Figure S1. Map-based cloning and complementation testing for ZOP1. (A) ZOP1 was mapped to a ~273-kb interval on Chromosome 1. In the interval, a single-nucleotide G to A substitution in AT1G49590 was identified by whole-genome sequencing of ros1zop1. (B) The G to A substitution creates a premature stop codon in AT1G49590 as indicated. AT1G49590 is the ZOP1 gene. The ZOP1-encoded protein includes a C-terminal C2H2-type zinc finger domain and an octamer repeat (OCRE) domain. (C) The ZOP1 transgene complements the RD29A-LUC silencing. Three independent T2 ZOP1 transgenic lines were used for complementation testing. (D) The ZOP1 transgene complements the developmental defect of ros1zop1.

Figure S2. Alignment of ZOP1 and its conserved homologs. ZOP1 and its homologs are evolutionarily conserved from unicellular green algae to plants.

Figure S3. Effect of zop1 on Pol IV-dependent siRNA accumulation at the whole-genome level. (A-E) Normalized Pol IV-dependent siRNA reads in 100-Kb windows of Arabidopsis genomes are plotted for ros1 (read lines), ros1nrpd1 (blue lines), and ros1zop1 (green lines).

Figure S4. Analysis of DNA methylation at RdDM target loci by quantitative chop-PCR. Genomic DNA from the indicated genotypes was digested by the DNA methylation sensitive restriction enzyme HaeIII and subjected to quantitative PCR at RdDM target loci AtSN1 and IGN23. Undigested DNA was amplified as an internal control.

Figure S5. Effect of zop1 on DNA methylation patterns at CG, CHG, and CHH sites on Arabidopsis chromosomes. The DNA methylation patterns in ros1, ros1nrpd1, and ros1zop1 were plotted for all five Arabidopsis chromosomes. The DNA methylation levels of ros1 and ros1nrpd1 or ros1zop1 are represented by black lines and red lines, respectively. The DNA methylation at CG, CHG, and CHH sites is separately diagrammed in three different charts in the top panel. The reduction of CG, CHG, and CHH DNA methylation in ros1nrpd1 or ros1zop1 compared to ros1 is shown in the charts in the bottom panel. (A, C, E, G, I) DNA methylation patterns of ros1nrpd1 compared to ros1 on Chromosome 1, 2, 3, 4, and 5 are separately shown. (B, D, F, H, J) DNA methylation patterns of ros1zop1 compared to ros1 on Chromosome 1, 2, 3, 4, and 5.

Figure S6. Analysis of the DNA methylation differences that are identified by whole-
genome bisulfite sequencing. The identified NRPD1- and ZOP1-dependent loci AT5G35540, AT1G54750, and AT2G14247 were subjected to sequence-specific bisulfite sequencing.

**Figure S7.** Effect of zop1 on DNA methylation of genes and TEs at CG, CHG, and CHH sites. DNA methylation patterns are plotted for (A) genes and (B) TEs. In the charts, ros1, ros1nrpd1, and ros1zop1 are represented by green, red, and blue lines, respectively.

**Figure S8.** Detection of RNA transcripts at RdDM target loci by quantitative RT-PCR. (A, B) The RNA transcripts of AtSN1 A, AtGP1, solo LTR, and ROS1 were measured by quantitative RT-PCR. The relative expression and standard deviation are shown in the charts as bars and lines, respectively. (C) Detection of the Pol V-dependent RNA transcripts from AtSN1 B, IGN5B and IGN23 by semiquantitative RT-PCR. Actin gene was amplified as an internal control. No RT represents amplification of RNA without reverse transcription.

**Figure S9.** Analysis of the expression of RdDM components in ros1zop1 relative to ros1. Quantitative RT-PCR was carried out to compare the transcript levels of RdDM components in ros1 and ros1zop1. The transcript levels of RdDM components in ros1 were set as 1.0. The transcript levels of RdDM components in ros1zop1 relative to those in ros1 are shown in the chart. The standard deviation is indicated with lines.

