Individuals with progranulin haploinsufficiency exhibit features of neuronal ceroid lipofuscinosis

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Heterozygous mutations in the GRN gene lead to progranulin (PGRN) haploinsufficiency and cause frontotemporal dementia (FTD), a neurodegenerative syndrome of older adults. Homozygous GRN mutations, on the other hand, lead to complete PGRN loss and cause neuronal ceroid lipofuscinosis (NCL), a lysosomal storage disease usually seen in children. Given that the predominant clinical and pathological features of FTD and NCL are distinct, it is controversial whether the disease mechanisms associated with complete and partial PGRN loss are similar or distinct. We show that PGRN haploinsufficiency leads to NCL-like features in humans, some occurring before dementia onset. Noninvasive retinal imaging revealed preclinical retinal lipofuscinosis in heterozygous GRN mutation carriers. Increased lipofuscinosis and intracellular NCL-like storage material also occurred in postmortem cortex of heterozygous GRN mutation carriers. Lymphoblasts from heterozygous GRN mutation carriers accumulated prominent NCL-like storage material, which could be rescued by normalizing PGRN expression. Fibroblasts from heterozygous GRN mutation carriers showed impaired lysosomal protease activity. Our findings indicate that progranulin haploinsufficiency caused accumulation of NCL-like storage material and early retinal abnormalities in humans and implicate lysosomal dysfunction as a central disease process in GRN-associated FTD and GRN-associated NCL.

INTRODUCTION

Neurodegenerative diseases of adulthood are usually thought to occur via mechanisms distinct from those of childhood. Neuronal ceroid lipofuscinosis (NCL) is the most common neurodegenerative disease of childhood. Progranulin-related frontotemporal dementia (FTD), a neurodegenerative syndrome of older adults, is the most common cause of dementia in those <60 years old. Individuals affected by FTD usually present with behavioral changes, language difficulties, or motor symptoms (1). Heterozygous mutations in the progranulin (GRN) gene are common causes of familial FTD. Such mutations lead to progranulin (PGRN) haploinsufficiency, reducing PGRN expression by 60 to 70% via nonsense-mediated mRNA decay (2). PGRN has been reported to regulate numerous cellular processes, including cell survival, inflammation, and protein clearance (3–12). However, it is not understood why partial loss of PGRN causes neurodegeneration.

Recently, we discovered that retinal degeneration occurs in preclinical heterozygous GRN mutation carriers (13), a phenotype also observed in Grn knockout mice (13, 14). Grn knockout mice also develop prominent deposits of autofluorescent aggregates known as lipofuscin throughout the central nervous system (CNS) (7, 15, 16). Ultrastructurally, this storage material bears a striking resemblance to storage material found in NCL, a disease clinically characterized by early vision loss and retinal atrophy. Individuals with homozygous GRN mutations develop a clinical syndrome consistent with NCL (vision loss followed by seizures and ataxia) (17–19). Electron microscopy (EM) analysis of lymphocytes from one of these subjects revealed NCL-like storage material (18).

Classically, NCL encompasses a family of related genetic syndromes with shared clinical and pathologic features, which together are the major cause of neurodegeneration in childhood. Vision loss is often an early clinical feature of NCL and can precede development of other neurological symptoms, which usually include seizures, ataxia, cognitive dysfunction, and early death (20). Most of the known mutations associated with NCL cause loss of function in genes necessary for normal lysosome function. In keeping with this, deposition of autofluorescent intralysosomal storage material known as lipofuscin is a pathologic hallmark of NCL (21).

Although it is generally agreed that NCL can be caused by complete loss of PGRN, whether FTD caused by partial loss of PGRN shares similar clinical, pathologic, or mechanistic features with NCL remains controversial. Unlike the Grn knockout mice, heterozygous Grn mice do not develop CNS lipofuscinosis (7). Furthermore, the classic clinical and pathological findings considered characteristic of GRN-associated FTD appear distinct from those in NCL (namely, early behavioral/language abnormalities with pathologic TDP-43 mislocalization in FTD versus vision loss, seizures, and ataxia with pathologic lipofuscin accumulation in NCL). However, a comprehensive evaluation of humans with PGRN haploinsufficiency for clinicopathological features of NCL has not been conducted.

