Antisense oligonucleotides targeting the miR-29b binding site in the GRN mRNA increase progranulin translation

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Heterozygous GRN (progranulin) mutations cause frontotemporal dementia (FTD) due to haploinsufficiency, and increasing progranulin levels is a major therapeutic goal. Several microRNAs, including miR-29b, negatively regulate progranulin protein levels. Antisense oligonucleotides (ASOs) are emerging as a promising therapeutic modality for neurological diseases, but strategies for increasing target protein levels are limited. Here, we tested the efficacy of ASOs as enhancers of progranulin expression by sterically blocking the miR-29b binding site in the 3′ UTR of the human GRN mRNA. We found 16 ASOs that increase progranulin protein in a dose-dependent manner in neuroglioma cells. A subset of these ASOs also increased progranulin protein in iPSC-derived neurons and in a humanized Grn mouse model. In FRET-based assays, the ASOs effectively competed for miR-29b from binding to the GRN 3′ UTR RNA. The ASOs increased levels of newly synthesized progranulin protein by increasing its translation, as revealed by polysome profiling. Together, our results demonstrate that ASOs can be used to effectively increase target protein levels by partially blocking miR binding sites. This ASO strategy may be therapeutically feasible for progranulin-deficient FTD as well as other conditions of haploinsufficiency.

Progranulin is a lysosomal and secreted protein with pleiotropic effects, including promoting neuronal survival, neurite outgrowth, wound healing, tumor cell growth, and modulating inflammation (1, 2). In humans, heterozygous GRN mutations cause frontotemporal dementia (FTD) due to progranulin haploinsufficiency (3, 4) and reduced progranulin levels are a risk factor for Alzheimer’s disease (5–11). Therefore, increasing progranulin levels is a therapeutic goal for these forms of dementia (12, 13). Gene therapy studies in mice provide proof of concept that restoring progranulin in heterozygous Grn mice improves FTD-associated neuropathology and behavioral deficits (14). Current therapeutic efforts are focused on small molecules that increase progranulin expression (15–18), gene therapies (14, 19), monoclonal antibodies that modulate progranulin trafficking (20), and protein replacement (21). However, there are currently no approved therapies for progranulin-deficient FTD.

Antisense oligonucleotides (ASOs), short synthetic oligonucleotides used to modulate target RNAs, are emerging as a promising therapeutic modality for neurological diseases (22, 23). The most commonly used ASO strategies involve RNase H1-mediated degradation of the target mRNA and modulation of splicing of the target mRNA (22). ASO-based strategies for increasing target protein levels are still relatively limited. Several strategies have been reported, including targeting upstream ORFs (uORFs) and regulatory elements in the 5′ UTR (24, 25). However, these approaches are limited by the fact that not all genes possess uORFs and/or 5′ regulatory elements.

MicroRNAs (miRs) have been estimated to regulate more than 60% of all human proteins (26). These miRs typically bind to the 3′ UTR of target mRNAs and decrease protein levels through translational repression or mRNA decay (27, 28). For progranulin, three miRs have been reported to negatively regulate progranulin protein levels: miR-29b, miR-107, and miR-659 (29–33). The binding sites of miR-29b and miR-659 have been mapped to the GRN 3′ UTR (29–32). Notably, the miR-659 binding site overlaps with the rs5848 SNP (31), which is a risk factor for FTD (31) and Alzheimer’s disease (9, 34). In silico analyses predict that this SNP affects the binding affinity of miR-659 to the GRN mRNA, with the T allele having a stronger binding and therefore greater translational repression. Consistent with this, individuals with the TT genotype (minor allele) at rs5848 have ~30% decreased progranulin protein in brain tissue and in CSF (31, 35), suggesting that miR-mediated...
modulation of progranulin levels contributes to the risk of these forms of dementia. Thus, these miRs present as potential targets for increasing progranulin protein.

In the current study, we tested a therapeutic strategy for increasing progranulin protein levels by using ASOs to target the miR-29b binding site in the human GRN mRNA. We show that these ASOs effectively increase progranulin protein in cultured cells, in iPSC-derived neurons, and in mouse brains. Additionally, we determined the mechanism of action is that these ASOs displace miR-29b from its binding site and thereby derepress translation, resulting in increased synthesis of progranulin protein.

Results

ASOs targeting the miR-29b binding site of the GRN mRNA increase progranulin protein levels

Previous studies have reported that broad inhibition of miR-29b increases progranulin protein levels in cells (29, 30). We confirmed that similar treatment with a miR-29b inhibitor increases progranulin protein levels in differentiated SH-SY5Y human neuroblastoma cells (Fig. S1) and in H4 human neuroglioma cells (Fig. 1A), which have moderate expression of miR-29b (Fig. S2A). Data from the Human miRNA Tissue Atlas (36) show that miR-29b is expressed in the human brain (Fig. S2B).