**Figure S10.** Affinity purification of ZOP1-Flag from ZOP1-Flag transgenic plants. Total protein extracts were isolated from wild-type and ZOP1-Flag transgenic plants and subjected to affinity purification of ZOP1-Flag. The purified proteins were run on an SDS-PAGE gel and visualized by silver staining.

**Figure S11.** Subcellular localization of ZOP1-Flag by immunolocalization. Root meristem tissue was used for immunolocalization with the anti-Flag antibody.

**Figure S12.** The ZOP1 C-terminal domain binds both double-stranded DNA and double-stranded RNA. (A) Analysis of the nucleic acid-binding ability of ZOP1 by EMSA. The bacterially expressed full-length ZOP1 was purified and used in EMSA. The nucleic acids used included single-stranded RNA, double-stranded RNA, 5’ overhanging double-stranded RNA, single-stranded DNA, and double-stranded DNA. The single-stranded DNA and RNA probes were RD29A-DNA-F and RD29A-RNA-F. The 5’ overhangs of 5’ overhanging double-stranded RNA were 2 nt. (B) The double-stranded DNA-and double-stranded RNA-binding assay was carried out with increasing amounts of ZOP1 (0.10, 0.15, 0.20, 0.25, 0.30, 0.35 μg). (C) Increasing
amounts of competitive unlabeled probes were added to the reaction system. Double-stranded DNA- and double-stranded RNA-binding abilities of ZOP1 were separately analyzed. (D) Diagrams of the full-length and truncated ZOP1 proteins. The proteins were expressed and purified from *E. coli*. (E) The proteins that have the ZOP1 C-terminal domain (ZOP1-full, ZOP1-P2, and ZOP1-P5) can bind both double-stranded DNA and double-stranded RNA, whereas the proteins without the C-terminal domain (ZOP1-P1, ZOP1-P3, and ZOP1-P4) have no nucleic acid-binding ability.

**Figure S13.** Detection of AGO4 expression in WT, *ros1, ros1zop1, ros1nrpd1*, and *ros1nrpe1* using anti-AGO4 antibody. Ponceau S-stained rubisco proteins are shown as a loading control.

**Figure S14.** Detection of the interaction between ZOP1 and canonical RdDM proteins by coimmunoprecipitation assay. *ZOP1-Myc* or *ZOP1-Flag* transgenic plants were crossed to NRPD1-Flag, NRPE1-Flag, DRM2-Flag, or RDM4-Myc transgenic plants, and the offspring plants that harbored both transgenes were harvested. Total protein extracts from indicated plants were immunoprecipitated with anti-Myc- or anti-Flag-conjugated agarose beads. Beads without conjugated antibodies were used as a negative control. The precipitated proteins were run on SDS-PAGE gels and subjected to western blotting with anti-Flag or anti-Myc antibodies. The interaction of ZOP1 with (A) NRPD1, (B) NRPE1, (C) DRM2, and (D) RDM4 was separately detected.

**Figure S15.** The interaction between ZOP1 and NRPB1 was tested by coimmunoprecipitation in the presence of RNase and DNase.

**Figure S16.** The negative controls for Flag and Myc signals in immunofluorescence assay.

**Figure S17.** Analysis of splicing defects in *zop1, mac3a3b, mos4, mos12*, and *mos14* by RT-PCR. The *zop1*-affected splicing sites and the splicing sites affected by the other four splicing mutants are included in the analysis.

**Figure S18.** Quantitative RT-PCR analysis of RdDM component-encoding genes in WT, *mac3a3b, mos4, mos12*, and *mos14*.

**Supplemental Tables**

**Table S1.** Quantitative results of small RNA northern blotting for *zop1*.

**Table S2.** List of the 100-bp loci that produce Pol IV-dependent siRNA and the number of normalized siRNA reads in *ros1, ros1nrpd1, ros1zop1*, and *ros1nrpe1*.

