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Receptor-interacting serine/threonine protein kinase 1 (Ripk1) regulates progranulin levels

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Receptor-interacting serine/threonine protein kinase 1 (Ripk1) regulates progranulin levels

by

Amanda Rose Mason

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Developmental and Stem Cell Biology

in the

GRADUATE DIVISION
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by
Amanda Rose Mason
DEDICATION AND ACKNOWLEDGEMENTS

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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 is an attractive 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. 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 support targeting Ripk1 as a therapeutic strategy in both AD and FTD.
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CHAPTER 1.

INTRODUCTION

1.1 PROGRANULIN OVERVIEW

Progranulin (PGRN), also known as granulin-epithelin precursor, proepithelin, acrogranin, and GP88/PC-cell derived growth factor, is a secreted growth factor with diverse roles in inflammation, cancer, and metabolic disease\(^1\)\(^-\)\(^5\). PGRN is found widely among the eukaryotes and is present in worms, zebrafish, mice, and humans, but is absent in flies. It is a highly glycosylated, highly cysteine-bonded proprotein composed of seven and one half conserved granulin domains. Progranulin can be cleaved into individual granulins by metalloproteinases (MMP-9\(^6\), MMP-14\(^7\), and ADAMTS-7\(^8\)) and neutrophil-secreted proteases (elastase and proteinase 3\(^9\)). PGRN is expressed highly in epithelial tissues, macrophages, and the brain. In the brain, PGRN expression increases over the course of development\(^10\), and the primary sources of PGRN are neurons and microglia\(^10\),\(^11\). Microglia activated by insult or injury are an especially abundant source of PGRN as they exhibit a significant upregulation in PGRN expression. Indeed, increased progranulin expression (3- to 8-fold, by qPCR or immunostaining) has been observed in activated microglia in mouse models of spinal cord injury\(^12\),\(^13\), axotomy\(^14\), traumatic brain injury (TBI)\(^15\), amyotrophic lateral sclerosis (ALS)\(^16\), Alzheimer’s disease (AD)\(^17\), and multiple sclerosis (MS)\(^18\). However, at baseline, most of the brain’s PGRN may be produced by neurons, as demonstrated by selective knock-out experiments: when PGRN was excised using a neural-specific (CamKII or Nestin) Cre recombinase there was a 30-65% decrease in brain PGRN, but there was no detectable decrease in PGRN when PGRN was excised using a microglial-specific (LysM) Cre (Andrew Arrant, Erik Roberson lab, University of Alabama at Birmingham, oral communication).
In the periphery, PGRN acts as a growth factor to promote cell division of epithelial, endothelial, and cancer cells\textsuperscript{19} and to spur wound healing\textsuperscript{20}. PGRN is upregulated in many forms of cancer including breast\textsuperscript{21}, ovarian\textsuperscript{22}, and gastrointestinal cancer\textsuperscript{23}. It is also a key adipokine and mediates insulin resistance induced by a high fat diet\textsuperscript{24}. PGRN also plays an important role in immunity, as demonstrated by the increased susceptibility to bacterial infection in PGRN null animals\textsuperscript{25}. Interestingly, full-length PGRN and individual granulins display opposing effects on inflammation: granulins promote inflammation by increasing the expression of IL-1β, IL-8, and TNFα\textsuperscript{26,27} whereas PGRN inhibits TNFα and upregulates anti-inflammatory cytokines such as IL-10\textsuperscript{28}.

Many binding partners for PGRN have been identified, including extracellular (Dlk1\textsuperscript{29}, COMP\textsuperscript{30}, perlecan\textsuperscript{31}), cytoplasmic (hexokinase\textsuperscript{32}, TPM3\textsuperscript{33}), and nuclear (cyclin-T1\textsuperscript{34}, Tat\textsuperscript{34}) proteins. Signaling pathways identified to be activated by PGRN include MAPK/ERK\textsuperscript{22,35,36} and PI3K/Akt\textsuperscript{35–37}. The identity of the receptor(s) that mediate PGRN signaling remain somewhat elusive, but one receptor has been identified, sortilin\textsuperscript{38}, which will be discussed further below (see Section 1.2). Another possible receptor, TNFR1, was reported to bind PGRN\textsuperscript{39} but others have failed to replicate this result\textsuperscript{40}.

1.2 PROGRANULIN FUNCTION IN NEURONS

In neurons, PGRN seems to exert a trophic effect, promoting survival of cortical and motor neurons in culture\textsuperscript{41}, neurite outgrowth in culture\textsuperscript{42}, and motor neuron axonal outgrowth in zebrafish\textsuperscript{43}. In addition, PGRN may play an important role in synapse biology. PGRN knockdown leads to
increased synaptic vesicle number and increased vesicle release probability in cultured hippocampal neurons\textsuperscript{44}. FTD patients with \textit{GRN} mutations also exhibit an increase in synaptic vesicle number\textsuperscript{44}. Moreover, PGRN is secreted in an activity-dependent manner\textsuperscript{45}.

PGRN has also been shown to play a role in protecting neurons against different forms of cell stress including A\beta toxicity in culture\textsuperscript{46} and stroke induced by middle cerebral artery occlusion \textit{in vivo}\textsuperscript{47}. In addition, lack of PGRN makes neurons more susceptible to several cell stressors, including H\textsubscript{2}O\textsubscript{2}\textsuperscript{48}, NMDA\textsuperscript{48}, PI3K and MEK inhibitors\textsuperscript{49}, and MPTP \textit{(in vivo)}\textsuperscript{50}. In worms, lack of PGRN confers resistance to ER stress, osmotic stress, and heat shock\textsuperscript{51}.

The only PGRN receptor widely accepted in the field is sortilin, a neuronal scavenger receptor. PGRN binds to sortilin through its C-terminus, and is internalized and targeted to the lysosome after binding\textsuperscript{38,52}. Importantly, sortilin regulates extracellular PGRN levels; knock-out of sortilin resulted in a 2.5- to 5-fold increase in extracellular PGRN with a concomitant decrease in intracellular PGRN\textsuperscript{38}. However, at least some functions of PGRN, including neurite outgrowth\textsuperscript{42} and improvement of neuronal survival\textsuperscript{41}, do not require sortilin.

### 1.3 PROGRANULIN FUNCTION IN MICROGLIA

PGRN functions as a chemoattractant for microglia: lentiviral overexpression of PGRN increased the number of microglia surrounding the viral injection site in mice\textsuperscript{28}. PGRN deficiency in microglia leads to a hyperinflammatory state with cultured PGRN knock-out microglial cells having highly increased expression of inflammatory genes upon LPS stimulation compared to wild-type controls\textsuperscript{50}. PGRN may also affect the phagocytic properties of microglia. Macrophages
from PGRN knock-out mice exhibited increased rates of phagocytosis\textsuperscript{53}; however, another group found that recombinant PGRN increased the endocytosis of Aβ peptide in wild-type primary mouse microglia\textsuperscript{28}. While these two results seem somewhat at odds, it is clear that PGRN can regulate phagocytosis.

1.4 PROGRANULIN IN FRONTOTEMPORAL DEMENTIA

Frontotemporal dementia (FTD) is a devastating neurodegenerative disease which affects approximately 25,000-45,000 individuals in the United States\textsuperscript{54,55}. Symptoms usually begin in the fifth or sixth decade of life and include loss of social awareness, apathy, disinhibition, poor executive function, impaired verbal comprehension, and/or impaired speech production. The average time from onset to death is less than ten years and there are currently no treatments\textsuperscript{56}.

The brain pathology underlying the severe and progressive symptoms of FTD is equally striking. Pronounced neuronal loss is observed in the frontal and temporal cortices; at the time of death many patients have such pronounced neurodegeneration that it can be appreciated macroscopically as a brain with shrunken and sharp rugae. Gliosis is another prominent pathological feature. Approximately 40% of FTD cases show familial inheritance\textsuperscript{57,58}, and the most common inheritance pattern is autosomal dominant. Mutations in \textit{GRN}, the gene encoding PGRN, are one of the most common causes of FTD\textsuperscript{59,60}, representing about 15-20\% of familial patients\textsuperscript{61–64}. These patients exhibit TDP-43 pathology, an abnormal accumulation of hyperphosphorylated cytoplasmic TDP-43 protein, in affected brain regions such as the frontal and temporal cortices\textsuperscript{65}. TDP-43 pathology is also found in many other forms of neurodegeneration, including non-\textit{GRN} FTD, ALS, and AD\textsuperscript{66}. Mutations in \textit{GRN}, unusually among mutations causing neurodegenerative disease, are
haploinsufficiency mutations: patients have half the normal level of *GRN* mRNA and less than half the normal level of PGRN protein in blood, CSF, and unaffected brain regions\textsuperscript{67–69}. Almost all mutations in *GRN* are nonsense or frameshift mutations which lead to the production of an early stop codon and the initiation of nonsense-mediated decay\textsuperscript{61}; this explains the 50% reduction in *GRN* mRNA seen in patients. *GRN* mutations are autosomal dominant and fully penetrant, with 90% of patients exhibiting symptoms by age \textsuperscript{70}. However, the disease course is quite variable, even among patients with the same mutation. In a cohort of 37 patients with PGRN Arg493X mutations, age of onset was 44–69 and clinical diagnoses included FTD, primary progressive aphasia (a language variant of FTD), corticobasal syndrome, and AD\textsuperscript{70}. All patients who came to autopsy had FTD pathology with TDP-43 inclusions.

