Long-term, efficient inhibition of microRNA function in mice using rAAV vectors

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Understanding the function of individual microRNA (miRNA) species in mice would require the production of hundreds of loss-of-function strains. To accelerate analysis of miRNA biology in mammals, we combined recombinant adenovirus (rAAV) vectors with miRNA ‘tough decoys’ (TuDs) to inhibit specific miRNAs. Intravenous injection of rAAV9 expressing anti–miR-122 or anti–let-7 TuDs depleted the corresponding miRNA and increased its mRNA targets. rAAV producing anti–miR-122 TuD but not anti–let-7 TuD reduced serum cholesterol by >30% for 25 weeks in wild-type mice. High-throughput sequencing of liver miRNAs from the treated mice confirmed that the targeted miRNAs were depleted and revealed that TuDs induced miRNA tailing and trimming in vivo. rAAV-mediated miRNA inhibition thus provides a simple way to study miRNA function in adult mammals and a potential therapy for dyslipidemia and other diseases caused by miRNA deregulation.

miRNAs repress the expression of mRNAs with which they can partially base pair; miRNAs are predicted to regulate more than half of all protein-coding genes in mammals1,2, but few miRNA-target interactions have been experimentally validated, especially in vivo.

Genetic disruption of a miRNA gene is a powerful strategy to study miRNA function, but many miRNA genes have the same seed sequence, the 6–8 nucleotide (nt) miRNA region that defines its target repertoire, and therefore one member of a miRNA family may compensate for loss of another. Creating an animal model in which all members of a miRNA family are deleted is daunting. Moreover, humans and mice have in common more than 276 miRNA species in mice would require the production of hundreds of loss-of-function strains. To accelerate analysis of miRNA biology in mammals, we combined recombinant adenovirus (rAAV) vectors with miRNA ‘tough decoys’ (TuDs) to inhibit specific miRNAs. Intravenous injection of rAAV9 expressing anti–miR-122 or anti–let-7 TuDs depleted the corresponding miRNA and increased its mRNA targets. rAAV producing anti–miR-122 TuD but not anti–let-7 TuD reduced serum cholesterol by >30% for 25 weeks in wild-type mice. High-throughput sequencing of liver miRNAs from the treated mice confirmed that the targeted miRNAs were depleted and revealed that TuDs induced miRNA tailing and trimming in vivo. rAAV-mediated miRNA inhibition thus provides a simple way to study miRNA function in adult mammals and a potential therapy for dyslipidemia and other diseases caused by miRNA deregulation.

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Plasmid DNA vectors that express miRNA ‘sponges’, which comprise multiple tandem miRNA binding sites designed to competitively inhibit miRNA function, have been used in cultured cells9 and in flies10. Depletion of miR-223 using a sponge-expressing lentiviral vector to stably modify hematopoietic stem cells ex vivo, followed by bone marrow reconstitution in mice, produced a phenotype similar to that observed in a genetic miRNA knockout11. Lentiviral vectors delivered by stereotaxic injection into the brain have been used to express miRNA-inhibiting sponges to study miRNA function in neurons in vivo12. However, the risk of insertional mutagenesis and the requirement for ex vivo manipulation may limit the use of lentiviral vector–based miRNA inhibition for functional genomics studies and human therapy.

Delivery of miRNA inhibitors using the 4.7-kilobase (kb) single-stranded DNA parvovirus AAV promises to circumvent many of the risks associated with lentiviral vectors13. In the past decade, new rAAV vectors have been created from natural AAV serotypes, providing efficient gene transfer vehicles that target diverse tissues in mice and non-human primates14. Local delivery by direct injection of AAV2-expressed miRNA sponges into the eye has enabled inhibition of miR-96, miR-182 and miR-183 in the mouse retina15. The same miRNA cluster has been inhibited by expressing sponges from a transgene specifically transcribed in...
the mouse retina\textsuperscript{16}. More recently, compact, RNA polymerase III–driven miRNA decoys have been reported, including TuDs and miRZips, both of which inhibit miRNA function in cultured cells and in vivo\textsuperscript{17,18}. The relative potencies of these inhibitors and their ability to stably and efficiently inhibit miRNAs over the long term in adult mammals have not yet been tested.

