PROGRANULIN BIOLOGY: SMALL MOLECULE ENHANCERS OF PROGRANULIN EXPRESSION AND BIOCHEMICAL ANALYSIS OF GRANULIN RECEPTORS

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DEDICATION

This work is dedicated to Dr. Ayse Cenik and Dr. Ziya Cenik. I am honored to have followed in their footsteps.

I am grateful to my mentors Joachim Herz and Gang Yu for their support and patience. Their intellectual curiosity and enthusiasm are contagious. I appreciate the guidance of my dissertation committee: Ilya Bezprozvanny and Jonathan Terman. I thank Eyup Akarsu and Emine Demirel-Yilmaz, my first academic mentors, for imparting my scientific foundation and supporting my decision to pursue graduate studies in the US.

I thank my fellow lab members, especially Chantelle Sephton, who is a co-author on all of my recent publications, and Dan Dries, who has been both an excellent mentor and a dear friend. This work would not have been possible without the superb support of our lab manager, Cong Yu. Colleen Dewey, Yu-Hong Han, Paul Mayer, Xunde (Daniel) Xian and Yun Wang provided critical reagents, technical expertise and candid critique, for which I am grateful.

Science nowadays is a collaborative effort and I have been very lucky to work with many outstanding scientists. In particular, I thank Shuxin Li, Bruce Posner, Shuguang Wei, Eric Dammer, Giovanni Coppola, Daniel Geschwind, Laura Mitic, Robert Farese, and Bruce Miller for rewarding research collaborations.

I thank Can Cenik, my brother and fellow scientist, for countless hours of stimulating discussions and his statistical expertise, which has been invaluable.

Finally, I thank my wife, Bercin, for her love and support. She has been my inspiration for a long time.

PROGRANULIN BIOLOGY: SMALL MOLECULE ENHANCERS OF PROGRANULIN EXPRESSION AND BIOCHEMICAL ANALYSIS OF GRANULIN RECEPTORS

by

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

December, 2012

PROGRANULIN BIOLOGY: SMALL MOLECULE ENHANCERS OF PROGRANULIN EXPRESSION AND BIOCHEMICAL ANALYSIS OF GRANULIN RECEPTORS

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Frontotemporal dementia (FTD) is the second most common presenile dementia syndrome. Mutations in the *GRN* gene account for about 20% of patients with familial FTD. The protein encoded by *GRN*, progranulin, is a secreted glycoprotein with growth factor-like and immunomodulatory activities. Human progranulin contains seven granulin domains (denoted granulins A through F) that can be individually liberated following proteolytic cleavage. It is uncertain whether the holoprotein, the granulins or both mediate the biological effects of progranulin. All pathogenic *GRN* mutations result in haploinsufficiency and decreased extracellular progranulin. Therefore, increasing progranulin expression from the wild-type allele or (pro)granulin receptor agonists may be therapeutic in FTD. The overall goals of the work presented here were to identify small molecule enhancers of progranulin expression and (pro)granulin receptors that can be drug targets for the treatment and prevention of *GRN* deficient FTD. As described

here, I discovered that suberoylanilide hydroxamic acid (SAHA), an FDA-approved histone deacetylase (HDAC) inhibitor, enhances *GRN* expression and nearly normalizes progranulin levels in haploinsufficient primary human cells from *GRN* mutation carriers. I also discovered that granulin A binds three proteins in solubilized extracts of rodent brain membranes: wolframin, excitatory amino acid transporter 1 (EAAT1), and the α_3 subunit of the Na^+/K^+ ATPase. I argue that these proteins are candidates for a putative granulin receptor.

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PRIOR PUBLICATIONS

Cenik B, Sephton CF, Kutluk Cenik BK, Herz J and Yu G (2012). "Progranulin: a proteolytically processed protein at the crossroads of inflammation and neurodegeneration." J Biol Chem **287**(39): 32298-32306.PMID:3463300

Demirel-Yilmaz E, **Cenik B**, Ozcan G and Derici MK (2012). "Various phosphodiesterase activities in different regions of the heart alter the cardiac effects of nitric oxide." J Cardiovasc Pharmacol **60**(3): 283-292

Dewey CM, **Cenik B**, Sephton CF, Johnson BA, Herz J and Yu G (2012). "TDP-43 aggregation in neurodegeneration: are stress granules the key?" Brain Res **1462**: 16-25.PMID:3372581

Sephton CF, **Cenik B**, Cenik BK, Herz J and Yu G (2012). "TDP-43 in central nervous system development and function: clues to TDP-43-associated neurodegeneration." Biol Chem **393**(7): 589-594

Cenik B, Sephton CF, Dewey CM, Xian X, Wei S, Yu K, Niu W, Coppola G, Coughlin SE, Lee SE, Dries DR, Almeida S, Geschwind DH, Gao FB, Miller BL, Farese RV, Jr., Posner BA, Yu G and Herz J (2011). "Suberoylanilide hydroxamic acid (vorinostat) up-regulates progranulin transcription: rational therapeutic approach to frontotemporal dementia." J Biol Chem **286**(18): 16101-16108.PMID:3091219

Dewey CM, **Cenik B**, Sephton CF, Dries DR, Mayer P, 3rd, Good SK, Johnson BA, Herz J and Yu G (2011). "TDP-43 is directed to stress granules by sorbitol, a novel physiological osmotic and oxidative stressor." Mol Cell Biol **31**(5): 1098-1108.PMID:3067820

Sephton CF, Cenik C, Kucukural A, Dammer EB, **Cenik B**, Han Y, Dewey CM, Roth FP, Herz J, Peng J, Moore MJ and Yu G (2011). "Identification of neuronal RNA targets of TDP-43-containing ribonucleoprotein complexes." J Biol Chem **286**(2): 1204-1215.PMID:3020728

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FTD – frontotemporal dementia

FTLD – frontotemporal lobar degeneration

PNFA – progressive nonfluent aphasia

SD – semantic dementia

CBS – corticobasal syndrome

PSP – progressive supranuclear palsy

HTS – high-throughput screening

SAHA – suberoylanilide hydroxamic acid

DMSO – dimethyl sulfoxide

qPCR – quantitative polymerase chain reaction

HDAC – histone deacetylase

NCL – neuronal ceroid lipofuscinosis

TNF – tumor necrosis factor

TNFR – TNF receptor

LSC – liquid scintillation counting

TDP-43 – TAR DNA-binding protein 43

LS/MS/MS – liquid chromatography tandem mass spectrometry

LTP – long term potentiation

FBS – fetal bovine serum

SDS – sodium dodecyl sulfate

PGRN – progranulin

CHAPTER ONE

INTRODUCTION

Acknowledgement

Parts of this chapter, including figures, have been reproduced, with or without modifications, from my published work, Cenik et al. (2012).

Frontotemporal Dementia

Historical Overview and Terminology

A dementia disorder characterized by predominantly behavioral and language dysfunction was first described by Arnold Pick in 1892 (Pearce, 2003). Alois Alzheimer, in 1911, described a specific form of brain pathology characterized by solitary, round, argyrophilic neuronal cytoplasmic inclusions, which were later named Pick bodies. The clinical syndrome described by Pick and the neuropathology described by Alzheimer were merged into the clinicopathological entity "Pick's disease" in the 1920s (Brun and Gustafson, 2011). In the following decades, either Pick's disease or "senile dementia of Alzheimer type" were used to describe all neurodegenerative dementias. Later, the term "frontotemporal dementia" (FTD) has been used as an umbrella term to refer to the clinical syndromes of "non-Alzheimer type" neurodegenerative dementias. Clinical presentation of these syndromes, i.e. changes in social, behavioral, and language function, contrasts with the predominant and early impairment of episodic memory in Alzheimer disease (Blennow et al., 2006). The first international meeting dedicated to FTD was organized in Lund, Sweden in 1986 (Brun and Gustafson, 2011). Since then,

the terminology and diagnostic criteria for FTD spectrum disorders has undergone many revisions as more information about the neuropathology, genetics, and molecular pathogenesis became available. While the terms FTD and frontotemporal lobar degeneration (FTLD) have sometimes been used interchangeably, FTLD commonly refers to the underlying neuropathology, consisting of frontal and temporal lobe atrophy associated with neuronal loss and gliosis (Tartaglia, 2012). Classically, three clinical syndromes are grouped under FTLD: behavioral variant-FTD (bvFTD), progressive nonfluent aphasia (PNFA), and semantic dementia (SD). Three additional clinical syndromes, namely corticobasal syndrome (CBS), progressive supranuclear palsy (PSP), and FTD with motor neuron disease (FTD-MND), are also included in the FTD spectrum (Roberson, 2006). CBS and PSP are associated with distinctive neuropathology: corticobasal degeneration (CBD) refers to the pathology in CBS, while PSP refers to both the clinical entity and the neuropathology.

Clinical Features

FTLD is the second most common presenile dementia disorder after Alzheimer disease, representing 5-15% of all dementias (Ratnavalli et al., 2002; Rabinovici and Miller, 2010). The most common presentation of FTLD is bvFTD. Disease onset is most common in the sixth decade with a male preponderance of two-to-one (Tartaglia, 2012). Early symptoms of bvFTD include apathy, disinhibition, lack of empathy, odd, compulsive or unlawful behaviors, overeating, and personality changes. Executive function rapidly deteriorates. 10-15 % of patients also suffer from motor neuron disease (FTD-MND).

In PNFA, early expressive aphasia is the primary presenting complaint. SD patients are usually fluent but have great difficulty with finding words and comprehension. CBS and PSP generally present with rigidity and parkinsonism. Substantial overlap of symptoms is observed later in the course of FTD spectrum diseases such that some degree of behavioral, language, cognitive or motor dysfunction is not uncommon in any of the aforementioned clinical syndromes (Kertesz et al., 2005).

There are no disease-modifying treatments. Symptomatic and supportive treatment is usually attempted with selective serotonin reuptake inhibitors, antipsychotics and behavioral and environmental interventions. Median survival after diagnosis is shorter than 10 years (Hodges et al., 2003) and may be as short as 3-4 years for bvFTD (Tartaglia, 2012).

Neuropathology

The underlying neuropathology of FTD spectrum disorders is varied; however, there are some common themes. Atrophy of brain parenchyma is usually more severe than Alzheimer disease. While the frontal and the anterior temporal lobes are affected earlier and more severely, the atrophy can be asymmetrical and almost any region of the brain, including the parietal lobes, hippocampus and basal ganglia, can be affected (Kril et al., 2005). Neuronal loss, ballooned or achromatic neurons (Pick cells), spongiosis/microvacuolation of cortical layers II and III, astrogliosis and microgliosis are observed (Broe et al., 2004; Roberson, 2006).

At the subcellular level, abnormal cytoplasmic accumulation of proteins is the hallmark of FTLD. The main proteinaceous components of these inclusions have been

identified. The first protein shown to be abnormally deposited in FTLD inclusions was tau (Wilhelmsen et al., 1994; Clark et al., 1998; Goedert et al., 1998; Hutton et al., 1998; Poorkaj et al., 1998), a microtubule binding protein necessary for intracellular transport and maintaining cellular shape (Goedert et al., 1996; Rademakers et al., 2004). "Tauopathies" account for all PSP and most CBS cases, in addition to a substantial proportion of FTLD cases, termed FTLD-tau. In the brain, tau exists in two alternatively spliced isoforms, 3R and 4R, which contain 3 or 4 microtubule binding domains, respectively. In bvFTD cases caused by FTLD-tau, the underlying pathology is sometimes referred to as Pick's disease. The characteristic lesion in Pick's disease is round, intraneuronal inclusions enriched in 3R tau. CBS and PSP are characterized by accumulation of 4R tau (Dickson et al., 2011). Tau is also the main component of the classical neurofibrillary tangles in Alzheimer disease.

Neuronal inclusions that do not stain for tau typically stain for ubiquitin. These cases were historically termed FTLD-U. The major protein component of most ubiquitinated, tau-negative inclusions has been identified as TDP-43, an RNA binding protein that regulates splicing (Arai et al., 2006; Neumann et al., 2006; Lagier-Tourenne et al., 2010; Polymenidou et al., 2011; Sephton et al., 2011). This specific neuropathology is now termed FTLD-TDP. TDP-43 protein in these inclusions is ubiquitinated, hyperphosphorylated and may be proteolytically processed. Loss of normal, nuclear staining for TDP-43 is typical. FTLD-TDP is further classified: FTLD-TDP A is characterized by numerous TDP-43 positive inclusions and short dystrophic neurites in layer II of the cortex; FTLD-TDP B exhibits transcortical involvement with fewer inclusions and dystrophic neurites; FTLD-TDP C predominantly involves layer II, with

many long, dystrophic neurites; FTLD-TDP D displays numerous neuronal intranuclear inclusions and fewer intracytoplasmic inclusions (Tartaglia, 2012).

Ubiquitin positive neuronal cytoplasmic inclusions that do not stain for TDP-43 were recently shown to contain FUS (fused in sarcoma), an RNA/DNA binding protein involved in transcriptional regulation, mRNA processing and microRNA biogenesis (Neumann et al., 2009a; Neumann et al., 2009b). This subtype is termed FTLD-FUS. It is associated with a distinct clinical syndrome and neuropathology, characterized by early age of onset, obsessive-compulsive features, and caudate nucleus atrophy. A very small fraction of FTLD cases display no apparent inclusions and are termed dementia lacking distinctive histopathology.

Genetics

One of the most striking features of FTLD is the high proportion of familial cases: about 30-50%. Multiple causative genes have been identified. Mutations in the MAPT gene (chromosome 17q21.1), which encodes tau, cause FTLD-tau as well as other tauopathies in the FTD spectrum, i.e. CBS and PSP (Wilhelmsen et al., 1994; Hutton et al., 1998). The frequency of MAPT mutations in FTD seems to vary according to the population studied (Rademakers et al., 2004); MAPT mutations causing about 15-30 % of familial FTD cases (8-15 % of all FTD cases) is a reasonable estimate. At least 50 causative mutations have been identified, comprising partial deletions, missense and splice site mutations. As mentioned above, alternative splicing of MAPT exon 10 results in tau protein isoforms with either 3 (3R) or 4 (4R) microtubule binding domains in the C-terminus. Small deletions and splice site mutations change the ratio of these tau

isoforms (3R/4R) (Goedert and Jakes, 2005). Large deletions (exons 6-9) and some missense mutations probably diminish microtubule binding while other missense mutations induce tau aggregation. Almost all patients with documented MAPT mutation have a family history of dementia, indicating autosomal dominant transmission with high penetrance (See et al., 2010).

Mutations in three genes have been shown to produce FTLD-TDP. Missense mutations in VCP (valosin containing protein, chromosome 9p13.3) result in a clinical syndrome consisting of FTD, inclusion body myositis and Paget disease of the bone. The brain pathology is of the FTLD-TDP D subtype. VCP (also called Cdc48) is an ATP-driven chaperone involved in membrane physiology and ubiquitin-mediated protein degradation (Meyer et al., 2012). Mutations in *GRN* (chromosome 17q21.32), encoding a proteolytically processed growth factor and immune regulator named progranulin, also cause FTLD-TDP. Progranulin biology will be reviewed in the next section. Recently, a hexanucleotide repeat expansion in C9ORF72 (chromosome 9 open reading frame 72) was linked to FTD and amyotrophic lateral sclerosis (ALS) (DeJesus-Hernandez et al., 2011; Renton et al., 2011). The functions of this gene are currently unknown.

Mutations in CHMP2B (charged multivesicular body protein 2B, chromosome 3p11.2) also cause familial FTLD (FTD-3). This gene encodes a part of the endosomal sorting complex in the endosomal/lysosomal system. The neuropathology is characterized by widespread spongiform changes in the cortex and sparse ubiquitin and p62 positive neuronal inclusions. Since p62 is a part of the ubiquitin-proteasome system, this neuropathology is termed FTLD-UPS.

Progranulin Haploinsufficiency Causes FTLD-TDP

In 2006, mutations in *GRN* (encoding progranulin) were discovered to be a cause of FTLD-TDP (Baker et al., 2006; Cruts et al., 2006). More than 70 mutations in *GRN*, almost all of which result in a null allele, have been identified in FTLD patients. In hereditary cases, the mode of inheritance is autosomal dominant with incomplete penetrance (Gass et al., 2006; Rademakers et al., 2007). Serum progranulin levels are lower in mutation carriers and patients (<~60 ng/mL) than in controls (>~125 ng/mL) (Finch et al., 2009; Sleegers et al., 2009). A few causative missense mutations also result in reduced levels of progranulin (Shankaran et al., 2008). About 20% of familial FTD cases (5-10% of all FTD cases) are caused by *GRN* mutations.

Clinical manifestations of heterozygous, loss-of-function *GRN* mutations include all frontotemporal lobar degeneration subtypes (bvFTD, PNFA and SD), parkinsonism, and CBS (van Swieten and Heutink, 2008; Rabinovici and Miller, 2010). The neuropathological phenotype is FTLD-TDP with brain atrophy (most severe in the frontal cortex), variable loss of pigmentation of the substantia nigra, hippocampal sclerosis, and gliosis (van Swieten and Heutink, 2008). The characteristic cellular pathology is neuronal cytoplasmic inclusions staining for TDP-43 and dystrophic neurites. Interestingly, TDP-43 protein binds *GRN* mRNA (Sephton et al., 2011; Colombrita et al., 2012).

Homozygous GRN Mutations

All FTD patients from families with *GRN* mutations reported so far have been heterozygous for the *GRN* allele. The mechanism of pathogenesis is haploinsufficiency, evidenced by the dominantly inherited null mutations and lower (=< 50 %) levels of

circulating progranulin in patients and mutation carriers. Even though *GRN* knock-out mice are viable and fertile, it has been assumed that homozygous loss of *GRN* in humans might be lethal. However, two homozygous *GRN* deficient patients have recently been reported (Smith et al., 2012). These patients presented with adult onset neuronal ceroid lipofuscinosis (NCL), suffering from progressive loss of vision, retinal dystrophy, cerebellar ataxia and seizures. Circulating progranulin was undetectable. NCLs are genetic, progressive lysosomal storage diseases, characterized by accumulation of lipofuscin (Kohlschutter and Schulz, 2009). At least 10 related disorders are now classified as NCLs. Causative mutations occur in genes encoding lysosomal enzymes and several incompletely characterized membrane proteins. Lipofuscin, an aggregate of oxidized, cross-linked proteins and lipids, accumulates during normal aging but this is greatly accelerated in NCLs. Lipofuscin can be toxic to cells by chelating metals, enhancing oxidative damage and inhibiting mitochondrial and lysosomal function (Jung et al., 2007). Interestingly, increased accumulation of lipofuscin has not been reported in cases of FTLD-TDP, but has been detected in mouse models of the disease.

Progranulin Biology

Historical Overview

In 1990, while screening for growth-modulatory peptides in tissue extracts, Shoyab et al. (1990) isolated two 6 kDa, cysteine rich peptides from rat kidney that they named epithelins 1 and 2. The same year, Bateman et al. (1990) isolated several 6 kDa, cysteine rich peptides from leukocyte granule extracts and named them granulins. Epithelins 1 and 2 are identical to granulins A and B, respectively. Two years later, both

groups announced the cloning of the gene encoding these peptides: *GRN*, which is translated into a large precursor protein containing 7½ granulin (epithelin) repeats (Bhandari et al., 1992; Plowman et al., 1992). In the following years, this granulin precursor protein was isolated from various biological sources and alternatively called proepithelin, acrogranin (Anakwe and Gerton, 1990; Baba et al., 1993), PC-cell derived growth factor (PCDGF) (Zhou et al., 1993) or progranulin (Bhandari et al., 1992; He and Bateman, 1999). Initial investigations focused on the role of progranulin in cancer (Zhang and Serrero, 1998). In 2002, Zhu et al. (2002) discovered that granulins are released following proteolytic cleavage of progranulin by elastase and reported on the immunomodulatory activities of progranulin and granulins in their seminal article published in Cell. With the discovery of *GRN* mutations in FTLD in 2006 (Baker et al., 2006; Cruts et al., 2006), interest in this protein peaked in the neuroscience field.

Evolutionary Conservation and Tissue Expression

Progranulin is evolutionarily conserved in Animalia: homologues exist in vertebrates and *C.elegans* (Kao et al., 2011), but seemingly not in *Drosophila*. It has no robust sequence homology to any other known protein family. Progranulin is widely expressed in epithelia, bone marrow, immune cells, solid organs, and the nervous system, both during development and in adulthood (Bhandari et al., 1992; Daniel et al., 2000; Daniel et al., 2003; Mackenzie et al., 2006; Matsubara et al., 2012). In the brain, intracellular expression is highest in neurons and activated microglia (Ryan et al., 2009; Ahmed et al., 2010; Petkau et al., 2010). At the subcellular level, progranulin co-localizes with ER and Golgi markers in the secretory pathway and the lysosomal marker Lamp1

(Ryan et al., 2009; Hu et al., 2010; Almeida et al., 2011). Progranulin is a glycoprotein (Zhou et al., 1993) and readily detected in blood and cerebrospinal fluid (Ghidoni et al., 2008; Van Damme et al., 2008).

Biological Activities

Reported biological activities of progranulin fall into three broad categories: growth factor-like activities, modulation of immune responses, and neuronal effects. Progranulin is overexpressed in many human and experimental tumors, including carcinomas (Donald et al., 2001; Ong and Bateman, 2003; Serrero, 2003; Cuevas-Antonio et al., 2010), gliomas (Liau et al., 2000) and sarcomas (Matsumura et al., 2006). It may act akin to a growth factor, stimulating proliferation (He and Bateman, 1999; Monami et al., 2009), survival (He et al., 2002), and invasion (Tangkeangsirisin and Serrero, 2004). Progranulin has been reported to activate many of the typical cell proliferation signaling pathways, including extracellular regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K) and Akt pathways (He et al., 2002; Monami, 2006; Feng et al., 2010), not only in tumor cells but also in neurons (Gao et al., 2010; Xu et al., 2011). Progranulin may be a prognostic marker (Wang et al., 2011; Serrero et al., 2012) or a therapeutic target in cancer: progranulin overexpression confers an aggressive phenotype to adenocarcinoma (He and Bateman, 1999), immortalized ovarian epithelial cells (Miyanishi et al., 2007), breast cancer (Tangkeangsirisin and Serrero, 2004; Elkabets et al., 2011) and hepatocellular carcinoma (Cheung et al., 2004), and anti-progranulin treatment reduces in vivo tumorigenicity of teratoma (Zhang and Serrero, 1998) and breast cancer cell lines (Lu and Serrero, 2000). However, it should be

emphasized that while these early studies strongly linked progranulin to cancer, no cell surface receptor has been shown to mediate these effects.

Full-length progranulin is generally anti-inflammatory; while proteolytically released granulins may have the opposite effect. Progranulin reduced reactive oxygen species production by immune-complex activated neutrophils (Kessenbrock et al., 2008) and blocked TNF-α induced immune responses, namely respiratory burst, degranulation and spreading of adherent human neutrophils (Zhu et al., 2002). Progranulin also attenuated TNF-α induced IL-8 release (Kojima et al., 2009). A recent finding suggests that these activities may be mediated at the level of TNF receptors (Tang et al., 2011). Progranulin expression is induced by inflammatory stimuli in astrocytes (Suh et al., 2012). Consistent with its immunomodulatory role, progranulin expression was found to be induced in multiple sclerosis (MS), a classic example of neuroinflammation (Vercellino et al., 2011). However, no difference in CSF progranulin levels was reported in MS by an earlier study (De Riz et al., 2010). Pickford *et al.* (Pickford et al., 2011) reported that progranulin may also be a chemotactic cue for microglia. In this study, intracerebral injection of progranulin led to microgliosis in excess of that seen in a control lesion. This is somewhat dissimilar to the knock-out studies discussed above, where the deletion of progranulin was shown to lead to gliosis. In contrast, Kessenbrock *et al.*(Kessenbrock et al., 2008) have reported that recombinant progranulin reduced neutrophil infiltration in a reverse passive Arthus reaction model. These disparate results may perhaps be explained by differential in vivo proteolysis of progranulin into pro-inflammatory granulins or by differential effects on microglia and neutrophils.

Progranulin expression is stimulated in the early phases of wound healing together with pro-inflammatory mediators (Zhu et al., 2002). In this context, progranulin may be an attractant for neutrophils, monocytes, fibroblasts and endothelial cells. It also stimulates tube formation of endothelial cells (He et al., 2003). In mice with genetic deletion of an inhibitor of progranulin degradation (SLPI), exogenous progranulin was shown to enhance cutaneous wound healing (Zhu et al., 2002). Recently, progranulin was found necessary for efficient activation of toll-like receptor 9 (TLR9) by CpG oligodeoxynucleotides (CpG-ODNs) (Park et al., 2011). In this study, macrophages from *Grn*$^{-/-}$ mice had a muted response to CpG. However, it was somewhat unclear whether full-length progranulin or granulins mediated this effect.

Neuronal Effects

One of the first reports about neuronal effects of progranulin was an article by Van Damme *et al*. (Van Damme et al., 2008), in which they showed that progranulin induced neurite outgrowth. This has been replicated by Gao *et al*. (Gao et al., 2010) but could not be replicated by Hu *et al*. (Hu et al., 2010). Exogenous recombinant progranulin increased survival of motor neurons in serum-free conditions in the study by van Damme *et al*., but knocking-down progranulin to ~20% of normal levels did not affect survival of hippocampal neurons in another study (Tapia et al., 2011). Two recent studies investigated the effect of progranulin deficiency on neuronal morphology and synaptic transmission. Both genetic deletion of *Grn* in mice (Petkau et al., 2012) and siRNA mediated knock-down of progranulin (Tapia et al., 2011) lead to reduced dendritic length and reduced spine density in hippocampal neurons.

Electrophysiologically, the ratio of field excitatory post-synaptic potential (fEPSP) slope to afferent volley amplitude was diminished in hippocampal slices prepared from *Grn*$^{-/-}$ mice (Petkau et al., 2012). This study also reported that induction of LTP in *Grn*$^{-/-}$ slices was more difficult and that mean LTP amplitude (change in fEPSP slope) was diminished in *Grn*$^{-/-}$ slices. The authors interpreted these findings as suggesting reduced synaptic connectivity and impaired synaptic plasticity in *Grn*$^{-/-}$ mice. However, the variation from slice to slice was large, especially in the *Grn*$^{-/-}$ group.

Progranulin knock-down in cultured hippocampal neurons resulted in reduced numbers of co-localized pre- and post-synaptic markers (i.e. reduced synapse density), increased number of synaptic vesicles per synapse (as revealed by electron microscopy), and increased mEPSC frequency (Tapia et al., 2011). This suggests decreased synaptic connectivity but enhanced transmission at individual synapses, indicative of a homeostatic response.

Proteolytic Cleavage of Progranulin and the Granulin Peptides

Human progranulin contains seven full-length and one half-length granulin domains, which are released following proteolytic cleavage of progranulin. These granulins are named, from the N-terminus of progranulin to the C-terminus, granulins p, G, F, B, A, C, D, E, with "p" denoting the half-length "paragranulin" domain (Fig.1.1). The molecular structure of individual granulin domains has been solved. Each granulin domain consists of parallel, stacked β-hairpins held together by six disulfide bonds (Hrabal et al., 1996; Tolkatchev et al., 2008) (Fig.1.1).