Several mouse models of *GRN* deficient FTD have been reported\textsuperscript{25,71–76}. Unfortunately, heterozygous loss of PGRN causes little change in behavior or neuropathology, even in very aged mice (up to two years). One exception is a sociability defect reported by one group\textsuperscript{73}. Because of this, much more emphasis has been placed on studying the phenotypes of *Grn* homozygous null mice. Studies variously report changes in anxiety, coordination, depression, and memory/learning in *Grn* -/- mice, but no consistent changes in behavior are seen. On the other hand, all strains of *Grn* -/- mice exhibit microgliosis, astrogliosis, and strikingly increased deposition of lipofuscin, an autofluorescent byproduct of lysosomal degradation associated with aging. All these pathological features worsen as the mice age. However, none of the *Grn* -/- strains exhibit the major pathological hallmarks of *GRN*-FTD, TDP-43 inclusions and neuronal loss. Overall, the *Grn* deficient mice exhibit several FTD-like features but fail to recapitulate some of the major hallmarks of FTD.
All known cases of FTD resulting from \textit{GRN} mutation exhibit heterozygous mutations in \textit{GRN}. Two patients (sisters) with homozygous mutations in \textit{GRN} have been reported and, surprisingly, their clinical presentation is of neuronal ceroid lipofuscinosis (NCL)\textsuperscript{77}. Very distinct from FTD, NCL symptoms include vision loss and cerebellar ataxia. Pathology is that of a lysosomal storage disorder, with predominant lipofuscinosi. Clearly, gene dose is a very important determinant of phenotype in cases of \textit{GRN} mutation. These two patients call into question the suitability of \textit{Grn} \textsuperscript{-/-} mice for modeling FTD.

The discovery that homozygous loss of \textit{PGRN} causes NCL has sparked an interest in the possible role of \textit{PGRN} in supporting lysosome function. Several reports link lysosome biology and \textit{PGRN}. First, gene network analysis identified \textit{PGRN} as one of the top genome-wide candidates for regulating lysosome function\textsuperscript{78}. \textit{PGRN} also colocalizes with lysosomal markers. Additionally, the \textit{GRN} gene contains two binding sites for TFEB, an important transcription factor, which regulates the transcription of many lysosomal genes. \textit{PGRN} knock-out mice have increased TFEB translocation to the nucleus, which is required for TFEB transcriptional regulation, and increased staining for lysosomal markers\textsuperscript{79}. Finally, TMEM106B, a genetic modifier of \textit{PGRN} level and of age-of-onset in \textit{GRN}-FTD, controls lysosome size: TMEM106B knockdown decreases lysosome size and overexpression increases lysosome size\textsuperscript{80}. Together with the information that homozygous mutation in \textit{GRN} causes NCL, these data support a role for \textit{PGRN} in lysosome biology.
Because *GRN* mutations lead to halved PGRN protein levels, it is postulated that this form of FTD is a loss-of-function disease in which replacement of PGRN to normal levels would be an attractive therapeutic strategy. Conveniently, PGRN levels can be easily measured in both serum and CSF to track therapeutic efficacy. Experiments in both cellular and animal models of FTD showed rescue of FTD-related phenotypes by overexpressing or adding exogenous PGRN. For example, in zebrafish, knockdown of two PGRN homologs results in decreased motor neuron axon outgrowth and this phenotype could be rescued by human PGRN overexpression. In another study, primary mouse hippocampal neurons lacking PGRN exhibited a decrease in neurite length and branching which was rescued by lentiviral PGRN expression. Finally, primary microglia from mice lacking PGRN showed a dramatic increase in expression of proinflammatory genes which was rescued by lentiviral expression of PGRN. Taken together, these results suggest that restoring normal PGRN levels can reverse phenotypes associated with PGRN deficiency and therefore represents an attractive therapeutic strategy in FTD patients with *GRN* mutations.

### 1.5 Progranulin in Alzheimer’s Disease

AD affects 4 million individuals in the U.S. alone. The prevalence of AD is increasing rapidly as the global population ages and is expected to reach 80 million worldwide by 2040. AD symptoms include loss of memory, disorientation, and inability to perform self-care. There are no treatments that can slow or halt the disease progression, which is inevitably fatal. Pathologically, AD is defined by the presence of Aβ plaques and neurofibrillary tangles composed of the protein tau, as well as widespread and pronounced neuronal loss, especially in the posterior regions of the cortex and in the hippocampus.
Although familial cases of AD are quite rare, representing less than 5% of total cases, there is a genetic link between GRN and sporadic AD. First, a few patients with clinical AD and heterozygous null mutations in GRN have been reported\textsuperscript{82–84}. Second, a single nucleotide polymorphism (SNP) in the 3’ UTR of GRN is a risk factor for AD (odds ratio, 1.4)\textsuperscript{85}, and this SNP is associated with decreased serum PGRN levels\textsuperscript{86}. Moreover, in a mouse model of AD, overexpression of PGRN reduced plaque burden, improved memory, and decreased neuronal loss\textsuperscript{87}. These results suggest that reduced PGRN may contribute to AD pathophysiology and that increasing PGRN levels can reverse AD phenotypes. Therefore, increasing PGRN represents an attractive therapeutic strategy in AD patients in addition to FTD patients.

1.6 PROGRANULIN AS A THERAPEUTIC TARGET

There are several ongoing attempts to manipulate PGRN levels as a therapeutic strategy. First, PGRN plays a crucial role in tumor growth, chemoresistance, and tissue invasion in hepatocellular carcinoma (HCC)\textsuperscript{88,89}. A group of researchers at the University of Hong Kong have developed an antibody to PGRN, which can inhibit human HCC xenograft growth in mice when used alone, or eradicate the tumors entirely when used in combination with a chemotherapeutic agent\textsuperscript{90}. This anti-PGRN antibody may well move into clinical trials for HCC soon, especially considering that the group has patented the reduction of PGRN as a therapeutic strategy in HCC (US patent #20140356321 A1).

On the other hand, several pharmacologic strategies for increasing PGRN levels have been investigated for the treatment of FTD. First, inhibitors of the vacuolar ATPase (which controls intracellular, vesicular, and extracellular pH) increase PGRN levels through a non-transcriptional
mechanism\textsuperscript{91}, but these drugs have not been pursued further in human patients. Second, the histone deacetylase (HDAC) inhibitor vorinostat increases \textit{GRN} transcription \textit{in vitro}\textsuperscript{92}. Another HDAC inhibitor, FRM-0334, was used by FORUM Pharmaceuticals in a phase II clinical trial of FTD patients with \textit{GRN} mutations (ClinicalTrials.gov identifier NCT02149160). The trial ended in early 2016, but no results have been reported. Finally, based on preclinical data showing that the calcium channel blocker nimodipine increases PGRN levels (Li Gan, University of California, San Francisco, oral communication), a phase I clinical trial examining the effect of nimodipine on \textit{GRN}-FTD patients is underway (ClinicalTrials.gov identifier NCT01835665). Overall, there is enthusiasm for modulating PGRN levels as a therapeutic strategy, and this study seeks to add to the body of knowledge surrounding this exciting idea.

1.7 AIMS OF THIS STUDY

Because increasing PGRN level is an attractive therapeutic strategy in both FTD and AD, and because little is known about the genetic regulation of PGRN level, we decided to perform an siRNA-based genome-wide screen to uncover genetic modifiers of PGRN level. Here, we will report the results of the screen in the kinome, the subset of the genome encoding kinases (719 genes). The remainder of the genome-wide screen will be reported by Lisa Elia, Senior Research Associate, Finkbeiner lab. The goal of the present study was to discover genetic modifiers of PGRN levels as a way to (1) more fully understand PGRN biology and (2) uncover new pathways, which can be targeted to increase PGRN therapeutically.

Among the 719 kinases, we identified 24 hits (3.3\% hit rate) that increase PGRN levels. We prioritized receptor-interacting serine/threonine protein kinase 1 (Ripk1) as our top hit because of
(1) the large effect size of knocking down Ripk1, (2) the fact that Ripk1 is highly expressed in brain, and (3) the availability of tools for interrogating Ripk1 function, including knock-in mice and a specific inhibitor (Section 2.1). We verified that Ripk1 is a *bona fide* genetic regulator of PGRN by using eight different siRNAs against Ripk1 to increase PGRN by ELISA or western blot, using shRNA against Ripk1 to increase PGRN in mouse cortical neurons from wild-type or *Grn* heterozygous mice, and confirming that Ripk1 knockdown does not affect cell number or global secretion (Section 2.2). 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 (Section 2.3). We found that the effect of Ripk1 knockdown on PGRN is independent of Ripk1’s kinase activity and occurs through a novel signaling pathway (Section 2.4). These data support targeting Ripk1 as a therapeutic strategy in both AD and FTD.
CHAPTER 2.

RESULTS

2.1 KINOME SCREEN

In order to uncover genetic regulators of PGRN level, we designed an siRNA-based screen which reads-out extracellular PGRN level via sandwich enzyme-linked immunosorbent assay (ELISA). Because the sandwich ELISA format requires two PGRN-specific antibodies, we first raised a rabbit antibody against a peptide in the C-terminus of mouse PGRN; this antibody served as the capture antibody; we named this antibody “anti-PGRN CT”. We used a commercially available antibody for the detection antibody. We demonstrated the specificity of the anti-PGRN CT antibody by western blot; the antibody detects a single band, which is eliminated by siRNA against PGRN (Fig 2.1A). We also validated the sandwich ELISA protocol by testing media PGRN levels from wild-type, PGRN knock-out\(^5\), or PGRN overexpressing mouse primary cortical neurons. As expected, the ELISA assay showed that PGRN knock-out neurons have almost no detectable PGRN, and PGRN overexpressing neurons have more than 3-fold increased PGRN compared to wild-type neurons (Fig 2.1B).
Having established a robust PGRN ELISA protocol, we performed a screen of the 719 genes in the kinome (Fig 2.2A). In the primary screen, we reverse transfected a mouse neuroblastoma-derived cell line (neuro2a) with a mixture of 4 different siRNAs against each gene and assayed extracellular PGRN via our ELISA. Hits from the primary screen were defined as those siRNAs that resulted in an extracellular PGRN concentration at least 1.5-times above the plate mean (Fig 2.2B). Hits from the primary screen were repeated in triplicate in the secondary screen. Secondary screen hits were those siRNAs that gave a significant increase in PGRN compared to nontargeting control siRNA by ANOVA (Fig 2.2C). Secondary screen hits were evaluated in a tertiary screen, which assayed the effect on PGRN of each of the four siRNAs separately. Tertiary screen hits were those genes for which at least two of the four siRNAs led to an increase in secreted PGRN. 24 genes passed the tertiary screen (Table 2.1); Ingenuity Pathway Analysis reveals that these genes are involved in 15 significantly overrepresented pathways (Table 2.2). We prioritized
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 two-fold (Fig 2.2D), (2) Ripk1 is highly expressed in brain, and (3) there are many tools available for interrogating Ripk1 function, including knock-in mice and a specific inhibitor of Ripk1 kinase activity. Although a two-fold increase in PGRN may seem modest, we were looking for effects of this size. FTD patients have about half the normal level of PGRN and many cancer patients have PGRN overexpression. Therefore, we wanted to increase PGRN from the disease range (half) to the normal range, a factor of approximately two.

