

## LETTERS

# Null mutations in progranulin cause ubiquitin-positive frontotemporal dementia linked to chromosome 17q21

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Frontotemporal dementia (FTD) with ubiquitin-immunoreactive neuronal inclusions (both cytoplasmic and nuclear) of unknown nature has been linked to a chromosome 17q21 region (FTDU-17) containing *MAPT* (microtubule-associated protein tau)<sup>1-3</sup>. FTDU-17 patients have consistently been shown to lack a tau-immunoreactive pathology<sup>1-3</sup>, a feature characteristic of FTD with parkinsonism linked to mutations in *MAPT* (FTDP-17)<sup>4</sup>. Furthermore, in FTDU-17 patients, mutations in *MAPT* and genomic rearrangements in the *MAPT* region have been excluded by both genomic sequencing<sup>5</sup> and fluorescence *in situ* hybridization on mechanically stretched chromosomes<sup>6</sup>. Here we demonstrate that FTDU-17 is caused by mutations in the gene coding for progranulin (*PGRN*), a growth factor involved in multiple physiological and pathological processes including tumorigenesis<sup>7</sup>. Besides the production of truncated *PGRN* proteins due to premature stop codons<sup>8</sup>, we identified a mutation within the splice donor site of intron 0 (IVS0+5G>C), indicating loss of the mutant transcript by nuclear degradation. The finding was made within an extensively documented Belgian FTDU-17 founder family<sup>3</sup>. Transcript and protein analyses confirmed the absence of the mutant allele and a reduction in the expression of *PGRN*. We also identified a mutation (c.3G>A) in the Met1 translation initiation codon, indicating loss of *PGRN* due to lack of translation of the mutant allele. Our data provide evidence that *PGRN* haploinsufficiency leads to neurodegeneration because of reduced *PGRN*-mediated neuronal survival. Furthermore, in a Belgian series of familial FTD patients, *PGRN* mutations were 3.5 times more frequent than mutations in *MAPT*, underscoring a principal involvement of *PGRN* in FTD pathogenesis.

FTD is the second most common form of dementia in individuals <65 yr of age and manifests clinically with progressive behavioural and personality disturbances including disinhibition, perseveration and emotional blunting, evolving eventually into generalized cognitive impairment<sup>9</sup>. FTD results from severe neuronal degeneration of frontal and/or temporal brain regions. FTDU is the most common pathological subtype of FTD and is characterized by the presence of ubiquitin-immunoreactive neuronal inclusions in the absence of tau pathology<sup>10</sup>. In autosomal-dominant FTDU families linkage has been observed with chromosome 9p12-p13 (ref. 11), caused by mutations in

the valosin-containing protein VCP<sup>11,12</sup>, and 17q21 in the *MAPT* region<sup>13</sup>. Several FTDU families have been conclusively linked to 17q21 (ref. 14) (Fig. 1a), but the underlying gene remained unknown.

We reduced the minimal candidate region of FTDU-17 to 6.3 megabases (Mb) in one Dutch family, 1083 (Fig. 1a), and excluded simple mutations in *MAPT* by exon-based and genomic sequencing<sup>1,5</sup>. We also excluded the possibility that FTDU-17 resulted from a genomic rearrangement in the *MAPT* locus through using fluorescence *in situ* hybridization (FISH) on mechanically stretched chromosomes<sup>6</sup>—normal patterns were observed in patients of Dutch family 1083 and a recently identified Belgian FTDU-17 founder family<sup>3</sup> (Supplementary Fig. 1). Also, comparative genomic hybridization on oligo-based arrays confirmed the absence of a deletion or duplication in the *MAPT* region in both families (data not shown). In addition, biochemical studies of frozen brain tissue had shown normal *MAPT* messenger RNA and protein isoforms<sup>2,13</sup>. Together, these data supported the notion that FTDU-17 had to be a separate entity in which the ubiquitin-immunoreactive brain pathology was unrelated to tau.

Extensive mutation analyses of other genes from within the 17q21 candidate region identified nonsense and frameshift mutations in the progranulin gene (*PGRN*)<sup>8</sup>. In family 1083, a nonsense mutation Gln125X (exon 4, c.373C>T) was identified in patient III-26, and was absent from 190 control individuals (Supplementary Fig. 2). The mutation segregated with disease (Supplementary Fig. 2a), and nonsense-mediated mRNA decay produced a null allele (Supplementary Fig. 2c). In patients of the Belgian FTDU-17 founder family DR8 (Fig. 2), we identified a G-to-C transversion in intron 0 of *PGRN* at position +5 relative to the first non-coding exon 0 (IVS0+5G>C; Table 1, where IVS indicates intervening sequence), which segregated with disease (Fig. 2). We identified the Belgian founder family based on conclusive 17q21 linkage in one family (DR8 in Fig. 2, LOD score 3.49 at D17S931 (ref. 3)) and subsequent disease haplotype sharing in seven apparently unrelated familial FTD patients (Supplementary Fig. 2b), indicative of a distant common ancestor<sup>3</sup>. Mutation analysis of *PGRN* in 103 Belgian FTD patients identified the IVS0+5G>C mutation only in the eight probands belonging to the different branches of the Belgian founder family and not in 436 control individuals. In both families, 1083 and DR8, the pathology showed

