Small RNAs (sRNAs) associate with Argonaute (AGO) proteins in effector complexes, termed RNA-induced silencing complexes (RISCs), which regulate complementary transcripts by translation inhibition and/or RNA degradation. In the unicellular alga *Chlamydomonas*, several metazoans, and land plants, emerging evidence indicates that polyribosome-associated transcripts can be translationally repressed by RISCs without substantial messenger RNA (mRNA) destabilization. However, the mechanism of translation inhibition in a polyribosomal context is not understood. Here we show that *Chlamydomonas* VIG1, an ortholog of the *Drosophila melanogaster* Vasa intronic gene (VIG), is required for this process. VIG1 localizes predominantly in the cytosol and comigrates with monoribosomes and polyribosomes by sucrose density gradient sedimentation. A VIG1-deleted mutant shows hypersensitivity to the translation elongation inhibitor cycloheximide, suggesting that VIG1 may have a nonessential role in ribosome function/structure. Additionally, FLAG-tagged VIG1 copurifies with AGO3 and Dicer-like 3 (DCL3), consistent with it also being a component of the RISC. Indeed, VIG1 is necessary for the repression of sRNA-targeted transcripts at the translational level but is dispensable for cleavage-mediated RNA interference and for the association of the AGO3 effector with polyribosomes or target transcripts. Our results suggest that VIG1 is an ancillary ribosomal component and plays a role in sRNA-mediated translation repression of polyribosomal transcripts.

*Significance*

Small RNAs (sRNAs) are a class of noncoding RNAs that regulate complementary mRNAs, by triggering translation repression and/or transcript decay, and influence multiple biological processes. In animals, land plants, and some protists like the alga *Chlamydomonas*, sRNAs can repress translation of polyribosome-associated mRNAs, without or with only minimal transcript destabilization. However, the precise silencing mechanism is poorly understood. We found that *Chlamydomonas* VIG1, a homolog of the *Drosophila melanogaster* Vasa intronic gene and a member of a widely conserved protein family in eukaryotes, is involved in this process. VIG1 appears to be an ancillary ribosomal constituent. Additionally, VIG1 copurifies with core components of sRNA effector complexes and plays a key role in the sRNA-mediated translation repression of polyribosomal transcripts.
of miRNAs, suggest that this slicer-independent degradation of target transcripts may account for most of the miRNA-mediated stable repression in many postembryonic cells (1, 4, 5).

In contrast, in embryonic stem cells and during early embryogenesis of metazoans, miRISC binding to target messenger RNAs (miRNAs) mainly causes translation inhibition, with little effect on transcript stability (1, 7, 8). Similar observations have been reported in neurons, where reversible miRISC-mediated silencing regulates synaptic plasticity (9). Intriguingly, GW-repeat proteins are absent from the miRISC in the Caenorhabditis elegans germ line (7) and from the polyribosome-associated miRISC that strongly inhibits translation upon serum starvation in Drosophila melanogaster S2 cells (10). Moreover, in somatic mammalian cell lines, certain miRNAs binding to the protein-coding sequence (CDS) of transcripts repress translation, through a GW182/TNRC6-independent mechanism, without triggering mRNA destabilization (11). Thus, in metazoans, miRNAs appear to regulate target transcripts via several mechanisms that can be modulated by cellular context, miRISC composition, and subcellular localization, as well as target mRNA-specific effects (4, 12, 13).

In land plants, even the slicer mode of mRNA action appears to target (many) translating miRNAs. By isolating poly(A) miRNAs having a 5'-monophosphate (as a result of RNA cleavage/ degradation) and identifying the nucleotide positions of the 5' ends, a 3'-nt periodicity in the RNA degradation products within the CDS was observed for a number of miRNA targets, suggesting a 3-nt periodicity in the RNA degradation products within the Chlamydomonas reinhardtii S2 cells (10). Moreover, in somatic mammalian cell lines, certain miRNAs binding to the protein-coding sequence (CDS) of transcripts repress translation, through a GW182/TNRC6-independent mechanism, without triggering mRNA destabilization (11). Thus, in metazoans, miRNAs appear to regulate target transcripts via several mechanisms that can be modulated by cellular context, miRISC composition, and subcellular localization, as well as target mRNA-specific effects (4, 12, 13).

In plants and green algae lack orthologs of the metazoan GW182/TNRC6 proteins and, in A. thaliana, the known factors required for miRNA-mediated translation inhibition include the microtubule-severing enzyme KATANIN1, decapping enhancer VARICOSE, GW-containing protein SUO, endoplasmic reticulum (ER) membrane protein ALTERED MERISTEM PROGRAM1, and dsRNA-binding protein DRB2 (2, 4, 15, 16). Subcellular localization and fractionation studies indicated that miRNA-mediated translation repression mainly occurs on membrane-bound polyribosomes, likely associated with the ER (2, 4, 15). Studies with lysates from tobacco BY-2 cells suggested that the plant (mi)RISC, bound to the 5' UTR or the CDS of a transcript, can sterically block the recruitment or the movement of ribosomes (17). However, a preferential accumulation of ribosome footprints was not observed in the region upstream of CDS miRNA target sites in A. thaliana Ribo-seq (sequencing) or RNA degrade data (14, 18).