Because broad inhibition of miR-29b affects levels of other miR-29b targets and therefore has many other effects (37), we sought to test if ASOs targeting the miR-29b binding site in the GRN mRNA can increase progranulin protein levels in a more specific manner. To this end, we designed a panel of 18-mer ASOs with 2’-O-methoxyethyl (2’-MOE) ribose and phosphorothioate backbone modifications that span the entire miR-29b binding site with single nucleotide resolution (Fig. 1B and Table S1). Our initial ELISA-based screening at 5 μM in H4 cells identified 10 ASOs that significantly increased progranulin protein levels, compared to a non-targeting control ASO (Fig. 1C). We subsequently validated these 10 ASOs (M1, M2, M3, M5, M6, M10, M11, M31, M36, M39) and identified 6 additional ASOs (M4, M25, M28, M29, M38, M40) that increased progranulin protein levels at 10 μM, as determined by Western blot (Fig. S3). To ensure these effects were specific, we retested a subset of these ASOs (M5, M10, M36) against a panel of negative control ASOs with 2 bp or 4 bp mismatches (Table S2) and found that the introduction of these mismatch sequences largely abolished the ASOs’ activity (Fig. S4). These

Figure 1. ASOs targeting the miR-29b binding site in the human GRN mRNA increase progranulin protein levels. A, broad inhibition of miR-29b increases progranulin protein levels in H4 neuroglioma cells. ELISA for progranulin protein levels 24 h after transfection with 100 nM miR-29b inhibitor using Lipofectamine 3000. B, schematic of the GRN 3’ UTR indicating miR-29b binding site and region targeted by ASOs. C, validation of ASO hits from initial screening. H4 cells were treated with 5 μM ASO for 24 h, and progranulin levels were measured in cell lysates by ELISA. Data are presented as means ± SD; individual data points represent biological replicates. * indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001, and **** indicates p < 0.0001, as determined by one-way ANOVA with Dunnett post hoc test.

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results suggest that the activity of the progranulin-targeting ASOs are likely the result of on-target effects. Because progranulin is also secreted from cells, we tested if the ASOs similarly increased progranulin in the conditioned media. We found that ASOs that increased cellular progranulin levels also increased secreted progranulin levels (Fig. S5), suggesting that the observed increases in cellular progranulin levels are not due to altered trafficking or secretion.

We noted that the efficacious ASOs largely clustered in two regions: (1) overlapping with the seed sequence (M25–M40), and (2) toward the 5’ end of the miR-29b binding site (M1–M6, M10–M11). Notably, ASOs M1–M5 do not overlap with the binding site. The predicted secondary structure of the GRN mRNA (Fig. S6) reveals that this region forms a large loop, which could increase accessibility for ASO binding. Moreover, this loop is in close proximity to the miR-29b binding site and may interact with the miR-29b binding site, which could in part explain the efficacy of ASOs M1–M5. For subsequent mechanistic studies, we chose to focus on several representative ASOs: M5, M10, and M36.

Next, we carried out time course and dose curve experiments for selected ASOs in H4 cells. The time course studies revealed that increased progranulin protein levels are detectable within 1 h of treatment for ASO M10 and by 8 h for ASO M36 (Fig. 2A). The slower kinetics of ASO M36 may be due its binding properties, as it has a lower melting temperature than ASO M10. Overall, the time frame of these ASO effects is consistent with previous reports of rapid internalization of 2-MOE ASOs by cells that is detectable within 30 min (38) and of detectable depression of a microRNA let-7a Renilla luciferase reporter within 2 to 3 h (39). Dose curve experiments showed that the ASOs exhibit dose-dependent effects (Fig. 2B). The EC50 values (half maximal effective concentrations) of these ASOs were 1 to 6 μM, as calculated by nonlinear regression (1.7 μM for M5; 5.9 μM for M10; 1.0 μM for M36). These ASOs also increase progranulin levels in a dose-dependent manner in iPSC-derived cortical neurons (Fig. 2C). These ASOs exhibited no detectable toxicity in H4 cells, as determined by MTT assays (Fig. S7).