Figure 2.2. A screen of the kinome identifies Ripk1 as a genetic regulator of PGRN level. (A) Screen design. (B) Results of primary screen. Ripk1 result is highlighted with arrowhead. (C) Results of secondary screen. Ripk1 result is highlighted with arrowhead. (D) 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.
Table 2.1. List of genes that passed tertiary screen (final hit list).

| Gene Name | Accession Number | Fold-Change in [PGRN] | Number of siRNAs that Increased [PGRN] |
|-----------|------------------|-----------------------|----------------------------------------|
| Gmfb      | NM_022023        | 3.47                  | 3/4                                    |
| Prkcq     | NM_008859        | 2.82                  | 4/4                                    |
| Hipk2     | NM_010433        | 2.55                  | 4/4                                    |
| Ripk1     | NM_009068        | 2.32                  | 4/4                                    |
| Lrrk2     | NM_025730        | 2.27                  | 2/4                                    |
| Egfr      | NM_007912        | 2.21                  | 4/4                                    |
| Vegfr3    | NM_008029        | 2.13                  | 3/4                                    |
| Itgb1bp1  | NM_008403        | 2.07                  | 4/4                                    |
| Ttk       | NM_009445        | 1.95                  | 3/4                                    |
| Srpk1     | NM_016795        | 1.79                  | 3/4                                    |
| Stk38     | NM_134115        | 1.77                  | 4/4                                    |
| Prkg2     | NM_008926        | 1.69                  | 2/4                                    |
| Csnk1g1   | NM_173185        | 1.64                  | 3/4                                    |
| Pik3c2a   | NM_011083        | 1.64                  | 3/4                                    |
| Epha3     | NM_010140        | 1.62                  | 3/4                                    |
| Ick       | NM_019987        | 1.52                  | 3/4                                    |
| Pdgfra    | NM_011058        | 1.51                  | 2/4                                    |
| Ilk       | NM_010562        | 1.47                  | 3/4                                    |
| Mapk11    | NM_011161        | 1.47                  | 2/4                                    |
| Cdk7      | NM_009874        | 1.45                  | 2/4                                    |
| Gyk       | NM_008194        | 1.44                  | 2/4                                    |
| Tex14     | NM_031386        | 1.35                  | 4/4                                    |
| Ros1      | NM_011282        | 1.28                  | 3/4                                    |
| Prkci     | NM_008857        | 1.25                  | 2/4                                    |
Table 2.2. Significantly overrepresented pathways revealed through Ingenuity Pathway Analysis of final hit list.

| Signaling Pathway                                           | p-Value |
|-------------------------------------------------------------|---------|
| 1 Gap Junction Signaling                                    | 0.007   |
| 2 UVB-Induced MAP Signaling                                 | 0.007   |
| 3 Hepatic Fibrosis                                           | 0.011   |
| 4 UVC-Induced MAPK Signaling                                | 0.013   |
| 5 Nitric Oxide Signaling in the Cardiovascular System       | 0.018   |
| 6 HER-2 Signaling in Breast Cancer                          | 0.021   |
| 7 Dopamine-DARPP32 Feedback in cAMP Signaling               | 0.021   |
| 8 Thrombin Signaling                                        | 0.021   |
| 9 Glioma Signaling                                          | 0.023   |
| 10 VEGF Family Ligand-Receptor Interactions                 | 0.023   |
| 11 NF-kB Signaling                                          | 0.023   |
| 12 ErbB Signaling                                           | 0.027   |
| 13 NF-kB Activation by Viruses                              | 0.027   |
| 14 IL-8 Signaling                                           | 0.034   |
| 15 eNOS Signaling                                           | 0.041   |

2.2 RIPK1 IS A BONA FIDE GENETIC REGULATOR OF PGRN

Because the tertiary screen revealed that 4 different siRNAs against Ripk1 individually increased PGRN levels (Fig 2.3A), we knew that the possibility that these siRNAs affect PGRN levels through off-target effects was small. However, we decided to exclude this possibility by using another 4 Ripk1 siRNAs (for a total of 8 unique siRNAs). We found that the 4 new siRNAs also lead to increased PGRN levels (Fig 2.3B). Because 8 different Ripk1 siRNAs all lead to an increase in PGRN, it is statistically almost impossible that the effects are off-target. We also confirmed that all 8 siRNAs effectively knock down Ripk1 (Fig 2.3C-D).
Figure 2.3. Eight different Ripk1 siRNAs increase PGRN by ELISA. (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. (C-D) qPCR for Ripk1 on neuro2a cells transfected with NT siRNA, Grn siRNA, or eight different Ripk1 siRNAs. n = 4 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. Error bars represent SD.

The PGRN detected in our ELISA assay is native and undenatured (not boiled, no reducing agents present). Because PGRN can form a dimer\textsuperscript{93} and is highly glycosylated and cysteine-bonded, it
could be that the differences in PGRN levels seen comparing nontargeting siRNA and Ripk1 siRNA are due to changes in tertiary protein structure or post-translational modifications (which could affect antibody binding) rather than a change in total PGRN levels. To exclude this possibility, we checked PGRN levels after Ripk1 knockdown by western blot, which allows for size discrimination of PGRN. Using the ELISA capture antibody and a native gel, we detected only one band corresponding to native PGRN at approximately 180 kD, which was increased by Ripk1 knockdown (Fig 2.4A, C). Full-length PGRN runs at approximately 88 kD in a reducing gel, so this band may represent a dimer. This is hard to confirm as there is no known manipulation that disrupts dimerization. We next used an antibody raised against a peptide in linker 1 of PGRN (“anti-PGRN linker 1”) and a reducing gel and again saw an increase in PGRN with Ripk1 siRNA (Fig 2.4B, D). This PGRN band ran at the expected size for a monomer and showed no shift (which would indicate post-translational modification) with Ripk1 knockdown. Together, these data show that both native and fully denatured PGRN are increased by Ripk1 knockdown, suggesting that the change in PGRN level detected by ELISA is not due to a change in tertiary structure or post-translational modification.
Ripk1 knockdown could increase extracellular PGRN levels in many different ways: increased cell number, increased secretion of all secreted proteins, increased transcription, increased translation, increased secretion, or decreased degradation. To exclude the possibility that Ripk1 knockdown increased PGRN level by increasing cell number, which is especially important given Ripk1’s role as a mediator of cell death⁹⁴, we measured the number of viable cells at the time of ELISA assay. We found that the change in cell number with Ripk1 siRNA varied from -20% to +14% (Fig 2.5A-B), which cannot explain the change in PGRN protein level of up to +200% (Fig 2.3A-B). Thus, Ripk1 knockdown does not increase PGRN levels by increasing cell number. To exclude the possibility that Ripk1 knockdown increased PGRN level by increasing global secretion, we measured the secretion of another constitutively secreted protein, Collagen IV. We
found no change in the extracellular levels of Collagen IV upon Ripk1 knockdown (Fig 2.5C), suggesting that Ripk1 does not increase the secretion of all secreted proteins.

**Figure 2.5. 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.

We next tested whether Ripk1 knockdown affected PGRN levels in cell types other than neuro2a cells. Because brain PGRN is produced primarily by neurons and microglia, we first checked the effect of Ripk1 knockdown in a microglial-like cell line, BV-2. We found that 4 siRNAs against Ripk1 increased PGRN levels by ELISA in BV-2 cells (Fig 2.6A). 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. Additionally, at baseline, the majority of brain PGRN may be produced by neurons, as demonstrated by selective knock-out experiments: when PGRN was excised using a neural-specific (CamKII or Nestin) Cre recombinase, there was
a 30–65% decrease in brain PGRN versus no detectable decrease in PGRN when PGRN was excised using a microglial-specific (LysM) Cre (Andrew Arrant, Erik Roberson lab, University of Alabama at Birmingham, oral communication). 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 (Fig 2.6A). Next, we compared PGRN levels (by ELISA) in primary mouse cortical neurons infected with nontargeting, PGRN, or Ripk1 shRNA and found that Ripk1 shRNA increases extracellular PGRN levels approximately 2-fold (Fig 2.6B). For this experiment, we used both wild-type neurons and neurons from a mouse FTD model harboring a heterozygous mutation in PGRN. The PGRN +/- neurons have less than half the normal PGRN level, as expected. Importantly, we found that Ripk1 shRNA doubled PGRN in both wild-type and PGRN +/- mouse neurons. In fact, Ripk1 shRNA rescued PGRN levels in PGRN +/- neurons to wild-type baseline levels (Fig 2.6B). This is an extremely important result because it shows that Ripk1 knockdown can restore normal PGRN levels in an FTD model. Given the involvement of PGRN overexpression in many forms of cancer, it is crucial that this rescue is to wild-type PGRN levels, and not high above this level.
2.3 RIPK1 KNOCKDOWN INCREASES PGRN TRANSLATION

Having excluded an increase in cell number and an increase in global secretion, we determined that Ripk1 knockdown could be increasing extracellular PGRN level through increased transcription, increased translation, increased secretion, and/or decreased 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 are unchanged by Ripk1 knockdown (Fig 2.7A). Therefore, increased transcription is not the mechanism by which Ripk1 knockdown increases PGRN levels.
We next determined whether the increase in extracellular PGRN detected by ELISA and by western could be explained by increased PGRN secretion. PGRN is a secreted molecule, so an increase in the extracellular level of PGRN could theoretically be achieved 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 2.7B). 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 are several times higher than intracellular PGRN levels, meaning that even if all intracellular PGRN was converted to extracellular PGRN, it could not account for the doubling of PGRN after Ripk1 knockdown (as seen by ELISA, Fig 2.3) that we observed.

