analyses.** RNA isolation and hybridization for miRNAs and endogenous siRNAs were performed as described (29). 5'-End-labeled <sup>32</sup>P antisense DNA oligonucleotides or LNA oligonucleotides were used to detect miRNAs, ta-siRNAs, AtSN1 siRNA, siRNA02, and siRNA1003. For cluster 2 siRNAs, a DNA fragment was amplified from genomic DNA with forward and reverse primers (Table S1), gel-purified, and labeled by random priming. The detection of pre-miR166a and pre-miR164b was performed as described (12).

GST-DDL RNA binding assays were performed as described (30, 31). A DNA fragment corresponding to pri-miR162b was amplified by PCR from genomic DNA with primers miR162bp1, which contained a T7 promoter, and miR162bp2 (Table S1). The resulting PCR product was purified and used as a template for *in*

*vitro* transcription with T7 polymerase in the presence of [ $\alpha$ -<sup>32</sup>P]UTP to generate pri-miR162b.

**Transient Expression in *N. benthamiana* and coIP.** Transient expression of DCL1-YFP and HA-DDL was performed as described (32). For coIP between DDL and DCL1, the harvested leaves of *N. benthamiana* were ground in liquid nitrogen and homogenized in 3 vol of protein lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.2% Nonidet P-40, 2 mM DTT, 5% glycerol, complete protease inhibitor mixture (Roche)] and centrifuged for 15 min at 16,110 × *g*. After preclearing with protein-A agarose, half of the HA-DDL or HA-HEN1 lysate was mixed with the DCL1-YFP lysate. The mixed lysate or the HA-DDL or HA-HEN1 lysate alone was incubated with anti-GFP (and GFP variants) antibodies coupled to protein A agarose beads (Clontech) for 2 h. The immune complexes were then washed four times with 1 ml of lysis buffer. Proteins retained on the beads were resolved on SDS/polyacrylamide gels. Anti-YFP (Covance), anti-HA (Sigma-Aldrich), and anti-HEN1 antibodies were used to detect DCL1-YFP, HA-DDL, and HA-HEN1, respectively, by using Western blot analysis.

**siRNA-Mediated Knockdown of SNIP1.** HeLa cells were transfected with siRNAs targeting SNIP1 or a nontargeting control siRNA (Table S1) (Dharmacon) by using Oligofectamine (Invitrogen). Cells were harvested for protein and RNA analyses 3 days after transfection. Anti-SNIP1 antibodies were purchased from Bethyl Laboratories.

**Transient Expression in 293T Cells and CoIP.** For details, see *SI Materials and Methods*.

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# Supporting Information

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## SI Materials and Methods

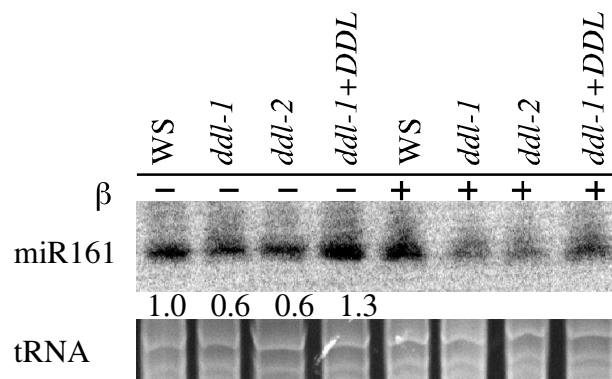
**Plasmid Construction.** To generate the GST-DDL plasmid, full-length *DDL* cDNA was amplified from a plasmid with forward and reverse primers (Table S1) and cloned into pGEX-2TK.

The promoter of *MIR172a* was amplified from genomic DNA with primers miR172ap5 and miR172ap6 (Table S1). The promoter of *MIR172b* was amplified from genomic DNA with primers miR172bp1 and miR172bp2 (Table S1). The fragments were cloned into pPZP211-GUS-NOS to generate the promoter-GUS fusion constructs.

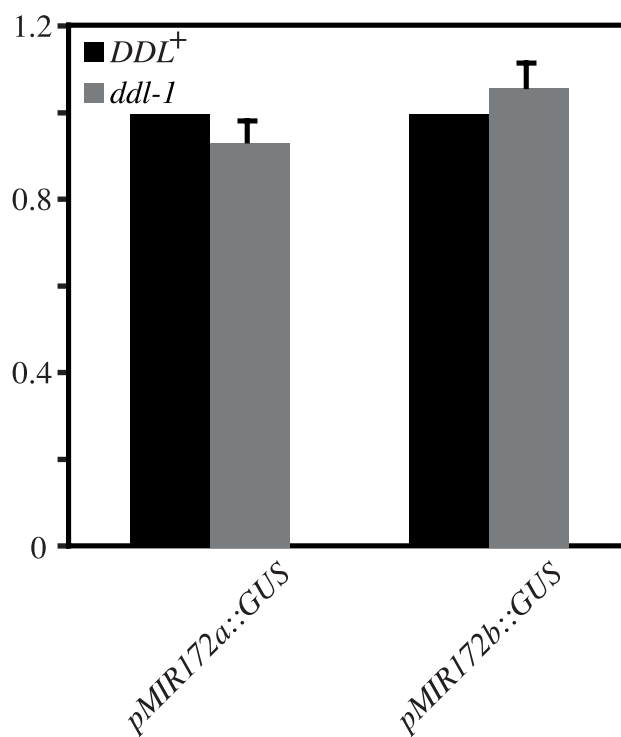
**Transient Expression in 293T Cells and CoIP.** Human 293T cells were transfected with a Drosha-Flag plasmid (pCK-3'flag-Drosha)