Here we report proof-of-concept studies using TuDs delivered systemically in mice via rAAV vectors. Each TuD depleted the target miRNA and increased the expression of the corresponding miRNA target genes. miRNA depletion in vivo was accompanied by the 3′ addition of nontemplated nucleotides as well as 3′-to-5′ shortening of the miRNA, a degradation pathway previously observed in flies and transformed, cultured human cells\textsuperscript{19}. Our data suggest that rAAV-expressing TuDs could enable the study of miRNA functions in adult mammals and perhaps even form the basis for stable therapy for hypercholesterolemia and other disorders caused by aberrant miRNA expression.

**RESULTS**

TuDs are more effective than sponges or miRZips

To test the efficacy of transcribed sponges\textsuperscript{9}, TuDs\textsuperscript{17} (Supplementary Fig. 1) and miRZips (http://www.systembio.com/microrna-research/microrna-knockdown/mirzip/), we targeted the abundant miRNA miR-122, which regulates cholesterol biosynthesis, and the let-7 miRNA (Supplementary Table 1). We expressed miRNA sponges using the RNA polymerase II SV40 promoter, the liver-specific TBG promoter or the RNA polymerase III U6 promoter; we used the U6 promoter to drive TuD and miRZip expression (Fig. 1a,b).

For each miRNA inhibitor, we measured its ability to derepress a nuclear-targeted nLacZ mRNA reporter (which encodes β-galactosidase; nβ-Gal) containing one or three fully complementary miR-122–binding sites in its 3′ untranslated region (UTR). Reporter expression was reduced by \textasciitilde 50% when one miR-122–binding site was present in the nLacZ 3′ UTR and \textasciitilde 80% when three sites were present (Fig. 1c). We transfected the nLacZ reporter plasmid along with each miR-122 inhibitor construct or with a control plasmid into HuH-7, a human hepatoma cell line expressing \textasciitilde 16,000 miR-122 molecules per cell\textsuperscript{20}. Among the RNA polymerase II–driven sponges to miR-122 (anti–miR-122 sponges), only the strong liver-specific TBG promoter increased expression of nLacZ with a single miR-122–binding site, indicating that the sponge partially inhibited miR-122. However, nLacZ expression was not significantly (\(P > 0.05\)) increased by the sponge when the reporter contained three miR-122–binding sites (Fig. 1c), suggesting that the change in miR-122 activity or concentration was too small to overcome the greater repression conferred by three sites.

In contrast, both the one- and three-site reporters were derepressed by the RNA polymerase III–driven anti–miR-122 TuD. For the one-site reporter, the TuD restored nLacZ expression to that observed when no miR-122 target sites were present in the reporter (Fig. 1c).

To remove potential promoter differences, we compared three
miRNAs that are extensively complementary to their targets direct Argonaute2 protein to cleave the mRNA, whereas less extensive complementarity generally decreases mRNA stability. To test whether the TuD can also inhibit repression directed by an miRNA with imperfect complementarity to its target, we designed a firefly luciferase (FLuc) reporter with seven copies of a bulged miR-122–binding site in its 3′ UTR; FLuc with seven mutant sites served as a control (Supplementary Table 1). We transfected the miR-122–responsive FLuc reporter, anti–miR-122, anti–let-7 or control TuD plasmid and, as an internal control, a Renilla reniformis luciferase (RLuc) plasmid into HuH-7 cells. The anti–miR-122 TuD, but not the control or anti–let-7 TuDs, fully derepressed FLuc expression (Fig. 1f). The TuD inhibitors were also specific: the anti–let-7, but not the anti–miR-122, TuD increased expression

accumulates in the nucleus rather than the cytoplasm9. Again, only the TuD significantly (P < 0.001) derepressed mLacZ repression by miR-122 in HuH-7 cells (Fig. 1d). The anti–miR-122 TuD expression construct but not an anti–let-7 TuD or an anti–miR-122 or anti–let-7 sponge, was similarly effective in human embryonic kidney (HEK) 293 cells artificially expressing pri–miR-122 from a transfected plasmid (Fig. 1e).

