The mevalonate pathway regulates microRNA activity in *Caenorhabditis elegans*

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Contributed by Gary Ruvkun, February 14, 2012 (sent for review January 24, 2012)

The mevalonate pathway is highly conserved and mediates the production of isoprenoids, which feed into biosynthetic pathways for sterols, dolichol, ubiquinone, heme, isopentenyl adenine, and prenylated proteins. We found that in *Caenorhabditis elegans*, the nonsterol biosynthetic outputs of the mevalonate pathway are required for the activity of microRNAs (miRNAs) in silencing their target mRNAs. Inactivation of genes that mediate multiple steps of the mevalonate pathway causes derepression of several miRNA target genes, with no disruption of the miRNA levels, suggesting a role in miRNA-induced silencing complex activity. Dolichol phosphate, synthesized from the mevalonate pathway, functions as a lipid carrier of the oligosaccharide moiety destined for protein *N*-linked glycosylation. Inhibition of the dolichol pathway of protein *N*-glycosylation also causes derepression of miRNA target mRNAs. The proteins that mediate miRNA repression are therefore likely to be regulated by *N*-glycosylation. Conversely, drugs such as statins, which inhibit the mevalonate pathway, may compromise miRNA repression as well as the more commonly considered cholesterol biosynthesis.

MicroRNAs (miRNAs) are ~22-nucleotide noncoding RNAs that repress gene expression posttranscriptionally. In animals, miRNAs cause repression by base pairing to the 3′ untranslated region of their target mRNAs, which contain perfect or near-perfect sequence complementarity to nucleotides 2–7 (the "seed region") of miRNAs and mismatches and bulges in other parts of the miRNA–mRNA duplex (1). miRNAs are processed to their mature form in a well-understood pathway involving the RNase III proteins Drosha and Dicer; mature miRNAs then associate with Argonaute proteins to form the core miRNA-induced silencing complex (miRISC). However, the exact mechanism by which miRNAs repress target mRNA stability or translation is still an open question (2). In addition, genetic and biochemical analysis has implicated multivesicular bodies in the repression of target mRNAs by miRNAs (3, 4), underscoring the need to better understand how membrane trafficking functionally intersects with the miRNA pathway. Genetic screens for mutants that phenocopy well-known miRNA mutants are a promising avenue to identify other components in the miRNA repression of target mRNAs.

The development of the nematode *Caenorhabditis elegans* proceeds through four larval stages (L1–L4) that are separated by molts, followed by the reproductive adult stage. During development, heterochronic genes regulate the timing of cell fate specification in several tissues, with the result that heterochronic mutants exhibit disrupted synchronization between tissues of developmental timing events (5). *lin-4* and *let-7* miRNAs are *C. elegans* heterochronic genes (6, 7). These miRNAs are generated at specific stages to repress their target genes, which encode regulators of cell fate specification (8–10).

The mevalonate pathway is present in all higher eukaryotes and many bacteria and mediates the production of isoprenoids. The isoprenoids feed into a wide range of biosynthetic pathways: sterols, primarily cholesterol; dolichol, which serves as the lipid carrier of the oligosaccharide moiety destined for protein *N*-linked glycosylation; ubiquinone and heme A, which function in the electron transport chain; prenylated proteins; and isopentenyl adenine, which is present at position 37 of tRNAs that read codons starting with U (11). Cholesterol, the bulk product of the mevalonate pathway in humans and many other organisms, is important for membrane structure and steroid hormone synthesis. *C. elegans* possesses a functional mevalonate pathway but lacks all enzymes for the synthesis of sterols, suggesting that mevalonate in *C. elegans* is an important precursor for other biosynthetic pathways (12). Although it does not synthesize cholesterol itself, *C. elegans* requires exogenously supplied cholesterol for growth and development (13, 14).

Here we present a link between the nonsterol biosynthetic products of the mevalonate pathway and miRNA activity in *C. elegans*. Inactivation of genes that function in rate-limiting steps of the mevalonate pathway causes desilencing of miRNA targets and retarded heterochronic defects that resemble the effects of mutations in *lin-4* and *let-7* miRNAs. We show that among the downstream branches of the mevalonate pathway, the biosynthesis of dolichol for protein *N*-linked glycosylation is important for miRNA activity. Biological functions of protein *N*-glycosylation include facilitating protein folding and stability, intracellular targeting, intercellular recognition, and others (15). Earlier studies identified Argonaute as a peripheral membrane protein located on the Golgi and/or endoplasmic reticulum (ER) in several mammalian cell lines, and the protein was originally named for this: GERp95 (Golgi ER protein 95 kDa) (16, 17). Our analyses suggest that *N*-glycosylation is required for miRISC function, for example via regulating the sorting of miRISC to the cellular membrane compartment.