Although increased secretion alone cannot explain the increase in PGRN seen upon Ripk1 knockdown, a change in secretion or uptake rate of PGRN could still occur after Ripk1 knockdown (in combination with some other mechanism). To check this possibility, we made recombinant mouse PGRN with an N-terminal FLAG tag. We applied the FLAG-PGRN to PGRN knock-out neurons and assayed the cell lysates for intracellular FLAG-PGRN over time. We found that PGRN knock-out neurons can take up FLAG-PGRN. We compared nontargeting and Ripk1 shRNA and found that Ripk1 knockdown does not decrease the rate of PGRN uptake (Fig 2.7C). If anything, Ripk1 shRNA increases the rate of PGRN uptake. Therefore, we can conclude that Ripk1 knockdown does not increase PGRN through increased secretion or decreased re-uptake.
We also noted from this experiment that, after PGRN wash out from the media, all intracellular PGRN is secreted within one hour. This indicates that PGRN cycles rapidly in and out of the cell.

To examine PGRN translation, we added $^{35}$S-methionine to the media and monitored the radioactive PGRN level (extracellular plus intracellular) 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 2.7D). These data indicate that Ripk1 knockdown increases PGRN levels by increasing translation rate. Interestingly, by 3 hours, the relative abundance of PGRN in nontargeting versus Ripk1 siRNA conditions is ~1.6, in line with what we saw by western blot and ELISA. Because this assay examines only full-length PGRN, one alternative possibility is that PGRN is cleaved into granulins more slowly in the Ripk1 knockdown condition. Although this experiment cannot exclude this possibility, we believe it is unlikely based on the fact that western blot using an antibody directed to linker 1 of PGRN reveals a prominent band representing full-length PGRN and no smaller species (Fig 2.4). This antibody would detect any poly-granulin species containing linker1 formed as individual granulins are cleaved off the full-length protein. Because these species are rare in neuro2a cells (in contrast to brain\textsuperscript{95}), we believe PGRN cleavage is slow to moderate in neuro2a cells and therefore unlikely to explain the change in PGRN build-up during $^{35}$S-methionine incubation.
We next examined PGRN degradation using metabolic pulse labeling with $^{35}$S-methionine on total (extracellular plus intracellular) PGRN. We found that one population of PGRN was degraded very rapidly (within 8 hours; Fig 2.8A-B) while another population of PGRN remained present for
up to 3 days (Fig 2.8A). However, results were variable between experimental replicates. Ripk1 siRNA caused PGRN to decay less rapidly than nontargeting siRNA in some experiments (e.g. Fig 2.8A) and more rapidly in others (e.g. Fig 2.8B). Additionally, the shape of the decay curve for the nontargeting siRNA condition did not fit a one-phase decay (as expected for metabolic pulse-chase experiments). On the other hand, the results for the Ripk1 siRNA condition could be fit to a one-phase decay curve and yielded a PGRN half-life of 2–11 hours, significantly shorter than the previously reported half-life of 40 hours (although the authors did not show any data to support this number). Therefore, we could not conclude that there is any change in PGRN turnover upon Ripk1 knockdown.

Figure 2.8. The effect of Ripk1 knockdown on PGRN degradation. (A-B) Autoradiography for $^{35}$S-PGRN in neuro2a cells transfected with NT or Ripk1 siRNA and pulsed with $^{35}$S-methionine (top) and quantification of autoradiograms (bottom). Samples were collected for PGRN pull-down and autoradiography 0, 8, 20, 28, 48, or 72 hours after $^{35}$S wash-out (A) or -1, 0, 1, 3, 5, or 7 hours after $^{35}$S wash-out (B). Representative of ≥3 independent experiments, with considerable variability between replicates. kD, kilodalton.
2.4 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 which inhibits all Ripk1 kinase activity, including autophosphorylation\textsuperscript{96,97}. 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 $\gamma^{32}$P-ATP followed by Ripk1 pulldown and autoradiography. Nec1 inhibited Ripk1 autophosphorylation (Fig 2.9A-B). Next, we checked whether Nec1 could increase PGRN. We found that, unlike Ripk1 knockdown, inhibition of Ripk1 by Nec1 did not increase PGRN (Fig 2.9C). We next assayed PGRN levels in mice with a homozygous knock-in for the D138N mutation in Ripk1, which confers loss of kinase activity\textsuperscript{98}. We found that brain PGRN levels were not changed by loss of Ripk1 kinase activity (Fig 2.9D). Together, these data indicate that the loss of Ripk1’s kinase activity is not sufficient to increase PGRN. This result may seem surprising; however, somewhat uniquely among kinases, many of the effects of Ripk1 are kinase-independent\textsuperscript{94}.
Figure 2.9. Loss of Ripk1 kinase activity does not replicate the effect of Ripk1 knockdown. (A) Western blot (WB) for Ripk1 on neuro2a cells transfected with nontargeting (NT) siRNA or Ripk1 siRNA, treated with Nec1 (+ 6 uM; ++ 18 uM) or DMSO (−), and pulsed with γ\(^{32}\)P-ATP. Samples were collected for Ripk1 pull-down and immunoblotting 48 hours after transfection. Representative of 2 independent experiments. (B) Autoradiography for \(^{32}\)P on samples from (A). Representative of 2 independent experiments. (C) PGRN ELISA on neuro2a cells treated with DMSO or Nec1 (0.1, 0.4, 1.6, or 6.4 uM) for 48 hours. Representative of 3 independent experiments. (D) PGRN ELISA on brain lysates from wild-type (+/+) or Ripk1 D138N/D138N kinase dead knock-in mice. n = 3 animals per condition. Error bars represent SD. kD, kilodalton.

Because many signaling pathways downstream of Ripk1 have previously been explored\(^{94,99–102}\), we wanted to check whether the Ripk1 acts through one of these pathways to affect PGRN. The three main signaling complexes formed downstream of Ripk1 are (1) Complex I which contains the TNF receptor and activates Nfkb-dependent transcription, (2) 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 Nfkb. Although siRNAs to Nfkb1 were effective at reducing Nfkb1 mRNA levels (Fig 2.10A), Nfkb1 siRNA did not increase PGRN levels (Fig 2.10B). Likewise, siRNAs to Casp8 and Ripk3 were effective in reducing target mRNA levels (Fig 2.10C, E), but neither siRNA increased PGRN levels (Fig 2.10 D, F). These data suggest that the three best-studied signaling pathways downstream of Ripk1 are not responsible for its effect.
Therefore, we conclude that the effect of Ripk1 knockdown on PGRN levels may proceed through a novel pathway.

**Figure 2.10. Knockdown of key genes in three pathways downstream of Ripk1 does not replicate effect of Ripk1 knockdown.** (A) qPCR for Nfkb1 in neuro2a cells transfected with nontargeting (NT) or Nfkb1 siRNA. n = 4 samples per condition. Representative of 2 independent experiments. (B) 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. (C) qPCR for Casp8 in neuro2a cells transfected with NT or Casp8 siRNA. n = 4 samples per condition. Representative of 2 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) qPCR for Ripk3 in neuro2a cells transfected with NT or Ripk3 siRNA. n = 6 samples per condition. Representative of 2 independent experiments. (F) 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. For panels B, D, and F, 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.
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 (Fig 2.11). We also tested whether PGRN and Ripk1 co-localize by immunocytochemistry. Unlike PGRN and Lamp1 (lysosomal marker), PGRN and Ripk1 do not significantly co-localize (Fig 2.12).

**Figure 2.11. Ripk1 and PGRN do not co-immunoprecipitate.** (A) Western blot (WB) for PGRN (sheep anti-PGRN antibody) on neuro2a cells transfected with nontargeting (NT) siRNA, Grn siRNA, or Ripk1 (Rip) siRNA then lysed and subjected to immunoprecipitation (IP) by rabbit anti-PGRN antibody, mouse anti-Ripk1 antibody, control IP with normal rabbit IgG (Rb IgG), or control IP with normal mouse IgG (Ms IgG). Note that a small amount of PGRN binds to the control Rb and Ms IgG, and that Ripk1 IP does not pull down any PGRN in excess of this background level. Representative of 2 independent experiments. (B) WB for Ripk1 (rabbit anti-Ripk1 antibody) on neuro2a cells transfected with nontargeting (NT) siRNA, Grn siRNA, or Ripk1 (Rip) siRNA then lysed and subjected to immunoprecipitation (IP) by rabbit anti-PGRN antibody, mouse anti-Ripk1 antibody, control IP with normal rabbit IgG (Rb IgG), or control IP with normal mouse IgG (Ms IgG). Note that the high background in lanes 1-4 is due to the presence of a very bright and somewhat smeared band corresponding to pull-down antibody heavy chain. Lanes 1-4 had an “origin species” match for pull-down and western blot primary antibodies (rabbit), unlike all other lanes in Fig 2.11. Representative of 2 independent experiments. kD, kilodalton.
Figure 2.12. PGRN and Ripk1, unlike PGRN and Lamp1, do not co-localize. Immunocytochemistry for Ripk1 (A), PGRN (B), and Lamp1 (C) in neuro2a cells. (D) Merge of (A) and (B) with nuclei (Hoechst) in blue. (E-G) Inset from (D) at greater magnitude; colocalization is shown in yellow. (H) Merge of (B) and (C) with nuclei (Hoechst) in blue. (I-K) Inset from (H) at greater magnitude; colocalization is shown in white. Representative cells from a population of over 100.
CHAPTER 3.

DISCUSSION

3.1 KINOME SCREEN

From the 719 genes in the kinome, we identified 24 genes (hit rate, 3.3%) that cause an increase in PGRN when knocked down by siRNA (Table 2.1). This hit rate is in line with those from other neurobiology-related siRNA screens, which ranged from 0.5% to 14%\textsuperscript{103}. Although this study focused on our top hit, Ripk1, other genes on the hit list are interesting targets for future investigation. However, as with most siRNA-based screens, we anticipate that some of the hits are false positives. For example, follow-up experiments with Lrrk2 siRNA revealed it to be acting through a probable off-target effect, as Lrrk2 is not detectably expressed in neuro2a cells. Nevertheless, one promising area of future research would be to validate and further investigate other hits from Table 2.1.