Figure 2 | Real-time monitoring of endogenous miRNA activity using an miRNA sensor system. (a) Schematic of Gluc-expressing vectors. CB, chicken β actin promoter with CMV enhancer. ITR, inverted terminal repeat. (b,c) Gluc activity measured 48 h after AAV vector plasmids were transfected into HuH-7 (n = 6 independent trials; b) or HeLa cells (n ≥ 17; c). Error bars, s.d. (d,e) Luciferase expression in C57BL/6J mice that were administered 1 × 10^{12} genome copies of scAAV9 per mouse by tail-vein injection. Blood was collected at the indicated times and assayed for Gluc activity relative to injected Gluc vector lacking both the TuD expression cassette and 3′ UTR miRNA-binding sites. Error bars, s.d.; n = 4 for each group.
of both Dicer (Dicer1) mRNA and protein; Dicer is an endogenous let-7 target (Fig. 1g,h and Supplementary Fig. 2). Among the three miRNA antagonists we evaluated in vitro—sponge, TuD and miRZip—a TuD transcribed from a U6 promoter was the most potent miRNA antagonist.

**Monitoring miRNA function in living mice**

To test the ability of TuDs to inhibit miRNA function in vivo, we constructed rAAV vector genomes expressing a miRNA-responsive *Gaussia princeps* luciferase (GLuc) reporter gene21 (Fig. 2a). GLuc is a secreted protein, enabling detection of the reporter in the blood or urine of live animals. We added seven bulged miR-122 or let-7 target sites to the 3′ UTR of the GLuc mRNA to render it miRNA-responsive and inserted the U6 promoter-driven TuD cassette targeting either miR-122 or let-7 into the GLuc intron. Reporters lacking either the seven miRNA-binding sites, the TuD expression cassette or both served as controls. In Huh7 cells, the anti–miR-122, but not anti–let-7, TuD derepressed the reporter bearing seven miR-122–binding sites (Fig. 2b). Similarly, in HeLa cells only the anti–let-7 TuD derepressed the reporter bearing seven let-7–binding sites (Fig. 2c).

Both miR-122 and let-7 are present in liver20, and let-7 is also found in heart22, miR-122 comprises 70% of total liver miRNA20, posing a stringent test for the ability of TuDs to inhibit the function of even the most abundant miRNA species. We packaged the rAAVs into the AAV9 capsid, which preferentially transduces liver and heart. To improve transduction, we prepared all rAAVs as self-complementary genomes23. We administered the vectors intravenously to adult male C57B/6 mice and monitored GLuc activity in blood.

Initially, GLuc activity was comparable among the mice injected with vectors expressing the miR-122–regulated reporters, irrespective of the presence of a TuD expression cassette (days 3 and 7). By week 2, GLuc activity declined in mice that received vectors lacking the anti–miR-122 TuD, whereas it increased in mice treated with the anti–miR-122 TuD–expressing vector (Fig. 2d). Similarly, GLuc activity was low in mice that received the let-7–regulated reporter and high in mice that received the same reporter containing the anti–let-7 TuD cassette (Fig. 2e). One notable difference was that the let-7–regulated reporter was silenced at the earliest time point (day 3), whereas the miR-122–regulated reporter showed an initial lag (Fig. 2d,e); we do not currently understand the source of this difference. Derepression of GLuc expression by either anti–miR-122 or anti–let-7 TuD was sustained for the 25-week duration of the study, (Fig. 2d,e), during which we detected no significant (*P > 0.05*) decrease in AAV genome copies (Supplementary Fig. 3).