On the basis of our previous observations of early retinal degeneration in humans with heterozygous GRN mutations—which could be consistent with an NCL phenotype—we suspected that other NCL-like
features may occur in these patients but may have been overlooked. We examined retinas of living symptomatic heterozygous GRN mutation carriers by noninvasive autofluorescence imaging and analyzed postmortem cortical tissues from FTD subjects with heterozygous GRN mutations. In addition, we imaged lymphoblasts from heterozygous GRN mutation carriers and their normal siblings by EM to quantify NCL-like storage material. We enhanced PGRN expression in lymphocytes from heterozygous GRN mutation carriers to assess the effects of PGRN on NCL-like structures and storage material. Finally, we directly assessed lysosomal function in fibroblasts from heterozygous GRN mutation carriers. Our findings indicate that core clinicopathological features of NCL occur in humans with PGRN haploinsufficiency caused by heterozygous GRN mutations, implicating lysosomal dysfunction as a potential disease process in GRN-associated FTD.

RESULTS
Lipofuscinosis and NCL-like storage material in retinal neurons from Grn knockout mice
Vision loss is often the first symptom noticed by patients with some forms of NCL, and it is accompanied by retinal atrophy and deposition of autofluorescent lipofuscin. We reported retinal degeneration accompanied by electrophysiological dysfunction in Grn knockout mice (13), which was subsequently reported by others (14). Similar to findings in cortical neurons from Grn knockout mice and in various NCL animal models (7), we observed NCL-like lysosomal storage material in retinal neurons from Grn knockout mice (Fig. 1A).

Age-dependent autofluorescent lipofuscin deposition was observed throughout the retina in Grn knockout mice, reminiscent of autofluorescent lipofuscin deposits in NCL (Fig. 1, B and C). In addition, subretinal drusen-like autofluorescent deposits were higher in aged Grn knockout mice than in control animals: 16.0 ± 2.7 versus 3.2 ± 1.9 deposits per retinal cross section (n = 5, P < 0.001, mixed-model linear regression). Pathologic retinal lipofuscinosis occurred in very young Grn knockout mice (1.5 months of age), preceding any other functional or degenerative phenotype in these animals (Fig. 1, C and D, and table S3) (7, 8, 13, 22, 23). Consistent with previous evaluations of the cortex (7), we did not see increased retinal lipofuscinosis in aged Grn +/− mice (figs. S1 and S2).

Noninvasive imaging of retinal autofluorescence is routinely performed in clinical settings with confocal scanning laser ophthalmoscopy (csLO) and can be used to detect retinal lipofuscin deposits. To determine whether retinal lipofuscinosis secondary to PGRN deficiency can be imaged in vivo, we used a modified csLO to image the retina of anesthetized Grn knockout mice. Similar to our observations in fixed tissue, we detected retinal lipofuscin using csLO in Grn knockout mice (Fig. 1, E and F). We simultaneously imaged Grn knockout mice with spectral domain optical coherence tomography (OCT) to determine whether individual lipofuscin deposits could be localized to specific retinal layers. Although most autofluorescent deposits were below the resolution of OCT in mice, we did note occasional aggregates of subretinal deposits in Grn knockout mice, consistent with our pathological observations (Fig. 1E, insets).

Noninvasive imaging of retinal lipofuscin in heterozygous GRN mutation carriers by autofluorescent retinal imaging
We previously reported retinal thinning in humans with heterozygous GRN mutations (13). We used csLO to image retinal autofluorescence in 11 heterozygous GRN mutation carriers and 22 age-matched and sex-matched healthy controls (tables S1 and S2). Retinal lipofuscinosis was seen more often in heterozygous GRN mutation carriers than in controls (Fig. 2, A to C). Most lipofuscin deposits were not visible on OCT scans through the same region (Fig. 2B). Occasional lipofuscin deposits correlated with subretinal drusen-like aggregates (Fig. 2C). Quantification of subretinal drusen revealed a trend toward increased drusen per eye in GRN mutation carriers, but this was not statistically significant (table S2). Heterozygous GRN mutation carriers were about twice as likely to have retinal lipofuscin deposits than controls (Fig. 2D and tables S1 and S3). In addition, both the average number and the area of lipofuscin deposits were substantially greater in GRN mutation carriers (Fig. 2, E and F), including those who were clinically asymptomatic (Fig. 2, E and F).

