FIERY1 promotes microRNA accumulation by suppressing rRNA-derived small interfering RNAs in Arabidopsis

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Plant microRNAs (miRNAs) associate with ARGONAUTE1 (AGO1) to direct post-transcriptional gene silencing and regulate numerous biological processes. Although AGO1 predominantly binds miRNAs in vivo, it also associates with endogenous small interfering RNAs (siRNAs). It is unclear whether the miRNA/siRNA balance affects miRNA activities. Here we report that FIERY1 (FRY1), which is involved in 5′−3′ RNA degradation, regulates miRNA abundance and function by suppressing the biogenesis of ribosomal RNA-derived siRNAs (risiRNAs). In mutants of FRY1 and the nuclear 5′−3′ exonuclease genes XRN2 and XRN3, we find that a large number of 21-nt risiRNAs are generated through an endogenous siRNA biogenesis pathway. The production of risiRNAs correlates with pre-rRNA processing defects in these mutants. We also show that these risiRNAs are loaded into AGO1, causing reduced loading of miRNAs. This study reveals a previously unknown link between rRNA processing and miRNA accumulation.
small RNAs (sRNAs) serve as sequence determinants in post-transcriptional gene silencing (PTGS) in plants. The two major types of PTGS small RNAs are microRNAs (miRNAs) and small interfering RNAs (siRNAs). Like miRNAs, PTGS siRNAs are usually 21–22 nt long, but unlike miRNAs, they are derived from double-stranded precursors from transgenes, viruses, and endogenous loci, such as the TRANS-ACTING SI RNA (TAS) loci. The machinery underlying the biogenesis and function of miRNAs and siRNAs contains shared and distinct components. miRNA precursors are processed by DICER-LIKE1 (DCL1) into mature miRNAs, whereas siRNAs from transgenes, viruses, and endogenous transcripts are generated by other DCLs. Both miRNAs and siRNAs undergo 3′ terminal methylation by HUA ENHANCER1 (HEN1). The partial sharing of the silencing machinery may crossstalk and potential mutual regulation between miRNAs and siRNAs.

RNA quality control (RQC) suppresses siRNA production from many endogenous transcripts. Among the RQC genes are those encoding 5′-3′ EXORIBONUCLEASE5 (XRN3) and XRN4; the decapping complex subunits DECAPRING (DCP1), DCP2, and VARIOC (VCS); the SUPERKILLER (SKI) complex components SKI2 and SKI3, which are involved in 3′-5′ exoribonucleolytic RNA degradation; Nonsense-mediated decay components UPFRESHIFT1/3 (UPF1/3); the 3′-5′ POLY(A)-SPECIFIC RIBONUCLEASE (PARN); and exosome subunits RIBOSOMAL RNA PROCESSING4 (RPP4) and RRP6. The sRNAs produced when these RQC genes are compromised are usually 21–22 nt long, phased, and dependent on the PTGS siRNA pathway. Zhang et al. proposed that aberrant RNAs accumulated in these mutants are bound by SUPPRESSOR OF GENE SILENCING3 (SGS3) and serve as templates for siRNA biogenesis. Interestingly, a study on the decapping complex demonstrated that DCP1, DCP2, and VCS are required for the accumulation of some miRNAs.

Ribosomal RNA (rRNA)-derived sRNAs have been observed in several organisms. In Schizosaccharomyces pombe, defects in TRAMP-mediated RNA silencing trigger the biogenesis of Ago1-associated, rRNA-derived siRNAs (rr-siRNAs). In Neurospora crassa, 20–21 nt qiRNAs are produced from aberrant rRNAs in an Rdrp (RNA-dependent RNA polymerase)-dependent manner, are enriched in 5′ U, and are loaded into the AGO protein QDE-2. qiRNAs are thought to function in DNA damage repair. In Caenorhabditis elegans, 22-nt rRNA-derived siRNAs (risiRNAs) corresponding to both strands of rDNA are generated under conditions that induce pre-rRNA processing defects. C. elegans risiRNAs are enriched in 5′ G and are thought to regulate rRNA abundance. In Arabidopsis, rDNA-derived 24-nt siRNAs were first described in 2006. These siRNAs are produced in a Pol IV-dependent and DCL3-dependent manner and guide DNA methylation. Later studies also found 21-nt small RNAs originating from bidirectional transcripts from the intergenic spacers (IGS) of RNA genes. A recent report found that viral infections trigger the production of siRNAs from rRNAs, but the molecular or biological impacts of these ribosomal small RNAs remain unknown.

In this study, a mutation in FIERY1 (FRY1) is isolated in a genetic screen aimed at uncovering new factors in the miRNA pathway. FRY1 encodes a dephosphorylating enzyme that converts 3′-phosphoadenosine 5′-phosphosulfate (PAPS) into adenosyl 5′-phosphosulfate (APS) in sulfur assimilation. FRY1 also converts 3′-phosphoadenosine 5′-phosphate (PAP) into 5′ AMP and Pi. FRY1 functions in various biological processes, such as stress signaling, drought tolerance, cell elongation, flowering time, leaf development, root development, and plant immunity. fry1 mutant phenotypes resemble those of higher-order xrn mutants, possibly because accumulated PAP suppresses XRN enzymatic activity, thereby compromising 5′-3′ RNA degradation. Several studies have shown that FRY1 and XRNs function in RNA degradation and suppress PTGS in Arabidopsis. In a fry1 mutant, 21-nt sRNAs from the 5′ external transcribed spacer (ETS) of rRNAs accumulate in a DCL2/4-dependent manner. In addition, miRNA processing intermediates accumulate in fry1, possibly due to compromised XRN activity.

We find that fry1 mutations lead to the accumulation of 21–22 nt sRNAs from miRNAs and rRNAs, transcripts that do not normally undergo siRNA biogenesis. The production of risiRNAs in fry1, as well as xrn2 xrn3 mutants correlate with pre-rRNA processing defects in these mutants. We show that the siRNAs depend on the PTGS siRNA pathway for biogenesis and are loaded into AGO proteins, AGO1 and AGO2. More importantly, risiRNAs compete with miRNAs for these AGO proteins, resulting in the compromised accumulation of miRNAs. Removal of risiRNAs partially rescues both the miRNA abundance defects and the plant phenotypes of fry1. Collectively, the findings provide insights into the biogenesis of endogenous siRNAs and the crosstalk between siRNAs and miRNAs.