Translation inhibition mediated by sRNAs also occurs in C. reinhardtii (6, 19, 20) and the translationally repressed transcripts remain associated with polyribosomes (6). Global ribosome profiling in parallel with RNA-seq and quantitative proteomics on a Dicer mutant and its Dicer-complemented strain revealed that Chlamydomonas miRNAs regulate endogenous targets mainly by pairing to the CDS of transcripts, leading to RNA degradation and/or translation repression (20). However, ribosome footprints were not piled up upstream of the miRNA-binding sites (20). Thus, sRNA-mediated translation repression of polyribosome-associated transcripts is a widespread phenomenon in eukaryotes but the actual molecular mechanism(s) remains largely uncharacterized. Here, we show that a Chlamydomonas ortholog of the D. melanogaster Vasa intronic gene (VIG) and mammalian SERPINE1 mRNA-binding protein 1 (SERBP1) is required for translation repression mediated by sRNAs/miRNAs. Chlamydomonas VIG1 is a component of the (mi)RISC, associates with translating ribosomes in an mRNA-independent manner, and may possibly modulate multiple steps of protein synthesis.

Results

VIG1 Is Required for Translation Repression Mediated by siRNAs/ miRNAs. In C. reinhardtii, the tryptophan synthase β-subunit (TSβ; encoded by the MAA7 gene) is required to convert the indole analog 5-fluorouridine (5-FI) into the toxic tryptophan analog 5-fluorotryptophan. Suppression of MAA7 by RNA interference (RNAi), triggered by dsRNAs produced from inverted-repeat (IR) transgenes, results in strains resistant to 5-FI (6, 21). In

Fig. 1. Chlamydomonas VIG1 is required for the siRNA-mediated translation repression of the MAA7 transcript. (A) Growth and survival of the indicated strains on TAP medium with or without 7 μM 5-fluorouridine. CC-124, wild-type strain; Maa7-IR44, CC-124 transformed with an IR transgene designed to induce RNAi of MAA7; vig1, VIG1 deletion mutant; vig1(tagVIG1)-3 and -6, transgenic strains of vig1 transformed with FLAG-CBP-VIG1 under the control of the PsaD promoter. (B) Immunoblot analysis of tryptophan synthase β-subunit levels. Immunodetection of histone H3 was used as a control for equivalent loading of the lanes. (C) Northern blot analysis of MAA7 transcript levels. The same filter was reprobed with the coding sequence of ACT1 (encoding actin) as a control for similar loading of the lanes. (D) Northern blot analysis of MAA7 siRNAs in the indicated strains. The same filter was reprobed with the U6 small nuclear RNA sequence to assess the amount of sample loaded per lane. (E) Northern blot analysis of sRNAs isolated from the indicated strains and detected with probes specific for Chlamydomonas miRNAs. (F) Immunoblot analysis of AGO3/2 proteins in the indicated strains. The asterisk indicates a nonspecific cross-reacting antigen.
several transgenic strains, like Maa7-IR44, MAA7 is silenced by siRNA-mediated translation repression of polyribosome-associated transcripts, without mRNA destabilization (6). In order to gain some insights into this process, we generated a library of RNAi-defective insertional mutants in the Maa7-IR44 background. One such mutant, designated vig1, contained a deletion of the Cre09.g393358 (VIG1) gene, encoding the only Chlamydomonas member of a conserved eukaryotic protein family that includes VIG and SERBP1 (SI Appendix, Figs. S1A and S2). The entire VIG1 gene is deleted in Chlamydomonas vig1 (SI Appendix, Fig. S3A) and no VIG1 transcript is detected, by Northern blotting, in the mutant background (SI Appendix, Fig. S3B).

The vig1 mutant showed sensitivity to 5-FI, indicating a defect in the RNAi-mediated suppression of MAA7 (Fig. 1A). Moreover, it contained TSβ-protein amounts quite similar to those in the wild-type CC-124 strain (Fig. 1B), without appreciable changes in the MAA7 transcript abundance (Fig. 1C). MAA7 siRNA levels were slightly reduced in the mutant background (Fig. 1D), as were the amounts of a few miRNAs (Fig. 1E) and of AGO3/2 (Fig. 1F), the main core components of the (mi)RISC in Chlamydomonas (19). However, the barely noticeable reduction in components of the RNAi machinery seemed insufficient to explain the defect in siRNA-mediated translation repression of MAA7. Transformation of the vig1 mutant with a transgene stably expressing FLAG-CBP-tagged VIG1 (Materials and Methods) reverted all described phenotypes (Fig. 1).