Results

***hmgs-1*/HMG-CoA Synthase Functions in the *let-7* miRNA Pathway.** In *C. elegans*, *let-7* miRNA regulates developmental timing events during the L4-to-adult transition. *let-7* down-regulates its target genes, such as *lin-41* and *hbl-1*, and this releases their repression of LIN-29, a key transcription factor that is required for the terminal specification of epidermal cells at the adult stage (9, 18, 19). Loss of *let-7* activity either by mutation of the *let-7* gene or inactivation of *dcr-1*/*Dicer* or *alg-1*/*Argonaute*, the core components in miRNA maturation and function, causes retarded heterochronic phenotypes in which larval developmental patterns are reiterated and adult-specific specializations do not occur (7, 20). To identify other proteins that act in the miRNA pathway, we screened for gene inactivations that enhance a weak *let-7 (mg279)* reduction-of-function mutation (21). One of the strong hits from this screen, *F25B4.6*/*hmgs-1*, encodes the *C. elegans* ortholog of HMG-CoA synthase. Inactivation of *hmgs-1* by RNAi causes *let-7*–like phenotypes in three independent assays: first, the burst-through-vulva phenotype characteristic of *let-7*

Author contributions: Z.S. and G.R. designed research; Z.S. performed research; Z.S. and G.R. analyzed data; and Z.S. and G.R. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1202421109/-/DCSupplemental.

strong loss-of-function mutations (Fig. 1*A*); second, the failure to express a reporter gene for an adult-specific collagen, *col-19::gfp* (Fig. 1*B*); and third, the failure to produce alae, an adult-specific cuticle structure, at the nominal adult stage (Table S1).

Two lines of evidence further support that *hmgs-1* functions in the *let-7*–regulated heterochronic pathway. First, although *hmgs-1* inactivation causes relatively weak or incompletely penetrant retarded phenotypes on its own, these phenotypes are strongly enhanced in sensitized genetic backgrounds with compromised *let-7* activity (Fig. S1 and Table S1). For example, upon *hmgs-1* inactivation, 9% of wild-type animals, but 100% of *alg-1(gk214)* and 67% of *ain-1(ku322)* mutants, fail to produce adult-specific lateral alae (Table S1). These mutations in genes that encode the ALG-1/Argonaute protein or the AIN-1/*A*LG-1 *in*teracting protein compromise miRNA function, and inactivation of *hmgs-1* is strongly synergistic with these mutations. Second, the retarded phenotypes caused by *hmgs-1* inactivation are suppressed by the loss of function of validated *let-7* target genes. For example, the retarded phenotypes of *hmgs-1* inactivation are completely suppressed by *lin-42(n1089)*, partially suppressed by *lin-28(n719)*, and weakly suppressed by *lin-41(ma104)*, a hypomorphic mutation that causes only weak precocious phenotypes (Fig. 1 *A* and *B*). These data suggest that *hmgs-1* functions in the *let-7* pathway via the regulation of the activity of validated *let-7* target genes *lin-28*, *lin-41*, and *lin-42*.

***hmgs-1* Is Required for *let-7* Family and *lin-4* miRNA Silencing of Target Genes.** To ask more directly whether *hmgs-1* functions in the miRNA pathway, we assayed whether miRNA target mRNAs become derepressed upon inactivation of this gene. We focused on the genetically verified targets of the *let-7* family and *lin-4* miRNAs. The hunchback factor *hbl-1* (*h*unch*b*ack *l*ike) is silenced synergistically by the *let-7* family of miRNAs (*mir-48*, *mir-241*, and *mir-84*) during the L2-to-L3 stage transition (22). Knocking down *hmgs-1* by RNAi prevents down-regulation of *hbl-1::gfp* at the L3 stage. This resembles the phenotype caused by mutations in the *let-7* family of miRNAs (Fig. 2*A*). We assayed whether the silencing of *lin-14* by *lin-4* miRNA during the late L1 stage is dependent on *hmgs-1*. LIN-14 protein levels become derepressed at the late L1 stage by approximately twofold comparing *hmgs-1* RNAi-treated to stage-matched control animals, and the derepression is still apparent at the L2 stage (Fig. 2*B*). To ask whether the desilencing of *lin-14* upon inactivation of *hmgs-1* is due to reduced *lin-4* miRNA repression of *lin-14* via its 3′ untranslated region (3′ UTR), we analyzed the down-regulation of *lin-14* in the *lin-14(n355)* gain-of-function mutant, which lacks all of the sites in the *lin-14* 3′ UTR that are complementary to *lin-4* and *let-7* and its paralogs (23, 24). *lin-14* is not further desilenced when *hmgs-1* is inactivated in the *lin-14(n355)* mutant background (Fig. 2*C*, *Upper*). *lin-14* is also not further desilenced in the *lin-4(e912)* null mutant upon inactivation of *hmgs-1* (Fig. 2*C*, *Lower*). This indicates that the derepression of *lin-14* after *hmgs-1* inactivation is dependent on the *lin-14* 3′ UTR and *lin-4* miRNA. Together, these results show that *hmgs-1* is required for silencing of *lin-14* by *lin-4* miRNA.

