The Receptor-Interacting Serine/Threonine Protein Kinase 1 (Ripk1) Regulates Progranulin Levels

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ABSTRACT

Progranulin (PGRN), a secreted growth factor, is a key regulator of inflammation and is genetically linked to two common and devastating neurodegenerative diseases. Haploinsufficiency mutations in GRN, the gene encoding PGRN, cause frontotemporal dementia (FTD) and a GRN SNP confers significantly increased risk for Alzheimer’s disease (AD). Because cellular and animal data indicate that increasing PGRN can reverse phenotypes of both FTD and AD, modulating PGRN level has been proposed as a therapeutic strategy for both diseases. However, little is known about the regulation of PGRN levels. In this study, we performed an siRNA-based screen of the kinome to identify genetic regulators of Pgrn levels in a rodent cell-based model system. We found that knocking down receptor-interacting serine/threonine protein kinase 1 (Ripk1) increased both intracellular and extracellular Pgrn protein levels by increasing the translation rate of Pgrn without affecting mRNA levels. We observed this effect in neuro2a cells, wild-type primary mouse neurons, and Grn-haploinsufficient primary neurons from an FTD mouse model. We found that the effect of Ripk1 on Pgrn is independent of Ripk1’s kinase activity and occurs through a novel signaling pathway. These data suggest that targeting Ripk1 may be a therapeutic strategy in both AD and FTD.

Frontotemporal dementia (FTD) and Alzheimer’s disease (AD), two devastating neurodegenerative diseases, affect approximately 40,000 and 4 million individuals, respectively, in the United States(1–3). Symptoms include apathy, disinhibition and/or language difficulty (FTD) or loss of memory, disorientation, and inability to perform self-care (AD). Pathologically, both are marked by pronounced synaptic and neuronal degeneration, as well as aberrant protein accumulation in affected brain regions. Unfortunately, no treatments halt or even slow the progression of these uniformly fatal diseases.

The secreted growth factor progranulin (PGRN) has roles in inflammation, cancer, metabolic disease, and neuronal health(4–8) and is a link between FTD and AD. First, mutations in the gene that encodes PGRN (GRN) are among the most common genetic causes of FTD(9, 10). These mutations are fully penetrant(11), autosomal dominant, haploinsufficiency mutations that lead to half the normal production of GRN mRNA and PGRN protein(12–14). On the other hand, a SNP in the 3′ UTR of GRN is a risk factor for AD (odds ratio, 1.4)(15) and is associated with decreased serum PGRN levels(16). Thus, halving the level of PGRN invariably causes FTD, and a reduction in PGRN increases the risk for AD.

Overexpressing Pgrn rescued disease phenotypes in cellular and animal models of FTD and AD. For example, in both an in vivo (zebrafish) and an in vitro (cultured mouse...
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neurons) FTD model, knockdown or deletion of Pgrn reduced neurite outgrowth, and overexpressing Pgrn rescued this deficit(17, 18). Likewise, hyperinflammatory primary microglia from mice lacking Pgrn were rescued (to normal expression of inflammatory genes) by lentiviral expression of Pgrn(19). Finally, in a mouse model of AD, overexpressing Pgrn reduced plaque burden, improved memory, and decreased neuronal loss(20). These results indicate that increasing Pgrn levels can reverse phenotypes associated with both FTD and AD. Therefore, increasing Pgrn represents an attractive therapeutic strategy for these two diseases.

Because little is known about the genetic regulation of Pgrn, we performed an siRNA-based screen of the kinome to uncover genetic modifiers of Pgrn level. Among the 719 kinases, we identified 24 hits (3.3% hit rate) that increase Pgrn levels. We identified receptor-interacting serine/threonine protein kinase 1 (Ripk1) as our top hit. We verified that Ripk1 is a bona fide genetic regulator of Pgrn in neuro2a cells, microglial-like BV-2 cells, wild-type primary neurons, and neurons from an FTD mouse model. We further found that knocking down Ripk1 increased both intracellular and extracellular Pgrn protein levels by increasing the translation rate of Pgrn without affecting mRNA levels. The effect of Ripk1 knockdown on Pgrn is independent of Ripk1’s kinase activity and occurs through a novel signaling pathway. These data support the possibility of targeting Ripk1 as a therapeutic strategy in both AD and FTD.

RESULTS

Kinome Screen—To uncover genetic regulators of Pgrn level, we knocked down each gene in the kinome with siRNA and assayed the extracellular Pgrn level via sandwich enzyme-linked immunosorbent assay (ELISA). The screen had three stages. In the primary screen, each gene was knocked down by a mixture of four siRNAs. In the secondary screen, each hit from the primary screen was repeated in triplicate. In the tertiary screen, each of the four siRNAs against a gene was tested individually. The results of the primary and secondary screen are shown in Fig. 1A, B. We found that 24 genes passed the tertiary screen (Table 1). Ingenuity Pathway Analysis reveals that these genes are involved in 15 significantly overrepresented pathways (Table 2). We identified receptor-interacting serine/threonine protein kinase 1 (Ripk1) as our top hit because (1) it was one of the most effective upregulators of Pgrn: in the secondary screen, a mixture of four Ripk1 siRNAs increased secreted Pgrn by more than twofold (Fig. 1C), (2) Ripk1 is highly expressed in brain, and (3) there are many tools available for interrogating Ripk1 function. FTD patients have about half the normal level of PGRN, and many cancer patients have supraphysiological PGRN expression(21–23). Therefore, we wanted to increase Pgrn from the disease range (half) to the normal range, a factor of approximately two.