**ASO effect requires miR-29b and involves competition of miR-29b from the GRN 3’ UTR**

We next tested if the ASOs act through miR-29b by using a miR-29b inhibitor. This inhibitor binds to miR-29b and thereby prevents miR-29b binding to its target RNAs. The experiments of Figure 3A demonstrate that ASOs M5, M10, and M36 increased progranulin levels similarly to the miR-29b inhibitor. Importantly, the ASOs’ ability to increase progranulin levels was largely absent in the presence of the miR-29b inhibitor, suggesting that the ASOs block miR-29b’s effect on progranulin. For ASO M36, co-treatment with the miR-29b inhibitor did further increase progranulin levels, likely due to the relatively weaker effect of ASO M36 compared to ASOs M5 and M10.

To test if the ASOs can compete for binding of miR-29b, we used a FRET-based assay to monitor the in vitro interaction between miR-29b and a GRN 3’ UTR RNA that contains the miR-29b binding site. Using this assay, we show that ASO M36 effectively competed off miR-29b (Fig. 3B), with an IC50 value of 0.53 μM. In contrast, the non-targeting control ASO was unable to displace miR-29b. Together, these experiments indicate that the ASOs’ effects require miR-29b and involve competition for miR-29b binding to the GRN mRNA.

**ASOs increase the translation of progranulin**

Despite increasing progranulin protein levels, the ASOs did not increase GRN mRNA levels in H4 cells, compared to a non-targeting control ASO or vehicle (Fig. 4A), suggesting that the ASOs increase the rate of progranulin translation. This would be consistent with the canonical effects of translational repression by many miRs (27, 28). To formally test this, we

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**Figure 2. Time course and dose curve experiments for selected ASOs.** A. ASOs increase progranulin protein levels within several hours. H4 cells were treated with 10 μM ASO for the indicated times, and progranulin levels in cell lysates were determined by Western blot. B. Dose curve of ASO treatment in H4 cells (24 h). Progranulin levels were measured in cell lysates by ELISA. Data from two experiments were normalized to the untreated control group of each respective experiment, and data are presented as means ± SD; individual data points represent biological replicates. C. ASOs increase progranulin protein levels in neurons. Western blot of progranulin in iPSC-derived neurons following ASO treatment at the indicated concentrations for 3 to 4 days.
performed polysome profiling to assess the amount of polysome-bound GRN mRNA, which reflects its rate of translation. We found that cells treated with ASOs M10 and M36 had marked enrichment of GRN mRNA in the heavy polyribosome fractions (11–16), compared to cells treated with a non-targeting control ASO (Fig. 4B), indicating that these

**Figure 3. ASOs act through blocking miR-29b binding.** A, ASOs require miR-29b to increase progranulin protein levels. ELISA for progranulin protein levels after co-treatment with miR-29b inhibitor (70 μM) and ASOs (20 μM) for 24 h. B, FRET assay demonstrating that ASO M36 can compete miR-29b from binding to a partial GRN 3′ UTR RNA. Data are presented as means ± SD; individual data points represent biological replicates. * indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001, and **** indicates p < 0.0001 as determined by two-way ANOVA with Tukey post hoc test. n.s., not significant.

**Figure 4. ASOs increase progranulin translation.** A, GRN mRNA levels in H4 cells following ASO treatment (5 μM, 24 h), as determined by qPCR. B, GRN mRNA distribution in each of 16 sucrose gradient fractions from ASO-treated H4 cells (5 μM, 22 h) analyzed by qPCR. Fraction 1: hydrolysoluble fraction; fractions 2, 3, and 4: 40S, 60S, and monosomes, respectively; fractions 5 to 10: light polyribosomes; and fractions 11 to 16: heavy polyribosomes. Data are plotted as a fraction of total mRNA on the gradient. UV traces are shown on the right side. C, Western blot showing immunoprecipitated, newly synthesized, AHA/biotin-labeled progranulin protein following ASO treatment in H4 cells (10 μM, 24 h). The streptavidin-bound fluorescence signal co-localizing with progranulin was quantified in three independent experiments. Data are presented as means ± SD; individual data points represent biological replicates. * indicates p < 0.05, as determined by one-way ANOVA with Dunnett post hoc test. n.s., not significant. PGRN, progranulin.
ASOs increase the rate of progranulin translation. This effect was specific to GRN mRNA, as it was not observed with FUS mRNA (data not shown), which is linked to amyotrophic lateral sclerosis (ALS) and FTD.

To determine if the ASOs increase the amount of newly synthesized progranulin protein, we used a clickable methionine analog azidohomoalanine (AHA) to label nascent proteins (40). As shown in Figure 4C, we treated H4 cells with ASOs for 21 h, then briefly removed methionine from the culture media, followed by the addition of AHA for incorporation into nascent proteins for 2 h. With the cell lysates, we performed a click reaction to conjugate biotin to AHA-labeled proteins. Following immunoprecipitation of progranulin, western blots using fluorescently labeled streptavidin confirmed that ASOs M5 and M36 increase the synthesis of progranulin protein (Fig. 4C). Together, these studies establish the mechanism of action is that these ASOs displace miR-29b from its binding site and thereby derepress translation, resulting in increased synthesis of progranulin protein.