***hmgs-1* Acts in miRNA Pathways in Other Cell Types as Well.** *hmgs-1* also modulates the activity of miRNAs whose functions are unrelated to developmental timing. *lsy-6* is an miRNA that regulates the specification of the taste neurons ASE left (ASEL) and ASE right (ASER) which, even though they are bilaterally symmetric, express distinct patterns of receptor genes based on the asymmetric activity of *lsy-6* miRNA (25, 26). Specifically expressed in less than 10 neurons including ASEL but not ASER, *lsy-6* down-regulates the *cog-1* transcription factor only in ASEL, thus distinguishing the gene expression profile of ASEL from ASER (25, 26). The ASEL neuron of *lsy-6(ot71)* null mutants fails to down-regulate *cog-1* and adopts the ASER pattern of gene expression as a result. On the other hand, animals bearing a hypomorphic allele of *lsy-6*, *ot150*, display the ASEL specification defect with incomplete penetrance. Inactivation of genes that are key for miRNA activity, for example *nhl-2*, significantly enhances the ASEL fate specification defect in the *lsy-6(ot150)* but not in the wild-type background (27). We asked whether knocking down *hmgs-1* causes an ASEL specification defect by scoring *lim-6^{pro}::gfp*, an ASEL-specific reporter.

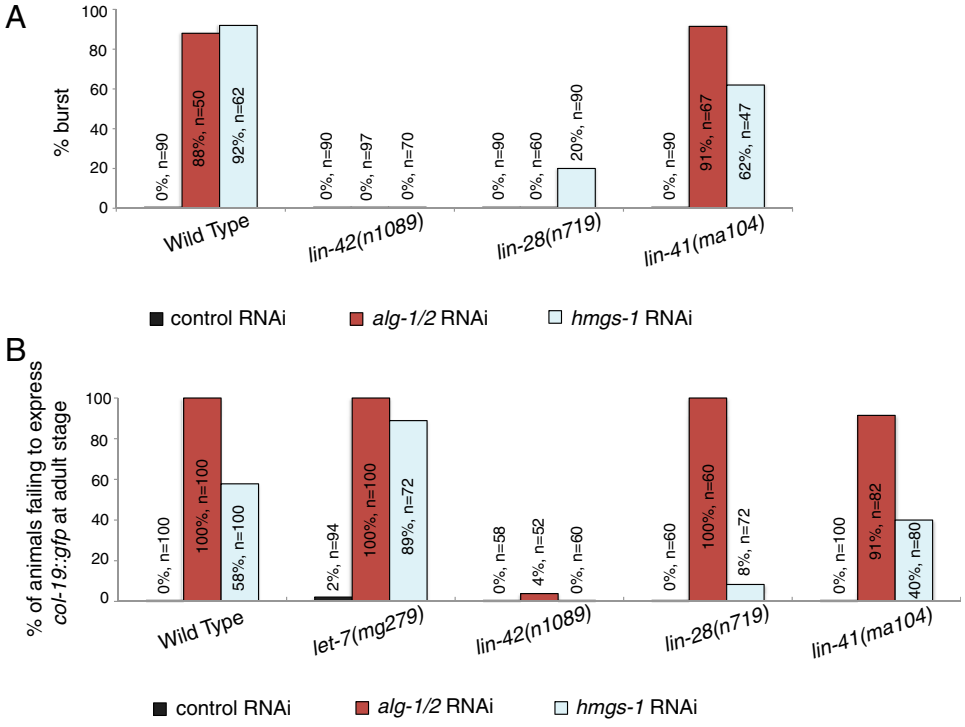

Fig. 1. Inactivation of *hmgs-1* causes *let-7*–like phenotypes. (*A*) Shown are the percentage of animals that burst after the L4-to-adult molt upon treatment with control, *alg-1/2*, or *hmgs-1* RNAi in the indicated genetic background. Inactivation of *alg-1/2* or *hmgs-1* causes bursting with high penetrance in the wild-type animals but lower penetrance in the *lin-42(n1089)* or *lin-28(n719)* loss-of-function mutants. (*B*) Inactivation of *hmgs-1* causes animals to not express *col-19::gfp* in hyp7 cells at the adult stage. The penetrance of this phenotype is elevated in the *let-7(mg279)* mutant and decreased in the *lin-42(n1089)*, *lin-28 (n719)*, or *lin-41(ma104)* loss-of-function mutants. Inactivation of *alg-1/2* also causes animals to not express *col-19::gfp* in hyp7 cells; this phenotype is suppressed in *lin-42(n1089)*.