Results

A mutation in FIERY1 was isolated from a suppressor screen. CTR1 is a negative regulator in the ethylene response pathway. ctrl mutants exhibit constitutive ethylene responses, resulting in shorter root and hypocotyl, tightened apical hook, and swollen hypocotyl. We took advantage of this conspicuous triple response phenotype to construct a visual reporter of miRNA biogenesis. We designed an artificial miRNA targeting CTR1 (amiR-CTR1) driven by a β-estradiol-inducible promoter (Fig. 1a). Upon induction, the transgenic line harboring amiR-CTR1 exhibited the ctrl mutant phenotype (Supplementary Fig. 1a), consistent with amiR-CTR1 accumulation and reduced CTR1 expression (Supplementary Fig. 1b, c). This reporter line was mutagenized by EMS, and M2 plants were screened for mutants resembling wild-type (WT) plants after induction. One mutant, T5520 (Fig. 1b), with a compromised triple response was isolated. As expected, analysis of amiR-CTR1 and CTR1 protein levels in T5520 revealed reduced accumulation of amiR-CTR1 (Fig. 1c) and partial recovery of CTR1 protein levels (Supplementary Fig. 1d) compared to the amiR-CTR1 parental line after β-estradiol induction. T5520 also had pleiotropic phenotypes, including round leaves and delayed flowering (Fig. 1d and Supplementary Fig. 1e). The leaf phenotypes differed from those of canonical miRNA biogenesis mutants such as ago1 and hy1.

To identify the causal mutation in T5520, we conducted genome re-sequencing using pooled plants with the mutant phenotypes in the F2 population of the T5520 x Col-0 cross. A G > A mutation in AT5G63980 (FIERY1/SLAL, FRY1 hereafter) was identified and verified by genotyping. The mutation was in the acceptor site of the second intron and resulted in the skipping of exon 3 and the creation of an early stop codon (Supplementary Fig. 1f–h). Thus, T5520 is likely a null mutant of FRY1. The FRY1 coding sequence rescued the developmental abnormalities of T5520 (Supplementary Fig. 1i).

FRY1 promotes the accumulation of miRNAs. The reduced accumulation of amiR-CTR1 in T5520 suggested that the fry1 mutation impacted miRNA biogenesis. Unfortunately, the amiR-CTR1 transgene was silenced in T3 and later generations, which prevented further studies of amiR-CTR1. To determine whether FRY1 promotes miRNA accumulation, we examined endogenous
performed sRNA sequencing of WT, fry1-6, the global effects of fry1-8 (previously named NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-019-12379-z | www.nature.com/naturecommunications

miRNAs in two FRY1 T-DNA insertion lines, SALK_020882 (previously named fry1-69) and SALK_151367 (designated as fry1-8 hereafter).

RNA gel blot analysis showed that miR156, miR166, miR390, and miR398 had reduced abundance in fry1-6 (Fig. 1e). To assess the global effects of fry1 mutations on endogenous miRNAs, we performed sRNA sequencing of WT, fry1-6, and fry1-8 (Supplementary Table 1 and Supplementary Fig. 2a). A general trend of miRNA downregulation was observed in both mutants (Fig. 2a, Supplementary Fig. 2b, and Supplementary Data 1), consistent with the initial finding of reduced amiR-CTR1 levels in T5520. Among the statistically significant differentially expressed miRNAs, 27 and 29 miRNAs were reduced in fry1-6 and fry1-8, respectively, which outnumbered upregulated miRNAs (Fig. 2b). The sRNA-seq data for the abundance changes of miR156, miR166, miR390, and miR398 were similar to those detected by RNA gel blot analysis (Figs. 2c and 1e). miR168 was among 8 upregulated miRNAs identified by sRNA-seq, and RNA gel blot analysis for miR168 showed a similar change in abundance (Fig. 2d). Other upregulated miRNAs included two miR395 family members targeting APS genes involved in sulfur metabolism, and the upregulation of miR395 probably might have occurred in response to the altered sulfonation pathway in fry1-69. A previous study also reported reduced miRNA levels in fry1 mutants, but the cause of the defect was not thoroughly investigated9.

To pinpoint the levels of endogenous pri/pre-miRNAs and the expression of major miRNA biogenesis factors by RT-qPCR and protein gel blot assays (Supplementary Fig. 2c–e). For six miRNAs showing reduced levels in fry1-6 and miR168, which increased in abundance in fry1-6, we quantified the corresponding pri/pre-miRNA levels. Only one (pri/pre-miR156a) had reduced accumulation, one (pri/pre-miR390b) had increased accumulation, and pri/pre-miR159b, pri/pre-miR166a, pri/pre-miR167a, pri/pre-miR168 and pri/pre-miR393b were unaffected in fry1-6 (Supplementary Fig. 2c). Thus, the changes in pri/pre-miRNA levels did not correlate with the abundance of the mature miRNAs, indicating that the global reduction in miRNA abundance in fry1-6 could not be explained by defects in MIR gene transcription. No substantial downregulation of the miRNA biogenesis genes was observed (Supplementary Fig. 2d). Consistent with the increased levels of miR168 and the co-regulation of miR168 and AGO143, AGO1 protein levels were increased in fry1-6 mutants (Supplementary Fig. 2e), suggesting a compensation mechanism for reduced accumulation of miRNAs. Taken together, these results suggest that the global reduction in miRNA levels in the fry1 mutants was not due to a general defect in MIR gene transcription or pri/pre-miRNA processing.

**FRY1 prevents the production of ectopic siRNA.** To further evaluate the changes of sRNAs in fry1 at a global level, all sRNA reads in the three genotypes were mapped to the genome, and their length distribution was examined. As expected, the WT distribution was characterized by a smaller 21-nt peak and a larger 24-nt peak. In both fry1 mutants, however, the 21-nt peak was enhanced with a concomitant reduction in the 24-nt peak (Fig. 3a), indicating an unexpected increase in 21-nt endogenous sRNAs.
As the global reduction in miRNA accumulation in the fry1 mutants could not explain the increase in the 21-nt sRNA peak, we investigated changes in other categories of sRNAs. First, we examined the composition of 21-nt sRNAs. In WT, miRNAs constituted the largest category of 21-nt sRNAs in terms of abundance, with rRNA fragments being the second most abundant, followed by sRNAs from genes, TAS loci, and transposable elements (TEs). In the fry1 mutants, miRNA abundance decreased, while the abundance of sRNAs from coding genes and rRNAs increased (Fig. 3b). Notably, rRNA fragments constituted the most abundant category of sRNAs in the mutants. To identify the sources of the differentially accumulating sRNAs, we compared sRNA abundance in 100-bp bins across the genome between fry1 and WT. Bins with higher and lower sRNA accumulation in fry1 were referred to as hyper and hypo DSRs (differential sRNA regions), respectively. We found that 21-nt hyper DSRs greatly outnumbered hyper DSRs of other lengths and hypo DSRs of all lengths in both mutants (Fig. 3c). Many miRNA loci were among the 21-nt hypo DSRs (Supplementary Data 2), and the large number of 21-nt hyper DSRs was consistent with the observed increase in total 21-nt sRNAs. Genomic classification of these 21-nt hyper DSRs revealed that most of them corresponded to RNA regions and non-miRNA genic regions, consistent with the changes in 21-nt sRNA composition (Fig. 3d).

