Identification of biological pathways regulated by PGRN and GRN peptide treatments using transcriptome analysis

Sara Rollinson,† Kate Young,† Janis Bennion-Callister and Stuart M. Pickering-Brown
Institute of Brain Behaviour and Mental Health, University of Manchester, AV Hill Building, Ackers Street, Manchester, M13 9PT, UK

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Abstract

Mutations in progranulin (PGRN) have been linked to two neurodegenerative disorders, heterozygote mutations with frontotemporal lobar degeneration (FTLD) and homozygote mutations with neuronal ceroid lipofuscinosis (NCL). Human PGRN is 593aa secreted growth factor, made up of seven and a half repeats of a highly conserved granulin motif that is cleaved to produce the granulin peptides A–G and paragranulin. While it is thought that PGRN protects against neurodegeneration through its role in inflammation and tissue repair, the role of PGRN and granulins in the nervous system is currently unclear. To better understand this, we prepared recombinant PGRN, granulin A–F and paragranulin, and used these to treat differentiated neuronal SH-SY5Y cells. Using RNA sequencing and bioinformatics techniques we investigated the functional effects of PGRN and the individual granulins upon the transcriptome. For PGRN treatment we showed that the main effect of short-duration treatments is the down-regulation of transcripts, supporting that signalling pathway induction appears to be dominant effect. Gene ontology analysis, however, also supports the regulation of biological processes such as the spliceosome and proteasome in response to PGRN treatment, as well as the lysosomal pathway constituents such as CHMP1A, further supporting the role of PGRN in lysosomal function. We also showed that the response to granulin treatments involves the regulation of numerous non-coding RNA’s, and the granulins cluster into groups of similar activity on the basis of expression profile with paragranulin and PGRN having similar expression profiles, while granulins B, D, E and G appear more similar.

Introduction

Frontotemporal lobar degeneration (FTLD) is a common neurodegenerative disease, often striking in late middle age, affected individuals displaying progressive changes in personality, behavioural and/or language abnormalities (Neary et al., 1998). Approximately 8% of FTLD is linked to the presence of a mutation in progranulin (PGRN) (Nguyen et al., 2013). Homozygous mutations in PGRN are associated with the distinct disease neuronal ceroid lipofuscinosis (NCL), characterized by the storage of abnormal lipopigment in lysosomes, while FTLD as a result of heterozygote PGRN mutations is associated with TDP–43 immunoreactive-positive neuron and glial inclusions (Smith et al., 2012).

Progranulin is a 593aa secreted growth factor made up of seven and a half repeats of a cysteine-rich motif in the order P–G–F–B–A–C–D–E, where A–G are full repeats and P is the half motif known as paragranulin (Hrabal et al., 1996). PGRN is thought to be cleaved into 6-kDa granulin (GRN) peptides by various proteases including proteinase 3, ADAMTS-7 and the matrix metalloproteinase-12 (Butler et al., 2008; Kessenbrock et al., 2008; Xu et al., 2008; Suh et al., 2012). PGRN has multiple functions, the regulation of inflammation, stress and wound healing (He & Bateman, 1999) (He et al., 2003; Yin et al., 2010), and its expression is increased in activated microglia in many neurodegenerative diseases including Creutzfeldt–Jakob disease, motor neuron disease, multiple sclerosis and Alzheimer’s disease (AD) (Malaspina et al., 2001; Baker & Manuelidis, 2003; Baker et al., 2006; Lopez de Munain et al., 2008; Vercellino et al., 2011), suggesting a role in neuroinflammation (Pickford et al., 2011). The relative functions of PGRN and the granulin peptides are not well distinguished, and it is unknown if progranulin is the main active molecule or a precursor protein for the granulin peptides. Both the full-length molecule and the peptides have been shown to exist in vivo (Bateman & Bennett, 2009) and their anti/pro-inflammatory activities suggest opposing functions (De Muynck & Van Damme, 2011). There is some evidence that PGRN modulates inflammation through the modulation of survival signalling, with the mitogen-activated protein (MAP) and kinase (MAPK), extracellular signal-related kinase (Erk1/2) and the phosphoinositide-3 (PI-3K) effectors Akt1 and p70-S6 kinase (He et al., 2002; Kamrava et al., 2005; Monami et al., 2006; Bateman & Bennett, 2009) being modulated by PGRN. The childhood forms...
of NCL are due to defects in genes believed to be involved in lysosomal processing, suggesting a lysosomal function for PGRN.

Given that mutations causing PGRN deficiency result in FTLD, functional information on the role of PGRN would add to the current knowledge of FTLD, identifying cellular processes affected by disease. To further this we produced a series of constructs expressing either full-length PGRN or the GRN peptides and purified recombinant proteins. Using SH-SY5Y cells differentiated to a mature neuronal phenotype and RNA sequencing methodologies we evaluated deregulated transcripts and their pathways affected as a result of a 30-min cell stimulation with PGRN or the granulin peptides.

Materials and methods

Constructs and protein purification

Full-length progranulin including the signal peptide was cloned into the pHTN (GHalotag CMV-neo) Halotag<sup>®</sup> vector from the origene clone RC202139 from the pCMV6-Entry vector. Sequences encoding the granulins were synthesised by MWG using the following template: EcoRI site-Granulin-His Tag-Stop codon-NoT1 site, and were then cloned into the pHTN Vector (for the sequences see Supporting Information). Constructs were transfected into HEK 293 cells grown in DMEM4 containing 10% heat-inactivated foetal bovine serum, 2 mM l-glutamine and 20 units/mL penicillin and 20 μg/mL streptomycin. Transfection was carried out using 5 μg of each construct per 2 × 10<sup>6</sup> cells using jetPRIME (polyplus). For the production of stable clones, 24 h post-transfection the media was replaced with fresh media containing 100 μg/mL G418. After selection of transfected cells, clone stocks were maintained in media supplemented with 100 μg/mL G418.

