Chemical Neurobiology of Progranulin-Deficient Frontotemporal Dementia

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Chemical Neurobiology of Progranulin-Deficient Frontotemporal Dementia

A dissertation presented

by

Angela A She

to

The Committee on Higher Degrees in Chemical Biology

In partial fulfillment of the requirements

for the degree of

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Chemical Neurobiology of Progranulin-Deficient Frontotemporal Dementia

Abstract

Frontotemporal dementia (FTD) is a presenile dementia presenting with a variety of clinical phenotypes arising from Frontotemporal Lobar Degeneration (FTLD), a family of neurodegenerative pathologies with a predeliction for the frontal, insular, and anterior temporal lobes. Known autosomal dominant causes of FTLD include heterozygous mutations in the *GRN* gene causing haploinsufficiency of progranulin (PGRN) protein. As mRNA from the mutated allele of *GRN* is degraded via nonsense-mediated mRNA decay mechanisms, one therapeutic avenue for PGRN-deficient FTD is to increase mRNA, and subsequently protein expression, of the ‘wild-type’ (non-mutated) copy of *GRN*. This dissertation presents the systematic assessment of the abilities of different classes of small molecules to enhance *GRN* mRNA and PGRN protein expression in human induced pluripotent stem cell (iPSC)-derived neuronal cells. The work explores selectivity and kinetic requirements of histone deacetylase (HDAC) inhibitors to enhance PGRN expression (Chapter 3), defines key epigenetic markers associated with HDAC inhibitor-mediated *GRN* regulation (Chapter 4), identifies inhibitors of bromodomain and extra-terminal domain containing proteins (BET inhibitors) as a novel class of *GRN*/PGRN enhancers (Chapter 5), and considers the implications of treatment with other classes of compounds that regulate *GRN*/PGRN expression (Chapter 6). Finally, the dissertation describes the generation of patient-derived, iPSC models of behavioral variant FTD (bvFTD) using reprogramming technologies that can
be used for modeling the pathophysiology and treatment of FTD. Taken together, these findings have implications for studies of the epigenetic and potential cis-regulatory mechanisms controlling GRN expression from the human genome and therefore may advance translational efforts to develop targeted therapeutics for treating PGRN-deficient FTD and other neurodegenerative disorders.
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Chapter 1

An Introduction to Frontotemporal Dementia and Progranulin
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1.1 Frontotemporal Dementia

Frontotemporal dementia (FTD) represents a spectrum of disorders that together make up the second most common form of presenile dementia after Alzheimer’s disease. With a diverse set of clinical phenotypes ranging from changes in personality and behavior (the behavioral variant) to language impairment (the aphasic variants) to movement deterioration (progressive supranuclear palsy, corticobasal degeneration, or amyotrophic lateral sclerosis), FTD encompasses 5-15% of all cases of dementia in individuals between the ages of 45 and 65 years. FTD symptoms are the result of a varied set of neuropathologies together known as Frontotemporal Lobar Degeneration (FTLD), which ultimately cause rapid and specific neurodegeneration of the frontal, insular, and anterior temporal lobes. Although the majority of FTD patients start showing symptoms in the 45-65 age group, age of onset can vary from the early 30s (very early onset) to the 80s (very late onset). Because clinical presentation of FTD is exceptionally heterogeneous and includes relative preservation of memory compared to other dementias, FTD is difficult to accurately diagnose clinically even with use of neuroimaging technologies such as magnetic resonance imaging (MRI). In particular, in the early states, FTD can often be misdiagnosed as depression, schizophrenia, and Parkinson’s disease, amongst others. In addition, variation in age of onset and speed of progression of disease can further obscure diagnoses. These challenges have led to significant interest in a better understanding of the biological and genetic underpinnings...
of FTD in order to understand the impact of FTD on patients and on the population and to develop new treatment strategies.

As the clinical symptoms of FTD are heterogeneous, so are the genetic origins of the disease. There is evidence of a strong genetic component to the onset of FTD – up to 50% of FTD patients report at least one family member with either FTD or another neurodegenerative disease, and in about 20% of cases, the disease segregates with an autosomal dominant inheritance pattern.6 Thus far, mutations in three major genes have been found to be associated with the highly-penetrant autosomal dominant variants of FTD – tau (MAPT), progranulin (GRN), and open reading frame 72 on chromosome 9 (C9ORF72).7-9 These genes have also been associated with other neurodegenerative diseases: MAPT with Alzheimer's disease,7 GRN with neuronal ceroid lipofuscinosis (NCL),10 and C9ORF72 with amyotrophic lateral sclerosis (ALS).9 In addition, mutations in other genes like transactive response (TAR) DNA binding protein (TARDBP), associated with ALS, valosin-containing protein (VCP), associated with Paget’s disease of bone, and charged multivesicular body protein (CHMP2B), associated with ALS, have also been associated with FTD.11, 12 The functional consequence of these mutations are a highly active area of investigation, as they can cause different cellular phenotypes that may ultimately lead to the specific frontal and/or temporal lobar degeneration characteristic of FTD. Investigation of these mutations also has the potential to uncover mechanistic details about disease onset and progression that may aid in the development of novel therapeutics for the prevention of FTD.

On the protein level, FTD patients often exhibit protein inclusion phenotypes within the cytoplasms of neurons, known as neuronal cytoplasmic inclusions (NCI).13 In
genetic forms of the disease, the identity of the misfolded proteins within the NCI are often dependent on the specific causal genetic mutation causing the disease. While mutations in MAPT lead to intracellular tau aggregation in neurons (FTD-tau), also seen in Alzheimer’s disease (although without mutations), mutations in GRN, C9ORF72, VCP, and to a lesser extent TARDBP, lead to accumulation of ubiquitinated TAR DNA-binding protein (TDP-43) in neurons (FTD-TDP). Although the precise role of TDP-43 in the brain is still unclear, in FTD-TDP patients, hyperphosphorylated, ubiquinated TDP-43 aggregates in the cytoplasm, and the presence of unmodified TDP-43 in the nucleus is drastically reduced. Interestingly, it is not mutations in TARDBP that are most associated with TDP-43 pathology, but mutations in GRN, implying that disruption to protein clearance mechanisms are more likely to lead to TDP-43 accumulation than misfolding of TDP-43 itself. More work must be done to ascertain the role of both tau and TDP-43 accumulation in FTD pathogenesis, but clearance of these protein inclusions may be used as a marker for wild-type phenotype rescue upon treatment with a therapeutic entity.

Today, although we are able to diagnose FTD more accurately than ever and thus are better able to appreciate the genetic causes and differences in FTD disease variants, the specific pathways involved in FTLD etiopathogenesis are only beginning to be understood. There has been increasing interest in drug development for FTD, but there are currently no Food and Drug Administration (FDA) approved drugs for slowing, stopping, or reversing disease progression. Further elucidation of FTLD pathophysiology and mechanisms that may be useful for intervention are necessary for the development of efficacious treatments for these devastating diseases.
1.2 Progranulin and FTD

One of the known autosomal dominant forms of FTD is caused by mutations in the \textit{GRN} gene on chromosome 17 encoding the multifunctional, secreted protein progranulin (PGRN), which leads to haploinsufficiency of PGRN in patients (PGRN-deficient FTLD).\textsuperscript{8, 17-20} PGRN, a 593 amino acid-long, 88 kDa glycoprotein, is expressed in epithelia, bone marrow, immune cells, solid organs, and the nervous system during both development and adulthood. Full-length PGRN is secreted, and the cysteine-rich precursor protein can be cleaved into different seven 6 kDa granulins (GRN-A – G) and paragranulin (GRN-P).\textsuperscript{21-24} The granulin repeats are defined by the unique and highly conserved 12-cysteine motif, $\text{CX}_{5-6}\text{CX}_5\text{CCX}_8\text{CCX}_6\text{CCXDXXHCCPX}_4\text{CX}_{5-6}\text{C}$, except for GRN-G which has 10 cysteine residues and GRN-P which has 6 cysteine residues.\textsuperscript{25} The granulins are separated by linker sequences which can be recognized by both intracellular and extracellular proteases including matrix metallopeptidase 9 (MM-9),\textsuperscript{26} MM-12,\textsuperscript{24} MM-14,\textsuperscript{27} a disintegrin and metalloproteinase with thrombospondin type 1 motif 7 (ADAMTS-7),\textsuperscript{28} neutrophil elastase (NE),\textsuperscript{29} and proteinase-3 (\textit{Figure 1-1}).\textsuperscript{29} These proteases can cleave PGRN into single granulins or combinations of granulins and the resulting cleavage products have been shown to have different functions in different tissues and cellular processes including embryogenesis, cell growth and survival, transcriptional regulation, wound repair, and inflammation.\textsuperscript{21}
Figure 1-1: Visualization of full-length human PGRN and proteolytically cleaved granulins. PGRN can be cleaved by a number of metalloproteinases (MMP-9, MMP-12, MMP-14, ADAMTS-7) and neutrophil elastases and proteinases, which result in granulin peptides, GRN A-G and paragranulin (P), or combinations.

In the adult brain, PGRN is predominately expressed in both neurons and microglia.\textsuperscript{30} Although PGRN is widely expressed in neural cells during development and is implicated in sexual differentiation of the brain,\textsuperscript{31} expression becomes limited to defined populations upon maturity, including cortical neurons, hippocampal pyramidal neurons, and Purkinje cells in the cerebellum.\textsuperscript{32} Full-length PGRN has been found to be neuroprotective and neurotrophic in cortical, hippocampal, and motor neurons through activation of glycogen synthase kinase 3-β (GSK3-β), a Wnt pathway player,\textsuperscript{33} and secreted PGRN is involved in multiple signaling pathways including the WNT, ERK, PI3K, and AKT pathways, which have been found to activate cell proliferation pathways in neurons.\textsuperscript{23} In addition, GRN-E has also been associated with neurite outgrowth and neuron proliferation in neurons.\textsuperscript{34}

In microglia, innate immunity cells found in the central nervous system, PGRN is known to manage inflammatory response, and the expression of PGRN is strongly
correlated with injury response mechanisms. While secreted, full-length PGRN has been found to be anti-inflammatory, GRN-B, cleaved within microglia by MMP-12, as well as exogenous recombinant PGRN applied to fresh wounds, have actually been found to increase inflammatory responses. In PGRN-deficient FTLD, the migration of microglia to a site of neurodegeneration is known to flood the area with PGRN secreted from the microglia. However, while this influx of microglia restores the local PGRN concentration to levels approaching that of a wild-type brain, even these levels of PGRN are not enough to compensate for the neurodegeneration that had occurred. This implies either that the PGRN secreted from neurons and microglia differs in a crucial way such that neurons cannot utilize PGRN secreted from microglia, that injury or neurodegeneration stimulates microglial secretion of other factors that inhibit neuronal uptake of the excess PGRN, or that the large number of activated microglia in the area of neurodegeneration is otherwise contributing to the neurodegeneration and that even excess PGRN cannot compete with the immune response. In any case, more work must be done to understand the relationship between neurons, microglia, and PGRN in unaffected and PGRN-deficient FTLD afflicted human brains.

In addition to being a signalling protein, PGRN is also strongly associated with lysosomal activity and homeostasis. In cortical neurons, it has been found that full-length PGRN can be endocytosed into the cell by binding to sortilin or lysosome-bound protein prosaposin and is quickly trafficked to the lysosome once in the cell. Expression of GRN is also transcriptionally coregulated with other lysosomal genes such as LAMP1 and LAMP2, and is upregulated in HeLa cells by known small molecule inducers of lysosome biogenesis and function, such as sucrose, as well as by overexpressing transcription
factor EB (TFEB), a transcription factor known to regulate genes associated with lysosomal biogenesis.\textsuperscript{39} In addition, knockout of Grn in mice and GRN in humans leads to lysosomal storage disorders, suggesting a pathological link between lack of PGRN and lysosomal dysfunction.\textsuperscript{10} Although the full role of PGRN in lysosomal biogenesis or homeostasis has yet to be confirmed and the question of whether once trafficked to the lysosome, PGRN is cleaved into granulins that have some other role in the lysosome or other part of the cell is still unanswered, the linkage of PGRN to lysosomal function has profound implications about PGRN-deficient FTD disease progression and possible drug targets within lysosomal pathways that may provide disease-modifying and possibly preventative treatments.

Over 70 distinct mutations have been found in the exons of GRN in FTD patients, which account for 5-15\% of all FTD cases (\textit{Figure 1-2}).\textsuperscript{17, 21} These mutations are overwhelmingly (~95\%) insertions, deletions, or point mutations leading to premature stop codons, and result in the loss of function of the GRN allele via truncation and functional nullification of the mutated mRNA by nonsense-mediated mRNA decay.\textsuperscript{21, 40, 41} Loss-of-function mutations in one copy of the GRN gene are sufficient to cause PGRN haploinsufficiency and the associated frontal and temporal lobe neurodegeneration, while mutations in both copies of GRN lead to neuronal ceroid lipofuscinosis (NCL), a progressive lysosomal storage disorder with childhood onset.\textsuperscript{10} PGRN-deficient FTD patients also exhibit characteristic accumulation of ubiquitinated TDP-43 in neurons (FTD-TDP).\textsuperscript{15} While the molecular mechanisms connecting PGRN and TDP-43 are not well established, it has been hypothesized that one or more granulins help to clear TDP-43 from cells through lysosomal pathways, and loss of one
copy of the gene could lead to protein accumulation within the cells.\textsuperscript{23} Loss of full length PGRN may also be responsible for the chronic low level of inflammation in the microglia as well as rapid neuronal degeneration in FTD patients.\textsuperscript{21, 30} It is currently unclear if loss of any one granulin is sufficient to cause FTD symptoms.\textsuperscript{21, 42}

**Figure 1-2: Representation of pathogenic loss-of-function and potential pathogenic missense mutations in GRN leading to FTD.** Mutations in GRN that have been linked to FTD with cytoplasmic inclusions of TDP-43, noted in a schematic of the genomic structure of GRN (orange, top) leading to PGRN protein (bottom). The gray boxes indicate noncoding exon 1 and 3’ untranslated region. The orange boxes indicate coding exons 2-13. The protein has been shown with granulin domains indicated (Figure 1-1). Mutations are denoted with the cDNA numbering relative to the full-length GRN transcript (GenBank accession number NM_002087.2) and have been compiled by Nicolson et. al.\textsuperscript{41} except for the mutation in blue, which is a novel GRN mutation discussed in Chapter 7. Pink lettering describe potential pathogenic missense mutations with their protein numbering according to the full-length PGRN isoform (GenPept accession number NP_002078.1).

In recent years, GRN has emerged as a gene of interest, not only in FTD, but also in Alzheimer's disease, ALS, and NCL.\textsuperscript{14, 23} Research into PGRN and its role in neuroprotection make it an attractive therapeutic target. Thus, insight into GRN modulators may help in the development of therapeutics for this subclass of FTD, as well as for other neurological diseases that affect the aging population.

9
1.3 Targeting PGRN haploinsufficiency by upregulation of endogenous PGRN

Since PGRN-deficient FTD is a disease of haploinsufficiency, one course of therapeutic action may be to increase PGRN protein levels by upregulating GRN expression from the remaining wild-type allele, allowing restoration of total PGRN levels and processed granulins.\textsuperscript{21, 23} Consistent with this notion, it has been shown that exogenous PGRN can rescue the wild-type neurite outgrowth phenotype in cultured \textit{Grn}\textsuperscript{−/−} mouse primary neural cultures, and increasing PGRN expression has proven to be beneficial in several PGRN-deficient animal models.\textsuperscript{43, 44} However, protein replacement therapies in the brain are challenging, and it has been found that treating fresh wounds or injury sites with exogenous PGRN can lead to increased inflammation, which is thought to exacerbate neurodegeneration.\textsuperscript{30} In addition, in brain regions with progressive neurodegeneration, migrating microglia already secrete copious amounts of PGRN which has not been found to slow or stop neuronal death. These observations have led to a hypothesis that different cell types secrete different pools of PGRN, and simply flooding an area with exogenous PGRN would not be sufficient to slow or stop neurodegeneration.\textsuperscript{35} Full-length PGRN also acts as a signaling molecule which may lead to unintended consequences in cell proliferation and tumorigenesis.\textsuperscript{44} In that case, a more precise modulation of gene expression would therefore be favorable, and small molecules that increase endogenous neuronal PGRN by upregulating the wild-type allele may help to restore a wild-type phenotype in neurons in a more cell-regulated manner. Small molecule probes that upregulate PGRN in human neuronal cell cultures may also be able to help elucidate the role of PGRN in PGRN-deficient FTD and this dissertation presents work to that end.
Chapter 2

Model Systems for Studying Progranulin-Deficient Frontotemporal Dementia
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Summary

Relevant model systems are essential in studying complex diseases such as FTD. In neurodegenerative diseases, the organ of interest in a living state cannot be biopsied (except in rare cases), and post-mortem, autopsied human brain samples are only able to allow study of the end stage of disease progression. We are only starting to understand the role that PGRN plays in neuronal (and other) cells and in FTD disease progression, and it is imperative that the model system used to study PGRN-deficient FTD reflects the disease progression and potential treatment results as accurately as possible. In studying PGRN, many cellular model systems, from mouse primary cells to human immortalized cell lines, and whole animal models from zebrafish to mice have been used. This chapter gives an overview of the advantages and disadvantages of current model systems used to study PGRN and FTD, and identifies human induced pluripotent stem cell (iPSC)-derived neuronal cells as a highly suitable model system for the work in this dissertation.

2.1 Animal models for studying PGRN-deficient FTD

Animal models are valuable in the study of neurodegenerative diseases and aging, as it is potentially possible to study nervous system development and disease progression in an animal with a known genetic background in a controlled environment. In addition, there are currently many well-validated genetic and molecular biological approaches in whole animal models that make it feasible to study the contributions of
particular genes or proteins in specific cells or systems in the animal. As progranulin is evolutionarily conserved in vertebrates and \textit{C. elegans},\textsuperscript{23} studying progranulin deficiency and the disrupted mechanisms that follow can provide insight into mechanisms behind PGRN-deficient FTD, even if neurodegenerative phenotypes are not present. For instance, in the transparent body of \textit{C. elegans} where the lineage of each of the 959 somatic cells has been well characterized and the fate of each cell can easily be followed, it has been found that absence of progranulin causes an increased rate of apoptotic cell clearance.\textsuperscript{45} This may help explain why PGRN-deficient FTD progresses so quickly, as microglia more readily clear out neurons that have undergone, or are on the verge of undergoing, apoptosis. However, while the use of \textit{C. elegans} in lineage mapping is unparalleled, and \textit{C. elegans} is instrumental in studying development, homozygous and heterozygous progranulin knockout \textit{C. elegans} mutants have not been found to have altered lifespans and do not exhibit striking signs of excess neuronal cell death.\textsuperscript{45} Thus, while \textit{C. elegans} remains an important tool for studying progranulin biology, it is an incomplete model for studying the role of PGRN in neurodegenerative processes relevant to FTD in humans.

Zebrafish, which are a beneficial model organism due to the transparent nature of the embryos and ability to be used in high-throughput screening, have also emerged as a model organism for FTD, first through the creation of transgenic zebrafish expressing human tau and then through the generation of progranulin knockout zebrafish models.\textsuperscript{46, 47} Zebrafish express two orthologues of \textit{GRN}: \textit{granulin A} (\textit{grna}) and \textit{granulin B} (\textit{grnb}), as well as two shorter paralogues known as \textit{granulin 1} (\textit{grn1}) and \textit{granulin 2} (\textit{grn2}) respectively.\textsuperscript{48} \textit{Gma}\textsuperscript{−/−}, \textit{grnb}\textsuperscript{−/−}, and \textit{grna}\textsuperscript{−/−}\textit{grnb}\textsuperscript{−/−} knockout zebrafish
have been generated by zinc-finger nuclease technology, but none of these knockout lines exhibited growth impairment, developmental issues, or FTD/NCL-related neuropathology, despite the fact that progranulin knockdown zebrafish lines were found to exhibit spinal motor neuron axonopathies. Thus far, knockout zebrafish have been used to study the role of PGRN in inflammation, wound repair, and tissue regeneration, but because zebrafish genetics in the case of progranulin is complex, more work must be done to ascertain conditions in which progranulin knockout zebrafish could be a viable model system for PGRN-deficient neurodegenerative diseases. *grn* knockout zebrafish may have to undergo small molecule treatment or further genetic manipulations before they exhibit signs of neurodegeneration that model FTD or NCL.

Rodent models have traditionally been the gold standard in modeling neurodegenerative diseases in animals, not only for gene homology and humanization potential, but because rodents can exhibit external behaviors analogous to human behaviors and standardized assessments exist for measuring learning and memory. To date, heterozygous *Grn*+/− and homozygous *Grn*−/− mice have been generated to model PGRN deficiency. Though learning and memory were not impaired, *Grn*+/− mice showed reduced socialness when introduced to other mice, were more socially dominant when forced to interact with other mice, and exhibited impaired fear conditioning responses. These behaviors became more pronounced as the mice aged, and in fact, mimic behaviors of reduced sociability and emotional defects in FTD patients. However, although amygdalal neuronal impairments became apparent in aging *Grn*+/− mice, potentially accounting for the social and emotional abnormalities seen
in the mice, no other FTD cellular phenotypes, including gliosis, neuronal loss, or TDP-43 accumulation, were found in the \textit{Grn}^{+/−} mouse brains.\textsuperscript{49} Thus, while the \textit{Grn}^{+/−} haploinsufficient mouse line may be used as a behavioral model for FTD, it is, in the end, still an incomplete model of FTD. \textit{Grm}^{∗/+} haploinsufficient mice may still be useful in the search for small molecule modulators of PGRN, and can be especially beneficial in safety and efficacy studies for probes and drugs used to upregulate systemic levels of PGRN.

\textit{Grm}^{−/−} mice, on the other hand, are known to exhibit lipofuscinosis, ubiquitinated protein accumulation, and vacuolization within neurons, and has been suggested to be an appropriate model for studying PGRN-deficient FTD due to the prevalence of cellular phenotypes in addition to the behavior phenotypes.\textsuperscript{51} However, the \textit{Grm}^{−/−} mouse line does not exhibit a discernable pattern of neurodegeneration that connects it to directly to FTLD pathology in humans, and is in fact, a much more appropriate model for NCL, known to be caused by homozygous null \textit{GRN} mutations in humans.\textsuperscript{51}

Though current progranulin-deficient animal models have not been able to recapitulate the \textit{in vivo} cellular phenotypes associated with PGRN-deficient FTD, advanced genetic manipulation technologies, such as CRISPR (clustered regularly-interspaced short palindromic repeats) and further insight into the differences between human and model organism gene homologues paint an optimistic future for the development of animal models for complex human diseases. It is easier now than ever to manipulate expression of multiple genes in a complex animal model like a mouse. Furthermore, understanding the differences between humans and model organisms in terms of gene expression profile and disease presentation can help to elucidate the
differences in drug efficacy between a mouse in the lab and a human during an expensive clinical trial, and may help to bridge the knowledge gap in translating drug discoveries from the bench to the clinic.