**Aberrant sRNA accumulation from coding genes.** Many 21-nt hyper DSRs overlapped with coding genes (Fig. 3d). To investigate the changes in genic sRNAs, we used annotated genes as units and identified genes with differential sRNA accumulation, which we referred to as DSGs, between WT and the fry1 mutants. Hyper DSGs, i.e., genes with higher levels of sRNAs in the fry1 mutants, constituted the vast majority of DSGs (Fig. 3e). The significant overlap in these hyper DSGs between fry1-6 and fry1-8 (Fig. 3f and Supplementary Data 3, super exact test P value = 0) indicated that FRY1 suppresses sRNA production from these genes. There were 228 21-nt hyper DSGs in both mutants combined, and although sRNAs derived from these DSGs constituted only ~4% of the total 21-nt sRNAs (Fig. 3g, Supplementary Fig. 3a), they represented over 10% of the total rRNA-depleted 21-nt sRNAs (Supplementary Fig. 3b). It should be noted that most sRNA analyses in the literature ignore rRNA-derived sRNAs, which were included in this study. We selected two hyper DSGs with highly abundant sRNAs to perform RNA gel blot validation of the sRNA-seq results, and the accumulation of 21-nt sRNAs from these two genes was indeed higher in fry1-6 relative to WT (Fig. 3h).

We next investigated the possible mechanisms of sRNA accumulation from these coding genes. The accumulation of genic sRNAs was previously reported in RNA decay-deficient mutants such as ein5-1 ski2-3, dcp2-1, vcs, and xrn3-8.21,26,33. We re-analyzed the published sRNA-seq data for these mutants using our own pipeline for comparison to WT and the mutants (Fig. 3i). First, we examined the 21-nt hyper DSRs in these mutants and confirmed the accumulation of aberrant 21-nt sRNAs from coding genes reported in the original studies (Supplementary Fig. 3c, d). Next, we compared 21-nt hyper DSGs between fry1 and these mutants (Fig. 3j). The overlap between fry1 and all of the analyzed mutants was statistically significant, except for the overlap between fry1 and xrn3-8 (Supplementary Data 4). As XRN3 is a nuclear exonuclease while the other proteins are thought to act in cytoplasmic RNA decay, the results indicated that the aberrant genic sRNA accumulation in the fry1 mutants likely occurred in the cytoplasm. It was previously reported that in the ein5-1 ski2-3 double mutant, 21-nt sRNAs were generated from the 3' fragments of miRNA target transcripts resulting from miRNA-guided cleavage, due to insufficient exoribonucleolytic degradation of these fragments in the cytoplasm.12 We examined
several of these experimentally validated miRNA targets with aberrant 21-nt sRNA accumulation in ein5-1 ski2-3, but we found no accumulation of 21-nt sRNAs from these genes in fry1 (Supplementary Fig. 3e). In contrast, NIA1 and NIA2, which are not targeted by miRNAs, generated aberrant sRNAs in fry1 (Supplementary Fig. 3e). Moreover, only 17 of the 228 hyper DSGs in fry1 are predicted miRNA targets (Supplementary Data 5). We tried to identify common features of the genes that produced 21-nt sRNAs in fry1 and found that they tended to have fewer exons (Supplementary Fig. 3f) and longer gene length, transcript length, and UTR length (Supplementary Fig. 3g–j).
Aberrant sRNA accumulation from rRNAs. Besides the large number of 21-nt hyper DSRs from coding genes, over half of the 21-nt hyper DSRs were from rRNA regions. rRNA-derived sRNAs are usually considered degradation remnants of rRNAs and have typically been ignored in previous studies of sRNAs in plants. To confirm that the accumulated sRNAs arose from rRNAs and not from other overlapping features, we used the genome browser IGV to visualize the detailed changes in 21-nt sRNAs at rDNA loci in all genotypes. At an rDNA locus on chromosome 3, it was obvious that regions with abundant sRNAs expanded from mature rRNA regions found in WT to the ETS/ITS (external/internal transcribed spacer) regions in fry1 (Fig. 4a). Moreover, sRNAs were largely from the sense strand in WT, but antisense sRNAs were present in fry1 (Fig. 4a). To verify the sRNA-seq data, we designed two probes to detect the antisense sRNAs by RNA gel blot analysis, which confirmed the accumulation of these antisense sRNAs from the rDNA locus (Fig. 4b). This finding suggested that the sRNAs were unlikely to be rRNA degradation products. We also wondered whether rRNA-derived sRNAs similarly accumulated in the aforementioned RNA decay mutants. However, in ein5-1 ski2-3, dcp2-1, vcs, and xrn3-8, no over-accumulation of sRNAs from rDNA loci (Supplementary Figs. 3d and 4a), particularly from the ETS/ITS regions and the antisense strand (Supplementary Fig. 4b), was observed. Although the sRNA library construction for ein5-1 ski2-3 and xrn3-8 included an rRNA removal step, rRNA fragments were still detectable due to incomplete removal. In addition, antisense rRNA fragments were not expected to have been removed by the filtering steps. Thus, the lack of ETS/ITS-derived and antisense strand-derived sRNAs suggested that the mutations in these RNA decay genes did not lead to the production of rRNA-derived sRNAs.