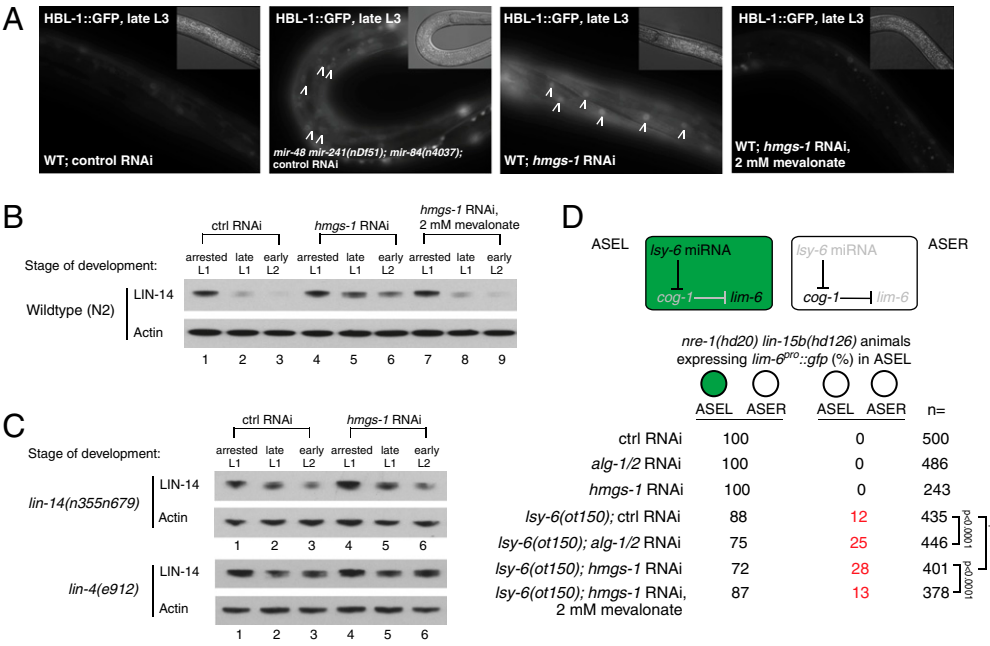

Fig. 2. Inactivation of *hmgs-1* causes desilencing of miRNA target genes. (*A*) Inactivation of *hmgs-1* causes defects in the down-regulation of *hbl-1::gfp* at the L3 stage. This resembles the phenotype caused by mutations in the *let-7* family of miRNAs: *mir-48*, *mir-241*, and *mir-84*. The desilencing of *hbl-1::gfp* upon *hmgs-1* RNAi is rescued by supplementation with 2 mM mevalonate. Images were captured using the same exposure settings and processed identically. Arrowheads point to the desilenced *hbl-1::gfp* in the nuclei of hyp7 cells. (*Insets*) Nomarski images. (*B* and *C*) Immunoblots. Actin was probed as a control for even loading. (*B*) Inactivation of *hmgs-1* causes defects in the down-regulation of *lin-14*, the target of *lin-4* miRNA, at the late L1 stage (lane 5) and early L2 stage (lane 6). Mevalonate supplementation rescues this phenotype (lanes 8 and 9). (*C*) Inactivation of *hmgs-1* does not further desilence *lin-14* in the *lin-14(n355n679)* mutant lacking the *lin-14* 3′ UTR or the *lin-4(e912)* null mutant (compare lanes 5 and 6 to lanes 2 and 3). (*D*) *lsy-6* miRNA is expressed in the ASEL but not ASER neuron in the wild type. It is required for ASEL specification, as judged by the expression of *lim-6^{pro}::gfp* in ASEL, which is promoted by down-regulation of *cog-1* by *lsy-6*. In a sensitized genetic background with a weak allele of *lsy-6*, *ot150*, inactivation of *alg-1/2* or *hmgs-1* significantly enhanced the ASEL specification defect. Supplementing 2 mM mevalonate rescued the phenotype of *hmgs-1* inactivation. Brackets indicate statistically significant difference as judged by a two-tailed χ^2 test.

To enhance the efficiency of RNAi in neurons, we crossed the *lim-6^{pro}::gfp* reporter into the RNAi-hypersensitive *nre-1(hd20) lin-15b(hd126)* mutant background (28). RNAi was initiated at the L3 stage of *lsy-6(ot150); nre-1(hd20) lin-15b(hd126)* parental (P_0) animals, and *lim-6^{pro}::gfp* was scored in the progeny. Twenty-eight percent of *hmgs-1* RNAi-treated animals (n = 401), compared with 12% of control RNAi-treated animals (n = 435), showed the ASEL specification defect (Fig. 2*D*). However, the progeny of *nre-1(hd20) lin-15b(hd126)* animals with the wild-type *lsy-6* gene did not show any ASEL specification defect upon inactivation of *hmgs-1*. Similar results were obtained by knocking down *alg-1/2* by RNAi. This result supports a requirement for *hmgs-1* for the efficient down-regulation of *cog-1* by *lsy-6* miRNA.

Taken together, these observations suggest that *hmgs-1* modulates the function of many and perhaps all miRNAs in multiple tissues, and at multiple stages during development.

***hmgs-1* Acts Downstream of miRNA Biogenesis and Loading of ALG-1/Argonaute.** We asked whether *hmgs-1* is required for miRNA biogenesis/accumulation or activity. To distinguish between these possibilities, we first assayed the mature miRNA levels by real-time PCR. The levels of *let-7*, *lin-4*, and *mir-55* all remained unchanged upon knocking down *hmgs-1* (Fig. 3*A*) despite the fact that the targets of *let-7* and *lin-4* became derepressed. We also assayed the *let-7* level in the *let-7(mg279)* mutant, which has a reduced level of mature *let-7* miRNA resulting from defects in the splicing and processing of the *let-7* transcript (29). Inactivation of *hmgs-1* did not reduce the level of mature *let-7* even in this sensitized genetic background (Fig. S2). In contrast, knocking down *alg-1/2* caused a significant reduction of miRNA levels, consistent with its role in miRNA biogenesis and stability.

We also surveyed whether *hmgs-1* regulates the protein level and/or cellular localization of the core miRNA cofactors. We monitored ALG-1/Argonaute and AIN-1/ALG-1 interacting protein. Neither the overall expression level nor the subcellular localization of GFP::ALG-1 or AIN-1::GFP was altered upon knocking down *hmgs-1* (Fig. S3).