2.2 Human iPSC-derived neuronal cells as a model for studying FTD

While model organisms have been essential in studying PGRN in a systemic way and on a developmental time scale, it remains a challenge to find a model organism that can exhibit cellular phenotypes similar to that of a neurodegeneration-prone human brain. Knowledge about the way that aging differentially affects different model organisms compared to humans is still incomplete and questions still exist as to whether the complex phenotypes of a human neurodegenerative disease can be recapitulated in another species. In addition, animal cell models are often inadequate to completely capture the nuanced genetic background of a neurodegenerative disease, especially when the goal of small molecule treatment is to transcriptionally regulate a gene via epigenetic mechanisms that may differ between species.

Because PGRN is expressed and has known functions in many cell types, many human cell lines have been used to study PGRN, including HEK293T (human embryonic kidney cell line),\textsuperscript{38} HeLa (immortalized human cervical cancer cell line),\textsuperscript{52} fibroblasts,\textsuperscript{53} immune cells,\textsuperscript{24} and iPSC-derived neuronal cells.\textsuperscript{54, 55} Research using these cell lines have revealed critical information about the different roles that PGRN plays in different cell types, but because PGRN is a multifunctional protein, it is especially critical that PGRN be studied in the cell type most related to the disease of interest. In studying FTD, a human neurodegenerative disease in which neurons, in
particular, are degenerating, a highly relevant model system in which to study PGRN and its role in the human neurons may, in fact, be human neurons. As the live human brain cannot be biopsied except in rare conditions, and primary human neurons cannot be routinely obtained on a scale to support biochemical and pharmacological studies, one way to culture human neuronal cells is by differentiating human iPSCs obtained through the reprogramming of more readily available patient somatic cells (i.e. skin biopsy fibroblasts) (Figure 2-1). These neuronal cells would have a known genetic background, and have the potential to exhibit phenotypes similar to the neuronal cells of the fibroblast donor. It has also been shown via the ENCODE project that the promoter region of GRN in different cell types can contain different epigenetic marks, so confirming epigenetic regulatory effects of small molecule treatment in human neuronal cells is especially important for FTD drug development. This would avoid the scenario where the effects of a compound that was efficacious in a mouse primary cell line or human immortalized cancer cell line are not recapitulated in a human brain during clinical investigation.

The majority of the following work uses neural progenitor cells (NPCs) and differentiated neurons obtained from previously reprogrammed iPSCs derived from a clinically unaffected human fibroblast cell line GM08330, with Chapter 7 describing efforts to reprogram patient and familial control fibroblast cell lines into iPSCs for subsequent derivation into NPCs and neurons.
Figure 2-1: General iPSC reprogramming and differentiation scheme from patient skin punches to iPSC-derived NPCs and neurons. Patient skin punch graphic adapted from Mayo Clinic. Transmitted light images from work summarized in Chapter 7 to create bVFTD patient-derived iPSC models.
Chapter 3

Chemogenomic Profiling in Human Neurons Reveals Selectivity & Kinetic Requirements of HDAC Inhibitors as Progranulin Enhancers for Treating Frontotemporal Dementia

This chapter contains work in part published in the following manuscript:

Chapter 3: Chemogenomic Profiling in Human Neurons Reveals Selectivity & Kinetic Requirements of HDAC Inhibitors as Progranulin Enhancers for Treating Frontotemporal Dementia

Abstract

Because PGRN-deficient FTD is a disease of haploinsufficiency, one therapeutic strategy is to identify small molecule enhancers of the wild-type allele of \textit{GRN}. This chapter details the findings that in human iPSC-derived neuronal cells, Class I HDAC inhibitors are sufficient to affect not only \textit{GRN} mRNA enhancement, but increase PGRN intracellular and secreted protein levels as well. In addition, only inhibitors with fast-binding kinetics to their HDAC target complexes are capable of potently enhancing PGRN expression and increasing PGRN secretion. These findings have implications for studies of the epigenetic mechanisms controlling \textit{GRN} expression from the human genome and therefore may advance translational efforts to develop targeted therapeutics for treating PGRN-deficient FTD.
3.1 A directed mRNA screen for GRN regulators

We began by screening a selection of known bioactive small molecules in human iPSC-derived NPCs and differentiated neurons with the L1000 assay, an mRNA expression profiling technique developed at the Broad Institute. The method utilizes multiplexed, Luminex bead-based technology to measure the expression of 1000 selected 'landmark' genes from cells in a single well of a 384-well plate, and is based on the reduced representation model of the genome, where the whole human transcriptome can be computationally inferred from the measurements of the 1000 landmark transcripts. We noted that GRN was amongst the measured landmark genes, making this a powerful assay to use in our studies, if the results could be validated by orthogonal means at both the mRNA and protein levels.

Analysis of the L1000 data sets in NPCs and neurons revealed histone deacetylase (HDAC) inhibitors as a class of small molecules that strongly upregulated GRN mRNA (Figure 3-1). Indeed, around 85% of the top 25 GRN enhancers in either cell type were Zn$^{2+}$-dependent HDAC inhibitors. To validate these results, we conducted a more targeted GRN mRNA screen using quantitative PCR (qPCR), the gold standard for quantitating gene expression levels in cells. In human control iPSC-derived NPCs, we found that HDAC inhibitors increased GRN mRNA levels significantly compared to other classes of small molecules such as WNT pathway modulators (Figure 3-2).
Figure 3-1: L1000 screen for GRN enhancers in iPSC-derived NPCs (A and B) and neurons (C and D). An L1000 screen of bioactive compounds was carried out in collaboration with the L1000 platform at the Broad Institute in NPCs (A) and neurons (C), with enhancers defined as compounds with a $Z > 2.5$ and suppressors defined as $Z < -2.5$. The top 25 hits in each cell line are shown in (B) and (D) with red stars denoting compounds known to target Zn$^{2+}$-dependent HDACs. Z-scores are normalized to vehicle (DMSO) z-scores.
Figure 3-2: Targeted small molecule screen for GRN mRNA enhancement. HDAC inhibitors were shown to enhance GRN mRNA levels, as measured by qPCR, compared to Wnt pathway modulators. Activities of non-HDAC epigenetic regulators are discussed in Chapter 5.
These results corroborated previous findings that HDAC inhibitors, in particular the potent HDAC inhibitor SAHA (Vorinostat), has been found to increase GRN transcription in a mouse Neuro-2A GRN luciferase reporter cell line, Epstein-Barr virus-immortalized, human lymphoblastoid cell lines from a healthy control and a FTD subject with a nonsense GRN R493X mutation, and in a human SH-SY5Y neuroblastoma cell line. In addition, crebinostat, another potent HDAC inhibitor, had been shown to enhance GRN mRNA expression in mouse cortical neurons. More recently, in the case of SAHA, these studies have been extended to the context of human iPSC-derived cortical neurons where SAHA has been demonstrated to enhance PGRN protein expression, albeit at doses that caused altered regulation of many other genes. Collectively, these observations regarding the epigenetic regulation of GRN has led to interest in using HDAC inhibitors as a rational therapeutic approach to treat PGRN-deficient FTD.

3.2 HDACs and HDAC inhibitors

Lysine acetylation on histones are dynamic post-translational epigenetic modifications that modulate chromatin structure and can dictate states of gene transcription. Hyperacetylated lysines on histones often mark areas of active gene transcription, as the acetyl groups help to decondense the chromatin and contribute to maintenance of the unfolded state of the DNA around the gene as well as recruitment of acetyl-lysine binding proteins, as discussed in Chapter 5 in the context of bromodomains. These acetylation marks are regulated by the activities of histone acetyltransferases (HATs), which acetylate histones, and histone deacetylases
(HDACs), which remove acetyl groups from histones and may also be found in corepressor complexes on the promoter region of the gene.\textsuperscript{63, 64} Although most HDACs operate primarily on histones, some HDACs have also been found to deacetylate non-histone proteins, such as tubulin, p53, and yin yang 1 (YY1).\textsuperscript{64} In neurological diseases, where genetic mutations can result in transcriptional dysregulation and protein accumulation phenotypes, HDACs have emerged as drug targets both for their ability to epigenetically regulate genes of interest and to modulate post-translational modifications in non-histone proteins within the cell.\textsuperscript{64, 65} HDAC inhibitors, which effectively promote acetylation of HDAC substrates, have become as an important class of small molecules that have been used both as chemical probes in the lab and as FDA-approved drugs in the clinic for oncology.\textsuperscript{66}

In mammals, 18 HDACs have been identified and have been divided into four classes based on their sequence homology to yeast HDACs, the first enzymes of the class to be discovered (\textit{Figure 3-3}). Class I, II, and IV HDACs are dependent on a chelated Zn\textsuperscript{2+} for activity and are known as the classical HDAC family, and Class III HDACs are dependent on a nicotinamide adenine dinucleotide (NAD\textsuperscript{+}) cofactor for activity and consists of 7 sirtuins, related to Sir2 in yeast.\textsuperscript{64} All Zn\textsuperscript{2+}-dependent HDACs are expressed in the brain and are highly expressed in neurons,\textsuperscript{67} with Class I HDACs (HDAC1/2/3/8), related to yeast Reduced Potassium Dependency 3 (RPD3) localized primarily to the nucleus and Class II (HDAC4/5/6/7/9/10), related to yeast Histone Deacetylase 1 (HDA1), shuttling between the nucleus and the cytoplasm.\textsuperscript{64} Class IV HDACs, comprised solely of HDAC11, are also expressed in the central nervous system and are thought to regulate inflammatory responses.\textsuperscript{65} Because Zn\textsuperscript{2+}-dependent HDAC
isoforms have similar active sites, but different functions within the cell, the development of selective HDAC inhibitors has been important in creating a chemogenomic toolkit to understand the role that HDACs play in gene expression in a cell.

Figure 3-3: Classification of HDACs with general scheme of HDAC activity. HDACs are divided into Zn$^{2+}$-dependent and NAD$^+$-dependent HDACs, and further subdivided into four classes, the catalytic domains of which are denoted in the legend, along with other relevant recognition domains. General mechanisms of HDACs involve identifying acetyl-lysine moieties on histones and other proteins and subsequently deacetylating them. HDAC inhibitors compete for the catalytic site.
Recently, HDAC inhibitors like SAHA have been identified as small-molecule enhancers of PGRN expression, although the mechanisms through which GRN is epigenetically regulated and the requirement for inhibition of specific HDAC family members remain poorly understood. Since SAHA is a potent, but relatively non-selective HDAC inhibitor targeting Class I (HDAC1/2/3/8), IIb (HDAC6/10) and IV (HDAC11) HDAC isoforms, the precise molecular mechanisms behind PGRN regulation in human neurons have yet to be elucidated. In particular, it is unknown if SAHA, or other HDAC inhibitors, directly affect histone acetylation within the GRN locus in a manner related to transcriptional changes, which of the 11 members of the Zn$^{2+}$-dependent family of HDACs are the relevant targets of inhibition, or possibly whether engagement of other targets and mechanisms are critical for the enhancing PGRN production. Furthermore, recent developments in the pharmacology of HDAC inhibitors have revealed that the binding kinetics of certain chemotypes can be tuned to control target residence time and therefore selectivity. Thus, beyond selectivity for different HDAC isoforms, determining the effect of HDAC inhibitors with different kinetic profiles on the epigenetic regulation of GRN in the context of human neuronal HDAC complexes is essential to advance the development of next-generation HDAC inhibitors as a targeted therapeutic for PGRN-deficient FTD and overcome potential limitations of first-generation HDAC inhibitors, like SAHA, that lack isoform and functional selectivity.

To address these knowledge gaps, we used a chemogenomic strategy to systematically dissect the role of different HDAC isoforms in GRN mRNA and PGRN regulation using a collection of small molecule probes differentially targeting Class I and Class II HDAC isoforms and with different binding kinetics in a human iPSC-based
neuronal culture system with robust and scalable mRNA and protein level assays. Overall, our data provide strong evidence to support the conclusion that pharmacological inhibition of Class I HDACs is sufficient to enhance PGRN protein production in human neurons and proliferating neural progenitor cells. Moreover, besides selectivity requirements, we show that only fast-binding HDAC inhibitors are capable of potently enhancing GRN mRNA expression and PGRN protein secretion, whereas HDAC inhibitors that exhibit prolonged slow-on/slow-off binding kinetics, are largely inactive despite measurable effects on histone acetylation and other gene expression.

### 3.3 Hydroxamic acid epigenetic probes for GRN mRNA and PGRN protein expression in human NPCs and neurons

![Figure 3-4: Model and experimental scheme for measuring the effect of HDAC inhibitors on GRN mRNA and PGRN protein expression](image)

**Figure 3-4: Model and experimental scheme for measuring the effect of HDAC inhibitors on GRN mRNA and PGRN protein expression** along each phase of expression, from mRNA (Assay 1 – qPCR) to intracellular protein (Assay 2 – Western Blot and ELISA) to secreted protein (Assay 3 – ELISA). With the HDAC complex, $S_n = $ subunit and TF = transcription factor.
Previously, SAHA and crebinostat, hydroxamic acid HDAC inhibitors known to target Zn\(^{2+}\)-dependent Class I and Class IIb HDACs, were found to increase GRN gene expression in mouse proliferative Neuro-2A cells and in post-mitotic mouse primary neurons.\(^{53, 62}\) To begin translating these findings to a human neuronal context and enable further dissection of the underlying mechanism, we initially sought to confirm these observations in cultured human iPSC-derived NPCs and in post-mitotic, differentiated neuronal cultures using assays that reported on GRN mRNA levels, intracellular PGRN protein, and extracellular secreted PGRN protein (Figure 3-4). In screening for small molecule enhancers GRN transcription, it is imperative that all levels of progranulin (mRNA, intracellular, and secreted protein) are enhanced.

Treatment of human NPC and neuronal cultures with SAHA and crebinostat caused a robust and consistent induction of both GRN mRNA and PGRN protein levels (Figure 3-5). Because PGRN is a secreted protein, we also investigated the concentration of the protein secreted into the media at the end of the treatment time and determined that the increase in secreted PGRN correlates with the increase in GRN mRNA and intracellular PGRN levels (Figure 3-5). In addition, we demonstrated that treatment with panobinostat (LBH589), also a highly-potent, cinnamic hydroxamic acid, broad spectrum, HDAC inhibitor,\(^{75}\) likewise significantly increased GRN mRNA and PGRN protein levels in NPCs and differentiated neurons and that the increase in secreted PGRN protein after treatment is proportional to the increase in mRNA and intracellular protein levels (Figure 3-5). Consistent with past literature about the potency of panobinostat compared to other hydroxamic acid HDAC inhibitors, panobinostat was significantly more potent than SAHA or crebinostat at inducing GRN mRNA and PGRN.
protein expression in human neuronal cultures, with efficacious doses in the sub-micromolar range compared to micromolar range of SAHA or crebinostat.\textsuperscript{62, 76, 77}

Although SAHA significantly enhances \textit{GRN} mRNA and PGRN protein levels in NPCs, it is known to have poor brain penetrance.\textsuperscript{78} In collaboration with the Hooker lab at the Martinos Center for Biomedical Imaging at Massachusetts General Hospital/Harvard Medical School, we showed that the HDAC positron emission tomography (PET) probe [(11)C]Martinostat, a potent, broad spectrum brain-penetrant hydroxamic acid HDAC inhibitor, is capable of enhancing \textit{GRN} mRNA and PGRN protein levels in NPCs (\textit{Figure 3-6}).\textsuperscript{79} Future optimization of brain penetrant HDAC inhibitors that are able to enhance PGRN expression using the panel of assays described here, along with agents like [(11)C]Martinostat to measure HDAC target occupancy in animal models and ultimately in humans, will enable a precise understanding of the pharmacokinetics of HDAC inhibition and pharmacodynamic regulation of PGRN.
Figure 3-5: Profiling hydroxamic acid HDAC inhibitor effects on GRN mRNA and PGRN protein expression in human iPSC-derived NPCs and neurons. A) Chemical structures for fast on/off HDAC inhibitors: (1) SAHA, (2) crebinostat, and (3) panobinostat. IC$_{50}$ values for (1) from Bradner et al.,$^{68}$ (2) from Fass et al.,$^{62}$ and (3) from Khan et al.$^{80}$ All are shown to increase GRN mRNA expression and PGRN intracellular and secreted protein levels in human NPCs (black) and 18-day neurons (gray) as shown in B (SAHA, 10 µM), C (crebinostat, 2.5 µM), and D (panobinostat, 0.5 µM); vehicle = DMSO. All cells were treated for 24 hours. mRNA quantification was done relative to housekeeping gene GAPDH. Protein quantification is shown relative to vehicle and was measured by ELISA. Dividing lines on Western blots indicate samples from separate gels. Each condition shown is the average of n=3 biological replicates x 3 technical replicates with significance calculated by unpaired t-test. *** p < 0.001 **** p < 0.0001
Figure 3-6: Broad-spectrum HDAC inhibitor, PET probe [(11)C]Martinostat increases GRN mRNA and PGRN protein levels in NPCs. A) Chemical structure for (4) [(11)C]Martinostat. IC$_{50}$ values for Martinostat from Schroeder et al.$^{76}$ In these studies, the non-radiolabeled compound was used. Martinostat significantly increases GRN mRNA (B) and PGRN protein levels (C) in iPSC-derived NPCs. D) Global acetyl-histone levels in NPCs treated with Martinostat. Data from (B) and (D) has been published and figure was adapted from Wey et al.$^{79}$ Vehicle = DMSO. All cells were treated for 24 hours. mRNA quantification was done relative to housekeeping gene GAPDH. Protein quantification is shown relative to vehicle and was measured with ImageJ. Each condition shown is the average of n=3 biological replicates x 3 technical replicates (mRNA), and 3 replicates (protein) #Because treatment with 5.0 μM Martinostat was toxic to cells, whole-cell lysates from three replicates were combined into one pool to obtain sufficient protein for this dose. Significance calculated by unpaired t-test. Graphs shown as mean ± SD. ** p < 0.01 **** p < 0.0001
While these broad specificity-HDAC inhibitors increase PGRN levels upon treatment, the simultaneous inhibition of multiple members of the HDAC family of enzymes is known to have significant effects on not only GRN but also many other genes.\textsuperscript{54, 62} Understanding the inhibition of which specific HDAC isoforms are necessary and sufficient for upregulation of PGRN will help answer questions about the suitability of HDAC inhibitors as viable therapeutics for FTD and other disorders of PGRN-deficiency given concerns related to dose-limiting toxicities seen with broad-spectrum HDAC inhibitors in the context of oncology. To this end, we first tested the Class IIb HDAC6-selective inhibitors, ACY-1215 (rocilinostat) and tubastatin A, and a Class I HDAC8-selective inhibitor, PCI-34051, in human NPCs and neurons at a range of concentrations previously reported to be effective in the literature.\textsuperscript{81-83} Neither tubastatin A nor PCI-34051 increased GRN mRNA or PGRN protein expression, although tubastatin A had a robust effect on H3K9 acetylation (Figure 3-7).

Although tubastatin A did not increase PGRN expression, ACY-1215, the additional reported HDAC6-selective inhibitor, significantly increased both GRN mRNA and PGRN protein levels (Figure 3-7). Here, we optimized the dose of ACY-1215 for maximal PGRN enhancement activity, although even at 10-fold lower concentrations, ACY-1215 still showed comparable PGRN enhancement (Figure 3-7). It may be that ACY-1215 at the cellular concentrations tested of 5 µM has lost its selectivity for HDAC6 since its IC\textsubscript{50} for HDAC6 is 5 nM, which is only ~10x selective over HDAC 1, 2, 3 based upon in vitro potencies.\textsuperscript{84} Consistent with this notion, ACY-1215 treatment at doses that were effective for increasing PGRN protein levels (5 µM) caused as strong of induction of H3K9 acetylation as SAHA that also potently inhibit Class I HDACs (Figure 3-7).
Figure 3-7: HDAC6 and HDAC8-selective inhibitor effects on GRN mRNA and PGRN protein expression in human iPSC-derived NPCs and neurons. A) Chemical structures for HDAC6-selective inhibitors (5) ACY-1215 and (6) Tubastatin A and HDAC8-selective inhibitor (7) PCI-34051. IC_{50} values for (5) from Santo et al., 84 (6) from Butler et al., 82 and (7) from Balasubramanian et al. 83 B) HDAC6 inhibitor effects on GRN mRNA expression and PGRN protein expression in human NPCs (black) and 18-day neurons (gray). ACY-1215 (5 µM) had a significant effect on GRN and PGRN expression. Tubastatin A (10 µM) was not effective. C) HDAC8 inhibitor PCI-34051 (10 µM) did not have a significant effect on GRN and PGRN expression in human NPCs (black) or 18-day neurons (gray). Vehicle = DMSO. All cells were treated for 24 hours. mRNA quantification was done relative to housekeeping gene GAPDH. Protein quantification is shown relative to vehicle and was measured by ELISA. Each condition shown is the average of n=3 biological replicates x 3 technical replicates with significance calculated by unpaired t-test. **** p < 0.0001
3.4 Class IIa HDAC inhibitor treatment does not increase GRN mRNA

SAHA does not inhibit Class IIa HDACs, so to test whether Class IIa HDAC inhibition is sufficient to enhance PGRN expression, we treated NPCs with a Class IIa-selective HDAC inhibitor TMP269. We found that TMP269, which contains a trifluoromethyloxadiazole (TFMO) metal-binding group, was not effective at increasing GRN mRNA in NPCs (Figure 3-8), implying that inhibition of Class IIa HDACs is not sufficient to enhance PGRN in neuronal cells.

Figure 3-8: TMP269 does not increase GRN mRNA in NPCs. A) Chemical structure for (8) TMP269, a Class IIa HDAC inhibitor. IC50 values from Lobera et al. B) TMP269 (10 µM) did not increase GRN mRNA, while positive control SAHA (10 µM) did. Vehicle = DMSO. All cells were treated for 24 hours, with 2 biological replicates x 3 technical replicates. mRNA quantification was done relative to housekeeping gene GAPDH. Graphs are shown as mean + SEM with significance calculated by unpaired t-test. **** p < 0.0001
3.5 Class I, slow-binding HDAC inhibitors, do not increase PGRN expression in human neuronal cultures.

To further probe the contribution of different HDAC isoforms on PGRN expression, we tested the effects of Class I-selective, ortho-aminoanilide HDAC inhibitors, CI-994 (HDAC1/2/3) and Cpd-60 (HDAC1/2) on human NPCs and neurons. Surprisingly, given their biochemical potency and ability to significantly increase H3K9 acetylation levels in treated human iPSC-derived NPCs and neurons, neither CI-994 nor Cpd-60 significantly increased GRN mRNA or PGRN levels (Figure 3-9). Rather, in the case of CI-994 treatment in neurons, a statistically significant decrease of GRN mRNA and intracellular PGRN protein, and in NPCs a significant decrease of intracellular PGRN as well as secreted extracellular PGRN, is observed (Figure 3-9). To ensure that this lack of enhancement by CI-994 was not unique to human neuronal cells, we also repeated these assays in another human, non-neuronal cell line, HEK293T, producing the same results of a lack of GRN mRNA or PGRN induction whereas SAHA was effective at doing both (Figure 3-10). In contrast, both CI-994 and Cpd-60 significantly increased FXN (Frataxin) mRNA levels (Figure 3-9), another gene known to be under epigenetic control of Class I HDACs in human iPSC-derived neuronal cells that has been shown to be enhanced by other ortho-aminoanilide HDAC inhibitors.70, 72
Figure 3-9: Profiling ortho-aminoanilide HDAC inhibitors on GRN mRNA and PGRN protein expression in human iPSC-derived NPCs and neurons. 