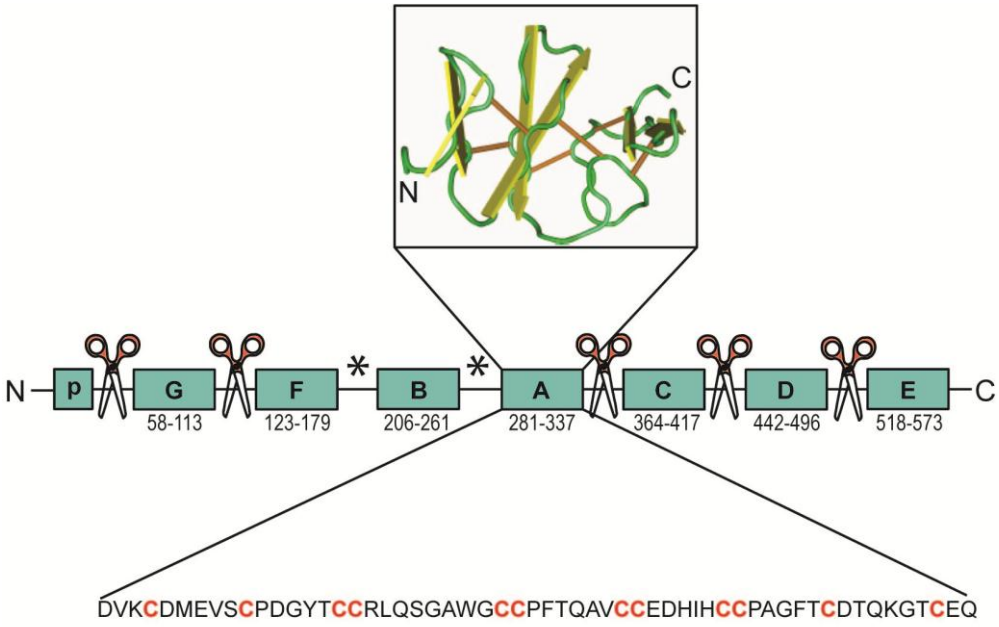


FIGURE 1.1 Domain Structure of Progranulin. Boxed letters denote granulin domains. The NMR structure of granulin A according to coordinates deposited by Tolkatchev et al. (2008) (MMDB ID 63884) is shown on top. Disulfide bridges are shown in orange, β-sheets in yellow, and the peptide backbone in green. Scissors denote elastase cleavage sites according to data presented in (Zhu et al., 2002). Asterisks denote linker regions where proteolytic cleavage also takes place, but the protease that releases granulins A and B has not been conclusively identified. The amino acid sequence of granulin A is shown at the bottom. Cysteines are highlighted in red. Numbers denote approximate positions of granulin domains relative to full-length human progranulin (593 residues).

Progranulin is proteolytically cleaved by neutrophil elastase (Zhu et al., 2002), proteinase 3 (a neutrophil protease) (Kessenbrock et al., 2008), MMP-12 (matrix metalloproteinase 12; macrophage elastase) (Suh et al., 2012), MMP-14 (matrix metalloproteinase 14) (Butler et al., 2008) and ADAMTS-7 (a disintegrin and metalloproteinase with thrombospondin motifs 7) (Bai et al., 2009). Zhu et al. (2002) have mapped the neutrophil elastase cleavage sites and shown that cleavage occurs in the linker regions between granulin domains. However, they did not detect any cleavage sites between granulins F, B, and A (Fig. 1.1) even though granulins A and B had been individually purified from various sources. Notably, incubation of recombinant progranulin with these proteases does not always result in the release of solely 6-12 kDa fragments as would be expected if the ~ 80 kDa precursor protein was completely processed to 7½ granulin domains. At least 5 intermediate products larger than 15 kDa seem to be present after over-digestion with either of these proteases according to data presented in published reports (Zhu et al., 2002; Kessenbrock et al., 2008; Suh et al., 2012). However, Kojima et al. observed mostly <16 kDa bands after 16h digestion at 37°C with elastase (Kojima et al., 2009). After in vitro incubation with MMP-12, several fragments (15-45 kDa) were still detectable with an antibody against the C-terminus of progranulin (Suh et al., 2012).

Secretory leukocyte protease inhibitor (SLPI) protects progranulin from proteolysis by elastase. Interestingly, SLPI binds progranulin directly and this interaction is protective against proteolysis even when the active site of SLPI is mutated (Zhu et al., 2002). Apolipoprotein A-I similarly inhibits progranulin proteolysis (Okura et al., 2010).

Biological effects have been attributed to the granulin peptides. Granulin A has been reported to induce anchorage-independent growth of cultured keratinocytes and fibroblasts while apparently inhibiting proliferation of other cancer cell lines (Shoyab et al., 1990; Plowman et al., 1992; Culouscou et al., 1993). The effect of granulin B was generally inhibitory and antagonistic to granulin A. At least in one case (Plowman et al., 1992), full-length progranulin did not have the same activity as granulin A. An independent group reported that granulin D increased DNA synthesis in cultured astrocytes and, to a limited extent, in primary glioblastoma cells (Liau et al., 2000). Granulin-E was reported to act similarly to progranulin and support neuronal survival in cell culture (Van Damme et al., 2008). Notably, purified, recombinant granulins do not have the anti-inflammatory activities of full-length progranulin but, on the contrary, seem to be pro-inflammatory (Zhu et al., 2002); elastase-digested progranulin induced IL-8 release from A549 cells whereas recombinant granulin B induced IL-8 release from both A549 and SW-13 cells.

Progranulin Receptors

Progranulin has a classic N-terminal signal peptide and several N-glycosylation sites (Songsrirote et al., 2010). It is readily secreted in cell culture and detected in serum and cerebrospinal fluid in animals. Exogenous, recombinant progranulin has many biological effects as detailed above. Hence several groups have searched for progranulin receptors. In 1993, Culouscou et al.(Culouscou et al., 1993) reported that radioactively labeled granulin A (epithelin 1) bound a 140-145 kDa protein on breast cancer cells. Later, Xia and Serrero (Xia and Serrero, 1998) reported that full-length progranulin

(referred to as PCDGF) bound to a 120 kDa protein on mink lung epithelial cells. However, the identities of these putative receptors were not revealed.

The first cell surface protein conclusively shown to bind progranulin was sortilin (Hu et al., 2010). Sortilin has diverse biological functions, including prosaposin trafficking to lysosomes (Zeng et al., 2009), hepatic VLDL secretion (Musunuru et al., 2010) and proneurotrophin-induced apoptosis (Lee et al., 2001). It is a regulator of extracellular progranulin levels. Sortilin knock-out mice are grossly normal, have increased levels of extracellular progranulin, and are resistant to motor neuron injury (Jansen et al., 2007). Sortilin does not seem to be a signal transducing receptor itself but acts as a co-receptor for the low affinity neurotrophin receptor $p75^{NTR}$. Neurotrophins are overexpressed in cancer (Akil et al., 2011) and implicated in neurodegeneration and inflammation (Wong et al., 2010). Progranulin binds sortilin via the C-terminus of the former, suggesting that the C-terminal granulin domain can potentially mediate the binding even after proteolytic cleavage. However, the precise role of progranulin binding in sortilin function remains unknown.

Perhaps the most promising candidates for the elusive progranulin receptor are the tumor necrosis factor receptors (TNFRs). In 2011, Tang et al. (2011) reported that progranulin bound to TNFRs with high affinity and blocked the binding of TNF-α. They also showed that progranulin or a synthetic progranulin fragment was therapeutic in an arthritis model , essentially acting as an endogenous TNF-α antagonist. Indeed, progranulin has anti-inflammatory effects. Zhu et al. (2002), had previously reported that full-length progranulin suppressed TNF-α induced neutrophil activation. However, these results have not been independently replicated (see chapter 3).

Antagonism of TNF-α can potentially explain some of the cancer-promoting effects of progranulin. TNF-α was first discovered as a humoral factor that caused rapid hemorrhagic necrosis of experimental tumors. It is cytotoxic to several tumor cell lines in vitro, increases endothelial permeability, may stimulate an anti-tumor immune response, and is in clinical use for immunotherapy of limb sarcomas (Verhoef et al., 2007). By over-expressing progranulin, a presumed TNF-α antagonist, tumors may escape TNF-α toxicity.

TNF-α is involved in synaptic scaling, maintaining synapses in a plastic state (Steinmetz and Turrigiano, 2010). After silencing, TNF-α enables up-regulation of surface AMPA receptor expression and mEPSC amplitudes (Stellwagen and Malenka, 2006). If progranulin is acting as a TNF-α receptor antagonist at the synapse, we would expect that progranulin deficiency would be functionally equivalent to over-abundance of TNF-α. However, this does not seem to be the case (Tapia et al., 2011).

Mouse Models of Progranulin Deficiency

Several independent mouse lines with genetic *Grn* deletions have been generated. Behaviorally, the most consistent finding is social interaction deficits (Yin et al., 2010b; Ghoshal et al., 2012; Petkau et al., 2012). In a classic test of hippocampal learning and memory (Morris water maze) $Grn^{-/-}$ mice had mild deficits at old age (18 months) in one study (Yin et al., 2010b) but no deficits at 8 months of age in another study (Petkau et al., 2012). Other reported mild behavioral deficits include depression-like behavior and either increased (Kayasuga et al., 2007; Petkau et al., 2012) or decreased anxiety (Yin et al.,

2010b). These behavioral phenotypes are fairly consistent with the clinical manifestations of FTLD, which include early behavioral problems and later deficits in memory.

Histopathologically, robust microgliosis, astrogliosis and increased ubiquitin staining is observed in the brains of aged *Grn*$^{-/-}$ mice (Ahmed et al., 2010; Yin et al., 2010a; Yin et al., 2010b; Ghoshal et al., 2012; Petkau et al., 2012). Ahmed et al. recently showed that intracytoplasmic ubiquitinated aggregates observed in these mice are probably composed of lipofuscin (Ahmed et al., 2010). Although some vacuolation was observed in habenula and hippocampus in very old (23 months) *Grn*$^{-/-}$ mice (Ahmed et al., 2010), overt neuronal loss seems to be very mild or absent (Ghoshal et al., 2012), in contrast with the severe atrophy observed in human FTLD. Yin *et al.* observed increased staining with an antibody against phosphorylated TDP-43 in the brains of 18 months old *Grn*$^{-/-}$ mice (Yin et al., 2010b); however, none of the other studies detected overt TDP-43 proteinopathy. Gliosis, commonly observed post-mortem in human FTLD, is robustly recapitulated in knock-out animals. However, the signature TDP-43 pathology has not been observed. Interestingly, none of these findings were reported in heterozygous *Grn*$^{+/-}$ mice which would be analogous to the haploinsufficient condition in human FTLD-TDP.

Progranulin-deficient mice display dysregulated immune responses in the brain (Yin et al., 2010a). Macrophages from *Grn*$^{-/-}$ mice express higher levels of pro-inflammatory cytokines (MCP-1, CXCL1, IL-6, IL-12p40 and TNF-α) in response to LPS, but they express less IL-10. Microglia cultured from these animals have toxic effects on co-cultured wild-type neurons. However, the immunomodulatory role of progranulin in the periphery may be different. In a recent study, *Grn*$^{-/-}$ mice on a high-fat diet had reduced IL-6 concentrations in blood and adipose tissue. Interestingly, *Grn*

ablation was protective against insulin resistance (Matsubara et al., 2012). Loss of the progranulin homolog results in accelerated clearance of apoptotic cells in *C.elegans* (Kao et al., 2011) and disruption of motor neuron development in zebrafish (Chitramuthu et al., 2010).

CHAPTER TWO

HIGH-THROUGHPUT SCREENING FOR SMALL MOLECULE ENHANCERS OF PROGRANULIN EXPRESSION

Abstract

Progranulin (*GRN*) haploinsufficiency is a frequent cause of familial frontotemporal dementia (FTD), a currently untreatable, progressive neurodegenerative disease. By screening the Prestwick Chemical Library®, we identified suberoylanilide hydroxamic acid (SAHA), an FDA-approved histone deacetylase inhibitor, as an enhancer of *GRN* expression. SAHA dose-dependently increased *GRN* mRNA and protein levels in cultured cells and restored near-normal *GRN* expression in haploinsufficient cells from human subjects. We conducted preliminary pharmacokinetic studies of SAHA in mice, which revealed a short half-life and relatively poor blood brain barrier penetration. We also screen a chemical diversity library of ten thousand additional compounds, discovering ~45 additional lead compounds that increase *GRN* reporter expression. SAHA has demonstrated therapeutic potential in other neurodegenerative diseases and represents a rational therapeutic approach for the prevention and treatment of *GRN* deficient FTD.

Acknowledgement

Data presented in this chapter, in part or in whole, has been published (Cenik et al., 2011). The text and the figures are reproduced, with or without modifications, from this publication.

Introduction

Several lines of evidence show that FTLD-TDP with *GRN* mutations is caused by haploinsufficiency. All *GRN* mutations reported so far are either null mutations (non-sense or frame-shift) or have been shown to lead to a non-functional allele. Deletion of the mouse homologue, *Grn*, recapitulates many aspects of FTLD. Furthermore, the concentration of progranulin in the serum is lower in patients and mutation carriers, compared to healthy controls. Based on the rationale that reduced progranulin expression causes FTLD-TDP, we hypothesized that increasing progranulin expression from the wild-type allele may prevent or slow down disease progression. This line of reasoning has also been followed by Capell et al. (2011), who recently reported that alkalizing drugs and vacuolar ATPase inhibitors increase progranulin expression through a post-transcriptional mechanism.

Many drugs in clinical use induce complex changes in gene expression (Iorio et al., 2010). One of the earliest and most successful examples of altering gene expression for therapeutic benefit is the case of hydroxymethylglutaryl-CoA reductase inhibitors, commonly known as statins, which induce the expression of the LDL receptor in the liver, thus clearing cholesterol from the blood (Ma et al., 1986). In addition to changing gene expression through signaling pathways, therapeutics may also act through chromatin remodeling. Thus, the role of epigenetics in the pathogenesis and therapy of neuropsychiatric disorders is an expanding area of research (Narayan and Dragunow, 2010).

Our objective in this project was to find small molecule enhancers of progranulin transcription by high-throughput screening of chemical libraries. We first screened the

Prestwick library, comprising 1200 marketed drugs, assuming the identification of an FDA-approved compound would accelerate the search for a cure for FTLD. We found that an FDA-approved drug, suberoylanilide hydroxamic acid (SAHA; Vorinostat) enhances progranulin expression robustly. Since, we have expanded our screening efforts to a chemical diversity library comprising 10,000 compounds. We have also carried out preliminary pharmacokinetic studies of SAHA in mice.

Methodology

Reagents and Antibodies

Cell culture reagents and TRIzol® were obtained from Invitrogen; SAHA (Vorinostat), MS-275, and CAY10591 from Cayman Chemical; Resveratrol, M344 *[4-(Dimethylamino)-N-[7-(hydroxyamino)-7-oxoheptyl]-benzamide]*, PTACH, dimethyl sulfoxide **(**DMSO), sodium butyrate, droxinostat, trichostatin A, leflunomide, and sodium valproate from Sigma; SRT1720 and MC1568 from Selleck Chemicals; Tubastatin A from BioVision Research Products (Mountain View, CA, USA). Tubacin was a kind gift from Stuart L. Schreiber (funded by Initiative for Chemical Genetics - National Cancer Institute).

Rabbit antibodies were generated against *Linker-3* anti-mouse progranulin peptide ([C]VPWMKKVIAPLRLPDPQIL, amino acid residues 353-371) conjugated to keyhole limpet hemocyanin.

Plasmids and Reporter Cell Lines

The firefly luciferase coding sequence was fused by bacterial recombination to the authentic human *GRN* start codon in exon 2 (NM_002087.2) on a bacterial artificial chromosome (BACPAC RP11-812N09) and stably transfected Neuro-2a cells (N2a) were derived utilizing co-transfection with a plasmid encoding Zeocin (Invitrogen) resistance.

Cell Culture and Drug Treatments

Neuro2a (N2a) and HEK 293 cells were grown in DMEM/10% FBS. Sodium valproate was dissolved in PBS. All other drugs were dissolved in DMSO (10-50 mM stock solutions kept at -80° C) and diluted in cell culture medium to a final DMSO concentration of 0.2-0.5%.

Human Cell Lines

All experiments pertaining to collection of human samples were approved by the UCSF Committee on Human Research. The human subjects and family members were recruited at UCSF Memory and Aging center and written informed consent was obtained. Genotypes were confirmed by direct sequencing. To obtain human dermal fibroblasts, skin biopsy samples were cut into small pieces, placed under a coverslip and grown in DMEM containing glutamine, sodium pyruvate, non-essential amino acids, 10% FBS, penicillin, streptomycin and Amphotericin B for about 3 weeks. Amphotericin B was omitted for further passages. The cells were used at 3rd or 4th passage.

Immortalized human lymphoblastoid cells were prepared as described(Neitzel, 1986). Briefly, white blood cells were obtained by Ficoll gradient centrifugation of the buffy coat from donor blood and transformed in growth medium containing 25% FCS,

1% phytohaemagglutinin and 10% Epstein-Barr virus supernatant. Rapidly growing cultures were maintained in RPMI-1640/10% FBS.

Library Screening, Luciferase Reporter Assays and Determination of Cell Viability

N2a cells were assayed in 384-well plates, 3000 cells per well. 6 hours after cell plating, 1200 Prestwick Chemical Library® compounds in DMSO, including internal controls, were dispensed using a BioMekFX to final concentrations of 2.5 µM compound and 1% DMSO (unless indicated otherwise). Sodium butyrate (9 mM) was used as positive control on each plate for initial screening. Luciferase activity was measured 24h after compound addition using Bright-Glo™ (Promega) reagent (20 µl per well). Each well was normalized to the average luminescence from DMSO-treated wells on the same plate. N2a cells were seeded in 384-well plates, 3000 cells per well. After 24h drug treatment, ATP content of each well was measured with the CellTiter-Glo® Luminescent Cell Viability Assay from Promega according to the manufacturer's instructions.

RNA Extraction and qPCR

Cells in 6-well plates were lysed in 500 µl TRIzol® reagent/well. cDNA was reverse transcribed with MultiScribe™ (Applied Biosystems). For some experiments, Quick-RNA MiniPrep system from Zymo Research (Irvine, CA 92614, USA) was used to isolate total RNA. Primer sequences were: human (h) U36B-F 5´-CGAGGGCACCTGGAAAAC-3´, hU36B-R 5´-CACATTCCCCCGGATATGA-3´, hGRN-S 5´-CAGGGACTTCCAGTTGCTGC-3´, hGRN-A 5´-GCAGCA-GTGATGGCCATCC-3´, mouse(m) cyclophilinQF1S 5´-GGAGA-

TGGCACAGGAGGAA-3′, mCyclophilinQR1A 5′-GCCCGTAGTGCTTCAGCTT-3′, mGRNS 5′-AGTTCGAATGTCCTGACTCCGCCA-3′ mGRNA 5′-AAGC-CACTGCCCTGTTGGTCCTTT-3′. Intronic(i)GRN_F1 5′-CCGGCT-ACTGTCCAGAGGTCC-3′, iGRN_R1 5′-CTAGGGGAGTTTCAAGAGGCAGGT-3′. qPCR reactions (10 µl) contained 20 ng cDNA, 150 nM primer and 5 µl SYBR FastGreen PCR Master Mix (Applied Biosystems), performed in triplicate on an Applied Biosystems Prism 7500 Fast sequence detection system. Relative mRNA levels were calculated using U36B or cyclophilinQ primers as internal controls.

SAHA Pharmacokinetics

18 female CD-1 mice (5 wks.) were administered 40 mg/kg SAHA ip, 0.2 ml/mouse formulated as 10% DMSO,10% cremophor EL, 80% D5W (5% Dextrose in water). Plasma was processed from whole blood by centrifugation of the ACD treated blood for 10' at 10000 rpm in a standard centrifuge. Brains were weighed and snap frozen in liquid nitrogen. Brain homogenates were prepared by mincing the brain tissue and homogenizing in a 3-fold volume of PBS (total volume of homogenate in ml = 4X weight in g.) 100 microliters plasma or brain homogenate was mixed with 200 microliters of acetonitrile containing 0.1% formic acid and 100 ng/ml IS (final concentration of IS = 20 ng/sample). The samples were vortexed 15 sec, incubated at room temp for 10' and spun 2 X 13,200 rpm in a standard microcentrifuge. The supernatant was then analyzed by LC/MS/MS. Buffer A: Water + 0.1% formic acid; Buffer B: MeOH + 0.1% formic acid; flow rate 1.5 ml/min; column Agilent C18 XDB column, 5 micron packing 50 X 4.6 mm size ; 0-1.5 min 3% B, 1.5 - 2.5 min gradient to

100% B, 2.5-3.5 min 100% B, 3.5 - 3.6 min gradient to 3% B, 3.6 - 4.5 min 3% B; IS N-benzylbenzamide (transition 212.1 to 91.1); compound transition 265.1 to 232.3.

Immunoblotting and Quantification

Cells were lysed in RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, Roche complete protease inhibitor cocktail) and cleared by centrifugation (20,000xg,10 min). 0.5 ml cell culture supernatants containing 1% FBS were concentrated by centrifugation (14,000xg, 50 minutes) in Millipore Amicon Ultra devices (3 kDa cut-off). 15 - 20 μg of total protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 5% milk for 1 h and probed overnight with the primary antibodies at 4º C. Mouse progranulin was detected with linker-3 AP antibody at 1:5000 dilution; β-actin, with Sigma anti-β-actin antibody (cat #A2228) at 1:5000; human progranulin, with Invitrogen anti-PCDGF antibody at 1:1000; GAPDH, with Sigma anti-GAPDH antibody at 1:10000. Bound IgG was detected by ECL. For quantitative immunoblotting, secondary antibodies labeled with IRDye® infrared dyes and an Odyssey® Infrared Imager (LI-COR® Biosciences) were used and manufacturer's instructions were followed.

Results

Identification of Potential Enhancers of Progranulin Expression by Chemical Library Screening

To generate a reporter of *GRN* promoter activity, the firefly luciferase gene was inserted by homologous recombination into the human *GRN* gene on a bacterial artificial

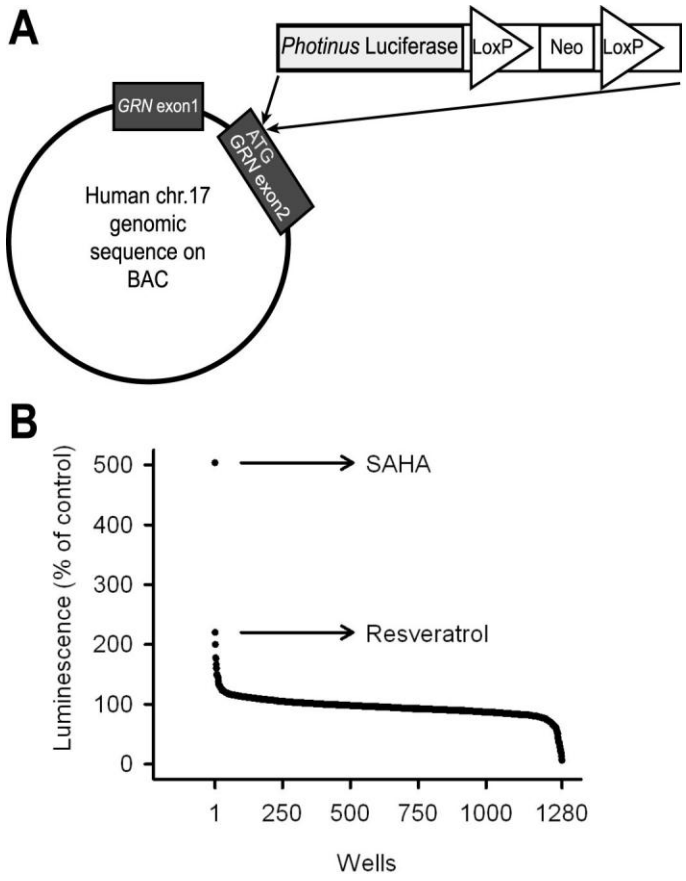
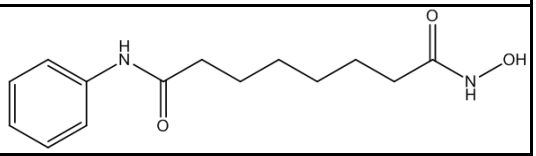
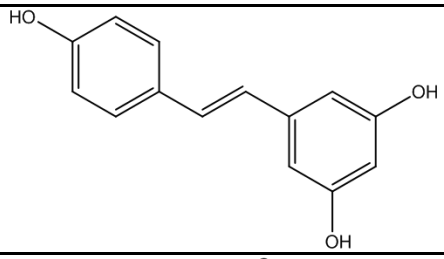
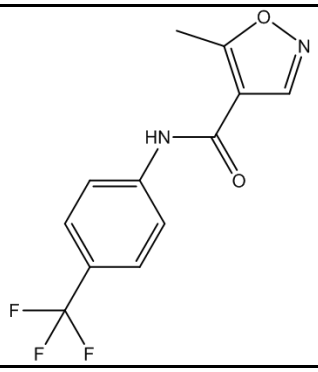
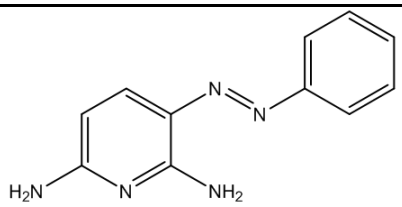
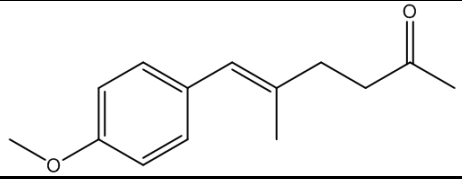


FIGURE 2.1 Luciferase Reporter Based, High-Throughput Screening For Small Molecule Enhancers of Progranulin Expression. *A.* Schematic representation of BAC based reporter construct. Luciferase coding sequence was inserted into the second exon of the human *GRN* gene, replacing the original start codon with the first codon of luciferase cDNA. *B.* Results from screening 1200 compounds comprising the Prestwick Chemical Library®. Neuro-2a cells stably expressing the reporter were treated with the compounds at 2.5 µM concentration for 24h and luciferase activity was measured afterwards. Arrows indicate the compounds that resulted in the top two highest activities.

chromosome (Fig. 2.1A). Utilizing co-transfection with a plasmid encoding Zeocin resistance, we stably integrated this construct into the neuronally derived mouse cell line N2a. Several independent clones robustly and stably expressed luciferase from the *GRN* promoter/enhancer under baseline conditions (Fig. 2.2). Next, we sought to validate our automated, 384-well, high throughput screening (HTS) strategy using sodium butyrate, a histone deacetylase (HDAC) inhibitor, as a positive control. The coefficients of variation were 5.3% for treatment wells and 8% for DMSO control, while Z´ value was 0.68, indicating a favorable signal/noise ratio. Utilizing this HTS assay, we screened the Prestwick Chemical Library ®, comprising 1200, marketed drugs and identified multiple compounds that increased *GRN* promoter activity more than three standard deviations above the mean, as summarized in Figure 2.1B and Table 2.1. >98% of the compounds in this chemical library had no significant effect on luciferase activity, indicating high specificity of the screening assay. The highest levels of activation resulted from addition of suberoylanilide hydroxamic acid (SAHA), a class I and class II HDAC inhibitor; resveratrol, a putative class III HDAC activator; and leflunomide, a pyrimidine synthesis inhibitor. We established the dose response curves for these three compounds, as shown in Figure 2.3A. SAHA and resveratrol dose-dependently increased luciferase activity in the reporter assay with sub-micromolar EC_{50} values: 0.51 μM for SAHA and 0.24 μM for resveratrol. Leflunomide had minimal activity and was not pursued further.