For the purification of granulin peptides, 40 × 150 mm<sup>2</sup> culture dishes were seeded with the appropriate stable clonal line, once at 90–100% confluence the media was aspirated and the cells were rinsed with ice-cold PBS. Cells were harvested and the pellet collected by centrifugation at 425 g for 10 min at 4 °C. Pellets were lysed with 10 mL of mammalian lysis buffer containing 1× Protease/Phosphatase Inhibitor Cocktail (Promega) and 200 μL RQ1 DNase for 15 min with end to end rotation at 4 °C. The supernatant was diluted 1 : 3 with PBS/1 mM dithiothreitol/0.05% Nonidet P40 and centrifuged for 30 min at 19 357 g at 4 °C. The resulting supernatant was then added to 1.25 mL equilibrated Halotag<sup>®</sup> Resin (Promega) and the cell supernatant incubated for 2 h at room temperature. Mature full-length progranulin contains a signal peptide with the protein secreted into the media, thus this was used for purification rather than cells. The media was centrifuged at 425 g to remove any cell debris, concentrated in a 30-kDa molecular weight cut-off centrifugal filter (Amicon) to approximately 15 mL and bound to the Halotag<sup>®</sup> resin as described previously. Resin washing and cleavage of the granulins and progranulins using the TEV protease were carried out as per the manufacturer’s standard conditions (Promega), with cleavage reactions allowed to proceed overnight at 4 °C. The cleaved protein lysates were further purified using gel filtration chromatography to remove any contaminating proteins present after the Halotag cleavage. Eluates were polished using a Superdex Peptide 10/300GL column run on an Etan LC machine (GE) in PBS containing 1 mM dithiothreitol. Fractions containing protein were pooled and the protein concentration determined using the Qubit protein assay (Life Technologies). The purity of the peptides and full-length progranulin were assessed using electrophoresis and silver staining (see Supporting Information).

Cell treatments and RNA sequencing

SH-SY5Y human neuroblastoma cells were semi-differentiated to a neural phenotype using 1 μM retinoic acid for 3 weeks (Forsby, 2011). Cells were treated by replacing the media in each dish with fresh media containing either 0.5 μg/mL progranulin or 50 ng/mL for the individual granulins or no additive for the control. After treatment cells were washed with ice-cold PBS and the total RNA extracted following the Qiagen RNeasy mini kit protocol. RNA samples were checked for integrity using an Agilent Bioanalyzer before proceeding with library construction (RIN number of > 7).

Five μg of each RNA sample was spiked with control ERCC RNA Spike-In Mix 1 and depletion of ribosomal RNA carried out using the RibominusTM Eukaryote Kit (Ambion). Library construction was carried out using the Life Technologies Ion Total RNA-Seq Kit v2. The prepared libraries were sent to the Clinical Research Centre in Edinburgh where they were run on Ion PI Chips Kit v2 using the Ion Proton Machine. A single library was prepared for each of the granulin and control treatments, while a duplicate sample was prepared for the progranulin treatment.

Real-time PCR

Real-time PCR was carried out using the Applied Biosystems 7900HT Fast Real-Time PCR system. Primers were designed using the Applied Biosystems PRIMER EXPRESS software (version 3.0), against the sequence corresponding to the annotated gene (genome build hg19). Briefly, RNA from both the original RNA sequencing experiment and from replicate cell treatments was taken and converted into mRNA using random hexamers with Transcriptor reverse transcriptase (Roche) using the manufacturer’s protocol. Standard curves for each gene were constructed using undifferentiated SH-SY5Y mRNA, while four replicates of 50 ng each were amplified for each gene in a reaction volume of 10 μL using standard reaction cycling conditions and using Applied Biosystems Power Sybr green master mix. The standard curve method of analysis was used (Pfaffl, 2001), with both GAPDH and β-actin being used as reference genes (see Supporting Information for primer sequences).

RNA sequencing analysis

Analysis was undertaken using the pipeline suggested by Life Technologies for RNA-seq data generated via ion torrent technology of Life Technologies (2012). The ERCC spike-in mix transcript levels were used to assess library preparation and sample processing quality control, all samples had a R<sup>2</sup> in the range of 0.88–0.94 passing the minimum QC value of 0.8. Using the Fastx toolkit (http://hannon-lab.cshl.edu/fastx_toolkit/index.html) sequences were filtered if they did not meet the minimum requirement of a phred quality of 17 in > 80% of the bases. Sequences below 35 bp were also discarded as they are likely adaptors or poor quality reads. Sequence quality was assessed using the FASTQ program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), sequence over 250 bp in length was found to have a poor GC distribution and thus was trimmed to that length. Sequences were mapped to the genome (human version hg19) using a two-step approach. First, sequences were mapped using STAR aligner (Dobin et al., 2013), then the unmapped reads taken and aligned using Bowtie2 (Langmead, 2010). The aligned files were merged using Picard tools (http://broadinstitute.github.io/picard/) and the mapped reads converted to gene counts using HTseq using the union mode (Anders et al., 2015). Gene expression
comparisons were carried out using DESeq2 package run through R (Love et al., 2014). The log-transformed normalized library data dChip was used for visualization of data sets by heatmap construction (Li & Wong, 2001). Cluster 2.11 (Eisen et al., 1998) was used to generate Eigen values for principal components analysis. Pathway and gene ontology analysis were carried out using David (Jiao et al., 2012), P values were corrected for multiple testing using the false discovery rate (Benjamini & Hochberg, 1995). Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-4500 (http://www.ebi.ac.uk/array-express/experiments/E-MTAB-4500).

Results

Identification of differentially expressed genes from granulin and progranulin treatments

Using DeSeq2 and a cut-off of \( P \leq 0.01 \) comparisons were carried out against the 30-min sham treatment (media change only) and each of the GRN peptide or PGRN treatments. PGRN affected more changes in gene expression than any of the individual GRN peptides (\( N = 447 \) v 60–169) (Table 1). In comparison to the control the GRN’s fell into two approximate groups with GRN’s B, C, F and paragranulin exhibiting a greater number of differentially expressed transcripts (\( N = 102, 131, 169 \) and 161), than GRN’s A, D, E and G with \( N = 60, 86, 80 \) and 80 genes respectively. A similar grouping can also be seen from the degree of up-regulation, with low to mid numbers of genes being up-regulated by GRN’s B, C, F, G and paragranulin (57.8, 53.4, 47, 47.5, 39.1%), while GRN’s A, D and E brought about more up-regulation (80, 75.5 and 76.2% respectively). These lists roughly correspond to the order of the GRN’s occurring in PGRN. PGRN treatment itself resulted in only 34% transcripts being up-regulated. The full list of significant genes can be found in the Supporting Information.