A) Chemical structures for slow-on/off ortho-aminoanilide HDAC inhibitors (9) CI-994 and (10) Cpd-60. IC$_{50}$ values for (9) from Bradner et al.$^{68}$ and (10) from Schroeder et al.$^{86}$

B) Neither CI-994 nor Cpd-60 had a significant effect on GRN mRNA expression or PGRN protein expression in human NPCs (black) or 18-day neurons (gray); 3 biological x 3 technical replicates.

C) CI-994 and Cpd-60 are shown to increase H3K9 acetylation in 18-day neurons despite not enhancing PGRN protein levels.

D) Slow on/off HDAC inhibitors CI-994 and Cpd-60 increase Frataxin (FXN) mRNA expression in 18-day neurons, while fast on/off inhibitor SAHA does not; 3 biological x 2 technical replicates. Cells were treated for 24 hours with vehicle (DMSO), SAHA (10 µM), CI-994 (10 µM) or Cpd-60 (5 µM). mRNA quantification was done relative to housekeeping gene GAPDH. Protein quantification is shown relative to vehicle and was measured by ELISA. All graphs are shown as mean + SEM, with significance relative to vehicle calculated by unpaired t-test. ** p < 0.01 **** p < 0.0001
Figure 3-10: GRN mRNA and PGRN intracellular and secreted protein in MSR293 cells treated with HDAC inhibitors. SAHA (10 µM) enhanced GRN mRNA and PGRN intracellular and secreted protein in MSR293 cells while CI-994 (10 µM) did not. All cells were treated for 24 hours, with 3 biological replicates. mRNA quantification was done relative to housekeeping gene GAPDH. Representative western blots are shown here. Graphs are shown as mean + SEM with significance calculated by unpaired t-test. *** p < 0.001 **** p < 0.0001

Besides varying in their isoform selectivity from the hydroxamates, the ortho-aminoanilides CI-994 and Cpd-60 also differ in terms of their binding kinetics.69-71, 74 Whereas hydroxamates have fast-on/fast-off mechanisms of action, ortho-aminoanilides like CI-994 and Cpd-60 both have slow-on/slow-off mechanisms of action.70, 86, 87 This mechanism of action of slow-on/slow-off inhibitors in the case of the FXN gene has been shown to be essential for its induction, as fast-on/fast-off inhibitors like SAHA are inactive.70, 88 Taking these observations into consideration, we further treated NPCs with CI-994 for up to 72 hours to account for slow-binding properties and still found that CI-994 did not significantly enhance GRN mRNA or PGRN protein levels in neuronal cells (Figure 3-11). These results indicate that simply extending the treatment time for the slow-binding HDAC inhibitors CI-994 and Cpd-60 is not sufficient to enhance GRN mRNA or PGRN protein levels. Thus, in addition to isoform selectivity, the specific interaction mechanism and kinetics of HDAC inhibition appears to be important factors
for obtaining optimal enhancement of GRN mRNA expression and PGRN protein expression in the context of the human neurobiology.

**Figure 3-11: NPC time course treatment with CI-994.** Despite longer treatment times, CI-994 did not (A) increase GRN mRNA or (B) PGRN protein, and in fact, (C) decreased PGRN secreted protein expression in NPCs compared to vehicle; 2 biological x 3 technical replicates. Cells were treated for 24, 48, or 72 hours with vehicle (DMSO), CI-994 (1 µM), or CI-994 (5 µM). mRNA quantification was done relative to housekeeping gene GAPDH. Representative Western blot shown. All graphs are shown as mean ± SEM, with significance relative to vehicle calculated by unpaired t-test. * p < 0.05 ** p < 0.01

In contrast to the inactivity of CI-994 in our human iPSC-derived neuron culture system even when treated for multiple days, recent findings demonstrate that CI-994 is capable of enhancing PGRN expression in cultured mouse N2a cells and *in vivo* in brain tissues when it is directly infused intracerebroventricularly for multiple days. These differences may reflect species-specific differences in the epigenetic regulation of GRN gene expression, particularly considering the fact that in HEK293T cells, a human embryonic kidney cell line, CI-994 did not increase PGRN expression (*Figure 3-10*).
3.6 Tetrapeptide Class I HDAC inhibitor apicidin enhances PGRN expression

As an alternative test of whether Class I HDAC inhibition is sufficient to enhance PGRN expression using inhibitors with different binding kinetics than the slow-binding ortho-aminoanilides represented by CI-994 and Cpd-60, we turned to additional compounds in our chemogenomic toolkit. We first tested the efficacy of Class I-selective HDAC inhibitor apicidin, a cyclic tetrapeptide natural product that contains a ketone group rather than the strong metal-chelating hydroxamate group found within SAHA, crebinostat, panobinostat, and ACY-1215. Using recombinant human Class I HDACs in an enzymatic assay measuring deacetylase activity, we first determined that apicidin has an in vitro kinetic profile similar to panobinostat since its potency did not increase with prolonged incubation as compared to the slow-binder CI-994 (Figure 3-12). Consistent with the hypothesis that Class I HDAC inhibition is sufficient and its isoform selectivity and kinetic profile, in human NPCs and human neurons, apicidin potently enhanced both GRN mRNA and PGRN protein expression (Figure 3-13). These data decouple the hydroxamic acid moiety from being necessary for efficacy in increasing PGRN levels in human neurons and provide evidence that targeting HDAC1/2/3 with fast-binding inhibitors is sufficient to increase GRN mRNA levels as well as intracellular and secreted PGRN protein.
Figure 3-12: In vitro HDAC enzymatic assay for Apicidin and Valproate. A) Schematic of experimental design measuring effect of preincubation on inhibitor potency for B) HDAC1, C) HDAC2, and D) HDAC3. The appropriate inhibitor and enzyme were added to a well of a 96-well plate and incubated for a 0-3 hours. After incubation, fluorogenic Class I HDAC substrate MAZ1600 was added and the reaction was left to incubate for 40 minutes before it was quenched and read. Maximum HDAC inhibition occurs at 3 hours, and graphs are plotted as % inhibition at given time of maximum inhibition. Apicidin (100 nM) and Valproate (2.5 mM) both show, for HDACs 1, 2, 3, similar binding kinetics to positive fast-on/fast-off control panobinostat (25 nM), whereas CI-994 (100 nM), positive slow-on/slow-off control shows an increase in inhibition with prolonged precubation. Mean data is shown.
Figure 3-13: Apicidin increases GRN mRNA and PGRN protein expression in human iPSC-derived NPCs and neurons.  

A) Chemical structure for tetrapeptide (11) apicidin, IC50 values from Huber et al.90  

B) Apicidin significantly increases GRN mRNA and PGRN intracellular and secreted protein expression human NPCs (black) and 18-day neurons (gray). Cells were treated for 24 hours with vehicle (DMSO), apicidin (2.5 µM), or positive control SAHA (10 µM). mRNA quantification was done relative to housekeeping gene GAPDH. Protein quantification is shown relative to vehicle and was measured by ELISA. Each condition shown is the mean + SEM of n=3 biological replicates x 3 technical replicates with significance calculated by unpaired t-test.  

**** p < 0.0001
3.7 FDA-approved, Class I HDAC inhibitor valproate enhances PGRN expression

As the next test of our model that Class I HDAC inhibition is sufficient to enhance PGRN expression using inhibitors with different binding kinetics than the long-residence time *ortho*-aminoanilides represented by CI-994 and Cpd-60, we tested the efficacy of valproate (valproic acid), an FDA-approved, Class I HDAC-selective\textsuperscript{77} drug used for the treatment of epilepsy and bipolar disorder. Similar to apicidin and panobinostat, valproate also has fast-on/fast-off binding kinetics since its potency did not increase with prolonged incubation as compared to the slow-binder CI-994 (Figure 3-12). As predicted based upon its selectivity and kinetic profile, in human NPCs and human neurons, valproate indeed enhanced both *GRN* mRNA and PGRN protein expression, albeit at much higher dose (1-5 mM) than any of the hydroxamates or apicidin (Figure 3-14). However, these efficacious doses correlate with previously reported values in mouse neuronal assay, as well as correspond to the relative lack of potency of carboxylic acids compared to hydroxamic acids in HDAC biochemical assays.\textsuperscript{53, 77}

With chronic treatment in humans, valproate reaches serum concentration of 300-700 µM\textsuperscript{66} with the brain concentration estimated to have a C\textsubscript{max} = 400 µM.\textsuperscript{91} Although in our studies valproate at doses lower than 1 mM only increased PGRN protein expression by 1.3-1.5x compared to vehicle in cultured NPCs and neurons (Figure 3-14), it is possible that sustained treatment (i.e. weeks to months) with valproate may cumulatively have a beneficial effect on PGRN levels. Additionally, it may be feasible to find other agents that synergize or further enhance the effects of valproate toward PGRN while minimizing undesired effects.
Figure 3-14: Valproate increases GRN mRNA and PGRN protein expression in human iPSC-derived NPCs and neurons.  

A) Chemical structure for the carboxylic acid (12) valproate. IC$_{50}$ values from Fass et al.  

B) Valproate (5 mM) significantly increases GRN mRNA and PGRN intracellular and secreted protein expression human NPCs (black) and 18-day neurons (gray). 

C) Dose response for valproate effect on GRN mRNA expression. Cells were treated for 24 hours with vehicle (ddH$_2$O) or valproate (at indicated doses). mRNA quantification was done relative to housekeeping gene GAPDH. Protein quantification is shown relative to vehicle and was measured by ELISA. Each condition is the average of n=3 biological replicates x 3 technical replicates. All graphs are shown as mean ± SEM, with significance relative to vehicle calculated by unpaired t-test. * p < 0.05  ** p < 0.01  **** p < 0.0001

Questions around the effective dose of a drug like valproate highlight more general points of consideration raised by our studies when attempting to relate in vitro IC$_{50}$’s for HDAC inhibitors to their effective concentrations in cellular studies in order to discern the selectivity of target engagement. In general, in the studies reported here we observed a shift of ~500-1000X from the in vitro IC$_{50}$’s toward recombinant HDACs before we saw an effective biological response. While potency shifts are commonly seen with many small molecule probes, the magnitude is not always as great as that
which has been our consistent experience in the context of functional assays of HDAC inhibitors in primarily rodent and human neuronal assays. Based upon the findings of Bantscheff et al. and our unpublished studies (Hennig et al. manuscript in preparation) this shift may in part be due to the fact that certain HDAC inhibitors exhibit differential potency toward the endogenous, large multi-subunit complexes that HDACs are part of in cells in comparison to the recombinant enzymes most traditionally used for in vitro HDAC assays. In the case of enhancement of GRN mRNA levels through HDAC inhibition, the panel of functional human neuronal assays we described here in which endogenous HDAC complexes are present will enable the further refinement of the structure-activity relationship for epigenetic regulation of PGRN production.

3.8 Relationship between GRN mRNA and PGRN protein levels in HDAC inhibitor-treated human NPCs and neurons

Having tested a set of HDAC inhibitors with a range of efficacies toward enhancing GRN expression, we sought to determine the correlation between changes in GRN mRNA levels and levels of intracellular and secreted PGRN protein. We found that increases in GRN mRNA levels were highly positively correlated with increases in both PGRN protein expression and secretion into the extracellular media (Figure 3-15). In addition, these correlations were similar when comparing between proliferative NPCs and post-mitotic neurons. Taken together, these data suggest that multiple human cell types of the neural lineage have a conserved mechanism of epigenetic regulation involving sensitivity to Class I HDAC inhibitors with fast-binding kinetics.
3.9 Comparison of RNAi-mediated targeting of individual HDAC isoforms to small molecule inhibitors of HDACs

To probe the mechanism through which small molecule inhibitors of HDACs regulate GRN mRNA and PGRN protein production, we sought to complement our chemogenomic profiling with functional genomic studies using RNA interference (RNAi). Using lentiviral-mediated delivery of short hairpin RNAs (shRNAs) to selectively target individual Class I HDACs predicted to be relevant based upon the inhibitor sensitivities, along with four separate control shRNAs (RFP, lacZ, luciferase, and GFP), we created a panel of stable human NPC lines designed to have selective silencing of HDACs. Upon
selection and expansion of NPC lines we were able to obtain robust silencing of each of HDAC1, HDAC2, and HDAC3 compared to the four control shRNAs (*Figure 3-16*). However, RNAi-mediated silencing of none of these HDAC isoforms had a statistically significant effect on *GRN* mRNA or PGRN protein expression in human NPCs (*Figure 3-16*).

These results suggest that either multiple Class I HDACs need to be inhibited simultaneously or that the loss of HDAC protein from RNAi-mediated silencing does not faithfully recapitulate the same functional consequence of small molecule inhibition. In support of the latter being a relevant consideration, it is well known that HDACs are part of large, macromolecular complexes with intermolecular interactions with other HDAC family members as well as other complex subunits.69, 93 Accordingly, the loss of one component of these chromatin-modifying complexes may differentially impact epigenetic regulation compared to the functional consequences of the interaction of substrate competitive inhibitor within the HDAC active site that otherwise leaves the complex intact.

Furthermore, analysis of Western blot results showed evidence for compensatory effects from single HDAC knockdown in the form of enhanced expression of other Class I HDACs. Most notable, we observed elevated HDAC1, and to a lesser extent HDAC3, levels upon HDAC2 silencing (*Figure 3-16*). Thus, we conclude that rebalancing of HDAC activity due to loss of a given HDAC isoform in human NPCs may confound the straightforward comparison of single HDAC genetic perturbations to small molecule inhibitor treatments.
Figure 3-16: GRN mRNA and PGRN protein expression in stable HDAC 1, 2, and 3 shRNA lines or control shRNA lines. A) Western blot showing knockdown of HDAC1 (HD1), HDAC2 (HD2), or HDAC3 (HD3) after lentiviral shRNA knockdown compared to controls. Ctrl1 = RFP; Ctrl2 = lacZ; Ctrl3 = luciferase; Ctrl4 = GFP When compared to control lines, knockdown of HDAC1, HDAC2, or HDAC3 independently showed no strong GRN mRNA (B) or PGRN intracellular (A, C) or secreted (D) protein enhancement in NPCs. mRNA quantification was done relative to housekeeping gene GAPDH. Protein quantification is shown relative to vehicle and was measured by ELISA. Each condition shown is the average of n=3 biological replicates x 3 technical replicates (mRNA) or 2 technical replicates (protein). All graphs are shown as mean + SEM, with significance relative to vehicle calculated by unpaired t-test.
3.10 Summary and future studies

Here, we provide evidence that one potential mechanism of increasing PGRN levels in human NPCs and neurons is through inhibition of Class I HDACs (HDAC1/2/3) and that inhibitors of these HDACs with fast binding kinetics robustly increase GRN mRNA, intracellular PGRN protein, and secreted PGRN protein. Concentrations of compounds used in our studies were optimized for maximal effect in enhancing PGRN expression, capitalizing on the different potencies and selectivities of different compounds within the expanding chemogenomic toolkit for dissecting Zn\textsuperscript{2+}-dependent HDACs. We show here that selectively inhibiting Class I HDACs with fast-on/fast-off binding kinetics could be an efficacious way of correcting PGRN haploinsufficiency by increasing expression of the endogenous, wild-type allele of the GRN gene in FTD patients. By directly targeting gene expression in this way, we expect not only an increase in gene expression but also in protein expression and action. Indeed, we observed a strong correlation between GRN mRNA levels and intracellular PGRN protein as well as secreted PGRN (Figure 3-15). The secreted full length PGRN is involved in many signaling pathways including the WNT, ERK, and PI3K pathways,\textsuperscript{21, 22} and cleaved, intracellular granulins are involved in many neuronal functions, such as neurite outgrowth,\textsuperscript{21} so targeting pathways that solely increase protein lifetime within the cell might not recapitulate wild-type cell activity in PGRN-deficient cells.

Contrary to our initial expectations, our functional genomic studies using RNAi-mediated silencing of HDAC1, HDAC2, and HDAC3 failed to provide evidence that loss of any one of these Class I HDACs is sufficient to enhance GRN/PGRN expression (Figure 3-16). Since our functional genomic methods did not further narrow down a
single targetable Class I HDAC isoform, further studies must be performed to determine whether small molecule inhibition of a single (or subset of) Class I HDAC isoforms is sufficient for increasing \(\text{GRN}/\text{PGRN}\) expression. The current classes of inhibitors that target a subset of Class I HDACs are ortho-aminoanilides, which we show here in the case of CI-994 and Cpd-60, are unable to significantly increase \(\text{GRN}\) expression in human iPSC-derived NPCs and neurons. Indeed, even at concentrations that significantly increased H3K9 acetylation, CI-994 and Cpd-60 did not increase \(\text{GRN}\) mRNA or PGRN protein levels (Figure 3-9). Importantly, these results demonstrate it is possible to decouple \(\text{GRN}\) gene expression from global effects on genome-wide acetylation level with HDAC inhibitors. Based upon the strong induction of PGRN expression with the ketone-containing Class I HDAC selective inhibitor apicidin, these results indicate it is possible to retain the selectivity to Class I HDACs, with potential in the future for fine tuning of the binding kinetics. Further research into HDAC inhibitor development leading to the synthesis of a panel of HDAC inhibitors that are isoform-selective and exhibit a range of binding kinetics would contribute to the power of chemogenomic toolkits like the one we used here, and would allow us to probe more specifically for the HDAC isoform and kinetic requirements for a potential PGRN-enhancing drug. In particular, it will be important to test additional ortho-aminoanilide HDAC inhibitors with varying binding kinetics to determine whether the conclusions we draw from both CI-994 and Cpd-60 hold for additional members of this chemotype.

In addition, the effects of CI-994 and Cpd-60 on human cells seems to differ from the effects on rodent \textit{in vitro} and \textit{in vivo} models, and future studies with an expanded set of HDAC inhibitors comparing levels of enhancement between human and rodent
neurons will be important for future translational of next-generation HDAC inhibitors for enhancing PGRN levels. Besides comparison of human to rodent culture systems, further work must also be done to assess the degree to which Class I selective, fast-binding HDAC inhibitors increase PGRN in patient-derived neuronal cell lines where the levels of PGRN expression are already low. There is evidence that SAHA increases GRN significantly in FTD patient fibroblasts, and recent findings have extended this to FTD-patient neuronal cell lines. Because many patients with PGRN-deficient FTD exhibit accumulation of TDP-43 within neurons and Grn-/- mice are known to exhibit lysosomal defects, future studies can be directed towards understanding whether HDAC inhibitors can help to alleviate these phenotypes in cultured patient-derived NPCs and neurons, in rodent models, and ultimately, in patients.

3.11 Materials and Methods

L1000 assay. L1000 assay was done with human iPSC-derived neural progenitor cells (NPCs) and 18-day differentiated neurons (cultured as described below) in conjunction with the L1000 platform at the Broad Institute. Processed data was provided after the ‘roasting’ procedure and mean replicate (n=3) data was provided and used in analysis.

Human iPSC-Derived Neural Progenitor Cell Culture. All NPCs used were obtained from differentiated iPSCs previously reprogrammed from a clinically unaffected human fibroblast cell line GM08330 (Coriell Institute for Medical Research, Camden, NJ) as described in Sheridan et al., 2011 and were cultured as described in Zhao et al., 2012. The 8330-8 NPCs were cultured on plastic tissue culture ware (T75 flasks,
Falcon #353133 and 6-well plates, Falcon #353046) which were coated first with 20 
µg/mL poly-ornithine (Sigma Aldrich #P3655) in ddH₂O for 2 hours and then with 5 
µg/mL laminin (Sigma Aldrich #L2020) in Dulbecco’s Phosphate-Buffered Saline (DPBS 
1X; Gibco #14190-144) to provide suitable extracellular adherence and growth factors 
for NPCs. Coated tissue culture ware was stored for at least 48 hours at 4°C or 
overnight at 37°C in laminin-DPBS before use.

Media for NPC culture (NPC media) was composed of 70% DMEM (Dulbecco's 
modified Eagle's Medium, Gibco #11995), 30% Ham's F12 with L-glutamine (Modified 
Cellgro/Mediatech #10-080-CV), 1X penicillin/streptomycin (100X, Gibco #15140-122), 
and 1X B27 Supplement (50X, Gibco #17504-044). NPC media was then supplemented 
with EGF (20 ng/mL, Epidermal Growth Factor, Sigma Aldrich #E9644, prepared as 
1000X stock in DMEM), bFGF (20 ng/mL, basic Fibroblast Growth Factor, ReproCELL 
#03-0002, prepared as 1000X stock in PBS), and heparin (5 µg/mL, Sigma Aldrich 
#H3149, prepared as 1000X stock in Ham's F12) just before use.

NPCs were maintained in complete NPC media at 37°C with 5% CO₂ in a 
humidified atmosphere and passaged twice per week at a 1:3 ratio or 4 x 10^6 cells per 
T75 flask. For passaging, confluent cultures in T75 flasks were washed once with 10 mL 
DPBS and then treated with 1 mL TrypLE Select (Life Technologies #12563029) until 
cells detached. TrypLE treatment was stopped by adding 9 mL NPC media. Cells were 
gently triturated to obtain a single-cell suspension and were centrifuged at 1000 rpm 
(700 x g) for 5 min and then resuspended in complete NPC media.
Human iPSC-derived Neuron Culture. All human iPSC-derived neurons were derived from above stock of NPCs by growth factor withdrawal. The 8330-8 NPCs were grown on plastic tissue culture ware in 6-well plates (Falcon #353046) that were coated with a combination of 20 µg/mL polyornithine (Sigma Aldrich #P3655) and 5 µg/mL laminin (Sigma Aldrich #L2020) in DPBS. The resulting plates were stored at 4°C for at least 48 hours before use and were washed with 1 mL DPBS before cells were plated.

NPCs were plated at 0.5x10^6 cells per well in a poly-ornithine/laminin single coated 6-well plate in 2 mL NPC media with above growth factors. The cells were allowed to incubate at 37°C for 48-72 hours to grow to confluency. The media was aspirated and the cells were incubated in NPC media with no growth factors for desired amount of time, with media changes every 3-4 days.

MSR293 Cell Culture. Human MSR293 cells (a derivative of HEK293 cells with superior adherence technology) were cultured as described by commercial protocols (ThermoScientific #R79507). For treatment, cells were grown in uncoated 6-well plates (Falcon #353046) with application of Geneticin at 0.8 mg/mL (ThermoScientific #10131027) upon plating.

Compound Preparation of HDAC inhibitors. All compounds were purchased from commercial vendors. Stock concentrations of all compounds were made at 1000x in DMSO (Sigma Aldrich #D2438), except for sodium valproate that was dissolved in ddH2O. Upon treatment, the stock compounds were diluted 1:1000 in NPC media.
(DMSO concentration 0.1%) and was applied to confluent MSR293 cells, NPCs or 18-day differentiated neurons for indicated time period.