To assay whether *hmgs-1* regulates the competence of ALG-1/Argonaute in loading miRNAs, we purified miRISC from synchronized L4-stage *alg-1(gk214)* mutants rescued with an HA-ALG-1 single-copy construct. HA-ALG-1–bound *let-7*, *lin-4*, and *mir-55* levels remained unchanged upon inactivation of *hmgs-1* (Fig. 3 *B* and *C*), indicating ALG-1 loading is unaltered. We also found that the guide:passenger strand ratio of these miRNAs remained unchanged when *hmgs-1* was inactivated (Fig. S4). Together, these results position *hmgs-1* downstream of miRISC loading and duplex unwinding. It suggests that one or multiple downstream steps, for example the competence of miRISC in finding and silencing its target mRNAs, are dependent on *hmgs-1*.

Noncholesterol Output of the Mevalonate Pathway Modulates miRNA Activity. Humans and some other organisms have two forms of HMG-CoA synthase: the cytosolic form, which acts in the mevalonate pathway, and the mitochondrial form, which functions in the production of ketone bodies during starvation. *C. elegans* bears just the *hmgs-1* ortholog of HMG-CoA synthase, which is predicted to be cytosolic. Therefore, we hypothesized that the isoprenoid output of the mevalonate pathway has a role in miRNA activity. Three strands of evidence support this hypothesis, as follows.

First, we reasoned that if the retarded phenotypes caused by *hmgs-1* inactivation are due to reduced biosynthetic outputs of the mevalonate pathway, then supplementing mevalonate, the downstream product of HMG-CoA synthase, should rescue these phenotypes. Indeed, mevalonate supplementation completely rescued all retarded phenotypes caused by inactivation of *hmgs-1* (Fig. 4*A* and Table S2) but did not rescue the retarded phenotypes induced, for example, by inactivation of the Argonaute gene *alg-1/2* (Table S2). Mevalonate supplementation also rescued the desilencing of *hbl-1::gfp* and *lin-14* in *hmgs-1* RNAi-treated animals (Fig. 2 *A* and *B*). Furthermore, the ASEL specification defect upon knocking down *hmgs-1* was also rescued by mevalonate (Fig. 2*D*). In contrast, supplementation to even 50 μg/mL cholesterol (the standard nematode growth

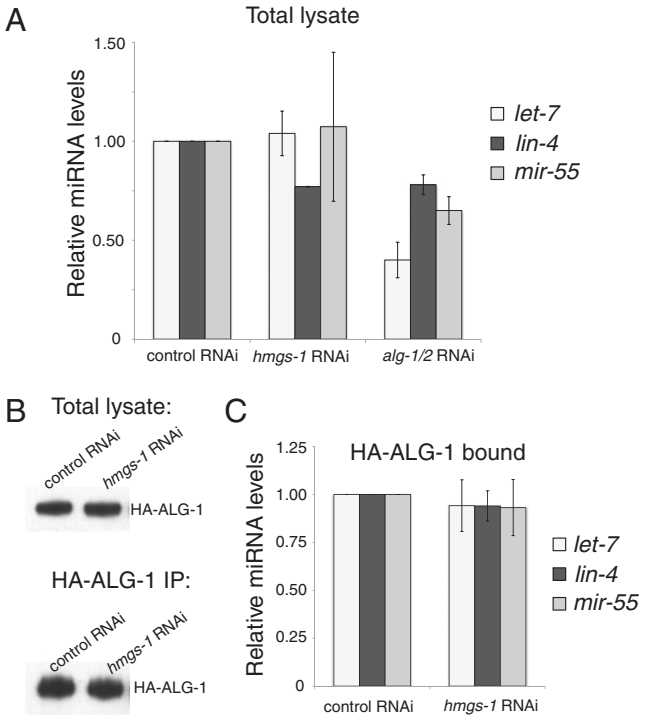

Fig. 3. *hmgs-1* acts downstream of miRNA biogenesis/accumulation and loading of ALG-1. (*A*) Shown are the mature miRNA levels in total worm lysate, determined by real-time PCR. The miRNA levels are reduced upon *alg-1/2* inactivation but remain unchanged upon *hmgs-1* inactivation. (*B* and *C*) HA-ALG-1 was immunoprecipitated from animals treated with control or *hmgs-1* RNAi, and the level of HA-ALG-1–bound miRNAs was determined. (*B*) Equal amounts of HA-ALG-1 were purified from control and *hmgs-1* RNAi-treated animals. Shown is the Western blot of HA-ALG-1 in total lysate (*Upper*) and from HA-ALG-1 IP (*Lower*). (*C*) Relative levels of *let-7*, *lin-4*, and *mir-55* bound by HA-ALG-1; they remain unchanged upon *hmgs-1* inactivation. In *A* and *C*, for each miRNA, the result is shown relative to its level in animals treated with control RNAi. The mean and SD were calculated from three biological replicates. Error bars represent SEM.