To evaluate the expression of the GRNs vs. progranulin we performed a cluster analysis of the log2 fold change values produced for each analysis, the list of genes showing the most variance across the samples (with a significance of \( P < 0.01 \)) were taken and used for Spearman’s rank clustering (Fig. 1A). Eigen values were generated using cluster 2.11 (Eisen et al., 1998) and a principal components plot produced using components 1 and 2 (Fig. 1B). In both sets of analysis paragranulin and progranulin clustered together, suggesting that this peptide may share function with the parent molecule. GRNs B, D, E and G cluster together suggesting there may be some commonality of function for these peptides, while GRNs A, C and F separate from all other treatments and appear to have their own gene expression signatures.

Non-coding RNA gene expression as a result of GRN/PGRN treatment

A large number of non-protein-coding (RNA only) transcripts were identified as being differentially expressed (Table 2). The majority of the RNA genes found differentially expressed were those of the

| Treatment         | Overall genes | Up-regulated (%) | RNA genes | Micro RNA | Small nuclear RNA | Y RNA | IncRNA | Antisense | Pseudogene | Protein coding |
|-------------------|---------------|------------------|-----------|-----------|------------------|-------|--------|-----------|-------------|----------------|
| GRN A             | 60            | 48 (80)          | 15        | 1         | 7                | 2     | 4      | 0         | 17          | 14             |
| GRN B             | 102           | 59 (57.8)        | 19        | 4         | 7                | 0     | 3      | 3         | 20          | 46             |
| GRN C             | 131           | 70 (53.4)        | 23        | 8         | 6                | 1     | 2      | 0         | 16          | 75             |
| GRN D             | 86            | 65 (75.5)        | 10        | 7         | 4                | 3     | 1      | 5         | 38          | 18             |
| GRN E             | 80            | 61 (76.2)        | 10        | 3         | 2                | 4     | 4      | 2         | 33          | 22             |
| GRN F             | 169           | 80 (47)          | 19        | 12        | 11               | 2     | 2      | 2         | 46          | 75             |
| GRN G             | 118           | 38 (47.5)        | 7         | 7         | 2                | 0     | 1      | 1         | 18          | 44             |
| Paragranulin      | 161           | 63 (39.1)        | 11        | 14        | 5                | 3     | 3      | 3         | 54          | 68             |
| Progranulin       | 447           | 153 (34)         | 39        | 14        | 8                | 8     | 17     | 10        | 55          | 296            |

*Transcript numbers reflect differential expression for each gene feature at \( P < 0.01 \) as determined using DESeq2. IncRNA long non-coding RNA.

![Fig. 1.](image-url)
small nuclear and tRNA class, parts of the cellular machinery for transcription and translation. Also numerous were pseudogene transcripts, the majority being of the tRNA class incapable of coding mRNA. However, several transcripts have the potential to produce a processed gene product, possibly causing dysregulation by affecting mRNA levels through acting as a decoy for regulatory factors or as a source of endogenous siRNA’s (Poliseno et al., 2010), though the potential function of those identified in this study is unknown. Between 2 and 11 small nuclear RNA’s were differentially expressed depending on the treatment, responsible for post-transcriptional modification of RNA targets, though most have not been functionally characterized due to redundancy (Bratkovic & Rogelj, 2014). PGRN treatment resulted in the differential expression of five Y RNA’s, part of the Ro60 ribonuclease Y RNA’s are putatively thought to be essential regulators of cell fate (Kohn et al., 2013). A small number of micro-RNAs were found to be differentially regulated, with between N = 1 and 14 depending on the treatment. MiR-34a was down-regulated after both PGRN and paragranulin treatment (−2.45 and −2.397 fold respectively). MiR-34a has been shown to be up-regulated in AD and targets multiple transcripts including Cdk4, cyclin E2, hepatocyte growth factor receptor MET, Bcl-2 and endogenous tau proteins levels in vitro (Bommer et al., 2007; He et al., 2007; Tazawa et al., 2007; Welch et al., 2007; Dickson et al., 2013). Down-regulation of mir-34a has also been demonstrated to confer a growth advantage as the miR induces cell growth retardation (Wang et al., 2009). Paragranulin treatment also down-regulated MiR-132 (−3.45 log fold change), down-regulation has been associated with AD in the hippocampus, cerebellum and medial frontal gyrus tissue. MiR-132 has been shown to be a functional regulator of acetylcholinesterase (AChE) which suppresses peripheral inflammation in the brain by intercepting cytokine production, down-regulation associated with an increase in AChE expression (Shaked et al., 2009). Further the inhibition of mir-132 induces apoptosis in cultured primary neurons, and has been shown to directly regulate PTEN, FOXO3a and P300, all key elements of the AKT signalling pathway (Wong et al., 2013). Eighteen long non-coding RNA’s (lincRNA) were found differentially expressed after PGRN treatment, with the individual GRN’s treatments regulating between 1 and 4 transcripts, unfortunately the majority of these transcripts are not well characterized. LincRNA’s are known to be involved in epigenetic regulation — functioning in the recruitment of protein factors, modulating transcription by targeting transcription factors, co-repressors and the basal machinery itself. LincRNA’s are also capable of acting as microRNA’s, siRNA’s, splicing and affecting epigenetic regulation (Kung et al., 2013). Eleven antisense RNA (AsRNA) transcripts were affected by PGRN treatment. AsRNA cis-antisense transcripts are often translated along with associated protein-coding genes by region and may affect splicing regulation; however, the exact function of these molecules is unknown. As with all the non-protein-coding transcripts identified, the possible scope for regulation is enormous, though whether these transcripts have biological functions remains unknown.