_Treatment of NPCs with HDAC inhibitors._ NPCs were plated at 0.5 x 10^6 cells per well in a poly-ornithine/laminin double-coated 6-well plate, as described above, in 2 mL NPC media with above growth factors. The cells were allowed to incubate at 37°C for 48-72 hours to grow to 95-100% confluency. The media was aspirated and the cells were treated with compound in fresh NPC media for 24 hours, or otherwise indicated amount of time.

_Treatment of Neurons with HDAC inhibitors._ Human iPSC-derived neurons were generated as described above. On day 18, the media was aspirated and the cells were treated with compound in fresh NPC media for 24 hours.

_Determination of Gene Expression Changes in Human Cell Culture._ RNA was generated from each well of a 6-well treated plate of NPCs or neurons. Media was aspirated. The wells were washed with 1 mL of DPBS and then lysed with 1 mL TRIzol Reagent (ThermoFisher #11596026). The cells in TRIzol were incubated at room temperature for 5 minutes and RNA was extracted with the DirectZol RNA MiniPrep Kit (Zymo Research #R2052). RNA was stored at -80°C until ready to use. cDNA was generated using the High Capacity cDNA synthesis Kit with RNase inhibitor (ThermoFisher #4368814) with 1000-1200 ng RNA per reaction. cDNA was used
immediately or stored at -20°C until ready to use. Before use, cDNA was diluted 1:4 with DNase/RNase-free H₂O (Invitrogen #10977-015).

qPCR was conducted on the Roche 480 Light Cycler in a 384-well plate. Into each well was added 5 µL of TaqMan 2X Gene Expression Master Mix (ThermoFisher #4369510), 0.5 µL of 20X commercial TaqMan primer probe (ThermoFisher, GRN: Hs00963707_g1, FXN: Hs00175940_m1, GAPDH: Cat.#432924E), 0.5 µL of DNase/RNase-free H₂O, and 4 µL of above diluted cDNA. Results were normalized to GAPDH and replicate mean values and standard error of the mean are reported. Significance was determined with unpaired t-tests with GraphPad Prism software.

Western Blot Antibodies and Analysis. Cell pellets were collected from each well of a 6-well plate, frozen in dry ice, and stored at -80°C until ready to use. Cell pellets were lysed in radio immunoprecipitation assay (RIPA) buffer (Boston BioProducts #BP-115) with EDTA-free protease inhibitors (Sigma Aldrich #4693159001) and rocked at 4°C for 30 minutes. The lysates were centrifuged at 14,000 rcf at 4°C for 25 minutes and the supernatant was collected. Protein quantification was done with the Pierce bicinchoninic assay (BCA) (ThermoFisher #23227). Lysates were diluted to 800 ng/µL in RIPA Buffer with protease inhibitors and stored between -20 and -80°C until ready for use. Before use, lysates were boiled at 95°C with SDS loading buffer (New England BioLabs #B7703S) + DTT (New England BioLabs #B7705S) for 5 min.

In probing for PGRN and HDACs, proteins were separated on NuPAGE 4-12% Bis-Tris gels (ThermoFisher #NP0335BOX) in MOPS SDS Running buffer (ThermoFisher #NP0001). For H3K9 acetylation blots, proteins were separated on 16%
Tricine gels (ThermoFisher #EC6695BOX) in Tricine running buffer (ThermoFisher #1691442). To each well, 8 µg total protein was loaded and gels were run at 125V for 1 hour. Gels were then transferred onto 0.45 µm PVDF membranes (ThermoFisher #88518). Membranes were blocked in 5% milk in TBST for 1 hour and probed overnight at 4°C with primary antibodies in 5% BSA + 0.02% sodium azide (PGRN: Invitrogen #40-3400, 1:1000; H3K9ac: Sigma Aldrich #H9286, 1:5000; HDAC1: Abcam #ab7028, 1:10,000; HDAC2: Abcam #ab7029, 1:10,000; HDAC3: BD Transduction #611125, 1:1000; GAPDH: Abcam #ab8245, 1:10,000). Membranes were then washed with PBS, incubated with secondary antibody in TBST containing 5% milk for 1 hour. Secondary antibodies were as follows: for all proteins except HDAC3 and GAPDH: anti-rabbit-HRP, Cell Signaling #7074S, 1:2000; for HDAC3 and GAPDH: anti-mouse-HRP, Cell Signaling #7076S, 1:2000. Membranes were then washed with PBS for 1 hour, and developed with chemiluminescence reagents (Pierce ECL Western Blotting Substrate, ThermoFisher #PI32106; SuperSignal West Dura Extended Duration Substrate, ThermoFisher #PI34076).

**ELISA.** All ELISA was done with the Progranulin (human) ELISA kit (AdipoGen #AG-45A-0018YPP-KI01) at the manufacturer's instructions. Cell culture supernatant was collected from treated cells after 24 hours and incubated with protease inhibitors (Sigma Aldrich #4693159001). The collected media was spun down to remove debris and supernatant was collected and stored at -80°C until ready to use. Supernatant was diluted 1:5 in provided ELISA buffer, and protein lysates (as prepared above) were diluted 1:100 in ELISA buffer. ELISA results were collected with SpectraMax Plus 384
Microplate Reader (Molecular Devices). ELISA data was normalized to control values. Replicate mean values and standard error of the mean are reported. Significance was determined with unpaired t-tests with GraphPad Prism software.

In vitro HDAC binding assay. The in vitro HDAC binding assay was done with HDAC1, HDAC2, and HDAC3 using an endpoint assay with fluorogenic HDAC substrate MAZ1600 (gift from Dr. Ralph Mazitschek, MGH) in HDAC assay buffer (in Milli-Q water, 100 mM KCl; 50 mM HEPES, GIBCO #15630-114; 0.05% BSA, Invitrogen #P2489; 0.001% Tween-20, Zymed #00-3005). To a well of a 96-well plate was added 20 µL of 6X HDAC inhibitor stock in HDAC assay buffer (compounds purchased from commercial vendors) and 60 µL enzyme at appropriate concentration in HDAC assay buffer (HDAC1, BPS Biosciences #50051, 30 ng; HDAC2, BPS Biosciences #50052, 45 ng; HDAC3, BPS Biosciences #50003, 12 ng) and left to incubate for desired amount of time (0-3 hours). After desired incubation time, 40 µL 3X MAZ1600 in HDAC assay buffer (at 3X substrate $K_m$ per enzyme) was added to each well. The reaction was left to proceed for 40 minutes whereupon a background read was taken with an Envision Multiwell plate reader (Perkin-Elmer; excitation 355 nm, emission 460 nm). The reaction was then quenched with 150 nM Trypsin (Worthington Biochemical Corporation #LS003744) + 10 µM Panobinostat (Broad Institute) and was allowed 30 minutes to develop after which a fluorescent read was taken on the Envision Multiwell plate reader (Perkin-Elmer; excitation 355 nm, emission 460 nm).
HDAC shRNA Studies. NPCs were seeded at 50% confluency in poly-ornithine/laminin coated 12-well plastic dishes (1.6 x 10^5 cells/well). After a 24 hour incubation, the cells were infected at an MOI = 10 with lentivirus packaged with shRNAs cloned into lentiviral vector pLK0.1 (http://www.broadinstitute.org/rnaí/public/vector/details?vector=pLKO.1) targeting the following sequences: HDAC1 – CCTAATGAGCTTCCATACAAT; HDAC2 – CAGTCTCACCATTTCAGAAA; HDAC3 – CAAGAGTCTTAATGCCTTCAA; RFP (control) — CTCAGTTCCAGTACGGCTCCA; LacZ (control) – TCGTATTACAACGTCCGTGACT; luciferase (control) — ACGCTGAGTACTTCCGAAATGT; GFP (control) – ACAACAGCCAACAACGTCTATA. The cells were spun down at 2000 rpm for 30 minutes at room temperature and incubated at 37°C for 24 hours. After 24 hours, the media was changed, complete NPC media was added and the cells were incubated at 37°C for 24 hours. When the cells were 90-100% confluent, complete NPC media with puromycin (0.8 µg/mL; Sigma #P8833) was added to select for infected cells. Cells were thereafter grown and split as denoted above with puromycin in the media.
Chapter 4

Mechanisms of HDAC Inhibitor-Mediated Progranulin Enhancement in Human Neuronal Cultures
Chapter 4: Mechanisms of HDAC Inhibitor-Mediated Progranulin Enhancement in Human Neuronal Cultures

Summary

While the delineation of the selectivity and kinetic requirements of HDAC inhibitors as PGRN enhancers in human neuronal cultures is important to the development of HDAC inhibitor-based therapeutics for PGRN-deficient FTD, it is also crucial to understand the mechanisms behind HDAC inhibitor-mediated PGRN enhancement, both from an academic understanding of the ways HDAC inhibitors with different kinetics act upon epigenetic regulators in a human cellular context and from the standpoint of efforts to develop next generation epigenetic therapies for FTD. This chapter dives into potential mechanisms of HDAC inhibitor-mediated PGRN enhancement and provides evidence for elevated acetylation of specific histone lysine (H3K27ac) residues in the promoter region of the GRN locus that occur only with HDAC inhibitors that cause elevation of GRN mRNA levels. In addition, transcription factor EB (TFEB), a master regulator of lysosomal and autophagy gene expression, is implicated in the response to HDAC inhibitors in a manner correlated with elevated GRN mRNA expression. Taken together, these findings have implications for both epigenetic and potential cis-regulatory mechanisms controlling GRN expression.

4.1 Mapping of epigenetic modifications of the GRN promoter upon HDAC inhibitor treatment

To elucidate the mechanism through which small molecule inhibitors of HDACs that enhance GRN mRNA levels are effective, we sought to address the questions of
whether HDAC inhibitors directly affect the acetylation state of histones located within chromatin on the GRN promoter. Although global histone acetylation was not measurably different when NPCs were treated with fast-on or slow-binding HDAC inhibitors (Figures 3-6, 3-7, 3-9, 3-10), the resolution of specific acetylation sites associated with the promoter proximal region of the GRN locus may reveal HDAC inhibitor changes that are obscured when analyzing global histone acetylation levels by Western blotting. For this purpose, chromatin immunoprecipitation (ChIP)-qPCR assays studies were done to understand potential differential acetylation changes in the GRN promoter given treatment with fast- or slow-binding HDAC inhibitors. To guide these ChIP-qPCR studies, we focused on the promoter-enhancer region of GRN, as computationally defined by enrichment of H3K27 acetyl marks, a histone post-translational modification known to be associated with active transcription, according to the Human Epigenome Atlas data from human embryonic stem cell (H9)-derived NPCs and neurons, as well as the dorsolateral prefrontal cortex and inferior temporal brain regions (Figure 4-1).96 This promoter proximal region corresponds to transcription factor binding and histone modification sites from previously generated global ChIP-seq data from the ENCODE (Encyclopedia of DNA Elements) project, which revealed that across several non-neuronal human cell lines, the GRN locus also contains peaks exhibiting an enrichment of H3K27 acetylation marks, as well as binding sites for multiple Class I HDAC complex members and HDAC-interacting proteins (Figure 4-1).56, 97 On the basis of these epigenomic maps, we selected three regions of the GRN locus with predicted dynamic changes in H3K27 acetylation to query whether they played a regulatory role in controlling active transcription (Figure 4-1).
Figure 4-1: ChIP-qPCR of H3K27 acetylation on GRN promoter/enhancer region. A) Visualization of the promoter/enhancer region of GRN in two human brain regions and human embryonic stem cell H9-derived NPCs and neurons, with measured H3K27 acetylation marks in the brain regions and imputed H3K27 acetylation in the H9-derived cell lines as compiled by the WashU EpiGenome Browser. 96 TSS = transcriptional start site. B) Promoter/enhancer region of GRN, showing H3K27 acetylation and binding sites for selected ENCODE Transcription Factors for up to 7 cell lines (indicated above). Image was modified from UCSC Genome Browser and shown to scale with (A). 96 H3K27Ac data is layered, so some colors may not be reflected in the legend and more information may be found on the UCSC Genome Browser. Continued on pg. 58.
4-1: ChIP-qPCR of H3K27 acetylation on GRN promoter/enhancer region, continued from pg. 57.

**B)** Transcription factors were selected based on binding affinity for HDAC complexes or known HDAC-modulated genes. Transcription factors are color coded in grayscale where the darkness of the box is proportional to maximum ChIP data value seen in any cell line in the region. The ChIP regions denote regions of interest for ChIP-qPCR. **C)** H3K27Ac ChIP-qPCR data in NPCs for the regions on the GRN promoter denoted in (A and B). Cells were treated with vehicle (DMSO), panobinostat (0.5 µM), or CI-994 (10 µM) for 24 hours whereupon cells were collected for ChIP-qPCR studies. Panobinostat increases H3K27Ac in regions 1 and 3, compared to vehicle, while CI-994 increases H3K27ac in region 3 compared to vehicle. Each condition is shown as the average of 3 technical replicates, with significance calculated by unpaired t-test. **p< 0.01 *** p < 0.001

These ChIP-qPCR assays revealed that in NPCs, treatment with panobinostat or CI-994 differentially affected H3K27 acetylation within chromatin in the GRN promoter (Figure 4-1). Specifically, panobinostat significantly increased H3K27 acetylation in “ChIP Region 1” on the GRN promoter while CI-994 did not. Neither compound treatment increased H3K27 acetylation in “ChIP Region 2,” which is consistent with the WashU EpiGenome data that reports minimal acetylation marks in this region of the characterized brain sections.96 Finally, both compounds increased H3K27 acetylation in “ChIP Region 3,” to varying levels. Taken together, these data suggest that the increased H3K27 acetylation in “ChIP Region 1” may play a significant role in GRN expression in human neuronal cells. While other genomic loci may be similarly differentially affected by HDAC inhibitors that elevate GRN mRNA levels, these data provide the first demonstration of a region of chromatin in the GRN locus in the human genome that responds to HDAC inhibition in a manner correlated with GRN mRNA induction. This provides a potential mechanistic explanation for why slow-binding HDAC inhibitors like CI-994 are ineffective at regulating GRN mRNA—they are unable to regulate the acetylation state of histones within key regulatory regions of the GRN gene promoter. This may be because the kinetics of chromatin and protein complex rearrangement at specific sites on the GRN promoter are incompatible with the binding
kinetics of a slow-binding HDAC inhibitor. That is, the chromatin is rearranging faster than a slow-binding HDAC inhibitor like CI-994 can affect HDAC binding to the chromatin and its activity. Future experiments with compounds with fast-on/slow-off kinetics would contribute to this hypothesis.

Thus, the post-translational state of chromatin in “ChIP Region 1” may predict whether an HDAC inhibitor will be effective at increasing \( GRN \) mRNA levels irrespective of the effect of the inhibitor on global histone acetylation. Since the H3K27 acetylation patterns in this region differ even in different human cell types analyzed in the ENCODE genome wide ChIP studies\(^97\) (Figure 4-1), these results stress the importance of understanding species-, cell type-, and gene-specific small molecule-mediated changes when trying to understand the role of epigenetic mechanisms in controlling \( GRN \) gene expression. Further studies on the effects of different HDAC inhibitors on HDAC corepressor complex binding on the \( GRN \) promoter (i.e. Sin3A, CoREST) could also reveal other differential effects of HDAC inhibitor treatment, leading to insight on the mechanism behind HDAC inhibitor-mediated PGRN enhancement.

### 4.2 The role of TFEB in HDAC-mediated \( GRN \) regulation in human neuronal cells

Besides directly affecting histone acetylation, HDACs are also known to epigenetically regulate many transcription factors, and it is possible that different classes of HDAC inhibitors with different binding kinetics differentially affect key transcription factors involved in the \( cis \)-regulatory mechanisms governing transcription at the \( GRN \) locus. For instance, the transcription factor EB (TFEB), a member of the MiT/TFE subfamily of basic helix–loop–helix (bHLH) leucine zipper transcription factors
and master regulator of autophagy-lysosomal gene expression, is implicated in \textit{GRN} expression through its specific recognition and binding to multiple existing enhancer box (E-box) consensus sequences (5'-CANNTG-3') in the \textit{GRN} promoter region.\textsuperscript{98-101} On the \textit{GRN} promoter/enhancer regions, denoted by ENCODE ChIP-verified transcription factor binding sites and regions of H3K27 acetylation, computational analysis revealed 45 E-box sequences to which TFEB can potentially bind, a proportion of which overlap with other known regions of transcription factor binding (\textit{Figure 4-2}). It must be noted, however, that the short E-box sequences, specific DNA response elements that recruit transcription factors, are common in the genome to begin with, even in areas where there is no known transcription factor binding. In addition, as a master regulator of lysosomal gene expression, TFEB has been linked to the expression of members of the computationally-derived CLEAR (Coordinated Lysosomal Expression and Regulation) network, and is thought to bind to either a full CLEAR sequence (5'-TCACGTGA-3') or a partial CLEAR motif (5'-TCACG-3'). Although the promoter region of human \textit{GRN} does not contain any full CLEAR sequences, computational analysis revealed there are three partial CLEAR motifs to which TFEB may bind, two of which occur within DNA regions known to recruit other transcription factors (\textit{Figure 4-2}).
Figure 4-2: ChIP-qPCR of TFEB on the GRN promoter/enhancer region. A) Potential TFEB binding sites on the GRN promoter/enhancer region shown relative to H3K27 acetylation in 7 cell lines from the ENCODE project and the UCSC Genome Browser. H3K27Ac data is layered, so some colors may not be reflected in the legend and more information may be found on the UCSC Genome Browser. E-box sites (5'-CANNTG') are shown with blue lines, while CLEAR motifs (5'-TCACG-3') are shown in orange. These sites are overlaid with the ENCODE transcription factor binding regions (yellow boxes). Portions of the genome where both E-box sites and CLEAR motifs occur near known transcription factor binding sites are shown, with the start site of GRN 5' UTR marked in bold, highlighted in purple. B) TFEB ChIP-qPCR data in NPCs for the regions on the GRN promoter denoted in (A). Cells were treated with vehicle (DMSO), panobinostat (0.5 µM), or CI-994 (10 µM) for 24 hours whereupon cells were collected for ChIP-qPCR studies. Panobinostat significantly increases TFEB occupancy, compared to vehicle. Each condition is shown as the average of 2 replicates, with significance calculated by unpaired t-test. * p< 0.05 *** p < 0.001
As TFEB has been implicated in GRN expression and there are potential binding sites for TFEB on the GRN promoter, it is possible that HDAC inhibitor modulation of TFEB may contribute mechanistically to the enhancement of GRN/PGRN expression in human neuronal cultures. TFEB overexpression has been shown to be sufficient to enhance GRN mRNA and PGRN protein levels in human cells, and we hypothesized that HDAC inhibitors that affect GRN/PGRN expression would have a different effect on TFEB levels compared to those HDAC inhibitors that do not. In support of this hypothesis, we observed that HDAC inhibitors overall increase TFEB protein in human NPCs, and that the level of this enhancement in TFEB levels correlated with an increase in PGRN in a dose-dependent manner (Figure 4-3), though phosphorylated-TFEB (pTFEB), the inactive, cytoplasmic-localized form of TFEB, remained constant. HDAC inhibitors that surpassed a ‘threshold’ of ~10-fold induction of TFEB protein levels were capable of enhancing PGRN levels (e.g. panobinostat), whereas HDAC inhibitors below this level (e.g. the slow-binder CI-994) were not (Figure 4-3). In addition, TFEB ChIP-qPCR studies showed that upregulation of GRN/PGRN correlate with increased TFEB occupancy on the GRN promoter/enhancer region (Figure 4-2). This is the first time that TFEB has been shown definitively to bind to the GRN promoter in a human neuronal context and suggests that in addition to differential histone acetylation marks at key positions on the chromatin, differential TFEB induction by different classes of HDAC inhibitors may also contribute to the difference between different HDAC inhibitor chemotypes on GRN expression through its cis-regulatory functions.
Figure 4-3: TFEB vs. PGRN protein expression in NPCs upon HDAC inhibitor treatment. A) TFEB, pTFEB(Ser142), and PGRN protein levels in NPCs treated with HDAC inhibitors. B) TFEB protein enhancement is correlated with PGRN protein enhancement in a dose-dependent manner, with the graph showing the best-fit curve with 95% confidence interval (dotted line). Phospho-TFEB (pTFEB) levels were constant. NPCs were treated with vehicle (ddH2O for valproate, DMSO for all others), SAHA (10 µM), panobinostat (0.5 µM), crebinostat (2.5 µM), ACY-1215 (5 µM), Tubastatin A (10 µM), PCI-34051 (10 µM), apicidin (2.5 µM), CI-994 (10 µM), Cpd-60 (5 µM), or valproate (5 mM) for 24 hours whereupon cells were collected. Representative Western blots are shown for each condition. Quantification was done with ImageJ and results are shown as means relative to GAPDH and normalized to vehicle.
It must be noted, however, that TFEB occupancy on the GRN promoter is still only correlative to GRN/PGRN upregulation and may be due to effective overexpression of TFEB in the cell. The use of CRISPR-Cas9 genome editing technologies will afford the opportunity to knockout TFEB to allow determination of whether its presence is necessary for HDAC inhibitor induced PGRN expression. These experiments will require careful consideration of the potential role for compensation by other TFEB family members that by upregulated in the absence of TFEB as well as other compensatory changes that may occur by deleting a critical regulator of lysosomal biogenesis.

4.3 The role of the P-TEFb complex in HDAC-inhibitor mediated GRN expression in human neuronal cells

Having generated evidence for differential post-translational modification of histones in the GRN promoter, as well as differential induction of TFEB as a potential cis-regulatory factor, as a result of treatment of human neuronal cells with fast-on/off versus slow-on/off HDAC inhibitors, we sought to determine if the mechanistic basis for these two types of HDAC inhibitors might also involve differential regulation on the level of transcription elongation of GRN. It has been observed that treatment with some HDAC inhibitors, including SAHA, prompts the release of positive transcription elongation factor b (P-TEFb) – composed of CDK9/CyclinT – from its inhibitory complex resulting in changes in the levels of phosphorylated CDK9\textsuperscript{102}. Once phosphorylated and part of the freed P-TEFb complex, pCDK9 becomes capable of phosphorylating RNA Polymerase II (RNA Pol II), releasing it from its promoter proximal paused state and allowing transcriptional elongation to occur.
We found, however, that in the case of human NPCs, treatment with fast-on/off HDAC inhibitors did not differentially enhance pCDK9 levels (Figure 4-4). Instead, we observed that CI-994 significantly decreased the level of the 55 kDa isoform of pCDK9 (pCKD9-55). As we have shown that CI-994 subtly, but statistically significantly, decreases PGRN expression (Figures 3-9, 3-11), the correlative decrease in pCDK9-55 levels suggests that maintaining pCDK9-55 levels may be necessary for HDAC inhibitors to enhance GRN transcription levels. It must be noted, however, that pCDK9 levels may not be the optimal measure to quantify P-TEFb activity and further experiments correlating RNA Pol II occupancy and activity state on the GRN gene are necessary to more precisely understand the role of P-TEFb-mediated control of transcriptional elongation at the GRN promoter.
**Figure 4-4: pCDK9 vs. PGRN protein expression in NPCs upon HDAC inhibitor treatment.**

**A)** pCDK9(Thr186), and PGRN protein levels in NPCs when treated with HDAC inhibitors, with CDK9 shown as a baseline of the protein; PGRN and GAPDH repeated from *Figure 4-3* as reference.