Sequence features, such as length and 5′ nucleotide identity, are highly related to Dicer processing and AGO sorting. We therefore examined these two features of the sRNAs that mapped to rDNA loci to determine whether they are siRNAs. As expected, sRNAs of every length (18–30 nt) arising from the sense strand were nearly equally abundant, suggesting that they correspond to rRNA degradation products. However, only 21-nt sRNAs from both strands dramatically accumulated in fry1 (Supplementary Fig. 4c), consistent with the finding that the “rRNA” feature was enriched in 21-nt hyper DSRs but not in other size classes (Fig. 3b, c). These findings suggested that these sRNAs might be produced by DCL1 or DCL4, which generate 21-nt sRNAs in the mutants. U was the most common 5′ nucleotide among sense sRNAs, while C was the preferred 5′ nucleotide among antisense sRNAs (Supplementary Fig. 4d). The 5′ nucleotide preferences therefore suggested that the aberrantly accumulated sRNAs were siRNAs that loaded into AGO proteins.

Defects in 5′-3′ rRNA processing lead to sRNA accumulation. rRNA-derived sRNAs were observed when Arabidopsis plants were infected with viruses. The production of these siRNAs depends on RDR1, which is induced by viral infection. We sought to determine the source and biogenesis requirements of rRNA-derived sRNAs in fry1 mutants. XRN2, XRN3, and FRY1 are known to be involved in rRNA processing; in xrn2, xrn2 xrn3, and fry1 mutants, various forms of aberrant rRNAs accumulate. We therefore investigated the integrity of the rRNA processing pathway in the fry1 mutants and the relationship between aberrant rRNAs and the biogenesis of 21-nt sRNAs. For the analysis, we included the rRNA processing mutants xrn2-1 and atprmt3-2 as positive controls and xrn3-2, which exhibits normal rRNA processing, as the negative control. In all of the genotypes, the abundance of mature 25S and 18S rRNAs was similar (Supplementary Fig. 5a). Next, we used well-established probes to examine the ITS regions by RNA gel blot analysis. Consistent with previous reports, both xrn2-1 and atprmt3-2 showed changes in rRNA intermediates containing rRNA sequences, while xrn3-2 did not have obvious differences compared to WT (Supplementary Fig. 5b). Interestingly, both fry1-6 and fry1-8 had greater accumulation of 35S, 27S, and pre-5.8S rRNAs compared to xrn2-1 (Supplementary Fig. 5b). These differences may be the consequence of defects in both XRN2 and XRN3 function in fry1, as the accumulation of aberrant rRNAs in the xrn2 xrn3 double mutant was similar to that of fry1 mutants.

We also analyzed the accumulation of miRNAs and rRNA-derived sRNAs in the xrn2, xrn3, and xrn2 xrn3 mutants. rRNA-derived sRNAs only accumulated in fry1-6 and xrn2 xrn3 (Fig. 4c). In addition, the abundance of miR166 and miR398 was only reduced in fry1-6 and xrn2 xrn3 (Fig. 4d), suggesting a negative correlation between the levels of miRNAs and rRNA-derived sRNAs. Intriguingly, changes in the abundance of miRNAs and rRNA-derived sRNAs correlated with the phenotypic severity of the mutants (Supplementary Fig. 5c). However, miR168 showed similar changes in fry1-6 and xrn4 and was not affected by mutations in XRN2 or XRN3, suggesting that low XRN4 activity caused the increase of miR168 in fry1 (Fig. 4e).

A recent study demonstrated that defects in 3′-5′ rRNA processing induced the accumulation of antisense siRNAs from rRNA loci in C. elegans. We examined the accumulation of rRNA-derived sRNAs in the Arabidopsis exosome mutant mtr4, which exhibits defects in 3′-5′ rRNA processing and has marginal effects on PTGS. Using the same probes, however, we could...
not detect any sRNAs from the analyzed rDNA locus in the mtr4 mutant (Supplementary Fig. 5d). These findings indicated that the biogenesis of 21-nt sRNAs from aberrant rRNAs in Arabidopsis resulted specifically from defects in 5′-3′ exonuclease activity.

Rogue 21-nt sRNAs are products of the PTGS pathway. To further investigate whether the coding-gene-derived and rRNA-derived sRNAs were siRNAs, we examined whether their biogenesis required RDR and/or DCL proteins. We crossed fry1-6 with known siRNA biogenesis mutants, including rdr1-1, rdr2-1, rdr6-11, dcl2-1, dcl3-1, and dcl4-2. Intriguingly, the biogenesis of coding-gene-derived sRNAs and rRNA-derived sRNAs was different. The accumulation of rRNA-derived antisense sRNAs in fry1-6 was completely suppressed by rdr6-11, weakly affected by rdr2-1 and unaffected by rdr1-1 (Fig. 5a). Meanwhile, although 21-nt rRNA-derived sRNAs were eliminated in fry1-6 dcl4-2, sRNAs predominantly 22 nt accumulated from the same loci (Fig. 5a). This was due to DCL2, as in the fry1-6 dcl2-1 dcl4-2 triple mutant, 21–22-nt sRNAs were almost gone (Fig. 5a). However, for coding-gene-derived sRNAs, RDR1 was crucial for their biogenesis as rdr1-1 suppressed the enhanced sRNA accumulation in fry1-6 (Fig. 5b). Moreover, these sRNAs accumulated even more in the fry1-6 rdr6-11 mutant than in fry1-6, which might be the consequence of the slightly upregulated RDR1 expression in the absence of RDR6.
function (Supplementary Fig. 6a). Similar to rRNA-derived sRNAs, 
dcl2-1 and dcl4-2 together completely suppressed the over-accumulation of coding-gene-derived sRNAs (Fig. 5b).

As the biogenesis of these 21-nt rogue sRNAs required RDR1/6 and DCL2/4, we concluded that these sRNAs were siRNAs and, more specifically, ribosomal siRNAs (risiRNAs) for the rRNA-derived ones. The biogenesis requirements of these rogue siRNAs are similar to those of another class of endogenous siRNAs, namely phasiRNAs, which exhibit a head-to-tail phasing signature\(^3\). Hence, we next investigated the phasing of rogue

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**Figure 6a**

- **Panel a**: Images showing gel electrophoresis results for different genotypes of TAS1A and TAS3 genes. The genotypes include WT, fry1-6, fry1-8, dcl4-2, dcl2-1, dcl3-1, and rdr2-1. The phasing scores are indicated above each lane.

**Figure 6b**

- **Panel b**: Images showing gel electrophoresis results for AT1G74100 and AT3G59940 genes. The phasing scores are indicated above each lane.

**Figure 6c**

- **Panel c**: Graphs showing phasing scores for TAS1A and TAS3 genes across different positions. The x-axis represents the position on the genome, and the y-axis represents the phasing score. Line colors indicate WT, fry1-6, and fry1-8 genotypes.