Ripk1 is a Bona Fide Genetic Regulator of Pgrn—Because the tertiary screen revealed that four different siRNAs against Ripk1 individually increased Pgrn levels (Fig. 2A), the possibility that these siRNAs co-incidentally all affect Pgrn levels through off-target effects was small. However, to further exclude this possibility, we tested a new set of four Ripk1 siRNAs (for a total of eight unique siRNAs), which were not in the original screen and were directed against different portions of the Ripk1 sequence than the original siRNAs. We found that the four new siRNAs also lead to increased Pgrn levels (Fig. 2B). We also confirmed that all eight siRNAs effectively knock down Ripk1 (Supplementary Fig. 1A, B). Based on these observations, we conclude that Ripk1 is a bona fide genetic regulator of Pgrn levels.

The Pgrn detected in our ELISA assay is native, and Pgrn can form a dimer(24) and is highly glycosylated and contains disulfide bonds. Ripk1 knockdown might alter Pgrn tertiary protein structure or post-translational modification, leading to changes in antibody detection of Pgrn and apparent differences in Pgrn levels. To assess this possibility, we evaluated Pgrn produced after Ripk1 knockdown by western blot, which allows for size discrimination of Pgrn. Using the ELISA capture antibody directed to the C-terminus of Pgrn and native gel electrophoresis, we detected only one band corresponding to native Pgrn at approximately 180 kD, which was increased by Ripk1 knockdown (Supplementary Fig. 1A, B). Based on these observations, we conclude that Ripk1 is a bona fide genetic regulator of Pgrn levels.

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band ran at the expected size for a monomer and showed no shift to indicate post-translational modification with Ripk1 knockdown. Together, these data show that both native and fully denatured Pgrn are increased by Ripk1 knockdown, indicating that the change in Pgrn level is real and unlikely to be an apparent change due to altered detection by ELISA.

Ripk1 plays an important role as a mediator of cell death(25), so we wondered whether the Ripk1 knockdown increased Pgrn levels by blocking cell death and increasing the number of cells producing Pgrn. To exclude the possibility that Ripk1 knockdown increased Pgrn level by increasing cell number, we measured the number of viable cells at the time of ELISA assay. Four of the Ripk1 siRNAs had no effect at all on cell number, three modestly reduced cell number, and one modestly increased it (-20% to +14%; Fig. 3A, B). Thus, there was no consistent effect of Ripk1 knockdown on cell number, and none of the effects of Ripk1 siRNAs on cell number could account for the change in Pgrn protein level of up to +200% induced by Ripk1 knockdown (Fig. 2A, B). We conclude that Ripk1 knockdown does not increase Pgrn levels by increasing cell number.

Next, we considered the possibility that Ripk1 knockdown affected Pgrn levels in cell types other than neuro2a cells. Because brain Pgrn is produced primarily by microglia and neurons(26, 27), we first checked the effect of Ripk1 knockdown in a microglial-like cell line, BV-2. We found that the four siRNAs against Ripk1 increased Pgrn levels by ELISA in BV-2 cells (Fig. 4A). We next focused on cortical neurons. Cortical neurons are an extremely important cell type in FTD and AD since very pronounced loss of these cells occurs during disease. To check whether Ripk1 knockdown increases Pgrn levels in cortical neurons, we first produced lentiviruses containing nontargeting shRNA, shRNA against Pgrn, or shRNA against Ripk1 and confirmed target knockdown in neurons (Supplementary Fig. 2A, B). Next, we compared Pgrn levels in primary mouse cortical neurons transduced with nontargeting, Pgrn, or Ripk1 shRNA and found that Ripk1 shRNA increases extracellular Pgrn levels approximately twofold (Fig. 4B). For this experiment, we used both wild-type and Grn“+" mouse neurons. In fact, Ripk1 shRNA rescued Pgrn levels in Grn“+" neurons to wild-type baseline levels (Fig. 4B).

Ripk1 Knockdown Increases Pgrn Translation—Having excluded an increase in cell number and an increase in global secretion, we considered the possibility that Ripk1 knockdown could be increasing extracellular Pgrn level by increasing transcription, increasing translation, increasing secretion, and/or decreasing degradation. We tested each possibility in turn.

To examine Pgrn transcription, we measured Pgrn mRNA levels by qPCR, with and without Ripk1 knockdown. We found that steady-state Pgrn mRNA levels were unchanged by Ripk1 knockdown (Fig. 5A). Therefore, increased transcription is not the mechanism by which Ripk1 knockdown increases Pgrn levels.

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We next determined whether the increase in extracellular Pgrn detected by ELISA and by western blot could be explained primarily by increased secretion of preexisting Pgrn. Since Pgrn is a secreted molecule, an increase in the extracellular level of Pgrn could theoretically be achieved solely by increased secretion, resulting in decreased intracellular and increased extracellular Pgrn. To check if this was occurring in our system, we measured the intracellular and extracellular Pgrn levels by ELISA. We found that intracellular Pgrn levels increased to nearly the same degree as extracellular Pgrn levels (Fig. 5B). This experiment demonstrates that increased secretion alone cannot explain the increase in Pgrn seen after Ripk1 knockdown. Rather, Ripk1 knockdown causes an increase in the total amount of Pgrn. Supporting this idea, we measured the absolute level of Pgrn inside and outside neuro2a
cells and primary neurons and found that extracellular Pgrn levels were several times higher than intracellular Pgrn levels at baseline (Supplementary Fig. 3A, B), meaning that even if all intracellular Pgrn was secreted and became extracellular Pgrn, it could not produce the doubling of Pgrn that we observe after Ripk1 knockdown (Fig. 2A).