Gene expression changes induced by GRN treatments

Our analysis of the GRN treatments revealed similarities in differential expression between the peptide treatments, which is expected given the fact that they share a conserved structure as well as differences. The main commonalities between the GRN’s were in genes involved in transcriptional regulation, stress response and cytoskeleton maintenance. The top four up- and down-regulated genes from each treatment are shown in Table 3. In GRN A, treatment appeared to down-regulate genes involved in signalling such as sprout-related EVH1 domain containing 3 (−3.16 log2 fold change) and versican (−1.11 log2 fold change). GRN B treatment down-regulated both VGF (−2.98 log2 fold change) and chromogranin (−2.07 log2 fold change), both secretory granule proteins for other peptides. While their exact functions are unknown, both are involved in stress responses and have been associated with synaptic degeneration in AD. Two proteins implicated in negative regulation TGF beta signalling, FAM89B (−log2 fold change) and protein phosphatase 1 (regulatory subunit 15A) (−1.96 log2 fold change), were found. GRN C treatment down-regulated several proteins involved in folate metabolism, cystathionine-beta-synthase (−0.8 log2 fold change), methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2 (−0.87 log2 fold change) and serine hydroxymethyltransferase 2 (mitochondrial) (−1.23 log2 fold change). Folate deficiency has been linked to an increase in the inflammatory response, and in macrophages an increase in expression of the inflammatory mediators, IL1beta, IL6 and TNFalpha (Ryan et al., 2009), suggesting this may be an indirect response to inflammation by the GRN peptide. GRN D and E treatments were notable for their predominance of RNA genes in particular non-expressed rRNA pseudogenes. Parathyromosin, a gene involved in the innate immune response, and proprotein convertase, which regulates the cleavage of neuroendocrine peptide precursors, were both down-regulated by GRN D treatment (−1.47 and −1.74 fold respectively). In the GRN F gene list transcriptional regulation either as transcription factors, activators or repressors was noted, the majority of which down-regulated by treatment. Activating transcription factor 5 (−2.73), CCAAT/enhancer binding protein beta (−2.13), coiled-coil domain containing 85B (−3.04) and empty spiracles homeobox 2 (EMX2) (−8.02). C1D nuclear receptor co-repressor was up-regulated (1.91 log2 fold change). GRN G also appeared to regulate several transcription factors including several those seen in GRN F including the CCAAT/enhancer binding protein beta (−2.5) and EMX2 gene (−5.8). Several solute carrier genes involved in membrane transport were also found to be differentially expressed; solute carrier family 1 member 4 (−1.96), solute carrier family 38 member 6 (1.74), solute carrier family 7 member 11 (−1.72), solute carrier organic anion transporter family member 1C1 (3.24) and solute carrier organic anion transporter family member 5A1 (2.77). Paragranulin treatment again was associated with the differential expression of transcription factors, including activating transcription 4 (−1.13), basic transcription factor 3-like 4 (−1.19) and X-box binding protein 1 (−1.36).

Gene expression changes induced by PGRN treatment

Our analysis of the PGRN treatment data set revealed the largest number of differentially expressed transcripts of all our treatments with a total of 447 transcripts being differentially expressed, 296 of these being known protein coding, Table 4 lists the top 10 up- and down-regulated protein-coding genes. In common with GRN treatments many of the genes were involved in transcriptional regulation, response to stress and cytoskeleton maintenance. Indeed the transcription factor EMX2 was down-regulated by almost all treatments (−1.9 log2 fold change). No PGRN treatment specific genes were seen, likely a reflection of the homology between the peptides and the parent molecule. Several genes of unknown function, C1orf183 (5.21 log2 fold change), C4orf53 (−2.36 log2 fold change) and C1orf134 (−1.52 log2 fold change) were found in this list, as were several proteins involved in signalling (EPH receptor 5.49 log2 fold change).
| Gene                                | Symbol/accession | Log2 fold change | P-value |
|-------------------------------------|------------------|-----------------|---------|
| GRN A                               |                  |                 |         |
| Known lincRNA                       | RP1-74M1.3       | -2.67           | 0.0041  |
| Novel lincRNA                       | ENSG00000261452  | -1.96           | 0.0074  |
| Long intergenic non-protein-coding  | LINC00641        | -1.83           | 0.0097  |
| RNA 641                             |                  |                 |         |
| Small nucleolar RNA SNORAI1         |                  | 1.85            | 0.0075  |
| Small nucleolar RNA, C/D box 3B-1   | SNORD3B-1        | 2.09            | 0.0046  |
| Y RNA                               |                  | 2.42            | 0.0093  |
| Small nucleolar RNA host gene 8     | SNHG8            | 2.52            | 0.0004  |
| Small nucleolar RNA, C/D box 3B-2   | SNORD3B-2        | 2.58            | 0.0005  |
| GRN B                               |                  |                 |         |
| Chaperonin containing TCP1, subunit 5| CCT5P1           | -5.32           | 0.0218  |
| MicroRNA 197                        | MIR197           | -3.04           | 0.0231  |
| MicroRNA 99b                        | MIR99B           | -2.82           | 0.008   |
| Small nucleolar RNA host gene 8     | SNHG8            | 3.04            | 0.002   |
| Small nucleolar RNA, C/D box 3B-2   | SNORD3B-2        | 3.11            | 0.002   |
| HYI antisense RNA 1                 | HYI-AS1          | 3.28            | 0.0433  |
| Novel antisense                     |                  | 5.35            | 0.0069  |
| GRN C                               |                  |                 |         |
| MicroRNA 182                        | MIR182           | -4.396          | 1.22E-10|
| MicroRNA 197                        | MIR197           | -4.003          | 0.0002  |
| MicroRNA 183                        | MIR183           | -3.475          | 0.0012  |
| MicroRNA 99b                        | MIR99B           | -3.268          | 1.19E-09|
| Small nucleolar RNA host gene 15    | SNHG15           | 0.8831          |         |
| Small nucleolar RNA, C/D box 3B-2   | SNORD3B-2        | 1.2029          | 0.0070  |
| Small nucleolar RNA host gene 7     | SNHG7            | 1.3299          | 6.88E-06|
| Small nucleolar RNA host gene 8     | SNHG8            | 1.9519          | 5.13E-10|
| GRN D                               |                  |                 |         |
| MicroRNA 99b                        | MIR99B           | -2.799          | 4.80E-05|
| MicroRNA 182                        | MIR182           | -2.768          | 0.00012 |
| MicroRNA 183                        | MIR183           | -3.76           | 0.00550 |
| MicroRNA 320a                       | MIR320A          | -2.653          | 0.0063  |
| Y RNA                               | ENSG00000207161  | 5.7846          | 0.00307 |
| Y RNA                               | ENSG00000206959  | 6.5436          | 5.83E-11|
| AGBL1 antisense RNA 1               | AGBL1-AS1        | Inf             | 0.00042 |
| Antisense                           |                  | Inf             | 8.19E-05|
| GRN E                               |                  |                 |         |
| MicroRNA 182                        | MIR182           | -3.042          | 0.0001  |
| MicroRNA 197                        | MIR197           | -2.867          | 0.004   |
| MicroRNA 99b                        | MIR99B           | -2.388          | 0.0013  |
| Novel lincRNA                       | RP11-309L24.4    | -2.254          | 0.0094  |
| Small nucleolar RNA, C/D box 3B-2   | SNORD3B-2        | 3.1532          | 9.19E-06|
| Small nucleolar RNA host gene 8     | SNHG8            | 3.1601          | 5.11E-06|
| (non-protein coding)                |                  |                 |         |
| Small nucleolar RNA U3              | RNA U3           | 4.2547          | 0.0085  |
| Y RNA                               | ENSG00000206959  | 4.5071          | 0.001   |
| GRN F                               |                  |                 |         |
| MicroRNA let-7e                     | MIRLET7E         | -5.487          | 0.0025  |
| MicroRNA 99b                        | MIR99B           | -5.22           | 6.70E-09|
| MicroRNA 182                        | MIR182           | -4.764          | 2.43E-07|
| MicroRNA 1269a                      | MIR1269A         | -4.644          | 0.00315 |
| Small nucleolar RNA, C/D box 3B-2   | SNORD3B-2        | 4.1253          | 7.84E-09|
| Y RNA                               | ENSG00000206959  | 6.0748          | 1.42E-07|
| AGBL1 antisense RNA 1               | AGBL1-AS1        | Inf             | 0.0013  |
| Long intergenic non-protein-coding  | LINC00284        | Inf             | 0.0098  |
| RNA 284                            |                  |                 |         |