We also examined how the deletion of VIG1 affected the expression of Chlamydomonas genes regulated by endogenous miRNAs/siRNAs. We already demonstrated that Cre16.g683650 (encoding a predicted protein kinase) is translationally repressed by the miR_C sRNA (22). The miR_C binding site in the Cre16.g683650 mRNA overlaps the stop codon and contains a mismatch with...
nucleotide 10 of the sRNA, which would hinder AGO-mediated cleavage (Fig. 2A). As with MAAT, Cre16.g638650 was translationally derepressed in the vig1 mutant without any obvious alteration in its transcript level (Fig. 2B). In contrast, the regulation of 2 miRNAs, corresponding to Cre17.g697550 and Cre12.g532950, targeted for cleavage by Chlamydomonas miRNAs and/or endogenous siRNAs (22, 23) was not defective in the mutant background. Their transcript abundance did not change in vig1 relative to the wild-type or the complemented strains (SI Appendix, Fig. S4A). In addition, the steady-state levels of several miRNAs, including miR_C, and of the transcripts for the 3 AGOs encoded in the Chlamydomonas genome were not substantially altered in the vig1 mutant (SI Appendix, Fig. S4 B and C).

These results, taken together, suggest that Chlamydomonas VIG1 is required for sRNA-mediated translation repression but seems entirely dispensable for sRNA-mediated target RNA cleavage/degradation. We have previously reported that sRNA-repressed transcripts were found associated with polyribosomes by sucrose density gradient centrifugation (6). Although ribosome-profiling data on individual transcripts tend to be noisy without very deep coverage, we nevertheless also examined, by Ribo-seq, the association with ribosomes of the miR_C translationally repressed Cre16.g638650 transcript. Ribosome-protected mRNA fragments (RPFs) of typical length distribution and with the 3-nt periodicity indicative of translationally active ribosomes were clearly detected (Fig. 2A). Moreover, the RPFs were fairly uniformly spread along the CD53, without any obvious ribosome stalling upstream of the miR_C binding site (Fig. 2B).

**VIG1 Copurifies with (mi)RISC Core Components.** VIG/SERBP1 has been previously identified as a component of the (mi)RISC in D. melanogaster, C. elegans, and human cells (24, 25). However, its role has remained somewhat enigmatic since its downregulation caused a slight (cleavage-mediated) RNAI defect in D. melanogaster and no obvious RNAI deficiency in C. elegans (24). To explore whether Chlamydomonas VIG1 might be an (mi)RISC component, we affinity purified FLAG-CBP–tagged VIG1 from RNase A-treated cell lysates of a complemented vig1 transgenic strain and identified associated proteins by mass spectrometry (Fig. 3A). As a negative control, we carried out similar purifications from a transgenic strain expressing a FLAG-CBP–tagged bleomycin resistance protein (Ble) from Streptomyces hindustanus. In 3 independent experiments, VIG1 copurified with AGO3 and DCL3 (Fig. 3A and SI Appendix, Table S1), 2 well-characterized core RNAI components in Chlamydomonas (19, 20, 22). In addition, VIG1 associated, in at least 2 experiments, with putative mRNA splicing factors (SART1, squamous cell carcinoma antigen recognized by T cells 1; SRP23, serine arginine rich-like protein 23), several components of the protein translation machinery (eIF3A, eukaryotic translation initiation factor 3 subunit A; eIF3M, eukaryotic translation initiation factor 3 subunit M; eIF4A, eukaryotic translation initiation factor 3 subunit A; eS4 [RPS4], 40S ribosomal protein S4e; eS7 [RPS7], 40S ribosomal protein S7e; eL13 [RPL13], 60S ribosomal protein L13e; uL13 [RPL13A], 60S ribosomal protein L13A), a putative ATP-dependent RNA helicase (HEL61), and protein arginine N-methyltransferase 2 (PRMT2) (Fig. 3A and SI Appendix, Table S1). The associations appear to be specific, since none of these proteins was detected in 3 equivalent purifications with FLAG-CBP-Ble, and RNAI-independent, since the cell lysates were treated with RNase A.

The (mi)RISC can catalyze the endonucleolytic cleavage of RNA substrates, as directed by complementarity to its associated siRNAs/miRNAs. Since FLAG-CBP-VIG1 and associated proteins were purified from cells containing MAAT siRNAs, which we have previously characterized by sequencing (6, 21), we designed a complementary MAAT RNA substrate to test whether the isolated complex showed sequence-specific nuclease activity in vitro. In this assay, expected cleavage products were observed for the full reactions with or without ATP (Fig. 3B). However, the endonucleolytic activity was abolished by the addition of EDTA, which chelates the Mg2+ cofactors required for AGO slicer activity. As controls, no specific cleavage was observed by incubation of the homologous RNA substrate with FLAG-CBP-Ble or

![image](https://example.com/image.png)
by incubation of a nonhomologous RNA substrate with FLAG-CPB-VIG1 (Fig. 3B). Thus, C. reinhardtii VIG1 appears to be an (mi)RISC component, part of a complex with AGO3 that is capable of siRNA-directed sequence-specific RNA cleavage. It is possible, but currently untested, that VIG1 may affect to some degree (mi)RISC loading/stability, explaining the very minor reduction in AGO3/2 and certain siRNA/miRNA levels in the vig1 strain (Fig. 1D–F). However, it is unlikely that VIG1 is required for target RNA cleavage since, as already mentioned, its absence in the vig1 mutant does not result in deficient regulation of endogenous sRNA targets subject to cleavage/degradation (SI Appendix, Fig. S4A). Interestingly, in its normal cellular context, the RBM represses the MA47 transcript at the translational level without mRNA cleavage/degradation (Fig. 1C). In contrast, the in vitro purified RISC can cleave an MA47 homologous RNA (Fig. 3B). We speculate that some associated factor(s) and/or posttranslational modification(s), which might suppress AGO3 endonucleolytic activity in vivo, may have been lost during the purification.