To examine Pgrn translation, we added 35S-methionine to the medium, allowing it to incorporate into newly translated Pgrn, and monitored the amounts of radioactive Pgrn, combining extracellular and intracellular compartments, over the course of 3 hours. As expected, radioactive Pgrn levels increased during the course of the experiment, representing the synthesis of new Pgrn. We found that Ripk1 knockdown increased the rate of Pgrn synthesis, compared to nontargeting siRNA (Fig. 5C). These data indicate that Ripk1 knockdown increases Pgrn levels by increasing translation rate. Interestingly, by 3 hours, the abundance of Pgrn in Ripk1 siRNA condition is ~1.6-fold greater than in the nontargeting condition, in line with what we saw by western blot and ELISA.

Having found that Ripk1 knockdown increases Pgrn translation, we next wanted to determine whether the effect of Ripk1 on translation was specific to Pgrn or more general (genome-wide or secretome-wide). To assess global protein synthesis, we used a fluorescence-based method, the OPP reaction(28). We found that total protein synthesis, as measured by GFP fluorescence, was not different between nontargeting siRNA and Ripk1 siRNA-transfected cells (Fig. 5D). We therefore concluded that the effect of Ripk1 on translation is not global. We also found no change in the steady-state extracellular levels of Collagen IV upon Ripk1 knockdown (Fig. 3C), suggesting that Ripk1 does not increase the translation of all secreted proteins; instead, its effect on Pgrn may be specific.

We also examined Pgrn degradation by metabolic pulse labeling with 35S-methionine. We observed no consistent differences in the rate of Pgrn turnover with or without Ripk1 knockdown (data not shown).

**Ripk1 Knockdown Increases Pgrn Through a Novel, Kinase-Independent Signaling Mechanism**—Because Ripk1 is a kinase, we wanted to check whether its effect on Pgrn depends on its kinase activity. 7-Cl-O-Necrostatin 1 (Nec1) is a selective inhibitor of Ripk1 that inhibits all Ripk1 kinase activity, including autophosphorylation(29, 30). First, we checked Nec1 target engagement in our system by showing that addition of Nec1 to neuro2a cells inhibited Ripk1 autophosphorylation. We tracked phosphorylation using a pulse of radioactive γ32P-ATP, followed by Ripk1 pulldown and autoradiography. Nec1 reduced the ratio of phosphorylated Ripk1 (measured on autoradiograph) to total Ripk1 (measured on Western blot) (data not shown). We next tested whether Ripk1 inhibition by Nec1 increased Pgrn. We found that, unlike Ripk1 knockdown, inhibition of Ripk1 by Nec1 did not increase Pgrn (Fig. 6A).

Although Nec1 is a highly specific inhibitor of Ripk1, it is difficult to exclude the possibility that some off-target effect of Nec1 might be affecting Pgrn levels and masking the effect of Ripk1 inhibition on Pgrn. Therefore, we took a complementary genetic approach to determine whether Ripk1 knockdown increased Pgrn levels by reducing its kinase activity. We assayed Pgrn levels in mice with a homozygous knock-in for the D138N mutation in Ripk1, which confers loss of kinase activity(31). We found that brain Pgrn levels were not changed by loss of Ripk1 kinase activity (Fig. 6B). Together, these data indicate that the loss of Ripk1’s kinase activity is not sufficient to increase Pgrn. This suggests that Ripk1 regulates Pgrn in a kinase-independent manner, similar to many other effects of Ripk1(25).

In an effort to more clearly define the pathway connecting Ripk1 and Pgrn, we tested whether Ripk1 and Pgrn directly interact using co-immunoprecipitation (co-IP). Under the conditions used here, we did not detect any Pgrn being pulled down by Ripk1 IP or any Ripk1 being pulled down by Pgrn IP (Supplementary Fig. 4). We also tested whether Pgrn and Ripk1 co-localize by immunocytochemistry. Whereas Pgrn strongly co-localized with the lysosomal marker Lamp1, Pgrn and Ripk1 did not significantly co-localize (Supplementary Fig. 5). In summary, we found no evidence to support the hypothesis that Ripk1 regulates Pgrn through a direct interaction.

Having shown that Ripk1 knockdown increased Pgrn levels in a kinase-independent
manner, evidently without directly interacting with Pgrn, we next tested whether known functions of Ripk1, including its key kinase-independent ones, were mechanisms of Pgrn regulation. Many signaling pathways downstream of Ripk1 have been described\(^{(25, 32–35)}\). The three main signaling complexes formed downstream of Ripk1 are (1) Complex I which contains the tumor necrosis factor (TNF) receptor and activates Nfkβ-dependent transcription, (2) death-inducing signaling complex (DISC) which contains Caspase-8 (Casp8), and (3) the Necrosome, which contains Ripk3. We determined whether knocking down an essential component of each signaling arm could replicate the effect of Ripk1 knockdown. First, we knocked down Nfkb1, one of the subunits of Nfkβ. Although siRNAs to Nfkb1 were effective at reducing Nfkb1 mRNA levels (Supplementary Fig. 6A), Nfkb1 siRNA did not increase Pgrn levels (Fig. 6C). Likewise, siRNAs to Casp8 and Ripk3 were effective in reducing target mRNA levels (Supplementary Fig. 6B, C), but neither siRNA increased Pgrn levels (Fig. 6D, E). These data suggest that the three best-studied signaling pathways downstream of Ripk1 are not responsible for its effect on Pgrn.

Next we considered the fact that Pkr, a major antiviral protein, phosphorylates eIF2 to inhibit mRNA translation during viral infection, and that Pkr and Ripk1 co-IP\(^{(36, 37)}\). Therefore, we reasoned that Ripk1 could cooperate with Pkr to down-regulate translation of Pgrn. To test this possibility, we knocked down Pkr using a mixture of four different siRNAs against this target, and we found that Pgrn levels did not increase (Fig. 6F). Therefore, we believe that Ripk1 is not acting through Pkr to change Pgrn levels and we conclude that the effect of Ripk1 knockdown on Pgrn levels may proceed through a novel pathway.