TABLE 2.1 Activating Compounds Identified By Screening the Prestwick Chemical Library®.

Compound	Fold change	Z-score*	Structure
SAHA** (Vorinostat)	5.0	21.3	
Resveratrol	2.2	6.5	
Leflunomide	2.0	5.4	
Phenazopyridine HCl	1.8	4.3	
Nabumetone	1.8	4.2	

* Z score=(compound normalized activity - average normalized activity)/population standard deviation
** SAHA= Suberoylanilide hydroxamic acid

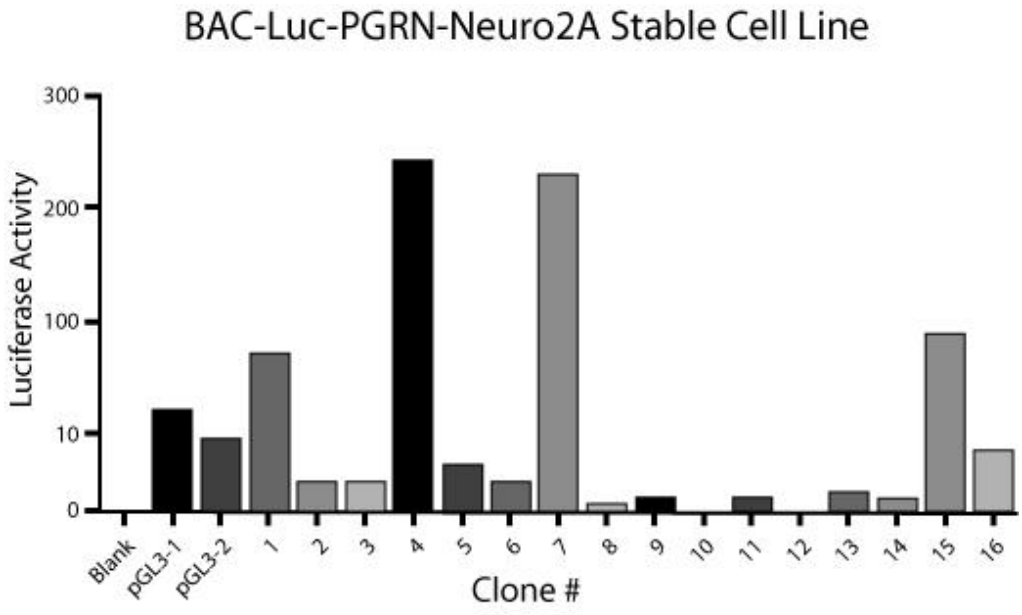


Figure 2.2 N2a Cell Lines Stably Expressing Luciferase Reporter. The cell lines were derived by co-transfection of the BAC based reporter construct and pcDNA4 encoding Zeocin resistance followed by limiting dilution in Zeocin containing medium. Luciferase activity is shown in arbitrary units. pGL3-1 and pGL3-2 indicate positive control samples: N2a cells transfected with a plasmid encoding luciferase.

Pan-HDAC Inhibition Is Necessary For Reporter Activation

Since SAHA is a known HDAC inhibitor, we tested whether other HDAC inhibitors would have the same effect on our reporter assay. Pan-HDAC inhibitors TSA, PTACH and M344 significantly increased luciferase activity (Fig. 2.3B), although the effect of TSA was limited by toxicity at concentrations ≥ 0.1 μM.

Next, we tested whether selective inhibitors of class I HDACs would be effective in the luciferase reporter assay. Valproate and MS-275 only enhanced luciferase activity at the highest end of pharmacological concentrations (Fig. 2.3C). This effect further

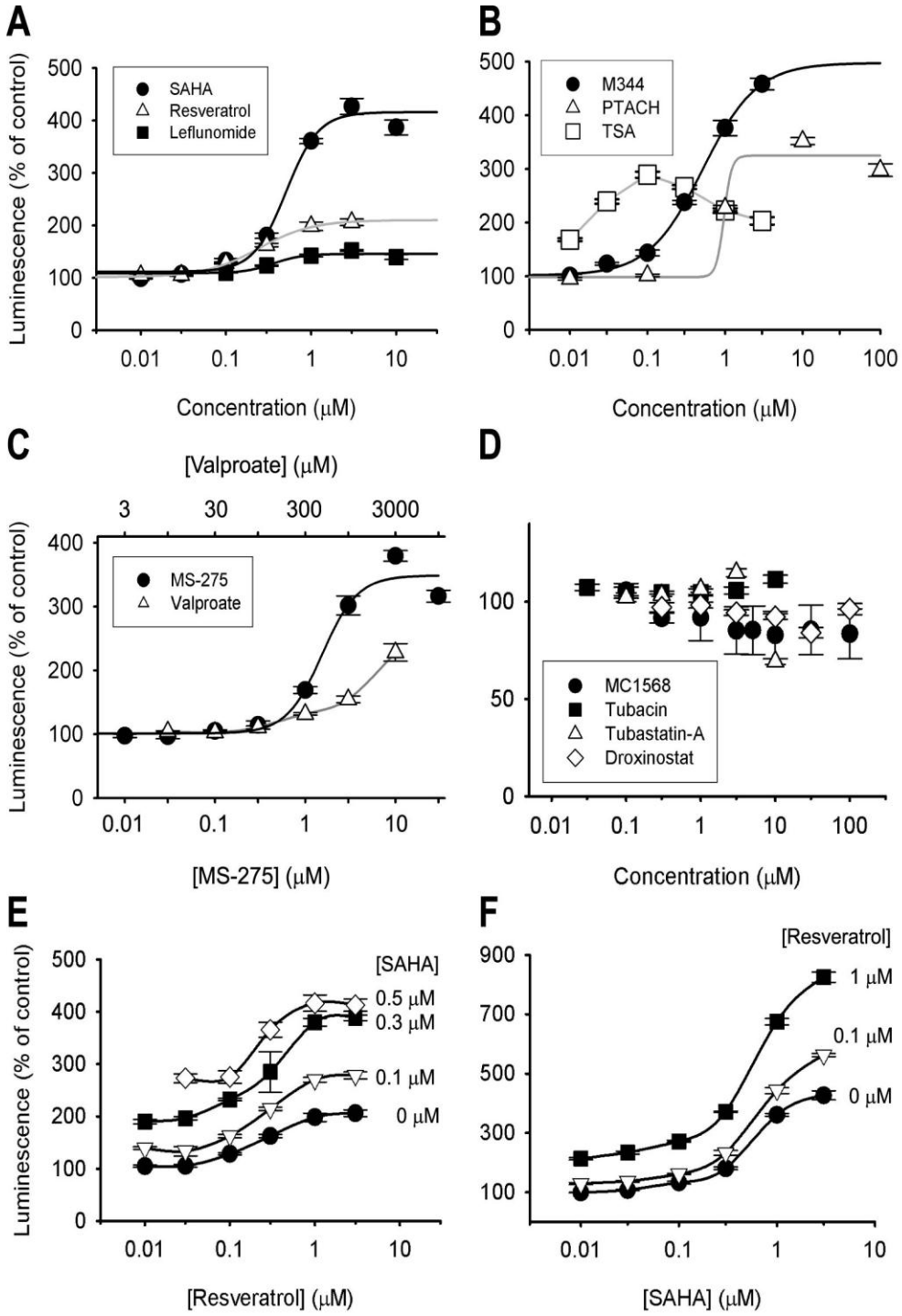

A
Luminescence (% of control)
SAHA
Resveratrol
Leflunomide
Concentration (μM)
B
M344
PTACH
TSA
Concentration (μM)
C
[Valproate] (μM)
MS-275
Valproate
[MS-275] (μM)
D
MC1568
Tubacin
Tubastatin-A
Droxinostat
Concentration (μM)
E
[SAHA]
0.5 μM
0.3 μM
0.1 μM
0 μM
[Resveratrol] (μM)
F
[Resveratrol]
1 μM
0.1 μM
0 μM
[SAHA] (μM)

FIGURE 2.3 HDAC Inhibitors And Resveratrol Dose-Dependently Increase Luciferase Reporter Activity. N2a luciferase reporter cell line used for screening was also utilized in follow-up experiments. *A*. Dose-response relationships for HTS hits SAHA (•, black line), resveratrol (△, gray line) and leflunomide (■, light gray line). EC_{50} values were 0.51 μM for SAHA and 0.24 μM for resveratrol. *B*. Dose-response relationships for HDAC inhibitors M344 (•, black line), PTACH (△, gray line) and trichostatin A (□, light gray line). *C*. Dose-response relationships for class I specific HDAC inhibitors MS-275 (•, black line), and sodium valproate (△, gray line). *D*. Selective HDAC inhibitors MC1568 (class II selective, •), tubacin (HDAC6 specific, ■), tubastatin A (HDAC6 specific, △) and droxinostat (HDAC3, 6, and 8 selective, ◇) did not increase *GRN* promoter reporter activity. *E*. SAHA and resveratrol have additive effects. Resveratrol concentrations tested are shown on the x-axis. SAHA concentrations tested are noted next to the dose-response curves. •, resveratrol alone; ▽, resveratrol + 0.1 μM SAHA; ■, resveratrol + 0.3 μM SAHA; ◇, resveratrol + 0.5 μM SAHA. *F*. SAHA and resveratrol have additive effects. SAHA concentrations tested are shown on the x-axis. Resveratrol concentrations tested are noted next to the dose-response curves. •, SAHA alone; ▽, SAHA + 0.1 μM resveratrol; ■, SAHA + 1 μM resveratrol.

supports our hypothesis that HDAC inhibition is the mechanism of action responsible for enhanced progranulin expression. Since these compounds could inhibit HDACs less selectively at these concentrations we cannot conclude that class I HDAC inhibition is sufficient for enhanced expression.

We also tested M344, a putatively selective inhibitor of HDAC6, and observed dose-dependent activation of the luciferase reporter at sub-micromolar concentrations. The EC_{50} (0.52 μM) and maximum effect size (about 4-fold) of M344 were similar to values observed for SAHA (Fig. 2.3B). However, two other HDAC6 specific inhibitors, tubacin (16) and tubastatin A (17) did not change luciferase activity (Fig 2.3D). M344, although 3-fold selective for HDAC6 over HDAC1, nevertheless inhibits HDAC1 with

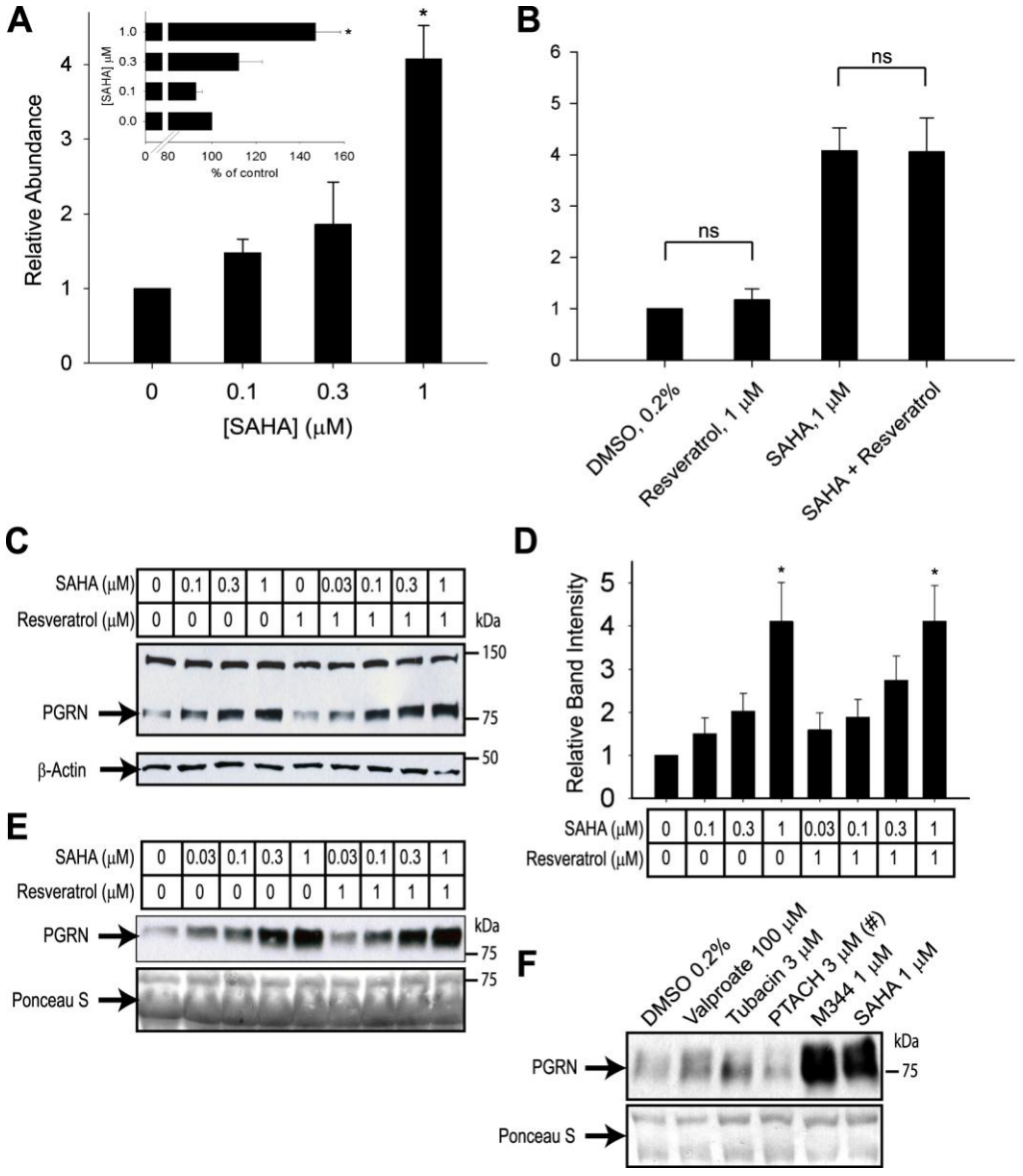


FIGURE 2.4 SAHA Enhances Progranulin Expression in Cultured Cells. *A & B.* Quantitative PCR analysis measuring relative abundance of *GRN* mRNA in Neuro-2a cells treated with SAHA or resveratrol for 24h. 1 μM SAHA significantly increased *GRN* expression while 1 μM resveratrol did not have any effect either alone or in combination with 1 μM SAHA. *A, inset.* The same samples were analyzed using qPCR primers designed to detect pre-mRNA. * denotes $p<0.05$, ns denotes $p>0.05$ versus vehicle control or for the

indicated comparisons. *C, D, E & F*. Neuro-2a whole cell lysates (*C,* quantified in *D*) and, cell culture supernatants (*E & F*) analyzed for progranulin protein expression by immunoblotting after 24h application of indicated drugs. (#) indicates that significant toxicity was observed with the indicated treatment.

an IC_{50} of 250 nM (Heltweg et al., 2004). Therefore, we conclude that HDAC6 inhibition is not sufficient to enhance progranulin reporter activity.

We furthermore tested droxinostat (a selective inhibitor of HDAC3, HDAC6, and HDAC8), and MC1568 (a class II selective HDAC inhibitor), but we did not observe any effects on luciferase reporter activity (Fig. 2.3D).

Since activation of sirtuins is one of the supposed biological effects of resveratrol, we also tested SRT1720 (0.01-1 μM) and CAY10591 (0.5 or 5 μM), other putative activators of sirtuins. These compounds had no effect on luciferase activity in the progranulin reporter assay (data not shown), suggesting that resveratrol may be acting through a different pathway.

SAHA and Resveratrol Have Additive Effects in the Progranulin Promoter Activity Assay

We tested the interaction between these two compounds, utilizing the reporter assay. As shown in Figure 2.3E and 2.3F, we observed an upward shift of dose-response curves when a fixed, sub-maximal concentration of either drug was tested in combination with a range of different concentrations of the other. Maximum effect size was about 4-fold with SAHA alone, about 2-fold with Resveratrol alone and about 8-fold with the combination of the maximal effective concentrations of both (Fig. 2.3F).

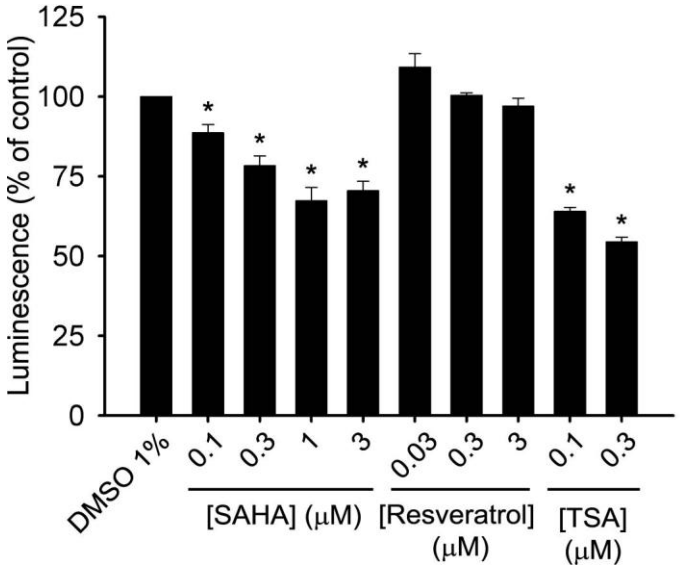


FIGURE 2.5. HDAC Inhibitors Moderately Decrease Cell Viability. Neuro-2a cells were treated with the indicated drugs for 24 hours and cell titers were measured with a luciferase based assay. Lower luminescence values correspond to lower ATP concentrations in the well. Error bars indicate SEM, * denotes $p<0.05$ versus vehicle control.

SAHA Increases Progranulin mRNA and Protein Levels in Neuro-2a Cells

To test whether SAHA and resveratrol augment transcription from the endogenous *GRN* promoter, we treated N2a cells with these drugs for 24h and performed qPCR to measure the relative levels of progranulin mRNA. 1 μM SAHA significantly increased the relative abundance of *GRN* mRNA (Fig. 2.4A). We repeated this experiment with primers designed to amplify *GRN* pre-mRNA but not mature mRNA and saw the same trend we observed using regular qPCR primers (Fig. 2.4A, inset). This suggests the increase in *GRN* mRNA is at least partially due to increased transcription.

In contrast to the luciferase reporter assay, we did not observe increased *GRN* mRNA expression with resveratrol treatment, either alone or in combination with SAHA (Fig. 2.4B). It is possible that resveratrol may have direct effects on luciferase enzyme

stability or activity in the reporter assay. Alternatively, the reporter may have been artificially inserted nearby a regulatory element responsive to resveratrol. These results emphasize the general importance of secondary validation of HTS hits.

We next tested whether increased *GRN* mRNA expression translates into increased protein levels. We analyzed total cell lysates from N2a cells treated with SAHA, resveratrol or a combination of both by Western blotting. After 24h treatment with SAHA alone or with SAHA plus resveratrol, the intensity of the progranulin immunoreactive band was dose-dependently increased compared to the loading control (Fig. 2.4C and 2.4D). In line with our qPCR results, the effect of resveratrol was muted. Progranulin is a secreted protein; therefore we also tested whether increased *GRN* expression translates into increased secretion. For this experiment, we treated N2a cells as indicated in Figure 2.4E, collected and concentrated conditioned medium and analyzed it by Western blotting. Treatment with increasing concentrations of SAHA resulted in more intense progranulin reactive bands, replicating our results from our qPCR and total cell lysate Western blot analyses.

We also tested whether the effects we observed in the luciferase reporter assay with different HDAC inhibitors would correlate with increased progranulin levels. As shown in Figure 2.4F, M344, but not PTACH, robustly increased secreted progranulin levels. At the concentrations required to increase transcription from the GRN promoter, PTACH reduced cell viability by approximately 20% and exhibited overt toxicity on remaining cells (data not shown). This pronounced toxicity readily accounts for the reduced accumulation of progranulin in the medium.

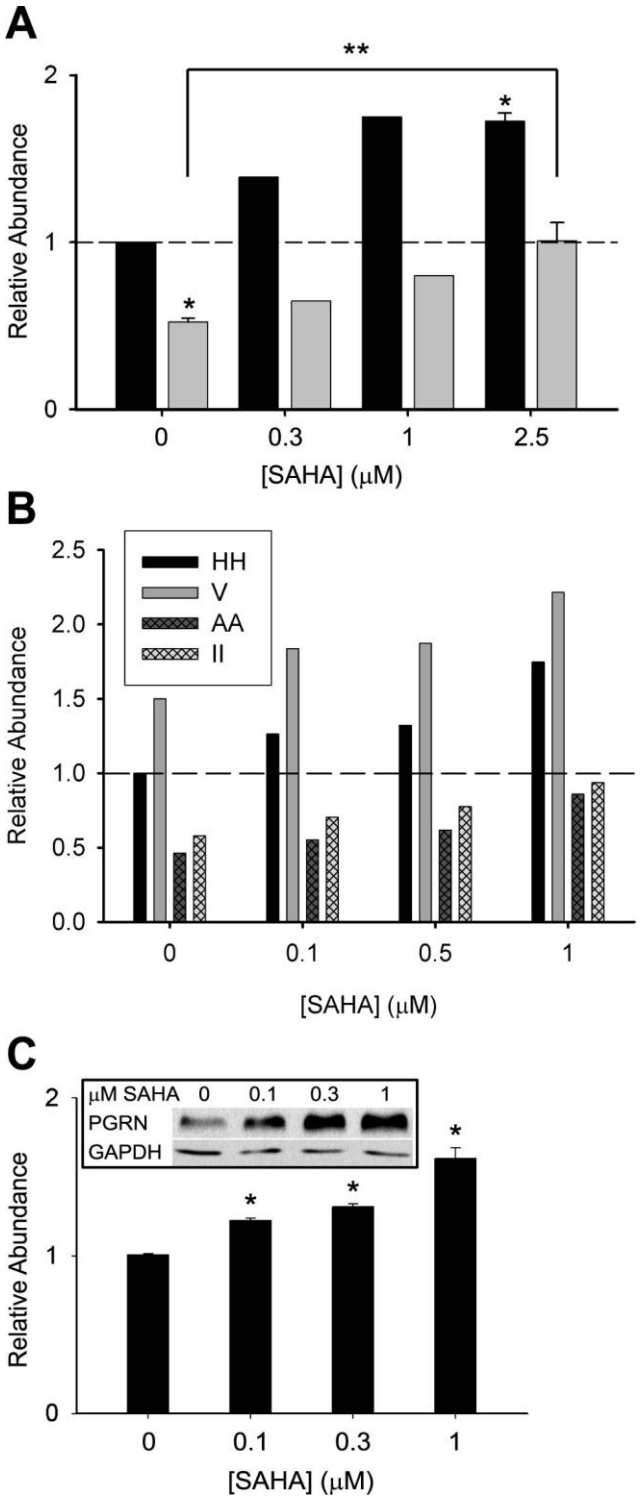

A
**
*
*
Relative Abundance
2
1
0
0
0.3
1
2.5
[SAHA] (μM)
B
HH
V
AA
II
Relative Abundance
2.5
2.0
1.5
1.0
0.5
0.0
0
0.1
0.5
1
[SAHA] (μM)
C
μM SAHA 0 0.1 0.3 1
PGRN
GAPDH
Relative Abundance
2
1
0
*
*
*
0
0.1
0.3
1
[SAHA] (μM)

FIGURE 2.6 SAHA Enhances Progranulin Expression in Haploinsufficient Human Cells. *A.* EBV-immortalized human lymphoblastoid cells from a subject with a non-sense *GRN* R493X mutation (gray bars) and from a relative carrying wild-type alleles (black bars) were treated with SAHA for 24h and relative abundance of *GRN* mRNA was measured by qPCR. . n=2 for 0.3 and 1 μM SAHA, n=3 for control and 2.5 μM SAHA. * denotes $p<0.05$ versus *GRN* +/+ control, ** denotes $p<0.05$ for the indicated comparison. Dotted line indicates relative abundance of 1. *B.* Human dermal fibroblast (HDF)s from two subjects heterozygous for *GRN* non-sense mutations (AA, and II, crosshatched bars) and from two control subjects (HH, and V) were treated as indicated and relative abundance of *GRN* mRNA was measured by qPCR. All samples were processed in parallel and $\triangle\triangle C_t$ values were normalized to one of the control samples (HH). Dotted line indicates relative abundance of 1. *C.* Cumulative data from the four cell lines shown in *B*, plus an independent HDF line with a heterozygous R493X mutation is shown. n=6 for 0 and 1 μM SAHA, n=4 for 0.1 and 0.3 μM SAHA. * denotes $p<0.05$ versus vehicle control. Inset shows western blot analysis of total cell lysates from HEK293 cells treated with the indicated concentrations of SAHA for 24h.

To assess the toxicity of SAHA, resveratrol and TSA, we treated N2a cells with these compounds in 384-well plates and measured cell titers 24h later with a luciferase based assay that measures ATP content of the wells. SAHA in concentrations ≥ 0.3 μM reduced cell viability by about 25% (Fig. 2.5), consistent with previously reported (Yin et al., 2007) effects.

SAHA Treatment Can Normalize Progranulin mRNA Levels in GRN +/- Human Cells

Since *GRN* null mutations lead to familial FTD through *GRN* haploinsufficiency, we tested whether SAHA could correct this progranulin deficit in human cells that contain only one wild-type allele of the *GRN* gene. For the experiment summarized in Figure 2.6A, we used EBV-immortalized human lymphoblastoid cells derived from a subject with a *GRN* null mutation (R493X) and similarly prepared cells from a wild-type

relative. *GRN* mRNA expression was reduced by about 50% in heterozygous lymphoblasts compared to control cells. Treatment with SAHA dose-dependently increased *GRN* mRNA levels in both haploinsufficient and control lymphoblasts (Fig. 2.6A). After 24h treatment with 2.5 µM SAHA, relative abundance of *GRN* mRNA in haploinsufficient cells was normalized to wild-type levels.

Furthermore, we obtained human dermal fibroblasts (HDFs) from two subjects with nonsense or frameshift *GRN* mutations (Q300X [II] and S226WfsX28 [AA]) and their unaffected siblings. We treated these fibroblasts with SAHA or vehicle and measured the relative abundance of *GRN* mRNA 24h later. Four cell lines were treated in parallel and, as shown in Figure 2.6B, the heterozygous cell lines had a relative insufficiency of *GRN*. SAHA dose dependently increased *GRN* mRNA in all cell lines, nearly normalizing *GRN* levels in the heterozygous cells at a concentration of 1 µM. Cumulative data from all 4 cell lines, plus another HDF line from a subject with a R493X mutation treated with only vehicle or 1 µM SAHA is shown in Figure 2.6C. Statistically significant increases in *GRN* mRNA were observed at all tested concentration (0.1-1 µM). The qPCR results from primary human cells were replicated at the protein level. Because basal expression of progranulin in the primary cells was too low to obtain consistent results with immunoblotting, we treated human HEK 293 with SAHA. As shown in Figure 2.6C inset, progranulin protein expression was robustly increased by the drug.