**VIG1 Is Mainly Located in the Cytosol and Associates with Bona Fide Translating Ribosomes.** VIG1 is predicted to be a structurally disordered protein, with intrinsically disordered regions covering ∼83% of the polypeptide length (SI Appendix, Fig. S1B). Some of these regions consist of arginine/glycine-rich (RG/RGG) repeats (SI Appendix, Fig. S1A). However, 2 sequence domains are well-conserved in putative VIG1 orthologs from a wide spectrum of eukaryotes: the Stm1_N domain, found in the Saccharomyces cerevisiae suppressor of Tom 1 (Stm1) protein (SI Appendix, Figs. S1A and S5), and the HABP4/SERBP1 (PAI-RBP1) domain, found in the HABP4 family of hyaluronan-binding proteins and in SERBP1 (SI Appendix, Figs. S1A and S6). Cryo-EM structures of eukaryotic 80S ribosomes revealed that yeast Stm1, D. melanogaster VIG2, and mammalian SERBP1 interact directly with inactive ribosomes (26, 27). Stm1 and SERBP1 have been proposed to function as clamping factors that prevent ribosome subunit disassembly and preclude their degradation when translation is massively slowed down under nutrient deprivation/stress conditions (27–30).

To begin assessing the association of Chlamydomonas VIG1 with ribosomes, we examined its subcellular localization by immunofluorescence imaging. FLAG-CPB-VIG1 was found to localize mainly in the cytosol, with some preference for perinuclear regions (Fig. 4 and SI Appendix, Fig. S7). The parental Maa7-IR44 strain was used as a negative control, to verify the absence of background fluorescence in the Alexa Fluor 488 channel corresponding to FLAG-CPB-VIG1. We also tested conditions that might potentially alter VIG1 localization, by virtue of its association with the (mi)RISC and/or with ribosomes. Heat stress triggers translational arrest and the formation of stress granules, cytoplasmic aggregates of stalled translational preinitiation complexes (which can include 40S ribosomal subunits and translation initiation factors), after polyribosome disassembly (31). In contrast, cycloheximide treatment “freezes” translating ribosomes on transcripts (6, 32). However, the localization of FLAG-CPB-VIG1 did not change appreciably under any of the conditions analyzed (Fig. 4 and SI Appendix, Fig. S7).

To examine the possible association of VIG1 with translating ribosomes, we carried out polyribosome profiling by sucrose density gradient sedimentation. A fraction of VIG1 comigrated with monoribosomes and polyribosomes, suggesting that VIG1 does associate with actively translating ribosomes (Fig. S4). Interestingly, a fraction of AGO3/2 also comigrated with polyribosomes (Fig. S4),

![Fig. 4. Immunofluorescence localization of FLAG-CPB-tagged VIG1. Phase-contrast images of the cells, immunolocalization of epitope-tagged VIG1 (detected with an antibody conjugated to Alexa Fluor 488), DAPI staining of nuclear and organellar DNA, and merged images. Representative images are shown, with the location of the nucleus indicated by “N.” Cells were grown to middle logarithmic phase in TAP medium, collected by centrifugation, and then processed for immunofluorescence microscopy. Aliquots of cells were also exposed to 42 °C for 45 min (heat shock) or incubated in the presence of 50 μg/mL cycloheximide for 2 h prior to preparation for immunofluorescence microscopy. vig1(tagVIG1)-3, VIG1 deletion mutant expressing a transgene of FLAG-CPB-VIG1 under the control of the PsaD promoter. Maa7-IR44, parental strain. (Scale bars, 5 μm.)](https://www.pnas.org/doi/10.1073/pnas.1904840117)
supporting a connection of the (mi)RISC with translating ribosomes and consistent with the previously reported association of sRNAs with polyribosomes (6). To determine further whether the fast-sedimenting VIG1 and AGO3/2 were indeed associated with polyribosomes, we treated lysates with EDTA, known to chelate Mg$^{2+}$ and dissociate translating cytosolic ribosomes into their 40S and 60S subunits (6). This caused, as expected, redistribution of all tested proteins to the subpolyribosomal region of the gradient (SI Appendix, Fig. S8A). Thus, VIG1 localizes predominantly in the cytosol of C. reinhardtii and associates with monoribosomes and polyribosomes.