**ASOs increase progranulin protein levels in vivo**

Lastly, we tested ASO M5 in vivo using a recently developed humanized GRN mouse model (41). This model is a BAC transgenic mouse line in which the human GRN gene (including the 3’ UTR) was inserted in the Hprt locus on the mouse X chromosome. At 3 weeks following intracerebroventricular (ICV) administration of 500 μg ASO (42), we observed increased human progranulin protein by ELISA in the cortex of mice that received ASO M5 (Fig. 5A). Progranulin levels were increased by 63% and 61% in male and female mice, respectively, compared to mice of the same sex that received the non-targeting control ASO. By Western blot, we also increased human progranulin levels in the cortex, thalamus, and hippocampus of male mice that received ASO M5 (Fig. 5B). These effects are consistent with the ASO distribution in these brain regions (Fig. S8), as detected by immunofluorescence using an antibody that recognizes the ASO backbone (43). ASO M5 did not increase GRN mRNA levels in the cortex (Fig. 5C), suggesting that it increases progranulin translation, similar to our findings in cells (Fig. 4). Levels of inflammation were comparable in mice that received ASO M5 and the control ASO (Fig. 5D). Additionally, ASO M5 was tolerated in wild-type mice when tested at a higher 700 μg dose, based on post-injection observation and assessments of inflammation in the CNS (Fig. S9). Together, these results demonstrate that ASOs targeting the miR-29b binding

**Figure 5. ASOs increase progranulin protein levels in mouse brains.** A–B, human progranulin levels in brains of humanized GRN mice (2–5 months old) at 3 weeks after ICV administration of 500 μg ASO. A, Human progranulin levels in cortex, as determined by ELISA. B, Western blot of human progranulin in cortex, thalamus, and hippocampus. C–D, qPCR analysis of cortical tissue showing that ASO M5 does not increase GRN mRNA levels (C) and is tolerated (D). Levels of inflammation were comparable in mice that received ASO M5 and the control ASO. Non-Tg, non-transgenic. * indicates p < 0.05, as determined by one-way ANOVA with Dunnett post hoc test.
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site can increase human progranulin protein levels in cultured cells, neurons, and in vivo.

Discussion

In the current study, we show that ASOs targeting the miR-29b binding site can increase human progranulin protein levels in cultured cells, in iPSC-derived neurons, and in the brains of humanized GRN mice. In cells, the ASO effects are dose-dependent and are detectable within several hours. Our mechanistic studies revealed that the ASOs act through miR-29b, compete for miR-29b binding to the 3’ UTR of the GRN mRNA, increase the rate of progranulin translation, and ultimately increase the amount of newly synthesized progranulin protein. To our knowledge, this is the first in-depth characterization of using ASOs to increase the translation of a target protein by sterically blocking a miR binding site. Several previous studies used oligonucleotides to block miR binding sites (44–49), but to our knowledge, this is the first report using this strategy to increase progranulin levels.

This ASO-based strategy has several notable advantages. The increased progranulin protein is endogenously produced from the intact GRN allele, and therefore is expected to have identical properties and function; this avoids unexpected complications from ectopic GRN expression and/or modified forms of recombinant progranulin used in other therapeutic strategies. Additionally, this strategy increases progranulin protein levels within a narrow physiological window and thus would avoid issues arising from high-level overexpression of progranulin, such as possible tumorigenesis (50, 51). Moreover, by targeting regions partially overlapping with the miR-29b binding site in the GRN mRNA increases the specificity of our approach by avoiding other miR-29b target genes due to sequence differences between genes in the regions flanking the binding site. Lastly, this ASO strategy targeting the miR-29b binding site is agnostic to the specific disease mutation and could be used in the context of any of the more than 70 FTD-associated, loss-of-function GRN mutations that have been identified (52).