Increased storage material in postmortem cortex of heterozygous GRN mutation carriers
We next asked whether storage material deposition also occurred in the frontal cortex of heterozygous GRN mutation carriers, because degeneration of this region of the CNS causes many of the clinical symptoms of FTD. Formalin-fixed postmortem human cortex samples from 15 GRN mutation carriers, 16 nondemented control subjects, and 6 Alzheimer's disease (AD) subjects were imaged by EM. We noted a marked increase in storage material accumulation in cortical neurons of GRN mutation carriers but not AD subjects compared to nondemented controls (Fig. 2, G and H). Morphologically, the storage material found in the formalin-fixed postmortem cortex from GRN mutation carriers resembled granular osmiophilic deposits. Such deposits occur in a broad range of NCL subtypes, including recessive infantile-onset forms caused by PPT1 mutations (NCL type 1) and an adult-onset form caused by dominant mutations in DNAJC5 (NCL type 4). Both the percentage of neurons that contained storage material and the number of storage material deposits per neuron were increased in heterozygous GRN mutation carriers (Fig. 2H). Storage material was also present in microglia in heterozygous GRN mutation carriers (fig. S3). We did not observe an increase in NCL-like storage material in postmortem cervical spinal cord tissue from heterozygous GRN mutation carriers, an area of the CNS that does not typically degenerate in GRN-related neurodegeneration (fig. S4). Consistent with the results from EM, we observed an increase in autofluorescent lipofuscin deposits in the frontal cortex tissue from GRN mutation carriers by fluorescence microscopy (Fig. 2I). Lipofuscin staining using Sudan black showed a similar result (Fig. 2, J and K).

NCL-like storage material in lymphoblasts from heterozygous GRN mutation carriers
The presence of intracellular storage material with characteristic ultrastructural patterns is a defining pathological feature of NCL. Such storage material accumulates in neurons and certain non-neuronal cells in NCL patients. Evaluation of peripheral blood lymphocytes for storage material is commonly performed in clinical settings when a diagnosis of NCL is under consideration. Recently, a patient diagnosed with NCL was posthumously found to carry a heterozygous GRN mutation (c.1477C>T p.Arg493X), but neither electron micrographs nor tissue samples were available at the time to confirm the original diagnosis (17). We obtained lymphoblasts that had been banked from this patient and processed them for EM to evaluate the presence or absence of NCL-like storage material. We simultaneously evaluated lymphoblasts from a healthy individual and a patient with juvenile NCL (CLN3 homozygous −1-kb deletion). As expected, lymphoblasts carrying a CLN3 mutation had numerous enlarged vacuolated structures, many containing multilamellar fingerprint profile patterns or onion skin-like storage material (Fig. 3A).
Strikingly, lymphoblasts from the heterozygous GRN mutation carrier had prominent vacuolated structures containing storage material that was morphologically similar to that found in lymphoblasts carrying the CLN3 mutation. Storage material was largely absent in lymphoblasts from a healthy control (Fig. 3A, inset).

We next asked whether NCL-like storage material also occurred in heterozygous GRN mutation carriers lacking a clinical diagnosis of NCL. We cultured lymphoblasts from five heterozygous GRN mutation carriers and processed them for EM. Siblings with WT GRN alleles of similar age served as controls. Lymphoblasts from these GRN mutation carriers had membrane-bound multilamellar NCL-like storage material deposits, similar in morphology to the GRN mutation carrier clinically diagnosed with NCL (Fig. S5A). When analyzed as a group, we saw more storage material in lymphoblasts from GRN mutation carriers than in those from controls (Fig. 3B). When analyzed as matched sibling pairs, three of the GRN mutation carriers had lymphoblasts with more storage material than their control siblings (Fig. S5, B and C). A similar but less pronounced difference was observed in the other two pairs (Fig. S5, B and C).