DISCUSSION

Some mutations in \(GRN\) cause autosomal dominant FTD and a \(GRN\) SNP is a risk factor for AD. Because cellular and animal data indicate that increasing Pgrn can reverse phenotypes of both FTD and AD, modulating Pgrn level is an attractive therapeutic strategy for both diseases. Therefore, the goal of this study was to identify genetic regulators of Pgrn levels. We performed a screen of the kinome and identified 24 genes that upregulate Pgrn (Table 1).

The hits ranged from extremely well-studied genes known to have a role in brain disease (Lrrk2) to relatively poorly studied genes thought to play a role in sperm development (Tex14). Our initial prioritization of the hits was based on effect size, and we focused on approximately the top ten hits. Of these, we were initially most enthusiastic about Lrrk2 due to its known role in Parkinson’s disease; however, we found that Lrrk2 expression is not detectable in Neuro2a cells. Therefore, we concluded that the effect on Pgrn levels of the pooled siRNAs against Lrrk2 was most likely an off-target effect and instead focused on the other top hits. Among these, we prioritized Ripk1 because of (1) the fact that Ripk1 is highly expressed in brain, and (2) the availability of tools for interrogating Ripk1 function, including knock-in mice and a specific inhibitor\(^{(30–32)}\). Future studies will investigate the role of other top hits in regulating Pgrn; in particular, we find Egfr and Vegfr3 compelling due to their close relationship and the incredible depth of knowledge about Vegf family signaling.

We found many compelling lines of evidence that Ripk1 knockdown affects Pgrn level. First, eight different siRNAs against Ripk1 led to an increase in Pgrn by ELISA in neuro2a cells (Fig. 2A, B) without causing an increase in cell number or global secretion (Fig. 3). Second, Ripk1 knockdown increased Pgrn by both native and reducing western blot (Fig. 2C, F), indicating that the increase in Pgrn detected is not due to changes in tertiary structure or protein modification, but a true change in Pgrn level. Finally, we found that Ripk1 knockdown increased Pgrn level in microglial-like cells, mouse primary cortical neurons from wild-type mice, and most importantly, FTD model (\(Grn +/-\)) mice (Fig. 4). Importantly, Ripk1 knockdown approximately doubled Pgrn levels in each of our model systems and restored Pgrn to wild-type levels in an FTD model. It is potentially important from a therapeutic perspective that the induction of Pgrn was not greater since supraphysiological levels of Pgrn are linked to cancer progression and invasion.

We further found that Ripk1 knockdown increased the rate of Pgrn translation without affecting Pgrn transcription, secretion, or turnover (Fig. 5). Although we were surprised to discover that Ripk1 knockdown affects the translation of
Pgrn, there is precedent to the idea that Ripk1 can regulate the rate of translation of immunomodulatory genes. For example, the increase in IL-6 translation which occurs downstream of TNFα requires Ripk1(38). Additionally, translational regulation of Pgrn level has been previously reported: there is a splice variant of Pgrn with a long 5′UTR, which is translated less than the variant with a short 5′UTR(39). Thus, although we have not identified the exact mechanism by which Ripk1 knockdown leads to an increase in Pgrn translation, the idea that Ripk1 could change Pgrn translation rate is not unprecedented.

We found that loss of Ripk1 kinase activity, either by small-molecule inhibition or mutation, did not replicate the effect of Ripk1 knockdown (Fig. 6A, B), indicating that the effect of Ripk1 on Pgrn level does not depend on its kinase activity. Although this result may seem surprising for a kinase, many of the effects of Ripk1 do not depend on its kinase activity(25, 31, 33). In fact, of the three major signaling pathways downstream of Ripk1 (Nfkb transcription, Caspase-mediated apoptosis, and Ripk3-mediated necroptosis), two do not require Ripk1 kinase activity. Unfortunately, because inhibiting the kinase activity of Ripk1 does not increase Pgrn, use of the specific Ripk1 inhibitor Nec 1 in humans is unlikely to be a successful therapeutic approach.

An alternative approach would be small-molecule inhibition of another member of the pathway linking Ripk1 and Pgrn; however, discovering such a target will require further investigation. Still, there are options other than small molecules for reducing Ripk1 levels in humans, including antisense oligonucleotides (ASOs). Although genetic therapies, such as ASOs, are much newer and less common than traditional small-molecule drugs, ASOs are already being tested in other neurodegenerative diseases, including spinal muscular atrophy (SMA; Phase 3 trial, ClinicalTrials.gov identifier NCT02193074) and amyotrophic lateral sclerosis (ALS; Phase 1 trial, ClinicalTrials.gov identifier NCT02623699) and hold great promise. In fact, in August 2016, the SMA trial was halted early because the treatment was so effective that it was unethical to continue with a control arm(40). On the other hand, the long-term effects of reducing Ripk1, an essential gene(31), are not known and may be deleterious. One obvious alternative to a genetic therapy targeting Ripk1 is direct overexpression of PGRN. Whether a viral transduction strategy could deliver PGRN to all the areas of the brain that are affected in FTD and whether it could moderately upregulate PGRN to physiological levels without overshooting and promoting cancer is not known.

We also found no evidence that the three major signaling pathways downstream of Ripk1 mediate its effect on Pgrn levels (Fig. 6C-E). Of course, we cannot definitively exclude a contribution from these complex and intertwined pathways, but these data indicate the effect of Ripk1 knockdown on Pgrn levels may proceed through a novel mechanism.

In conclusion, the data presented here represent the first report linking Ripk1 and Pgrn and support targeting Ripk1 as a potential therapeutic strategy for increasing PGRN levels in both AD and FTD.