3.2 RIPK1 IS A BONA FIDE GENETIC REGULATOR OF PGRN

We found many compelling lines of evidence that Ripk1 knockdown affects PGRN level. First, 8 different siRNAs against Ripk1 lead to an increase in PGRN by ELISA in neuro2a cells (Fig 2.3) without causing an increase in cell number or global secretion (Fig 2.5). Second, Ripk1 knockdown increased PGRN by both native and reducing western blot (Fig 2.4), 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 increases PGRN level in microglial-like cells, mouse primary cortical neurons from wild-type mice, and, most importantly, FTD model (\textit{Grn} +/-) mice (Fig 2.6). Since an increase in PGRN is closely linked to
cancer progression and invasion, we hoped that Ripk1 knockdown would cause approximately a
doubling of PGRN, which would rescue haploinsufficiency but not greatly exceed wild-type
levels. Importantly, Ripk1 knockdown approximately doubles PGRN levels in each of our model
systems (neuro2a cells with Ripk1 siRNA by ELISA and western blot, BV-2 cells with Ripk1
siRNA by ELISA, mouse neurons with Ripk1 shRNA by ELISA) and leads to the restoration of
wild-type level in an FTD model.

There are several caveats for this set of experiments. First, although we attempted to reverse the
increase in PGRN seen upon Ripk1 knockdown using siRNA-resistant version of Ripk1, we were
unable to detect such a reversal. This was due to the fact that Ripk1 overexpression was toxic to
neuro2a cells. Therefore, we were unable to demonstrate that Ripk1 siRNAs were acting in an on-
target manner through this canonical “siRNA-resistant rescue” experiment. However, we were
able to demonstrate that 8 different siRNAs directed against Ripk1 all led to an increase in PGRN.
Because these 8 sequences were all distinct, and because the likelihood of those sequences arising
together in another gene is infinitesimally small, the only way our results could be due to off-target
effects is if each of the 8 siRNAs target different genes which all happen to affect PGRN levels.
It is much more likely that these 8 siRNAs are working through effects on Ripk1, so much so that
we are very confident in our results in spite of the lack of the “siRNA-resistant rescue” experiment.

Another caveat from this set of experiments is that we noted toxicity from the Grn and Ripk1
shRNA lentiviruses compared to the nontargeting shRNA virus. We found a decrease in cell
number of 7-14% with Grn shRNA virus and 35-40% with the Ripk1 shRNA virus, compared to
nontargeting shRNA (Fig 3.1). Therefore, it could be that the effect of Ripk1 shRNA on PGRN
levels is due to cell death/lysis releasing PGRN into the media or causing upregulation of PGRN as an inflammatory response. However, we think that this is unlikely since Grn shRNA virus also leads to a decrease in cell number, albeit a smaller one, while causing a significant decrease in PGRN level (Fig 2.6). If cell death alone could increase PGRN, we would expect the Grn shRNA condition to fail to knock down PGRN, or knock down PGRN poorly, but it knocks it down robustly. Of note, even though cell number is decreased by Ripk1 shRNA, total PGRN level is still approximately doubled (Fig 2.6), so per cell PGRN is increased even more.

Figure 3.1. The effect of shRNA lentiviruses on mouse neuron viability. Cell numbers (determined by CellTiterGlo assay) in mouse primary cortical neurons infected with nontargeting (NT), Grn, or Ripk1 shRNA viruses on day \textit{in vitro} (DIV) 2 and assayed DIV 7. **** \( p<0.0001 \) versus +/- with NT shRNA by ANOVA. # \( p<0.05 \); #### \( p<0.0001 \) versus +/- with NT shRNA by ANOVA. \( n = 6 \) samples per condition. Representative of \( \geq 3 \) independent experiments. Error bars represent SD.

One final caveat to this set of experiments is that we discovered that cell type is very important when examining the effect of Ripk1 knockdown on PGRN levels. For example, while we saw a robust increase in PGRN upon Ripk1 knockdown in neuro2a cells, BV-2 cells, and primary cortical neurons, we could not detect an increase in PGRN in mouse embryonic fibroblasts upon Ripk1 knockdown by siRNA (data not
shown). Neurons and fibroblasts have very distinct gene expression, and we speculate that fibroblasts may lack a critical component of the pathway linking PGRN and Ripk1. We also found that Ripk1 knockdown in human iPS-derived forebrain neurons did not affect PGRN levels (see Appendix and Fig A.6). However, in these cells, we only achieved approximately 20-30% knockdown of Ripk1 (versus ~75% in neuro2a cells and mouse neurons), so we believe this discrepancy between neuro2a cells / BV-2 cells / mouse neurons and iPS-derived neurons may be due to poor function of the human Ripk1 shRNAs. However, we cannot rule out the possibility that an increase in PGRN level upon Ripk1 knockdown is a feature of only neuro2a cells, BV-2 cells, and mouse primary cortical neurons.

3.3 RIPK1 KNOCKDOWN INCREASES PGRN TRANSLATION

We found that Ripk1 knockdown increases the rate of PGRN translation without affecting PGRN transcription, secretion, or turnover (Fig 2.7, 2.8). Because the assay we used to measure PGRN translation examines only full-length PGRN, one alternative possibility is that PGRN is cleaved into granulins more slowly in the Ripk1 knockdown condition, compared to the nontargeting siRNA condition. Although this experiment cannot exclude this possibility, we believe it is unlikely based on the fact that western blot using an antibody directed to linker 1 of PGRN reveals a prominent band representing full-length PGRN and no smaller species (Fig 2.4). This antibody would detect any linker 1-containing poly-granulin species formed as individual granulins are cleaved off the full-length protein. This finding demonstrates that these species are rare in neuro2a cells, in contrast to brain. Likewise, the autoradiography used to measure PGRN translation revealed only nonspecific bands (not lessened by PGRN siRNA) of lower molecular weight than full-length PGRN (Fig 2.7). The C-terminally directed antibody used to pull-down PGRN should, like the linker 1 western blot antibody, recognize any poly-granulins containing the peptide used to raise this antibody. Because of these data, we believe PGRN cleavage is slow to moderate in
neuro2a cells and therefore unlikely to explain the change in PGRN build-up during $^{35}$S-methionine incubation.

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\textsuperscript{104}. Additionally, the p38/MAPK pathway is well-known for regulating the translation of several pro-inflammatory cytokines\textsuperscript{105,106}. Since Ripk1 knock-out reduces p38 activation\textsuperscript{104}, Ripk1 may be an important mediator of p38 and, in turn, the translation of cytokines. However, these effects of Ripk1 on inflammatory genes are opposite from the effect of Ripk1 on PGRN: loss of Ripk1 leads to less translation of IL-6 and possibly other cytokines, but our data show that Ripk1 knockdown leads to increased translation of PGRN. Therefore, the idea that Ripk1 can regulate translation is not completely novel, but clearly the regulation of PGRN is distinct from the known effects of Ripk1 on IL-6 and possibly other cytokines.

There may be other mechanisms by which Ripk1 could regulate translation. PKR, a major antiviral protein, phosphorylates eIF2 to inhibit mRNA translation during viral infection. PKR and Ripk1 co-IP\textsuperscript{107,108}. Therefore, Ripk1 could cooperate with PKR to regulate translation. Additionally, translational regulation of PGRN level has been 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\textsuperscript{109}. 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.
In our experiments examining PGRN secretion, re-uptake, and turnover, we were surprised to find that at least one sub-population of PGRN decays rapidly over the course of only 8 hours (Fig 2.8). We also found that neurons rapidly (within one hour) release all intracellular PGRN when PGRN is removed from the media (Fig 2.7). Thus, PGRN levels and localization may be very tightly regulated, emphasizing the importance of PGRN for cell health. Given PGRN’s important roles in synaptic transmission, neurite outgrowth, and stress resistance (see Introduction), it is not surprising that PGRN is tightly regulated.

3.4 RIPK1 KNOCKDOWN INCREASES PGRN THROUGH A NOVEL, KINASE-INDEPENDENT SIGNALING MECHANISM

We found that loss of Ripk1 kinase activity either by small molecule inhibition or mutation did not replicate the effect of Ripk1 knockdown (Fig 2.9), indicating that the effect of Ripk1 on PGRN level does not depend on its kinase activity. Although this result may seem surprising, somewhat unusually among kinases, many of the effects of Ripk1 are not dependent on its kinase activity\(^94,98,100\). In fact, out of the three major signaling pathways downstream of Ripk1 (Nfkbeta transcription, Caspase-mediated apoptosis, and Ripk3-mediated necroptosis), two do not require Ripk1 kinase activity. Although the original goal of our screen was to identify kinase-dependent effects on PGRN level, we were surprised and excited to explore the kinase-independent effects of Ripk1.

We found no evidence that PGRN and Ripk1 directly interact or colocalize (Fig 2.11, 2.12). We also found no evidence that the three major signaling pathways downstream of Ripk1 mediate its effect on PGRN levels (Fig 2.10). Of course, we cannot definitively exclude a contribution from
these complex and intertwined pathways to PGRN regulation, but these data indicate the effect of Ripk1 knockdown on PGRN levels may proceed through a novel mechanism. This idea warrants future study.

3.5 CLINICAL IMPLICATIONS

Both FTD and AD have a genetic link to PGRN: haploinsufficiency mutations in GRN cause FTD, and a GRN SNP confers significantly increased risk 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. Excitingly, several ongoing clinical trials attempting to increase PGRN in FTD (see Introduction) may provide the first human evidence to support this therapeutic approach. However, many important questions remain before successful PGRN-modulating therapies, including possibly therapies targeting Ripk1, can be rolled out in humans.