Several lines of evidence support that the increases in progranulin protein levels observed following treatment with the GRN-targeting ASOs are the result of on-target effects mediated by sterically blocking miR-29b binding to its binding site in the GRN 3’ UTR. First, the ASOs are able to physically compete for miR-29b binding to the GRN 3’ UTR RNA in a FRET-based assay (Fig. 3B). Second, the ASOs’ effects are dependent on miR-29b, as the ASOs do not increase progranulin protein levels when miR-29b is blocked (Fig. 3A). Furthermore, the ∼70% maximal increases in the progranulin levels observed with the GRN-targeting ASOs in H4 cells (ASOs M5 and M10 in Fig. 2B) are comparable to the ∼70% maximal increases observed with a broad miR-29b inhibitor (Fig. 3A). Third, in polysome profiling experiments, the ASOs specifically increase the rate of GRN translation (Fig. 4B); such an effect was not observed with FUS mRNA (data not shown), which is linked to ALS and FTD. Fourth, in silico analysis found no predicted off-target RNAs in the human genome with zero mismatches for any of the ASOs used in this study. Further analyses allowing for 1 bp mismatch revealed a total of seven potential off-target RNAs within exonic sequences; however, three are not expressed in the human brain based on the GTEx Portal (53), and none of the remaining four potential targets are shared by multiple ASOs. Lastly, this effect on progranulin protein levels was observed with multiple efficacious GRN-targeting ASOs (16 in total), when compared to multiple mismatch negative control ASOs (Fig. S4) and many non-efficacious GRN-targeting ASOs with identical chemistry (Fig. 1, B and C), further strengthening the case that these are on-target effects.

It is unclear how ASOs M1–M5 increase progranulin levels, despite not overlapping with the binding site. Based on the predicted secondary structure of the GRN mRNA (Fig. S6), the region targeted by ASOs M1–M5 harbors a large loop, which could provide increased accessibility for ASO binding. Moreover, this loop is in close proximity to the miR-29b binding site and may interact with the miR-29b binding site, which could in part explain the efficacy of ASOs M1–M5. Our data suggests that, similar to ASOs that overlap with the binding site, ASO M5 increases progranulin translation (Fig. 4C) and acts in a miR-29b-dependent manner (Fig. 3A).

Overall, ASOs are proving to be a promising therapeutic modality for CNS diseases, as ASOs are versatile modulators of endogenous RNAs, well tolerated in humans, and stable in the CNS with a long duration of action (22, 23). Future studies will focus on testing if these ASOs targeting the miR-29b binding site can delay or prevent FTD-associated neuropathology and behavioral deficits in mouse models. While the current study focuses on ASOs that target the miR-29b binding site, we have also tested a smaller panel of ASOs targeting the miR-659 binding site (31, 32) in the GRN 3’ UTR (see Fig. 1B) and identified ASOs that similarly increase progranulin protein levels (Fig. S10A). Moreover, ASOs targeting the miR-659 and miR-29b binding sites have additive effects (Fig. S10B); this may provide an opportunity to further increase progranulin in vivo. Our results suggest that this ASO strategy may be broadly applicable for pharmacologically increasing levels of target proteins and could be particularly useful for the development of ASO-based therapies for diseases of haploinsufficiency.

Experimental procedures

ASOs

ASOs used in these studies were 18-mer ASOs uniformly modified with 2’-O-methoxyethyl (2’-MOE) sugars with a phosphorothioate backbone, with the following exceptions: ASOs used in the experiment shown in Fig. S10A were modified with 2’-O-methyl (2’-O-Me) sugars with a full phosphorothioate backbone; ASO M5 in Figure 5 was modified with 2’-MOE with a mixed phosphorothioate backbone; and the miR-29b inhibitor is a 23-mer ASO containing 2’-MOE sugar and phosphorothioate backbone modifications. ASO sequences and chemistries are provided in Tables S1 and S2. ASOs used for in vitro and cell-based studies were
dissolved in water and stored at −20 °C. For in vivo studies, lyophilized ASOs were dissolved in sterile PBS without calcium or magnesium (Gibco, 14190-250) and sterilized by passing through a 0.2 μm filter. The following mirVana miRNA inhibitors were obtained from ThermoFisher: miR-29b inhibitor (4464084, Assay ID: MH110103) and Negative Control #1 (4464076). Off-target candidate genes with complementary RNA sequences were predicted using GGGenome with RefSeq human RNA release 205 (March 2021).