**EXPERIMENTAL PROCEDURES**

**Neuro2a Culture**—Neuro2a cells were grown in DMEM high glucose (Mediatech) + 10% FBS (Life Technologies) + 1% pen/strep (Life Technologies) + 1% GlutaMAX (Life Technologies) in 5% CO₂ at 37 °C and passaged as needed, approximately two times per week.

**Screen**—The screening assay was an ELISA for detecting extracellular mouse Pgrn. The ELISA protocol was as described(20). The screening method was also described elsewhere(41). Briefly, neuro2a cells were reverse transfected in 96-well plates with an siRNA library against the kinome (GE/Dharmacon). Each gene was targeted by a mixture of four siRNAs at a concentration of 12.5 nM per siRNA (50 nM total). Cells (20,000 cells per well) were transfected for 3 hours, and then the medium was changed to DMEM + 1% FBS (150 µL per well). After 24 hours, media were collected and assayed for Pgrn concentration by a sandwich ELISA. Hits from primary screen were any wells at least 1.5 standard deviations above the plate mean. Hits from the primary screen were re-assayed in triplicate for the secondary screen. Secondary hits were those siRNAs that gave a significant increase in Pgrn compared to nontargeting siRNA by ANOVA. For the tertiary screen, hits from the secondary screen were re-assayed (six replicates...
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per condition) with each of the four siRNAs assayed separately instead of as a mixture. Hits for the tertiary screen were those genes for which at least two of four siRNAs significantly increased Pgrn (by ANOVA).

Transfection of Neuro2a cells—After we identified Ripk1 as the top hit, we optimized the transfection protocol. The modifications from the screening protocol are as follows: 9,000 instead of 20,000 neuro2a cells per well; 90 minutes instead of 3 hours of transfection time; 200 µL instead of 150 µL media change; 72 instead of 24 hours of incubation time after transfection but before ELISA.

siRNA—All siRNAs were obtained from GE/Dharmacon. Catalog numbers were: nontargeting siRNA (D-001206-14-05), Grn siRNA (M-062134-01-0005), Ripk1 siRNA (#1: D-040150-01-0002, #2: D-040150-02-0002, #3: D-040150-03-0002, #4: D-040150-04-0002, #5: J-040150-05-0002, #6: J-040150-06-0002, #7: J-040150-07-0002, #8: J-040150-08-0002), Nfkβ1 siRNA (D-047764-01-0005), Casp8 siRNA (D-043044-01-0005), Ripk3 siRNA (D-049919-04-0002).

Determination of Cell Number—Each time an ELISA was performed on neuro2a cells, except during the initial screen, the number of cells per well at the end of the experimental period was determined using the CellTiter-Glo system (Promega), following the manufacturer’s instructions. Briefly, cells were washed once with PBS, then 100 µL PBS was added per well, and finally cells were lysed with 100 µL per well of CellTiter-Glo reagent. Plates were rocked at room temperature for approximately 5 minutes. Lysed samples were added to an opaque plate and luminescence was read for 500 ms. Finally, relative cell numbers in each well were calculated.

Bioinformatics—The 24 hits obtained from the tertiary screen were inputted into Ingenuity Pathway Analysis software (QIAGEN) and significantly over-represented pathways were determined, using the kinases (719 genes) as the reference gene set.

Western Blotting—Samples for western blotting included lysates, concentrated media, or beads from a pull-down. Lysates were prepared as described below, assayed for protein concentration, and equal protein was loaded in each lane. Media samples were concentrated approximately 30-fold using Millipore Amicon columns (50 kD cut-off) and assayed for protein concentration. Equal amounts of total protein were loaded in each lane, resulting in the equivalent of approximately 250 µL of unconcentrated media being loaded per well. Pull-down beads were obtained as described below. All samples were diluted with Laemmli sample buffer with BME and boiled for 5 minutes before gels were loaded.

Gels (8% or 4–12% tris-glycine, Life Technologies) were run in standard tris/glycine/SDS running buffer and then transferred with 20% methanol at 4 °C. Membranes were blocked for 30 minutes in 5% nonfat dry milk in TBS + 0.1% Tween-20 then incubated overnight with primary antibody diluted in 5% nonfat dry milk in TBS + 0.1% Tween-20. Membranes were then washed three times for five minutes in TBS + 0.1% Tween-20 with rocking. Secondary antibody was diluted in 5% nonfat dry milk in TBS + 0.1% Tween-20 and added for approximately one hour at room temperature. After three more washes, blots were developed using Western Lightning Plus ECL (PerkinElmer) for 30 seconds to 2 minutes and exposed to film. Quantification of western blots was performed using densitometry.

Primary antibodies were rabbit anti-Pgrn raised against a peptide in linker 1 of Pgrn (Consortium for Frontotemporal Dementia Research, 8.4 µg/mL), sheep anti-Pgrn (R&D AF2557, 1/1000), rabbit anti-Ripk1 (Cell Signaling 3493, 1/1000), and mouse anti-FLAG (Sigma F1408, 1/5000).

Secondary antibodies were goat anti-rabbit IgG conjugated to HRP (Dako P0448, 1/2000), goat anti-mouse IgG conjugated to HRP (Dako P0447, 1/2000), and donkey anti-sheep IgG conjugated to biotin (R&D BAF016, 1/1000) followed by streptavidin conjugated to HRP (Pierce 1/10,000).

Native western blots were performed as above but with the following modifications: sample buffer was Tris-Glycine Native Sample Buffer (Life Technologies); samples were not boiled; running buffer was Tris-Glycine Native Running Buffer (Life Technologies); primary antibody was rabbit anti-Pgrn CT (0.9 µg/mL).