(1), a human Dicer plasmid (pCDNA3-hDicer), a Myc-exportin 5 plasmid (pKmyc-Exp5) (2), or an HA-SNIP1 plasmid (pCD-5'-HA-c19fulllength) (3) by using Lipofectamine 2000 (Invitrogen). Two days after transfection, cells were harvested and lysed in buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.2% Nonidet P-40, 2 mM DTT, 5% glycerol, complete protease inhibitor mixture (Roche)]. IP was performed by using an immobilized anti-HA monoclonal antibody (Roche). After IP, protein samples were resolved on 8% SDS/polyacrylamide gels. Monoclonal anti-Flag, anti-Myc, and anti-HA antibodies were used to detect Drosha-Flag, Myc-exportin 5, and HA-SNIP1, respectively, by using Western blot analysis.

1. Lee Y, et al. (2003) The nuclear RNase III Drosha initiates microRNA processing. *Nature* 425:415–419.
2. Yi R, Doehle BP, Qin Y, Macara IG, Cullen BR (2005) Overexpression of exportin 5 enhances RNA interference mediated by short hairpin RNAs and microRNAs. *Rna* 11:220–226.
3. Kim RH, et al. (2000) A novel smad nuclear interacting protein, SNIP1, suppresses p300-dependent TGF-beta signal transduction. *Genes Dev* 14:1605–1616.



**Fig. S1.** The methylation of miR161 is not affected by the *ddl* mutations. Total RNAs from *Ws* (WT), *ddl* mutants, or a *ddl-1* line rescued with *DDL* genomic DNA (*ddl-1 + DDL*) were either subjected (+) or not subjected (–) to the  $\beta$  elimination reactions ( $\beta$ ) and probed for miR161. Because the mobility of miR161 did not change after the  $\beta$  elimination reactions, miR161 was methylated in all four genotypes. The numbers below the hybridization image indicate the relative abundance of miR161 among the four genotypes. A portion of the stained gel is shown below the hybridization image.



**Fig. S2.** RT-PCR analysis of the levels of *GUS* mRNA in *DDL*<sup>+</sup> and *ddl-1* plants harboring a *pMIR172a::GUS* or a *pMIR172b::GUS* transgene. The levels of *GUS* mRNA in the *ddl-1* plants were normalized to those of *UBIQUITIN5* and compared with *DDL*<sup>+</sup> plants.





**Table S1. DNA and RNA oligonucleotides used in this study**

Oligonucleotide name	Sequence
GST-DDL primers	
GST-DDL-F	5'-cgcgatccgcgatggctcctagttctagggtccc-3'
GST-DDL-R	5'-cgcgatccgcgtcactcggcagaattctcgtgc-3'
<i>MIR172a</i> promoter primers	
miR172ap5	5'-taactgcagtagattgggttagttaaacgagcc-3'
miR172ap6	5'-attctgcagagaaagacctggtgagatctagaa-3'
<i>MIR172b</i> promoter primers	
miR172bp1	5'-gtactgcagcagagaaaggtagactacaagtgcc-3'
miR172bp2	5'-agactgcagagagaaaggtagtgagagtgcaagtg-3'
pri-miRNA primers	
miR159a-p1	5'-ggagctctacttccatcgta-3'
miR159a-p2	5'-ccagttctcatcaaaactttc-3'
miR166a-p1	5'-gactctggctcgtctattca-3'
miR166a-p2	5'-tggtccgaagacgctaaaac-3'
miR167a-p1	5'-gaagctgccagcatgatcta-3'
miR167a-p2	5'-gggtttatagaagggtgcga-3'
miR171a-p1	5'-ccgcgccaatatctcagta-3'
miR171a-p2	5'-tgtctccatttcaacacacaca-3'
miR172a-p1	5'-ccgcgccaatatctcagta-3'
miR172a-p2	5'-aatagtcgttgattgccgatg-3'
miR172b-p1	5'-tctcttgtcgtgcgtaaat-3'
miR172b-p2	5'-cgctacaaacaacgacaga-3'
miR162b-p1	5'-taatacgactcactataggatctatccactctctgtaa-3'
miR162b-p2	5'-taacaacaacaatttcattttatcc-3'
<i>UBQUITIN5</i> primers	
N.UBQ5	5'-ggtgctaagaaggaagaat-3'
C.UBQ5	5'-ctccttcttctggtaaacgt-3'
Cluster 2 primers	
cluster2-f	5'-ttgctgatttgattttatgcat-3'
cluster2-r	5'-cttttcaaacataaaccagaaa-3'
SNIP1 siRNAs	
siRNA-SNIP1-1S	5'-gaaccgagcccaggaguuuu-3'
siRNA-SNIP1-1A	5'-aacuccucggcucgguucuu-3'
siRNA-SNIP1-2S	5'-gcaagucuccucgagaaguu-3'
siRNA-SNIP1-2A	5'-cuucugcaggagacuugcuu-3'
siRNA-control-S	5'-uucgcuugcagagagaucuu-3'
siRNA-control-A	5'-gauucucucgcaagcgaauu-3'