First, when in the disease course will modulation of PGRN level be most beneficial? Unfortunately, most FTD patients are diagnosed only after years of exhibiting symptoms, and significant neuronal loss may already have occurred by that time. Indeed, average duration of FTD is 6 years and average time to diagnosis is 3 years, meaning the average patient only survives 3 years after diagnosis\textsuperscript{110}. It is not known whether increasing PGRN will only prevent disease before onset, slow the progress of existing disease, halt disease progress altogether, or reverse existing deficits. If an increase in PGRN can only slow FTD, it may have little benefit in patients who are already profoundly disabled at the time of diagnosis. Fortunately, patients with mutations in GRN
can be identified and possibly treated before they are symptomatic if they have a family history of FTD.

Next, it is not known where an increase in PGRN would be most beneficial. Presumably brain PGRN levels are most relevant for AD and FTD, but which cell type(s) should produce the PGRN? In this study, we found that Ripk1 knockdown increases PGRN in primary cortical neurons, a cell type that degenerates in both AD and FTD. Though we did find that Ripk1 knockdown increases PGRN in a microglial-like cell line (Fig 2.6), we did not examine the effect of Ripk1 knockdown in primary microglia. Activated microglia produce many times more PGRN than neurons, so may be an important source of brain PGRN. Additionally, FTD and AD patients exhibit microgliosis, highlighting the possible importance of this cell type in disease. It is not known whether therapies that increase PGRN in neurons, microglia, or both will be most beneficial to FTD patients, but it would be interesting to test the effect of Ripk1 knockdown in primary microglia.

A therapeutic strategy that increases PGRN in the brain may also increase peripheral PGRN. It is not known what the effects of an increase in peripheral PGRN will be in humans, but it may be an increase in the risk of malignancy, considering the upregulation of PGRN in many forms of cancer (see Introduction). This highlights the possible need for therapeutic options that affect PGRN specifically in the brain. Unfortunately, Ripk1 is expressed in many peripheral cell types, so Ripk1-directed therapies may affect global PGRN levels.

Another question surrounding PGRN-modulating therapies is the amount of PGRN increase that is desirable. Our goal in this study was to approximately double PGRN levels in the
haploinsufficient state to rescue PGRN to wild-type levels. However, it is not known what dose of PGRN is required to support and protect neurons, or whether the neurotrophic effects of PGRN are even dose-dependent. On top of that, because full-length PGRN and individual granulins can exert opposing effects on inflammation (see Introduction), the balance between PGRN and granulins may be an important therapeutic consideration. This is difficult to track as granulin-specific antibodies are unavailable. Given the extreme importance of PGRN dose demonstrated by haploinsufficiency causing FTD, homozygous loss causing NCL, and overexpression causing cancer, it is likely that tight regulation of PGRN, and perhaps granulin, levels will be required for an effective therapeutic.

There are also questions that remain to be answered about the suitability for Ripk1 as a therapeutic target. Ripk1 is an essential gene, as demonstrated by the perinatal lethality of Ripk1 knock-out \(^{111}\). It is not known whether a reduction in Ripk1 levels in humans would cause any negative effects in addition to any related to an increase in PGRN. Also, because inhibition of the kinase activity of Ripk1 is not enough to increase PGRN, the simplest method for blocking Ripk1 in humans (kinase inhibition with a known drug) is not feasible. On the other hand, there are many options for reducing Ripk1 levels in humans, including antisense oligonucleotides (ASOs), shRNA delivered via virus, or perhaps small molecule inhibition of another member of the pathway linking Ripk1 and PGRN. Discovering such a target will require further investigation.

Finally, one last challenge to developing PGRN-modulating therapies for FTD is that preclinical models of GRN-haploinsufficient FTD are very mild phenotypically. Although many PGRN +/- mice have been reported\(^{25,71-76}\), none have robust deficits in behavior or neuropathology. The most
convincing PGRN +/- phenotype is a sociability deficit reported by one group\textsuperscript{73}. Thus, it may be hard to detect the benefits of increased PGRN. Using PGRN homozygous knock-out mice is not a good alternative because most strategies for increasing PGRN require an endogenous PGRN locus, and because PGRN knock-out models NCL, not FTD (see Introduction). On the other hand, if one of the first clinical trials seeking to modulate PGRN levels demonstrates a benefit from increased PGRN, proving that a therapy can increase PGRN levels in mice, without examining behavior or pathology, may be enough to drive the initiation of a clinical trial.

In conclusion, the data presented here represent the first report linking Ripk1 and PGRN and support targeting Ripk1 as a therapeutic strategy for increasing PGRN levels in both AD and FTD.
CHAPTER 4.

METHODS

4.1 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.

4.2 SCREEN

For the primary screen, neuro2a cells were reverse transfected in 96-well plates with an siRNA library against the kinome (GE/Dharmacon). Each gene in the kinome was represented by a mixture of four siRNAs. First, stock siRNAs were resuspended in siRNA Buffer (GE/Dharmacon) at 2 uM. Next, lipofectamine (0.5 uL per well) and Opti-MEM (Life Technologies, 24.5 uL per well) were added to 96-well plates. After five minutes at room temperature, 22.5 uL Opti-MEM and 2.5 uL of 2 uM siRNAs were added to each well. After incubating the transfection complexes for 20 minutes at room temperature, neuro2a cells were resuspended in low serum media (DMEM + 1% FBS) and added to transfection complexes (50 uL of cells resuspended at 400,000 cells/mL). Thus, the final concentration of siRNAs during transfection was 50 nM total siRNAs (12.5 nM each siRNA). Cells were incubated with transfection complexes for three hours at 37 °C. After incubation, media was changed to DMEM + 1% FBS (150 uL per well). After 24 hours, media was collected for ELISA.
To assay extracellular mouse PGRN, we developed a sandwich ELISA against PGRN. First, we raised an antibody against a 15-amino acid peptide in the C-terminal of mouse PGRN (RWMFLRDPVPRPLL) in rabbits; this antibody served as the capture antibody (called “anti-PGRN CT” from here on). With the Farese lab (Harvard University Medical School), we raised two batches of this antibody that were indistinguishable in terms of performance. High protein-binding plates (Corning) were coated with the anti-PGRN CT capture antibody (10 ug/mL in PBS; 100 uL per well) overnight at 4 °C. Then, plates were washed three times (100 uL PBS) and then blocked with 1% BSA (in PBS) for one hour at 37 °C. Next, plates were washed three times (100 uL PBS) and media samples from transfected neuro2a cells and standards (0 – 16 ng/mL recombinant mouse PGRN from R&D diluted in DMEM + 1% FBS) were added to assay plate, 100 uL per sample, and incubated at 37 °C for one hour. Next, plates were washed three times and the detection sheep anti-mouse PGRN antibody was added (R&D AF2557; 0.2 ug/mL; 100 uL per well; diluted in PBS + 0.4% Tween-20 + 0.1% BSA) and incubated at 37 °C for one hour. Then plates were washed 3-5 times and biotinylated anti-sheep IgG antibody (R&D BAF016; 0.2 ug/mL; 100 uL per well; diluted in PBS + 0.4% Tween-20 + 0.1% BSA) was added and the plate was incubated at 37 °C for one hour. Finally, after three washes, high-sensitivity streptavidin-HRP (Pierce; 1/10,000 in PBS + 0.4% Tween-20 + 0.1% BSA; 100 uL per well) was added and incubated at 37 °C for one hour. After three final washes, plates were developed by adding 100 uL per well TMB/E (Millipore) and waiting for blue color change (1-5 minutes). Reaction was stopped with 1 N hydrochloric acid (100 uL per well). Absorbance was read at 450 nm on a plate reader. ODs were converted to concentrations using the standard curve. For all plates included in final analysis, t-test comparing control wells (nontargeting siRNA versus PGRN siRNA) was significant. For each plate, a “plate mean” was determined by averaging the PGRN concentrations.
of all non-control wells. 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 by transfecting neuro2a cells and performing ELISA as above (secondary screen). In the secondary screen, we performed ANOVA comparing each condition on the plate to nontargeting siRNA control. Hits were those siRNAs that gave a significant increase in PGRN compared to control by ANOVA. For the tertiary screen, hits from the secondary screen were re-assayed (six replicates 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 2 of 4 siRNAs significantly increased PGRN (by ANOVA).

4.3 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), Nfkb1 siRNA (D-047764-01-0005), Casp8 siRNA (D-0443044-01-0005), Ripk3 siRNA (D-049919-04-0002).

4.4 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.
4.5 TRANSFECTION OF NEURO2A CELLS
After we identified Ripk1 as the top hit, we optimized the transfection protocol for Ripk1. The modifications from the screening protocol (Section 4.2) are as follows: 9,000 instead of 20,000 neuro2a cells per well; 90 minutes instead of 3 hours of transfection time; 200 uL instead of 150 uL media change; 72 instead of 24 hours of incubation time after transfection but before ELISA.

4.6 MOUSE PGRN ELISA
After we identified Ripk1 as the top hit, we optimized the ELISA protocol. ELISA was performed as described above (Section 4.2) with the following modifications: media samples were diluted ¼ (neuro2a cells) or ½ (primary neurons) in PBS + 0.1%BSA + 0.4%Tween-20; detection antibody was sheep anti-mouse PGRN biotinylated antibody (R&D BAF2557). For ELISA on lysate samples, cells were 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 approximately 5 minutes with nutation and used undiluted.

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 uL PBS was added per well, and finally cells were lysed with 100 uL per well of CellTiter-Glo reagent. Plates were rocked at room temperature for approximately five minutes. Last, lysed samples were added to an opaque plate and luminescence was read for 500 ms. Finally, relative cell numbers in each well were calculated.
For ELISA with brain lysate, wild-type and Ripk1 D138N/D138N mouse brains were obtained from the Kelliher lab at the University of Massachusetts. Cortices were dissected and homogenized 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) and then spun down at maximum speed for 30 minutes at 4 °C. The supernatant was transferred to a fresh tube and analyzed for protein content. 20 ug total protein was loaded per well of the 96-well ELISA plate.

4.7 WESTERN BLOTTING

Samples for western blotting included lysates, concentrated media, or beads from a pull-down. Lysates were prepared as described elsewhere, 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 uL of unconcentrated media being loaded per well. Pull-down beads were obtained as described elsewhere. 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 two 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 ("anti-PGRN linker 1," Consortium for Frontotemporal Dementia Research, 8.4 ug/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 ug/mL).