Primary Neurons —Dissociation media with kynurenic acid (DM/KY) was prepared as
follows: 82 mM Na$_2$SO$_4$, 30 mM K$_2$SO$_4$, 20 mM glucose, 5.8 mM MgCl$_2$, 1 mM Hepes, 1 mM kynurenic acid, 0.25 mM CaCl$_2$, 0.001% phenol red, pH 7.4. Mice aged P0 to P1 were decapitated and their brains were dissected to isolate cortices. Cortices were digested with 10 units per mL papain (Worthington) plus 17 mM cysteine (diluted in DM/KY) for 15 minutes at 37 °C. Next, papain was inactivated using trypsin inhibitor from egg white (Sigma) at 10 mg/mL in DM/KY for 15 minutes at 37 °C. Next, cortices were rinsed once with Opti-MEM (Life Technologies) + 4 mM glucose and then dissociated by pipetting up and down. After tissue chunks settled, live single cells in suspension were counted using trypan blue a hemacytometer. Cells were plated at 125,000 cells per well of a 96-well plate. After two hours, media was changed to Neurobasal (Life Technologies) + 2% B27 supplement (Life Technologies) + 1% pen/strep (Life Technologies).

Lentivirus—shRNAs against mouse Grn and Ripk1 and a non-targeting shRNA were obtained from Sigma (GRN: CCGGCCTAGAATAACGAGCCATCATCTCG AGATGATGGCTCCTATTTAGGTTTTTG; non-targeting: CCGGCAACAAGATGAAGAGCACCAACTCG AGTTGCTCCTATTATCGTTTTTG). shRNAs were subcloned into FUGW using the following primers F ATATGCTAGCTTTCCCATGATTCCTTCAT, R ATATTTAATTAACCATTTGTCTCGAGGTCG A and digestion/ligation at NheI, PacI sites. Vectors containing shRNAs were packaged into lentivirus by the UCSF ViraCore. Viruses were titrated using QuickTiter Lentivirus Titer Kit (Cell Biolabs) and the manufacturer’s instructions. Lentiviruses were used for infection at a concentration of 5x10$^5$ TU/mL and washed off after 24 hours.

qPCR—RNA was isolated using RNeasy micro kit (Qiagen) and manufacturer’s instructions. Next, RT-PCR was performed using the Superscript III First Strand Synthesis Supermix and following manufacturer’s instructions (Invitrogen). Finally, cDNA was diluted 1/4. For qPCR, 4.5 µL cDNA was combined with 5 µL SYBR green master mix (Applied Biosystems) and 0.5 µL primer mix (20 µm each primer). Samples were run on an Applied Biosystems 7900HT. Data were analyzed using the delta delta CT method. Values were normalized to housekeeping gene cyclophilin. Primers were as follows: mouse Cyclophilin F TGGGAAGACCAAGACACATA, mouse Grn F TGCCCGGAGTCGACATGAT, mouse Ripk1 F GAAAGACAGGAGAGCCG, mouse Nfkb1 F ATGGCAGAGCATGTCCCTAC, mouse Casp8 F TGCTGAGACTACATCC, mouse Ccnc3 F TTGGTAGACTACATCC.

Measuring Pgrn Translation Rate—To measure translation, neuro2a cells were reverse transfected with nontargeting or Ripk1 siRNA as described above (1x10$^6$ cells/well of a six-well plate). After 48 hours, media were changed to methionine-free DME (Life Technologies) for 1 hour. Next, media were changed to methionine-free DME (Life Technologies) plus 0.2 mCi/mL $^{35}$S-methionine (EasyTag EXPRESS, PerkinElmer). At 0.5, 1, 1.5, 2, 2.5, and 3 hours after adding radioactivity, media were removed and saved, and cells were washed twice with PBS and then lysed in lysis buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.25% deoxycholate) with protease inhibitors (Pierce) for 5 minutes, followed by spinning at full-speed for 3 minutes. Media and lysate (minus pellet) were combined for each well of cells and Pgrn was pulled down anti-Pgrn CT at 4.4 µg/mL for 60 minutes at room temperature with rotation. Protein A/G agarose beads (Pierce) were added for 30 minutes at room temperature with rotation. Beads were washed three times with lysis buffer, then diluted in Laemmli sample buffer with BME and boiled for 5 minutes. Equal sample volumes were run on 8% tris-glycine gels. Gels were run in standard tris/glycine/SDS running buffer and then fixed for 1 hour at room temperature in 20% MeOH, 10%
acetic acid, 3% glycerol with rocking. Gels were then dried onto Whatman paper in a gel drier with 3.5 hours vacuum and 2 hours heat (65 °C). Dried gels were exposed to film (Biomax MS, Kodak) with an enhancer screen (Kodak BioMax TranScreen LE) at -80 °C for 16–18 hours. Bands were quantified using densitometry.

**Measuring Total Translation Rate**—To measure total protein translation, we reverse transfected neuro2a cells with siRNA as above, and used the Click-iT® Plus OPP Protein Synthesis Assay Kit (Life Technologies) 48 hours later, following the manufacturer’s instructions.