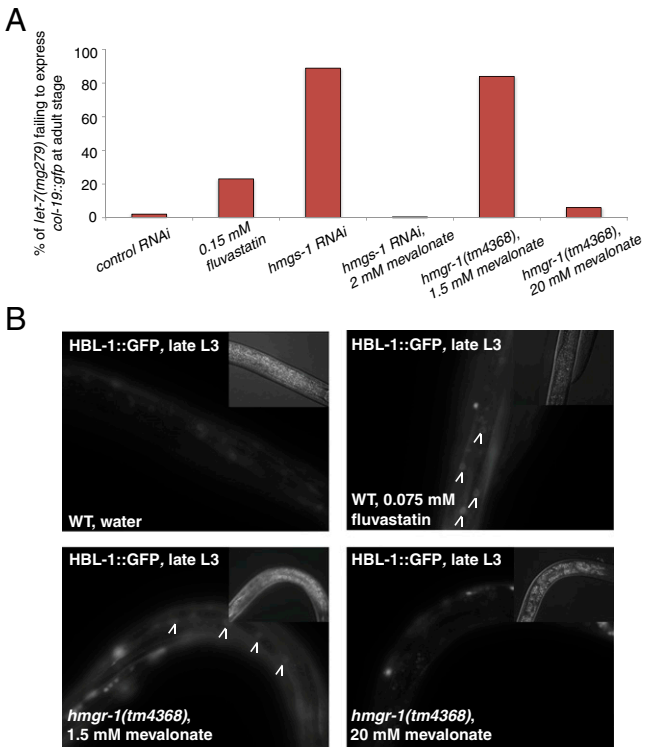

Fig. 4. The mevalonate pathway modulates miRNA activity. (*A*) Inactivation of the mevalonate pathway by either application of fluvastatin, inactivation of *hmgs-1* by RNAi, or mutation of *hmgr-1*, with a low level of mevalonate (1.5 mM) supplied in the medium, causes *let-7(mg279)* mutants to fail to express *col-19::gfp* in hyp7 cells. This phenotype can be rescued by supplementing mevalonate [2 mM for *hmgs-1* RNAi and 20 mM for the *hmgr-1(tm4368)* mutant]. (*B*) Inactivation of the mevalonate pathway by application of fluvastatin or mutation of *hmgr-1*, with a low amount of mevalonate (1.5 mM) supplied in the medium, causes defects in the down-regulation of *hbl-1::gfp* (targeted by the *let-7* family of miRNAs) at the L3 stage. However, *hmgr-1(tm4368)* animals growing on high mevalonate (20 mM) show wild-type down-regulation of *hbl-1::gfp*. Images were captured using the same exposure settings and processed identically. Arrowheads point to the desilenced *hbl-1::gfp* in the nuclei of hyp7 cells. (*Insets*) Nomarski images.

medium contains 5 μg/mL cholesterol) did not rescue any of the retarded phenotypes caused by knocking down *hmgs-1* (Table S2). This indicates that instead of cholesterol, other biosynthetic products of the mevalonate pathway modulate miRNA activity.

Second, HMG-CoA reductase (encoded by *F08F8.2*/*hmgr-1*) also acts in the miRNA pathway. HMG-CoA reductase is the rate-limiting enzyme that acts immediately downstream of HMG-CoA synthase in the production of mevalonate. The *C. elegans hmgr-1 (tm4368)* mutant strain bears a 620-bp deletion that spans the first three exons, causing a likely null mutation in this gene. The homozygous *hmgr-1(tm4368)* mutants that segregate from a heterozygote arrest at the L1 stage. However, if mevalonate is added to the growth media to 20 mM final concentration, the homozygous *hmgr-1(tm4368)* mutants are viable and fertile. We found that *hmgr-1(tm4368); let-7(mg279)* mutants grown with low (1.5 mM) mevalonate failed to express *col-19::gfp*, a defect that was not observed when mevalonate was increased to 20 mM (Fig. 4*A*). In addition, *hmgr-1(tm4368)* mutants grown with 1.5 mM mevalonate failed to properly down-regulate *hbl-1::gfp* at the L3 stage, a phenotype that was also rescued by a higher concentration of mevalonate (Fig. 4*B*).

Third, we found that statins, cholesterol-lowering drugs that inhibit HMG-CoA reductase activity, can compromise *let-7* activity. When fluvastatin was added to the growth medium, it caused *let-7(mg279)* animals to fail to express *col-19::gfp* at the adult stage (Fig. 4*A*). Fluvastatin also prevented the proper down-regulation of *hbl-1::gfp* at the L3 stage (Fig. 4*B*), similar to RNAi depletion of *hmgs-1* or the *hmgr-1(tm4368)* mutation.

Together, the above results strongly indicate that in *C. elegans*, the nonsterol outputs of the mevalonate pathway modulate miRNA activity.

The Dolichol Pathway for Protein *N*-Linked Glycosylation Is Required for miRNA Activity. Isoprenoids, the end products of the mevalonate pathway, feed into a wide range of downstream pathways in addition to the better-known synthesis of sterols (11). This includes protein prenylation; tRNA isopentenylation; and biosynthesis of ubiquinone, heme A, and dolichol (Fig. S5*A*). To further delineate which downstream biosynthetic output of the mevalonate pathway modulates miRNA activity, we inactivated genes corresponding to each step in the production and use of isoprenoids and surveyed the phenotypes. We screened for which of these gene inactivations caused *let-7(mg279)* animals to fail to express *col-19::gfp* (Table S3).