**B)** CI-994 decreases the 55 kD isoform of phospho-CDK9 (pCDK9-55). NPCs were treated with vehicle (ddH2O for valproate, DMSO for all others), SAHA (10 µM), panobinostat (0.5 µM), rebinostat (2.5 µM), ACY-1215 (5 µM), Tubastatin A (10 µM), PCI-34051 (10 µM), apicidin (2.5 µM), CI-994 (10 µM), Cpd-60 (5 µM), or valproate (5 mM) for 24 hours whereupon cells were collected. Representative Western blots are shown for each condition. Quantification was done with ImageJ and results are shown as means relative to GAPDH and normalized to vehicle.
4.4 Conclusions

In light of our observations regarding the effectiveness of various types of HDAC inhibitors, it is of interest to gain deeper insight into the mechanistic basis behind HDAC inhibitor-mediated GRN/PGRN enhancement and specifically to understand why, in human iPSC-derived neuronal cultures, fast-on/off binding Class I HDAC inhibitors preferentially increase GRN/PGRN expression as compared to compounds with slow-on/off kinetics. Targeted ChIP studies of H3K27 acetylation within chromatin spanning the GRN promoter proximal region of the genome revealed differential H3K27 acetylation enhancement on a specific region of the GRN promoter when treated with a fast-on/off vs. slow-on/off binding HDAC inhibitor. Importantly, these studies revealed that treating with the fast-binding HDAC inhibitor panobinostat increased H3K27 acetylation in the GRN promoter proximal region, while the slow-on/off binder CI-994 did not (Figure 4-1). In terms of mechanism, we now link observed changes in GRN mRNA induction to changes in specific histone acetylation sites located within the promoter proximal region of GRN locus.

Second, in addition to understanding direct HDAC activity on the GRN promoter proximal region, our mechanistic studies comparing fast-on/off vs. slow-on/off binding HDAC inhibitors revealed that enhancement of protein levels of TFEB, a known master regulator of lysosomal and autophagy gene expression, including that of GRN itself, were correlated with PGRN enhancement in human neuronal cell cultures (Figure 4-3). TFEB occupancy on the GRN promoter region, determined by targeted ChIP-qPCR studies on the GRN promoter region, was also correlated with GRN/PGRN
expression, suggesting a cis-regulatory role for TFEB in GRN expression and another mechanistic layer to HDAC inhibitor-mediated progranulin enhancement.

Finally, as it has been shown that HDAC inhibitors may also affect transcriptional elongation via the release of P-TEFb and subsequent RNA Pol II activation, we sought to determine if the regulation of P-TEFb complex activity may underlie the differential effect of fast-on/off binding Class I HDAC inhibitors as compared to compounds with slow-on/off kinetics. Although we did not find an enhancement of P-TEFb via measurement of pCDK9 in NPCs treated with HDAC inhibitors, there was some indication that the P-TEFb complex is necessary for GRN expression given that the 55 kD isoform of pCDK9, pCDK9-55, is significantly decreased upon treatment with slow-binding HDAC inhibitor, CI-994 (Figure 4-4).

Taken together, these data provide evidence for multiple mechanisms underlying the differential regulation of GRN/PGRN levels in response to treatment with various chemotypes of Zn\textsuperscript{2+}-dependent HDAC inhibitors.

4.5 Materials and Methods

*Human iPSC-Derived NPC Culture.* All NPCs used were differentiated from iPSCs previously reprogrammed from human fibroblast line GM08330 (Coriell Institute for Medical Research, Camden, NJ) and cultured described in Chapter 3.11.

*Compound Preparation and Treatment with HDAC Inhibitors.* All compounds were purchased from commercial vendors. Stock concentrations of compounds were made at 1000x in DMSO (Sigma Aldrich #D2438). For treatment, the stock compounds were
diluted 1:1000 in NPC media (DMSO concentration 0.1%) and was applied to confluent NPCs for 24 hours.

**Chromatin immunoprecipitation – quantitative PCR studies.** NPCs were grown in 15-cm dishes and treated with vehicle (DMSO), panobinostat (0.5 µM), or CI-994 (10 µM) for 24 hours. For Ac-H3K27 studies, 4 x 10^7 cells were used per immunoprecipitation. Cells were fixed with NPC media containing 1% formaldehyde (Tousimis #1008A) for 10 minutes at room temperature and quenched with glycine at a final concentration of 125 mM. Cells were collected, lysed in ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH8 in ddH_2O), and sonicated with EpiShear Probe Sonicator for 12 cycles of 20 seconds each at 50% amplitude. The sonicate was spun down and the supernatant was diluted 1:10 in ChIP Dilution Buffer (0.01% SDS, 0.275% TritonX-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH8, 0.167 M NaCl in ddH_2O) and incubated with anti-H3K27Ac antibody (Abcam #ab4729) or Normal Rabbit IgG antibody (Cell Signaling Technology #2729) at a final concentration of 3 µg/mL overnight at 4°C. 140 µL of Dynabeads Protein A (ThermoFisher #10001D) were washed twice with 280 µL ChIP dilution buffer and resuspended in 140 µL ChIP dilution buffer. The beads were then added to the antibody-lysate mixture and incubated for 1 hour at 4°C, after which the beads were collected and washed for 20 minute intervals at 4°C, twice with 1 mL Low Salt Buffer (0.1% SDS, 1% TritonX-100, 2 mM EDTA, 20 mM Tris-HCl pH8, 150 mM NaCl in ddH_2O), twice with 1 mL High Salt Buffer (0.1% SDS, 1% TritonX-100, 2 mM EDTA, 20 mM Tris-HCl pH8, 500 mM NaCl in ddH_2O), twice with 1 mL Lithium Chloride Buffer (250 mM LiCl, 10 mM Tris-HCl pH8, 1 mM EDTA, 1% deoxycholate, 1% Igepal...
CA-630 in ddH₂O), and twice with 1 mL 1X TE Buffer pH8. The DNA-protein complex was eluted from the beads at 65°C using ChIP Elution Buffer (1% SDS, 150 mM NaCl in 1X TE Buffer pH8) with 1:200 1.25 M DTT added just before use. This elution was performed twice with 125 µL of ChIP Elution Buffer. The eluates were combined and underwent reverse crosslinking at 65°C overnight. The DNA was then purified using the MinElute reaction cleanup kit (Qiagen #28206), and quantified using Qubit 3.0 fluorometer. qPCR was done directly with the eluted DNA with Taqman primer-probe sets as follows: ChIP Region 1: Forward primer: AGGATAGAAAGGCGAGCACA, Reverse primer: CACCCCATTTCTAGGGATCA, Probe: TTCATAACACTCCCTCGCACT; ChIP Region 2: Forward primer: CCACCCCACTGAAGCTAGCTG, Reverse primer: GCCCTTGCCTCTCCATCTAT, Probe: TAGCTGAGCCTGGAGAAGA; ChIP Region 3: Forward primer: CTCACGTTTGTGCTCTCCTTCC, Reverse primer: CCACAGAGCCCTGTAAGGT, Probe: TGGTTCTACCTGCTGTGAGCT. qPCR was conducted on the Roche 480 Light Cycler in a 384-well plate with a standard Taqman protocol. To a well of a 384-well plate was added 5 µL of TaqMan 2X Gene Expression Master Mix (ThermoFisher #4369510), 0.18 µL of 50 µM forward primer, 0.18 µL of 50 µM reverse primer, 0.25 µL of 10 µM probe, 2.39 µL DNase/Rnase-free H₂O, and 2 µL eluted DNA. Results were normalized to input DNA used per qPCR reaction and statistical significance was determined with unpaired t-tests with GraphPad Prism software.

For TFEB ChIP studies, NPCs were plated, treated, fixed, and collected as above and the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology #9003) was
used to conduct chromatin immunoprecipitations on 4x10^5 cells per immunoprecipitation according to manufacturer’s instructions. Antibodies used were as follows: anti-TFEB (Cell Signaling Technology #37785), Normal Rabbit IgG (Cell Signaling Technology #2729), positive control Histone H3, data not shown (Cell Signaling Technology #4620). qPCR was done with eluted DNA with Taqman primer-probe sets as follows: ChIP Region 1: Forward primer: AGGATAGAAAGGCAGCACA, Reverse primer: CACCCCCATTCTAGGGATCA, Probe: TTCATAACTCCCTCGCCTACCT; ChIP Region 2: Forward primer: CCACCCACTGACTAGCTG, Reverse primer: GCCCTTGCTCTCCATCTAT, Probe: TAGCTGAGCCTGGGAGAAGA. qPCR was conducted on the Roche 480 Light Cycler in a 384-well plate as above. Results were obtained as percent of input DNA and normalized to vehicle. Significance was determined with unpaired t-tests with GraphPad Prism software.

**Western Blot Antibodies and Analysis.** Cell pellets were collected from each well of a 6-well plate, frozen in dry ice, and stored at -80°C until ready to use. Cell pellets were lysed in radio immunoprecipitation assay (RIPA) buffer (Boston BioProducts #BP-115) with EDTA-free protease inhibitors (Sigma Aldrich #4693159001) and a phosphatase inhibitor cocktail (Sigma Aldrich #P5726) and prepared as described in Chapter 3.11. Lysates were diluted to 800 ng/µL in RIPA Buffer with protease and phosphatase inhibitors and stored between -20°C and -80°C until ready for use. Before use, lysates were boiled at 95°C with SDS loading buffer (New England BioLabs #B7703S) + DTT (New England BioLabs #B7705S) for 5 min.
Proteins were separated on NuPAGE 4-12% Bis-Tris gels (ThermoFisher #NP0335BOX) in MOPS SDS Running buffer (ThermoFisher #NP0001) and Western blots were performed as in Chapter 3.11. Primary antibodies used were as follows: PGRN: Invitrogen #40-3400, 1:1000; TFEB: Bethyl #A303-673M, 1:1000; pTFEB: ThermoFisher #ABE1971MI, 1:5000; CDK9: Santa Cruz #SC-8338, 1:1000; pCDK9: Cell Signaling #2549S, GAPDH: Abcam #ab8245, 1:10,000. Secondary antibodies used were as follows: for all proteins except GAPDH: anti-rabbit-HRP, Cell Signaling #7074S, 1:2000; for GAPDH: anti-mouse-HRP, Cell Signaling #7076S, 1:2000. Membranes were developed with chemiluminescence reagents (Pierce ECL Western Blotting Substrate, ThermoFisher #PI32106; SuperSignal West Dura Extended Duration Substrate, ThermoFisher #PI34076). Western blots were quantified with ImageJ.
Chapter 5

BET Bromodomain Inhibitors Reveal a Novel Target for Developing an Epigenetic Therapy for PGRN-Deficient FTD
Chapter 5: BET Bromodomain Inhibitors Reveal a Novel Target for Developing an Epigenetic Therapy for PGRN-Deficient FTD

Summary

While HDAC inhibitors encompass a set of well-validated probes and several FDA-approved drugs used in the clinic, it is known that HDAC inhibitors like SAHA affect the expression of a significant proportion of the genome and that clinical doses are often limited by toxicity. In neurodegenerative disease, where a patient may be exposed to a drug for years either as a treatment after onset or as a prophylactic measure, large-scale genome changes are less desirable and the safety profile of the treatment is of paramount importance. As such, the search for new epigenetic targets that affect PGRN enhancement is important and may affect future drug development directions for PGRN-deficient FTD and other neurodegenerative diseases. In this chapter, we provide evidence for a novel class of PGRN modulators: bromodomain and extra-terminal domain (BET) inhibitors, which have been shown to have less cellular toxicity in neuronal cells and overall affect expression of many fewer genes than HDAC inhibitors.

5.1 A directed mRNA screen for GRN regulators reveals pan-BET inhibitors enhance GRN expression

In the previously mentioned targeted GRN mRNA screen (Chapter 3.1), we found that in addition to HDAC inhibitors, a number of non-HDAC inhibitor small molecule epigenetic regulators also upregulated GRN mRNA expression compared to
known Wnt modulators (Figure 3-2). These compounds were identified to be BET (bromodomain and extra-terminal domain) family inhibitors and robustly increased GRN mRNA expression in human NPCs (Figure 5-1). As bromodomain inhibitors had not previously been identified as GRN modulators in any system, and the GRN mRNA upregulation was consistent across multiple structurally different bromodomain inhibitors, we elected to further validate these findings.

Figure 5-1: BET inhibitors identified to enhance GRN mRNA levels in NPCs. In the targeted small molecule screen described in Figure 3-2, BET inhibitors bromosporine, PFI-1, and I-BET151 were found to upregulate GRN expression compared to vehicle. All graphs are shown as mean + SD.

5.2 Bromodomains and bromodomain inhibitors

Acetylated lysines on histone tails have long been linked to active gene transcription due to their association with decondensed chromatin and contributions to
the maintenance of the unfolded state of the DNA around the gene of interest. This lysine acetylation is highly regulated by epigenetic “writers,” such as histone acetyltransferases (HATs) that acetylate the lysines, and epigenetic “erasers,” such as histone deacetylases (HDACs), which remove the acetyl groups (Figure 5-2).\textsuperscript{104} Besides changing the chromatin architecture around a particular gene, lysine acetylation can also recruit epigenetic “readers” with acetyl-lysine binding motifs, such as bromodomains, that target chromatin-modifying enzymes to specific sites, further preparing the gene and its promoter region for transcription (Figure 5-2).\textsuperscript{104}

Figure 5-2: Classes of epigenetic regulators. Chromatin is dynamically regulated by epigenetic writers, readers, and erasers, which add, bind to, and remove post-translational modifications respectively. Based on these epigenetic marks, chromatin condenses and decondenses, affecting gene transcription. Ac = acetyl, Me = methyl, P = phosphate
The bromodomain label, so called from the first discovery of the structural motif in relation to the *Brahma* gene in drosophila, refers to an acetyl-lysine binding module contained within a conserved left-handed bundle of four α-helices linked by variable loop regions. The binding pocket contains a conserved asparagine residue which hydrogen bonds to acetylated lysines on histones and other proteins. Thus far, in the human genome, 61 bromodomains, which have been found in 46 different proteins including HATs, histone methyltransferases, transcriptional coactivators and mediators, and other proteins associated with chromatin remodeling, have been identified.

The BET family of bromodomain-containing proteins (BRD2, BRD3, BRD4, and BRDT), is a group of proteins involved in gene transcription and cellular differentiation that has been identified as targets for cancer and diseases of the central nervous system. BET proteins contain two tandem bromodomains (BDI and BDII) and an extra-terminal (ET) domain that recruits specific regulatory effector proteins to the chromatin. BRD2, a known nuclear Ser/Thr kinase that promotes cell cycle progression, has been linked with cell growth and neuronal and adipose tissue differentiation. BRD3 is thought to associate with GATA1, a transcription factor involved in erythroid, megakaryocyte, and mast cell lineage gene activation in hematopoietic cells. BRD4 is known to interact with the positive transcription elongation factor b (P-TEFb), a complex consisting of Cdk9 and CyclinT1, and promotes the transition of RNA Polymerase II (RNA Pol II) from a promoter-proximal paused state into productive elongation state in part through phosphorylation of the C-terminal tail of RNA Pol II by CDK9/CyclinT1. Although BET family members have been found to have specific functions in the cell, BRD2, BRD3, and BRD4 can also more generally act
as nucleosome chaperones to assist in transcriptional elongation by facilitating RNA Pol II movement along hyperacetylated regions of chromatin. Finally BRDT is found exclusively in the ovaries and testes and is involved in spermatogenesis.

Small molecule bromodomain inhibitors generally operate via two mechanisms: 1) non-acetylated lysine mimetics, which target the bromodomain binding pocket without engaging the asparagine residue, instead sterically excluding hyperacetylated peptide binding, and 2) acetylated lysine mimetics which competitively form hydrogen bonds directly with the conserved asparagine. BET-specific inhibitors encompass a variety of chemical scaffolds but are generally acetyl-lysine mimetics that cause the bromodomain-containing protein to dissociate from the chromatin. Due to their effectiveness in inhibiting the transcription of cancer-related genes, such as MYC, their relatively low toxicity in cell-based assays, their ability to cross the blood brain barrier, and their well-established medicinal chemistry scaffolds, bromodomain inhibitors have garnered much interest for clinical use. To date, several clinical trials using bromodomain inhibitors have been initiated in the cancer, cardiology, and diabetes fields, with results forthcoming.

5.3 BET inhibitors enhance PGRN expression in human iPSC-derived neuronal cells

Given the results of our directed small molecule GRN mRNA screen (Figure 5-1), we probed the effects of several classes of BET inhibitors on all levels of progranulin expression. First, we found that pan-bromodomain inhibitor bromosporine.
significantly enhanced GRN mRNA and PGRN protein levels in human iPSC-derived NPCs and differentiated neurons (Figure 5-3).

**Figure 5-3:** Pan-bromodomain inhibitor bromosporine increases GRN mRNA and PGRN protein expression in human iPSC-derived NPCs and neurons. **A)** Chemical structure for pan-bromodomain inhibitor, (13) bromosporine **B)** Bromosporine significantly increases GRN mRNA and PGRN protein expression human NPCs (black) and 18-day neurons (gray). Bromosporine was also found to increase secreted PGRN in NPCs. Cells were treated for 24 hours with vehicle (DMSO) or bromosporine (2.5 µM). mRNA quantification was done relative to housekeeping gene GAPDH with n=3 biological replicates x 3 technical replicates. Representative Western blot shown. Protein quantification is shown relative to vehicle, with 3 replicates and was measured by ImageJ. All graphs are shown as mean ± SEM, with significance relative to vehicle calculated by unpaired t-test. * p < 0.05, ** p < 0.01, **** p < 0.0001
Although bromosporine promiscuously inhibits bromodomain modules in different bromodomain-containing proteins, our directed GRN screen also indicated that inhibition of the BET family of proteins was sufficient to enhance GRN mRNA expression. To validate these results, we tested other well-studied BET family specific inhibitors: PFI-1, a quinazoline derivative, (+)-JQ1, a thienotriazolodiazepine, and I-BET151, an isoxazole-based BET inhibitor. Each of these compounds significantly increased GRN mRNA and PGRN protein expression in NPCs and neurons (Figure 5-4), while inactive enantiomer (-)-JQ1 does not (Figure 5-5). In addition, both (+)-JQ1 and I-BET151 increased GRN mRNA and PGRN intracellular and secreted protein expression in a time-dependent manner, with steady-state enhancement between 12 and 24 hours (Figure 5-6). In all treatment with BET inhibitors, there was no noticeable cell toxicity at efficacious doses.

Members of the BET protein family have two tandem bromodomains that are thought to recognize specific patterns of multiple acetylated lysines. We sought to understand whether inhibiting one or more of these bromodomains was sufficient to enhance PGRN expression. RVX-208 (apabetalone), an orally bioavailable quinazolone that specifically inhibits BDII in BET proteins,\textsuperscript{110} was found to significantly increase GRN mRNA and PGRN protein levels in NPCs and neurons, albeit at a higher concentration than BET inhibitors which inhibited both BDs (Figure 5-6). This is consistent with the literature, which cites that at cellular concentration shown to affect BET protein binding to chromatin, RVX-208 is a weaker transcription regulator than (+)-JQ1.\textsuperscript{111} Gene transcription that is strongly up- or down-regulated by (+)-JQ1 is only weakly up- or
down-regulated by RVX-208,\textsuperscript{111} implying while inhibiting BDII in a BET protein may be sufficient for upregulating GRN, it may not be the most efficient method to do so.

**Figure 5-4:** Pan-BET inhibitors increase GRN mRNA and PGRN protein expression in human iPSC-derived NPCs and neurons. A) Chemical structures for BET inhibitors, (14) PFI-1, (15) (+)-JQ1, and (16) I-BET151. B) BET inhibitors significantly increase GRN mRNA and PGRN protein expression human NPCs (black) and 18-day neurons (gray). They were also found to increase secreted PGRN in NPCs. Cells were treated for 24 hours with vehicle (DMSO), PFI-1 (10 µM), (+)-JQ1 (1 µM), or I-BET151 (2.5 µM). mRNA quantification was done relative to housekeeping gene GAPDH with n = 3 biological replicates x 3 technical replicates. Representative Western blot shown, with lines denoting cutting of gel. Protein quantification was measured by ImageJ, with n=2 biological x 2 technical replicates. All graphs are shown as mean + SEM, with significance relative to vehicle calculated by unpaired t-test. * p < 0.05, ** p < 0.01, **** p < 0.0001
Figure 5-5: Inactive enantiomer (-)-JQ1 does not increase PGRN expression in NPCs. **A** Chemical structure for the inactive JQ1 enantiomer, (17) (-)-JQ1. **B** (-)-JQ1 does not increase GRN mRNA and PGRN protein expression human NPCs compared to vehicle. Cells were treated for 24 hours with vehicle (DMSO), (+)-JQ1 (1 µM), or (-)-JQ1 (1 µM). mRNA quantification was done relative to housekeeping gene GAPDH with n = 3 biological replicates x 3 technical replicates. Representative Western blot shown, with lines denoting cutting of gel. Graphs are shown as mean ± SEM, with significance relative to vehicle calculated by unpaired t-test. *** p < 0.001

Figure 5-6: GRN mRNA and PGRN protein time course for NPCs treated with BET inhibitors. **A** GRN mRNA and PGRN intracellular and secreted protein levels in NPCs treated with (+)-JQ1 or I-BET151 for up to 24 hours compared to vehicle. Maximum enhancement seems to occur between 12 and 24 hours. Graphs show means. **B** Western blot of PGRN protein expression in NPCs treated with (+)-JQ1 or I-BET151 over time. Cells were treated for up to 24 hours with vehicle (DMSO), (+)-JQ1 (1 µM), or I-BET151 (2.5 µM). Representative Western blot shown, with lines denoting different gels.
Figure 5-7: RVX-208 increases GRN mRNA and PGRN protein expression in human iPSC-derived NPCs and neurons. A) Chemical structure for BET BDII-selective inhibitor, (18) RVX-208 B) RVX-208 significantly increases GRN mRNA and PGRN protein expression in human NPCs (black) and 18-day neurons (gray). RVX-208 was also found to increase secreted PGRN in NPCs. Cells were treated for 24 hours with vehicle (DMSO) or RVX-208 (25 µM). mRNA quantification was done relative to housekeeping gene GAPDH with n=3 biological replicates x 3 technical replicates. Representative Western blots shown. Protein quantification is shown relative to vehicle, with 2 replicates and was measured by ImageJ. All graphs are shown as mean ± SEM, with significance relative to vehicle calculated by unpaired t-test. ** p < 0.01, **** p < 0.0001.
5.4 Correlation between $GRN$ mRNA and PGRN protein level in human NPCs treated with BET inhibitors

Similar to treatment with HDAC inhibitors (Chapter 3.8), we found that increases in $GRN$ mRNA levels were highly positively correlated with increases in both PGRN intracellular and secreted protein levels in BET inhibitor-treated NPCs (Figure 5-8). Increases in $GRN$ mRNA were also positively correlated with PGRN protein levels in post-mitotic neurons. These data imply that BET inhibitors, like HDAC inhibitors, have conserved mechanism of epigenetic regulation in different neuronal cell types.