**scAAV9-delivered TuDs target miRNAs for destruction in mice**

Four weeks after self-complementary (sc)AAV administration, we analyzed miRNA expression in the liver using quantitative reverse transcription (RT)-PCR. We observed an ~80% reduction in miR-122 in mice that received the anti–miR-122 TuD vector compared to anti–let-7 TuD or control lacking a TuD (Fig. 3a). Northern hybridization confirmed the reduction of miR-122 in mice that received the anti–miR-122 TuD and Supplementary Fig. 4). let-7 was similarly reduced in mice treated with the anti–let-7 TuD (Fig. 3b and Supplementary Fig. 4). (Our let-7 northern blot probe cannot distinguish among the nine mouse let-7 isoforms.) In contrast, we detected no reduction for miR-26a or miR-22, two abundant liver miRNAs (Fig. 3b and Supplementary Fig. 4).

High-throughput sequencing of miRNAs from the treated livers supports the view that scAAV9-delivered TuDs effectively and specifically trigger the destruction of complementary miRNAs. The TuD targeting miR-122 reduced the abundance of full-length, 23-nt miR-122 4.3-fold (0.233 of the initial amount), consistent with our qRT-PCR results (Figs. 1b and 3a,c). Abundance of 21- and 22-nt miR-122 isoforms decreased to a lesser extent, whereas the amount of 20-, 19- and 18- nt isoforms increased, suggesting that the TuD triggered 3′-to-5′ exorucleolytic trimming of miR-122 (Fig. 3c). As is the case with antisense-directed destruction of miRNAs in cultured human cells19, the anti–miR-122 TuD promoted the addition of nontemplated nucleotides to the 3′ end of miR-122 (Fig. 3d). Prefix-matching reads, which are sequences that initially match the mouse genome but end with nontemplated nucleotides, doubled in the anti–miR-122 TuD mouse compared to the control (Fig. 3d). The 3′ nontemplated nucleotides...
TuDs delivered with scAAV9 increase endogenous miRNA targets

TuD inhibitors delivered using scAAV9 derepressed miR-122– and let–7–regulated miRNAs in vivo (Fig. 5). We used qRT-PCR and western blotting (Fig. 5a,b and Supplementary Fig. 8) to analyze the expression of known miR-122 and let–7 targets in liver and heart four weeks after injection of the scAAV9 vectors. Mice injected with scAAV9 expressing the GLuc reporter but no TuD served as a control. For anti–miR-122 TuD-treated mice, we detected in the liver a 2.5- to 4.2-fold increase in Aldoa (3.3 ± 0.5; P < 0.04), Tmed3 (4.2 ± 1.5; P < 0.01), Hfe2 (3.3 ± 1.0; P < 0.02), and Ccng1 (2.5 ± 0.4; P < 0.001) miRNAs, all previously shown to be regulated by miR-122; expression of these was unaltered in the heart, which lacks miR-122 (Fig. 5a). We found no significant (P > 0.05) change in these four miR-122–regulated miRNAs in either liver or heart from mice that received the vector expressing anti–let–7 TuD (Fig. 5a). We also detected a significant increase in Hfe2 (1.5 ± 0.1; P < 0.02) and Tmed3 (1.6 ± 0.1; P < 0.008) proteins in the livers of anti–miR-122 TuD–treated mice (Fig. 5b and Supplementary Fig. 8).

The miRNA encoding the miRNA-producing enzyme Dicer is itself repressed by let–7. Using qRT-PCR we measured Dicer mRNA abundance in mice that received scAAV9 vector expressing either anti–miR-122 or anti–let–7 TuDs (Fig. 5a). When let–7 was inhibited, Dicer mRNA increased in both liver (1.9 ± 0.2; P ≤ 0.001) and heart (2.4 ± 0.4; P ≤ 0.003). Similarly, Dicer protein levels increased in the liver (2.0 ± 0.6; P < 0.05; Fig. 5b). (We did not examine protein levels in the heart.) The RAS family genes Hras, Nras and Kras have also been reported to be repressed