**VIG1 Appears to Be an Auxiliary Protein of Translating Ribosomes but is Not Required for AGO3 Interaction with Polyribosomes or Target Transcripts.** In wild-type C. reinhardtii, VIG1 is an abundant protein, similar in relative levels to ribosomal proteins and at least 15 times more abundant than AGO3 (SI Appendix, Fig. S9A). This implies that the majority of cellular VIG1 is unlikely to be part of the (mi)RISC. The affinity-purification experiments indicated that VIG1 interacts (directly or indirectly but in an RNA-independent manner) with several translation initiation factors and ribosomal proteins (Fig. 3A). Additionally, the vigl mutant is hypersensitive to exposure to cycloheximide, which inhibits translation elongation (6), but shows lower sensitivity to exposure to hygromycin B or rapamycin (SI Appendix, Fig. S9B). Hygromycin B seems to affect decoding fidelity as well as translational location of mRNA and tRNAs on the ribosome (6), whereas rapamycin is an inhibitor of the mTOR (mechanistic target of rapamycin) kinase. Together with the described VIG1 comigration with mono/polyribosomes on sucrose density gradients (Fig. 5A), these observations suggested that this protein may have an ancillary role(s) in ribosome function/structure (besides being a putative

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**Fig. 5.** VIG1 is not required for AGO3 association with polyribosomes or target transcripts. (A) Polyribosome profile of a vig1(tagVIG1)-3 lysate treated with 150 μg/mL cycloheximide and fractionated by sucrose density gradient centrifugation (Top). The distribution of the AGO3/2, RPL37, and FLAG-CBP-VIG1 proteins in the gradient fractions was examined by immunoblotting (Bottom). The location of monoribosomes (M) and polyribosomes (Poly) in the profile is shown above the blots. The asterisk indicates a nonspecific cross-reacting antigen. (B) Polyribosome profiles of the Maa7-IR44 and vig1 strains and immunological detection of AGO3/2 and RPL37 distribution in the gradient fractions. (C) RNA-binding protein immunoprecipitation and subsequent RT-PCR detection of AGO3-associated transcripts. RIP with anti–FLAG-M2 agarose beads (FLAG-IP) was performed from lysates of the indicated strains. Input RNA corresponded to 5% of the total purified amount. ACT1 was examined as a control transcript, not targeted by an sRNA-mediated mechanism. Maa7-IR44(FLAG-AGO3)-56, transgenic strain of Maa7-IR44 transformed with FLAG-tagged AGO3; vig1(FLAG-AGO3)-31, transgenic strain of vig1 transformed with FLAG-tagged AGO3; Maa7-IR44(FLAG-Ble), transgenic strain of Maa7-IR44 transformed with FLAG-tagged Ble.
[mi]RISC component). A nonessential role of VIG1 in ribosome function is supported by the fact that overall protein abundance did not change appreciably in the vig1 mutant relative to the wild-type or the complemented strains (SI Appendix, Fig. S10). Moreover, under standard laboratory conditions in TAP (Tris-acetate-phosphate) medium, lack of VIG1 does not appear to affect cell growth (Fig. L4 and SI Appendix, Fig. S9B), suggesting that VIG1 may only play a minor role (if any) in general protein synthesis.

To examine in more detail the function of VIG1 in sRNA-mediated translation inhibition, we tested whether it was required for association of AGO3/2 with polyribosomes or with translationally repressed target RNAs. However, the distribution of AGO3/2 by sucrose gradient sedimentation was very similar in both vig1 and its parental strain (Fig. 5B), suggesting that AGO3/2 interaction with polyribosomes is VIG1-independent. Our anti-AGO2 antibody was raised against an AGO2 peptide (21), but, in immunoblots, it also recognizes the much less abundant AGO2 homolog (19). However, its binding affinity appears to be too low for the immunoprecipitation of the native AGO proteins. Thus, in order to examine AGO3–target RNA interactions by RNA-binding protein immunoprecipitation (RIP), we generated transgenic strains expressing FLAG-tagged AGO3 in the vig1 and the Ma7-IR44 backgrounds. We selected transgenic strains expressing FLAG-AGO3 at similar levels and where the introduced tagged protein replaced a fraction of the endogenous AGO3, rather than overexpressing it to unphysiological levels (SI Appendix, Fig. SSB; Maa7-IR44[FLAG-AG03]-56 and vig1[FLAG-AG03]-31). As in mammalian cells (33), AGO3/2 and (most) miRNA/siRNA levels are positively correlated in C. reinhardtii (19, 21), suggesting that AGO3/2 proteins are stabilized by association with sRNAs and vice versa. As a further indication that FLAG-AGO3 proteins were not exceedingly overexpressed, we also verified that the transgenic strains had similar levels of endogenous miRNAs and equivalent to those in the wild-type strain (SI Appendix, Fig. S8C). RIP from the selected strains and subsequent reverse-transcriptase (RT) PCR analysis revealed that FLAG-AGO3 binding to its target miRNAs was not appreciably defective in the vig1 mutant (Fig. S5C). Hence, the association of AGO3-(mi)RISC with target RNAs is largely independent of VIG1.