**Table S3.** Statistics of whole-genome bisulfite sequencing in each ecotype.
Table S4. Percentage of DNA methylation at CG, CHG, and CHH sites in protein-coding genes.

Table S5. Percentage of DNA methylation at CG, CHG, and CHH sites in TEs.

Table S6. List of the intron-retention events in *ros1zop1* relative to *ros1*.

Table S7. List of the intron-retention events in *ros1* relative to *ros1zop1*.

Table S8. List of the genes that are upregulated or downregulated by *zop1*.

Table S9. List of purified proteins obtained by affinity purification of ZOP-Flag.

Table S10. List of DNA and RNA oligonucleotides that were used in this study.
Supplemental Figure S1

A

Chr. 1

Marker coordinates:
17347437
18287819
18560923
19526693

Recombination:
19/381
3/381
5/381
11/381

18354786 - 18356642 bp
AT1G49590 G57 to A

B

ZOP1

zop1-1
G57 to A

W to stop code

Protein
ZnF-C2H2
OCRE domain

C

D

Luminescence

WT
ros1
ros1zop1

ros1zop1+ ZOP1 transgene
T2-1 T2-2 T2-3

WT
ros1
ros1zop1
ros1zop1+ZOP1
Supplemental Figure S4

AtSN1

IGN23

HaeIII Digest / Undigest

C24, ros1, ros1zop1, ros1nrpe1, ros1nrpd1, Col-0, mac3a3b, mos4-1, mos12-1, mos14-1, nrpd1-3
G

Chr4. CG

Chr4. CHG

Chr4. CHH

H

Chr4. CG

Chr4. CHG

Chr4. CHH
Chr5. CG

Chr5. CHG

Chr5. CHH

DNA methylation (%) log₂(ros1zop1/ros1)

DNA methylation (%) log₂(ros1nrpd1/ros1)

Chr5. CG

Chr5. CHG

Chr5. CHH

DNA methylation (%) log₂(ros1zop1/ros1)

DNA methylation (%) log₂(ros1nrpd1/ros1)
Supplemental Figure S6

A

AT5G35540

DNA methylation

WT
ros1
ros1zop1
ros1nrpd1

CG
CHG
CHH

B

AT1G54750

DNA methylation

WT
ros1
ros1zop1
ros1nrpd1

CG
CHG
CHH

C

AT2G14247

DNA methylation

WT
ros1
ros1zop1
ros1nrpd1

CG
CHG
CHH
Supplemental Figure S7

A

- **Genes CG**
  - DNA methylation (%)
  - Position (Kb)
  - Graphs for different transcription factors (ros1, ros1nrpd1, ros1zop1)

- **Genes CHG**
  - DNA methylation (%)
  - Position (Kb)

- **Genes CHH**
  - DNA methylation (%)
  - Position (Kb)

B

- **Transposons CG**
  - DNA methylation (%)
  - Position (Kb)

- **Transposons CHG**
  - DNA methylation (%)
  - Position (Kb)

- **Transposons CHH**
  - DNA methylation (%)
  - Position (Kb)
Supplemental Figure S8

A. Relative expression level of AtSN1 A, AtGP1, and solo LTR genes across different conditions.

B. Relative transcript levels of AtGP1 and solo LTR in WT, ros, ros1zop1, ros1nrpe1, and zop1 conditions.

C. Gel electrophoresis showing expression levels of AtSN1B, IGN5B, IGN23, Actin2, no RT, and rRNA across different conditions.
Supplemental Figure S10
Supplemental Figure S11
**Figure S12**

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Figure S13
Supplemental Figure S15

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Figure S16

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[Images of fluorescence microscopy results for DAPI, α-Myc, α-Flag, α-Flag + α-Myc, and merged images]
Supplemental Figure S17