SAHA Pharmacokinetics in Mice

We carried out preliminary experiments to optimize *in vivo* administration of

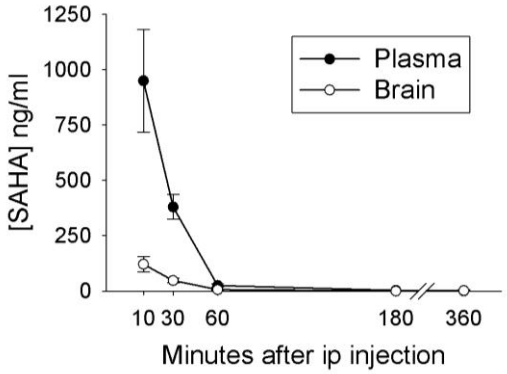


FIGURE 2.7 SAHA Pharmacokinetics in Mice. Female CD1 mice were injected SAHA 40 mg/kg ip. Blood and brain samples were collected at the indicated time points. SAHA concentrations in plasma and brain lysates were determined by LS/MS/MS. The following parameters were calculated: C_{max}: 950.3 ng/ml, T_{max}: 10 min, $T_{1/2}$: 61.6 min, blood/brain ratio: 0.11.

SAHA. We found out that the solubility of SAHA, contrary to a published report (Guan et al., 2009), is limited in saline-based aqueous vehicles, but workable in a mixture of 10% DMSO, 10% cremophor EL, and 80% D5W (5% dextrose in water). We tested the feasibility of intraperitoneal (ip) injection as a delivery method in mice. After 40 mg/kg ip administration to CD1 female mice, we collected blood and brain samples and measured SAHA concentrations by LC/MS/MS (Fig. 2.7). The following pharmacokinetic parameters were obtained: C_{max}= 950.3 ng/ml, T_{max}=10 min, $T_{½}$=61.6 min. The blood-brain ratio was 0.11. No gross acute toxicity was observed. In our cellular assays, the EC_{50} for *GRN* reporter up-regulation was ~130 ng/mL, which is easily achievable in plasma with ip injections.

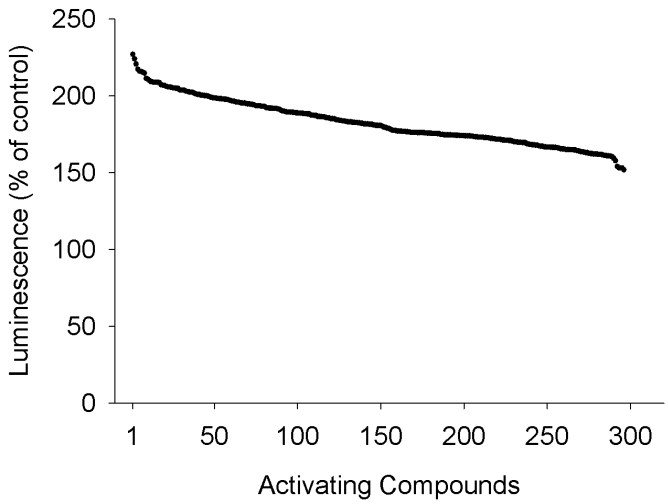
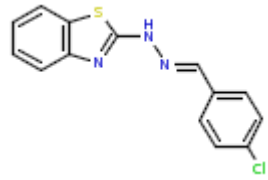
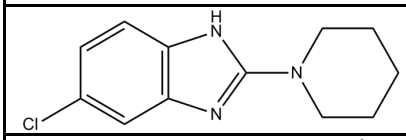
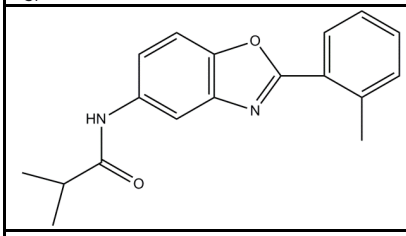
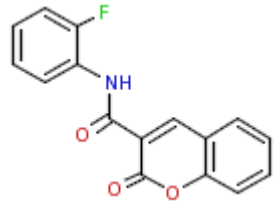
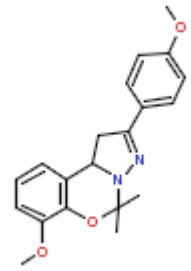


FIGURE 2.8 Activating Compounds Identified By HTS. In a screen of 10,000 compounds, 296 were identified as enhancing *GRN* luciferase reporter activity by more than 3 SD above the mean for a hit rate of ~3%.

10,000-Compound Chemical Diversity Library Screening

We utilized our progranulin-luciferase reporter to screen approximately 10,000 compounds for enhancers of progranulin expression. These compounds were part of a chemical diversity library, kindly provided by Dr. Bruce Posner and the HTS facility at the Simmons Cancer Center. We identified approximately 45 compounds that increased reporter activity at least two-fold (Fig. 2.8 and Table 2.2). Although these initial leads are promising, none of these compounds increased progranulin expression as strikingly as SAHA. However, some of these hits have reported biological activities (Table 2.2).

Table 2.2 Chemical Structures of Selected Small Molecules Identified in A Screen of 10,000 Compounds

Compound Structure	Z-score*	% Activation	Putative Biological Activity
	6.64	211	
	6.61	211	Reportedly active in a screen for lifespan altering molecules
	6.38	224	Reportedly active in a screen for potential muscular dystrophy and cachexia treatments
	5.94	216	
	5.92	215	

***** Z score=(compound normalized activity - average normalized activity)/population standard deviation

Discussion

Frontotemporal dementia is a devastating and fatal disease. Identification of drug targets that can be exploited to slow down or reverse the cognitive decline will probably depend on a better understanding of the molecular pathogenesis. Familial forms of the disease with known pathogenic mutations provide an opportunity for fast-track development of new therapeutics for FTD.

We chose the Prestwick library, comprising 1200 marketed drugs, for our initial screening, assuming the identification of an FDA-approved compound would accelerate the search for a cure for this disease. We discovered that SAHA, an HDAC inhibitor currently in clinical use for cutaneous T-cell lymphoma (Mann et al., 2007), enhances progranulin expression in a variety of relevant cell types in culture.

Physiological regulation of progranulin expression is largely unknown. Two recent studies have identified microRNAs miR-107 (Wang et al., 2010) and miR-29b (Jiao et al., 2010) as negative regulators of *GRN* expression. Also, a common variant in a putative miR-659 binding site of *GRN* was identified as a risk factor for FTD (Rademakers et al., 2008). Previously, Bhandari et al. had analyzed the promoter region of *GRN* (Bhandari et al., 1996) and identified various possible binding sites for transcription factors. Interestingly, two of the most prominent of these, GATA1 and SP1 have been reported to interact with HDACs (Watamoto et al., 2003; Liu et al., 2010), SP1 also being involved in the same regulatory network with miR-29b.

Recently, Capell et al. reported that an inhibitor of vacuolar ATPase, bafilomycin A1, and alkalizing drugs chloroquine, bepridil, and amiodarone increased secreted progranulin levels from haploinsufficient primary human lymphoblasts, although only

bafilomycin A1 increased the protein to near-normal levels (Capell et al., 2011). Bafilomycin exerts its effect through a post-transcriptional mechanism, suggesting a potential synergistic application with SAHA for FTD prevention, depending on toxicity and tolerated dose.

Small molecule inhibitors of HDACs are being developed as drugs for cancer and neurological disorders, such as Rubenstein-Taybi syndrome, Friedrich's ataxia and Fragile-X syndrome (Kazantsev and Thompson, 2008). Several compounds are now in clinical trials for cancer therapy and SAHA has been approved by the FDA for treatment of cutaneous T-cell lymphoma. SAHA has been investigated in a very similar paradigm for the treatment of motor neuron disease, and it was shown to enhance the expression of *SMN2* in brain slices, potentially substituting for the loss of *SMN1* expression, the gene mutated in spinal muscular atrophy (Hahnen et al., 2006). SAHA was shown to improve rotarod performance in a mouse model of Huntington's disease (Hockly, 2003). Furthermore, inhibition of HDAC2 by SAHA was reported to enhance memory formation in mice (Guan et al., 2009). HDAC inhibitors also have anti-inflammatory properties (Glauben et al., 2006; Lin et al., 2007; Halili et al., 2010). Interestingly, changes in inflammatory markers induced by SAHA treatment in a rodent septic shock model (Finkelstein et al., 2010) was similar to the effects of recombinant progranulin and a progranulin-mimetic peptide in a rodent inflammatory arthritis model . These findings suggest two possibilities; first that increasing the expression of progranulin, a protein with anti-inflammatory, growth promoting activities, may underlie some of the therapeutic effects observed in other conditions. Alternatively or in addition, SAHA may

have secondary beneficial effects in frontotemporal dementia, besides normalizing progranulin deficiency.

The second most efficacious hit in our screen was resveratrol, a plant polyphenol currently marketed as a dietary supplement. Resveratrol is implicated in neuroprotection against diverse types of injury (Araki, 2004; Vingtdeux et al., 2008; Zhang et al., 2010). Although resveratrol has been suggested to be an activator of sirtuins, this has been called into question recently (Pacholec et al., 2010) and the mechanism of action remains poorly understood (Calamini et al., 2010; Tang, 2010). We confirmed that resveratrol was effective in the luciferase based assay; however, we did not observe a significant increase in progranulin mRNA or protein levels by qPCR or Western blotting after treatment with resveratrol in any cell type. There can be several explanations for this discrepancy. Firstly, resveratrol may have direct effects on the luciferase enzyme stability or activity, thus representing a false positive hit. Given the small number of hits from our library screen, we opted not to do a counter-screen for luciferase activation from a different promoter, opting instead to validate hand-picked hits directly as we show here for SAHA. Secondly, resveratrol is reported to have diverse effects on gene expression and chromatin re-modeling (Yang et al., 2009; Kai et al., 2010), therefore the actual integration site of the reporter into the genomic DNA of the stable cell line may have resulted in a false-positive result. Lastly, albeit unlikely, the sensitivity of qPCR and Western blotting may not be high enough to detect less than 2-fold changes in progranulin levels under our experimental conditions.

HDAC inhibitors have a good track record as potential therapeutics. Our preliminary pharmacokinetics data shows that SAHA penetrates the blood brain barrier to

some extent. The next step will be to replicate our findings in vivo. Furthermore, we reasoned that other classes of compounds could also increase progranulin expression. Indeed, our 10,000-compound screen yielded some interesting hits. These hits will need to be verified. Since there is little information about their biological activities, medicinal chemistry efforts may be required to optimize pharmacodynamics and pharmacokinetics of these compounds. Ultimately, we hope that SAHA, another HDAC inhibitor, or a compound from a different class identified in our follow-up screen may be useful in the treatment of *GRN* deficient FTD.

Attributions and Publications

Data presented in this chapter has been published (Cenik et al., 2011). All qPCR experiments were done by Dr. Chantelle Sephton. The BAC-based luciferase reporter plasmid was generated by Dr. Wenze Niu. Dr. Xunde (Daniel) Xian helped with the derivation of N2a reporter cell line. Dr. Bruce Posner supervised and Dr. Shuguang Wei provided expert technical assistance for the HTS experiments. Drs. Giovanni Coppola, Suzee E. Lee, Daniel H. Geschwind, Bruce L. Miller, and Robert V. Farese, Jr provided human cell lines from FTD patients. Dr. Noelle Williams measured SAHA concentrations for the pharmacokinetics study. All other experiments described in this chapter were designed and conducted by me.

CHAPTER THREE

BIOCHEMICAL ANALYSIS OF PROGRANULIN AND GRANULIN RECEPTORS

Abstract

Progranulin is an extracellular protein with growth factor-like and immunomodulatory activities. Granulins are also released extracellularly and may have biological activities. We undertook a biochemical approach to the identification of progranulin and granulin receptors. Nogo-A was previously detected in the Yu laboratory as a potential progranulin interactor. However, we failed to confirm a physiologically significant interaction between progranulin and Nogo-A. Additional experiments revealed three promising candidates for granulin A receptors: glial glutamate transporter EAAT1, ER protein wolframin, and the α_3 subunit of the Na^+/K^+ ATPase. Finally, we critically examined the veracity of a reported interaction between progranulin and tumor necrosis factor receptors (TNFRs), finding no evidence that progranulin antagonizes TNFRs, supporting recent unpublished findings from the Tansey and Kukar laboratories.

Acknowledgement

Parts of this chapter have been reproduced, with or without modifications, from my published work (Cenik et al., 2012).

Introduction

The Search for Progranulin Receptors

Progranulin is a secreted protein readily detected in blood and cerebrospinal fluid in animals. Exogenous, recombinant progranulin has many biological effects in cell culture. Even though progranulin was discovered in the 1990s, no cell receptor had been shown to account for its biological activities until very recently. Since 2010, sortilin and TNFRs have been identified as cell surface receptors for progranulin. According to one report (Tang et al., 2011), progranulin is an endogenous TNF-α antagonist; therefore progranulin haploinsufficiency may lead to an over-abundance of TNF-α activity. However, it is somewhat confusing that an excess of TNF-α activity would lead to a specific neurodegenerative phenotype, rather than any of the other inflammatory conditions associated with this cytokine (Croft et al., 2012), most notably arthritis. A recent report suggested that serum levels of IL-6, TNF-α and IL-18 were unchanged in *GRN* mutation carriers compared to controls (although IL-6 levels were increased in symptomatic *GRN* mutation carriers) (Bossu et al., 2011). To the best of our knowledge, there are no published reports detailing the prevalence of a TNF-α related autoimmune disease (e.g. rheumatoid arthritis) in *GRN* mutation carriers or FTLD patients. Furthermore, the direct binding of progranulin to TNFRs could not be replicated by another group (Chen et al., 2012, unpublished manuscript).

Progranulin is made up granulin domains that can be proteolytically released. These granulins have been reported to be biologically active (see chapter 1 for details). In 1993, Culouscou et al. (Culouscou et al., 1993) reported that granulin A (then termed epithelin-1) bound a 140-145 kDa protein on the surface of MDA-MB-468 breast carcinoma cells. Granulins B and C competed with the binding of granulin A to this receptor. However, the identity of this receptor was not described.

Identification of cell surface receptors and downstream signaling pathways of progranulin and granulins will open up the possibility to develop therapeutics acting at the receptor level, i.e. classical agonists and antagonists. Therefore, we first followed up on experiments previously carried out in the Yu lab, undertaking a biochemical approach to identification of progranulin receptors. We also critically analyzed whether progranulin has an antagonistic effect at its putative receptor (TNFR) as reported. We also searched for granulin receptors, undertaking a biochemical approach.

Methodology

Reagents, Plasmids and Antibodies

Purified recombinant human and mouse progranulin (rhPGRN, rmPGRN), N-Nogo-Fc and Nogo-66-Fc fusion proteins, recombinant human TNF-α, and non-specific human IgG were purchased from R&D Systems; Protein A agarose beads from Roche; TEV protease, ultra-low IgG FBS and other cell culture reagents from Invitrogen; electrophoresis reagents from Bio-Rad.

Mouse monoclonal antibody against neuron specific βIII tubulin (TUJ-1) was obtained from Abcam; anti-EAAT1 (D44E2) rabbit monoclonal antibody from Cell Signaling; mouse monoclonal antibody against α_3 Na^+/K^+ ATPase subunit from Novus Biologicals; anti-c-Myc monoclonal antibody (9E10) from Pierce.

Plasmids encoding α_3 or β_1 subunits of Na^+/K^+ ATPase and Slc1a3 (EAAT1) were purchased from Open Biosystems. WFS1-Flag-pcDNA3 plasmid was from Addgene. Tritium-labeled glutamate was purchased from Perkin Elmer. Lipopolysaccharides (LPS) from E.coli O55:B5, tissue culture grade L-glutamate, choline

and general lab chemicals were from Sigma. Rap-Fc was a kind gift from Dr. Hong Choi in the Herz lab.

Neurite Outgrowth Assay

Four to six week old C57/BL6 mice were deeply anaesthetized with Avertin and quickly decapitated. Following superficial dissection, bilateral laminectomies were performed along the spinal column to expose the spinal cord. Dorsal root ganglia (DRGs) were quickly dissected in cold Hank's balanced salt solution (HBSS). DRGs were treated with collagenase (60 U) in F12 medium containing 5 mM $CaCl_2$ for 90 min at 37^0 C followed by 0.25% trypsin for 35 min at 37^0 C. Fresh medium (F12 + 10%FBS + antibiotics) was added and cells were dissociated by trituration. Dissociated cells were plated on coverslips coated with poly-D-lysine (0.1mg/ml), laminin and inhibitory peptides (where indicated). After 24 h in culture, the coverslips were fixed in paraformaldehyde (PFA) 30 min at 4^0 C, washed three times with Tris-buffered saline (TBS), blocked in 1% goat serum for 1 h and stained for neuron specific tubulin. Primary antibody TUJ-1 was used at 1:1000 dilution and Alexa-488 conjugated secondary antibody was used at 1:200 dilution. Images were taken on a Nikon fluorescence microscope with the 10x objective, 7 or more frames per coverslips. Images were quantified by one of the two methods: manual tracing of neurites followed by quantification in ImageJ software or automatic quantification of neurite lengths using Imaris (Bitplane) software. Briefly, *slice/measure*, and *surpass/statistics/filament/segment length* options were used in Imaris.

Protein Purification

cDNA encoding mouse progranulin (PGRN) was obtained from the IMAGE consortium. cDNA sequences corresponding to granulins (GRN) A, B, and C were amplified by PCR. A plasmid encoding part of the Fc portion of human Ig was a kind gift from Dr. Thomas Sudhof. These were used to subclone into pcDNA4 (Invitrogen) and generate an Fc-tagged progranulin and granulins, using standard molecular cloning techniques. Individual clones of HEK 293 cells stably overexpressing PGRN-Fc, granulin A-Fc, granulin B-Fc, and granulin C-Fc were generated using Zeocin (Invitrogen) selection. A plasmid encoding APP-Fc was kindly provided by Dr. Yu-Hong Han. This plasmid was transiently transfected into HEK cells using polyethylenimine (PEI, Polysciences).

-Fc tagged proteins were purified from conditioned medium (DMEM/10% ultra-low IgG FBS) by affinity chromatography utilizing Protein A agarose beads (Roche). Briefly, conditioned medium was adjusted to pH 8 with 1M Tris-HCl; -Fc tagged protein was bound to the beads overnight at 4° C; the beads were collected by passing through a chromatography column and washed with buffer containing 100 mM Tris-HCl (pH 8), 0.5 M NaCl, 0.1% Triton X-100; the protein was eluted with 100 mM glycine pH 3, immediately neutralized with Tris, buffer-exchanged into PBS supplemented with 10% glycerol and 0.5 mM EDTA, and stored at - 80 °C until use in biochemical experiments.

A plasmid encoding human growth hormone (hGH), 8xHis affinity tag and TEV protease cleavage site (pSGHVΦ) was kindly provided by Dr. Charles Dann III. Progranulin cDNA lacking N-terminal signal sequence was subcloned into the MluI site on pSGHVΦ to generate N-terminal tagged progranulin. Stable expression in DHFR-/-

CHO cells was obtained as detailed in (Leahy and D., 2000). His tagged proteins were purified using Ni-NTA purification system (Invitrogen).

Pull-down Assays

HEK 293 cells were transfected with a plasmid encoding EAAT1, collected non-enzymatically, and kept at -80^0 C until use. Cell pellets were lysed in extraction buffer (20 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM EDTA, 10% glycerol, Roche protease inhibitors) supplemented with 1% Triton X-100 and sonicated for 5 x 30 seconds. The lysate was cleared by centrifugation and pre-cleared by incubation with Protein A agarose beads for 1 h. ~7.5 µg of –Fc tagged proteins were added and incubated overnight at 4^0 C. Then, 40 µl of Protein A agarose beads were added into each sample and incubated for 1 h. The beads were washed three times with PBS. Bound proteins were eluted by boiling the beads in Laemmli buffer.

Brain Membrane Pull-Down Assays

10 rat brains (Pel-Freez) or 20 mouse brains were pulverized under liquid nitrogen cooling. A hand-held Dounce homogenizer was used to homogenize pulverized tissue in 75 mL of homogenization buffer (20 mM HEPES (pH 7.4) 0.25M Sucrose, 3 mM $MgCl_2$, 2 mM EGTA, Roche protease inhibitors). The homogenate was centrifuged at 280 x g (all centrifugation steps were carried out at 4° C); the pellet was re-homogenized in 30 mL homogenization buffer and centrifuged at 280 x g. Combined supernatants from the 280 x g spins were centrifuged at 1500 x g, for 10 min and the supernatant from the 1500 x g spin was centrifuged at 100,000 x g for 1 h (LE-80

Beckman centrifuge, Ti45 rotor). The pellet (P100) was washed with 15 mL extraction buffer (20 mM HEPES (pH 7.4) 150 mM NaCl, 2 mM EDTA, 10% glycerol, Roche protease inhibitors**)** and again centrifuged at 100,000 x g. The pellet was resuspended in 35 mL extraction buffer. Protein concentration was measured with BCA method (Pierce) and the suspension was diluted to ~10 mg/mL. Excess membrane fraction was kept at -80^{0}C. Equal volumes of 10 mg/ml membrane suspension and extraction buffer containing 2 % Triton X-100 were mixed to obtain final concentrations of 5 mg/mL protein and 1% detergent, and mixed gently for 2 h at 4^o C. The detergent extracted fraction was centrifuged at 100,000 x g for 1 h; the supernatant was kept and pre-cleared by incubating with washed Protein A agarose beads (1/100 volume of 50% slurry) for 1 h at 4°C. Beads were collected by centrifugation at 500 x g for 10 min and discarded. Protein concentration was again determined by BCA assay. Bait proteins (2.5 µg bait/mg detergent extracted protein) and Protein A agarose beads (5µl slurry/mg protein) were mixed in 10 mL extraction buffer and incubated for 1 h at 4^o C, with gentle rocking in 50 mL conical tubes. Beads were collected by centrifugation (500 x g, 5 min) and washed twice. 22mL of detergent extracted membrane fraction was added into each tube, $CaCl_2$ and $MgCl_2$ were also added to 5mM final concentration and the mixture was gently rocked overnight at 4^o C. The beads were collected by passing through chromatography columns at 4^o C and washed with 100 bed volumes of wash buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, 2 mM $CaCl_2$, 2mM $MgCl_2$). To elute bound proteins, beads were incubated with 1 bed volume of 1x Laemmli buffer at 65^o C for 5

min. The samples were concentrated by lyophilization before mass spectrometric analysis.

Online Reverse Phase Liquid Chromatography and LC-MS/MS

Immunoprecipitation eluates were desalted via loading and briefly resolving protein bands in a 10% polyacrylamide SDS gel. After staining with Coomassie blue, each gel lane was cut into bands which were subjected to in-gel digestion (12.5 μg/ml trypsin). Extracted peptides were loaded onto a C_{18} column (100 μm internal diameter, 12 cm long, ~300 nl/min flow rate, 5 μm, 200Å pore size resin from Michrom Bioresources, Auburn, CA) and eluted during a 10-30% gradient (Buffer A: 0.4% acetic acid, 0.005% heptafluorobutyric acid, and 5% AcN; Buffer B: 0.4% acetic acid, 0.005% heptafluorobutyric acid, and 95% AcN) for 90 minutes. The eluted peptides were detected by Orbitrap (350-1500 *m/z*, 1,000,000 AGC target, 1,000 ms maximum ion time, resolution 30,000 fwhm) followed by 9 to 10 data-dependent MS/MS scans in LTQ (2 *m/z* isolation width, 35% collision energy, 5,000 AGC target, 200 ms maximum ion time) on a hybrid mass spectrometer (Thermo Finnigan, San Jose, CA).

Acquired MS/MS spectra were extracted and searched against mouse and rat reference databases from the National Center for Biotechnology Information using the SEQUEST Sorcerer algorithm (version 2.0, SAGE-N). Searching parameters included mass tolerance of precursor ions (±50 ppm) and product ion (±0.5 *m/z*), partial tryptic restriction, with a dynamic mass shift for oxidized Met (+15.9949), four maximal modification sites and three maximal missed cleavages. Only *b* and *y* ions were considered during the database match. To evaluate false discovery rate (FDR), all original

protein sequences were reversed to generate a decoy database that was concatenated to the original database. The FDR was estimated by the number of decoy matches (n_d) and the total number of assigned matches (n_t). FDR = $2*n_d/n_t$, assuming the mismatches in the original database were the same as in the decoy database. To remove false positive matches, assigned peptides were grouped by a combination of trypticity (fully, partial and non-tryptic) and precursor ion-charge state (1+, 2+, and 3+). Each group was first filtered by mass accuracy (10 ppm for high-resolution MS), and by dynamically increasing XCorr (minimal 1.8) and ΔCn (minimal 0.05) values to reduce protein FDR to less than 5% (and less than 3% for proteins identified by a single peptide match). Proteins were quantified using the Abundance Index, which is defined as the spectral counts divided by the number of peptides per protein.

NF-κB-Luciferase Reporter Assays

NF-κB-luciferase reporter plasmid (NFκB-Luc) was a kind gift from Dr. James Chen (UT Southwestern). A mammalian expression plasmid encoding β-galactosidase (β-gal) was kindly provided by Dr. Paul Dutchak (UT Southwestern). Etanercept was a kind gift from Dr. Malu Tansey (Emory University). These plasmids were purified using NucleoBond® (Takara) endotoxin-free maxi-prep kits according to manufacturer's instructions. The plasmid encoding mouse *GRN* was generated by inserting full-length *GRN* cDNA into pcDNA4 (Invitrogen) by standard molecular cloning techniques and verified by sequencing. rhPGRN and rhTNF-α were obtained from R&D Systems; stock solutions were prepared in sterile, cell culture grade PBS. HEK 293A cells were cultured as described in chapter 2 and transiently transfected with FUGENE 6 (Roche) reagent,

48h before NF-κB-luciferase reporter assays. In relevant experiments, NFκB-Luc, β-gal, and test plasmids were transfected in 1:1:3 ratios. The cells were plated on 96-well plates and serum-starved for 12 hours. Etanercept or rhPGRN was applied 30 min before TNF-α treatment. All reagents were diluted and applied in serum-free DMEM. 5-6h after TNF-α treatment, cells were lysed in luciferase assay buffer (Promega) + 1% Triton X-100 (100 µL/well). Luciferase activity was measured with Victor3 Multi-label Counter (Perkin-Elmer). Then, 120 µL/well β-gal buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$, 0.4 mg/mL ONPG, 0.28% 2-Mercaptoethanol) was added and OD_{420} was measured ~30 min later with a spectrophotometer.

Mixed Primary Glial Cultures

Three or four P2-P5 mouse pups were anesthetized by placing on ice for 10 min and quickly decapitated. Cortices were quickly dissected in cold DMEM supplemented with penicillin, streptomycin and amphotericin B. Single cell suspension was obtained by papain (400 U) digestion at 37° C for 10 min followed by trituration. Cells were grown on tissue culture dishes in DMEM/10% FBS.

Glutamate Uptake Assay

HEK 293A cells were kindly provided by Dr. Chantelle Sephton and grown in 6-well tissue culture plates under DMEM/10% FBS. Cells were transfected with a plasmid encoding EAAT1 24 h before experiments using FUGENE 6 (Roche) transfection reagent. Cells were washed twice in warm PBS or warm glutamate-free, Na^+-free assay buffer. Test proteins were applied at 2x indicated concentrations in 500 µl glutamate-free

assay buffer (140 mM NaCl, 2.5 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES (pH 7.4), 5.5 mM glucose) for 15 min. Then, 500 µl assay buffer supplemented with 0.1 mM L-glutamate and 1 µCi/mL ^{3}H-glutamate was added. Glutamate uptake was stopped at indicated time points by quickly washing wells twice with ice-cold PBS and lysing cells in 1% SDS in water. Lysates were cleared by centrifugation. 500 µl of cleared lysate was mixed with 5 mL of Ultima Gold scintillation cocktail (Perkin Elmer). Intracellular radioactivity was measured by liquid scintillation counting and normalized to the protein concentration of the sample determined with the BCA method.