4.8 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) + 1% GlutaMAX (Life Technologies).

4.9 LENTIVIRUS

shRNAs against mouse *Grn* and *Ripk1* and a non-targeting shRNA were obtained from Sigma (GRN: CCGGCCTAGAATAACGAGCCATCATCTCGAGATGATGGCTCGTTATTCTAGGTTTTTG; Ripk1: CCGGGCATTGTCCTTTGGGCAATATCTCGAGATATTGCCCAAAGGACAATGCTTTTT; non-targeting: CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTT). shRNAs were subcloned into FUGW using the following primers F ATATGCTAGCTTTCCCATGATTCCTTCAT, R ATATTTAATTAACCATTTGGTCAGGTCGA and digestion/ligation at NheI, PacI sites. Vectors containing shRNAs were packaged into lentivirus by the UCSF ViraCore. Viruses were titered 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.

4.10 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 ¼. For qPCR, 4.5 uL cDNA was combined with 5 uL SYBR green master mix (Applied Biosystems) and 0.5 uL primer mix (20 uM each primer). Samples were run on an Applied Biosystems 7900HT. Data was analyzed using the delta delta CT method. Values were normalized to housekeeping gene cyclophilin. Primers were as follows: mouse Cyclophilin F TGGAAGAGCACCAAGACAACA, mouse Cyclophilin R TGCCGGAGTCGACAATGAT, mouse Grn F TGGTTCACACAGTGCCGTTTCAC, mouse Grn R AAAGGCAGACACTGCCCTGTGTG, mouse Ripk1 F GAAGACAGACCTAGACAGCGG, mouse Ripk1 R CCAGTAGCTTCACCAGCTCGAC, mouse Nfkb1 F ATGGCAGACGATGATCCCTAC, mouse Nfkb1 R TGTTGACAGTGGTATTTCTGGTG, mouse Casp8 F TGCTTGGACTACATCCCAC, mouse Casp8 R TGCAGTCTAGGAAGTTGACCA, mouse Ripk3 F GTGCTACCTACACAGCTTGAAC, mouse Ripk3 R CCCTCCCTGAAACGTGGAAC.

4.11 MEASURING PGRN TRANSLATION AND DEGRADATION RATE

To measure translation, neuro2a cells were reverse transfected with nontargeting or Ripk1 siRNA as described above (Section 4.5, 1x10^6 cells/well of a 6-well plate). After 48 hours, media was changed to methionine-free DME (Life Technologies) for 1 hour. Next, media was changed to methionine-free DME plus 0.2 mCi/mL ^{35}S-methionine (EasyTag EXPRESS, PerkinElmer). At 0.5, 1, 1.5, 2, 2.5, and 3 hours after addition of radioactivity, media was 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 ug/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.

Degradation rate (half-life) was measured as above with the following changes: after a 3 hour incubation with $^{35}$S-methionine, media was changed to DMEM + 1% FBS + 1% pen/strep + 1% GlutaMAX and then media/lysate samples were collected at 1, 2, 4, 6, 8, 20, 28, 48, and/or 72 hours.

4.12 RIPK1 AUTOPHOSPHORYLATION ASSAY

Neuro2a cells were reverse transfected with nontargeting or Ripk1 siRNA as described above (Section 4.5, 1x10$^6$ cells/well of a 6-well plate). After 24 hours, Nec1 or DMSO control was added to culture media. 48 hours after transfection, media was changed to phosphate-free DME (Life Technologies) with DMSO or Nec1. After 60 minutes at 37 °C, the media was changed to phosphate-free DME with DMSO or Nec1 plus 0.1 mCi/mL of $\gamma^{32}$P-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 NaVO$_3$)
with protease and phosphatase inhibitors (Pierce). Lysates were spun down at maximum speed for 10 minutes and pellet was discarded. Ripk1 was immunoprecipitated using mouse anti-Ripk1 antibody (BD 610458, 2.5 ug/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. Samples were run in duplicate on an 8% tris-glycine gel. The gel was cut in half and one half was transferred and western blotted for Ripk1 as described above (Section 4.7) while the other half was fixed, dried, and exposed to film as described above (Section 4.11).

4.13 CO-IMMUNOPRECIPITATION

Neuro2a cells were reverse transfected with nontargeting or Ripk1 siRNA as in Section 4.5 (1x10^6 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 NaVO_3) with protease inhibitors (Pierce) for 20 minutes. Lysates were spun down at max speed for 10 minutes and pellet was discarded. PGRN or Ripk1 were pulled down using mouse anti-Ripk1 (BD 610458, 2.5 ug/mL) or rabbit anti-PGRN CT (4.4 ug/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 (Section 4.7).
4.14 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.

4.15 PRODUCTION OF RECOMBINANT PGRN

A vector containing mouse PGRN with an N-terminal FLAG tag was obtained from the Strittmatter lab (Yale University). HEK cells were transfected with this plasmid at approximately 60% confluence using lipofectamine 2000 (Thermo) and the manufacturer’s instructions. Transfection complexes were incubated with cells for 4 hours before media was changed to DMEM + 2% FBS + 1% pen/strep + 1% GlutaMAX. 72 hours later, media was collected from
the transfected cells and concentrated approximately 4-fold using a 20 kD molecular weight cut-off concentrator column (Pierce) and spinning at 4000xg for 30 minutes. Protease inhibitors were added to the concentrated media and FLAG-PGRN was pulled down using 500 µL magnetic FLAG beads per 15 mL concentrated media (Sigma) overnight at 4 °C with rotation. Beads were then washed three times with TBS and FLAG-PGRN was eluted using 3XFLAG peptide (Sigma) at 500 ng/mL for 30 minutes at 4 °C with rotation. The eluant was concentrated using a 10 kD molecular weight cut-off column (Millipore) to remove FLAG peptide. Purification was verified using western blot for PGRN and FLAG. To test for the uptake of this recombinant FLAG-PGRN into cells, FLAG-PGRN was added to the media and cells were incubated at 37 °C. Cells were then washed with PBS and lysed as above (Section 4.11). FLAG was pulled down using anti-FLAG magnetic beads (Sigma) as above. Western blot for FLAG was carried out as above (Section 4.7).
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APPENDIX

EFFORTS TO DISCOVER PGRN +/- PHENOTYPES

A.1 OVERVIEW

During the course of this study, one of our goals was to uncover robust phenotypes to assay in PGRN +/- cells. We hoped this would allow us to examine the effect of Ripk1 knockdown on PGRN +/- functional phenotypes. Unfortunately, the phenotypes found in PGRN +/- animals and cells are often mild or absent. We were not able to discover any robust PGRN +/- phenotypes. This appendix summarizes the negative data obtained while examining differences between PGRN +/+ and PGRN +/- cells.

A.2 METHODS FOR THE APPENDIX

Survival Analysis

The method for survival analysis has been described elsewhere\textsuperscript{112,113}.

Lysosome Analysis

Primary neurons from wild-type or PGRN +/- mice were cultured until day \textit{in vitro} 10 as described above (Section 4.8) and then stained by immunocytochemistry for rat anti-Lamp1, sheep anti-PGRN, and chicken anti-MAP2 (Abcam ab5392, 1/10,000) as described above (Section 4.14). The number and size of lysosomes was quantified using Fiji. First, the Lamp1 channel was smoothened, then thresholded, then the watershed algorithm was applied. Finally, the automated particle counter was used to detect particles of size >10 pixel units and of any circularity.
Measures of Autophagy

Autophagy induction was measured via LC3 western blot. Western blot was carried out as described above (Section 4.7) using a rabbit anti-LC3 antibody raised in the Finkbeiner lab. Autophagy flux was measured as described elsewhere\textsuperscript{113}.

Differentiation of Human iPS-Derived Neurons

iPS cells from one FTD patient (S116X mutation) and one control were obtained from the Gao lab (University of Massachusetts Medical School) and maintained on Matrigel (Corning) in mTeSR (STEMCELL Technologies). They were differentiated to forebrain neurons using the following protocol: from day 1-11, cells were fed daily with N3 media (1:1 mixture of DMEM/F12 with GlutaMAX (Life Technologies) and Neurobasal (Invitrogen) plus 1\% B27 supplement (Life Technologies), 0.5\% N2 supplement (Life Technologies), 1 \(\mu\)g/mL insulin, 0.5\% NEAA (Life Technologies), 55 \(\mu\)M BME, and 1\% pen/strep) + 1.5 \(\mu\)M dorsomorphin + 10 \(\mu\)M SB431542. From day 12-15, cells were fed every day with N3 media. From day 16-20, cells were fed every day with N3 + 0.05 \(\mu\)M retinoic acid. On day 20, cells were rinsed with PBS, and dissociated using Enzyme-Free Dissociation Buffer (Gibco) for 15 minutes at 37 °C. Cells were triturated and replated in N4 media (N3 media with 2 ng/mL BDNF, 2 ng/mL GDNF, and 0.05 \(\mu\)M retinoic acid) + 10 \(\mu\)M ROCK inhibitor at a ratio of 1:2 on plates coated with poly-D-lysine (50 \(\mu\)g/mL) and laminin (5 \(\mu\)g/mL) overnight at 37 °C. From day 21-30, cells were fed every other day with N4 media. On day 30, cells were dissociated with Accutase (Thermo) for 10 minutes at 37 °C, then frozen in synthafreeze (Thermo, 15 million cells per mL). When needed, cells were thawed into N4 media + 10 \(\mu\)M ROCK inhibitor onto plates coated with poly-D-lysine and laminin. Media (N4) was changed every other day. On the fourth day after thawing, cells were replated to a new
poly-D-lysine/laminin-coated plate at 200,000 cells per well of a 96-well plate using Accutase and N4 media + ROCK inhibitor + 10 uM DAPT. Media (N4) was changed every other day. Cells were stained or media was collected for ELISA 12 days after thawing. If applicable, shRNA viruses were added at 5x10^5 TU/mL one day after replating to 96-well plate.

shRNA lentiviruses targeting human GRN and human RIPK1 were produced as above (Section 4.9). Sequences were human RIPK1 CCGGGCAGTCTTCAGCCCATTAAATCTCGAGATTATGGGCTGAAGACTGCTTTTTTG, human PGRN CCGGCCTAACCAAATTCTCCCTGGACTCGAGTCCAGGGAGAATTTGGTTAGGTCTTTGG.