**Figure 6d**

- **Panel d**: Graph showing phasing scores for rRNA across different positions (5’ETS, 18S, 5.8S, ITS1, ITS2, 25S, and 3’ETS). The y-axis represents the phasing score.
siRNAs using established methods\textsuperscript{32}. Surprisingly, the phasing scores at TAS and genes known to generate phased siRNAs were slightly reduced in the two fry1 mutants, while those at the rDNA locus and many 21-nt hyper DSGs were drastically increased (Fig. 5b–d, Supplementary Fig. 6b). These findings reinforced the conclusion that rogue siRNAs, including risiRNAs, were produced from double-stranded RNAs by processive DCL activity.

21-nt risiRNAs are loaded into AGO1. To address whether the 21-nt risiRNAs had any biological impacts in vivo, we first examined whether these siRNAs are loaded into AGO1 by immunoprecipitation (IP) of AGO1 in WT and fry1-6 (Supplementary Fig. 7a), followed by sequencing of the siRNAs from the IP products (Supplementary Data 6). Three independent experiments were performed. As expected, 21-nt siRNAs were enriched and 24-nt siRNAs were depleted in the AGO1 IP sRNA-seq (Fig. 6a). In addition, 5′ U was a predominant feature of sRNAs in both IP products (Supplementary Fig. 7b). This indicated that AGO1 IP sRNA-seq was successful.

As a preliminary analysis, we used the genome browser to examine AGO1 binding of siRNAs. At the rDNA locus on chromosome 3, 21-nt siRNAs were depleted in AGO1 IP from WT but enriched on both strands, including the ETS/ITS regions, in AGO1 IP from fry1-6 (Supplementary Fig. 7c). We then conducted IP enrichment analysis in WT and fry1-6: IP-enriched bins were defined as 100-bp bins with a statistically significant increase in sRNA abundance in IP versus input. In WT, most enriched bins were from miRNA and other genes, including TAS genes, and there were only 7 enriched bins from rRNA regions (Fig. 6b). Compared to WT, the numbers of enriched bins from miRNA and TAS genes were similar, but the number of enriched bins from rRNA regions increased in fry1-6 (Fig. 6b). We also examined the 5′ nucleotides of these risiRNAs (Fig. 6c). In WT, 5′-U siRNAs constituted about 40% of sense and over 80% of antisense 21-nt siRNAs associated with AGO1. In fry1-6, the 5′-U percentages were over 90 and 80% for sense and antisense 21-nt siRNAs, respectively. These findings suggested that in WT, many of these sense siRNAs might not be loaded into AGO1 despite their association with it, whereas rogue rRNA-derived siRNAs from both strands in fry1-6 could be loaded.

Notably, there were also more 21-nt siRNAs and more enriched 21-nt bins from coding genes in fry1-6 (Fig. 6b). Therefore, we conducted a similar IP enrichment analysis for genes and identified 193 and 1224 genes as those enriched for AGO1-associated 21-nt siRNAs in WT and fry1-6, respectively (Fig. 6d). As expected, the 1224 genes in fry1-6 included most of the 193 genes identified in WT. The 1224 genes also included most of the 228 hyper DSGs identified in fry1. Interestingly, the low overlap between the 228 hyper DSGs (i.e., genes with rogue 21-nt siRNAs in fry1-6 and fry1-8) and the 193 genes with AGO1-bound 21-nt siRNAs in WT suggested that the genes suppressed by FRY1 for sRNA production were distinct from those that generated siRNAs in WT. We also analyzed the sequence features of the 1224 IP-enriched genes in fry1-6, and the results supported the hypothesis that longer genes with fewer exons tended to generate 21-nt siRNAs in fry1 (Supplementary Fig. 7d–h).

The loading of miRNAs into AGO1 is compromised in fry1-6. Because most miRNAs associate with AGO1 under normal conditions, the miRNA-binding capacity of AGO1 might be compromised by the excessive accumulation of risiRNAs and siRNAs from other coding genes in fry1. To support the hypothesis that rogue siRNAs compete with miRNAs for loading into AGO1, we conducted small RNA-seq following AGO1 IP in fry1-6 rdr6-11, in which risiRNAs were barely detectable (Fig. 5a) (Supplementary Table 1 and Supplementary Data 6). As expected, around 50% of the 21-nt siRNAs associated with AGO1 were derived from rRNA in fry1-6, while the corresponding proportion in WT was less than 5%, according to the genomic classification of AGO1-associated 21-nt siRNAs (Fig. 7a). Consistent with results from the RNA gel blot assay (Fig. 5a), the proportion of risiRNAs in AGO1 was substantially reduced in fry1-6 rdr6-11 (Fig. 7a and Supplementary Fig. 8a). In addition, the fraction of AGO1-associated miRNAs decreased to less than 30% in fry1-6, and was restored to over 50% by the rdr6-11 mutation (Fig. 7a).

Although the numbers of enriched miRNAs in AGO1 IP were similar in WT, fry1-6 (Fig. 6b), and fry1-6 rdr6-11 (Supplementary Fig. 8b), the loading efficiency of miRNAs, as represented by the ratio of miRNA levels in IP and input, was slightly decreased in fry1-6 and restored in fry1-6 rdr6-11 (Supplementary Fig. 8c, d and Supplementary Data 7). Specifically, for the 20 most abundant miRNAs in WT, the IP/input ratios were significantly reduced in fry1-6 (Fig. 7b and Supplementary Fig. 8e). pair Wilcoxon test $P = 0.001718$ and recovered in fry1-6 rdr6-11 (paired Wilcoxon test $P = 0.003654$ between fry1-6 rdr6-11 and fry1-6). These results indicated that in fry1-6 AGO1’s binding to miRNAs declined, and AGO1 associated with risiRNAs. The reduced miRNA abundance in fry1 mutants was likely due to the compromised loading of miRNAs into AGO1.