Results

Progranulin Does Not Interact With Nogo-A

To identify brain proteins that interact with progranulin, a "pull-down" strategy had been used in the Yu lab. In experiments conducted by Dr. Wenze Niu, detergent solubilized rat brain membrane fractions were incubated with PGRN-Fc or an Fc-tagged control protein (APP-Fc) and precipitated with Protein A agarose beads. Bound proteins were eluted and visualized with silver staining. A predominant band at approximately 120 kDa was observed in the PGRN-Fc pull-down sample but not in the control sample. Bands corresponding to rat brain proteins that co-purified with PGRN-Fc were cut out of SDS-PAGE gels and identified by mass spectrometry at UT Southwestern Core Facilities. The ~120 kDa protein was identified as Reticulon 4 (Nogo-A). Nogo-A is a membrane protein component of myelin that inhibits neurite outgrowth. Both the N-terminus (N-Nogo) and the extracellular loop (Nogo-66) are bioactive, potently inhibiting neurite

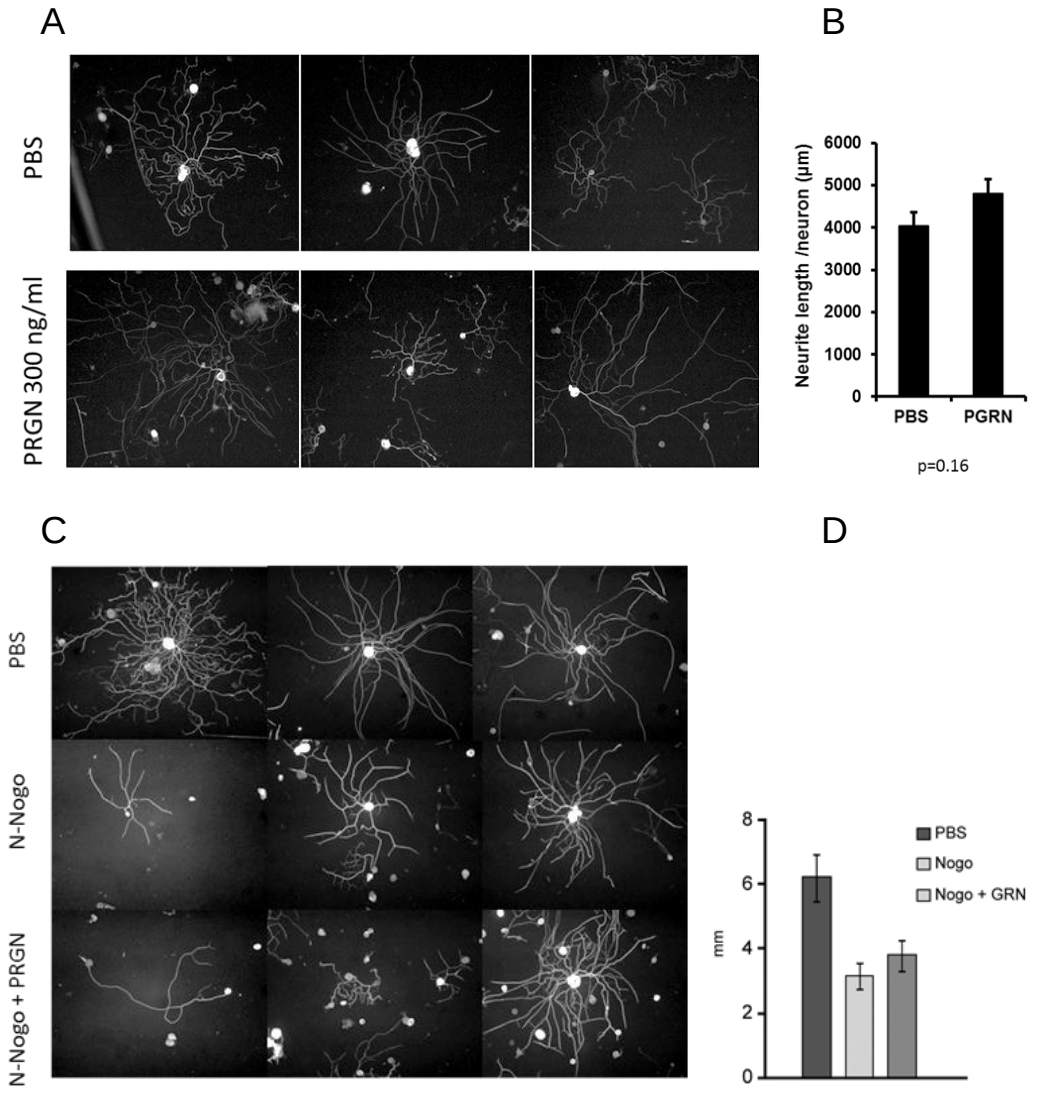


FIGURE 3.1 Progranulin Does Not Antagonize the Neurite Outgrowth Inhibitory Effects of N-Nogo. A. Dissociated DRG neurons were plated on coverslips either in the presence (bottom row) or absence (top row) of 300 ng/mL progranulin in the culture medium. Fixed cells were stained for neuron specific tubulin III 24 h later. B. Total neurite length per neuron was quantified with Imaris software. Error bars indicate SEM. C. Coverslips were coated with N-Nogo (3 µg/mL) (middle and bottom rows) or PBS (negative control, top row). Neurons plated on N-Nogo coated coverslips had fewer and shorter neurites. Addition of 300 ng/mL progranulin in the culture medium did not abolish the inhibitory activity. D. Quantification with Imaris software. mm denotes total neurite length per neuron, in millimeters. $p > 0.05$ for the comprasion Nogo versus Nogo + GRN. GRN denotes progranulin treatment; error bars, SEM.

outgrowth and inducing growth cone collapse. Nogo-66 binds to the Nogo receptor and PirB (Paired immunoglobulin-like receptor B) to exert its inhibitory activity. Whether N-Nogo acts through the same receptors is uncertain. Progranulin was previously reported to induce neurite outgrowth (Van Damme et al., 2008). Therefore, we hypothesized that progranulin might be a Nogo antagonist. We tested whether progranulin rescued the inhibition of neurite outgrowth induced by N-Nogo. We used dissociated dorsal root ganglion (DRG) neuronal cultures from young mice (4-6 weeks of age). Both N-Nogo and myelin robustly inhibited neurite outgrowth (data not shown). We tested increasing concentrations on N-Nogo and found that maximum inhibition occurred at 6 μg/mL. We used the half-maximal concentration of 3 μg/mL for the rest of the experiments (data not shown). When neurons were cultured in the presence of rmPGRN, we observed a slight trend towards increased neurite outgrowth that did not reach statistical significance (Fig. 3.1A&B). However, progranulin did not block the neurite outgrowth inhibitory effect of N-Nogo (Fig. 3.1C&D). The length the longest neurite was also similar between groups. The inhibitory effect of Nogo-66 was weaker than N-Nogo. Nevertheless, progranulin had no effect on Nogo-66 mediated inhibition either (data not shown).

These results called the physiological significance of the progranulin-Nogo interaction into question. Therefore, we decided to re-test the interaction between Nogo and progranulin. We incubated Fc-tagged N-Nogo and Nogo-66 with recombinant progranulin fused to human growth hormone on the N-terminus of the former (hGH_PGRN). We pulled-down Fc-tagged Nogo proteins on Protein A agarose beads and assayed whether progranulin co-purified. Interestingly, progranulin did not bind either N-Nogo-Fc or Nogo-66-Fc even in mild buffer conditions (0.5% Triton X-100)

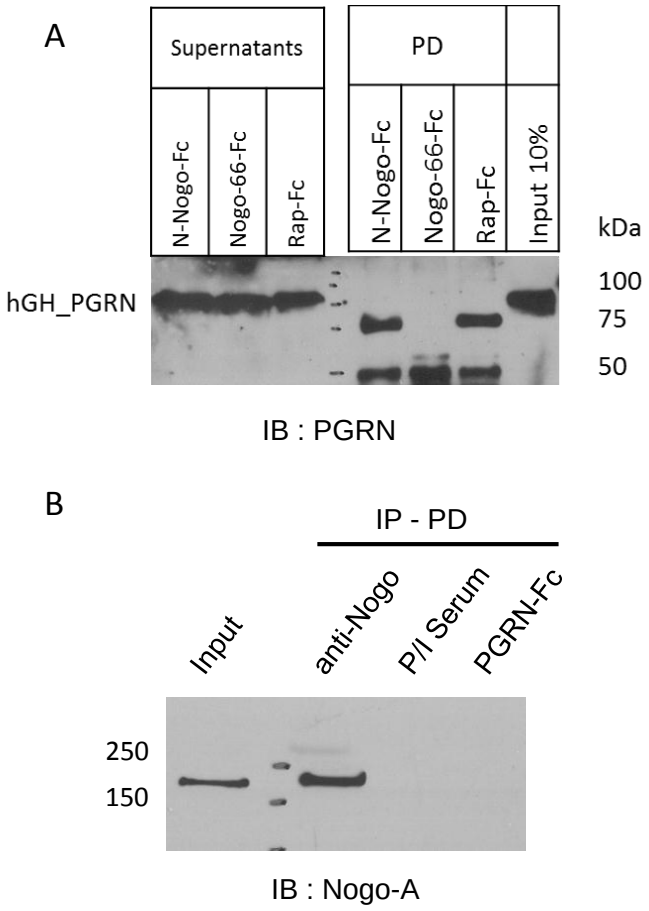


FIGURE 3.2 Progranulin Does Not Directly Interact with Nogo-A. A. 250 ng/mL human growth hormone-progranulin fusion protein (hGH_PGRN) was incubated with the indicated –Fc tagged baits (PD, pull-down) in PBS supplemented with 0.5% Triton X-100. Receptor associated protein (Rap) fused to –Fc is a negative control. PGRN denotes progranulin. hGH_PGRN is detectable in the input but does not co-precipitate with any of the baits. B. Detergent-solubilized brain membrane extract was incubated with the indicated reagents (IP, immunoprecipitation; PD, pull-down). Nogo-A can be immunoprecipitated with anti-Nogo Ig but does not co-precipitate with PGRN-Fc. P/I denotes pre-immune serum. IB denotes the primary antibody used for Western blot analysis.

(Fig. 3.2A). This result can be explained in several ways: the affinity tags (Fc and hGH) may have interfered with the binding; the binding might be dependent on a co-factor, e.g. magnesium, that was not included in the binding buffer; progranulin might be binding the C-terminus of Nogo; or the original finding of direct binding between progranulin and Nogo might have been an artifact.

Next, we tried to replicate the original finding that Nogo-A can be pulled down from brain lysates by PGRN-Fc. We found that Nogo-A can be efficiently immunoprecipitated from solubilized rat brain membrane fractions with an antibody against Nogo but did not co-precipitate with PGRN-Fc (Fig.3.2B), contrary to the results of experiments previously carried out in the lab. It is possible that we failed to replicate the conditions of the original experiment faithfully. It is also possible that the binding between progranulin and Nogo-A is very weak, so that it can only be detected by mass spectrometric techniques. This data, combined with our failure to demonstrate any physiologically relevant interaction between progranulin and Nogo convinced us not to pursue this project further.

Progranulin Does Not Directly Antagonize TNFRs

The lack of a canonical receptor and a well-defined activity assay for progranulin has been a stumbling block for the field. When Tang et al.(Tang et al., 2011) reported that progranulin was a direct blocker of TNFRs; we assumed that any of the well-defined TNF-α activity assays, e.g. activation of NF-κB, could be adopted as a progranulin activity assay. However, we learned that the laboratories of Dr. Malu Tansey and Dr. Thomas Kukar could that replicate the findings of Tang et al.. Specifically, they did not

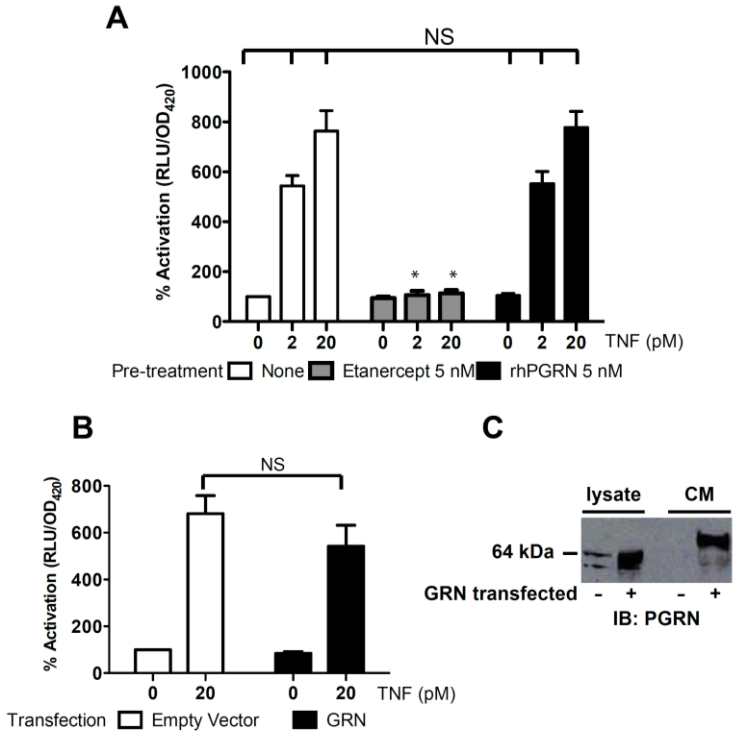


FIGURE 3.3 Progranulin Fails To Diminish NF-κB Reporter Activation in Response to TNF. A. HEK 293A cells were transfected with NF-κB-luciferase reporter and β-galactosidase expression plasmids. Pre-treatments and recombinant human TNF were applied at the indicated concentrations. Luciferase reporter activity in relative light units (RLU) was normalized to β-galactosidase activity (measured by optical density at 420 nm, OD_{420}) and expressed as percentage of activity observed in the untreated wells (left-most column). Error bars indicate SEM, n=4-5. * indicates $p<0.05$ versus no pre-treatment for the same TNF concentration. n.s. indicates $p > 0.05$ for the same TNF concentration. B. HEK 293A cells were transfected with NF-κB-luciferase reporter and β-galactosidase expression plasmids, plus either control plasmid (empty vector) or a plasmid encoding full-length mouse progranulin (GRN), as indicated. NF-κB reporter activity was measured and analyzed as in A. Error bars indicate SEM, n=5. C. Western blot analysis was performed on cells transfected in parallel to cells used in panel B to confirm progranulin expression. PGRN, progranulin.

detect a direct physical or functional interaction between progranulin and TNF receptors (Chen et al., 2012, unpublished manuscript). Therefore, we examined whether progranulin antagonized TNF-induced NF-κB activation using a luciferase reporter assay in HEK 293A cells. HEK 293A cells were transfected with an NFκB-luciferase reporter and a β-galactosidase expression plasmid (transfection control) to measure TNF-dependent NFκB transcriptional activity in the presence or absence of human progranulin (rhPGRN) or the TNFRII decoy receptor etanercept. We found that while etanercept completely abolished the response to TNF, rhPGRN had no effect (Fig3.3A). In another set of experiments, HEK 293A cells were transfected with the NF-κB-luciferase reporter and the β-galactosidase plasmids, plus either a control plasmid (empty vector) or a plasmid encoding full-length mouse progranulin (mPGRN). In the absence of TNF, there was a slight trend towards lesser reporter activation in the cells transfected with the mPGRN plasmid; however, progranulin did not have a statistically significant effect either in the presence or absence of exogenous TNF-α (Fig3.3B). Western blot analysis confirmed that the cells transfected with mPGRN were in fact expressing progranulin protein: Transfection with the mPGRN plasmid greatly enhanced mouse progranulin immunoreactivity in cell lysates and conditioned medium (CM) (Fig3.3C). While low levels of endogenous human progranulin expression are also evident in control cell lysates, the level of endogenous human progranulin in the CM is undetectable by this assay. We concluded that TNF-induced NF-κB signaling is not antagonized by recombinant progranulin or progranulin overexpression in mammalian cells.

Table 3.1 Proteins Highly Enriched From Brain Membrane Extracts By Granulin-A-Fc Pull-Down.

Protein	Ref_Seq IDs (Rat/Mouse)	Enrichment versus control, experiment-1	Enrichment versus control, experiment-2
Wolfram syndrome 1 (Wolframin)	NP_114011 NP_035846	*	*
Na^+/K^+ ATPase α_3 subunit	NP_036638 NP_659170	*	*
Solute carrier family 1, member 3 (EAAT1)	NP_062098 NP_683740	*	6x

*** not detected in control sample**

Granulin A Binds Several Brain Membrane Proteins

Granulins are released upon proteolysis of progranulin and are presumably bioactive. It is unlikely that granulins act via the TNFRs, though. Granulins A and B did not inhibit TNF-α-mediated effects but increased IL-8 secretion in one study (Zhu et al., 2002). Moreover, epithelins 1, 2 and 3 were suggested to specifically bind to a cell-surface proteins, and this binding did not compete with TNF-α (Culouscou et al., 1993). However, the identity of these putative receptors has remained elusive. It is possible that the vital importance of progranulin in the central nervous system is related to the biological activities of granulins. Since proteolytic cleavage of progranulin occurs extracellularly and granulins are stable in the inflammatory milieu, we hypothesized that granulins bind specific cell surface receptors in the brain. Granulin A was prioritized because of strong conservation, the above mentioned putative bio-activity and stable structure in solution as determined by NMR (Tolkatchev et al., 2008).

To test this hypothesis, we performed pull-down experiments with Fc-tagged granulin A as bait and detergent-extracted rat brain membrane proteins as input. An

unrelated protein (extracellular domain of APP), also fused to Fc, served as a control. We also used a truncated construct (-Fc construct) comprised of a small portion of granulin B fused to Fc and non-specific IgG as controls. As input, we used either mouse or rat brain membrane fractions solubilized by detergent treatment.

In-solution mass spectrometry identified several membrane proteins that were enriched in the granulin A pull-down sample, compared to controls (Table 3.1). Among our most enriched candidates were wolframin, mutations of which cause Wolfram syndrome, and excitatory amino acid transporter 1 (EAAT1), which is expressed on astrocytes (Liang et al., 2008) and on microglia during inflammation and may protect against excitotoxicity. We also pulled-down Na^+/K^+ ATPase subunits, which bind wolframin and EAAT1 and may have signaling functions themselves. These findings expand the rationale why progranulin (and therefore granulin) haploinsufficiency leads to a neurodegenerative phenotype.

In follow-up experiments, we confirmed that GRN-A-Fc pulls-down EAAT1 from mouse brain lysates (Fig. 3.4A). We expressed EAAT1 and EAAT1-myc-His fusion protein in HEK 293 cells and confirmed GRN-A-Fc pull-down from HEK cell lysates (Fig. 3.4B). Various control proteins, including an APP-Fc fusion protein, a truncated GRN-Fc fusion peptide (Fc construct), non-specific IgG, and full-length PGRN-Fc failed to pull down EAAT1 in these experiments (Fig. 3.4C).

We expressed full-length Na^+/K^+ ATPase subunits and wolframin in mammalian cells and the wolframin N-terminal domain in bacteria and insect cells (data not shown). However, follow up experiments with α_3Na^+/K^+ ATPase and wolframin were limited by technical difficulties of solubilizing these multi-pass transmembrane proteins in mild

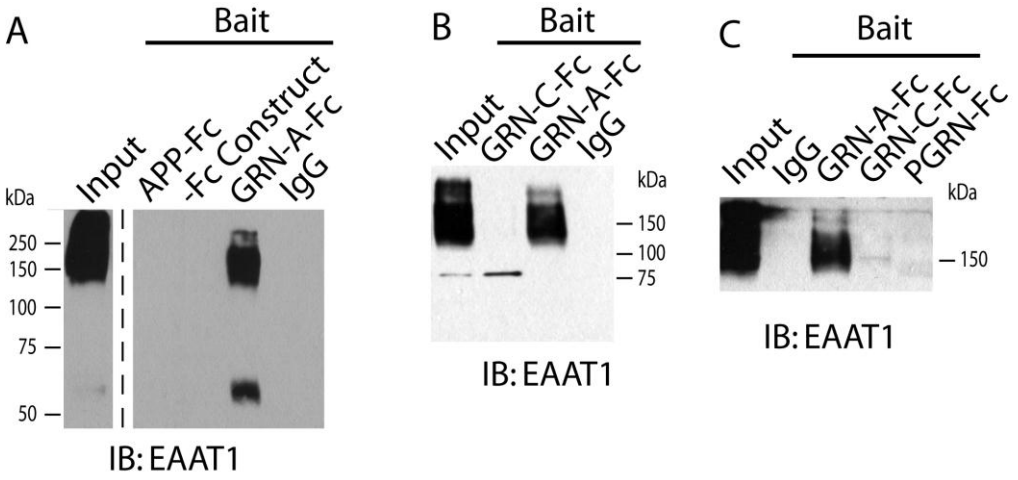


FIGURE 3.4. Biochemical Interaction between Granulin A-Fc and EAAT1. *A.* Indicated bait proteins were incubated with mouse brain detergent (1% Triton X-100) extracts (Input) and precipitated with Protein A agarose beads. After three washes, proteins remaining on the beads were eluted with Laemmli buffer and detected by western blotting. *B* and *C.* Pre-cleared lysates of HEK 293 cells over-expressing EAAT1 were used as input for the procedure described in *A*. IB indicates primary antibody used for Western blot analysis.

buffers suitable for protein-protein interaction studies. Therefore, we focused our efforts on elucidating the physiological significance of granulin A-EAAT1 interaction.

Granulin A Does Not Acutely Alter EAAT1 Mediated Glutamate Uptake

EAAT1 (also denoted as Slac1a3 or GLAST) is a member of a family of Na^+ dependent excitatory amino acid transporters. EAAT1 and EAAT2 are expressed in the brain. EAAT2 is constitutively expressed on astrocytes and is responsible for removing 90% of the glutamate released as a neurotransmitter at synapses. EAAT1 is expressed on Bergmann glia, reactive astrocytes and activated microglia in response to inflammation or increased glutamate concentration (Kim et al., 2003; Butchbach et al., 2004; Vallejo-Illarramendi et al., 2006). Removing excess glutamate from the synapse prevents excitotoxicity (Liang et al., 2008). On the other hand, EAAT1 mutation causes episodic ataxia and seizures (Jen et al., 2005).

First, we sought the replicate the finding that EAAT1 expression is induced during inflammatory stimulation. We treated mixed mouse glial cultures with lipopolysaccharide and assessed EAAT1 expression by Western blotting. As shown in Figure 3.5, EAAT1 expression was induced.

Progranulin is highly expressed by activated glia and proteolytic cleavage of progranulin is also induced during inflammation. Therefore, we hypothesized that granulin A may be an activator of EAAT1 during neuroinflammation. If this hypothesis were correct, it would explain why the loss of progranulin could lead to a neurodegenerative phenotype via excitotoxicity.

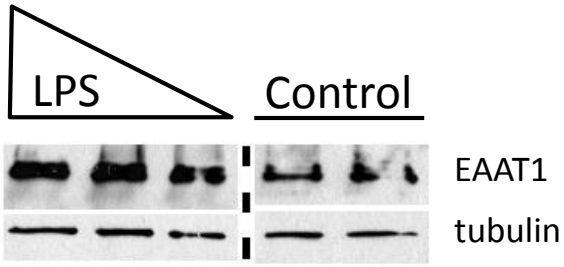


FIGURE 3.5 LPS Induces EAAT1 Expression In Mouse Mixed Glial Cultures.
Lipopolysaccharide (LPS) was dissolved in sterile PBS and applied at 1, 10, or 100 ng/mL (rightmost, middle and leftmost lanes, respectively) for about 18 h. Control treatment was sterile PBS. Primary antibodies used for Western blot analysis are shown on the right.

We established a glutamate uptake assay with tritium-labeled glutamate in HEK cells and demonstrated that transfected EAAT1 efficiently transports glutamate into the cells (Fig. 3.6A). The uptake was dependent on extracellular Na^+ and did not saturate for at least 7 minutes. Transfection with EAAT1 increased glutamate uptake by about 7-fold (data not shown) so that the majority of glutamate uptake in transfected cells was mediated by the transfected EAAT1. However, acute treatment with granulin-A-Fc did not have any effect on glutamate transport (Fig. 3.6B). We hypothesized that the Fc tag might be interfering with the physiological activity of granulin A, so we removed the tag by TEV protease incubation. Yet, TEV protease treated granulin A likewise failed to enhance glutamate transport acutely (Fig. 3.6C). We also tested the effect of full-length progranulin on EAAT1 mediated glutamate uptake. Up to 30 min of pre-incubation with progranulin had no effect on glutamate uptake (Fig 3.6D). Finally, we tested whether progranulin would alter glutamate uptake into mixed glial cells but observed no effect in this system either (data not shown).

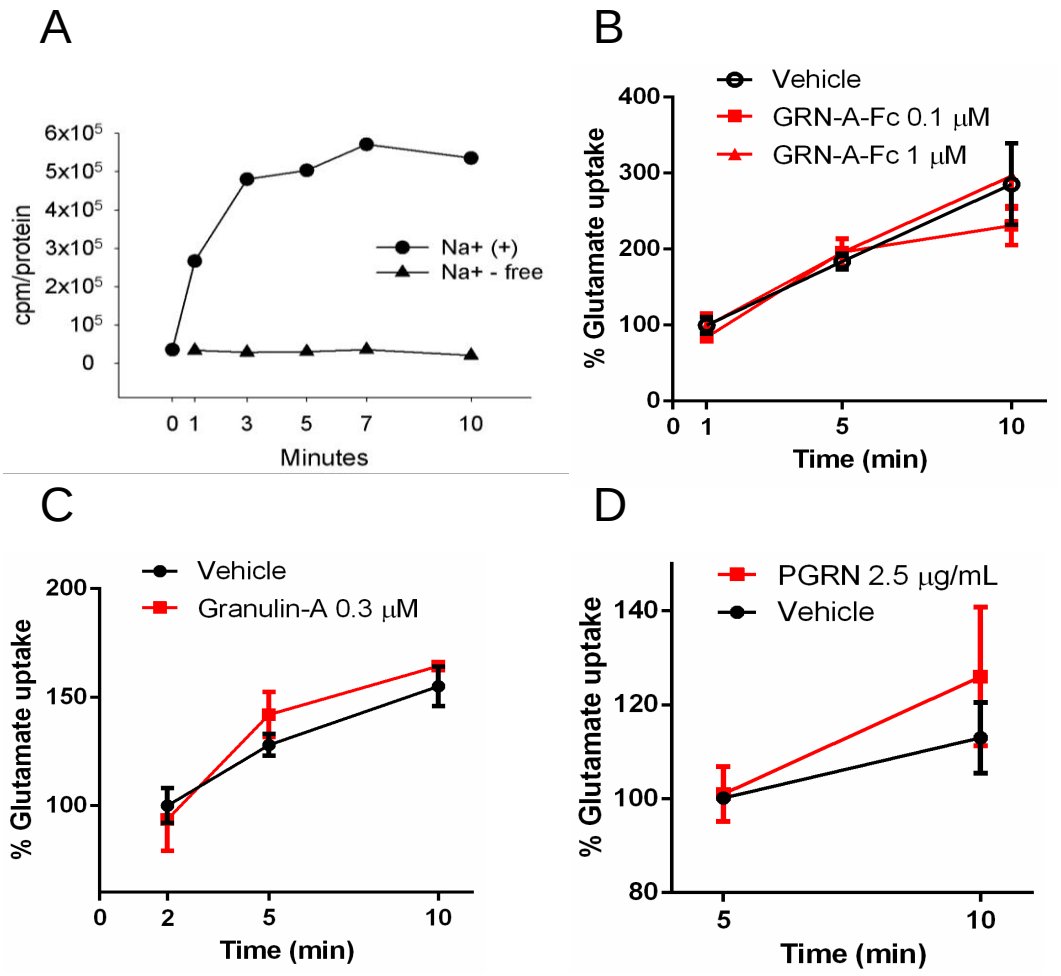


FIGURE 3.6 Neither Progranulin nor Granulin A Alters EAAT1 Mediated ^{3}H-Glutamate Uptake. A. HEK 293A cells were transfected with EAAT1 and incubated with glutamate uptake assay buffer containing ^{3}H labeled tracer glutamate, with or without 140 mM Na^{+} in the buffer. Cells were washed and lysed at indicated times and intracellular ^{3}H was measured by LSC. cpm/protein indicated counts per minute per mg of protein. B, C, and D. Indicated test proteins were applied 15-30 min before addition of tracer. Glutamate uptake was measured as in A and normalized. Error bars indicate SEM. $p > 0.05$ (Student's t-test) for vehicle versus treatment at every time point shown.