**Discussion**

The biogenesis and function of sRNAs have been the subject of extensive research in diverse eukaryotes. There is now good evidence that, besides triggering mRNA degradation (in a slicer-dependent or -independent manner), the (mi)RISC can also cause translation repression of polyribosomal transcripts, in some cases without or with minimal mRNA destabilization, in animals, plants, and some protists like the alga C. reinhardtii (2, 4, 6, 11, 13). However, the actual mechanism(s) of this translation inhibition remains poorly characterized. As already mentioned, in metazoans, GW182/TNRC6 proteins are not required for (cleavage-mediated) RNAi (24). Since the endogenous lin-4 transcript is translationally repressed by let-7 while associated with polyribosomes (13), it seems plausible that VIG1 is involved in this process. However, C. elegans vig1 mutants also show reduced levels of mature let-7 miRNAs, suggesting alternative (not mutually exclusive) VIG1 functions in miRISC loading and/or miRNA stability (25). Nonetheless, a growing body of evidence also supports the association of VIG/SERBP1 homologs with ribosomes. S. cerevisiae Stm1, D. melanogaster VIG2, and mammalian SERBP1 interact with inactive ribosomes as clamping factors that presumably prevent ribosome disassembly and decay when translation is inhibited by nutrient starvation/stress conditions (26–30). In addition, S. cerevisiae Stm1 and mammalian SERBP1 have been shown to associate with translating polyribosomes (30, 34, 35). Yeast Stm1 has been reported to perturb the association of elongation factor eEF3 with ribosomes and affect optimal translation elongation (34). Translation experiments using yeast extracts also suggested that Stm1 represses translation elongation, after formation of 80S ribosomes (30, 36). Additionally, yeast Stm1 has been genetically linked to mRNA decapping and degradation (30, 36). Mammalian SERBP1 has been implicated in multiple functions but recent findings indicate that most cytoplasmic SERBP1 is precipitated by ultracentrifugation and associates with translating ribosomes (as a substoichiometric component of the 40S subunit) (35). One of the A. thaliana VIG/SERBP1 homologs, ArtRGGGA, localizes in the cytoplasm and the perinuclear region (consistent with an association with ribosomes, although this has not been examined) and is involved in plant responses to osmotic stress (37).

The association of VIG/SERBP1 homologs with translating ribosomes may be mediated, at least partly, by RACK1 (receptor of activated protein C kinase 1), which is a component of the 40S ribosomal subunit and also acts as a scaffold for many proteins involved in diverse signaling pathways (38, 39). In yeast 2-hybrid assays, mammalian SERBP1 interacts with RACK1 (40) and proximity-dependent biotin identification analyses suggest that S. cerevisiae Stm1 is deposited close to RACK1, within the 40S ribosomal head region, when mRNAs are actively translated (29). Interestingly, RACK1 has also been found to affect different aspects of the miRNA pathway, from biogenesis to effector functions, in plants and metazoans (38, 39, 41). In C. elegans and human cells, RACK1 copurifies with AGOs and has been proposed to facilitate the recruitment of the miRISC to the ribosome (41). RACK1 was also found in complexes with AGO1 in A. thaliana, although its potential role in an sRNA effecter complex has not been examined (42).
Like some of its homologs, Chlamydomonas VIG1 localizes predominantly in the cytosol, with some preference for perinuclear regions (Fig. 4 and SI Appendix, Fig. S7), and comigrates with monoribosomes and polyribosomes by sucrose density gradient sedimentation (Fig. 5A). Affinity purification of FLAG-CBP-VIG1, from RNase A-treated cell lysates, indicated that the association of VIG1 with components of the translation machinery, including several translation initiation factors and ribosomal proteins, is RNA-independent (Fig. 3A). Moreover, the vig1-deleted mutant is hypersensitive to exposure to the translation elongation inhibitor cycloheximide (SI Appendix, Fig. S9B), suggesting that VIG1 may have some role(s) in ribosome function/structure. Chlamydomonas VIG1 also copurifies with AGO3 and DCL3 (Fig. 3A), consistent with it being a component of the (mi)RISC, and its depletion prevented the sRNA-mediated translation repression of polyribosomal transcripts (Figs. 1 and 2). However, this protein was dispensable for cleavage-mediated RNAi (SI Appendix, Fig. S5A) as well as for the association of the AGO3 effector with polyribosomes and with target transcripts (Figs. 5B and C). Intriguingly, we have previously reported that ribosomes associated with sRNA-repressed transcripts showed reduced sensitivity to translation inhibition by cycloheximide and suggested that sRNA-mediated repression of protein synthesis in C. reinhardtii may involve alterations to the translation/structural conformation of translating ribosomes (6). Together with current results, it is tempting to speculate that VIG1 is an ancillary ribosomal component and plays a direct role in the translation repression of sRNA/miRNA-targeted transcripts.