**Ripk1 Autophosphorylation Assay**—Neuro2a cells were reverse transfected with nontargeting or Ripk1 siRNA as described above (1x10⁶ cells/well of a six-well plate). After 24 hours, Nec1 or DMSO control was added to culture media. At 48 hours after transfection, media were changed to phosphate-free DME (Life Technologies) with DMSO or Nec1. After 60 minutes at 37 °C, the media were changed to phosphate-free DME with DMSO or Nec1 plus 0.1 mCi/mL of γ³²P-ATP (EasyTide, PerkinElmer). After 2 hours at 37 °C, cells were washed twice with PBS and lysed in lysis buffer (1% Triton-X, 150 mM NaCl, 20 mM HEPES, pH 7.3, 5 mM EDTA, 5 mM NaF, 0.2 mM NaVO₃) with protease and phosphatase inhibitors (Pierce). Lysates were spun down at maximum speed for 10 minutes, and the pellets were discarded. Ripk1 was immunoprecipitated using mouse anti-Ripk1 antibody (BD 610458, 2.5 µg/mL) for 16 hours at 4 °C with rotation. Protein A/G agarose beads were added for 1 hour at room temperature with rotation. Beads were washed twice with lysis buffer, beads were diluted with Laemmli sample buffer with BME and boiled for 5 minutes, run on an 8% Tris-glycine gel, and western blot for Pgrn or Ripk1 was performed as described above.

**Co-Immunoprecipitation**—Neuro2a cells were reverse transfected with nontargeting or Ripk1 siRNA as described above (1x10⁶ cells/well of a 6-well plate). After 72 hours, cells were washed once with PBS then lysed in lysis buffer (1% Triton-X, 150 mM NaCl, 20 mM HEPES, pH 7.3, 5 mM EDTA, 5 mM NaF, 0.2 mM NaVO₃) with protease inhibitors (Pierce) for 20 minutes. Lysates were spun down at max speed for 10 minutes, and pellets were discarded. Pgrn or Ripk1 was pulled down using mouse anti-Ripk1 (BD 610458, 2.5 µg/mL) or rabbit anti-Pgrn CT (4.4 µg/mL) for 16 hours at 4 °C with rotation. Controls were normal mouse IgG (Santa Cruz) or normal rabbit IgG (Santa Cruz). Protein A/G agarose (Pierce) was added for 60 minutes at room temperature with rotation, then beads were washed twice with lysis buffer. Finally, beads were diluted with Laemmli sample buffer with BME and boiled for 5 minutes, run on an 8% Tris-glycine gel, and western blot for Pgrn or Ripk1 was performed as described above.

**Immunocytochemistry**—Cells grown on glass coverslips in 24-well plates were fixed with 4% PFA for 15 minutes at room temperature, then washed with PBS, permeabilized with PBS + 0.1% Triton-X for 20 minutes, treated with 1 M glycine (in PBS) for 20 minutes, blocked (2% FBS, 3% BSA in PBS + 0.1% Triton-X) for 1 hour, and incubated with primary antibody (diluted in block) overnight at 4 °C. After three washes with PBS + 0.1% Triton-X, secondary antibodies (diluted in block) were added for 1 hour. After three more washes, Hoechst stain was added to visualize nuclei before coverslips were mounted on glass slides and imaged on a Nikon confocal microscope controlled by Micromanager.

Primary antibodies were sheep anti-Pgrn (R&D AF2557, 1/300), mouse anti-Ripk1 (BD 553792, 1/100), and rat anti-Lamp1 (BD 553792, 1/500). Secondary antibodies were donkey anti-mouse-Alexa488, donkey anti-sheep-Alexa555, and donkey anti-rat-Alexa647 (all from Life Technologies, 1/500). Specificity of Pgrn and Ripk1 antibodies for immunocytochemistry was confirmed by comparing staining in wild-type versus Grn knock-out or wild-type versus Ripk1 knock-out fibroblasts. Final staining experiments were performed in neuro2a cells.

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Howard for editorial assistance, and K. Nelson for administrative assistance. Study funding: 3R01 NS039074, R21 NS093236, CIRM TG2-01153, CIRM RB4-06079, NIH T32HD007470, NIH T32 GM007618, the Consortium for Frontotemporal Dementia Research, the Taube/Koret Center for Neurodegenerative Disease Research, the Hellman Foundation Program in Alzheimer’s Disease Research, and the J. David Gladstone Institutes. We dedicate this work to the memory of Ms. Nita Hirsch.

Conflicts of interest: The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Author contributions: ARM conducted the experiments, analyzed and interpreted the results, and wrote the paper. LPE designed the screen. SMF conceived the idea for the project, helped interpret results, and edited the paper with ARM. All authors reviewed the results and approved the final version of the manuscript.
REFERENCES


Ripk1 regulates progranulin levels

TABLE 1. List of genes that passed tertiary screen (final hit list).

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TABLE 2. Significantly overrepresented pathways revealed through Ingenuity Pathway Analysis of final hit list.

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<tr>
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FIGURE LEGENDS

FIGURE 1. A screen of the kinome identifies Ripk1 as a genetic regulator of Pgrn level. (A) Results of primary screen. Ripk1 result is highlighted with arrowhead. (B) Results of secondary screen. Ripk1 result is highlighted with arrowhead. (C) Secondary screen result for Ripk1 siRNA. NT, nontargeting. n = 3 samples per condition. ** p<0.01; **** p<0.0001 versus NT siRNA by ANOVA. Error bars represent SD.

FIGURE 2. Ripk1 siRNAs increase Pgrn by ELISA and Western Blot. (A, B) Pgrn ELISA on neuro2a cells transfected with nontargeting (NT) siRNA, siRNA against Grn, or eight different siRNAs against Ripk1. n = 6–12 replicates per condition. Representative of ≥3 independent experiments. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001 versus NT siRNA by ANOVA. (C) Western blot on concentrated media from neuro2a cells transfected with NT or Ripk1 siRNA. Native gel blotted with ELISA capture antibody. Representative of 2 independent experiments. (F) Western blot on concentrated media from neuro2a cells transfected with NT or Ripk1 siRNA. Reducing gel blotted with anti-Pgrn (linker 1) antibody. Representative of 2 independent experiments. (D, E) Quantification of gels shown in (C, F) by densitometry. ** p<0.01; *** p<0.001 compared to NT control by t-test. kD, kilodalton. Error bars represent SD.