Discussion

Numerous biological activities have been attributed to progranulin and granulins. Yet, it is still unclear how reduced levels of extracellular progranulin leads to neurodegeneration. Identification of cell surface receptors for progranulin and granulins would shed light on this issue.

An affinity pull-down approach yielded an interesting candidate, Nogo-A, as a potential progranulin receptor. However, follow-up experiments failed to confirm this interaction. Using an expression cloning approach, Strittmatter's group showed high-affinity binding between progranulin and sortilin (Hu et al., 2010). Sortilin regulates progranulin endocytosis and hence extracellular progranulin levels. Zheng et al. (2011) mapped the interaction between these two proteins and showed that the C-terminus of progranulin binds the β-propeller region of sortilin. Could sortilin be the canonical progranulin receptor? High affinity binding between progranulin and sortilin has been independently replicated (Chen et al., 2012, unpublished manuscript). Yet, the link between sortilin and neurodegeneration is unclear. Sortilin is the co-receptor for proNGF, which is strong inducer of apoptosis in neurons (Lee et al., 2001). However, there is no evidence that progranulin interferes with proNGF binding to sortilin or $p75^{NTR}$, the high affinity proNGF receptor. Sortilin is also involved in intracellular trafficking and lysosomal function. Given the recent report of lysosomal storage disease caused by GRN mutation, it is possible that sortilin-progranulin interaction regulates lysosomal biology in a yet unknown way.

The other cell surface receptors reported to have high affinity for progranulin were the TNFRs. Anti-inflammatory activities of progranulin are well documented and

inflammation can be a driver of neurodegeneration. However, our findings described in this chapter, together with the extensive studies conducted in the Tansey and Kukar laboratories (Chen et al., 2012, unpublished manuscript), argues against progranulin being a direct antagonist at the TNFRs. Progranulin probably exerts its anti-inflammatory activities through another receptor or indirectly.

Another possibility is that granulin peptides, rather than full-length progranulin, mediate the biological effects in the brain. Since progranulin proteolysis is known to take place extracellularly, we searched for cell surface granulin A receptors. We identified three major candidates, all of which might be related to neurodegeneration.

Our data suggests that granulin A or progranulin are not acute activators of EAAT1. Alternatively, granulin A may have a function in the regulation of EAAT1 expression or trafficking. HEK cells used in this assay express progranulin; it is possible that addition of exogenous granulin A is ineffective due to the saturation of the system. Experiments in Grn knock-out glial cultures may help address this issue.

Wfs1 (chromosome 4p16) encodes wolframin, a multi-pass ER membrane glycoprotein widely expressed in mouse brain (Hofmann, 2003). Recessive mutations in Wfs1 cause Wolfram syndrome characterized by diabetes mellitus, optic atrophy and diverse neurologic symptoms (Strom et al., 1998). Wfs1 knock-out mice have growth retardation and impaired behavioral adaptation in stressful environments (Ishihara, 2004; Kato et al., 2008; Luuk et al., 2009). The literature about wolframin function is not extensive. It has been suggested that wolframin may be involved in the unfolded protein response (Fonseca, 2005; Fonseca et al., 2010) and exocytosis (Hatanaka et al., 2011). What could be the link between wolframin, granulins and neurodegeneration? We have

not yet investigated the physiological significance of granulin A binding to wolframin. It is tempting to speculate that dysregulation of the unfolded protein response may be important for neurodegeneration given the accumulation of protein aggregates in FTLD and other neurodegenerative diseases.

The Na^+/K^+ ATPase is a universal cell surface pump essential for maintaining osmotic homeostasis in eukaryotic cells and the membrane potential in neurons. However, it may have signaling functions. The inhibitor oubain, at low concentrations, activates Src, IP_3**,** and MAPK pathways by binding the α_3 subunit (Tian, 2005; Zhang et al., 2008; Rodrigues-Mascarenhas et al., 2009; Karpova et al., 2010). α_3Na^+/K^+ ATPase has also been implicated in negative regulation of apoptosis in cultured neurons. Interestingly, α_3Na^+/K^+ ATPase is also an agrin receptor, regulating frequency of spontaneous action potentials in neurons and contractility in cardiac myocytes (Hilgenberg et al., 2006; Hilgenberg et al., 2009). This protein is known to interact with both EAAT1 and wolframin, potentially explaining why all three were pulled down together. Future experiments will explore this protein as a potential granulin A receptor.

Attributions and Publications

Dr. Shuxin Li performed parts of the neurite outgrowth assays: coating of coverslips, preparation of single cell suspension and TUJ-1 staining. He also helped with the design of the experiments and the interpretation of data. Mass spectrometry was done by Eric Dammer in Junmin Peng's lab at Emory University. Kimberley Yu and Phillip Yu performed pilot experiments with the NF-κB reporter system and helped with troubleshooting. Drs. Malu Tansey and Thomas Kukar shared their unpublished data

regarding progranulin-TNFR interaction. All other experiments described in this chapter were designed and performed by me.

CHAPTER FOUR

CONCLUSIONS AND RECOMMENDATION

Acknowledgement

Parts of this chapter, including the figure, have been reproduced, with or without modifications, from my published work (Cenik et al., 2012).

In Vivo Confirmation of HDAC Inhibitors as Enhancers of Progranulin Expression

SAHA is promising as a first generation therapeutic for *GRN* deficient FTLD since it is already FDA-approved for other indications. SAHA has also been used in animal models of neurodegeneration (Hockly, 2003; Faraco et al., 2006; Mielcarek et al., 2011), with promising results. The next logical step is to test the effectiveness of HDAC inhibitors in animal models of FTLD. Our pharmacokinetics data shows that the half-life of SAHA is short and its penetration of the blood-brain barrier is less than ideal. Therefore, continuous infusion with an osmotic pump is probably the best approach to SAHA delivery. SAHA inhibits HDAC6, resulting in increased tubulin acetylation which can easily be detected by Western blotting. This will be a practical surrogate endpoint since the secreted nature of progranulin makes it technically challenging to detect changes in CNS expression at the protein level. Nevertheless, qPCR can be used to assess brain progranulin expression after sc SAHA administration. If poor blood-brain barrier penetration proves problematic, the drug can the delivered directly into the CNS by placing an icv catheter connected to an osmotic pump (Pieper et al., 2010). The next step

would be testing SAHA in an animal model of *GRN* haploinsufficiency. *Grn*$^{+/-}$ mice represent a reasonable model to test the hypothesis that increasing progranulin expression from the wild-type allele can ameliorate behavioral deficits. *Grn* knock-out mice have been generated (Martens et al., 2012). *Grn*$^{+/-}$ mice are grossly normal and fertile; they display deficits in the tube test (Spencer et al., 2005) of social dominance (Robert Farese, personal communication) but their brains do not contain the characteristic phosphorylated, ubiquitinated NCIs. The advantage of this model is that the genetic manipulation closely resembles human FTLD with *GRN* mutations. Even though the mild phenotype may preclude demonstration of a dramatic therapeutic effect, it will be very informative to test whether SAHA can normalize progranulin levels in this model of haploinsufficiency.

Another approach would be to pursue the newer generation of HDAC inhibitors, which may have better pharmacokinetic properties. This approach would probably require collaboration with the drug industry. We have identified other lead compounds beyond SAHA in our 10K compound chemical diversity screen. Few of these compounds are commercially available. To pursue these leads further, the compounds will need to be synthesized. Then, they can be tested on our established cellular assays and ultimately in the in vivo system. Our high-throughput luciferase assay is amendable to scaling up, so even larger chemical libraries can screened to identify other lead compounds. If compounds acting through a non-HDAC dependent mechanism are identified, combination drug treatment will be an option.

Investigating How SAHA Modulates Progranulin Expression

In the experiments described in chapter 2, we were unable to elucidate in the last detail the mechanism of and the specific contribution of individual HDACs to the increased *GRN* expression resulting from SAHA treatment. A chemically similar (TSA) and a structurally unrelated (M344) HDAC inhibitor, as well as sodium butyrate, also increased *GRN* expression, supporting our conclusion that HDAC inhibition is critical to the underlying mechanism, although we cannot completely rule out off-target effects. HDAC inhibitors have global effects on gene expression. Although SAHA is FDA-approved, the targets responsible for its anti-cancer effects are also incompletely understood (Lane and Chabner, 2009).

Which HDAC isoform might be responsible for the effectiveness of SAHA? Histone deacetylases are classified into five classes (I-IIa-IIb-III and IV) (30). SAHA inhibits class I (HDACs 1-2-3-8), class IIa (HDACs 4-5-7-9) and class IIb (HDACs 6 and 10) enzymes. In the luciferase reporter assay, HDAC6 or class II specific inhibitors were not sufficient to enhance expression, while class I inhibitors MS-275 and valproate increased expression only at excessively high concentrations at which cross-inhibition of other HDACs may have occurred. Although we cannot rule out the possibility that class I HDAC inhibition might be sufficient, we interpret our data as supporting a complex mechanism requiring inhibition of several HDACs. RNAi-mediated knock-down of specific HDAC isoforms would help answer this question. This, in turn, will help the field design more specific inhibitors to enhance progranulin expression. However, our pharmacological data suggests that knock-down of a single HDAC isoform would be unlikely to account for increased progranulin expression.

Another approach to a more mechanistic understanding of HDAC inhibitor action

would be targeted analysis of histone acetylation around the *GRN* promoter. This line of inquiry will clarify whether HDAC inhibitors act directly on the *GRN* gene or have secondary effects and may lead to identification of new drug targets for GRN deficiency. Since HDAC inhibitors are being developed for several neurodegenerative diseases (Ferrante et al., 2003; Hockly, 2003; Herman et al., 2006; Thomas et al., 2008; Rai et al., 2010; Mielcarek et al., 2011), this approach will be relevant for the entire neurodegeneration field. The hypothesis that SAHA and other HDAC inhibitors directly increase histone acetylation at the progranulin promoter region can be tested with a chromatin immunoprecipitation (ChIP) assay utilizing acetyl-histone antibodies, followed by deep sequencing (ChIP-Seq). If we identify any regions near the GRN promoter that are differentially bound by acetylated histones after SAHA treatment, we would confirm these findings by qPCR utilizing specific primers. Then, we would test whether these regions are necessary and sufficient for the GRN transcription enhancing activity of SAHA, utilizing luciferase reporter assays. We can use bioinformatics tools to find out whether differentially acetylated regions contain any transcription factor binding sites. Then, we can experimentally verify TF binding at these sites by ChIP utilizing antibodies against these TFs. Overexpression and knock-down approaches can also be used to test whether these putative TFs (if discovered) affect *GRN* expression.

Lipidomics and the Role of Progranulin in Lysosomal Biology

An interesting question about the mechanism of *GRN* deficient neurodegeneration was raised by recent findings (Ward and Miller, 2011; Cenik et al., 2012; Smith et al., 2012): could *GRN* haploinsufficient FTLD-TDP and NCL with

homozygous *GRN* mutations be manifestations of the same spectrum of diseases? In NCL, time of disease onset varies from the neonatal period to young adulthood according to the mutated gene, but patients with "mild", hypomorphic mutations are known to present with later onset than those with "classical", loss-of-function mutations of the same genes (Kohlschutter and Schulz, 2009). The patients with homozygous loss-of-function mutations of progranulin reported by Smith et al. (Smith et al., 2012) presented in their 20s, which is quite late compared to other known NCL mutations. Perhaps, *GRN* haploinsufficient FTLD-TDP represents a very late onset form of atypical NCL. This hypothesis seems to be supported by the mouse models: $Grn^{-/-}$ mice display behavioral characteristics of FTLD together with lipofuscinosis. However, exaggerated lipofuscinosis has not been reported in FTLD-TDP patient brains (or other tissues), in which the main ubiquitinated protein is known to be TDP-43. Furthermore, different regions of the brain seem to be primarily affected in FTLD-TDP compared to NCL. Thus, it remains possible that FTLD-TDP and *GRN* mutant NCL are caused by disruptions of disparate pathways which are differentially sensitive to progranulin dosage.

Nevertheless, role of intracellular progranulin and granulins, especially in the lysosomal pathway, is an exciting new avenue of research. Given the recent finding of adult onset lysosomal storage disease caused by homozygous progranulin deficiency and the high affinity binding between progranulin and the lysosomal transport protein sortilin, an essential intracellular role for progranulin in lysosomal biology is a strong possibility. Moreover, another recently identified function of progranulin/granulins - binding of CpG-ODNs to TLR9 - takes place intracellularly in lysosomes. It is also likely that proteolytic cleavage of progranulin into granulins takes places in lysosomes (or

somewhere along the way from the ER to the lysosome). Could progranulin or granulins be acting as activators or transporters of lysosomal enzymes? Accumulation of the autophagy-related receptor p62 and the lysosomal protease cathepsin D were reported in *Grn*$^{-/-}$ mice. Perhaps progranulin functions akin to prosaposin which is likewise synthesized as a precursor protein, secreted, and taken up again into the cells, followed by proteolytic processing into small (8-11 kDa), bio-active saposin polypeptides (O'Brien and Kishimoto, 1991; Hiesberger et al., 1998) (Fig. 4.1). Saposins act as activators of lysosomal enzymes and genetic abnormalities of prosaposin causes lysosomal storage disorders of varying severity. Lysosomal enzymes also play roles in the regulation of immune responses, apoptosis and defense against pathogens (Hagen et al., 1991; Conus and Simon, 2008). Therefore, lysosomal functions of progranulin/granulins could explain their immunomodulatory properties.

The largest roadblock to testing the hypothesis that progranulin/granulins may act as activators or transporters of lysosomal enzymes is the diversity of lysosomal metabolism. A hypothesis-generating, "-*omics*" approach is will be practical to begin investigating the possible role of progranulin in lipid and lipoprotein metabolism. In collaboration with Dr. Jeff McDonald, we are in the process of conducting a lipidomics study of *Grn* -/- mice, which are models of FTLD behaviorally and NCL histopathologically. Infusion lipidomics using tandem mass spectrometry has been carried out with an AB SCIEX 5600 Triple TOF mass spectrometer; data analysis will be conducted in the near future. We expect to obtain semi-quantitative data detailing the abundance of several classes of lipids. If significant differences between *Grn* knock-outs and controls are observed, we will prioritize and confirm the hits with LC/MS/MS. Next,

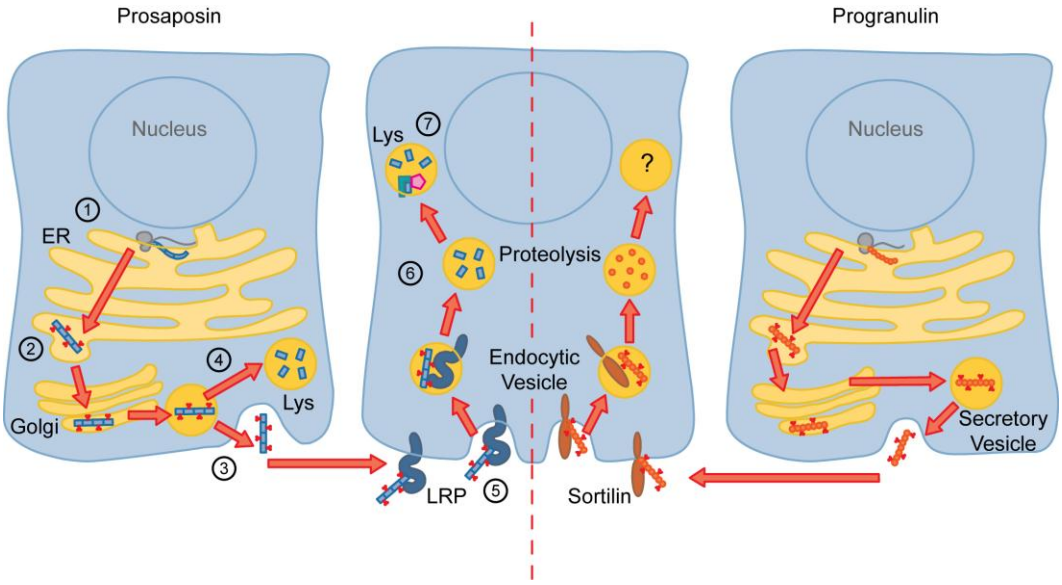


FIGURE 4.1 Trafficking of Prosaposin and Progranulin. Both prosaposin and progranulin consist of several repeats of saposin and granulin domains, respectively (1). Both proteins are N-glycosylated (2) and secreted (3). Prosaposin is also directly transported to the lysosomes via the mannose-6-phosphate receptor (4). Sortilin also plays a role in lysosomal trafficking of prosaposin (not shown). Re-uptake of progranulin is mediated by sortilin while prosaposin re-uptake is mediated by LRP, the mannose receptor (not shown) and the mannose-6-phosphate receptor (not shown) (5). Both proteins are probably proteolyzed intracellularly; although this has not been shown directly for progranulin (6). In the lysosome, the saposins activate lysosomal enzymes (pink pentagon) partly by lifting their substrates (green) out of the lipid bilayer (7). Lysosomal functions of granulins remain largely unknown. Prosaposin is shown in blue; progranulin, in red. Lys denotes lysosome.

we will generate hypotheses based on these results using pathway analysis. We will test whether *Grn* deficiency affects expression or activity of key lysosomal enzymes involved in the identified pathways. If a key enzyme regulated by progranulin or granulins is identified, we will test whether manipulating the expression of this enzyme can ameliorate FTLD-related phenotypes in mice or neurons.

Another supporting, hypothesis generating approach will be full transcriptome sequencing of $Grn^{-/-}$, $Grn^{-/+}$, and $Grn^{+/+}$ mice. Compensatory changes in endosomal/lysosomal pathway proteins may direct us to more specific hypotheses about the role of progranulin in lysosomal biology. One of the hits from our granulin A proteomics study, wolframin, incidentally points to the same pathway. Mutations in Wfs1 result in Wolfram syndrome, a disease with neurodegenerative characteristics. Wolframin is an ER protein that is probably involved in intracellular sorting, similar to sortilin (the progranulin receptor) and other proteins implicated in NCLs. It is possible that progranulin binding to wolframin plays a role in its intracellular transport, and perhaps sorting of lysosomal enzymes. Testing whether wolframin loss-of-function alters the expression or localization of progranulin or lysosomal markers will be informative. Wfs1 knock-out mice are available; a genetic interaction between Wfs1 and *Grn* can be tested by crossing these mice and scrutinizing neurodegenerative phenotypes.

Granulin Receptors

We have found that granulin-A-Fc binds solubilized EAAT1 in pull-down experiments but does not have an acute effect on EAAT1 mediated glutamate uptake. In light of this negative finding, the next step should be to confirm that the binding occurs in

physiologically significant conditions, i.e. on the surface of intact cells. For this purpose, we are in the process of generating myc-tagged granulin peptides. These will be used to test cell surface binding with immunocytochemistry. If cell surface binding is observed, competition by untagged granulin A will be tested as a control. Another approach for testing cell surface binding can be FACS.

The reason why we failed to observe a change in glutamate transport may be that the effect of progranulin deficiency takes place over the long term, perhaps interfering with EAAT1 expression and trafficking. To test this hypothesis, we will compare glutamate transport in mixed glial cultures prepared from wild-type or *Grn*$^{-/-}$ mice.

Na^+/K^+ ATPase, although ubiquitous, is also a promising candidate granulin interactor. It is an agrin receptor, regulating frequency of spontaneous action potentials in neurons and contractility in cardiac myocytes (Hilgenberg et al., 2006; Hilgenberg et al., 2009). Since wolframin, Na^+/K^+ ATPase and EAAT1 are known to bind each other, it will be important to confirm that granulin A directly binds this protein. Finally, our proven biochemical approach can be used to search for proteins interacting with other granulins (B-F).

BIBLIOGRAPHY

Ahmed, Z., H. Sheng, Y. f. Xu, W. L. Lin, A. E. Innes, J. Gass, X. Yu, H. Hou, S. Chiba, K. Yamanouchi, M. Leissring, L. Petrucelli, M. Nishihara, M. L. Hutton, E. McGowan, D. W. Dickson and J. Lewis (2010). "Accelerated Lipofuscinosis and Ubiquitination in Granulin Knockout Mice Suggest a Role for Progranulin in Successful Aging." American Journal Of Pathology **177**(1): 311-324.

Akil, H., A. Perraud, C. Melin, M. O. Jauberteau and M. Mathonnet (2011). "Fine-tuning roles of endogenous brain-derived neurotrophic factor, TrkB and sortilin in colorectal cancer cell survival." PLoS ONE **6**(9): e25097.

Almeida, S., L. Zhou and F. B. Gao (2011). "Progranulin, a glycoprotein deficient in frontotemporal dementia, is a novel substrate of several protein disulfide isomerase family proteins." PLoS ONE **6**(10): e26454.

Anakwe, O. O. and G. L. Gerton (1990). "Acrosome biogenesis begins during meiosis: evidence from the synthesis and distribution of an acrosomal glycoprotein, acrogranin, during guinea pig spermatogenesis." Biol Reprod **42**(2): 317-328.

Arai, T., M. Hasegawa, H. Akiyama, K. Ikeda, T. Nonaka, H. Mori, D. Mann, K. Tsuchiya, M. Yoshida, Y. Hashizume and T. Oda (2006). "TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis." Biochem Biophys Res Commun **351**(3): 602-611.

Araki, T. (2004). "Increased Nuclear NAD Biosynthesis and SIRT1 Activation Prevent Axonal Degeneration." Science **305**(5686): 1010-1013.

Baba, T., H. B. Hoff, 3rd, H. Nemoto, H. Lee, J. Orth, Y. Arai and G. L. Gerton (1993). "Acrogranin, an acrosomal cysteine-rich glycoprotein, is the precursor of the growth-modulating peptides, granulins, and epithelins, and is expressed in somatic as well as male germ cells." Mol Reprod Dev **34**(3): 233-243.

Bai, X. H., D. W. Wang, L. Kong, Y. Zhang, Y. Luan, T. Kobayashi, H. M. Kronenberg, X. P. Yu and C. J. Liu (2009). "ADAMTS-7, a direct target of PTHrP, adversely regulates endochondral bone growth by associating with and inactivating GEP growth factor." Mol Cell Biol **29**(15): 4201-4219.

Baker, M., I. R. Mackenzie, S. M. Pickering-Brown, J. Gass, R. Rademakers, C. Lindholm, J. Snowden, J. Adamson, A. D. Sadovnick, S. Rollinson, A. Cannon, E. Dwosh, D. Neary, S. Melquist, A. Richardson, D. Dickson, Z. Berger, J. Eriksen, T. Robinson, C. Zehr, C. A. Dickey, R. Crook, E. McGowan, D. Mann, B. Boeve, H. Feldman and M. Hutton (2006).

"Mutations in progranulin cause tau-negative frontotemporal dementia linked to chromosome 17." Nature **442**(7105): 916-919.

Bateman, A., D. Belcourt, H. Bennett, C. Lazure and S. Solomon (1990). "Granulins, a novel class of peptide from leukocytes." Biochem Biophys Res Commun **173**(3): 1161-1168.

Bhandari, V., R. Daniel, P. S. Lim and A. Bateman (1996). "Structural and functional analysis of a promoter of the human granulin/epithelin gene." Biochem J **319 (Pt 2)**: 441-447.

Bhandari, V., R. G. Palfree and A. Bateman (1992). "Isolation and sequence of the granulin precursor cDNA from human bone marrow reveals tandem cysteine-rich granulin domains." Proc Natl Acad Sci U S A **89**(5): 1715-1719.

Blennow, K., M. J. de Leon and H. Zetterberg (2006). "Alzheimer's disease." The Lancet **368**(9533): 387-403.

Bossu, P., F. Salani, A. Alberici, S. Archetti, G. Bellelli, D. Galimberti, E. Scarpini, G. Spalletta, C. Caltagirone, A. Padovani and B. Borroni (2011). "Loss of function mutations in the progranulin gene are related to pro-inflammatory cytokine dysregulation in frontotemporal lobar degeneration patients." J Neuroinflammation **8**: 65.

Broe, M., J. Kril and G. M. Halliday (2004). "Astrocytic degeneration relates to the severity of disease in frontotemporal dementia." Brain **127**(Pt 10): 2214-2220.

Brun, A. and L. Gustafson (2011). "The birth and early evolution of the frontotemporal dementia (FTD) concept." J Mol Neurosci **45**(3): 324-329.

Butchbach, M. E., G. Tian, H. Guo and C. L. Lin (2004). "Association of excitatory amino acid transporters, especially EAAT2, with cholesterol-rich lipid raft microdomains: importance for excitatory amino acid transporter localization and function." J Biol Chem **279**(33): 34388-34396.

Butler, G. S., R. A. Dean, E. M. Tam and C. M. Overall (2008). "Pharmacoproteomics of a metalloproteinase hydroxamate inhibitor in breast cancer cells: dynamics of membrane type 1 matrix metalloproteinase-mediated membrane protein shedding." Mol Cell Biol **28**(15): 4896-4914.

Calamini, B., K. Ratia, M. G. Malkowski, M. Cuendet, J. M. Pezzuto, B. D. Santarsiero and A. D. Mesecar (2010). "Pleiotropic mechanisms facilitated by resveratrol and its metabolites." Biochem J **429**(2): 273-282.

Capell, A., S. Liebscher, K. Fellerer, N. Brouwers, M. Willem, S. Lammich, I. Gijselinck, T. Bittner, A. M. Carlson, F. Sasse, B. Kunze, H. Steinmetz, R. Jansen, D. Dormann, K. Sleegers, M. Cruts, J. Herms, C. Van Broeckhoven and C. Haass (2011). "Rescue of Progranulin Deficiency Associated with Frontotemporal Lobar Degeneration by Alkalizing

Reagents and Inhibition of Vacuolar ATPase." Journal of Neuroscience **31**(5): 1885-1894.