To further support the above hypothesis, we examined the abundance of miRNAs in mutants whose risiRNAs were suppressed or partially suppressed, including fry1-6 rdr2-1, fry1-6 rdr6-11, and fry1-6 dcl4-2. In all these mutants, the downregulation of mir166 and mir398 was partially suppressed (Fig. 7c). However, the upregulation of mir168 was not affected in any of these double mutants compared to fry1-6 (Fig. 7d). Furthermore, the rdr6-11 mutation partially restored the fry1 mutant phenotype, especially the leaf shape phenotype (Fig. 7e). However, dcl4-2 did not rescue the fry1 phenotype, and the fry1-6 dcl4-2 double mutants were extremely small and died at about 22 days after germination (DAG) (Fig. 7e). This was similar to the phenotype of the ein5-1 ski2-3 dcl4 triple mutant, in which enhanced biogenesis of 22-nt siRNAs from endogenous genes by DCL2 led to further production of secondary siRNAs\textsuperscript{12}. However, while dcl2 dcl4 can fully rescue the ein5-1 ski2-3 mutant phenotype\textsuperscript{12}, the phenotype of the fry1-6 dcl2-1 dcl4-2 triple mutant was similar to that of fry1-6 but not WT (Fig. 7e). This also suggested that the developmental phenotypes of fry1 were not fully attributable to rogue siRNAs.
Because rogue siRNAs were loaded into AGO1, we sequenced the transcriptome of WT, fry1-6, fry1-6 rdr6-11, and fry1-6 rdr1-1 to understand if the siRNAs affected gene expression. Despite the large numbers of upregulated and downregulated genes (Supplementary Fig. 8f, g), we focused on known miRNA targets and the 200 genes generating siRNAs in fry1-6 (Fig. 3f). Among 10 representatives of known miRNA targets, 7 of them showed upregulated expression in fry1-6, and this upregulation was reduced in at least one of the fry1-6 rdr6-11 and fry1-6 rdr1-1 mutants (Supplementary Fig. 8h). This suggested that mutations in either RDR6 or RDR1 can partially restore miRNA activity in the fry1 mutant. 186 genes accumulating siRNAs in fry1-6 were detectably expressed by the RNA-seq and they showed different expression patterns in fry1-6 rdr1-1 and fry1-6 rdr6-11. In fry1-6 rdr1-1, where siRNA biogenesis from these genes was compromised, the expression of these genes was upregulated significantly as compared to WT. Congruously, their expression decreased in fry1-6 rdr6-11, in which siRNA biogenesis from these genes was enhanced (Supplementary Fig. 8i). This indicated that either siRNA biogenesis would eliminate transcripts from these genes or accumulated siRNAs would target the corresponding transcripts for cleavage.

The loading of miRNAs into AGO2 is compromised in fry1-6. We noticed that miR390, which is predominantly bound by AGO7 and AGO2, also decreased in abundance in fry1-6 (Fig. 1c and Fig. 2c). This may also be attributed to compromised loading of miR390 into AGO2/7 due to competition from rogue siRNAs. Thus, we tested the loading of AGO2-associated miRNAs by AGO2 IP followed by sRNA sequencing in WT, fry1-6, and fry1-6 rdr6-11 (Supplementary Data 6). The composition of 21-nt reads, the most abundant length in AGO2 IP samples (Supplementary Fig. 9a), was very different among the three genotypes. In WT, AGO2 mainly associated with miRNAs and trans-acting siRNAs (ta-siRNAs), while in fry1-6 siRNAs and coding-gene-derived siRNAs constituted the majority of AGO2-associated siRNAs (Supplementary Fig. 9b). Meanwhile, as rdr6-11 removed ta-siRNAs and a portion of risiRNAs, the proportion of AGO2-associated miRNAs increased in fry1-6 rdr6-11 compared to the fry1-6 single mutant (Supplementary Fig. 9b).

Next, we conducted analyses to identify genomic regions showing statistically higher levels of siRNAs in AGO2 IP relative to input. In fry1-6, the number of enriched bins corresponding to coding genes and Pol IV-dependent siRNA regions drastically decreased while that of rRNA bins increased as compared to WT (Supplementary Fig. 9c). Consistent with the findings from AGO1 IP (Fig. 6b and Supplementary Fig. 8b), the fry1-6 rdr6-11 mutant also showed a partial restoration of AGO2’s siRNA binding profile in terms of the numbers of enriched bins (Supplementary Fig. 9c). We specifically examined known AGO2-bound miRNAs, including miR159, miR390, and miR408 (Supplementary Data 8). The levels of the miRNAs in AGO2 IP decreased in fry1-6 but were slightly restored in fry1-6.
rdr6-11 (Supplementary Fig. 9d). We then examined the coding gene-derived siRNAs and risiRNAs. AGO2 bound siRNAs from 154 of the 228 genes with rogue siRNAs in fry1 (Supplementary Fig. 9e). Also, 5’ A, which is preferred by AGO221, was enriched in risiRNAs from both strands (Supplementary Fig. 9f). We noticed that, although rdr6-11 suppressed the loading of risiRNAs into AGO2, there were still 80 bins of risiRNAs in fry1-6 rdr6-11 (Supplementary Fig. 9c, g), and most of them were from the 5’ ETS (Supplementary Fig. 9g). These results suggested that the loading of sRNAs into AGO2 was affected in fry1-6.

Discussion
Because sRNAs from rDNA regions are usually considered degradation products of mature rRNAs, many previous sRNA-seq analyses have often excluded these sequences by removing them during library construction or after read mapping during data analysis. Still, some studies have reported the existence of rRNA-derived siRNAs and implicated their functions. In S. pombe, because rr-siRNAs preferentially begin with 5’ U and associate with Ago1, they may sequester Ago1 and interfere with its function17. Recent studies in C. elegans have described similar phenomena: under conditions such as 3’-5’ exonuclease impairment, cold stress, and deficient rRNA processing, 22-nt siRNAs with a 5’-G preference accumulated from both strands of rRNA. These risiRNAs can be loaded into NRDE-3, a nuclear Ago protein, and potentially target rRNA precursors19,20. In Arabidopsis, 24-nt siRNAs from rRNA involved in the RNA-directed DNA methylation pathway was first identified21,22. These siRNAs are not derived from rRNA precursors or mature rRNAs as they...