**Reduced NCL-like storage material in lymphoblasts from GRN mutation carriers treated with PGRN-expressing retrovirus**

We next determined whether the NCL-like storage material phenotype in lymphoblasts from GRN mutation carriers could be rescued by increasing...
PGRN expression in these cells. A PGRN-expressing retrovirus was used to transduce lymphoblasts carrying the GRN<sup>1200Q6X18</sup> mutation to increase PGRN expression to WT levels or higher (Fig. 3, C and D, and table S3). The NCL-like storage material was reduced in GRN<sup>1200Q6X18</sup> lymphoblasts infected with PGRN-expressing retrovirus compared to control cells transduced with an empty retrovirus (Fig. 3, E to G).
Fig. 3. GRN haploinsufficiency results in NCL-like storage material in lymphoblasts. (A) NCL-like storage material accumulates in lymphoblasts from a heterozygous GRN mutation carrier who clinically presented with NCL symptoms. Representative images of lymphoblasts from a healthy non-mutation-carrying control subject, an NCL patient (homozygous CLN3 mutation carrier), and a heterozygous GRN mutation carrier are shown. Note the similar ultrastructural patterns of the storage material observed in the CLN3 mutation carrier and the GRN mutation carrier, consisting of prominent vacuolated structures containing storage material with a fingerprint profile pattern (insets). (B) Quantification of NCL-like storage material in lymphoblasts from heterozygous GRN mutation carriers without clinical evidence of NCL versus noncarrier sibling controls. Five paired sets of lymphoblasts from control and heterozygous GRN mutation siblings were imaged with EM, and the number of vacuoles with storage material per cell and cross-sectional area occupied by storage material containing vacuoles was quantified in a blinded manner. **p < 0.001 via mixed-effect multivariate linear regression model. (C to G) Restoring normal PGRN expression in lymphoblasts from heterozygous GRN mutation carriers rescued the NCL-like storage material phenotype. (C) Western blot of intracellular PGRN and tubulin showing reduced expression of PGRN in heterozygous GRN exon 8 (IVS7-1g) mutation carrier lymphoblasts, which was rescued by treatment with a PGRN-expressing retrovirus. Control (empty vector) retrovirus did not alter PGRN expression. GRN exon 8 (IVS7-1g) mutation carrier lymphoblasts transduced with the PGRN-retrovirus that expressed PGRN at two different levels (endogenous, +; high, ++ ) were used for EM analysis. (D) Quantification of PGRN expression (normalized to tubulin). n = 3 wells per group; *p < 0.05, **p < 0.01, one-way ANOVA with Sidak’s multiple comparison test. (E and G) Representative electron micrographs of heterozygous GRN exon 8 (IVS7-1g) mutation carrier lymphoblasts transduced with control (empty vector) or huPGRN retrovirus expressing PGRN at an endogenous level. Lymphoblasts transduced with PGRN-expressing retrovirus had less storage material than did those that were treated with empty vector (insets). (F and G) Quantification of the number of vacuoles with storage material per lymphoblast (F) or total cross-sectional area of storage material per lymphoblast (G) in lymphoblasts transduced with control or PGRN-expressing retrovirus. n = 45 to 65 cells per group, *p < 0.05, **p < 0.01, one-way ANOVA with Sidak’s multiple comparison test. Means ± SEM are shown (B, D, F, and G).
Impaired lysosomal function in fibroblasts from heterozygous GRN mutation carriers

In NCL, lipofuscin accumulation is thought to be the pathological consequence of dysfunctional lysosomal proteolysis. We directly assessed lysosomal protease activity in human fibroblasts from heterozygous GRN mutation carriers and control siblings. We measured the activity of cysteine cathepsin proteases in living patient fibroblasts using a quenched fluorescent activity-based probe (24) and found that cathepsin activity in fibroblasts from GRN mutation carriers was reduced by ~40% compared to that in fibroblasts from control siblings (Fig. 4, A and B, fig. S6A, and table S3). We also saw reduced cathepsin D activity in fibroblast lysates from GRN mutation carriers (Fig. 4C and fig. S6B).

DISCUSSION

Our study shows that FTD caused by PGRN haploinsufficiency shares several fundamental clinicopathological features with NCL. Similar to other areas of the CNS, lipofuscin deposits formed in the retina in Grn knockout mice in a panretinal pattern similar to that seen in human NCL. Retinal lipofuscin deposition was an early phenotype in these mice, preceding neuron loss, and could be imaged in vivo. Retinal lipofuscin deposits also occurred in humans with heterozygous GRN mutations, including cognitively normal individuals, and could be detected with imaging technology used in clinical settings. Autofluorescent NCL-like storage material was present in the frontal cortex of human subjects with heterozygous GRN mutations. EM analysis of lymphoblasts from heterozygous GRN mutation carriers revealed the presence of NCL-like storage material, regardless of whether their clinical syndrome had NCL-like features. Transduction of lymphoblasts from GRN mutation carriers with PGRN-expressing retrovirus reduced storage material accumulation. Finally, lysosomal protease activity was impaired in fibroblasts from heterozygous GRN mutation carriers.

Whereas patients with homozygous GRN mutations have a clinical syndrome that closely resembles NCL, those with heterozygous GRN mutations usually present with FTD. Because the primary symptoms and pathological features of FTD and NCL are distinct, homozygous and heterozygous GRN mutations may cause neurodegeneration via divergent pathophysiological processes (17). In contrast, our study provides direct evidence that core clinicopathological phenotypes of NCL occur in heterozygous GRN mutation carriers. Vision loss and retinal atrophy are early phenotypes in most patients with NCL. Our recent observations of moderate retinal thinning in heterozygous GRN mutation carriers, compared to nearly complete vision loss and severe retinal atrophy in homozygous GRN mutation carriers (18), suggest a relationship between GRN gene dosage and the severity of retinal involvement. We also found that lipofuscinosis and membrane-bound storage material deposition—pathologic hallmarks of NCL—occurred in heterozygous GRN mutation carriers, consistent with what has been seen in homozygous GRN mutation carriers. A recent finding that an NCL case caused by a homozygous GRN mutation segregated in a family with neuropathologically confirmed FTLD further supports the notion that PGRN-deficient NCL and FTLD are pathophysiologically related (19).