Cenik, B., C. F. Sephton, B. K. Cenik, J. Herz and G. Yu (2012). "Progranulin: a proteolytically processed protein at the crossroads of inflammation and neurodegeneration." J Biol Chem.

Cenik, B., C. F. Sephton, C. M. Dewey, X. Xian, S. Wei, K. Yu, W. Niu, G. Coppola, S. E. Coughlin, S. E. Lee, D. R. Dries, S. Almeida, D. H. Geschwind, F. B. Gao, B. L. Miller, R. V. Farese, Jr., B. A. Posner, G. Yu and J. Herz (2011). "Suberoylanilide hydroxamic acid (vorinostat) up-regulates progranulin transcription: rational therapeutic approach to frontotemporal dementia." J Biol Chem **286**(18): 16101-16108.

Chen, X., J. Chang, Q. Deng, J. Xu, T. T. Nguyen, L. H. Martens, B. Cenik, G. Taylor, K. Hudson, J. Chung, K. Yu, P. Yu, J. Herz, R. V. F. Jr., T. Kukar and M. G. Tansey (2012). PGRN does not bind Tumor Necrosis Factor (TNF) Receptors and is not a direct regulator of TNF- or LPS-dependent signaling or bioactivity. *(unpublished manuscript)*

Cheung, S. T., S. Y. Wong, K. L. Leung, X. Chen, S. So, I. O. Ng and S. T. Fan (2004). "Granulin-epithelin precursor overexpression promotes growth and invasion of hepatocellular carcinoma." Clin Cancer Res **10**(22): 7629-7636.

Chitramuthu, B. P., D. C. Baranowski, D. G. Kay, A. Bateman and H. P. Bennett (2010). "Progranulin modulates zebrafish motoneuron development in vivo and rescues truncation defects associated with knockdown of Survival motor neuron 1." Mol Neurodegener **5**: 41.

Clark, L. N., P. Poorkaj, Z. Wszolek, D. H. Geschwind, Z. S. Nasreddine, B. Miller, D. Li, H. Payami, F. Awert, K. Markopoulou, A. Andreadis, I. D'Souza, V. M. Lee, L. Reed, J. Q. Trojanowski, V. Zhukareva, T. Bird, G. Schellenberg and K. C. Wilhelmsen (1998). "Pathogenic implications of mutations in the tau gene in pallido-ponto-nigral degeneration and related neurodegenerative disorders linked to chromosome 17." Proc Natl Acad Sci U S A **95**(22): 13103-13107.

Colombrita, C., E. Onesto, F. Megiorni, A. Pizzuti, F. E. Baralle, E. Buratti, V. Silani and A. Ratti (2012). "TDP-43 and FUS RNA-binding proteins bind distinct sets of cytoplasmic messenger RNAs and differently regulate their post-transcriptional fate in motoneuron-like cells." J Biol Chem.

Conus, S. and H. U. Simon (2008). "Cathepsins: key modulators of cell death and inflammatory responses." Biochem Pharmacol **76**(11): 1374-1382.

Croft, M., W. Duan, H. Choi, S. Y. Eun, S. Madireddi and A. Mehta (2012). "TNF superfamily in inflammatory disease: translating basic insights." Trends Immunol **33**(3): 144-152.

Cruts, M., I. Gijselinck, J. van der Zee, S. Engelborghs, H. Wils, D. Pirici, R. Rademakers, R. Vandenberghe, B. Dermaut, J.-J. Martin, C. van Duijn, K. Peeters, R. Sciot, P. Santens, T. De Pooter, M. Mattheijssens, M. Van den Broeck, I. Cuijt, K. l. Vennekens, P. P. De Deyn, S. Kumar-Singh and C. Van Broeckhoven (2006). "Null mutations in progranulin cause ubiquitin-positive frontotemporal dementia linked to chromosome 17q21." Nature **442**(7105): 920-924.

Cuevas-Antonio, R., C. Cancino, F. Arechavaleta-Velasco, A. Andrade, L. Barron, I. Estrada, R. L. Fernandez, V. Olguin, S. Ruiz, F. Imani, M. Zeferino-Toquero, A. Ulloa-Aguirre, G. L. Gerton and L. Diaz-Cueto (2010). "Expression of Progranulin (Acrogranin/PCDGF/Granulin-Epithelin Precursor) in Benign and Malignant Ovarian Tumors and Activation of MAPK Signaling in Ovarian Cancer Cell Line*." Cancer Investigation **28**(5): 452-458.

Culouscou, J. M., G. W. Carlton and M. Shoyab (1993). "Biochemical analysis of the epithelin receptor." J Biol Chem **268**(14): 10458-10462.

Daniel, R., E. Daniels, Z. He and A. Bateman (2003). "Progranulin (acrogranin/PC cell-derived growth factor/granulin-epithelin precursor) is expressed in the placenta, epidermis, microvasculature, and brain during murine development." Dev Dyn **227**(4): 593-599.

Daniel, R., Z. He, K. P. Carmichael, J. Halper and A. Bateman (2000). "Cellular localization of gene expression for progranulin." J Histochem Cytochem **48**(7): 999-1009.

De Riz, M., D. Galimberti, C. Fenoglio, L. M. Piccio, D. Scalabrini, E. Venturelli, A. Pietroboni, M. Piola, R. T. Naismith, B. J. Parks, G. Fumagalli, N. Bresolin, A. H. Cross and E. Scarpini (2010). "Cerebrospinal fluid progranulin levels in patients with different multiple sclerosis subtypes." Neurosci Lett **469**(2): 234-236.

DeJesus-Hernandez, M., Ian R. Mackenzie, Bradley F. Boeve, Adam L. Boxer, M. Baker, Nicola J. Rutherford, Alexandra M. Nicholson, NiCole A. Finch, H. Flynn, J. Adamson, N. Kouri, A. Wojtas, P. Sengdy, G.-Yuek R. Hsiung, A. Karydas, William W. Seeley, Keith A. Josephs, G. Coppola, Daniel H. Geschwind, Zbigniew K. Wszolek, H. Feldman, David S. Knopman, Ronald C. Petersen, Bruce L. Miller, Dennis W. Dickson, Kevin B. Boylan, Neill R. Graff-Radford and R. Rademakers (2011). "Expanded GGGGCC Hexanucleotide Repeat in Noncoding Region of C9ORF72 Causes Chromosome 9p-Linked FTD and ALS." Neuron **72**(2): 245-256.

Dickson, D. W., N. Kouri, M. E. Murray and K. A. Josephs (2011). "Neuropathology of frontotemporal lobar degeneration-tau (FTLD-tau)." J Mol Neurosci **45**(3): 384-389.

Donald, C. D., A. Laddu, P. Chandham, S. D. Lim, C. Cohen, M. Amin, G. L. Gerton, F. F. Marshall and J. A. Petros (2001). "Expression of progranulin and the epithelin/granulin precursor acrogranin correlates with neoplastic state in renal epithelium." Anticancer Res **21**(6A): 3739-3742.

Elkabets, M., A. M. Gifford, C. Scheel, B. Nilsson, F. Reinhardt, M. A. Bray, A. E. Carpenter, K. Jirstrom, K. Magnusson, B. L. Ebert, F. Ponten, R. A. Weinberg and S. S. McAllister (2011). "Human tumors instigate granulin-expressing hematopoietic cells that promote malignancy by activating stromal fibroblasts in mice." J Clin Invest **121**(2): 784-799.

Faraco, G., T. Pancani, L. Formentini, P. Mascagni, G. Fossati, F. Leoni, F. Moroni and A. Chiarugi (2006). "Pharmacological Inhibition of Histone Deacetylases by Suberoylanilide Hydroxamic Acid Specifically Alters Gene Expression and Reduces Ischemic Injury in the Mouse Brain." Molecular Pharmacology **70**(6): 1876-1884.

Feng, J. Q., F. J. Guo, B. C. Jiang, Y. Zhang, S. Frenkel, D. W. Wang, W. Tang, Y. Xie and C. J. Liu (2010). "Granulin epithelin precursor: a bone morphogenic protein 2-inducible growth factor that activates Erk1/2 signaling and JunB transcription factor in chondrogenesis." FASEB J **24**(6): 1879-1892.

Ferrante, R. J., J. K. Kubilus, J. Lee, H. Ryu, A. Beesen, B. Zucker, K. Smith, N. W. Kowall, R. R. Ratan, R. Luthi-Carter and S. M. Hersch (2003). "Histone deacetylase inhibition by sodium butyrate chemotherapy ameliorates the neurodegenerative phenotype in Huntington's disease mice." J Neurosci **23**(28): 9418-9427.

Finch, N., M. Baker, R. Crook, K. Swanson, K. Kuntz, R. Surtees, G. Bisceglio, A. Rovelet-Lecrux, B. Boeve, R. C. Petersen, D. W. Dickson, S. G. Younkin, V. Deramecourt, J. Crook, N. R. Graff-Radford and R. Rademakers (2009). "Plasma progranulin levels predict progranulin mutation status in frontotemporal dementia patients and asymptomatic family members." Brain **132**(3): 583-591.

Finkelstein, R. A., Y. Li, B. Liu, F. Shuja, E. Fukudome, G. C. Velmahos, M. deMoya and H. B. Alam (2010). "Treatment with histone deacetylase inhibitor attenuates MAP kinase mediated liver injury in a lethal model of septic shock." J Surg Res **163**(1): 146-154.

Fonseca, S. G. (2005). "WFS1 Is a Novel Component of the Unfolded Protein Response and Maintains Homeostasis of the Endoplasmic Reticulum in Pancreatic -Cells." Journal of Biological Chemistry **280**(47): 39609-39615.

Fonseca, S. G., S. Ishigaki, C. M. Oslowski, S. Lu, K. L. Lipson, R. Ghosh, E. Hayashi, H. Ishihara, Y. Oka, M. A. Permutt and F. Urano (2010).

"Wolfram syndrome 1 gene negatively regulates ER stress signaling in rodent and human cells." Journal of Clinical Investigation.

Gao, X., A. P. Joselin, L. Wang, A. Kar, P. Ray, A. Bateman, A. M. Goate and J. Y. Wu (2010). "Progranulin promotes neurite outgrowth and neuronal differentiation by regulating GSK-3beta." Protein Cell **1**(6): 552-562.

Gass, J., A. Cannon, I. R. Mackenzie, B. Boeve, M. Baker, J. Adamson, R. Crook, S. Melquist, K. Kuntz, R. Petersen, K. Josephs, S. M. Pickering-Brown, N. Graff-Radford, R. Uitti, D. Dickson, Z. Wszolek, J. Gonzalez, T. G. Beach, E. Bigio, N. Johnson, S. Weintraub, M. Mesulam, C. L. White, 3rd, B. Woodruff, R. Caselli, G. Y. Hsiung, H. Feldman, D. Knopman, M. Hutton and R. Rademakers (2006). "Mutations in progranulin are a major cause of ubiquitin-positive frontotemporal lobar degeneration." Hum Mol Genet **15**(20): 2988-3001.

Ghidoni, R., L. Benussi, M. Glionna, M. Franzoni and G. Binetti (2008). "Low plasma progranulin levels predict progranulin mutations in frontotemporal lobar degeneration." Neurology **71**(16): 1235-1239.

Ghoshal, N., J. T. Dearborn, D. F. Wozniak and N. J. Cairns (2012). "Core features of frontotemporal dementia recapitulated in progranulin knockout mice." Neurobiol Dis **45**(1): 395-408.

Glauben, R., A. Batra, I. Fedke, M. Zeitz, H. A. Lehr, F. Leoni, P. Mascagni, G. Fantuzzi, C. A. Dinarello and B. Siegmund (2006). "Histone hyperacetylation is associated with amelioration of experimental colitis in mice." J Immunol **176**(8): 5015-5022.

Goedert, M., R. A. Crowther and M. G. Spillantini (1998). "Tau mutations cause frontotemporal dementias." Neuron **21**(5): 955-958.

Goedert, M. and R. Jakes (2005). "Mutations causing neurodegenerative tauopathies." Biochim Biophys Acta **1739**(2-3): 240-250.

Goedert, M., R. Jakes, M. G. Spillantini, M. Hasegawa, M. J. Smith and R. A. Crowther (1996). "Assembly of microtubule-associated protein tau into Alzheimer-like filaments induced by sulphated glycosaminoglycans." Nature **383**(6600): 550-553.

Guan, J. S., S. J. Haggarty, E. Giacometti, J. H. Dannenberg, N. Joseph, J. Gao, T. J. Nieland, Y. Zhou, X. Wang, R. Mazitschek, J. E. Bradner, R. A. DePinho, R. Jaenisch and L. H. Tsai (2009). "HDAC2 negatively regulates memory formation and synaptic plasticity." Nature **459**(7243): 55-60.

Hagen, F. S., F. J. Grant, J. L. Kuijper, C. A. Slaughter, C. R. Moomaw, K. Orth, P. J. O'Hara and R. S. Munford (1991). "Expression and characterization of recombinant human acyloxyacyl hydrolase, a leukocyte enzyme that deacylates bacterial lipopolysaccharides." Biochemistry **30**(34): 8415-8423.

Hahnen, E., I. Y. Eyupoglu, L. Brichta, K. Haastert, C. Trankle, F. A. Siebzehnrubl, M. Riessland, I. Holker, P. Claus, J. Romstock, R. Buslei, B. Wirth and I. Blumcke (2006). "In vitro and ex vivo evaluation of second-generation histone deacetylase inhibitors for the treatment of spinal muscular atrophy." J Neurochem **98**(1): 193-202.

Halili, M. A., M. R. Andrews, L. I. Labzin, K. Schroder, G. Matthias, C. Cao, E. Lovelace, R. C. Reid, G. T. Le, D. A. Hume, K. M. Irvine, P. Matthias, D. P. Fairlie and M. J. Sweet (2010). "Differential effects of selective HDAC inhibitors on macrophage inflammatory responses to the Toll-like receptor 4 agonist LPS." Journal of Leukocyte Biology **87**(6): 1103-1114.

Hatanaka, M., K. Tanabe, A. Yanai, Y. Ohta, M. Kondo, M. Akiyama, K. Shinoda, Y. Oka and Y. Tanizawa (2011). "Wolfram syndrome 1 gene (WFS1) product localizes to secretory granules and determines granule acidification in pancreatic -cells." Human Molecular Genetics.

He, Z. and A. Bateman (1999). "Progranulin gene expression regulates epithelial cell growth and promotes tumor growth in vivo." Cancer Res **59**(13): 3222-3229.

He, Z., A. Ismail, L. Kriazhev, G. Sadvakassova and A. Bateman (2002). "Progranulin (PC-cell-derived growth factor/acrogranin) regulates invasion and cell survival." Cancer Res **62**(19): 5590-5596.

He, Z., C. H. P. Ong, J. Halper and A. Bateman (2003). "Progranulin is a mediator of the wound response." Nature Medicine **9**(2): 225-229.

Heltweg, B., F. Dequiedt, B. L. Marshall, C. Brauch, M. Yoshida, N. Nishino, E. Verdin and M. Jung (2004). "Subtype selective substrates for histone deacetylases." J Med Chem **47**(21): 5235-5243.

Herman, D., K. Jenssen, R. Burnett, E. Soragni, S. L. Perlman and J. M. Gottesfeld (2006). "Histone deacetylase inhibitors reverse gene silencing in Friedreich's ataxia." Nature Chemical Biology **2**(10): 551-558.

Hiesberger, T., S. Huttler, A. Rohlmann, W. Schneider, K. Sandhoff and J. Herz (1998). "Cellular uptake of saposin (SAP) precursor and lysosomal delivery by the low density lipoprotein receptor-related protein (LRP)." EMBO J **17**(16): 4617-4625.

Hilgenberg, L. G., B. Pham, M. Ortega, S. Walid, T. Kemmerly, D. K. O'Dowd and M. A. Smith (2009). "Agrin regulation of alpha3 sodium-potassium ATPase activity modulates cardiac myocyte contraction." J Biol Chem **284**(25): 16956-16965.

Hilgenberg, L. G., H. Su, H. Gu, D. K. O'Dowd and M. A. Smith (2006). "Alpha3Na+/K+-ATPase is a neuronal receptor for agrin." Cell **125**(2): 359-369.

Hockly, E. (2003). "Suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, ameliorates motor deficits in a mouse model of Huntington's

disease." Proceedings of the National Academy of Sciences **100**(4): 2041-2046.

Hodges, J. R., R. Davies, J. Xuereb, J. Kril and G. Halliday (2003). "Survival in frontotemporal dementia." Neurology **61**(3): 349-354.

Hofmann, S. (2003). "Wolfram syndrome: structural and functional analyses of mutant and wild-type wolframin, the WFS1 gene product." Human Molecular Genetics **12**(16): 2003-2012.

Hrabal, R., Z. Chen, S. James, H. P. Bennett and F. Ni (1996). "The hairpin stack fold, a novel protein architecture for a new family of protein growth factors." Nat Struct Biol **3**(9): 747-752.

Hu, F., T. Padukkavidana, C. B. Vægter, O. A. Brady, Y. Zheng, I. R. Mackenzie, H. H. Feldman, A. Nykjaer and S. M. Strittmatter (2010). "Sortilin-Mediated Endocytosis Determines Levels of the Frontotemporal Dementia Protein, Progranulin." Neuron **68**(4): 654-667.

Hutton, M., C. L. Lendon, P. Rizzu, M. Baker, S. Froelich, H. Houlden, S. Pickering-Brown, S. Chakraverty, A. Isaacs, A. Grover, J. Hackett, J. Adamson, S. Lincoln, D. Dickson, P. Davies, R. C. Petersen, M. Stevens, E. de Graaff, E. Wauters, J. van Baren, M. Hillebrand, M. Joosse, J. M. Kwon, P. Nowotny, L. K. Che, J. Norton, J. C. Morris, L. A. Reed, J. Trojanowski, H. Basun, L. Lannfelt, M. Neystat, S. Fahn, F. Dark, T. Tannenberg, P. R. Dodd, N. Hayward, J. B. Kwok, P. R. Schofield, A. Andreadis, J. Snowden, D. Craufurd, D. Neary, F. Owen, B. A. Oostra, J. Hardy, A. Goate, J. van Swieten, D. Mann, T. Lynch and P. Heutink (1998). "Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17." Nature **393**(6686): 702-705.

Iorio, F., R. Bosotti, E. Scacheri, V. Belcastro, P. Mithbaokar, R. Ferriero, L. Murino, R. Tagliaferri, N. Brunetti-Pierri, A. Isacchi and D. di Bernardo (2010). "Discovery of drug mode of action and drug repositioning from transcriptional responses." Proc Natl Acad Sci U S A **107**(33): 14621-14626.

Ishihara, H. (2004). "Disruption of the WFS1 gene in mice causes progressive -cell loss and impaired stimulus-secretion coupling in insulin secretion." Human Molecular Genetics **13**(11): 1159-1170.

Jansen, P., K. Giehl, J. R. Nyengaard, K. Teng, O. Lioubinski, S. S. Sjoegaard, T. Breiderhoff, M. Gotthardt, F. Lin, A. Eilers, C. M. Petersen, G. R. Lewin, B. L. Hempstead, T. E. Willnow and A. Nykjaer (2007). "Roles for the pro-neurotrophin receptor sortilin in neuronal development, aging and brain injury." Nat Neurosci **10**(11): 1449-1457.

Jen, J. C., J. Wan, T. P. Palos, B. D. Howard and R. W. Baloh (2005). "Mutation in the glutamate transporter EAAT1 causes episodic ataxia, hemiplegia, and seizures." Neurology **65**(4): 529-534.

Jiao, J., L. D. Herl, R. V. Farese and F. B. Gao (2010). "MicroRNA-29b regulates the expression level of human progranulin, a secreted glycoprotein implicated in frontotemporal dementia." PLoS ONE **5**(5): e10551.

Jung, T., N. Bader and T. Grune (2007). "Lipofuscin: formation, distribution, and metabolic consequences." Ann N Y Acad Sci **1119**: 97-111.

Kai, L., S. K. Samuel and A. S. Levenson (2010). "Resveratrol enhances p53 acetylation and apoptosis in prostate cancer by inhibiting MTA1/NuRD complex." Int J Cancer **126**(7): 1538-1548.

Kao, A. W., R. J. Eisenhut, L. H. Martens, A. Nakamura, A. Huang, J. A. Bagley, P. Zhou, A. de Luis, L. J. Neukomm, J. Cabello, R. V. Farese and C. Kenyon (2011). "A neurodegenerative disease mutation that accelerates the clearance of apoptotic cells." Proceedings of the National Academy of Sciences **108**(11): 4441-4446.

Karpova, L. V., E. R. Bulygina and A. A. Boldyrev (2010). "Different neuronal Na+/K+-ATPase isoforms are involved in diverse signaling pathways." Cell Biochemistry and Function **28**(2): 135-141.

Kato, T., M. Ishiwata, K. Yamada, T. Kasahara, C. Kakiuchi, K. Iwamoto, K. Kawamura, H. Ishihara and Y. Oka (2008). "Behavioral and gene expression analyses of Wfs1 knockout mice as a possible animal model of mood disorder." Neuroscience Research **61**(2): 143-158.

Kayasuga, Y., S. Chiba, M. Suzuki, T. Kikusui, T. Matsuwaki, K. Yamanouchi, H. Kotaki, R. Horai, Y. Iwakura and M. Nishihara (2007). "Alteration of behavioural phenotype in mice by targeted disruption of the progranulin gene." Behavioural Brain Research **185**(2): 110-118.

Kazantsev, A. G. and L. M. Thompson (2008). "Therapeutic application of histone deacetylase inhibitors for central nervous system disorders." Nature Reviews Drug Discovery **7**(10): 854-868.

Kertesz, A., P. McMonagle, M. Blair, W. Davidson and D. G. Munoz (2005). "The evolution and pathology of frontotemporal dementia." Brain **128**(Pt 9): 1996-2005.

Kessenbrock, K., L. Frohlich, M. Sixt, T. Lammermann, H. Pfister, A. Bateman, A. Belaaouaj, J. Ring, M. Ollert, R. Fassler and D. E. Jenne (2008). "Proteinase 3 and neutrophil elastase enhance inflammation in mice by inactivating antiinflammatory progranulin." J Clin Invest **118**(7): 2438-2447.

Kim, S.-Y., S.-Y. Choi, W. Chao and D. J. Volsky (2003). "Transcriptional regulation of human excitatory amino acid transporter 1 (EAAT1): cloning of the EAAT1 promoter and characterization of its basal and inducible activity in human astrocytes." Journal of Neurochemistry **87**(6): 1485-1498.

Kohlschutter, A. and A. Schulz (2009). "Towards understanding the neuronal ceroid lipofuscinoses." Brain Dev **31**(7): 499-502.

Kojima, Y., K. Ono, K. Inoue, Y. Takagi, K.-i. Kikuta, M. Nishimura, Y. Yoshida, Y. Nakashima, H. Matsumae and Y. Furukawa (2009). "Progranulin expression in advanced human atherosclerotic plaque." Atherosclerosis **206**(1): 102-108.

Kril, J. J., V. Macdonald, S. Patel, F. Png and G. M. Halliday (2005). "Distribution of brain atrophy in behavioral variant frontotemporal dementia." J Neurol Sci **232**(1-2): 83-90.

Lagier-Tourenne, C., M. Polymenidou and D. W. Cleveland (2010). "TDP-43 and FUS/TLS: emerging roles in RNA processing and neurodegeneration." Human Molecular Genetics **19**(R1): R46-R64.

Lane, A. A. and B. A. Chabner (2009). "Histone Deacetylase Inhibitors in Cancer Therapy." Journal of Clinical Oncology **27**(32): 5459-5468.

Leahy and D. (2000). "A Mammalian Expression Vector for Expression and Purification of Secreted Proteins for Structural Studies." Protein Expression and Purification **20**(3): 500-506.

Lee, R., P. Kermani, K. K. Teng and B. L. Hempstead (2001). "Regulation of cell survival by secreted proneurotrophins." Science **294**(5548): 1945-1948.

Liang, J., H. Takeuchi, Y. Doi, J. Kawanokuchi, Y. Sonobe, S. Jin, I. Yawata, H. Li, S. Yasuoka and T. Mizuno (2008). "Excitatory amino acid transporter expression by astrocytes is neuroprotective against microglial excitotoxicity." Brain Research **1210**: 11-19.

Liau, L. M., R. L. Lallone, R. S. Seitz, A. Buznikov, J. P. Gregg, H. I. Kornblum, S. F. Nelson and J. M. Bronstein (2000). "Identification of a human glioma-associated growth factor gene, granulin, using differential immuno-absorption." Cancer Res **60**(5): 1353-1360.

Lin, H. S., C. Y. Hu, H. Y. Chan, Y. Y. Liew, H. P. Huang, L. Lepescheux, E. Bastianelli, R. Baron, G. Rawadi and P. Clement-Lacroix (2007). "Anti-rheumatic activities of histone deacetylase (HDAC) inhibitors in vivo in collagen-induced arthritis in rodents." Br J Pharmacol **150**(7): 862-872.

Liu, S., L.-C. Wu, J. Pang, R. Santhanam, S. Schwind, Y.-Z. Wu, C. J. Hickey, J. Yu, H. Becker and K. Maharry (2010). "Sp1/NFκB/HDAC/miR-29b Regulatory Network in KIT-Driven Myeloid Leukemia." Cancer Cell **17**(4): 333-347.

Lu, R. and G. Serrero (2000). "Inhibition of PC cell-derived growth factor (PCDGF, epithelin/granulin precursor) expression by antisense PCDGF cDNA transfection inhibits tumorigenicity of the human breast carcinoma cell line MDA-MB-468." Proc Natl Acad Sci U S A **97**(8): 3993-3998.

Luuk, H., M. Plaas, S. Raud, J. Innos, S. Sütt, H. Lasner, U. Abramov, K. Kurrikoff, S. Kõks and E. Vasar (2009). "Wfs1-deficient mice display

impaired behavioural adaptation in stressful environment." Behavioural Brain Research **198**(2): 334-345.

Ma, P. T., G. Gil, T. C. Sudhof, D. W. Bilheimer, J. L. Goldstein and M. S. Brown (1986). "Mevinolin, an inhibitor of cholesterol synthesis, induces mRNA for low density lipoprotein receptor in livers of hamsters and rabbits." Proc Natl Acad Sci U S A **83**(21): 8370-8374.

Mackenzie, I. R., M. Baker, S. Pickering-Brown, G. Y. Hsiung, C. Lindholm, E. Dwosh, J. Gass, A. Cannon, R. Rademakers, M. Hutton and H. H. Feldman (2006). "The neuropathology of frontotemporal lobar degeneration caused by mutations in the progranulin gene." Brain **129**(Pt 11): 3081-3090.

Mann, B. S., J. R. Johnson, M. H. Cohen, R. Justice and R. Pazdur (2007). "FDA approval summary: vorinostat for treatment of advanced primary cutaneous T-cell lymphoma." Oncologist **12**(10): 1247-1252.

Martens, L. H., J. Zhang, S. J. Barmada, P. Zhou, S. Kamiya, B. Sun, S. W. Min, L. Gan, S. Finkbeiner, E. J. Huang and R. V. Farese, Jr. (2012). "Progranulin deficiency promotes neuroinflammation and neuron loss following toxin-induced injury." J Clin Invest.