Figure 5-8: Correlation between effects of BET inhibitors on $GRN$ mRNA expression, PGRN intracellular protein expression, and PGRN secreted protein in A) NPCs and B) 18-day differentiated neurons, with representative compound I-BET151. Data shows a positive correlation between mRNA expression, protein expression, and protein secretion, with correlation coefficient, $r$, and p-value enumerated above.
5.5 Comparison of BET bromodomain induced degradation (Degronimid) technology to conventional small molecule BET bromodomain inhibitors

The fact that BET inhibitors with five unique chemical scaffolds were all able to enhance GRN mRNA and PGRN intracellular and secreted protein expression in human neuronal cell cultures strongly implies that BET proteins are suitable epigenetic targets for PGRN enhancement in FTD. However, the members of the BET family that are necessary and sufficient for GRN regulation and the mechanism by which they do so are still open questions.

To address whether inhibition of one or more BET proteins is sufficient to upregulate PGRN, we first attempted, in NPCs, a pilot of the Degronimid platform technology, which utilizes small molecules to rapidly facilitate targeted protein degradation and clearance, to knock down BET proteins. The compound used here, dBET1, consists of (+)-JQ1 – which binds to BET proteins – conjugated to Phthalimide, which induces protein degradation through recruitment of cereblon, a component in the E3 ubiquitin ligase complex, and is found to specifically target BET proteins for ubiquitin-dependent degradation. When we treated NPCs with dBET1, we found that dBET1 reduced BRD2 by around 50% and almost completely eliminated BRD3 and BRD4 (Figure 5-9). Compared to vehicle, reducing the BET proteins did not significantly increase GRN mRNA expression, but did slightly increase PGRN protein expression (Figure 5-9). While further optimization must be done with this chemical degradation system, these initial studies indicate that reducing one or more of the BET proteins has a divergent effect from conventional BET inhibitor treatment at the level of mRNA but may still have a positive effect on PGRN protein expression. As dBET1 does not seem
to be specific for any one BET protein in NPCs, it may be necessary to knock down singular BET proteins via siRNA or CRISPR to confirm whether absence of any one BET protein, and its associated protein-specific epigenetic roles, can enhance PGRN expression. In addition, because dBET1 and BET inhibitors, such as I-BET151, competitively bind to BET proteins, it may be more challenging with Degronimid technology to assess whether the effect of BET inhibitors will be attenuated given degradation of the target protein in a combination experiment.

Figure 5-9: dBET1 effect on PGRN expression in NPCs. A) NPCs treated with dBET1 show lowered protein levels of BRD2, 3, and 4. B) dBET1 did not significantly increase GRN mRNA, but did slightly increase PGRN protein expression in NPCs. Cells were treated for 12 hours with vehicle (DMSO), I-BET151 (2.5 µM), or dBET1 (300 nM). mRNA quantification was done relative to housekeeping gene GAPDH with n=3 biological x 3 technical replicates. Representative Western blots shown. Protein quantification is shown relative to vehicle, with 3 replicates and was measured by ImageJ. All graphs are shown as mean ± SEM, with significance relative to vehicle calculated by unpaired t-test. * p < 0.05 ** p < 0.01, *** p < 0.001
5.6 Mechanisms of BET inhibitor-mediated GRN enhancement

As epigenetic readers, BET proteins promote gene expression by binding to acetylated lysines on the chromatin and recruiting transcription factors and transcriptional machinery, such as the co-activator Mediator, to bind to the chromatin. In addition, BRD4, in complex with P-TEFb (positive transcriptional elongation factor b complex), induces transcriptional elongation by relieving RNA Pol II from its paused state on the promoter. One understood mechanism of BET inhibitor gene regulation is that BET inhibitors promote displacement of BET proteins from the chromatin, preventing BET-dependent transcription complex formation. While through this mechanism, BET inhibitors are known to inhibit genes like MYC, the literature on BET protein-mediated mechanisms of transcriptional modulation demonstrates both suppression and enhancement of gene expression upon BET inhibitor treatment. For instance, pan-BET inhibitors, such as I-BET151, and more specific BET bromodomain inhibitors like RVX-208 have been found to upregulate genes in the cardiovascular network like APOA1, and it is possible that inhibition of BET-dependent genes cascades down regulatory pathways to upregulate genes like APOA1 and GRN. Other hypotheses around BET inhibitor-mediated gene enhancement come from studying reactivation of latent HIV with BRD4 inhibition and from profiling super-enhancer regions in mammalian genomes. In this model, inhibition of BRD4 and its subsequent displacement from the chromatin allows for a redistribution of BRD4 throughout the genome. In the case of HIV, freed BRD4 and its associated P-TEFb complex is recruited to the trans-active response (TAR) element in the viral genome via the viral protein Tat. In mammalian genomes, while the BRD4/Mediator containing
complex is found in thousands of transcriptionally active genes throughout the genome, a significant proportion of BRD4/Mediator containing complexes are found in large super-enhancer regions. These super-enhancers are disproportionately targeted by BET inhibitors, and the genes associated with super-enhancers, amongst them MYC, are disproportionately downregulated by BET inhibitor treatment. This allows BRD4, in both its roles in the Mediator complex and in the P-TEFb complex to be recruited to promoter/enhancer regions it would not populate at basal equilibrium. The sites of BRD4 redistribution can be directed by the activity of other transcription complexes, such as NF-κB, which may result in the upregulation of specific gene networks, but disruption of the transcription complexes in basal super-enhancer regions may also lead to BRD4 binding in promoter/enhancers regions poised for BET protein binding. In the case of GRN, where basal levels in neuronal cells are low, small changes in BRD4 binding to the GRN promoter/enhancer regions may cause relatively large levels of GRN enhancement.

BET inhibitors also seem to free the P-TEFb complex from its inactive state in the 7SK small nuclear ribonucleoprotein (snRNP) complex. Thus, BET inhibitor-mediated GRN enhancement may be a combination of these observed effects of BET inhibitor treatment. Binding of BET inhibitors to BRD4 causes displacement and redistribution of BRD4 from its basal chromatin occupancy. Displaced BRD4 can then interact with poised promoter/enhancer regions in complex with Mediator or associate with BET inhibitor-mediated freed P-TEFb. BRD4/Mediator induces transcriptional initiation and BRD4/P-TEFb promotes transcriptional elongation by phosphorylation of the C-terminal tail of RNA Pol II.
It has been shown that BET inhibitors can increase P-TEFb activity in the context of HIV transcript activation, as evidenced by increased phosphorylation at the CDK9 T-loop (pCKD9), which is required for CDK9 catalytic activity.\textsuperscript{118} To investigate whether BET inhibitors increase P-TEFb activity in the same manner in NPCs, we probed for pCDK9 after treatment with BET inhibitors. In the case of NPCs, there was no correlation between BET inhibitor-mediated enhancement of PGRN and pCDK9 (\textit{Figure 5-10}). It may be that the global level of pCDK9 is not a sufficient proxy for P-TEFb activity at the \textit{GRN} promoter or that increased phosphorylation of CDK9 upon treatment of BET inhibitors are mediated by viral protein Tat. To definitively understand the relationship between BET proteins and \textit{GRN} expression, it is necessary to determine BET protein occupancy on the \textit{GRN} promoter with and without BET inhibitor treatment. Future BET protein ChIP-PCR studies on regions of the \textit{GRN} promoter defined by \textit{Figure 4-2} would help to elucidate the role of BET proteins in \textit{GRN} expression.

\textbf{Figure 5-10: pCDK9 vs. PGRN protein expression in NPCs upon BET inhibitor treatment.} A) pCDK9(Thr186), and PGRN protein levels in NPCs when treated with BET inhibitors, with CDK9 shown as a baseline of the protein. B) Plot of protein levels of pCDK9 vs. PGRN protein levels shows no upregulation of pCDK9 upon treatment with BET inhibitors. NPCs were treated with vehicle (DMSO), I-BET151 (2.5 µM), (+)-JQ1 (1 µM), PFI-1 (10 µM), RVX-208 (25 µM), or negative control (-)-JQ1 (1 µM), for 24 hours whereupon cells were collected. Representative Western blots are shown for each condition. Quantification was done with ImageJ and results are shown as means relative to GAPDH and normalized to vehicle.
5.7 The role of TFEB in BET inhibitor-mediated \( GRN \) enhancement in human neuronal cells

Although BET proteins may directly modulate transcription activity on the \( GRN \) promoter, it is possible that they instead regulate key transcription factors that affect \( GRN \) expression. As discussed in previous chapters, TFEB has been implicated in \( GRN \) expression and it is possible that BET inhibitor modulation of TFEB may contribute mechanistically to the enhancement of PGRN expression in human neuronal cultures. Upon treating NPCs with BET inhibitors, we found that indeed, BET inhibitors increase TFEB protein and that this enhancement was correlated with PGRN enhancement. (Figure 5-11). However, these levels of TFEB enhancement were beneath 10-fold induction threshold model presented in Chapter 4 (Figure 5-10). This may mean that HDAC inhibitor- and BET inhibitor-mediated \( GRN \) expression necessitate different proportions of TFEB because of different transcriptional regulation mechanisms. For instance, HDAC inhibitors may cause an increase in histone acetylation or displacement of repressor complexes at the \( GRN \) promoter, but an increase in BET proteins to recruit transcription factors to the \( GRN \) promoter or increase transcriptional elongation may mean that less TFEB is needed to generate the same levels of \( GRN \) expression. However, these data also contribute to the hypothesis that PGRN plays a role in TFEB expression and that BET inhibitors may be increasing TFEB by increasing PGRN. Further investigation into nuclear localization of TFEB upon BET inhibitor treatment, as well as TFEB and BET protein occupancy on the \( GRN \) promoter upon BET inhibitor treatment is necessary to distinguish between these hypotheses.
Figure 5-11: TFEB vs. PGRN protein expression in NPCs upon BET inhibitor treatment. A) TFEB, pTFEB(Ser142), and PGRN protein levels in NPCs treated with BET inhibitors. B) TFEB protein enhancement is correlated with PGRN protein enhancement upon BET inhibitor treatment (left), though TFEB protein levels are below the TFEB threshold needed to upregulate PGRN with HDAC inhibitor treatment (right; superimposed on graph from Figure 4-3). Phospho-TFEB (pTFEB) levels were constant. NPCs were treated with vehicle (DMSO), I-BET151 (2.5 µM), (+)-JQ1 (1 µM), PFI-1 (10 µM), RVX-208 (25 µM), or negative control (-)-JQ1 (1 µM) for 24 hours whereupon cells were collected. Representative Western blots are shown for each condition. Quantification was done with ImageJ and results are shown as means relative to GAPDH and normalized to vehicle.
5.8 Combination treatment with HDAC inhibitors and BET inhibitors do not synergistically enhance GRN mRNA expression in NPCs

As HDACs and BET proteins are different classes of epigenetic regulators, inhibiting both epigenetic erasers and readers may have a synergistic effect on the expression of a gene that is upregulated by each separately. However, a pilot combination experiment with both HDAC inhibitors and BET inhibitors showed neither an additive nor synergistic effect on GRN mRNA expression. In fact, when treated with both panobinostat and I-BET151 at concentrations that separately significantly upregulate GRN mRNA and PGRN protein expression, NPCs showed less GRN upregulation than when treated with panobinostat and I-BET151 alone although global H3K9Ac was enhanced in all cells treated with panobinostat (Figure 5-12). This implies that there is some mechanism by which one compound attenuates the efficacy of the other. Further combination studies with other BET inhibitors and HDAC inhibitors may help illuminate whether this is a compound-specific or compound class-specific phenomenon. In addition, the combination of panobinostat and I-BET151 had a significant effect on normalization genes GAPDH and ACTB (data not shown), so further optimization of normalization genes and length of treatments must be achieved. Cotreatment effects on PGRN intracellular and secreted protein expression must also be ascertained before conclusions can be drawn about the combination treatment of HDAC inhibitors and BET inhibitors.
Figure 5-12: Combination with panobinostat and I-BET151 does not synergistically enhance GRN.

A) Heat map representation of GRN mRNA expression upon cotreatments with I-BET151 (y-axis) and panobinostat (x-axis). Color ranges from red (highest positive fold change) to white (baseline).

B) Scatterplot representation of GRN mRNA in NPCs treated with varying concentrations of panobinostat given a treatment of I-BET151. C) PGRN protein levels upon cotreatment with I-BET151 and panobinostat, with heat map and scatterplot representations of protein quantification. Protein quantification shown as mean + SEM and was done with ImageJ.
5.9 Summary and conclusions

Here, we introduce a novel mechanism of increasing PGRN levels in human NPCs and neurons: inhibition of BET proteins. Validated promiscuous bromodomain inhibitor bromosporine, pan-BET inhibitors, PFI-1, (+)-JQ1, and I-BET151, as well as BET BDII-selective inhibitor RVX-208, were all found to increase GRN mRNA and PGRN protein levels in human NPCs and post-mitotic neurons. Similar to HDAC inhibitor treatment of human neuronal cultures, we observed a strong correlation between GRN mRNA and PGRN intracellular and secreted protein levels upon BET inhibitor treatment indicative of an epigenetic regulatory mechanism.

Initial efforts to elucidate whether a particular BET protein is necessary and sufficient to enhance GRN mRNA and PGRN protein expression using Degronimid platform technology proved elusive, as treatment with dBET1 led to the degradation of multiple BET proteins in NPCs. Thus, optimization of Degronimid technology to target specific BET proteins or the knockdown of singular BET proteins with siRNA or CRISPR is necessary to ascertain the role of specific BET proteins in GRN expression. Further research into small molecule inhibitors specific to BET family members can also contribute to a chemogenomic toolkit that would be useful in profiling the role of specific BET proteins in gene expression. Of these probes used, I-BET151 has been found to have appropriate \textit{in vivo} half-life and oral bioavailability for a small molecule therapeutic.\textsuperscript{119} The isoxazole scaffold is also well studied in the medicinal chemistry field, making it an interesting scaffold on which to build BET inhibitor analogs toward potential therapeutics and fine-tune the specific moieties involved in BET protein specificity.
We have also attempted to probe the mechanism by which BET inhibitors enhance \textit{GRN} expression. BET inhibitors have been shown promote redistribution of BET proteins across the genome and, in the context of HIV activation, have been shown to increase P-TEFb activity indicated by increased phosphorylation of CDK9.\textsuperscript{117, 118} However, we did not find evidence of increased pCDK9 in BET inhibitor-treated NPCs (\textit{Figure 5-10}), perhaps because global pCDK9 levels may not definitively indicate P-TEFb activity at the \textit{GRN} promoter. BET-inhibitor enhancement of global pCDK9 may also require the viral protein Tat, which is not present in these NPCs. A better proxy for P-TEFb activity may be a study of RNA Pol II occupancy on the \textit{GRN} promoter.

We have also queried whether BET inhibitors indirectly modulate \textit{GRN} expression through upregulation of key transcription factors thought to be involved in \textit{GRN} expression. We found that in NPCs, treatment with BET inhibitors upregulated TFEB expression correlating with PGRN enhancement (\textit{Figure 5-11}). However, TFEB was not enhanced to the same degree it was upon HDAC inhibitor treatment and therefore does not fit into the TFEB threshold model in HDAC inhibitor-mediated \textit{GRN} enhancement (\textit{Figure 4-3}). To arrive upon a clearer mechanism of BET inhibitor-mediated \textit{GRN} enhancement, more work must be done to understand the occupancy of BET proteins and TFEB on the \textit{GRN} promoter, before and after treatment of BET inhibitors. These mechanistic insights combined with more targeted BET inhibitor development can ultimately lead to development of the most efficacious BET inhibitor-based therapies for PGRN-deficient patients with the fewest side effects.
5.10 Materials and Methods

Human iPSC-Derived NPC and Neuron Culture. All NPCs used were differentiated from iPSCs previously reprogrammed from human fibroblast line GM08330 (Coriell Institute for Medical Research, Camden, NJ) and cultured described in Chapter 3.11. Neurons were differentiated from NPCs via growth factor withdrawal as described in Chapter 3.11.

Compound Preparation and Treatment with Bromodomain Inhibitors. All compounds were purchased from commercial vendors. Stock concentrations of all compounds were made at 1000x in DMSO (Sigma Aldrich #D2438). For treatment, the stock compounds were diluted 1:1000 in NPC media (DMSO concentration 0.1%) and was applied to confluent NPCs or neurons for the allotted time. Treatment with bromodomain inhibitors was conducted with the same methodology as HDAC inhibitor treatment as described in Chapter 3.11.

Determination of Gene Expression Changes in Human Cell Culture. RNA was generated from each well of a 6-well treated plate of NPCs or neurons with TRIzol (ThermoFisher #11596026) and the DirectZol RNA MiniPrep Kit (Zymo Research #R2052) as described in Chapter 3.11. cDNA was synthesized with the High Capacity cDNA Synthesis Kit with RNase inhibitor (ThermoFisher #4368814) and qPCR was subsequently performed as in Chapter 3.11. Commercial Taqman primer probes used were as follows: GRN: Hs00963707_g1, GAPDH: Cat.#432924E. Results were normalized to GAPDH and replicate mean values and standard error of the mean are
reported. Significance was determined with unpaired t-tests with GraphPad Prism software.

**Western Blot Antibodies and Analysis.** Cell pellets were collected from each well of a 6-well plate, lysed in radio immunoprecipitation assay (RIPA) buffer (Boston BioProducts #BP-115) with EDTA-free protease inhibitors (Sigma Aldrich #4693159001) and a phosphatase inhibitor cocktail (Sigma Aldrich #P5726) and prepared as described in Chapter 3.11. Lysates were diluted to 800 ng/µL in RIPA Buffer and stored between -20 and -80°C until ready for use. Before use, lysates were boiled at 95°C with SDS loading buffer (New England BioLabs #B7703S) + DTT (New England BioLabs #B7705S) for 5 minutes, then spun down.

In probing for PGRN, proteins were separated on NuPAGE 4-12% Bis-Tris gels (ThermoFisher #NP0335BOX) in MOPS SDS Running buffer (ThermoFisher #NP0001). BRD2, BRD3, and BRD4 were separated on NuPAGE 7% Tris-Acetate gels (ThermoFisher #EA0358BOX). For H3K9 acetylation blots, proteins were separated on 16% Tricine gels (ThermoFisher #EC6695BOX) in Tricine running buffer (ThermoFisher #1691442). To each well, 8 µg total protein was loaded and gels were run at 125V for 1 hour. Gels were then transferred onto 0.45 µm PVDF membranes (ThermoFisher #88518) or 0.2 µm nitrocellulose membranes (Bio-Rad #1620112). Membranes were blocked in 5% milk in TBST for 1 hour and probed overnight at 4°C with primary antibodies in 5% BSA + 0.02% sodium azide (PGRN: Invitrogen #40-3400, 1:1000; BRD2: Bethyl #A302-583A, 1:1000; BRD3: Bethyl #A302-368A, 1:1000; BRD4: Cell Signaling #13440S, 1:1000; H3K9: Sigma Aldrich #H9286, 1:5000; GAPDH: Abcam
Membranes were then washed with PBS, incubated with secondary antibody in TBST containing 5% milk for 1 hour. Secondary antibodies were as follows: for all proteins except GAPDH: anti-rabbit-HRP, Cell Signaling #7074S, 1:2000; for GAPDH: anti-mouse-HRP, Cell Signaling #7076S, 1:2000. Membranes were then washed with PBS for 1 hour, and developed with chemiluminescence reagents (Pierce ECL Western Blotting Substrate, ThermoFisher #PI32106; SuperSignal West Dura Extended Duration Substrate, ThermoFisher #PI34076; SuperSignal West Femto Chemiluminescent Substrate, ThermoFisher #PI34096). Protein quantification was done with ImageJ and normalized to GAPDH.

**ELISA.** All ELISA was conducted with a Progranulin (human) ELISA kit (AdipoGen #AG-45A-0018YPP-KI01) at the manufacturer's instructions. Cell culture supernatant was collected from treated cells after indicated treatment time, incubated with protease inhibitors (Sigma Aldrich #4693159001), and spun down to remove debris. Supernatant was collected and stored at -80°C until ready to use. Supernatant was diluted 1:5 in provided ELISA buffer, and protein lysates (as prepared above) were diluted 1:100 in ELISA buffer. ELISA results were collected with SpectraMax Plus 384 Microplate Reader (Molecular Devices) and data was normalized to control values. Replicate mean values and standard error of the mean are reported. Significance was determined with unpaired t-tests with GraphPad Prism software.
Chapter 6

Exploration of Alternate Compound Classes for Progranulin Enhancement in Human iPSC-derived Neuronal Cultures
Chapter 6: Exploration of Alternate Compound Classes for Progranulin Enhancement in Human iPSC-derived Neuronal Cultures

Summary

The identification of fast-binding Class I HDAC inhibitors and BET inhibitors as GRN mRNA and PGRN protein enhancers has broad implications for clinical development or repurposing of FDA-approved therapeutics for PGRN-deficient FTD. However, these small molecule epigenetic regulator classes represent only a subset of potential drug targets, and studies into effects of other classes of small molecules on GRN and PGRN expression can lead to understandings about pathways that regulate PGRN expression as well as further insight into the role that PGRN plays in neuronal cells. Here, we describe the interesting effects on progranulin expression upon treatment with two alternate compound classes: mTOR inhibitors and S1PR antagonists.

6.1 The effect of mTOR inhibitors on GRN mRNA and PGRN protein expression in human neuronal cells

Transcription factor EB (TFEB), a master regulator of autophagy and lysosomal gene expression, has been implicated in GRN expression, and work denoted in Chapters 4 and 5 has shown a correlation between treatment with HDAC and BET inhibitors and significant enhancements in TFEB and PGRN expression. Because TFEB activity is highly regulated by mTORC1 phosphorylation, the inhibition of mTOR leads to TFEB activation through decreased TFEB phosphorylation and increased
TFEB nuclear translocation and TFEB-mediated gene expression. Inhibition of mTOR also activates autophagy pathways by affecting phosphorylation in multiple complexes within the autophagy pathway, \(^{120}\) which may promote clearance of protein aggregates, in the case of PGRN-deficient FTD: TDP-43. Thus, mTOR inhibition seems a viable therapeutic avenue for PGRN-deficient FTD, as treatment with mTOR inhibitors would hypothetically simultaneously increase PGRN expression through TFEB activation and clear protein aggregates through autophagy induction.