(continued)
| Gene                                      | Symbol/accession                  | Log2 fold change | P-value |
|-------------------------------------------|-----------------------------------|-----------------|---------|
| Paragranulin                              |                                   |                 |         |
| Antisense                                 | RP4-791M13.3                      | −4.944          | 0.0085  |
| Y RNA                                     | ENSG00000199378                   | −3.447          | 0.0063  |
| MicroRNA 132                              | MIR132                            | −3.435          | 0.0036  |
| MicroRNA 1269a                            | MIR1269A                          | −3.334          | 0.0006  |
| Long intergenic non-protein-coding RNA 1155 | LINC01155                        | 1.3306          | 0.0023  |
| Antisense                                 | AC007038.7                        | 1.6572          | 0.0057  |
| Y RNA                                     | ENSG00000199677                   | 2.1509          | 0.0008  |
| Y RNA                                     | ENSG00000222072                   | 2.6027          | 0.0033  |
| Programalin                               | ENSG00000199378                   | −4.233          | 3E-06   |
| MicroRNA 4477a                            | MIR4477B                          | −3.456          | 4E-06   |
| MicroRNA 34a                              | MIR34A                            | −2.458          | 0.0029  |
| LincRNA                                   | RP11-1263C18.1                    | −2.235          | 0.0043  |
| LincRNA                                   | CTD-2542L18.1                     | 3.1529          | 0.0014  |
| Antisense                                 | AC007970.1                        | 3.4565          | 0.0085  |
| LincRNA                                   | RP11-774O3.3                      | 3.5214          | 0.0007  |
| Long intergenic non-protein-coding RNA 598 | LINC00598                        | 3.679           | 0.0009  |

*Excluding rRNA, tRNA, mtRNA and pseudogenes.