**Cell culture**

H4 human neuroglioma cells (HTB-148) and SH-SY5Y human neuroblastoma cells (CRL-2266) were obtained from ATCC. H4 cells were cultured in DMEM (Dulbecco’s Modified Eagle Medium, high-glucose) (Gibco, 11995-073) supplemented with 10% fetal bovine serum (FBS) (Gibco, 26140-095), 10 μg/ml penicillin, and 10 μg/ml of streptomycin. SH-SY5Y cells were cultured in DMEM/F12 media (Gibco, 10565-018) supplemented with 10% heat-inactivated FBS, 10 μ/ml penicillin, and 10 μg/ml of streptomycin. All cell lines were free from mycoplasma contamination. For neuronal differentiation, SH-SY5Y cells were cultured in Neurobasal Plus media (Gibco, A3582901) supplemented with 1x B-27 Plus (Gibco, A3582801), 1x GlutaMax (Gibco, 35-050-061), and 10 μM all-trans retinoic acid (Alfa Aesar, 44540) for 6 days, with media changes every other day. For typical ASO treatments, cells were plated in 12-well plates (60,000 cells per well) or 6-well plates (150,000 cells per well), and then treated with ASOs as indicated on the following day. For transfection of miR-29b inhibitors, H4 cells were transfected using Lipofectamine 3000 (ThermoFisher, L3000015) and SH-SY5Y cells were transfected using Lipofectamine RNAiMAX (ThermoFisher 13778150). Human iPSCs harboring doxycycline-responsive Neurogenin-2 (NGN2) expression for differentiation into i3 neurons were treated with ASOs for 3 to 4 days. All ASO treatments were analyzed by quantitative RT-PCR using the comparative CT method. For RNA-seq analysis of miR levels, the small RNA fraction was isolated from triplicate 100-mm dishes of H4 cells using the mirVana miRNA Isolation Kit (Invitrogen, AM1561). Libraries were constructed using the Ion Total RNA-Seq Kit v2 (Life Technologies, 4475936) and sequenced on an Ion Torrent Proton at the Saint Louis University Genomics Core Facility. Reads were aligned to the human genome hg19 assembly, and data are presented as total normalized nucleotide coverage per miRNA. Data on miR expression levels in human tissues was from the Human miRNA Tissue Atlas (36). The predicted secondary structure of the human GRN mRNA was generated using the mfold program (56).

**ELISA and immunoblot analysis**

For progranulin measurements, cells were rinsed with PBS and then lysed in RIPA buffer containing protease inhibitors (Roche, cOmplete Mini EDTA-free Protease Inhibitor Cocktail). Cleared lysates were transferred to new tubes, and protein concentrations were determined using the Bio-Rad DC Protein Assay Kit II. For ELISA, progranulin concentrations were determined in duplicate using 10 to 15 μg of lysates per well (typically 8–20 μg of total protein per well) using a sandwich ELISA assay (R&D Systems, DPGRN0). For experiments analyzing secreted progranulin, conditioned media was collected and cleared at 10,000g for 10 min at 4 °C. For Western blot analysis, sample buffer was added to the lysates or conditioned media, and the samples were heated at 95 °C for 10 min. Equal amounts of protein lysates (10–20 μg) or equal volumes of conditioned media (10–20 μl) were separated on SDS–PAGE gels. Proteins were transferred to nitrocellulose membranes using the Bio-Rad Turbo-Blot transfer system. After blocking and antibody incubations, membranes were incubated with SuperSignal West Pico or Femto-enhanced chemiluminescent HRP substrate (ThermoFisher) and visualized using a Chemi-Doc system (Bio-Rad). Primary antibodies used for immunoblot analysis include an anti-human progranulin linker 5 polyclonal antibody #614 that recognizes an epitope between amino acids 497 to 515 (1:3000 dilution) (55), an anti-human vinculin monoclonal antibody (Cell Signaling Technology, 13901, 1:1000 dilution), and an anti-α-tubulin monoclonal antibody (Sigma, T9026, 1:2000 dilution). The HRP-conjugated secondary antibodies used were goat anti-rabbit IgG (H + L) (Jackson Immuno Research Labs, 111-035-144), donkey anti-mouse IgG (H + L) (Jackson Immuno Research Labs, 715035150), and donkey anti-sheep IgG (H + L) (Jackson Immuno Research Labs, 713035147).

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Total RNA was isolated from cultured cells using the RNeasy Mini kit (Qiagen, 74106) with on-column DNase digestion (Qiagen, 79256). RNA was reverse-transcribed to obtain cDNA using the iScript cDNA synthesis kit (Bio-Rad, 1708891), and qPCR was performed using PowerUp SYBR Green Master Mix (ThermoFisher, A25777) with a Bio-Rad CFX384 Real-Time System. Primers sequences for human genes were as follows: CYCLO-F, GGAGATGGCACA GGAGGAAA; CYCLO-R, CCGTAGTCCTCAGTGTGAA GTTCT; human GRN-F, AGGAGAACGCTACCACGGA; GRN-R, GCCGACCGAGTATGAGCACTCG; Results for qPCR were normalized to the housekeeping gene CYCLO, and evaluated by the comparative Ct method. For RNA-seq analysis of miR levels, the small RNA fraction was isolated from triplicate 100-mm dishes of H4 cells using the mirVana miRNA Isolation Kit (Invitrogen, AM1561). Libraries were constructed using the Ion Total RNA-Seq Kit v2 (Life Technologies, 4475936) and sequenced on an Ion Torrent Proton at the Saint Louis University Genomics Core Facility. Reads were aligned to the human genome hg19 assembly, and data are presented as total normalized nucleotide coverage per miRNA. Data on miR expression levels in human tissues was from the Human miRNA Tissue Atlas (36). The predicted secondary structure of the human GRN mRNA was generated using the mfold program (56).