A number of mTOR inhibitors, including rapamycin, wortmannin, and NVP-BEZ235, were found in our L1000 bioactive compound screen in NPCs to increase GRN mRNA with 24-hour treatment (data not shown). In validating these hits from the L1000 screen, we found that while ATP-competitive mTOR inhibitors like NVP-BEZ235 and AZD-8055 significantly increased GRN mRNA expression in human iPSC-derived NPCs and differentiated neurons, they were not successful at increasing PGRN intracellular or secreted protein levels (Figure 6-1). This is likely due to the fact that mTOR pathway is also highly involved in protein synthesis pathways through phosphorylation of, amongst other members of the protein translation initiation and elongation pathways, eukaryotic initiation factor 4G (eIF4G), eIF4E binding protein-1 (4E-BP1), ribosomal protein S6 kinase-1 (S6K1), and eukaryotic elongation factor 2 (eEF2).\(^{121, 122}\) Inhibition of mTOR prevents phosphorylation of protein synthesis pathway members, inhibiting cap-dependent protein synthesis. Thus, any increase in PGRN protein levels predicted by enhanced GRN mRNA expression due to mTOR inhibitor activation of TFEB may be suppressed by mTOR inhibitor inhibition of protein synthesis. It follows that although mTOR inhibitors would seem to stimulate both TFEB
transcription factor activity and autophagy pathways, targeting mTOR directly is not a viable avenue to increase PGRN protein expression.

Figure 6-1: Effect of mTOR inhibitors on PGRN expression in human neuronal cells. A) Chemical structures for mTOR inhibitors, (19) AZD-8055 and (20) NVP-BEZ235. B) AZD-8055 significantly increases GRN mRNA but does not significantly increase PGRN intracellular or secreted protein expression human NPCs (black) or 18-day neurons (gray). C) NVP-BEZ235 also significantly increases GRN mRNA but does not significantly increase PGRN protein levels in human NPCS (black) or 18-day neurons (gray). Cells were treated for 24 hours with vehicle (DMSO), positive control SAHA (10 µM), or compound at the given concentration. mRNA quantification was done relative to housekeeping gene GAPDH with n=3 biological replicates x 3 technical replicates. Protein quantification is shown relative to vehicle, with 2 replicates and was measured by ELISA. All graphs are shown as mean + SEM, with significance relative to vehicle calculated by unpaired t-test. * p < 0.05, *** p < 0.001, **** p < 0.0001
6.2 FTY720 (fingolimod) increases only secreted PGRN in human neuronal cultures

FTY720 (fingolimod), an immunomodulatory sphingosine analog that is FDA-approved for the treatment of multiple sclerosis, is rapidly phosphorylated in the cell nucleus by sphingosine kinase 2 (SphK2) to its active form, FTY720-phosphate (FTY270-p). While FTY720-p has been shown to modulate activity of sphingosine 1 phosphate (S1P) receptors and is a functional antagonist of S1P receptor 1 (S1PR1), it has also recently been reported to be a potent in vivo Class I HDAC inhibitor. Although FTY720 itself has not been shown to be an HDAC inhibitor, it is believed that phosphorylation by sphingosine kinase 2 (SphK2) is rapid enough in cells that any treatment with FTY720 effectively exposes the cell to FTY720-p. Because FTY720 is an approved drug for a neurological disease with a known safety profile, and because of our findings regarding the efficacy of Class I HDAC-selective inhibitors, we reasoned there was potential for repurposing of FTY720 to treat PGRN-deficient FTD if it would show the hypothesized effects on PGRN upregulation. Here, in human NPCs and differentiated neurons, we tested the effects of FTY720 and FTY720-p on GRN mRNA and PGRN intracellular and secreted protein expression, as well as on histone acetylation as a cellular marker of HDAC inhibition.

Contrary to our expectation, in NPCs and neurons, we found that neither FTY720 nor FTY720-p upregulated GRN mRNA or PGRN intracellular protein levels (Figure 6-2). We also did not find FTY720 or FTY720-p to have any HDAC inhibitor activity in these cells as indicated by the lack of any change in global H3K9Ac levels relative to the effects of known HDAC inhibitor control compounds (Figure 6-2). Because of lack of
HDAC inhibitor activity, it follows that upon treatment with FTY720 or FTY720-p, we would not expect HDAC inhibitor-mediated epigenetic regulation or HDAC inhibitor-induced GRN mRNA and PGRN enhancement. At this point, more research must be done to determine the reasons that FTY720 and FTY720-p differ in their HDAC inhibitor roles in different cell types.

Interestingly, although FTY720 and FTY720-p did not increase GRN mRNA and PGRN intracellular protein in NPCs or neurons, the compounds did increase the concentration of secreted PGRN in the extracellular media in NPCs (Figure 6-2). Because in breast cancer cells, Sph2K was found to rapidly convert FTY720 to FTY720-p and histone acetylation associated with HDAC inhibitor activity of FTY720-p was found to occur in less than 24 hours, we conducted a time course to see whether we could observe GRN enhancement at a time point earlier than 24 hours. We found that between 2 and 24 hours, FTY720 and FTY720-p do not significantly increase GRN mRNA expression, whereas positive control SAHA was found to increase GRN mRNA levels at around 6 hours (Figure 6-3). However, the time course profile of secreted PGRN in NPCs treated with FTY720 closely mimicked that of SAHA (Figure 6-3). FTY720-p did not show as significant an effect on secreted PGRN as its unphosphorylated counterpart, though there is still a modest increase in secreted PGRN upon treatment (Figure 6-3).
Figure 6-2: Effect of FTY720 and FTY720-p on progranulin expression in human neuronal cells. A) Chemical structures for (21) FTY720 and (22) FTY720-p. B) FTY720 and FTY720-p do not increase GRN mRNA or PGRN intracellular protein but do increase secreted PGRN in NPCs (black) and 18-day neurons (gray). Cells were treated for 24 hours with vehicle (DMSO), positive control SAHA (10 µM), FTY720 (10 µM) or FTY720-p (10 µM). mRNA quantification was done relative to housekeeping gene GAPDH with n=6 biological replicates x 3 technical replicates. Representative Western blots are shown here with vertical lines denoting cuts in gels. Protein quantification is shown relative to vehicle, with 3 replicates and was measured by ELISA. All graphs are shown as mean + SEM, with significance relative to vehicle calculated by unpaired t-test. ** p < 0.01, *** p < 0.001, **** p < 0.0001
Figure 6-3: Progranulin time course for NPCs treated with FTY720 and FTY720-p. *GRN* mRNA and PGRN secreted protein levels in NPCs treated with vehicle (DMSO), positive control SAHA (10 µM), FTY720 (10 µM), or FTY720-p (10 µM) for up to 24 hours. While FTY720 and FTY720-p do not significantly increase *GRN* mRNA levels throughout the time course, FTY720, and to a modest extent FTY720-p, increase secreted PGRN over the course of 24 hours.

Because *GRN* mRNA and PGRN secreted protein profiles for FTY720 differ significantly, and FTY720 was not shown to have HDAC inhibition activity in NPCs, it follows that the mechanism of PGRN secreted protein enhancement is not epigenetically regulated. One explanation could be through another function of FTY720: inhibition of expression of neurotrophin receptor p75NTR through inhibition of TNFα.124 Sortilin, a cell surface protein known to bind to PGRN and assist in PGRN trafficking to the lysosome, is thought to act as a coreceptor for p75NTR.23 It is therefore possible that when expression of p75NTR is low, the sortilin-p75NTR-PGRN complex could be unable to form, thus preventing PGRN from reentering the cell. This would explain the increased extracellular PGRN in NPCs treated with FTY720, while mRNA and intracellular protein remain constant. Future studies into the expression of p75NTR in NPCs upon treatment of FTY720 and the effect on other secreted proteins would help support this hypothesis.
6.3 Summary and Conclusions

Here, we described the effect of mTOR inhibitors and S1PR antagonists on GRN mRNA and PGRN protein expression. While mTOR inhibitors were found to enhance GRN mRNA expression, they were not able to significantly increase PGRN protein levels in human iPSC-derived neuronal cultures. This is likely due to the antagonistic effect that mTOR inhibitors have on protein synthesis, and implies that modulating mTOR directly may not be a viable therapeutic avenue for PGRN-deficient FTD. In addition, we tested S1PR antagonists FTY720 and FTY720-p and found that while the compounds increased extracellular concentrations of PGRN, there was no significant effect at the mRNA or intracellular protein levels. This is hypothesized to be due to an inhibition of PGRN endocytosis, which causes PGRN to accumulate in the extracellular media. In addition, we did not find evidence of Class I HDAC inhibition activity by FTY720 and FTY720-p, which are purported to be in vivo HDAC inhibitors.123

Overall, these experiments show that not all compound classes upregulate all stages of progranulin expression. Although we have shown in Chapters 3 and 5 that small molecule epigenetic regulators upregulate GRN mRNA and PGRN intracellular and secreted protein levels, this positive correlation is not found with treatment of mTOR inhibitors or FTY270. These experiments also imply that not all approved therapeutic avenues are appropriate for treating PGRN-deficient FTD, and sole treatment with mTOR inhibitors that activate autophagy may not be appropriate here, even when there is a protein inclusion phenotype. In addition, because PGRN is secreted and taken back into the cell, it is important in early stages of drug development to monitor levels of PGRN both intracellularly and extracellularly, lest a compound
affects reuptake and prevents PGRN from being able to carry out its role in cellular compartments. It is also imperative that mRNA studies are used only as a first step in identifying PGRN modulators, as compounds that positively affect GRN mRNA may not have such an effect on PGRN protein levels. Further research into the different classes of compounds that affect different modes of progranulin expression will help not only to illuminate compound classes that would be effective for treating PGRN-deficient FTD, but compound classes that may be detrimental to PGRN expression in FTD patients, both of which are important in development of therapeutics for FTD and diseases of PGRN deficiency.

6.4 Materials and Methods

Human iPSC-Derived NPC and Neuron Culture. All NPCs used were differentiated from iPSCs previously reprogrammed from human fibroblast line GM08330 (Coriell Institute for Medical Research, Camden, NJ) and cultured described in Chapter 3.11. Neurons were differentiated from NPCs via growth factor withdrawal as described in Chapter 3.11.

Compound Preparation and Treatment with Bromodomain Inhibitors. All compounds were purchased from commercial vendors. Stock concentrations of all compounds were made at 1000x in DMSO (Sigma Aldrich #D2438). For treatment, the stock compounds were diluted 1:1000 in NPC media (DMSO concentration 0.1%) and was applied to confluent NPCs or neurons for the allotted time.
**Determination of Gene Expression Changes in Human Cell Culture.** RNA was generated from each well of a 6-well treated plate of NPCs or neurons with TRIzol (ThermoFisher #11596026) and the DirectZol RNA MiniPrep Kit (Zymo Research #R2052) as described in Chapter 3.11. cDNA was synthesized with the High Capacity cDNA Synthesis Kit with RNase inhibitor (ThermoFisher #4368814) and qPCR was subsequently performed as in Chapter 3.11. Commercial Taqman primer probes used were as follows: GRN: Hs00963707_g1, GAPDH: Cat.#432924E. Results were normalized to GAPDH and replicate mean values and standard error of the mean are reported. Significance was determined with unpaired t-tests with GraphPad Prism software.

**Western Blot Antibodies and Analysis.** Cell pellets were collected from each well of a 6-well plate, lysed in radio immunoprecipitation assay (RIPA) buffer (Boston BioProducts #BP-115) with EDTA-free protease inhibitors (Sigma Aldrich #4693159001) and prepared as described in Chapter 3.11. Lysates were diluted to 800 ng/µL in RIPA Buffer and stored between -20 and -80°C until ready for use. Before use, lysates were boiled at 95°C with SDS loading buffer (New England BioLabs #B7703S) + DTT (New England BioLabs #B7705S) for 5 min, then spun down.

In probing for PGRN, proteins were separated on NuPAGE 4-12% Bis-Tris gels (ThermoFisher #NP0335BOX) in MOPS SDS Running buffer (ThermoFisher #NP0001). For H3K9 acetylation blots, proteins were separated on 16% Tricine gels (ThermoFisher #EC6695BOX) in Tricine running buffer (ThermoFisher #1691442). To each well, 8 µg total protein was loaded and gels were run at 125V for 1 hour. Gels were then
transferred onto 0.45 µm PVDF membranes (ThermoFisher #88518). Membranes were blocked in 5% milk in TBST for 1 hour and probed overnight at 4°C with primary antibodies in 5% BSA + 0.02% sodium azide (PGRN: Invitrogen #40-3400, 1:1000; H3K9ac: Sigma Aldrich #H9286, 1:5000; GAPDH: Abcam #ab8245, 1:10,000). Membranes were then washed with PBS, incubated with secondary antibody in TBST containing 5% milk for 1 hour. Secondary antibodies were as follows: for all proteins except GAPDH: anti-rabbit-HRP, Cell Signaling #7074S, 1:2000; for GAPDH: anti-mouse-HRP, Cell Signaling #7076S, 1:2000. Membranes were then washed with PBS for 1 hour, and developed with chemiluminescence reagents (Pierce ECL Western Blotting Substrate, ThermoFisher #PI32106; SuperSignal West Dura Extended Duration Substrate, ThermoFisher #PI34076; SuperSignal West Femto Chemiluminescent Substrate, ThermoFisher #PI34096).

ELISA. All ELISA was conducted with a Progranulin (human) ELISA kit (AdipoGen #AG-45A-0018YPP-KI01) at the manufacturer's instructions. Cell culture supernatant was collected from treated cells after indicated treatment time, incubated with protease inhibitors (Sigma Aldrich #4693159001), and spun down to remove debris. Supernatant was collected and stored at -80°C until ready to use. Supernatant was diluted 1:5 in provided ELISA buffer, and protein lysates (as prepared above) were diluted 1:100 in ELISA buffer. ELISA results were collected with SpectraMax Plus 384 Microplate Reader (Molecular Devices) and data was normalized to control values. Replicate mean values and standard error of the mean are reported. Significance was determined with unpaired t-tests with GraphPad Prism software.
Chapter 7

Generation of Induced Pluripotent Stem Cells (iPSCs) and Neuronal Cell Lines from FTD Patients
Chapter 7: Generation of Induced Pluripotent Stem Cells (iPSCs) and Neuronal Cell Lines from FTD Patients

Summary

Relevant model systems are essential in studying complex diseases such as FTD. In neurodegenerative diseases, in particular, where the organ of interest can only on rare occasions be biopsied and animal models have proved to be phenotypically incomplete models unable to completely capture the genetic background of a neurodegenerative disease, a highly relevant model system may be neurons derived through the reprogramming of readily available patient cells (Figure 2-1). These reprogrammed neuron lines will have a known genetic background, and are more likely to exhibit a phenotype similar to the patient's phenotype. Thus far, in this dissertation, all work in NPCs and neurons has been conducted in cells derived from an unaffected control patient. However, it is imperative that PGRN enhancers are able to adequately increase PGRN not only in control cells, but also in PGRN-deficient FTD patient lines. This chapter details the iPSC reprogramming from fibroblasts of bvFTD patients and familial controls and presents opportunities for future work in this area.

7.1 Summary of iPSC-reprogramming methods

The stem cell reprogramming field has been in a state of rapid technological development since Shinya Yamanaka introduced the first retrovirus reprogramming scheme in 2007. The general reprogramming scheme involves introducing to a sample of human fibroblasts a cocktail of transcription factors consisting of OCT4, SOX2, c-Myc, KLF4, and, introduced in later work, LIN28. OCT4, SOX2, and
LIN28 are core transcription factors that are highly expressed in embryonic stem cells and are instrumental in reprogramming and maintenance of pluripotency.\textsuperscript{126} The other factors, c-Myc and KLF4, restructure chromatin to better allow OCT4, SOX2, and LIN28 to bind to their targets, which would not occur in adult somatic cells otherwise.\textsuperscript{125} Although c-Myc is a well understood oncogene, efficiency of reprogramming falls when it is removed from the cocktail.\textsuperscript{125}

While the transcription factor cocktail has traditionally been introduced into cells via retroviruses and lentiviruses, which integrate the transcription factor DNA directly into the host cell’s genome, the very nature of the method introduces clonal variability into the cells. The viruses can introduce the transcription factor DNA, or even viral DNA, into unintended regions of the genome, within existing genes, or in constitutively active areas. Uncontrolled expression of c-Myc, for instance, can induce proliferation more akin to cancer than to iPSCs and could prevent or affect downstream differentiation potential.\textsuperscript{125}

Non-integrative methods of introducing the transcription factor cocktail were thus developed to solve this issue. For instance, replication-incompetent adenovirus, a non-integrating DNA virus, has been shown as a successful delivery vehicle for the transcription factor cocktail, but the efficiency of reprogramming has been a limiting factor for this method (0.0002% in human cells).\textsuperscript{127} The temperature-sensitive strain of non-replicative Sendai virus, an RNA virus which does not enter the nucleus, has been shown to reprogram human fibroblasts at a much higher efficiency than the adenoviral method.\textsuperscript{128} However, although Sendai virus can eventually be diluted out of cells because it is confined to the cytoplasm, it often takes more than 10 passages to fully
dilute the Sendai virus out of the cell, and due to the temperature-sensitive modifications made to the virus, the cells may have to be cultured at 39°C, somewhat higher than the ideal culture temperature. Episomal vector reprogramming, which involves the transient transfection of a plasmid containing the requisite transcription factors, has also shown successful iPSC reprogramming from human fibroblasts, albeit at low efficiency.

Footprint-free reprogramming methods, which include small molecules, protein cocktails, and mRNA cocktails, also do not leave trace DNA in the host cell genome. Although small molecules or proteins can augment efficiency of reprogramming when combined with other methods, even replacing some of the transcription factors in these methods, when used alone, they are not very effective. Small molecules can have unanticipated effects on cells from off-target activity in different pathways. It is also non-trivial to synthesize and traffic large amounts of bioactive proteins across the plasma membrane, where they can subsequently be rapidly degraded in the cell without carrying out the intended effect.

With this in mind, we choose to use a non-integrative mRNA reprogramming scheme. Here, we introduce the five transcription factors as an mRNA cocktail and incubate the cells with B18R, a type-1 interferon inhibitor that hinders the cell's innate immune response against foreign mRNAs. The cells are also treated with a commercially available cocktail of synthesized microRNA (miRNA) mimics that includes the human miR-302 family (hmiR-302a, hmiR302b, hmiR302c, hmiR302d and hmiR-367) along with additional proprietary components. These miRNAs have been shown to simultaneously knock down many of the cell-fate determining genes and upregulate
OCT4, improving reprogramming efficiency. Colonies with human embryonic stem cell (hESC) morphology (i.e. SSEA4+ cells) were picked, expanded, and characterized. Although relatively efficient, this protocol can be highly variable between patient lines, with no clear link between genetics and susceptibility to reprogramming. Thus, we varied several parameters that are known to affect reprogramming efficiency, such as passage number, starting cell seeding density and use of a feeder cell layer, in parallel to increase the rate of success early on.

### 7.2 Generation of patient-derived iPSC models of behavioral variant frontotemporal dementia (bvFTD)

Through the Massachusetts General Hospital Frontotemporal Dementia Unit and Massachusetts General Hospital Neurodegeneration Biorepository, a total of five fibroblast lines obtained from dermal punch biopsies from a family with a history of neurodegeneration on the paternal side were selected for reprogramming (Figure 7-1). The family consists of one cognitively normal mother and four siblings, two of whom were diagnosed with behavioral variant frontotemporal dementia (bvFTD). The five individuals were as follows: 1) MGH-2048: male proband with a clinical history of bvFTD and TDP-43 histopathology, identified by DNA sequencing to have a *GRN* mutation c.559_560insC (reference NM_002087.2 starting with c.1 at the “A” of the “ATG” initiator codon; g.42427906A>AC; Hg19/GRCh37). This insertion mutation in *GRN* is predicted to cause a frameshift mutation in PGRN leading to substitution of Leucine (L) to Proline (P) at amino acid 187 followed by 9 additional amino acids before a premature stop codon is encountered (p.L187Pfs9). This mutation is therefore predicted to cause
PGRN haploinsufficiency due to nonsense-mediated mRNA decay occurring as a result of the aberrant mRNA. 2) MGH-2050: brother of proband, also diagnosed with bvFTD, with the same c.559_560insC mutation. 3) MGH-2029: cognitively normal brother of proband MGH-2048, with wild-type GRN. 4) MGH-2047: cognitively normal sister of proband MGH-2048 with wild-type GRN. 5) MGH-2049: cognitively normal mother of proband MGH-2048 with wild-type GRN. After verifying the genotypes of all family members using next-generation sequencing, we reprogrammed the fibroblast lines via the transient mRNA reprogramming scheme described above.

Figure 7-1: Genetics of fibroblast lines for iPSC reprogramming. A) Pedigree of family from whom fibroblasts were obtained via dermal skin punch, with fibroblast line identity indicated below each symbol. Darkened squares indicate diagnosis of bvFTD and GRN mutation. B) GRN mutation for MGH-2048 and MGH-2050 on the GRN gene denoted with the cDNA numbering relative to the full-length GRN transcript (GenBank accession number NM_002087.2). The gray boxes indicate noncoding exon 1 and 3’ untranslated region. The orange boxes indicate coding exons 2-13.
Through our reprogramming efforts, one FTD line (MGH-2050; brother of proband MGH-2048, with c.559_560insC mutation in \textit{GRN}), yielded eight clonal colonies via the transient mRNA reprogramming scheme. These colonies exhibited typical iPSC colony morphology and stained positive for iPSC markers – SSEA-4, TRA-1-60, OCT4, NANOG, and alkaline phosphatase (\textit{Figure 7-2}).

The two most healthily growing clones, 2050-1 and 2050-8, were expanded and fully characterized by G-band karyotype, pyrosequencing analyses, and trilineage differentiation potential through a gene expression panel of differentiated embryoid bodies (EBs). The clones each had a normal karyotype, showed an expected downregulation of DNA methylation around the \textit{OCT4} and \textit{NANOG} promoters compared to fibroblasts, and exhibited the potential to differentiate into each of the three tissue lineages (\textit{Figure 7-3}).

Fibroblasts from the other four family members were reprogrammed with a similar transient mRNA reprogramming scheme by a commercial reprogramming vendor and were characterized by OCT4 and TRA-1-60 staining (\textit{Figure 7-4}).
Figure 7-2: Characterization of iPSC clones from MGH-2050 by iPSC marker staining. Eight iPSC clones were isolated and expanded from reprogramming of the MGH-2050 fibroblast line and stained for iPSC markers SSEA-4, TRA-1-60, NANOG, OCT4, and alkaline phosphatase.
Figure 7-3: Full characterization of iPSC clones 2050-1 and 2050-8. 

A) G-band karyotyping of 2050-1 and 2050-8 showed no clonal abnormalities in either line. 

B) Methylation on OCT4 and NANOG promoters, as shown by bisulfite sequencing, for fibroblasts and iPSC clones. Open and closed circles represent unmethylated and methylated CpG dinucleotides, respectively. 