On the basis of our findings, we propose that neurodegeneration is driven by a common central mechanism in both homozygous and heterozygous GRN mutation carriers but that the specific clinical syndrome is driven by GRN gene dosage. In homozygous GRN mutation carriers with complete PGRN loss, disease onset is in early adulthood and NCL symptoms predominate. In heterozygous GRN mutation carriers with partial PGRN loss, subclinical NCL features (such as retinal thinning) are present, but disease onset is in late adulthood and cognitive symptoms predominate. Additional subclinical features could also occur in heterozygous GRN mutation carriers that would require further investigation to detect. For example, although motor seizures are not commonly reported in GRN-associated FTD, prolonged electroencephalography might reveal subclinical epileptiform activity, similar to that seen in subjects with AD.
Other symptoms seen in homozygous GRN carriers, such as cerebellar dysfunction, have not been documented in heterozygous GRN mutation carriers. One possible explanation is that such symptoms can be notoriously difficult to detect in clinical settings when they are subtle and difficult to localize, for example, behavioral manifestations of cerebellar dysfunction. Also, potential cerebellar pathology is not always assessed in these cases at autopsy. It will therefore be of interest to determine whether structural changes to the cerebellum occur in these individuals, for example, through assessment of cerebellar volume loss or changes in neural tracts as evidence of disruption of normal cerebellar input/output. It remains unclear why heterozygous PGRN-deficient mice failed to exhibit NCL-like pathology, despite striking NCL-like pathology in Grn knockout mice. It is possible that mice do not live long enough to develop such pathology or that additional genetic modifiers are required for NCL-like pathology to emerge, for example, disease-related TMEM106b polymorphisms.

Notably, we observed NCL-like storage material in a heterozygous GRN mutation carrier who presented with symptoms compatible with late-stage NCL, for example, limb dystonia, gait abnormalities, and spasticity at the age of 46. Because whole-genome sequencing is more broadly used to assist in the diagnosis of unusual neurological cases, additional atypical clinical presentations of heterozygous GRN mutation carriers may be discovered. Alternatively, this subject may have had additional partial loss-of-function sequence variants in NCL-related genes that pushed their clinical syndrome toward an NCL-like phenotype.

PGRN has been reported to regulate a diverse range of cellular functions, including cell proliferation, cell survival, tumor invasion, protein turnover, inflammation, and synaptic plasticity, and more recently, lysosomal function. It has been unclear which of these functions is directly regulated by PGRN, and numerous mechanisms have been proposed to explain how partial PGRN loss causes FTD. PGRN is a secreted glycoprotein and, in neurons, localizes predominantly to endolysosomes (26). It binds directly to sortilin, a transmembrane receptor involved in receptor scavenging and protein trafficking from the Golgi to the lysosomes (26). In addition, mutations in the TMEM106b gene markedly influence the age of onset of FTD in heterozygous GRN mutation carriers (27, 28). TMEM106b localizes to endolysosomes and may regulate vesicular transport (29). On the basis of our observations of NCL-like phenotypes in heterozygous GRN mutation carriers, we propose that endolysosomal dysregulation is a potential central mechanism underlying neurodegeneration in humans with PGRN-deficient FTD.

Because nearly all heterozygous GRN mutations cause FTD through loss of PGRN expression, it has been speculated that increasing PGRN in GRN mutation carriers using therapeutic interventions should alter disease progression. However, one significant challenge in the development of effective therapeutics in FTD has been a lack of markers to monitor relevant pathophysiological processes. We found that PGRN-deficient lymphoblasts had increased NCL-like storage material and that increasing PGRN in these cells could rescue this phenotype, supporting a possible causal role of PGRN deficiency in endolysosomal dysfunction. It is largely accepted that the pathological accumulation of lipofuscin in NCL indicates ongoing endolysosomal dysfunction, but future studies are needed to clarify whether lipofuscin accumulation itself is pathogenic.

Our study further suggests that autofluorescent retinal imaging may have potential utility for GRN mutation carriers. Longitudinal autofluorescent retinal imaging could be an inexpensive, rapid, and non-invasive means of monitoring lipofuscinosis in the CNS, especially when paired with OCT analysis of retinal tissue loss in GRN mutation carriers. Future proof-of-concept studies in Grn knockout mice using serial in vivo retinal imaging could inform whether normalization of PGRN expression clears existing lipofuscin, slows future lipofuscin accumulation, and prevents retinal neuronal loss. Adaptation of OCT acquisition protocols may also permit the detection of abnormalities other than the subretinal deposits identified here.