| Gene                                      | Symbol/accession                  | Log2 fold change | P-value |
|-------------------------------------------|-----------------------------------|-----------------|---------|
| GRN A                                     |                                   |                 |         |
| Uncharacterized protein                   | ENSG00000199378                   | −4.233          | 3E-06   |
| Plexin domain containing 1                | PLXDC1                            | 2.11            | 0.00218 |
| NADH dehydrogenase (ubiquinone)           | NDUFA4                            | 2.19            | 0.00175 |
| l alpha subcomplex, 4, 9 kDa              |                                   |                 |         |
| Ribonuclease P RNA component H1            | RPHH1                             | 2.11            | 0.00218 |
| CD44 molecule (Indian blood group)        | CD44                              | −2.13           | 0.00785 |
| Processed pseudogene                      | ENSG00000225471                   | −2.51           | 0.00074 |
| Novel sense overlapping                   | ENSG00000224113                   | −2.66           | 0.00233 |
| Sprouty-related, EVH1 domain containing 3  | SPRED3                            | −3.16           | 0.00081 |
| GRN B                                     |                                   |                 |         |
| NADH dehydrogenase (ubiquinone)           | NDUFA4                            | 2.78            | 0.00441 |
| l alpha subcomplex, 4, 9 kDa              |                                   |                 |         |
| Histone cluster 1, H4k                    | HISTH4K                           | 2.58            | 0.00785 |
| Plexin domain containing 1                | PLXDC1                            | 2.53            | 0.01621 |
| Histone cluster 1, H4j                    | HISTH4J                           | 2.51            | 0.00933 |
| Microtubule-associated protein 1A         | MAP1A                             | −2.63           | 0.00727 |
| Chromosome 1 open reading frame 134       | C1orf134                          | −2.74           | 0.02523 |
| Zinc finger protein 579                   | ZNF579                            | −2.86           | 0.00969 |
| VGF nerve growth factor inducible         | VGF                               | −2.98           | 0.00235 |
| GRN C                                     |                                   |                 |         |
| Histone cluster 2, H2aa4                  | HIST2H2AA4                        | 2.26            | 0.00084 |
| Aquaporin 1 (Colton blood group)          | AQP1                              | 2.05            | 0.00010 |
| Histone cluster 2, H2aa3                  | HIST2H2AA3                        | 1.79            | 3.8E-05 |
| Histone cluster 2, H3c                    | HIST2H3C                          | 1.78            | 0.00706 |
| KIAA0125                                  | KIAA0125                          | −1.73           | 0.00588 |
| Family with sequence similarity 129, member A | FAM129A                         | −1.75           | 8.6E-07 |
| Chromosome 4 open reading frame 33        | C4orf33                           | −1.97           | 1.2E-07 |
| Empty spiracles homeobox                  | EMX2                              | −5.59           | 4.2E-50 |
| GRN D                                     |                                   |                 |         |
| Uncharacterized protein                   | ENSG000000268400                  | 3.45            | 6.2E-08 |
| Histone cluster 2, H4b                    | HIST2H4B                          | 2.11            | 0.00087 |
| Histone cluster 2, H3a                    | HIST2H3A                          | 2.04            | 0.00356 |
| Plexin domain containing 1                | PLXDC1                            | 1.95            | 0.00563 |
| Proprotein convertase subtilisin/kexin    | PCSK1N                            | −1.74           | 0.00696 |
| type 1 inhibitor                          |                                   |                 |         |
| Zinc finger protein 579                   | ZNF579                            | −2.03           | 0.00382 |
| Processed transcript                      | RP11-253E3.3                      | −2.41           | 0.00320 |
| Empty spiracles homeobox 2                | EMX2                              | −5.78           | 1.84-17 |
| Gene Symbol/accession | Log2 fold change | P-value |
|-----------------------|------------------|---------|
| GRN E                 |                  |         |
| Uncharacterized protein | MOQYU6         | 3.82    | 1.76E-07 |
| Adenosine A3 receptor | ADORA3          | 2.97    | 0.00656  |
| Oocyte expressed protein | OOE   | 2.77    | 0.00017  |
| POM121 transmembrane nucleoporin-like 2 | POM121L2 | 2.55    | 0.00908  |
| VGF nerve growth factor inducible | VGF | −1.90   | 0.0396   |
| Chromosome 4 open reading frame 33 | C4orf33 | −1.96   | 0.00437  |
| Zinc finger protein 579 | ZNF579      | −2.10   | 0.00619  |
| Empty spiracles homeobox 2 | EMX2   | −6.34   | 6.0E-15  |
| GRN F                 |                  |         |
| Apelin receptor | APLNR           | ∞       | 0.00141  |
| Histone cluster 2, H4b | HIST2H4B      | 3.05    | 1.6E-05  |
| Histone cluster 2, H2aa4 | HIST2H2AA4  | 2.57    | 0.00225  |
| Histone cluster 2, H3c | HIST2H3C      | 2.51    | 0.00173  |
| Inhibin, beta E | INHBE           | −3.65   | 8.9E-05  |
| Synaptic vesicle glycoprotein 2B | SV2B   | −3.72   | 0.00354  |
| ATPase, H+ transporting, lysosomal 13 kDa, V1 subunit G2 | ATP6V1G2 | −3.78   | 0.00609  |
| Empty spiracles homeobox 2 | EMX2   | −8.02   | 3.1E-20  |
| GRN G                 |                  |         |
| Solute carrier organic anion transporter family, member 1C1 | SLCO1C1 | 3.24    | 0.00094  |
| Solute carrier organic anion transporter family, member 5A1 | SLCO5A1 | 2.77    | 2.0E-06  |
| Origin recognition complex, subunit 2 | ORC2    | 2.53    | 1.7E-05  |
| HFM1, epipilakin 1 | HFM1           | 2.22    | 9.2E-05  |
| CCAAT/enhancer binding protein (C/EBP), beta | CEBPB   | −2.53   | 0.00025  |
| Inhibin, beta E | INHBE           | −3.01   | 0.00019  |
| Empty spiracles homeobox 2 | EMX2   | −5.85   | 7.8E-18  |
| Paragranulin          |                  |         |
| Bone morphogenetic protein 5 | BMP5    | 3.37    | 0.00864  |
| Thymosin beta 10 pseudogene 1 | TMSB10P1  | 2.57    | 2.0E-07  |
| Protein_coding |                  |         |
| Uncharacterized protein |                  |         |
| Chromosome 1 open reading frame 134 | C1orf134 | −1.88   | 0.00226  |
| Chromosome 4 open reading frame 33 | C4orf33  | −1.89   | 2.1E-05  |
| Empty spiracles homeobox 2 | EMX2   | −2.33   | 5.4E-08  |
| Synaptotagmin VI | SYT6            | −4.19   | 0.00731  |

Table 4. Top 10 up- and down-regulated genes after a 30-min progranulin treatment

| Gene Symbol/accession | Log2 fold change | P-value |
|-----------------------|------------------|---------|
| Phospholipase A2, group XIIA pseudogene 1 | PLA2G12AP1 | ∞       | 0.00878  |
| GRB2-related adaptor protein | GRAP            | 6.40    | 1.6E-11  |
| EPH receptor A3 | EPHA3           | 5.49    | 1.4E-06  |
| Chromosome 14 open reading frame 183 | C1orf183  | 5.21    | 0.00280  |
| Protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 2 | PPFIA2   | 3.77    | 0.00731  |
| ArfGAP with coiled-coil, ankyrin repeat and PH domains 1 | ACA1     | 3.46    | 0.00763  |
| Phosphotyrosine interaction domain containing 1 | PID1     | 2.86    | 1.1E-16  |
| Ankyrin repeat domain 30B-like | ANKRD30BL | 2.42    | 2.7E-10  |
| Solute carrier organic anion transporter family, member 5A1 | SLCO5A1  | 2.36    | 2.0E-12  |
| Complement component 2 | C2             | 2.34    | 0.0019   |
| Trans-2,3-enoyl-CoA reductase | TCR    | −1.51   | 1.8E-07  |
| Chromosome 1 open reading frame 134 | C1orf134  | −1.52   | 0.00284  |
| Family with sequence similarity 96, member B | FAM96B   | −1.56   | 5.6E-08  |
| CDC28 protein kinase regulatory subunit 1B | CKS1B    | −1.60   | 9.4E-08  |
| Family with sequence similarity 71, member E1 | FAM71E1  | −1.63   | 0.00265  |
| tRNA methyltransferase 11-2 homolog (S. cerevisiae) | TRMT112  | −1.64   | 7.8E-09  |
| Heat shock 70 kDa protein 1B | HSPA1B    | −1.65   | 5.7E-08  |
| Homer homolog 3 (Drosophila) | HOMER3   | −1.75   | 1.0E-07  |
| Empty spiracles homeobox 2 | EMX2     | −1.90   | 4.7E-11  |
| Chromosome 4 open reading frame 33 | C4orf33  | −2.36   | 8.5E-13  |
change, and protein tyrosine phosphatase receptor type F (3.77 log2 fold change).