**Polysome profiling**

H4 cells were cultured in 100-mm dishes and treated with 5 μM ASOs for 22 h. Five minutes prior to collecting cells, cycloheximide (CHX) was added to the cell culture media at a final concentration of 0.1 mg/ml. Cells were rinsed with ice-cold PBS and then lysed in Polyribosome Lysis Buffer containing 20 mM Tris–HCl pH 7.4, 100 mM KCl, 5 mM MgCl2, 1% NP-40, 0.1 mg/ml CHX, 1 mM DTT, 0.02 U/ml SUPERase In RNase Inhibitor (Invitrogen, AM2694), protease inhibitors (Roche, cOmplete Mini EDTA-free Protease Inhibitor...
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Cocktail), and phosphatase inhibitors (Roche, PhosSTOP) in nuclease-free water (Invitrogen, AM9937). Lysates were centrifuged (10,000g, 10 min, 4 °C), and the supernatant fractions were collected. Protein determination was performed using the Bio-Rad DC Protein Assay Kit II, and 1.35 mg per sample was loaded on a 15% to 45% sucrose density gradient (w/w sucrose, 20 mM Tris-HCl pH 7.4, 100 mM KCl, 5 mM MgCl₂, Ultrapure water). Samples were centrifuged for 2 h at 210,200g at 4 °C in a SW41 Ti rotor. Polysome profiling was performed using a BR-188 density gradient fractionation system (Brandel) (sensitivity setting of 1, baseline setting of 20, and flow rate of 1.5 ml/min) with upward displacement and continuous monitoring at 254 nm using a UA-6 detector. Polyribosome (13 fractions) and monosome (3 fractions) fractions were collected in a volume of 600 µl and the fractions were separated in two tubes (300 µl per tube). For subse-
quent RNA extraction, 600 µl Trizol Reagent (Invitrogen, 15-596-018) was added to each polyribosome fraction (300 µl), and samples were stored at ~80 °C. RNA was extracted by adding 200 µl chloroform (Fisher, C298) to each fraction and centrifuging at 18,213g for 15 min. The aqueous phase was transferred to a tube containing 500 µl of isopropanol (Fisher, A416P-4) and 5 µg of glycocen (Sigma, G1767-1VL). Then samples were pelleted at 18,213g for 15 min, dried, washed with 70% ethanol, and centrifuged at 18,213g for 15 min. The pellet was dried and resuspended in 20 µl of Ultrapure water. Samples were digested with DNase (Roche) for 40 min at 37 °C. cDNA was generated using 2 µg of RNA for each fraction with a High-Capacity cDNA Reverse Transcription Kit (ThermoFisher, 4368814). qPCR was performed using PowerUp SYBR Green Master Mix (ThermoFisher, A25776) using a QuantStudio 5 Real-Time PCR System. Primers se-
quences were as follows: human Actin-F, TCCGTGTGGA TCAGGGCTCCTCA; human Actin-R, CTGCTTGCTGATC-
CACATCTG; human GRN-F, AGGAGAACGCTACCAC 
TCGGCGGCTCCA; human Actin-R, CTGCTTGCTGATC-
CACATCTG. Increasing progranulin levels with ASOs

MTT assay

MTT assays were used to assess the effects of ASOs on cell viability. H4 cells were plated in 96-well plates at a density of 5000 cells per well. The following day, cells were treated with 10 µM ASO. After 21 h treatment, the media was removed and cells were incubated in 100 µl of 0.5 mg/ml thiazolyl blue tetrazolium bromide (MTT) (Sigma, M5655) solution was prepared in fresh culture media for 4 h. After incubation, the MTT solution was removed and 100 µl of DMSO was added to dissolve the crystals formed. The absorbance was measured at 570 nm using a BioTek Synergy H1 plate reader. Experiments were performed with triplicate wells, and the percent viability was normalized to cells treated with vehicle only (water).