It is important to note the limitations associated with this study. We found that PGRN-deficient lymphoblasts had increased NCL-like storage material and that increasing PGRN in these cells could rescue this phenotype. However, EM analysis of NCL-like storage material in lymphoblasts is too labor-intensive to be used in the clinic. It remains to be established whether other assays of impaired lysosome function in PGRN-deficient lymphoblasts could be used to measure disease progression or efficacy of potential treatments. We observed a robust difference in lipofuscin deposition in the retina and CNS of a single cohort of GRN mutation carriers compared to healthy controls. However, given their lack of specificity, these differences would not be sufficient for identification of presymptomatic individuals without an additional risk assessment, that is, genetics or family history. Replication of our findings in an independent cohort of GRN mutation carriers would reinforce our conclusions.

**MATERIALS AND METHODS**

**Study design**

The overall aim of the study was to determine whether cells and tissue from heterozygous GRN mutation carriers exhibited evidence of NCL-like accumulation of lysosomal storage material and lysosomal dysfunction. The objective of the first portion of the study was to determine whether NCL-like storage material occurred in Grn knockout mice using EM, histology, and live imaging techniques. The objective of the second portion of the study was to determine whether NCL-like storage material was present in the CNS of heterozygous GRN mutation carriers using retinal imaging of living subjects and analysis of postmortem human cortical tissue by EM. Subjects with heterozygous GRN mutations and age-matched and sex-matched control subjects without known neurological diseases were enrolled in the study through the University of California, San Francisco (UCSF) Memory and Aging Center in 2011–2014 as part of a larger study to evaluate retinal manifestations of neurodegenerative diseases. Predefined exclusion criteria included a history of ophthalmologic disease, including glaucoma, age-related macular degeneration (AMD), diabetic retinopathy, and ocular surgery (except cataract surgery). One GRN mutation carrier had AMD (dry) and was excluded from the analysis; the remainder did not have any of the above exclusion criteria. GRN mutation carriers also underwent a standardized neurologic evaluation by a board-certified neurologist with fellowship training in behavioral neurology. Clinical disease rating (CDR) scores were assigned to each subject on the basis of the result of this evaluation. For the purposes of this study, subjects were classified as asymptomatic (CDR = 0) or symptomatic (CDR > 0). Written informed consent was obtained from all participants with capacity. In those unable to provide informed consent because of diminished capacity, assent was obtained from the participant and written consent was obtained from a designated surrogate decision-maker. The study protocol was approved by the UCSF Committee on Human Research [IRB (Institutional Review Board) #11–05333]. The objective of the third portion of the study was to evaluate primary cells from heterozygous GRN mutation carriers for evidence of lysosomal dysfunction using EM analysis of storage material accumulation and functional readouts of lysosomal hydrolase activity. All data were included (there was no outlier exclusion).
Lymphoblast analysis

Epstein-Barr virus–transformed lymphocytes from deidentified human subjects were grown in RPMI + 10% fetal calf serum + 1% penicillin/streptomycin. Equal numbers of cells from each line were seeded into 10 ml of medium in T75 tissue culture flasks, and medium was doubled in volume each time cells reached saturation (determined by medium pH) until the total volume was 40 ml. Cells were again allowed to expand until saturation density and were then pelleted and gently resuspended in ice-cold EM fixative [2.5% glutaraldehyde, 2% paraformaldehyde, and 0.025% calcium chloride, in a 0.1 M sodium cacodylate buffer (pH 7.4)]. After a 15-min incubation in fixative on ice, cells were repelleted at 5000 rpm in a swinging-bucket rotor centrifuge for 10 min at 4°C. The fixative was removed, and fresh fixative was gently layered on top of the cell pellet. Samples were centrifuged again at 5000 rpm for 10 min at 4°C and then processed further for EM.

Retroviral transduction of lymphoblasts

Untagged human progranulin was cloned into a murine stem cell virus (MSCV) backbone vector that coexpressed mRuby as a fluorescent marker, with an empty vector serving as a control. 293T cells were cotransfected with the MSCV vector and pC3-Ampo via Lipofectamine 2000, and viral supernatant was collected 2 days later and filtered through a 0.45-μm filter. GRN mutation lymphoblasts were resuspended in empty vector or PGRN-expressing retroviral supernatant and monitored for expression via fluorescence microscopy. PGRN-expressing cells were sorted into two groups by fluorescence-activated cell sorting: “high PGRN-expressing” cells were the brightest 5% of cells sorted, and “low PGRN-expressing” cells were the remaining mRuby-positive cells. These groups were then expanded in parallel as described above and processed for EM.