**Biological pathway analysis**

To elucidate the function of PGRN and the GRN’s biological pathway analysis was carried out using DAVID (Huang et al., 2007; Jiao et al., 2012). Biological process categories significantly enriched in the data set in comparison to the genome were identified and the significant hits after multiple testing correction shown in Table 5. Genes involved in the ribosome pathway were found to be significantly enriched in all the individual granulin and PGRN treatments bar GRN G and paragranulin. The systemic lupus erythematosus (SLE) pathway was also found to be significantly enriched in the gene lists for most of the treatments (maximum \( P = 0.0014 \)). The genes responsible for this enrichment fell mostly in the histone class, histones consist of the basic building blocks of the nucleosome, a histone octamer surrounded by 146 bp of DNA. The N-terminal tail of the histones maybe post-translationally modified, by acetylation, methylation, ubiquitination, phosphorylation, sumoylation and adenosine diphosphate (ADP) ribosylation. The modification status alters the interaction between histones affecting DNA replication, transcription, alternate splicing, DNA repair and the relaxation or condensation of chromatin. Increased levels of serum PGRN have been associated with SLE, thought to be linked to disease status through the innate immune response Toll-like receptor 9 signalling pathway that is normally specific to microbial stimulus, which is also found to be aberrant in lupus, controlling in part anti-DNA production from B cells. Interestingly, the majority of the GRN treatments resulted in a down-regulation of histone expression, while PGRN and paragranulin treatments in up-regulation, for example HIST2H3A showed a 1.81 log2 fold increase with GRN G treatment and a \(-1.52 \) log2 decrease with a 30-min PGRN treatment. Also linked to this pathway was the finding that the complement component 2 gene showed a 2.34 log2 increase in expression after PGRN treatment. GRN peptides F and G were also found to show an enrichment of genes involved in glycine, serine and threonine metabolism (\( P = 0.009 \)), while PGRN treatment showed an enrichment in genes involved in cysteine and methionine metabolism (\( P = 0.006 \)). In cardiac endothelial cells cysteine, histidine and glycine have been shown to significantly reduce NF-κB activation and the degradation of its inhibitor IkBα in cells stimulated with TNFα. The amino acids also inhibited the expression of E-selectin and IL-6, suggesting that part of the anti-inflammatory effect of PGRN may be indirectly a result of modulating amino acid levels (Hasegawa et al., 2012). Both paragranulin and PGRN were both found to be enriched for genes involved in the spliceosome (maximum \( P = 0.007 \)). After PGRN treatment nine genes were found to be down-regulated, including the common component the heterogeneous nuclear riboprotein C (\( -0.88 \) log2 fold change) and the CWC15 spliceosome-associated protein homology (\( S. cerevisiae \)) (\( -0.82 \) log2 fold change). While the pathway only reached significance in these two treatments, the genes showed a trend to significance within most of the other treatments suggesting that the down-regulation of the spliceosome may be a common reaction to PGRN and its peptides. PGRN treatment was also associated with the enrichment of genes involved in the down-regulation of the proteasome pathway (\( P = 0.02 \)). A total of five genes were down-regulated, PSMC3, PSMD4, PSMA4, PSMA5 and PSMB6 proteasome components (\(-0.77 \) to \(-0.96 \) log2 fold change), comprising two regulatory and three core components of the proteasome. The proteasome regulates the concentrations of cellular proteins and degrades misfolded proteins and play a role in gene expression by the degradation of transcription factors. Both pyruvate metabolism (\( P = 0.01 \)) and glycolysis/glucose metabolism (\( P = 0.04 \)) were also pathways affected by PGRN treatment, both pathways again being down-regulated.

**Other genes of note**

While non-significant, 16 genes involved in membrane transport and Golgi and endosomal sorting were down-regulated by PGRN. These included the two charged multi-vesicular body proteins 1A and 5 (\(-0.81 \) and \(-1.17 \) log2 fold change), acyl-CoA dehydrogenase, very long chain (\(-0.77 \)), adaptor-related protein complex I sigma 1 subunit \((-0.83 \) activating transcription factor 4 \((-0.89 \)), B-cell receptor-associated protein 31 \((-0.79 \)), basic transcription factor 3 \((-0.95 \)), defender against cell death 1 \((-0.89 \)), developmentally regulated GTP binding protein 1 \((1.06 \) ER membrane protein complex subunit 3 \((-1.35 \)), lectin, mannose-binding 2-like \((-0.84 \)), membrane-associated ring
Fig. 2. Real-time validation of NGS data for progranulin treatments. Gene (RT, next gen RNA; rep, replicate treatment).

Quantitative real-time QRT-PCR validation

To validate gene expression changes found using transcriptome sequencing we performed QRT-PCR for a subset of up- and down-regulated genes. We used both GAPDH and β-actin for normalizing gene expression levels, as similar levels were found with both reference genes only the GAPDH normalized data are shown. Gene expression was determined on both the samples used for the transcriptome sequencing and experimental replicates, where new cells were differentiated and treated as before. For the PGRN treatments 15/19 genes passed validation (Fig. 2). The genes which failed validation (three on the original samples sequenced, the other on experimental replication) were generally those with smaller P values or fold changes. This could have been adjusted for by altering the P value threshold and filtering out genes with low levels of expression. However, it was decided to keep the gene lists as presented because interesting targets would be validated and deleting genes off the lists would result in false negatives. For the GRN treatments only one gene failed replication, PIDI, though smaller amounts of available RNA precluded testing more genes on each treatment (Fig. 3).

Discussion

While heterozygous mutations in PGRN are associated with FTLD with TDP-43 inclusions, homozygous mutations in PGRN are rather associated with neuronal ceroid lipofuscinosis (NCL), a lysosomal storage disease (Smith et al., 2012). Our findings support a function for PGRN in membrane transport as several genes involved in golgi/endosomal sorting were differentially regulated after PGRN treatment. Two proteins of the ESCRTIII (endosomal sorting complex required for transport III) were down-regulated by PGRN treatment, CHMP1A and CHMP5 (−0.81 and −1.17 log2 fold change respectively) (Schmidt & Teis, 2012). The ESCRTIII pathway has been previously implicated in FTLD, as mutations in CHMP2B have been reported in FTLD linked to chromosome 3 (Skibinski et al., 2005). The pathway itself is a key for the internalization and transport of neuronal and secreted growth factors as well as signalling molecules (Bronfman et al., 2007). A link between PGRN and the ESCRT pathway is intriguing; indeed an increase in level of CHMP1A was seen in brain tissue from GRN mutation-positive brains in comparison to other FTLD and control brains, suggesting that the protein is linked to FTLD and possibly more strongly to GRN-positive FTLD (see Supporting Information). Our data support the role for PGRN in down-regulating the ESCRTIII pathway as CHMP1A is one of the subunits required for the assembly of the complex (Bronfman et al., 2007).