FIGURE 3. Ripk1 knockdown does not increase cell number or global secretion. (A, B) Cell number determined by CellTiterGlo assay in neuro2a cells transfected with nontargeting (NT), Grn siRNA, or eight different Ripk1 siRNAs. n = 6–12 replicates per condition. Representative of ≥3 independent experiments. (C) Collagen IV level secreted by neuro2a cells transfected with NT, Grn, or Ripk1 siRNA, and assessed by Collagen IV ELISA (antibodies-online.com). n = 6 replicates per condition. Representative of 2 independent experiments. * p<0.05; ** p<0.01; **** p<0.0001 compared to NT control by ANOVA. Error bars represent SD.

FIGURE 4. Ripk1 knockdown increases Pgrn in microglial-like cells and rescues Pgrn level in FTD model neurons. (A) Pgrn ELISA on microglial-like BV-2 cells transfected with nontargeting (NT) siRNA, siRNA against Grn, or four different siRNAs against Ripk1. n = 6–12 replicates per condition. Representative of ≥3 independent experiments. Note, because Grn and Ripk1 siRNAs were toxic to BV-2 cells, all values are normalized to cell number. **** p<0.0001 compared to NT control by ANOVA. (B) Pgrn level in mouse cortical neurons transduced with NT, Grn, or Ripk1 shRNA lentivirus, assessed by ELISA. n = 6 replicates per condition. Representative of ≥3 independent experiments. Lentivirus was added on day in vitro (DIV) 2 and qPCR or ELISA samples were collected DIV 7. * p<0.05; **** p<0.0001 compared to +/+ NT control by ANOVA. # p<0.05; #### p<0.0001 compared to +/- NT control by ANOVA. Error bars represent SD.

FIGURE 5. Ripk1 knockdown increases Pgrn level by increasing Pgrn translation without affecting Pgrn transcription or secretion. (A) qPCR for Grn on neuro2a cells transfected with nontargeting (NT), Grn, or Ripk1 siRNA. n = 4 replicates per condition. Representative of ≥3 independent experiments. (B) Pgrn level in neuro2a cells transfected with NT, Grn, or Ripk1 siRNA, assessed by ELISA. The two graphs represent the cell lysate (right) and media (left) of the same samples. n = 6–12 replicates per condition. Representative of ≥3 independent experiments. (C) Autoradiography for 35S-Pgrn in neuro2a cells transfected with NT or Ripk1 siRNA and incubated with 35S-methionine (top, Pgrn band is indicated by *) and quantification of autoradiogram (bottom). Representative of ≥3 independent experiments. (D) Total protein synthesis (as measured by GFP fluorescence using the OPP assay) in neuro2a cells transfected with NT or Ripk1 siRNA. n = 149–192 cells per condition. ** p<0.01; *** p<0.001; **** p<0.0001 compared to NT control by ANOVA; * p<0.05 for comparison of slopes by linear regression. Error bars represent SD. kD, kilodalton.
FIGURE 6. The effect of Ripk1 knockdown on Pgrn is kinase-independent and proceeds through a novel signaling pathway. (A) Pgrn ELISA on neuro2a cells treated with DMSO or Nec1 (0.1, 0.4, 1.6, or 6.4 µM). Representative of 3 independent experiments. (B) Pgrn ELISA on brain lysates from wild-type (+/+) or Ripk1 D138N/D138N kinase dead knock-in mice. n = 3 animals per condition. (C) ELISA for Pgrn in neuro2a cells transfected with NT, Grn, Ripk1, or Nfkb1 siRNA. n = 6–12 samples per condition. Representative of 3 independent experiments. (D) ELISA for Pgrn in neuro2a cells transfected with NT, Grn, Ripk1, or Casp8 siRNA. n = 6–12 samples per condition. Representative of 3 independent experiments. (E) ELISA for Pgrn in neuro2a cells transfected with NT, Grn, Ripk1, or Ripk3 siRNA. n = 6–12 samples per condition. Representative of 3 independent experiments. (F) ELISA for Pgrn in neuro2a cells transfected with NT or Pkr siRNA. n = 2–8 samples per condition. For panels (C-E), results were normalized to cell number (from CellTiterGlo assay) because siRNAs to Nfkb1, Casp8, and Ripk3 caused a significant change in cell number. * p<0.05; ** p<0.01; **** p<0.0001 compared to NT control by ANOVA. Error bars represent SD.
Figure 1

A. Primary Screen

B. Secondary Screen

C. Secondary Screen - Ripk1

Figure legend:
- Standard Deviations from Plate Mean
- ANOVA Versus Control
- p-value
- Relative Extracellular [Pgrn]
Figure 2

A

ELISA

Relative Extracellular [PGRN]

NT, Grn, Ripk1 #1, Ripk1 #2, Ripk1 #3, Ripk1 #4

siRNA

B

ELISA

Relative Extracellular [PGRN]

NT, Grn, Ripk1 #5, Ripk1 #6, Ripk1 #7, Ripk1 #8

siRNA

C

Anti-Pgrn CT

Native Gel

NT si

Ripk1 #1 si

D

Native Gel

Reducing Gel

NT si

Ripk1 #1 si

E

Reducing Gel

NT si

Ripk1 #1 si

F

Anti-Pgrn Linker 1

Reducing Gel

NT si

Ripk1 #1 si
Figure 3

A. Cell Number

B. Cell Number

C. Collagen IV ELISA
Figure 4

A

ELISA

B

ELISA

![Graph A](image1)

![Graph B](image2)
A

**Grn mRNA**

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**Extracellular ELISA**

**Intracellular ELISA**

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**Autoradiography**

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**Total Protein Translation**

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Figure 5