C) Tri-lineage differential potential of iPSC clones. Embryoid bodies (EBs) generated from iPSC clones were submitted to a gene expression panel containing genes indicative of self-renewal processes and mesoderm, ectoderm, mesoderm, and endoderm tissues. Embryoid body samples for both iPSC lines showed upregulation of each tissue lineage compared to a standard, and iPSC samples scored positively in the self-renewal gene set. fc = fold change.
Figure 7-4: Characterization of iPSCs reprogrammed from fibroblasts lines 2048, 2029, 2047, and 2049, with OCT4 and TRA-1-60 staining. iPSC morphology is markedly different than fibroblast morphology and cultures look pure according to staining. All images were taken at 10x magnification.
7.3 Generation of NPCs from bvFTD-patient derived iPSCs

After generation of iPSCs from the patient fibroblast lines, we moved forward with NPC differentiation of the iPSC lines 2050-1 and 2050-8 using the Gibco Neural Induction system. Early passage NPCs showed positive staining for NPC markers, SOX1, SOX2, Nestin, and CXCR4 (Figure 7-5). However, upon passaging, it became apparent that other cell types were present in the cell culture and that FACS (fluorescence-activated cell sorting) or MACS (magnetic-activated cell sorting) purification is necessary to generate a pure NPC culture. Indeed, when comparing p.6 2050-8 NPCs to control 8330-8 NPCs, GRN mRNA expression was found to be comparable instead of deficient as expected for a PGRN-deficient cell line (Figure 7-6). This is likely because neural cells contain a proportionally lower amount GRN expression than other cell types\textsuperscript{135} and the presence of non-neuronal cell types with higher basal expression of GRN could obscure the measurement GRN mRNA. Further work must be done to generate a pure NPC cell culture before assessments can be made about basal GRN/PGRN levels in NPCs generated from PGRN-deficient patient skin punches and the efficacy of different compound classes on upregulating GRN mRNA and PGRN protein in PGRN-deficient cells.
Figure 7-5: Characterization of early passage NPCs generated from 2050-1 and 2050-8 iPSCs with staining of NPC markers SOX1, SOX2, Nestin, and CXCR4.

Figure 7-6: GRN expression in mixed cell population following NPC generation of 2050-8. A) Morphology in 2050-8 p.6 is mixed and visually different than the pure NPC population in 8330-8 cells. B) Because of mixed cell population, GRN mRNA in 2050-8 p.6 was similar to that in 8330-8, despite being a PGRN-deficient line.
7.4 Summary and Conclusions

For neurodegenerative diseases like FTD, where appropriate model systems are especially essential to understand both disease progression and therapeutic avenue, neuronal cell cultures differentiated from patient-derived iPSC cells provide a genetic and cell type relevant system in which to study neurodegenerative disease biology. Here, we have reprogrammed iPSCs from two patients diagnosed with bvFTD with a novel GRN mutation and from three cognitively normal familial controls without the mutation. Two of the FTD iPSC lines were then differentiated into NPCs, with future directions to purify the NPC cultures and differentiate into neurons. The neurons can then be phenotyped for TDP-43 inclusions, which should be evident by immunocytochemistry and high-content imaging. In addition, genome editing tools such as CRISPR-Cas systems, combined with homology-direct repair mechanisms can be used to generate genetically corrected control neuronal lines to phenotype alongside the PGRN-deficient lines.\textsuperscript{136, 137} These controls should not have the same protein aggregation phenotype as the PGRN-deficient lines and can be used to address line-to-line variability in control lines. Finally, the PGRN-deficient NPC and neuron cell lines can be used to test the hypothesis that small molecules found to enhance GRN mRNA and PGRN protein expression in control NPCs and neurons can upregulate PGRN expression in PGRN-deficient cells to approach wild-type levels. The PGRN-deficient neuronal cell lines can also be used to screen for small molecule PGRN enhancers that may be specific to PGRN-deficient cell lines.
7.5 Materials and Methods

*Human Fibroblast Derivation from Skin Biopsies.* Human Fibroblast (hFib) media consisted of Dulbecco’s Modified Eagle Medium (DMEM; Gibco #11995) containing 25 mM D-glucose, 10% (vol/vol) Fetal Bovine Serum (FBS; Gibco #16000-044), 11 mM sodium pyruvate, 100 U/mL penicillin, 100 ug/ml streptomycin, and 4 mM L-glutamine (L-Gln), that had been filtered through a 0.2-μm syringe filter prior to use. Penicillin (10,000 I.U./mL)/Streptomycin (10,000 ug/mL)/L-Glutamine (29.2 mg/mL) were added from a 100X stock (Cellgro, Mediatech, #30-009-CI). For splitting cells, 0.05% Trypsin/0.53 mM EDTA in Hank’s Balanced Salt Solution (HBSS) without sodium bicarbonate (Corning #25-052-CI) was used.

Human dermal fibroblasts were collected under a protocol approved by the Massachusetts General Hospital/Partners Healthcare Institutional Review Board (PI Dr. James Gusella). Informed written consent for generation of cell lines and stem cell models was obtained for all research subjects. A 1.5 - 3 mm dermal punch biopsy was taken from each subject under sterile conditions, transferred into a sterile tube with hFib media, and kept at 4°C until ready for processing. Under sterile conditions, media from each specimen was decanted or aspirated and the skin piece transferred to a 60 mm diameter, tissue-culture treated, polystyrene tissue culture dish (Falcon #353004) scored with a sterile scalpel to create a grid. The skin piece was then cut into smaller pieces using a scalpel and distributed across the plate. hFib media (4 mL) was then added to cover the tissue pieces and the dish was placed in an incubator at 37°C with 5% CO₂. The cultures were allowed to expand for a 1 – 4 week time period until
confluent with media removed and replaced with fresh media (4 mL) as needed, typically every 2 days. Fibroblasts typically emerged from culture and were visible after 1 week. Once confluent, cultures were passaged with dissociation with 0.05% Trypsin/0.53 mM EDTA in HBSS with 1 mL per 60 mm tissue culture dish or 1.5 mL per T75 flask followed by quenching by adding fresh hFib media and seeding to a new 60 mm tissue culture dish in 4 mL hFib media or T75 flask (Falcon #353136) in 14 mL hFib media. Individual vials of fibroblasts from each line were then cryopreserved at low passage numbers (<5) at a density of 3 vials per T75 flask in hFib media supplemented with 10% DMSO (Fisher #231-100) in Thermo Scientific Nunc CryoTube Vials (#366656).

Prior to reprogramming, fibroblast cultures were expanded in hFib Reprogramming media (hFib-R), consisting of DMEM (Gibco #11995), 10% vol/vol FBS (Gibco #16000-044), 1% GlutaMAX (Gibco #35050061), and penicillin/streptomycin that had been sterile filtered. Fibroblasts were cultured in T25 (Falcon #353109) and T75 flasks (Falcon #353133). Upon passaging, cells were briefly rinsed in D-PBS (Gibco #14190), then treated with 1 mL TrypLE (Gibco #12563) for 3-5 min. TrypLE was quenched by adding fresh hFib-R media. Cells were cryopreserved in hFib-R media with 10% DMSO (Sigma # D8418) at a density of 1/3 of a confluent T75 flask per vial. Another 1/3 of the T75 flask were frozen as cell pellets for genotype confirmation. For mycoplasma testing, fibroblasts were cultured in hFib-R media lacking antibiotics for at least 1 passage or 1 week period. Mycoplasma testing was performed using MycoAlert Mycoplasma Detection Kit (Lonza, #INCLT07-318) per the manufacturer's instructions.
Genotyping Patient Cells. Genomic DNA was extracted from cell pellets using the QIAGEN DNeasy Blood & Tissue kit (QIAGEN # 69504). PCR on genomic DNA was performed with the Phusion Hot Start II High Fidelity DNA Polymerase (ThermoScientific #F549L) with GC rich buffer and relaxed PCR conditions. Primers were designed to span the c.559_560 insertion mutation and were as follows: forward primer = GGGTGAAGACGGAGTCAGG, reverse primer = GAAGAGGAGCAAAACGTGAGG. PCR products were run on a 2% agarose gel. Bands were cut out and DNA was extracted with the QIAquick Gel Extraction Kit (QIAGEN # 28704) and sequenced with Sanger sequencing.

Reprogramming and Maintenance of iPSCs from Human Fibroblasts. Fibroblasts were reprogrammed using the Stemgent mRNA Reprogramming kit with some modifications (Stemgent #00-0071). Prior to reprogramming, mRNA reprogramming cocktail components (OCT4, SOX2, KLF4, c-Myc, LIN28, and nGFP) and B18R recombinant protein were aliquoted per the manufacturer's instructions. Fibroblasts were seeded into in a 6-well plate coated with Matrigel (Corning #354248) at three densities: 15,000 cells/well, 22,500 cells/well, and 30,000 cells/well. The cells were incubated overnight at 37°C and 5% CO2 in hFib-R media. On Day 1 of reprogramming, prior to the first transfection, cells were incubated in Nuff-conditioned Pluriton reprogramming medium (from the Stemgent kit) with B18R (300 ng/mL) and bFGF (basic Fibroblast Growth Factor, 20 ng/µL) for at least 2 hours in a low oxygen incubator (37°C, 5% CO2, 4% O2). From this point forward, cells were cultured in a low oxygen incubator. Then, the cells were transfected with a Stemgent microRNA reprogramming transfection complex (in
the Stemgent kit) as follows: one 35 µL aliquot of the microRNA reprogramming cocktail was thawed and 107.5 µL of the provided Stemfect Buffer was added to microRNA reprogramming cocktail. In a second sterile RNase free microcentrifuge tube was added 125 µL Stemfect Buffer and 20 µL Stemfect RNA transfection reagent. The microRNA solution and the transfection solution were mixed together with a P1000 pipet tip and incubated at room temperature for 15 minutes to create the microRNA transfection complex. In a dropwise fashion, 25 µL of the microRNA transfection complex was added to each well with the drops distributed evenly across the well. On Day 2 of reprogramming, cells were preincubated for up to 20 minutes with Nuff-conditioned Pluriotn medium containing B18R (300 ng/mL) and bFGF (20 ng/µL). The cells were then transfected with the mRNA transfection complex as follows: one 50 µL aliquot of the mRNA cocktail prepared previously was thawed on ice and 75 µL of the Stemgent Buffer was added to it. In a second sterile RNase-free microcentrifuge tube was added 125 µL Stemfect Buffer and 20 µL Stemfect RNA transfection reagent. The mRNA solution and the transfection solution were mixed with a P1000 pipet tip and incubated at room temperature for 15 minutes to form the mRNA transfection complex. To each well was added dropwise 25 µL of the mRNA transfection complex, distributed around the well. Daily mRNA transfections were performed as in Day 2 for 12 days with a microRNA transfection also performed on Day 5.

On Day 13 of reprogramming, media was changed to MEF-conditioned iPSC media with bFGF (20 ng/µL). MEF-conditioned iPSC media consists of DMEM/F12 (Gibco #11320033), 20% v/v KnockOut Serum Replacement (Sigma #12306C), 1% v/v NEAA
(Gibco #11140050), L-glutamine (Gibco #25030-081), penicillin/streptomycin, and 2-mercaptoethanol (7 µL per 1000 mL media; Bio-Rad #1610710), which had been incubated with mouse embryonic fibroblasts for 24 hours. Media was changed daily, cells were monitored until colonies were identified, which were livestained with SSEA4 (Stemgent #09-0097), and picked to be transferred to a MEF feeder layer in a 6-well plate. When colonies began growing robustly, they were picked and transferred off of the MEF feeder layer and MEF-conditioned iPSC media to 6-well plates coated with Geltrex (ThermoScientific #A1413302) and E8 media (ThermoScientific #A1517001). Cell media was changed daily. Upon passaging, iPSCs were incubated with Thiazovivin (Santa Cruz #sc-361380) for the first 24 hours after passaging. iPSCs were frozen one well of a 6-well plate per vial in E8 media with 10% DMSO.

**iPSC characterization.** Immunocytochemistry staining for iPSC cells was carried out as follows. For SSEA-4 and TRA-1-60, StainAlive antibodies (SSEA4: Stemgent #09-0097, TRA-1-60: Stemgent #09-0068) were used to livestain for the markers. Antibodies were diluted 1:200 in cell media and incubated 37°C for 15-20 minutes. The antibody solution was aspirated and fresh media was used to wash the cells for 5 minutes at 37°C, after which the media was aspirated and PBS + 1% FBS was added before imaging. For OCT4 and NANOG staining, cells were fixed with cold 90% methanol for 3-5 minutes, then washed with PBS and incubated with antibodies (OCT4: Santa Cruz #sc-101534; NANOG: Abcam #ab21624) in PBS + 1% FBS overnight at 4°C. Cells were washed with PBS for 10 minutes and incubated with secondary antibodies (OCT4: AlexaFluor antimouse 488, 1:500; NANOG: AlexaFluor antirabbit 555, 1:500) for 1 hour. The cells
were then washed with PBS with 1:2000 Hoecht 33342 for 10 minutes and again with PBS for 10 minutes, before imaging in PBS + 1% FBS. Alkaline phosphatase staining was conducted using Stemgent Alkaline Phosphatase Staining Kit II (Stemgent #00-0055) according to manufacturer's instructions. All cells were imaged on a Zeiss Axiosvert microscope and 10X objective equipped with a Zeiss Axiocam digital camera.

G-type karyotype analysis was conducted by the Cell Line Genetics Inc. OCT4 and NANOG methylation analysis was conducted by EpigenDx.

Embryoid bodies were generated by lifting iPSC colonies until they dislodged from the well and the colonies folded in on themselves to form embryoid bodies with smooth outlines. The embryoid bodies were cultured in Costar Ultra Low Cluster Plates (Corning #CLS3471) in iPSC media, with feedings every two days and daily agitation to prevent clumping. RNA was collected from the embryoid bodies via a conventional TRIzol organic phase extraction method and trilineage differentiation potential was assessed with the Taqman hPSC Scorecard according to the manufacturer's instructions (ThermoFisher #A15876).

*Neural Progenitor Cell Generation from human iPSCs.* Neural progenitor cells were generated from human iPSCs with the Gibco Neural Induction system (ThermoScientific #A1647801). iPSCs were split in single-cell suspension with Thiazovivin at around 15-20% confluency in a Geltrex-coated 6-well plate. Neural induction media (NIM; Gibco #A1647801) was applied to cells daily for 7-9 days until wells were confluent. The cells
were then passaged 1:6 – 1:10 using StemPro Accutase (ThermoScientific #A1110501) in NIM with Thaizovivin, after which the cells were transitioned to neural expansion media (NEM; 50% Neuralbasal medium, Gibco # 21103049; 50% Advanced DMEM, Gibco #12491015, with neural induction supplement from Gibco #A1647801). Thereafter, NPCs were grown in NEM on Geltrex-coated plasticware.

Immunocytochemistry was carried out for SOX1, SOX2, Nestin and CXCR4 by fixation in 4% paraformaldehyde, followed by a washing with PBS and incubation with primary antibodies (Nestin: EMD Millipore AB5922, SOX1: Cell Signaling #4194S, SOX2: Cell Signaling #3579, CXCR4: BD Biosciences #555976) in PBS + 1% FBS overnight at 4°C. Cells were washed with PBS for 10 minutes and incubated with secondary antibodies (CXCR4: AlexaFluor antimouse 488, 1:500; others: AlexaFluor antirabbit 555, 1:500) for 1 hour. The cells were then washed with PBS with 1:2000 Hoecht 33342 for 10 minutes and again with PBS for 10 minutes, before imaging in PBS + 1% FBS. Cells were imaged on a Zeiss Axiovert microscope and 10X objective equipped with a Zeiss Axiocam digital camera.

For qCPR, RNA was collected, cDNA synthesized, and qPCR conducted as described in Chapter 3.11. Taqman probes used are as follows: ThermoFisher, GRN: Hs00963707_g1, GAPDH: Cat.#432924E.
Chapter 8

Summary and Perspectives
Chapter 8: Summary and Perspectives

Frontotemporal dementia, the second most common form of presenile dementia after Alzheimer’s disease, is a devastating disease that afflicts 5-15% of individuals with dementia between the ages of 45 and 65 and their families and caretakers. Rooted in the neurodegeneration of frontal, insular, and anterior temporal lobe neural circuits, FTD strikes and progresses rapidly, with possible clinical phenotypes of personality and behavior changes, language impairment and progressive aphasia, and motor neuron diseases. Basic research around FTD epidemiology and genetics has led to characterizations in the biological and genetic underpinnings of the disease, but there are still no FDA-approved therapeutics to slow, stop, or reverse disease progression. As the field uncovers more on the etiology of the disease and therapeutically targetable pathways, more informed decisions around drug development can be made.

One of the known autosomal dominant forms of FTD, accounting for 5-15% of all FTD cases, is caused by mutations in the GRN gene encoding the multifunctional secreted glycoprotein, PGRN. Over 70 distinct mutations have been found in the exons of GRN, with the majority causing a frameshift leading to truncation and functional nullification of the gene by nonsense-mediated mRNA decay. Mutations in only one copy of the gene lead to PGRN haploinsufficiency and associated neurodegeneration with characteristic accumulation of ubiquitin and TAR DNA-binding protein (TDP). Increasing PGRN protein levels by upregulating GRN expression of the wild-type allele is one therapeutic avenue that has shown promise in several animal models. Thus, increasing PGRN levels in human neurons may help to restore a wild-type phenotype, and may be able to delay disease pathogenesis and neuron
degeneration. Small molecule probes that achieve this may also be able to help elucidate the role of PGRN in cellular pathways and in the context of FTD pathophysiology. This dissertation described efforts to identify small molecule modulators of PGRN using a human neuronal model and a panel of robust mRNA and protein expression assays, with future directions to further characterize effects of epigenetic modifiers of PGRN expression in human FTD patient iPSC-derived neuronal models generated using reprogramming technology.

**HDAC inhibitors**

Histone deacetylase (HDAC) inhibitors have previously been identified as small-molecule enhancers of PGRN expression, although the mechanisms through which GRN is epigenetically regulated and the requirement for inhibition of specific HDAC family members remain poorly understood. Using human induced pluripotent stem cell (iPSC)-derived neurons and a chemogenomic toolkit, we systematically assessed the ability of HDAC inhibitors to regulate GRN mRNA and PGRN levels both intracellularly and extracellularly. The data provide strong evidence that inhibition of Zn$^{2+}$-dependent Class I HDACs is sufficient to enhance PGRN expression in human neurons and that only inhibitors which exhibit fast-on kinetics to their HDAC targets are able to enhance GRN expression, potentially due to in part to kinetics of chromatin rearrangement and the shifting landscape around which HDACs and HDAC inhibitors must engage. These observations have important implications for the design of HDAC inhibitor-based small molecule therapies for PGRN-deficient FTD. If the kinetics of binding are a requirement for HDAC inhibitors to modulate GRN expression, this factor
must be taken into account when designing next generation HDAC inhibitor therapeutics for PGRN-deficient FTD. Moreover, because this kinetic distinction has not been found in animal models, it is important to understand the cell-type and species-specific differences between model systems when developing next-generation therapeutics.

We have also found upon studying the promoter/enhancer region of GRN that differential H3K27 acetylation enhancement occurred on a specific region of the GRN promoter upon treatment with a fast-binding HDAC inhibitor that did not occur with a slow-binding HDAC inhibitor, allowing is to link observed changes in GRN mRNA to changes in specific acetylation sites on the GRN promoter. Furthermore, our mechanistic studies comparing HDAC inhibitors with different binding kinetics revealed that enhancements of TFEB levels and TFEB occupancy on the GRN promoter were correlated with PGRN enhancement in human neuronal cell cultures, leading to another potential therapeutic target for PGRN-deficient FTD.

**BET bromodomain inhibitors**

This dissertation also introduces a novel mechanism of enhancing GRN mRNA and PGRN protein levels in human iPSC-derived NPCs and differentiated neurons: inhibition of bromodomain and extra-terminal domain (BET) proteins. BET-selective inhibitors with different chemical scaffolds were all found to increase GRN mRNA and PGRN intracellular and secreted protein levels in NPCs and neurons. We also found a strong correlation between GRN mRNA, PGRN intracellular protein, and secreted protein levels upon BET inhibitor treatment, implying an epigenetic regulatory mechanism. BET inhibitors have been compounds of interest in the treatment of many
diseases and, with known oral bioavailability and blood brain barrier penetrance, could be a fruitful therapeutic direction for PGRN-deficient FTD. However, more work must be done to elucidate the mechanism by which BET inhibitors enhance \textit{GRN} expression. Understanding the occupancy of BET proteins on the \textit{GRN} promoter before and after BET inhibitor treatment would greatly add to this mechanistic insight.

\textit{mTOR inhibitors and FTY720}

Although HDAC inhibitors and BET inhibitors upregulate both \textit{GRN} mRNA and PGRN protein expression, ostensibly due to epigenetic mechanisms, not all compound classes regulate \textit{GRN}/PGRN in the same way. Upon studying the effects of mTOR inhibitors and S1PR antagonist FTY720 on \textit{GRN} mRNA and PGRN protein expression, we found that mTOR inhibitors only upregulated \textit{GRN} mRNA expression, likely due to the antagonistic effect of mTOR inhibitors on protein synthesis, and FTY720 only enhances PGRN secreted protein, perhaps by downregulation of sortilin coreceptor \textit{p75NTR}. These results highlight a critical aspect of PGRN-deficient FTD therapeutic development: not all compound classes will be appropriate for treating PGRN-deficient FTD. Treatment with mTOR inhibitors may upregulate autophagy pathways which would help clear accumulated TDP-43 from PGRN-deficient neurons, but they would not treat the underlying deficits in PGRN protein, despite upregulating \textit{GRN} mRNA. In addition, FTY720, an orally bioavailable FDA-approved drug for multiple sclerosis that is known to cross the blood brain barrier, may also not be appropriate for PGRN-deficient FTD if it prevents reuptake of PGRN into neurons.
Generating human iPSC-derived neuronal cell models from bvFTD patients

The ultimate goal of working in human iPSC-derived neuronal cell models is to query the potential of a probe or therapeutic toward reversing a disease phenotype in a genetically relevant cell context. For diseases like FTD, where the organ of interest can rarely be biopsied, iPSC-derived neuronal cell cultures also give us the ability to track disease progression in neurons. Here, we have acquired fibroblasts from two patients diagnosed with bvFTD with a novel GRN mutation and three cognitively normal family members. We have reprogrammed these fibroblasts into iPSCs using a nonintegrative mRNA reprogramming method, and have differentiated two of the PGRN-deficient iPSC lines into NPCs. Future directions include purification of the NPC cultures, differentiation into neurons, examination of the neurons for PGRN-deficient FTD relevant phenotypes, creation of genetic controls using the CRISPR-Cas system, and validation of small molecule compounds found to upregulate PGRN in control NPC and neuron cell lines.

Although FTD has become more well known in the neurodegeneration community, and has even entered the wider discourse around brain health, it is still considered a rare disease, particularly because the different strains of FTLD seem to operate under different genetic mechanisms. However, in recent years, interest into FTD, in particular, PGRN-deficient FTD disease mechanisms and therapeutics, have been on the rise. With more FTD patients being diagnosed, we are able to understand more about disease genetics and progression and with technologies like iPSC-derived systems, CRISPR, and more complete chemogenomic toolkits, we are able to more rationally identify drug targets for FTD. In addition, GRN has been linked to other
neurodegenerative diseases like Alzheimer’s disease and NCL, and research into the role that PGRN plays in different brain tissues can help in the development of therapeutics for these and other related diseases. The research in this dissertation provides insight into therapeutic avenues, and with future studies, may help illuminate the barren therapeutic landscape for this devastating disease.
References


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