Matsubara, T., A. Mita, K. Minami, T. Hosooka, S. Kitazawa, K. Takahashi, Y. Tamori, N. Yokoi, M. Watanabe, E. Matsuo, O. Nishimura and S. Seino (2012). "PGRN is a key adipokine mediating high fat diet-induced insulin resistance and obesity through IL-6 in adipose tissue." Cell Metab **15**(1): 38-50.

Matsumura, N., M. Mandai, M. Miyanishi, K. Fukuhara, T. Baba, T. Higuchi, M. Kariya, K. Takakura and S. Fujii (2006). "Oncogenic property of acrogranin in human uterine leiomyosarcoma: direct evidence of genetic contribution in in vivo tumorigenesis." Clin Cancer Res **12**(5): 1402-1411.

Meyer, H., M. Bug and S. Bremer (2012). "Emerging functions of the VCP/p97 AAA-ATPase in the ubiquitin system." Nat Cell Biol **14**(2): 117-123.

Mielcarek, M., C. L. Benn, S. A. Franklin, D. L. Smith, B. Woodman, P. A. Marks and G. P. Bates (2011). "SAHA Decreases HDAC 2 and 4 Levels In Vivo and Improves Molecular Phenotypes in the R6/2 Mouse Model of Huntington's Disease." PLoS ONE **6**(11): e27746.

Miyanishi, M., M. Mandai, N. Matsumura, K. Yamaguchi, J. Hamanishi, T. Higuchi, K. Takakura and S. Fujii (2007). "Immortalized ovarian surface epithelial cells acquire tumorigenicity by Acrogranin gene overexpression." Oncol Rep **17**(2): 329-333.

Monami, G. (2006). "Proepithelin Promotes Migration and Invasion of 5637 Bladder Cancer Cells through the Activation of ERK1/2 and the Formation of a Paxillin/FAK/ERK Complex." Cancer Research **66**(14): 7103-7110.

Monami, G., V. Emiliozzi, A. Bitto, F. Lovat, S. Q. Xu, S. Goldoni, M. Fassan, G. Serrero, L. G. Gomella, R. Baffa, R. V. Iozzo and A. Morrione (2009). "Proepithelin Regulates Prostate Cancer Cell Biology by Promoting Cell Growth, Migration, and Anchorage-Independent Growth." American Journal Of Pathology **174**(3): 1037-1047.

Musunuru, K., A. Strong, M. Frank-Kamenetsky, N. E. Lee, T. Ahfeldt, K. V. Sachs, X. Li, H. Li, N. Kuperwasser, V. M. Ruda, J. P. Pirruccello, B. Muchmore, L. Prokunina-Olsson, J. L. Hall, E. E. Schadt, C. R. Morales, S. Lund-Katz, M. C. Phillips, J. Wong, W. Cantley, T. Racie, K. G. Ejebe, M. Orho-Melander, O. Melander, V. Koteliansky, K. Fitzgerald, R. M. Krauss, C. A. Cowan, S. Kathiresan and D. J. Rader (2010). "From noncoding variant to phenotype via SORT1 at the 1p13 cholesterol locus." Nature **466**(7307): 714-719.

Narayan, P. and M. Dragunow (2010). "Pharmacology of epigenetics in brain disorders." British Journal of Pharmacology **159**(2): 285-303.

Neitzel, H. (1986). "A routine method for the establishment of permanent growing lymphoblastoid cell lines." Hum Genet **73**(4): 320-326.

Neumann, M., R. Rademakers, S. Roeber, M. Baker, H. A. Kretzschmar and I. R. Mackenzie (2009a). "A new subtype of frontotemporal lobar degeneration with FUS pathology." Brain **132**(Pt 11): 2922-2931.

Neumann, M., S. Roeber, H. A. Kretzschmar, R. Rademakers, M. Baker and I. R. Mackenzie (2009b). "Abundant FUS-immunoreactive pathology in neuronal intermediate filament inclusion disease." Acta Neuropathol **118**(5): 605-616.

Neumann, M., D. M. Sampathu, L. K. Kwong, A. C. Truax, M. C. Micsenyi, T. T. Chou, J. Bruce, T. Schuck, M. Grossman, C. M. Clark, L. F. McCluskey, B. L. Miller, E. Masliah, I. R. Mackenzie, H. Feldman, W. Feiden, H. A. Kretzschmar, J. Q. Trojanowski and V. M. Lee (2006). "Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis." Science **314**(5796): 130-133.

O'Brien, J. S. and Y. Kishimoto (1991). "Saposin proteins: structure, function, and role in human lysosomal storage disorders." FASEB J **5**(3): 301-308.

Okura, H., S. Yamashita, T. Ohama, A. Saga, A. Yamamoto-Kakuta, Y. Hamada, N. Sougawa, R. Ohyama, Y. Sawa and A. Matsuyama (2010). "HDL/apolipoprotein A-I binds to macrophage-derived progranulin and suppresses its conversion into proinflammatory granulins." J Atheroscler Thromb **17**(6): 568-577.

Ong, C. H. and A. Bateman (2003). "Progranulin (granulin-epithelin precursor, PC-cell derived growth factor, acrogranin) in proliferation and tumorigenesis." Histol Histopathol **18**(4): 1275-1288.

Pacholec, M., J. E. Bleasdale, B. Chrunyk, D. Cunningham, D. Flynn, R. S. Garofalo, D. Griffith, M. Griffor, P. Loulakis, B. Pabst, X. Qiu, B. Stockman, V. Thanabal, A. Varghese, J. Ward, J. Withka and K. Ahn (2010). "SRT1720, SRT2183, SRT1460, and Resveratrol Are Not Direct Activators of SIRT1." Journal of Biological Chemistry **285**(11): 8340-8351.

Park, B., L. Buti, S. Lee, T. Matsuwaki, E. Spooner, M. M. Brinkmann, M. Nishihara and H. L. Ploegh (2011). "Granulin is a soluble cofactor for toll-like receptor 9 signaling." Immunity **34**(4): 505-513.

Pearce, J. M. (2003). "Pick's disease." J Neurol Neurosurg Psychiatry **74**(2): 169.

Petkau, T. L., S. J. Neal, A. Milnerwood, A. Mew, A. M. Hill, P. Orban, J. Gregg, G. Lu, H. H. Feldman, I. R. Mackenzie, L. A. Raymond and B. R. Leavitt (2012). "Synaptic dysfunction in progranulin-deficient mice." Neurobiol Dis **45**(2): 711-722.

Petkau, T. L., S. J. Neal, P. C. Orban, J. L. MacDonald, A. M. Hill, G. Lu, H. H. Feldman, I. R. A. Mackenzie and B. R. Leavitt (2010). "Progranulin expression in the developing and adult murine brain." The Journal of Comparative Neurology **518**(19): 3931-3947.

Pickford, F., J. Marcus, L. M. Camargo, Q. Xiao, D. Graham, J. R. Mo, M. Burkhardt, V. Kulkarni, J. Crispino, H. Hering and M. Hutton (2011). "Progranulin is a chemoattractant for microglia and stimulates their endocytic activity." Am J Pathol **178**(1): 284-295.

Pieper, A. A., S. Xie, E. Capota, S. J. Estill, J. Zhong, J. M. Long, G. L. Becker, P. Huntington, S. E. Goldman, C. H. Shen, M. Capota, J. K. Britt, T. Kotti, K. Ure, D. J. Brat, N. S. Williams, K. S. MacMillan, J. Naidoo, L. Melito, J. Hsieh, J. De Brabander, J. M. Ready and S. L. McKnight (2010). "Discovery of a proneurogenic, neuroprotective chemical." Cell **142**(1): 39-51.

Plowman, G. D., J. M. Green, M. G. Neubauer, S. D. Buckley, V. L. McDonald, G. J. Todaro and M. Shoyab (1992). "The epithelin precursor encodes two proteins with opposing activities on epithelial cell growth." J Biol Chem **267**(18): 13073-13078.

Polymenidou, M., C. Lagier-Tourenne, K. R. Hutt, S. C. Huelga, J. Moran, T. Y. Liang, S. C. Ling, E. Sun, E. Wancewicz, C. Mazur, H. Kordasiewicz, Y. Sedaghat, J. P. Donohue, L. Shiue, C. F. Bennett, G. W. Yeo and D. W. Cleveland (2011). "Long pre-mRNA depletion and RNA missplicing contribute to neuronal vulnerability from loss of TDP-43." Nat Neurosci **14**(4): 459-468.

Poorkaj, P., T. D. Bird, E. Wijsman, E. Nemens, R. M. Garruto, L. Anderson, A. Andreadis, W. C. Wiederholt, M. Raskind and G. D. Schellenberg (1998).

"Tau is a candidate gene for chromosome 17 frontotemporal dementia." Ann Neurol **43**(6): 815-825.

Rabinovici, G. D. and B. L. Miller (2010). "Frontotemporal Lobar Degeneration." CNS Drugs **24**(5): 375-398.

Rademakers, R., M. Baker, J. Gass, J. Adamson, E. D. Huey, P. Momeni, S. Spina, G. Coppola, A. M. Karydas, H. Stewart, N. Johnson, G. Y. Hsiung, B. Kelley, K. Kuntz, E. Steinbart, E. M. Wood, C. E. Yu, K. Josephs, E. Sorenson, K. B. Womack, S. Weintraub, S. M. Pickering-Brown, P. R. Schofield, W. S. Brooks, V. M. Van Deerlin, J. Snowden, C. M. Clark, A. Kertesz, K. Boylan, B. Ghetti, D. Neary, G. D. Schellenberg, T. G. Beach, M. Mesulam, D. Mann, J. Grafman, I. R. Mackenzie, H. Feldman, T. Bird, R. Petersen, D. Knopman, B. Boeve, D. H. Geschwind, B. Miller, Z. Wszolek, C. Lippa, E. H. Bigio, D. Dickson, N. Graff-Radford and M. Hutton (2007). "Phenotypic variability associated with progranulin haploinsufficiency in patients with the common 1477C-->T (Arg493X) mutation: an international initiative." Lancet Neurol **6**(10): 857-868.

Rademakers, R., M. Cruts and C. van Broeckhoven (2004). "The role of tau (MAPT) in frontotemporal dementia and related tauopathies." Hum Mutat **24**(4): 277-295.

Rademakers, R., J. L. Eriksen, M. Baker, T. Robinson, Z. Ahmed, S. J. Lincoln, N. Finch, N. J. Rutherford, R. J. Crook, K. A. Josephs, B. F. Boeve, D. S. Knopman, R. C. Petersen, J. E. Parisi, R. J. Caselli, Z. K. Wszolek, R. J. Uitti, H. Feldman, M. L. Hutton, I. R. Mackenzie, N. R. Graff-Radford and D. W. Dickson (2008). "Common variation in the miR-659 binding-site of GRN is a major risk factor for TDP43-positive frontotemporal dementia." Human Molecular Genetics **17**(23): 3631-3642.

Rai, M., E. Soragni, C. J. Chou, G. Barnes, S. Jones, J. R. Rusche, J. M. Gottesfeld and M. Pandolfo (2010). "Two new pimelic diphenylamide HDAC inhibitors induce sustained frataxin upregulation in cells from Friedreich's ataxia patients and in a mouse model." PLoS ONE **5**(1): e8825.

Ratnavalli, E., C. Brayne, K. Dawson and J. R. Hodges (2002). "The prevalence of frontotemporal dementia." Neurology **58**(11): 1615-1621.

Renton, A. E., E. Majounie, A. Waite, J. Simon-Sanchez, S. Rollinson, J. R. Gibbs, J. C. Schymick, H. Laaksovirta, J. C. van Swieten, L. Myllykangas, H. Kalimo, A. Paetau, Y. Abramzon, A. M. Remes, A. Kaganovich, S. W. Scholz, J. Duckworth, J. Ding, D. W. Harmer, D. G. Hernandez, J. O. Johnson, K. Mok, M. Ryten, D. Trabzuni, R. J. Guerreiro, R. W. Orrell, J. Neal, A. Murray, J. Pearson, I. E. Jansen, D. Sondervan, H. Seelaar, D. Blake, K. Young, N. Halliwell, J. B. Callister, G. Toulson, A. Richardson, A. Gerhard, J. Snowden, D. Mann, D. Neary, M. A. Nalls, T. Peuralinna,

L. Jansson, V. M. Isoviita, A. L. Kaivorinne, M. Holtta-Vuori, E. Ikonen, R. Sulkava, M. Benatar, J. Wuu, A. Chio, G. Restagno, G. Borghero, M. Sabatelli, I. Consortium, D. Heckerman, E. Rogaeva, L. Zinman, J. D. Rothstein, M. Sendtner, C. Drepper, E. E. Eichler, C. Alkan, Z. Abdullaev, S. D. Pack, A. Dutra, E. Pak, J. Hardy, A. Singleton, N. M. Williams, P. Heutink, S. Pickering-Brown, H. R. Morris, P. J. Tienari and B. J. Traynor (2011). "A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD." Neuron **72**(2): 257-268.

Roberson, E. D. (2006). "Frontotemporal dementia." Curr Neurol Neurosci Rep **6**(6): 481-489.

Rodrigues-Mascarenhas, S., A. D. S. de Oliveira, N. D. Amoedo, O. R. Affonso-Mitidieri, F. D. Rumjanek and V. M. Rumjanek (2009). "Modulation of the Immune System by Ouabain." Annals of the New York Academy of Sciences **1153**(1): 153-163.

Ryan, C. L., D. C. Baranowski, B. P. Chitramuthu, S. Malik, Z. Li, M. Cao, S. Minotti, H. D. Durham, D. G. Kay, C. A. Shaw, H. P. J. Bennett and A. Bateman (2009). "Progranulin is expressed within motor neurons and promotes neuronal cell survival." BMC Neuroscience **10**(1): 130.

See, T. M., A. K. LaMarre, S. E. Lee and B. L. Miller (2010). "Genetic causes of frontotemporal degeneration." J Geriatr Psychiatry Neurol **23**(4): 260-268.

Sephton, C. F., C. Cenik, A. Kucukural, E. B. Dammer, B. Cenik, Y. Han, C. M. Dewey, F. P. Roth, J. Herz, J. Peng, M. J. Moore and G. Yu (2011). "Identification of neuronal RNA targets of TDP-43-containing ribonucleoprotein complexes." J Biol Chem **286**(2): 1204-1215.

Serrero, G. (2003). "Autocrine growth factor revisited: PC-cell-derived growth factor (progranulin), a critical player in breast cancer tumorigenesis." Biochem Biophys Res Commun **308**(3): 409-413.

Serrero, G., D. M. Hawkins, B. Yue, O. Ioffe, P. Bejarano, J. T. Phillips, J. F. Head, R. L. Elliott, K. R. Tkaczuk, A. K. Godwin, J. Weaver and W. E. Kim (2012). "Progranulin (GP88) tumor tissue expression is associated with increased risk of recurrence in breast cancer patients diagnosed with estrogen receptor positive invasive ductal carcinoma." Breast Cancer Res **14**(1): R26.

Shankaran, S. S., A. Capell, A. T. Hruscha, K. Fellerer, M. Neumann, B. Schmid and C. Haass (2008). "Missense mutations in the progranulin gene linked to frontotemporal lobar degeneration with ubiquitin-immunoreactive inclusions reduce progranulin production and secretion." J Biol Chem **283**(3): 1744-1753.

Shoyab, M., V. L. McDonald, C. Byles, G. J. Todaro and G. D. Plowman (1990). "Epithelins 1 and 2: isolation and characterization of two cysteine-rich

growth-modulating proteins." Proc Natl Acad Sci U S A **87**(20): 7912-7916.

Sleegers, K., N. Brouwers, P. Van Damme, S. Engelborghs, I. Gijselinck, J. van der Zee, K. Peeters, M. Mattheijssens, M. Cruts, R. Vandenberghe, P. P. De Deyn, W. Robberecht and C. Van Broeckhoven (2009). "Serum biomarker for progranulin-associated frontotemporal lobar degeneration." Ann Neurol **65**(5): 603-609.

Smith, K. R., J. Damiano, S. Franceschetti, S. Carpenter, L. Canafoglia, M. Morbin, G. Rossi, D. Pareyson, S. E. Mole, J. F. Staropoli, K. B. Sims, J. Lewis, W. L. Lin, D. W. Dickson, H. H. Dahl, M. Bahlo and S. F. Berkovic (2012). "Strikingly different clinicopathological phenotypes determined by progranulin-mutation dosage." Am J Hum Genet **90**(6): 1102-1107.

Songsrirote, K., Z. Li, D. Ashford, A. Bateman and J. Thomas-Oates (2010). "Development and application of mass spectrometric methods for the analysis of progranulin N-glycosylation." J Proteomics **73**(8): 1479-1490.

Spencer, C. M., O. Alekseyenko, E. Serysheva, L. A. Yuva-Paylor and R. Paylor (2005). "Altered anxiety-related and social behaviors in the Fmr1 knockout mouse model of fragile X syndrome." Genes Brain Behav **4**(7): 420-430.

Steinmetz, C. C. and G. G. Turrigiano (2010). "Tumor necrosis factor-alpha signaling maintains the ability of cortical synapses to express synaptic scaling." J Neurosci **30**(44): 14685-14690.

Stellwagen, D. and R. C. Malenka (2006). "Synaptic scaling mediated by glial TNF-α." Nature **440**(7087): 1054-1059.

Strom, T. M., K. Hortnagel, S. Hofmann, F. Gekeler, C. Scharfe, W. Rabl, K. D. Gerbitz and T. Meitinger (1998). "Diabetes insipidus, diabetes mellitus, optic atrophy and deafness (DIDMOAD) caused by mutations in a novel gene (wolframin) coding for a predicted transmembrane protein." Hum Mol Genet **7**(13): 2021-2028.

Suh, H. S., N. Choi, L. Tarassishin and S. C. Lee (2012). "Regulation of Progranulin Expression in Human Microglia and Proteolysis of Progranulin by Matrix Metalloproteinase-12 (MMP-12)." PLoS ONE **7**(4): e35115.

Tang, B. L. (2010). "Resveratrol is neuroprotective because it is not a direct activator of Sirt1—A hypothesis." Brain Research Bulletin **81**(4-5): 359-361.

Tang, W., Y. Lu, Q. Y. Tian, Y. Zhang, F. J. Guo, G. Y. Liu, N. M. Syed, Y. Lai, E. A. Lin, L. Kong, J. Su, F. Yin, A. H. Ding, A. Zanin-Zhorov, M. L. Dustin, J. Tao, J. Craft, Z. Yin, J. Q. Feng, S. B. Abramson, X. P. Yu and C. J. Liu (2011). "The growth factor progranulin binds to TNF receptors

and is therapeutic against inflammatory arthritis in mice." Science **332**(6028): 478-484.

Tangkeangsirisin, W. and G. Serrero (2004). "PC cell-derived growth factor (PCDGF/GP88, progranulin) stimulates migration, invasiveness and VEGF expression in breast cancer cells." Carcinogenesis **25**(9): 1587-1592.

Tapia, L., A. Milnerwood, A. Guo, F. Mills, E. Yoshida, C. Vasuta, I. R. Mackenzie, L. Raymond, M. Cynader, W. Jia and S. X. Bamji (2011). "Progranulin Deficiency Decreases Gross Neural Connectivity But Enhances Transmission at Individual Synapses." Journal of Neuroscience **31**(31): 11126-11132.

Tartaglia, M. C. (2012). "Frontotemporal lobar degeneration: new understanding brings new approaches." Neuroimaging Clin N Am **22**(1): 83-97, viii.

Thomas, E. A., G. Coppola, P. A. Desplats, B. Tang, E. Soragni, R. Burnett, F. Gao, K. M. Fitzgerald, J. F. Borok, D. Herman, D. H. Geschwind and J. M. Gottesfeld (2008). "The HDAC inhibitor 4b ameliorates the disease phenotype and transcriptional abnormalities in Huntington's disease transgenic mice." Proc Natl Acad Sci U S A **105**(40): 15564-15569.

Tian, J. (2005). "Binding of Src to Na+/K+-ATPase Forms a Functional Signaling Complex." Molecular Biology of the Cell **17**(1): 317-326.

Tolkatchev, D., S. Malik, A. Vinogradova, P. Wang, Z. Chen, P. Xu, H. P. J. Bennett, A. Bateman and F. Ni (2008). "Structure dissection of human progranulin identifies well-folded granulin/epithelin modules with unique functional activities." Protein Science **17**(4): 711-724.

Vallejo-Illarramendi, A., M. Domercq, F. Perez-Cerda, R. Ravid and C. Matute (2006). "Increased expression and function of glutamate transporters in multiple sclerosis." Neurobiol Dis **21**(1): 154-164.

Van Damme, P., A. Van Hoecke, D. Lambrechts, P. Vanacker, E. Bogaert, J. van Swieten, P. Carmeliet, L. Van Den Bosch and W. Robberecht (2008). "Progranulin functions as a neurotrophic factor to regulate neurite outgrowth and enhance neuronal survival." The Journal of Cell Biology **181**(1): 37-41.

van Swieten, J. C. and P. Heutink (2008). "Mutations in progranulin (GRN) within the spectrum of clinical and pathological phenotypes of frontotemporal dementia." Lancet Neurol **7**(10): 965-974.

Vercellino, M., S. Grifoni, A. Romagnolo, S. Masera, A. Mattioda, C. Trebini, C. Chiavazza, L. Caligiana, E. Capello, G. L. Mancardi, D. Giobbe, R. Mutani, M. T. Giordana and P. Cavalla (2011). "Progranulin expression in brain tissue and cerebrospinal fluid levels in multiple sclerosis." Mult Scler **17**(10): 1194-1201.

Verhoef, C., J. H. de Wilt, D. J. Grunhagen, A. N. van Geel, T. L. ten Hagen and A. M. Eggermont (2007). "Isolated limb perfusion with melphalan and TNF-alpha in the treatment of extremity sarcoma." Curr Treat Options Oncol **8**(6): 417-427.

Vingtdeux, V., U. Dreses-Werringloer, H. Zhao, P. Davies and P. Marambaud (2008). "Therapeutic potential of resveratrol in Alzheimer's disease." BMC Neuroscience **9**(Suppl 2): S6.

Wang, M., G. Li, J. Yin, T. Lin and J. Zhang (2011). "Progranulin overexpression predicts overall survival in patients with glioblastoma." Med Oncol.

Wang, W. X., B. R. Wilfred, S. K. Madathil, G. Tang, Y. Hu, J. Dimayuga, A. J. Stromberg, Q. Huang, K. E. Saatman and P. T. Nelson (2010). "miR-107 Regulates Granulin/Progranulin with Implications for Traumatic Brain Injury and Neurodegenerative Disease." American Journal Of Pathology **177**(1): 334-345.

Ward, M. E. and B. L. Miller (2011). "Potential mechanisms of progranulin-deficient FTLD." J Mol Neurosci **45**(3): 574-582.

Watamoto, K., M. Towatari, Y. Ozawa, Y. Miyata, M. Okamoto, A. Abe, T. Naoe and H. Saito (2003). "Altered interaction of HDAC5 with GATA-1 during MEL cell differentiation." Oncogene **22**(57): 9176-9184.

Wilhelmsen, K. C., T. Lynch, E. Pavlou, M. Higgins and T. G. Nygaard (1994). "Localization of disinhibition-dementia-parkinsonism-amyotrophy complex to 17q21-22." Am J Hum Genet **55**(6): 1159-1165.

Wong, I., H. Liao, X. Bai, A. Zaknic, J. Zhong, Y. Guan, H. Y. Li, Y. J. Wang and X. F. Zhou (2010). "ProBDNF inhibits infiltration of ED1+ macrophages after spinal cord injury." Brain Behav Immun **24**(4): 585-597.

Xia, X. and G. Serrero (1998). "Identification of cell surface binding sites for PC-cell-derived growth factor, PCDGF, (epithelin/granulin precursor) on epithelial cells and fibroblasts." Biochem Biophys Res Commun **245**(2): 539-543.

Xu, J., M. Xilouri, J. Bruban, J. Shioi, Z. Shao, I. Papazoglou, K. Vekrellis and N. K. Robakis (2011). "Extracellular progranulin protects cortical neurons from toxic insults by activating survival signaling." Neurobiol Aging **32**(12): 2326 e2325-2316.

Yang, J., X. Kong, M. E. Martins-Santos, G. Aleman, E. Chaco, G. E. Liu, S. Y. Wu, D. Samols, P. Hakimi, C. M. Chiang and R. W. Hanson (2009). "Activation of SIRT1 by resveratrol represses transcription of the gene for the cytosolic form of phosphoenolpyruvate carboxykinase (GTP) by deacetylating hepatic nuclear factor 4alpha." J Biol Chem **284**(40): 27042-27053.

Yin, D., J. M. Ong, J. Hu, J. C. Desmond, N. Kawamata, B. M. Konda, K. L. Black and H. P. Koeffler (2007). "Suberoylanilide Hydroxamic Acid, a Histone Deacetylase Inhibitor: Effects on Gene Expression and Growth of Glioma Cells In vitro and In vivo." Clinical Cancer Research **13**(3): 1045-1052.

Yin, F., R. Banerjee, B. Thomas, P. Zhou, L. Qian, T. Jia, X. Ma, Y. Ma, C. Iadecola, M. F. Beal, C. Nathan and A. Ding (2010a). "Exaggerated inflammation, impaired host defense, and neuropathology in progranulin-deficient mice." J Exp Med **207**(1): 117-128.

Yin, F., M. Dumont, R. Banerjee, Y. Ma, H. Li, M. T. Lin, M. F. Beal, C. Nathan, B. Thomas and A. Ding (2010b). "Behavioral deficits and progressive neuropathology in progranulin-deficient mice: a mouse model of frontotemporal dementia." The FASEB Journal **24**(12): 4639-4647.

Zeng, J., J. Racicott and C. R. Morales (2009). "The inactivation of the sortilin gene leads to a partial disruption of prosaposin trafficking to the lysosomes." Exp Cell Res **315**(18): 3112-3124.

Zhang, F., J. Liu and J.-S. Shi (2010). "Anti-inflammatory activities of resveratrol in the brain: Role of resveratrol in microglial activation." European Journal of Pharmacology **636**(1-3): 1-7.

Zhang, H. and G. Serrero (1998). "Inhibition of tumorigenicity of the teratoma PC cell line by transfection with antisense cDNA for PC cell-derived growth factor (PCDGF, epithelin/granulin precursor)." Proc Natl Acad Sci U S A **95**(24): 14202-14207.

Zhang, L., Z. Zhang, H. Guo and Y. Wang (2008). "Na+/K+-ATPase-mediated signal transduction and Na+/K+-ATPase regulation." Fundamental & Clinical Pharmacology **22**(6): 615-621.

Zheng, Y., O. A. Brady, P. S. Meng, Y. Mao and F. Hu (2011). "C-terminus of progranulin interacts with the beta-propeller region of sortilin to regulate progranulin trafficking." PLoS ONE **6**(6): e21023.

Zhou, J., G. Gao, J. W. Crabb and G. Serrero (1993). "Purification of an autocrine growth factor homologous with mouse epithelin precursor from a highly tumorigenic cell line." J Biol Chem **268**(15): 10863-10869.

Zhu, J., C. Nathan, W. Jin, D. Sim, G. S. Ashcroft, S. M. Wahl, L. Lacomis, H. Erdjument-Bromage, P. Tempst, C. D. Wright and A. Ding (2002). "Conversion of proepithelin to epithelins: roles of SLPI and elastase in host defense and wound repair." Cell **111**(6): 867-878.