Pathway analysis also suggested a role for PGRN via the SLE pathway, SLE itself is an autoimmune disease in which the immune system attacks itself producing inflammation and tissue damage. PGRN serum levels have been shown to be elevated in SLE and are thought to enhance Toll-like receptor 9 signalling which is involved in the innate immune response (Tanaka et al., 2012), indeed PGRN has been shown to be a critical cofactor in enabling TLR9 signalling (Park et al., 2011). While the innate immune response pathway was not in itself enriched in our analysis, several pathway-related genes such as prothrombin alpha, plasminogen receptor, canopy FGF signalling regulator 4 and myeloid differentiation primary response 88 did appear in the significant gene list, adding support to the involvement of this pathway from the SLE data. Innate immunity provides an immediate defence against infection by the production of cytokines and the activation of the complement cascade, the down-regulation noted may in part be responsible for the protective effect of PGRN against neurodegeneration, as neuroinflammation may contribute to the development and progression of FTLD.

Paragranulin and PGRN were both found to be enriched for genes involved in the spliceosome. It should be noted that most of the treatments showed some down-regulation of the spliceosome which may indicate a common effect of PGRN and its peptides. FTLD itself is already linked with RNA processing dysfunction, with both TDP-43 and FUS sharing similar functional properties, likely both linked to RNA processing and metabolism, as such the effect of PGRN dysfunction on splicing could indeed predispose to disease. The ubiquitin proteosome system (UPS) pathway was also found to be down-regulated after PGRN treatment (P = 0.02). The proteosome is a protein complex found in the nucleus and cytoplasm of eukaryotic cells, responsible for the regulation of cellular protein concentrations and the degradation of misfolded proteins and transcription factors. One of the hallmarks of neurodegenerative disease is the accumulation of neurotoxic protein aggregates in the brain, e.g. tau and β-amyloid in AD and TDP-43 in FTLD. In this study,
PGRN treatment appears to down-regulate genes which are part of the UPS pathway responsible for the degradation of aberrant protein. If PGRN deficiency is responsible for the production of neurotoxic aggregation of TDP-43 and subsequent destruction of neuronal tissue, then one would expect PGRN treatment to up-regulate these genes. However, it is possible that this could be too simplistic an explanation and it is the relationship between the UPS and autophagic removal of TDP-43 that is important (Scotter et al., 2014).

The data does suggest, however, that the main function of PGRN at least for short treatments appears to be in slowing cell processes by via the down-regulation of the UPS, splicing and transport systems possibly as preparation for the main cellular response to PGRN via signalling. PGRN has been shown to stimulate signalling pathways including the mitogen-activated protein (MAP) and kinase (MAPK), extracellular signal-related kinase (Erk1/2) and the phosphoinositide-3 (PI-3K)/Akt1/mtor pathways (He et al., 2002; Kamrava et al., 2005; Monami et al., 2006; Bateman & Bennett, 2009). The loss of PGRN as a result of GRN mutation has also been linked to a dysregulation of Wnt signalling in FTLD suggesting that neuronal GRN deficiency is sufficient to significantly reduce neuronal survival (Rosen et al., 2011; Raitano et al., 2015). While these pathways were not directly transcriptionally affected, differential expression in genes related to these pathways was found. These included the B-cell CLL/lymphoma 9-like gene a transcriptional regulator that promotes beta catenin transcriptional activity linking in to the Wnt pathway. Also affected were brain expressed, X-linked 1, BUB3 mitotic checkpoint protein, calcium and integrin binding protein 1 and insulin-like growth factor 2 who all link PGRN treatment into the TOR signalling pathway, either directly or through the activation of erk1/erk2 and MAP kinase signalling.

While heterozygous mutations in PGRN are associated with FTLD with TDP-43 inclusions, the haploinsufficiency leads to less than half the expected amount of circulating protein (Finch et al., 2009; Sleegers et al., 2009), suggestive of cleavage of the PGRN into its constitutive GRN fragments. In addition, as GRN cleavage products are not present in NCL due to the absence of PGRN, either a deficiency in PGRN or the presence of GRN cleavage fragments maybe the critical factor in the development of FTLD-TDP. A degree of similarity was seen across the differential expression patterns of the granulin peptides, genes that reached significance for a
single treatment on examination of the raw values and fold changes revealed a similar trend for the majority of the other treatments, again likely a reflection of the homology between the peptides. Despite this our results suggest some possible functions for the individual peptides, with pro-inflammatory functions being suggested for GRN’s A and B via AKT/MAP kinase signalling, and GRN C via the folate pathway, while the other GRN’s appeared to mostly regulate transcription factors, activators or repressors. Within the GRN peptides tested, CHMP1A and CHMP5 only displayed a trend for significance for paragranulin, suggesting the N terminus may be the critical end of the molecule for endosomal functions, indeed paragranulin displayed an expression profile most similar to PGRN of all the GRN’s tested. GRN G treatment (the granulin adjacent to paragranulin in the intact PGRN molecule) affected a large number of soluble carrier family genes involved in membrane transport, and further suggests that this portion of the molecule maybe crucial for membrane transport, and that it is not only intact PGRN that may have a function within the ESCRT III pathway.

Despite the presence of cleavage sites within PGRN the abundance of GRN peptides in the brain is unknown, and it is unclear how single granulin peptides mediate biological function as most PGRN protein interactions require more than one granulin domain (Jian et al., 2013). Recently a PGRN cleavage product specific to brain regions with gliosis in AD and FTD specific for a GRN E antibody blotted at 33 kDa, suggesting that PGRN is cleaved to form a cleavage product containing GRN’s A–C–D–E in vivo (Sala- zar et al., 2015). This opens up the possibility of PGRN being cleaved into larger fragments that may retain more of the parent molecules functions, and further study is required to elucidate the existence and function of these peptides. Having established that the effects of progranulin and the granulins are multifactorial and often acting in a completely opposing manner, it is obvious that there is much work to be done to try and identify specific targets for putative treatment options.

Conflict of interests
The authors declare no competing financial interests.

Supporting Information
Additional supporting information can be found in the online version of this article:
Data S1. Details of granulin sequences used, real time PCR primers used, and additional CHMP1A data.

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Abbreviations
GRN, Granulin; FTLD, frontotemporal lobar degeneration; NCL, neuronal ceroid lipofuscinosis; PGRN, Progranulin.

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