Immunocytochemistry on iPS-derived neurons was performed as described above (Section 4.14). Primary antibodies were rabbit anti-GABA (Sigma A2052), rabbit anti-VGlut (Synaptic Systems 135 303), rabbit anti-GFAP (Dako), and mouse anti-MAP2 (Sigma M1406).

qPCR on iPS-derived neurons was performed as described above (Section 4.10). Primer sequences were human Cyclophilin F CCCACCGTGTTTCTTCGACATT, human Cyclophilin R GGACCCGTATGGCTTTAGGATGA, human PGRN F CCCTGGCAAAGAAGCTCCC, human PGRN R AGCTCACACGAGGTAGAAGCC, human RIPK1 F GGGAAGGTGTCTCTGTCTTTGC, human RIPK1 R CCTCGTGTGGCTCAATGCAG.

**Human PGRN ELISA**

The human PGRN ELISA was carried out as described for the mouse PGRN ELISA (Section 4.6) with the following modifications: capture antibody was raised against full-length recombinant
human PGRN and recognizes the C-terminus of PGRN (antibody C46.1 from Consortium for Frontotemporal Dementia Research) and used at 4 ug/mL, detection antibody was raised against recombinant human PGRN and recognizes the N-terminus of PGRN (antibody A21.1 from Consortium for Frontotemporal Dementia Research) and used at 0.7 ug/mL, standards were made using recombinant human PGRN (R&D), and samples were not diluted. Detection antibody was biotinylated by dialyzing in NaHCO₃ pH 8.5 using the Slide-A-Lyzer system (Pierce) overnight, then binding to 12 mg/mL NGS-biotin (Pierce) for 1.5 hours. Finally, biotinylated antibody was dialyzed back into PBS.

Mouse Embryonic Fibroblast Culture

Embryos from wild-type, PGRN +/-, or PGRN -/- mice were obtained at day 14 of gestation. Embryos were decapitated and eviscerated, then chopped up using a sterile razor blade. Tissue pieces were digested in 0.25% Trypsin (Life Technologies) for 2 hours at room temperature, then centrifuged at 1000 rpm for 5 minutes. The pellet was resuspended in DMEM with 10% FBS, 1% GlutaMAX, 1% pen/strep and plated at approximately 1x10⁶ cells per 10 cm dish. Cells were passaged twice per week. To test for staurosporine susceptibility, 500 nM staurosporine was added for 24 hours in serum-free media and then the MTS assay (Promega) was performed following the manufacturer’s instructions to check number of viable cells.

A.3 MOUSE CORTICAL NEURONS

We performed automated survival analysis on PGRN +/+, PGRN +/-, and PGRN -/- primary mouse cortical neurons. We did not find a difference in survival (Fig A.1A). We also performed survival analysis on PGRN +/+, PGRN R493X/+, and PGRN R493X/R493X knock-in mice.
(obtained from Farese lab, Harvard University Medical School); R493X is one of the most common human mutations in GRN. We did not find a difference in survival (Fig A.1B). We also examined the neuritic arbor of rat primary cortical neurons transfected with either nontargeting siRNA or PGRN siRNA by counting up the total pixels in neurites (identified by an automated algorithm). The PGRN siRNA significantly decreased the total length of the neurite arbor (Fig A.2A). We next examined the neuritic arbor of PGRN wild-type, heterozygous, and knock-out primary cortical neurons. We did not find a difference in total neurite length (Fig A.2B). Previous reports have found decreased neurite outgrowth in PGRN knock-out primary hippocampal neurons.42

Figure A.1. Primary cortical neurons from mice with homozygous or heterozygous Grn null mutations have no survival deficit. Kaplan-Meier survival plots for primary mouse cortical neurons from PGRN +/+, +/-, or +/- neurons (A) or PGRN +/-, +/-R493X, or R493X/R493X neurons (B). Neurons were transfected with EGFP as a transfection indicator and tracked longitudinally using robotic microscopy. n = 100-400 neurons per condition. Representative of ≥3 independent experiments.
Given that the effect of homozygous GRN mutation in humans is a lysosomal storage disease, NCL (see Section 1.4), we hypothesized that PGRN may play an important role in lysosome biology. We examined the size and number of lysosomes found in primary cortical neurons from PGRN +/+ and PGRN +/- mice. We found no change in lysosome size (Fig A.3A) but a significant increase in lysosome number (Fig A.3B). However, this effect was not present in neurons infected with nontargeting shRNA (Fig A.3C), so rescue via RIPK1 shRNA could not be attempted.
Figure A.3. PGRN-deficient primary neurons have normal lysosome size and increased lysosome number, which is absent in neurons infected with virus. Lysosome size (A) and number per cell (B) in wild-type and PGRN heterozygous (Het) primary cortical neurons, as measured by immunocytochemistry for Lamp1 followed by Fiji automated particle analysis. (C) Same as (B), except the neurons were infected with nontargeting (NT) shRNA lentivirus. Virus was added on day in vitro (DIV) 4 and cells were fixed on DIV 10. n = 7-13 cells per condition. * p<0.05 versus WT by t-test. Representative of 2 independent experiments. Error bars represent SD.

To further interrogate the autolysosomal pathway, we next tested autophagy induction in primary mouse cortical neurons from wild-type and PGRN knock-out mice using western blot for LC3. We found no difference in autophagy induction (Fig A.4). Finally, we tested autophagy flux in wild-type rat cortical neurons infected with a PGRN-overexpressing virus. We found that overexpression of both PGRN (wild-type) and PGRN R493X (truncated by premature stop codon at position 493) increased autophagic flux. However, another secreted protein not involved in neurodegeneration, secreted alkaline phosphatase (SEAP), also increased flux, perhaps indicating that increased flux is a response to the overexpression of a secreted protein (Fig A.5). Together, these data indicate that neither PGRN deletion nor overexpression affect autophagic flux.
Figure A.4. Neurons lacking PGRN have normal autophagy induction. Western blot for LC3 using primary cortical neuron lysate from wild-type (WT) or PGRN knock-out (KO) neurons treated with vehicle (Veh, DMSO), 100 mM ammonium chloride (NH4Cl), or 5 uM fluphenazine (FPZ) plus NH4Cl. FPZ treatment was 22 hours; NH4Cl treatment was 6 hours. LC3II (lower band on LC3 blot) was normalized to GAPDH and vehicle control. # p=0.065; * p<0.05; **** p<0.0001 by ANOVA. n = 4 samples per condition. Combined data from 2 independent experiments. Error bars represent SD.

Figure A.5. Neurons infected with secreted proteins exhibit increased autophagic flux. Density plot of half lives for primary rat cortical neurons infected with empty virus, PGRN virus, PGRN R493X virus, or secreted alkaline phosphatase (SEAP) virus and subjected to automated robotic autophagic flux assay. * p<0.05; *** p<0.001, **** p<0.0001 versus empty virus by KS test. n = 50-100 cells per condition. Representative of 2 independent experiments. Note, although PGRN R493X is subject to nonsense-mediated decay in vivo, in this in vitro system, it is abundantly overexpressed (data not shown).
A.4 HUMAN iPS-DERIVED NEURONS

FTD patient (carrying GRN S116X mutation) and control iPS cells were differentiated to excitatory forebrain neurons (iPSN) which stained positive for MAP2 (Fig A.6A) and VGlut (data not shown). The cultures had rare glia (GFAP+) and inhibitory neurons (GABA+) (data not shown). We found that FTD patient iPSN produced significantly less PGRN than control iPSN (Fig A.6B). We also made shRNA lentiviruses against human PGRN and human RIPK1, and found that they significantly knocked down target gene expression (Fig A.6C-F). However, PGRN levels were not affected by RIPK1 knockdown (Fig A.6G). This may be due to inadequate knockdown as only 20-30% knockdown of RIPK1 was achieved (compared to ~70% for mouse siRNAs in neuro2a cells).
Figure A.6. RIPK1 knockdown in human iPS-derived neurons from FTD patients. (A) Representative MAP2 immunostaining of FTD patient iPS cells differentiated to forebrain neurons (iPSN). Nuclei (Hoechst) in blue. (B) ELISA for human PGRN on wild-type (WT) and PGRN S116X FTD patient iPSN. n = 18 samples per condition. Representative of ≥3 independent experiments. (C-F) qPCR for GRN (C,E) or RIPK1 mRNA (D, F) in WT (C,D) or GRN S116X iPSN (E,F) infected with nontargeting (NT) shRNA, GRN shRNA, or RIPK1 shRNA virus. n = 6 samples per condition. (G) ELISA for human PGRN on WT or PGRN S116X patient iPSN infected with NT, GRN, or RIPK1 shRNA virus. n = 6 samples per condition. *** p<0.001 versus WT (B) or NT shRNA (C-F) by t-test. **** p<0.0001 by ANOVA vs WT NT shRNA; # p<0.05 by ANOVA vs S116X NT shRNA (G). Error bars represent SD.

A.5 FIBROBLASTS

Mouse embryonic fibroblasts from wild-type, PGRN heterozygous, or PGRN knock-out mice were tested for sensitivity to staurosporine, an ER stressor. PGRN knock-out cells were killed at a
higher rate than wild-type or heterozygous cells in response to staurosporine. There was no
difference between wild-type and heterozygous cells (Fig A.7).

**Figure A.7.** Homozygous, but not heterozygous, loss of PGRN sensitizes fibroblasts to
*staurosporine*. Mouse embryonic fibroblasts from wild-type (WT), PGRN heterozygous (Het), or PGRN
knock-out (KO) mice were treated with DMSO or staurosporine (500 nM) and tested for cell viability
using the MTS assay (Promega) and manufacturer’s instructions. n = 8 samples per condition. ***
p<0.001, **** p<0.0001 versus DMSO control (neighboring black bar) by ANOVA. ## p<0.01 versus
WT + Staurosporine by ANOVA. Error bars represent SD.
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