Electron microscopy

Lymphoblast pellets were generated as described above, mouse retinal tissue was fixed directly after harvest in EM fixative. Formalin-fixed postmortem human cortex was postfix in EM fixative before further processing. For lymphoblasts and retinal tissue, subsequent processing was performed in a Leica Lynx automatic tissue processor. Briefly, the tissue was postfixed in osmium tetroxide, stained en bloc with uranyl acetate, dehydrated in graded ethanol solutions, infiltrated with propylene oxide/Epon mixtures, embedded in pure Epon, and polymerized overnight at 60°C. One-micrometer sections were cut, stained with toluidine blue, and examined by light microscopy. Representative areas were chosen for electron microscopic study, and the Epon blocks were trimmed accordingly. Thin sections were cut with an LKB 8801 ultramicrotome and diamond knife, stained with lead citrate, and examined in an FEI Morgagni transmission electron microscope.

Quantification of lymphoblast storage material

EM images were blindly acquired by an experienced EM technologist, who, to reduce the chance of bias, was instructed to take whole-cell images at ×1200 magnification of the first 20 to 30 cells encountered during imaging, in which the nucleus was bisected at the plane of section. Blinded quantification of lymphocyte storage material was then carried out. The total number of vacuoles per lymphocyte with NCL-like storage material (defined by the presence of multilamellar, fingerprint profile storage material or the granular osmiophilic deposit profile storage material) was determined. Simultaneously, regions of interest around each vacuole containing storage material were traced, and the summed area of storage material–containing vacuoles per lymphocyte was determined.

Quantification of cathepsin activity (fluorescent-based and cathepsin D activity assay)

Human-derived fibroblasts (HDFs) were passaged into 10- or 15-cm tissue culture–treated culture dishes, allowed to expand to confluency, and cultured in 10% fetal bovine serum (FBS)/Dulbecco’s modified Eagle’s medium (DMEM) for 1 month. For experiments, 0.25% trypsin-EDTA solution was used to dissociate cells, and the cells were plated in 100-μl aliquots to a density of 2000 cells per well in TPP 96-well tissue culture plates. After adhesion of cells for 24 hours, the medium was removed from wells, and a probe, BMV109, was added to each well (0.25 μM BMV109 in 100 μl of FBS/DMEM). The probe was incubated with cells for 2 hours, after which point the probe-containing medium was removed, and wells were washed with nonfluorescent DMEM three times. Cells were incubated with Hoechst stain (1.6 μM) in nonfluorescent DMEM. The plates were imaged at ×10 magnification on Cellomics ArrayScan, with an exposure of 1 s. Two channels were used for imaging: 386 nm for nuclear stain and 650 nm for BMV109. The signal was quantified after imaging with Cellomics software using Cell Health Profiling. Wells with no probe were used as blanks. For cathepsin D activity assays, a fluorometric cathepsin D assay kit was used (BioVision). Briefly, HDFs were lysed with CD lysis buffer, incubated on ice for 10 min, and clarified with 100,000 g for 5 min, and total protein levels were normalized across samples. Lysates were added to the master assay mix, mixed, and incubated for 1 hour at 37°C. Fluorescence was read on a fluorescence plate reader (excitation, 328 nm; emission, 460 nm).

In vivo retinal imaging in mice

All animals were maintained in full-barrier facilities free of specific pathogens on a 12-hour light/dark cycle with food and water ad libitum. Experiments were conducted in compliance with the ARVO (Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Visual Research, and all protocols were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco (#AN087501-02A). Grn knockout mice were fully backcrossed into C57BL/6j, and WT mice of the same genetic background served as controls. Mice were anesthetized with a steady flow of 1.5 to 3% isofluorane, eyes were anesthetized with propocaine, dilated with 1% tropicamide and 2.5% phenylephrine, and corneas were kept moist with 2.5% methylcellulose. A contact lens was placed over the mouse eye to improve the optics of the system. Retinal images were taken with a modified confocal scanning laser ophthalmoscope/OCT device (Heidelberg Spectralis, Heidelberg Engineering). After adjusting centered beam placement by infrared cSLO and 90° position of the retina to the beam in the vertical and horizontal planes by OCT, autofluorescent retinal images were then taken at 457-nm excitation and 500-nm long-pass filter detection using TruTrack eye-tracking software, averaging at least 80 frames for each image, followed by OCT volume scans (100 automatic real-time, high resolution) through selected areas of interest.

Retinal lipofuscin quantification in mice

Eyes were enucleated and fixed in 4% paraformaldehyde overnight. Retinas were dissected out and mounted between slides and coverslips in a whole-mount configuration. Five separate fields of view, equally spaced around the perimeter of the retina, were imaged in each retina, located about 500 μm from the peripheral edge of the retina. Full-thickness confocal images were obtained at 2-μm step sizes using a Nikon Spinning Disk confocal microscope in the green fluorescent protein channel to image autofluorescent lipofuscin deposits. Individual lipofuscin deposits were marked with the cell counter feature by a blinded technician.
and the total number of lipofuscin deposits per z-stack was quantified. The average number of lipofuscin deposits per z-stack across all five fields of view was determined for each mouse.