Fig. 7 rdr6-11 partially rescues the fry1 phenotypes. a Genomic classification of 21-nt AGO1-associated sRNAs. In WT, miRNAs and ta-siRNAs constitute the majority of AGO1-associated 21-nt sRNAs. In fry1-6, there is a drastic increase in rRNA-derived sRNAs, consistent with the total sRNA composition. The rdr6 mutation results in a partial removal of risiRNAs and a concomitant partial restoration of miRNAs. The Y axis shows the cumulative RPM values for sRNAs corresponding to different genomic features. b The AGO1 loading efficiency of the 20 most abundant miRNAs in WT. The loading efficiency is represented by the ratio of RPM in immunoprecipitated samples to that in input. The efficiencies in WT and fry1-6 are significantly different based on a paired Wilcoxon test (P value = 0.001718). The efficiencies are recovered by the rdr6 mutation (fry1-6 rdr6-11 vs. fry1-6; P value = 0.003654; fry1-6 rdr6-11 vs. WT; P value = 0.114). c, d miRNA accumulation in rdr and dcl mutants. The internal control U6 snRNA was used to determine the relative miRNA levels. e Partial rescue of the fry1 phenotypes by rdr and dcl mutations. Plants shown are at 22 days after germination. rdr6-11 can partially rescue the mutant phenotypes of fry1-6. Meanwhile, DCL4 is necessary for the survival of fry1 mutants, probably due to the enhanced activity of DCL2 in dcl4-2. This was supported by the fry1-6 dcl2-1 dcl4-2 triple mutant. The restoration of leaf shape by rdr6-11 is shown by the enlarged leaves in the insets. Source data are provided as a Source Data file.
AGO (AGO1 and AGO2) proteins are associated with miRNAs and function in miRNA-directed target regulation. In degrades PAP to ensure the activation of XRN2/3 function. XRNs degrade aberrant RNAs to prevent the biogenesis of rogue siRNAs. As a result, most altered partitioning of AGO between miRNAs and siRNAs leads to reduced abundance of miRNAs.

Based on the present findings, we propose a competition model as shown in Fig. 8. In WT plants, FRY1 degrades PAP to ensure the activities of XRN4 in the cytoplasm and XRN2/3 in the nucleus. XRN4 and XRN2/3 efficiently degrade aberrant RNAs in both the cytoplasm and nucleus, respectively, which prevents the biogenesis of siRNAs. Thus, most AGO1 proteins are occupied by miRNAs. In the fry1 mutants, PAP accumulates and inhibits the activity of XRNs. Aberrant miRNAs and rRNAs accumulate and are captured by the siRNA pathway, which consequently generates rogue siRNAs that compete with miRNAs to occupy AGO1 and AGO2 proteins. Perhaps as an attempt to reach miRNA homeostasis, AGO1 levels were increased in fry1 (Supplementary Fig. 2e) However, excessive risiRNAs and siRNAs from coding genes still outcompeted miRNAs, resulting in their low loading efficiency and reduced abundance. Thus, the proper partitioning of AGO1 for miRNA and siRNA binding requires RQC.

Consistent with previous findings in cytoplasmic RNA decay mutants, the fry1-6 dcl4-2 double mutant was not viable but viability could be restored by the dcl2-1 mutation (Fig. 7e). Nevertheless, the fry1-6 dcl4-2 dcl2-1 triple mutant, like fry1-6 rdr2-1 and fry1-6 rdr6-11, still exhibited abnormal phenotypes compared to WT plants (Fig. 7e), indicating only a partial rescue of fry1 by mutations in the siRNA pathway. Indeed, the abundance of miR166 and miR398 was only partially restored in fry1-6 rdr6-11 that lacked rogue siRNAs (Fig. 7c). FRY1 and XRN2/3 facilitate the turnover of excised pre-miRNA loops in Arabidopsis. This defect in miRNA processing may also affect the abundance of mature miRNAs. The partial rescue of fry1 by rdr6-11 or dcl2-1 dcl4-2 differs from the full phenotypic rescue of ein5-1 sk2-3 by the same mutations, but resembles the minimal rescue of dcp2-1 and vcs-6 by rdr6-11. We also noticed that there were still abundant risiRNAs from the 5′ ETS in all dcl6 samples (Supplementary Fig. S8a and S9g); the biogenesis of these risiRNAs was RDR6-independent. These 21-nt risiRNAs may have originated from the bidirectional transcription of rDNA, as previously reported and may account for the partial rescue of fry1 phenotypes by rdr6. Despite this, the fry1 mutant phenotypes may not be entirely attributable to the accumulation of risiRNAs and 21-nt siRNAs from coding genes. Other processes affected in fry1, such as RNA processing, RNA decay, and sulfur metabolism, may also contribute to the phenotypes.

In summary, we show that RNA silencing activity is impacted by competition between miRNAs and siRNAs for AGO1 in Arabidopsis, and RNA quality control maintains small RNA homeostasis to ensure proper miRNA activities.

Methods

Plant materials and growth. Arabidopsis thaliana wild-type (accession Columbia-0) and T-DNA insertion lines of FRY1 (ATSG63980, fry1-6: SALK_00882, fry1-8: SALK_151367), XRN2 (ATSG42540, xrn2-1: SALK_041148), XRN3 (AT1G75660, xrn3-2: SAIL_782H09), XRN4 (AT1G4490, xrn4-9: SAIL_681E01), PRMT3 (AT3G12270, atpremt3-2: WISCSLOX91A01), RDR1 (AT1G14790, rdr1-1: SAIL_627F11), RDR2 (AT4G11130, rdr2-1: SAIL_1277H08), RDR6 (AT3G49500, rdr6-11: CS2485), DCL2 (AT3G03300, dcl2-1: SALK_064627), DCL3 (AT3G49290, dcl3-1: SALK_005512), and DCL4 (AT3G20320, dcl4-2: GABI_160G05) were used in this study. Seeds were germinated on 1/2 MS medium under short-day conditions (8 h light and 16 h dark) at 22°C, and seedlings were
either collected for analyses or transferred to soil for phenotypic observation on day 12.

EMS mutagenesis and mutant identification. amir-CTR1 (CGGGUUGG-GAAUAUUAGUAU) was designed using WMD tools (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) then inserted into the MIR191a backbone58. The amir-CTR1 fragment was recombined into the plasmid pER10 containing a β-estradiol-induction cassette using the XhoI and SpeI restriction sites (Supplementary Data 9). The construct was transformed into WT Arabidopsis by Agrobacterium transformation. To locate the T-DNA insertion site, we re-sequenced the amir-CTR1 transgenic plants on the HiSeq 2000 platform at the genomics core facility at UCR. By mapping the resulting reads to the Arabaport11 Arabidopsis thaliana genome (https://www.araport.org), we determined that the insertion was between nucleotides 18214427 and 18214448 on chromosome 5. We also confirmed that there was only a single T-DNA insertion in the genome, as no chimeric reads from two T-DNA borders (LB/RR) were found. About 2 mL freshly collected amir-CTR1 seeds were used for ethyl methanesulfonate (EMS) mutagenesis. Seeds were incubated overnight, rinsed with ddH2O several times, then washed in ddH2O for 4h. Finally, the seeds were sown in soil.