Our data suggest that TuDs act, at least in part, by targeting miRNAs for destruction via the tailing and trimming pathway. The mRNA encoding the miRNA-producing enzyme Dicer is itself repressed by let-7. Using qRT-PCR we measured Dicer mRNA abundance in mice that received scAAV9 vector expressing either anti–miR-122 or anti–let–7 TuDs (Fig. 5a). When let-7 was inhibited, Dicer mRNA increased in both liver (1.9 ± 0.2; P ≤ 0.001) and heart (2.4 ± 0.4; P ≤ 0.003). Similarly, Dicer protein levels increased in the liver (2.0 ± 0.6; P < 0.05; Fig. 5b). We did not examine protein levels in the heart. The RAS family genes Hras1, Nras and Kras have also been reported to be repressed...
by let-7. We detected increased expression of Nras in both liver (1.3 ± 0.1; P ≤ 0.01) and heart (1.3 ± 0.1; P ≤ 0.02) and of Hras1 (1.3 ± 0.1; P ≤ 0.04) in heart in anti–let-7 TuD–treated, but not anti–miR-122 TuD–treated mice (Fig. 5a).

**Anti–miR-122 TuD reduced cholesterol levels**

miR-122 is required for normal cholesterol biosynthesis; inhibition of miR-122 with AMOs decreases cholesterol metabolism in adult mice26,27 and non-human primates6,7. In wild-type mice, a single intravenous injection of scAAV9 expressing anti–miR-122 TuD significantly reduced total serum cholesterol (45 ± 5%; P ≤ 0.001) and high-density lipoprotein (HDL; 42 ± 5%; P ≤ 0.001) beginning 2 weeks after injection, and this reduction was sustained for the 25-week duration of the study. LDL levels also decreased (88 ± 102%; P ≤ 0.05) by the third week and remained depressed throughout the study (Fig. 6a). Total serum cholesterol, HDL and LDL levels were unaltered in mice that received the anti–let-7 TuD. We detected no significant

**DISCUSSION**

Our data suggest that, in mice, TuDs inhibited their miRNA targets via the target RNA–directed tailing and trimming pathway19. This work, in addition to observations in flies and cultured cells, suggests that the pathway is widely conserved among animals. Targeted miRNA destruction triggered by TuDs was sequence-specific for the nine let-7 isoforms. Compared to let-7a, for which the TuD was fully complementary, a single purine:purine mismatch between the TuD and let-7e reduced miRNA degradation by more than threefold, and four mismatches (between the TuD and let-7i) reduced miRNA destruction eightfold. We are therefore optimistic that future studies will reveal the rules for designing TuD inhibitors that target individual miRNA isoforms that differ by just one or two nucleotides.

The GLuc sensor system described here provides a simple means to detect changes in specific miRNA function, such as those caused by miRNA inhibitors, in live adult mammals over time. This system allows one to assess the activity of a specific miRNA in a cell line, tissue or organ, providing a quantitative measure of the effectiveness of an miRNA antagonist.

Retrospective profiling has linked aberrant miRNA expression to a variety of diseases, suggesting that miRNAs may provide new targets for therapy. Indeed, miR-122 inhibition by AMOs5–8 or scAAV-delivered TuDs lowered both HDL and LDL. However, the current view that HDL protects against heart attack argues that therapy for dyslipidemia should lower LDL but raise HDL levels. Recently, miR-33 was identified as a repressor of HDL biogenesis; miR-33 inhibition raises serum HDL level18. Perhaps simultaneous inhibition of miR-122 and miR-33 by a pair of TuDs expressed from a single scAAV vector may achieve a more balanced and healthy cholesterol profile and provide long-lasting therapy for familial hypercholesterolemia.

Low miR-122 levels have been associated with hepatocellular carcinoma in rodents and humans25–27, although no direct causal link has been established26,27. Because AAV vector expression is stable for years in rodents and primates, animals treated with scAAV9 expressing anti–miR-122 should enable testing the safety of prolonged miR-122 inhibition in general and the increased risk of developing hepatocellular carcinoma in particular.

**METHODS**

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

**Accession code.** Short Read Archive: GSE25971.

Note: Supplementary information is available on the Nature Methods website.