FRET-based assay for miR-29b binding

A FRET-based assay was developed to monitor the interaction between miR-29b (labeled with Cy3 at the 5’ end) and a partial GRN 3’ UTR RNA (nucleotides 189-246, labeled with Cy5 at the 3’ end). Reactions were set up in duplicate wells in black 384-well plates with 1 µM miR-29b and 1 µM GRN 3’ UTR in 20 µl of 1X SSC buffer, pH 7.0 containing 0.005% Tween-20. ASOs were added to the reactions in the same PBS containing 0.1 mg/ml CHX, and then lysed in Triton lysis buffer containing 1% Triton X-100, 25 mM sodium py-
rophosphate, 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 5 mM EGTA, 0.5% sodium deoxycholate, 20 mM HEPES pH 7.4, 10 mM β-glycerophosphate, 1 mM Na₃VO₄, 0.1 mg/ml CHX, and protease inhibitors (Roche, cOmplete Mini EDTA-free Protease Inhibitor Cocktail). Lysates were centrifuged (16,260g, 10 min, 4 °C), and the supernatant fractions were collected. Click reactions were performed using Click-iT Protein Reaction Buffer Kit (Invitrogen, C10276) according to the manufacturer’s protocol in order to label the AHA-incorporated proteins with biotin alkyne (ThermoFisher, B10185). The Click reaction samples were incubated on a rotator at 4 °C for 1 h, and diluted in lysis buffer at a 1:2 ratio before being subjected to anti-progranulin immunoprecipita-

AHA labeling

H4 cells were cultured in DMEM high glucose (Gibco, 11965-092) supplemented with 10% FBS (Gibco, 10010-023). Cells were cultured in 60-mm plate until about 80% conflu-
ence and treated with 10 µM ASOs for a total of 24 h. For optimal incorporation of the amino acid azidohomoalanine (AHA), cells were methionine-deprived for 2.5 h in methionine-free medium (DMEM without methionine, gluta-
mine, and cysteine (Gibco, 21013024), supplemented with 0.5% FBS, 1X GlutaMAX (Gibco, 35050061), and 63 µg/ml L-Cysteine) containing 10 µM ASO. Click-IT AHA (Invitro-
gen, C10102) was then added to the cell culture medium at a final concentration of 50 µM for the last 2 h of treatment to label de novo proteins. Cells were washed once with ice-cold
buffer at final concentrations between 0.64 nM–50 μM. Plates were read using a BioTek Synergy H1 plate reader with excitation at 540 nm and emission at 570 nm, and with excitation at 540 nm and emission at 665 nm. The ratio of fluorescence emissions at 665 nm/570 nm was calculated for each well, and data were transformed with baseline correction.

Mouse studies

Humanized GRN mice (41) were on the C57BL/6 background and were genotyped either by PCR as described (41) or by real-time PCR (Transnetyx). Mice were housed in a pathogen-free barrier facility with a 12-h light/12-h dark cycle by real-time PCR (Transnetyx). Mice were housed in a ground and were genotyped either by PCR as described (41) or by real-time PCR. Mice were anesthetized with isoflurane, as previously described (57). After 3 weeks, mice were sacrificed and brain tissues were collected for protein and RNA analyses. For ELISA, progranulin levels were determined in duplicate using 150 μg of protein lysates per well using a sandwich ELISA assay (abcam, ab252364). Western blot and qPCR analyses were carried out as described above. Immunofluorescence was performed as previously described (58). Briefly, fixed brains were frozen in OCT solution and sectioned using a cryostat. Free-floating sections were blocked, and then sequentially incubated with a pan-ASO antibody that recognizes the ASO backbone (43), Alexa Fluor Plus 647 goat anti-rabbit IgG, and DAPI. After washing, sections were mounted onto slides, and images were acquired on an Andor Dragonfly spinning disk confocal microscope with a 10x objective.

Ethics statement

All experiments using animals were approved by the Institutional Animal Care and Use Committee at Saint Louis University and followed NIH guidelines.

Statistical analyses

Data are presented as means ± SD or as means ± SEM, as indicated in the figure legends. Data were analyzed with GraphPad Prism software using the statistical tests described in the figure legends. p values < 0.05 were considered significant.

Data availability

The RNA-seq data used in this study are available in the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under accession code GSE188498. All the other data supporting the findings of this study are available within the article and its supporting information files.

Supporting information—This article contains supporting information.

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Conflict of interest—K. L., P. J.-N., and F. R. are paid employees of Ionis Pharmaceuticals. Ionis Pharmaceuticals and Saint Louis University have filed for patents based on using GRN ASOs to treat frontotemporal dementia. The authors declare that they have no other conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: 2′-O-Me, 2′-O-methoxymethyl; 2′-O-Me, 2′-O-methyl; AHA, azidohomoalanine; ALS, amyotrophic lateral sclerosis; ASO, antisense oligonucleotide; FTD, frontotemporal dementia; ICV, intracerebroventricular; iPSC, induced pluripotent stem cell; miR, microRNA; PGRN, progranulin; uORF, upstream open reading frame; UTR, untranslated region.

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