To find the causal mutation in T3520, the CTAB method was used to extract DNA from ~50 plants with the T3520 phenotype from the F2 population of the T5520 x Col-0 crosses. A DNA library was prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina (E7645S, NEB) according to the manual. The library was sequenced on the HiSeq 2000 platform at the genomics core facility at UCR, and the PE150 reads were mapped to the Arabidopsis genome. SNPs were called using SAMtools v1.397 then analyzed to identify the causal mutation using an online software NGM (http://bar.utoronto.ca/ngm/index.html). From the NGM result, we narrowed the location of the mutation to a region consisting of 10 candidate genes (AT5G49770, AT5G52170, AT5G54330, AT5G55330, AT5G57060, AT5G567270, AT5G63450, AT5G63980, AT5G64390, and AT5G64430) that contained SNPs with discordant chisq statistics over 0.95. We designed dCAPS primers for all of the candidate genes and analyzed another batch of F2 plants with the mutant phenotype50. This analysis pinpointed the mutation to AT5G64390 (FRY1).

RNA extraction and northern blotting. Total RNA was extracted from 12-day-old WT and mutant seeds using TRI reagent (MRC, TR118) according to the manufacturer’s instructions. For each sample, 10 μg total RNA was run on a 15% urea-PAGE gel and transferred to a HybridX nylon membrane. The RNA was cross-linked with the EDC cross linking buffer (0.16 M EDC, 0.13 M Methyleneimidazole at pH 8.0) at 65 °C for 90 min. Biotin-labeled probes were added to the hybridization buffer (5X SSC, 20 mM Na2HPO4 at pH 7.2, 7% SDS, 2X Denhardt’s solution) and incubated with the membrane at 55 °C overnight. After two wash steps (2X SSC, 0.1%SDS, 55 °C, 20 min each time) to remove excess probe, the membrane was processed using the Chemiluminescent Nucleic Acid Detection Module Kit (ThermoFisher, 98980) according to the instruction manual with probes described in Supplementary Data 9. The relative expression level in the RNA gel blots was calculated against the internal control U6 using Fiji41.

Protein detection. Aerial tissues (25 mg) of 12~14 day-old seedlings were harvested for protein extraction using 1x SDS buffer (100 mM Tris at pH8.6, 4% SDS, 20% Glycerol, 0.2% Bromophenol blue). The samples were loaded onto a 10% SDS-PAGE gel and proteins were then transferred to a nitrocellulose membrane after electrophoresis. Anti-AGO1 antibody (1:3,000, Agrisera, AS13 2650) and served as the internal control.

Data availability

The raw sequence data generated during this study were deposited into the NCBI GEO database under the accession code GSE133461. The source data for Figs. 1b, 1c, 2a, d, 3a–c, e, g, h, 4b–e, 5a, b, 6a, 7a–e, and Supplementary Figs. 1b–d, 2b–e, 3a–d, f–j, 4a, c, 5a–d, 6a, 7a, b, d–h, 8d, e, h, i, and 9a, b, d, f are provided as a Source Data File. The authors declare that any other supporting data is available from the corresponding author(s) upon request.

Code availability

All bioinformatic analyses in this study were performed by an integrated pipeline for next-generation sequencing analysis, pRNASeqTools v0.6 [https://github.com/grubbybio/pRNASeqTools/]. This pipeline can be used freely under the MIT license.

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Analysis of sRNA-seq data. Our sRNA-seq data and published datasets (GSE57936, GSE65056, GSE95473) were analyzed using a publicly available pipeline, pRNASeqTools v0.6. The sRNA-seq raw reads were trimmed to remove the 3' adapter sequences (AGATCGGAAGAGC) then size-selected (18–42 nt) using cutadapt v1.9.1. The trimmed reads were mapped to the Arabaport11 genome using ShortStack v3.4 and parameters ‘bowtie2_m 1000-maxmatch 50-mismatch 0’. To calculate and compare sRNA abundance in the WT and mutant libraries, the Arabidopsis genome was tiled into 100-bp bins (or bins based on specific features, e.g., miRNAs, TE's, genes, and 1000-bp gene-upstream sequences), and read counts for each single-stranded RNA modulate post-transcriptional gene silencing in plants.

AGO IP and sRNA-seq. Total protein was extracted from WT, fyrl-6, and fyrl-6 rdr6-11 seedlings using IP buffer (50 mM Tris 7.5, 150 mM NaCl, 0.1% NP-40, and 1x proteinase inhibitor cocktail). AGO1 antibody (8 μL/g) or AGO2 antibody (16 μL/g) and protein A Dynabeads (Invitrogen) were used to pull-down the superantagonist to obtain the AGO protein complex. For WT, fyrl-6, and fyrl-6 rdr6-11, the immunoprecipitation, RNA extraction, library construction, sequencing, and data analysis were performed in three independent experiments as described above for total sRNAs.

mRNA-seq and data analysis. RNA-seq libraries were constructed using NEBNext® Ultra® RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer’s instructions. The libraries were pooled and sequenced on the Illumina Novaseq 6000 system (paired-end, 150 bp) at Berry Genomics (Beijing, China). The RNA-seq data were analyzed using the pRNASeqTools. Briefly, raw reads were mapped to the Arabaport11 genome using STAR v2.7 and the parameters ‘--alignIntronmax 5000 --outSAMmultNmax 1 --outFilterMultimapNmax 50 --outFilterMismatchNoverLmax 0.17’. Mapped reads were counted by featureCounts v1.6.4.28 and comparison was performed using the R package DESeq2.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Code availability

All bioinformatic analyses in this study were performed by an integrated pipeline for next-generation sequencing analysis, pRNASeqTools v0.6 [https://github.com/grubbybio/pRNASeqTools/]. This pipeline can be used freely under the MIT license.

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Author contributions
W.H., C.Y., J.C., and X.Chen designed the research. H.G. provided the CTR1 antibody. W.H. performed the screening, and W.H. and C.Y. identified the mutant. W.H. and J.C. prepared the sequencing libraries, and C.Y. conducted the data analyses. J.C., C.Z., R.H., and C.W. performed genetic and biochemical experiments. C.Y., X.Chen, B.M., and X. Cao wrote the paper.

Competing interests
The authors declare no competing interests.

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