We hypothesize that the core (mi)RISC may interact (directly or indirectly) with Chlamydomonas VIG1 to trigger translation inhibition. Yet, VIG1 is predicted to be an intrinsically disordered protein (SI Appendix, Fig. S1B). This type of protein does not assume a single folded structure but instead rapidly interconverts between heterogeneous conformational states depending on environmental conditions and/or partner interactions (43, 44). Recent studies established that phase separation and protein–protein (and/or protein–RNA) interactions of a number of intrinsically disordered proteins are also regulated by posttranslational modifications, in particular arginine methylation and serine/threonine/tyrosine phosphorylation (43, 44). Indeed, the RG/RGG motifs (45, 46) in VIG1, which are extensively modified in eukaryotic ribosomes, may conceivably be required for proper interaction with the core (mi)RISC and/or the actual translation repression. In S. cerevisiae, which has lost all core components of the RNAi machinery (47), the VIG1 homolog, Stm1, lacks RG/RGG repeats (SI Appendix, Fig. S1A), consistent with these motifs being related to an sRNA-associated function(s).

We propose a model of sRNA-mediated translation repression of polyribosomal transcripts whereby VIG1 is generally associated with ribosomes (SI Appendix, Fig. S1A) and may play a minor (nonessential) role(s) in modulating ribosome function/structure under normal growth conditions. In S. cerevisiae, Stm1 seems to influence the association of elongation factor eEF3 with ribosomes, a protein that both stimulates eEF1A-dependent binding of a cognate aminoacyl-tRNA to the ribosomal A site and facilitates the release of deacylated tRNA from the ribosomal A site (34, 48). Although eEF3 was initially thought to be a fungal-specific translation factor, eEF3-like (eEF3L) proteins have now been identified in a wider range of eukaryotes (49). Chlamydomonas encodes 2 eEF3L homologs, containing the conserved domains involved in the interactions of yeast eEF3 with eEF1A and with the ribosome near the E site (48, 49). Given the vig1 mutant hypersensitivity to cycloheximide, VIG1 may possibly modulate translation elongation (e.g., by influencing the activity/interactions of eEF3L factors) and/or the accessibility of cycloheximide to its site of action at the ribosomal E site (32). Alternatively, VIG1 may associate with translating ribosomes mainly in “standby mode,” until it is required for clamping ribosomal subunits under nutrient starvation (29). Upon core (mi)RISC binding to a target transcript in a polyribosomal context, we hypothesize that it interacts (directly or indirectly) with VIG1 and switches this protein into an alternative conformational state leading to translation repression at potentially multiple steps (SI Appendix, Fig. S11B). Since VIG1 copurifies with eEF3 subunits, which are known to associate with the 40S ribosomal subunit (29, 50), it could conceivably affect the recruitment/interaction of components of the translation initiation machinery. Additionally, VIG1 associated with translating 80S ribosomes could inhibit translation elongation, as reported for yeast Stm1 (30, 36). However, in an in vitro assay with rabbit reticulocyte lysates, mammalian SERBP1 was able to bind to multiple ribosomal complexes but did not interfere with translation initiation or elongation (51), possibly reflecting the proposed need of an interaction with the core (mi)RISC for (nonfunctional) VIG1 homologs to trigger translation repression.

We favor the above model, characterized by an overall reduction (slowdown) of both translation initiation and elongation rates, because, in Chlamydomonas, ribosomes remain associated with sRNA-repressed transcripts, without noticeable changes in ribosome occupancy, and appear to be actively translating although with reduced sensitivity to inhibition by cycloheximide (6). Intriguingly, pretreatment of the mammalian ECV-304 cell line with cycloheximide also partly relieved the miRNA-mediated repression of a Renilla luciferase reporter (52). Yet, alternative models are also consistent with our observations. For instance, mRNA-bound ribosomes could be simultaneously frozen on the transcript, conceivably through liquid–liquid phase separation triggered by conformational changes in intrinsically disordered VIG1 interacting with the core (mi)RISC. Thus, the exact mechanism by which VIG1 may contribute to translation repression will require further investigation.

Materials and Methods
Culture Conditions, Transgenic Strains, and Mutants. Unless noted otherwise, C. reinhardtii cells were grown photoheterotrophically in Tris-acetate-phosphate medium or photoautotrophically in minimal high-salt (HS) medium (53, 54). For phenotypic analyses, cells grown to logarithmic phase in TAP or HS media were serially diluted, spotted on plates of the appropriate media (see figure legends), and incubated for 7 to 15 d under dim lights (6, 23, 54). Mutants and transgenic strains are described in SI Appendix, Materials and Methods.

RNA Analyses. Total cell RNA was purified with TRI Reagent (Molecular Research Center), following the manufacturer’s instructions. For Northern blot analyses of mRNAs, the isolated RNA was separated by agarose/formaldehyde gel electrophoresis, blotted onto nylon membranes, and hybridized with 32P-labeled probes (6, 21, 55). For small RNA analyses, total RNA samples were resolved on 15% polyacrylamide/7 M urea gels and electroblotted to Hybond-XL membranes (GE Healthcare) (6, 21, 55). Blots were hybridized with 32P-labeled DNA probes at 40 °C for 48 h using the High Efficiency Hybridization System (Molecular Research Center). Specific miRNAs were detected by hybridization with complementary DNA oligonucleotides labeled at their 5′ termini with γ32PATP and T4 polynucleotide kinase (6, 21). RNA-seq and Ribo-seq data for wild-type C. reinhardtii were reanalyzed from Chu et al. (56) (accession nos. ERR558436 and ERR558438).