The gene inactivations that caused a *let-7*–like phenotype, the failure to up-regulate *col-19::gfp* expression at the adult stage, all correspond to proteins that act in the dolichol pathway for protein *N*-linked glycosylation (Table S3; for a diagram of the dolichol pathway, see Fig. S5*B*). The *C. elegans* oligosaccharyltransferase (OST) complex, which carries out protein *N*-glycosylation, has five

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subunits: the catalytic subunit T12A2.2/STT3 and four accessory subunits: T22D1.4/ribophorin I, OSTB-1, OSTD-1, and DAD-1. Depleting any of these subunits by RNAi caused a defect in *col-19::gfp* expression in the *let-7(mg279)* mutant but not the wild-type background (Fig. 5*A*), suggesting a strong genetic interaction with *let-7*. To further validate this result, we performed the same assay using tunicamycin. Tunicamycin is an antibiotic that blocks the reaction of UDP-GlcNAc and dolichol phosphate in the first step of the dolichol pathway and thus inhibits the synthesis of *N*-linked glycoproteins. When tunicamycin was added to the worm growth medium, it caused *let-7(mg279)* adult animals to fail to express *col-19::gfp* in a dose-dependent manner (Fig. 5*B*).

More directly, we found that inactivation of *T12A2.2/STT3* disrupted down-regulation of the *hbl-1::gfp* reporter at the L3 stage (Fig. 5*C*). Furthermore, knocking down *T12A2.2/STT3* also exacerbated the ASEL neuron specification defect in the *lsy-6 (ot150)* mutant (Fig. 5*D*), similar to the inactivation of the mevalonate pathway.

Because *N*-glycosylation facilitates protein folding in the ER, blockage of *N*-glycosylation by RNAi, mutation, or drugs causes protein misfolding, which induces ER stress and the unfolded protein response (UPR). We asked whether the desilencing of miRNA target genes is an element of the unfolded protein response. We found that several gene inactivations that strongly induce ER UPR did not prevent proper down-regulation of *hbl-1::gfp* at the L3 stage (Fig. S6*A*). However, several gene inactivations that induce ER UPR did enhance the failure to express *col-19::gfp* in the *let-7(mg279)* mutant, and a mutation in the unfolded protein response factor *xbp-1* (30) suppressed this defect (Fig. S6*A*). The *xbp-1(zc12)* mutation also suppressed the failure to express *col-19::gfp* induced by inactivation of *T12A2.2/STT3*, but not the failure to express *col-19::gfp* induced by inactivation of *alg-1/2* or *hmgs-1* (Fig. S6*B*). This suggests that the mevalonate pathway is required for miRNA activity for more than one reason: first, a relatively direct role of *N*-glycosylation in miRISC function possibly via regulating the sorting of miRISC to the appropriate cellular membrane compartment; and second, a relaying signaling cascade downstream of ER UPR in opposing miRNA activity (Fig. S6*C*).

Discussion

Here we report that the dolichol phosphate/protein *N*-glycosylation output of the mevalonate pathway is required in the miRNA repression of target mRNAs in *C. elegans*. Mechanistically, our analyses positioned HMG-CoA synthase downstream of miRISC loading and duplex unwinding. This suggests that one or multiple downstream steps, for example the competence of miRISC in finding and silencing its target mRNAs, are dependent on the isoprenoid output of the mevalonate pathway. Support for this idea also emerged from plant miRNA genetics, although that work points to cholesterol output (31).

Several studies have identified Argonaute as a peripheral membrane protein, and its membrane association might be crucial for its function (16, 17, 31). Therefore, our study suggests that *N*-linked glycosylation could have a relatively direct role in the regulation of assembly, sorting, or turnover of miRISC on the cellular membrane compartment. Meanwhile, disruption of *N*-glycosylation elicits the ER UPR signaling cascade, which also mildly opposes *let-7* activity. However, it is unknown whether the ER UPR intersects a specific subset or a broad group of miRNAs.

Whereas the efficacy of statins to lower cholesterol and in this manner treat atherosclerosis is the general view of their molecular mechanism, the role of the mevalonate pathway in miRNA repression of target mRNAs suggests an miRNA axis in the treatment of heart disease with statins. There are tantalizing hints of the involvement of miRNAs in heart disease and the effects of statins on miRNAs. The mammalian cardiac miRNA miR-208 has been implicated in cardiac fibrosis: Deletion of miR-208 suppresses the high-blood-pressure induction of fibrosis by the