**Human retinal imaging**

After pupillary dilation with 1% tropicamide ophthalthic solutions (Akorn), study participants underwent retinal autofluorescence imaging with a cSLO and OCT (Heidelberg Spectralis). cSLO imaging was performed using the standard autofluorescence settings of the device using TruTrack eye-tracking software, averaging at least 80 frames for each image. Trained technologists who were blinded to the mutation status acquired the images using standardized illumination and gain settings. Image quality was assessed by a blinded neuro-ophthalmologist, and only eyes with in-focus retinal images of sufficient quality to assess for the presence or absence of lipofuscin were analyzed further. Blinded quantification of retinal lipofuscin was performed jointly by a neuro-ophthalmologist (A.J.G.) and behavioral neurologist (M.E.W.). For each eye, the number of individual lipofuscin deposits was counted, and regions of interest were drawn around each lipofuscin deposit. The total number of lipofuscin deposits and the summed number of lipofuscin deposits were then calculated for each eye.

**Quantification of lipofuscin in postmortem human cortical tissue**

Formalin-fixed, paraffin-embedded, deidentified postmortem human frontal cortex and cervical spinal cord from heterozygous GRN mutation carriers, AD subjects, and age- and sex-similar control subjects without a clinical or pathological diagnosis of neurodegenerative disease were obtained from the University of British Columbia, the UCSF Neurodegenerative Disease Brain Bank, and Northwestern University. The work was deemed not human-subjects research per UCSF Committee on Human Research guidelines. 7.5-µm-thick sections were cut, dewaxed, and counterstained with DAPI (4′,6-diamidino-2-phenylindole). Autofluorescence imaging of lipofuscin and imaging of Sudan black–stained sections was performed by a blinded technician using a 20× objective and Cy5 filter set and bright field. Six to 30 nonoverlapping fields of view of superficial cortex were imaged, and regions of interest were drawn around lipofuscin deposits via an automated algorithm (Volocity software). The total area of cortex occupied by lipofuscin deposits was then calculated for each field of view. For EM analysis of storage material from postmortem human cortex, formalin-fixed cortex was post-fixed in EM chromosome 17. *Nature* 442, 916–919 (2006).

**Statistics**

We used unpaired two-tailed *t* tests for normally distributed data and Mann-Whitney *U* tests for nonparametric data in experiments in which the two comparison groups were involved. In experiments in which more than two comparison groups were involved, we used an ANOVA test with post hoc testing. A mixed-effects multivariable linear regression analysis, when used, accounted for intramouse and intraindividual variability. Statistical testing was performed using Stata 12.0 and Prism 6.

**SUPPLEMENTARY MATERIALS**

www.sciencetranslationalmedicine.org/cgi/content/full/9/385/eaah5642/DC1

Fig. S1. Increased lipofuscin in retinas from aged Gm−/− but not Gm+/− mice.

Fig. S2. Increased subunit C deposition in retinas of Gm−/− mice.

Fig. S3. Progranulin-deficient microglia accumulate NCL-like lysosomal storage material.

Fig. S4. EM analysis of postmortem spinal cord from control subjects and heterozygous GRN mutation carriers. Fig. S5. Increased NCL-like storage material in lymphoblasts from heterozygous GRN mutation carriers versus sibling controls.

Table S1. Patient characteristics and quantification of human OCT macular cross-sectional scans.

Table S2. Patient characteristics and quantification of human OCT macular cross-sectional scans.

**REFERENCES AND NOTES**


Individuals with progranulin haploinsufficiency exhibit features of neuronal ceroid lipofuscinosis


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Connecting the dots in neurodegenerative disease

Heterozygous GRN mutations lead to progranulin haploinsufficiency and cause frontotemporal dementia (FTD) in the elderly population, whereas homozygous GRN mutations cause neuronal ceroid lipofuscinosis (NCL), a lysosomal storage disease that mainly affects children. The underlying relationship between progranulin-deficient FTD and NCL remains unexplored. Now, Ward et al. show that patients with heterozygous GRN mutations exhibit clinical and pathological features that are strikingly similar to NCL. Like NCL patients, individuals with heterozygous GRN mutations accumulate storage material throughout the central nervous system, and their cells exhibit signs of lysosomal dysfunction. These findings implicate lysosomal dysfunction as a central mechanism in both GRN-associated FTD and NCL.