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ONLINE METHODS

Construction of miRNA antagonist and sensor plasmids. The siFluc fragment in pRNA-U6.1/Neo-siFluc (GenScript) was replaced with TuD mir-122, TuD let-7, mir-122–Zip, let-7–Zip, mir-122 sponge and let-7 sponge that were as previously described29 (http://www.systembio.com/microrna-research/microrna-knockdown/mirzip/) to generate U6-driven expression cassettes for expression of different miRNA antagonists. The XbaI–Apal linker was generated by annealing oligonucleotide pairs, XbaI–Apal linker F and XbaI–Apal linker R (Supplementary Table 1), followed by cloning into the Apal site after the Fluc gene in pGL3-control plasmid. The chemically synthesized mir-122 or let-7 sponge sequence flanked with XbaI and Apal sites was digested and cloned into the XbaI–Apal polyn linker of pGL3 to create SV40 promoter–drive sponge expression cassettes. Then, the fragment containing the Fluc gene and mir-122 or let-7 sponge was isolated by NcoI and Apal double digestions from pGL3 mir-122 sponge or pGL3 let-7 sponge and cloned into the Kpn1 site of pAAVCB vector plasmid or between PstI and MluI sites of pAAVTBGVI plasmid to generate CB and TBG promoter–drive sponge expression vectors, respectively.

One or three copies of perfectly complementary miRNA target sites were designed based on the annotated miRNA sequences in miRBase28 and inserted into the BstBI restriction site in the 3′ UTR of the nLacZ expression cassette of the ubiquitously expressed pAAVCB nuclear-targeted β-galactosidase (nLacZ) plasmid, or into the 3′ UTR of RLuc in the psiCHECK-2 plasmid (Promega) using synthetic oligonucleotides (Supplementary Table 1). To express mir-122, a pri-mir-122-122 fragment was amplified by PCR from mouse genomic DNA with specific oligonucleotides (Supplementary Table 1) and cloned into the XbaI restriction site of pAAVCB. The identity of the XbaI–Apal linker was verified by sequencing. scAAV9 vectors used in this study were generated, purified and titered as previously described29.

To create AAV vectors, seven copies of bulged target sites for mir-122 or let-7 were synthesized and cloned into the BclI site of the GLuc reporter gene in the pscAAVCB plasmid. The identity of the pri-mir-122-122 was verified by sequencing. scAAV9 vectors used in this study were generated, purified and titered as previously described29.

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Cell culture. HEK 293, HuH-7 (ref. 30) and HeLa cells were cultured in Dulbecco’s Modified Eagle Medium supplemented with 10% FBS and 100 mg/l of penicillin-streptomycin (HyClone). Cells were maintained in a humidified incubator at 37 °C and 5% CO2. Plasmids were transiently transfected into cells using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer’s instructions.

Reporter assays. nLacZ reporters were assayed by staining for nβGal activity 48 h after transfection and counting the number of blue cells. FLuc and RLuc were assayed by lysing the cells with passive lysis buffer (Dual-Glo Luciferase Assay System, Promega), and 10 μl lysate was used for the assay. FLuc and RLuc activities were assessed using the Dual-Glo Luciferase Assay System according to the manufacturer’s instructions. The GLuc assay was performed as described21 using 10 μl culture medium from each transfection. To monitor GLuc expression in vivo, mice were bled from a superficial cut on a facial vein made with a 5.5 mm animal lancet (MEDIpoint); 5 μl blood was assayed.

Mice. C57BL/6J mice (Charles River Laboratories) were maintained and used according to the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School. Four- to six-week-old wild-type C57BL/6J male mice were treated by tail vein injection with AAV vectors at 1 × 1012 genome copies/mouse or 5 × 1013 genome copies/kg. To monitor lipid profiles, serum samples were collected and analyzed for total cholesterol, HDL and LDL using a COBAS C 111 analyzer (Roche Diagnostics). For RNA analyses, mice were necropsied four weeks after the treatment; liver and heart tissues were collected for RNA isolation. rAAV genome copy numbers in total liver DNA were determined as described previously31.

qRT-PCR analysis. RNA was extracted using Trizol (Invitrogen), according to the manufacturer’s instructions. Total RNA (0.5–1 μg) was primed with random hexamers and reverse-transcribed (Multiscribe Reverse Transcriptase, Applied Biosystems). Quantitative PCR reactions were performed in triplicate with 0.3 μM gene-specific primer pairs (Supplementary Table 1) using the GoTaq qPCR master mix (Promega) in a StepOne Plus Real-time PCR instrument (Applied Biosystems). The expression of mature mir-122 and U6 was assayed using the TaqMan microRNA Assay (Applied Biosystems).