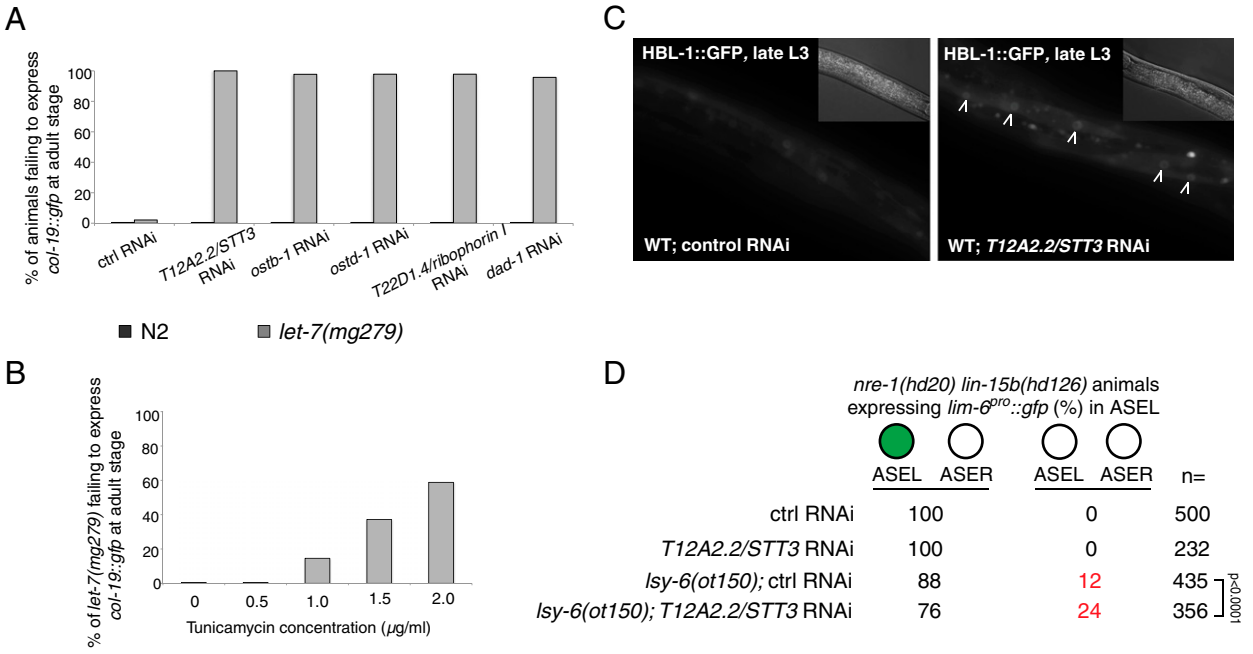

Fig. 5. The dolichol pathway for protein *N*-glycosylation is required for miRNA activity. (*A*) Inhibiting OST activity by RNAi depletion of any of its five subunits causes defects in the nominal adult-stage up-regulation of *col-19::gfp* in hyp7 cells in a *let-7(mg279)* but not wild-type background. (*B*) Tunicamycin causes defects in the nominal adult-stage up-regulation of *col-19::gfp* in the *let-7(mg279)* mutant in a dose-dependent manner. (*C*) Inhibiting OST activity by RNAi depletion of its catalytic subunit, *T12A2.2/STT3*, causes defects in the down-regulation of *hbl-1::gfp* at the L3 stage. Images were captured using the same exposure settings and processed identically. Arrowheads point to the desilenced *hbl-1::gfp* in the nuclei of hyp7 cells. (*Insets*) Nomarski images. (*D*) RNAi depletion of *T12A2.2/STT3* enhances the ASEL specification defect in the *lsy-6*(*ot150)* mutant but not wild-type genetic background. Brackets indicate a statistically significant difference as judged by a two-tailed χ^2 test.

heterochronic misexpression of the fetal myosin heavy-chain gene (32). Moreover, the mammalian miRNA miR-122 activates the replication of the hepatitis C virus (HCV) via complementary sequences in the 5′ UTR of the virus (33–36). Statins emerged from drug screens for inhibition of HCV replication and act via blocking the geranylgeranyl pyrophosphate output of the mevalonate pathway (37, 38), which possibly mediates the membrane localization of a particular viral replication protein or down-regulates mir-122 function in a manner similar to the inhibition of *let-7* and *lin-4* miRNA function in *C. elegans* we have found. Because statins are so broadly prescribed, it is important to determine whether the therapeutic activities or side effects of statin treatment are due to reduced miRNA activity.

Materials and Methods

C. elegans strains used in this study are listed in *SI Materials and Methods*. The bursting and alae assays were performed on carefully staged worms as previously described (21). Animals were scored as young gravid adults for expression of *vels13[col-19::gfp]* in hyp7 cells. Fluvastatin (EMD) was dissolved in deionized water to produce a 100 mM stock solution, and then added to nematode growth media to final concentrations mentioned in the text. Cholesterol (in ethanol), mevalonic acid [mevalonolactone powder (Sigma), dissolved in deionized water], and tunicamycin (Sigma; dissolved in DMSO) were used similarly, with concentrations mentioned in the text. Details for feeding RNAi, LIN-14 Western blots, miRNA real-time PCR, and ALG-1 immunoprecipitation (IP) are provided in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank G.R. laboratory members, in particular G. Hayes, for constructive suggestions and thoughtful critiques of the work; T. Montgomery for kindly providing the worm strain for ALG-1 IP; M. Newman for helping with mass spectrometry; and Y. Kang for help with sequencing RNAi clones. We thank F. Slack for the [*alg-1p::gfp::alg-1*] strain, and the *Caenorhabditis* Genetics Center and the Mitani laboratory for other worm strains. Z.S. is supported by an American Heart Association predoctoral fellowship. This work was supported by National Institutes of Health Grant R01-GM44619 (to G.R.).

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