Immunoblot Analyses. Approximately 5 x 106 cells, grown to logarithmic phase, were pelleted by centrifugation and resuspended in 50 μL of SDS gel running buffer. Ten-microliter aliquots of boiled samples were separated by SDS/PAGE and electrophoretically transferred to nitrocellulose membranes (54, 55). Tryptophan synthase β-subunit was immunodetected by overnight incubation at 4 °C with a 1:5,000 dilution of a rabbit antibody raised against the Campotheca acuminata enzyme (a generous gift from Thomas McKnight, Texas A&M University, College Station, Texas) (6, 21). AGO3 was detected by incubation with a 1:10,000 dilution of a goat antibody raised against a C-terminal peptide (ASRSGRGAGAAEGG) conjugated to keyhole limpet hemocyanin (KLH) (21). This antibody also cross-reacts with Chlamydomonas AGO2 (19), but the steady-state level of this protein is, at least

1 order of magnitude lower than that of AGO3 [PaxDb (57)]. The Cre16.663630 protein was immunodetected by overnight incubation at 4 °C with a 1:10,000 dilution of a rabbit antibody raised against a C-terminal peptide (GIPSKASHKGGVRM) conjugated to KLH (22). Commercially available antibodies were used to detect histone H3 (Abcam; ab1791), RPL37 (Agrisera; AS12 2115), COX2B (Agrisera; AS06 151), RPS14 (Agrisera; AS12 2111), RBCS1/2 (Agrisera; AS07 259), and the AcV5 epitope tag (baculovirus envelope gp64 protein; ebioscience; 14-6995), which was engineered as part of the FLAG-CBP tag.

Affinity FLAGGING OF FLG-CBP-Tagged VIG1 and Protein Identification by Mass Spectrometry. To purify VIG1-associated polypeptides, a complemented vig1 transgenic strain [vig1tag(VIG1)-3] was grown to midlogarithmic phase in TAP medium (containing 7 μM 5-F) and cells were collected by centrifugation. For each experiment, ∼2 × 1012 cells were resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM MgCl2·6H2O, 2.5 mM CaCl2, 1 mM imidazole, 10 mM β-mercaptoethanol, and 10% glycerol) supplemented with 2.0 mM benzamidine, 0.2 mM phenylmethanesulfonyl fluoride (PMSF), 5 μL/mL plant protease inhibitor mixture (Sigma), and 30 μg/mL RNase A. Cells were broken by 2 passages through a French press at 5,000 psi and the lysate was clarified by centrifugation at 16,000 × g for 30 min at 4 °C. The extract was then incubated with buffer-equilibrated calmodulin-Sepharose beads (GE Healthcare) for 16 h at 4 °C. Subsequent purification steps were performed as previously described (58). FLAG-CBP-Ble purification, following the same protocol, was used as a negative control. Isolated proteins were fractionated by 10% SDS/PAGE, stained with Sypro Ruby (Bio-Rad), digested in-gel with trypsin, and identified by tandem mass spectrometry as described (58). We observed that VIG1 migrates anomalously on SDS/PAGE and Sepharose beads (GE Healthcare) for 16 h at 4 °C. Subsequent purification and the lysate was clarified by centrifugation at 16,000 × g for 30 min at 4 °C.

In Vitro RISC Activity. To test for sequence-specific cleavage activity, VIG1 and associated proteins were purified as described above. After the final regular wash, the beads were rewash and resuspended with RISC activity buffer (30 mM Hapes, pH 7.5, 100 mM NaCl, 5 mM MgCl2·6H2O, 2 mM CaCl2, and 0.5 mM DTT). The in vitro reactions contained 17 μL resuspended beads in RISC activity buffer, 1 μL 5′-labeled synthetic MAL7 RNA (1 pmol/μL), SAGGACAGCGUGUGCAAGAACGAGCGCC-3′; IDT), 1 μL α-U1 snRNA (Promega), and 1 μL of a mixture of 0.2 mM GTP and 1 mM ATP (final volume 20 μL). For EDTA treatments, the reactions were supplemented with 8 mM EDTA. A nonhomologous, 3′-labeled RNA (5′-GGGAUUGAUCUCCAGCGGAAA-3′; IDT) was also used as a negative-control substrate. All reactions were allowed to proceed at room temperature for 0, 20, or 40 min. At each time point, an aliquot was transferred to a new tube on ice and an equal volume of formamide loading buffer (Sigma) was added. The samples were then denatured at 65 °C for 3 min, quickly cooled on ice, and 3 μL was then resolved on 15% denaturing polyacrylamide gels and detected by autoradiography.