Northern blot analysis. To detect mir-122, miR-26a, miR-22 and let-7 in total liver RNA, 10 μg total RNA was resolved by denaturing 15% PAGE, transferred to Hybond N+ membrane (Amersham BioSciences), and cross-linked with 254-nm light (Stratagen). Synthetic DNA oligonucleotides (Supplementary Table 1), 5′ end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (NEB), were used as probes for miR-122, miR-26a, miR-22 and let-7 and U6 (Supplementary Table 1) and hybridized in Church buffer (0.5 M NaHPO4, pH 7.2, 1 mM EDTA and 7% (w/v) SDS) at 37 °C. Membranes were washed using 1× SSC, 0.1% (w/v) SDS buffer and then visualized using a FLA-5100 Imager (FUJIFILM). High-resolution northern analysis was performed as described previously19.

Small RNA sequencing. Small RNA libraries were constructed and sequenced as described previously19. Briefly, 50 μg total RNA was isolated with the mirVana kit (Ambion), and 19–29 nt small RNAs were purified by electrophoresis through a 15% polyacrylamide/urea gel (SeqLab Gel, National Diagnostics). IDT miRNA cloning linker-1 was ligated to the 3′ end of small RNAs using truncated T4 RNA ligase 2 (NEB) and the products gel purified; a 5′ RNA adapter was ligated to the 3′ ligation products using T4 RNA ligase. The final ligation products were used as templates for reverse transcription using the small RNA RT primer. The cDNA was amplified with small RNA PCR primer 1 and RNA PCR primer 2. The PCR product was gel-purified and submitted for high-throughput sequencing. Supplementary Tables 2 and 3 summarize the sequencing results. Small RNA analyses were as previously described19. The sum of genome matching and prefix-matching (tailed) reads were used to determine sequencing depth, which in
turn was used for normalization, allowing direct comparison of the relative abundance of genome- and prefix-matching reads. Because deep sequencing of small RNA from liver of mice expressing anti–let-7 TuD exhibited a general reduction in miRNA abundance after normalization to sequencing depth, we used northern hybridization to determine the change in abundance of miR-21, miR-29a, miR-29b, miR-126 and miR-16 in total liver RNA from three biological replicates each for AAV-control– and AAV-TuD-let-7–treated mice. The average relative change of these miRNAs between AAV-control and AAV-TuD-let-7 samples was used to correct the normalized miRNA abundance in the corresponding high-throughput sequencing dataset.

**Western blot analysis.** Proteins were extracted with RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP40 (v/v), 1% sodium deoxycholate (w/v) and 0.1% SDS (w/v)) containing a protease inhibitor mixture (Boston BioProducts). Protein concentration was determined by Bradford assay. Protein samples, 50 µg each, were loaded onto 10% polyacrylamide gels, electrophoresed, and transferred to a nitrocellulose membrane (Amersham BioSciences). Briefly, membranes were blocked with blocking buffer (LI-COR) at room temperature for 2 h, followed by incubation with anti-GAPDH (1:10,000; Millipore), anti-Hfe2 (1:1,000), anti-CyclinG1 (1:1,000), anti-Dicer (1:300; Santa Cruz Biotechnology), anti-Ras (1:1000; Millipore) or anti-Tmed3 (1:400; Sigma-Aldrich) for 2 h at room temperature. After three washes with PBS plus 0.1% Tween-20 (v/v), membranes were incubated for 1 h at room temperature using secondary antibodies conjugated to LI-COR IRDye. Signals were detected using an Odyssey Imager (LI-COR).

**Statistical analysis.** Results are reported as mean ± s.d. and compared between groups using the two-tailed Student’s t-test, except for Figure 6c, where the Mann-Whitney U test was used.

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