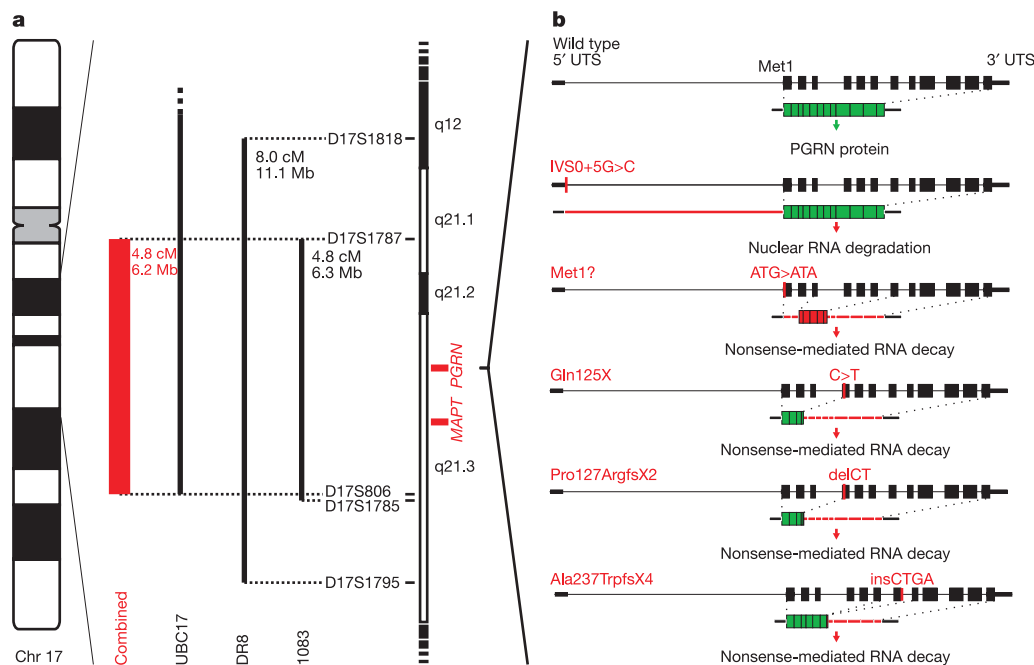
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the characteristic ubiquitin-immunoreactive neuronal cytoplasmic and nuclear inclusions in the temporal and frontal cortices<sup>1,3</sup> (Supplementary Table 1 and Supplementary Fig. 3). Rarely, inclusions also involved glia cells, but none of the inclusions was shown to have an appreciable presence for any protein from almost 30 possible proteins tested<sup>15</sup>. Also, patients of both families met the clinical criteria of FTD<sup>10,16</sup>, without associated signs of motor neuron disease<sup>1,3</sup>.

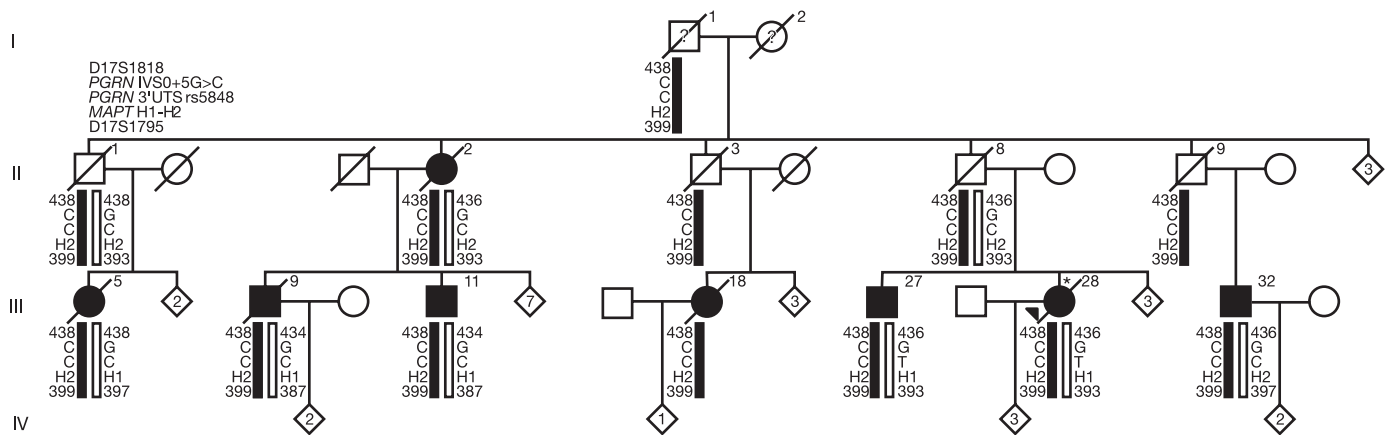
The IVS0+5G>C mutation is located in the splice donor site of the first *PGRN* intron (intron 0) following the non-coding exon 0 (Table 1 and Fig. 1); *in silico* analysis predicted a marked drop in binding efficiency of the U1 snRNP complex. Furthermore, analysis of full-length *PGRN* complementary DNA in lymphoblasts and brain of probands DR8 (III-28, Fig. 2) and DR27 (III-4, Supplementary Fig. 2b) did not identify aberrant transcripts (Fig. 3a). However, DR8 III-28 and DR27 III-4—who were heterozygous C/T for single-nucleotide polymorphism (SNP) rs5848 located in the 3′ untranslated sequence (UTS), with the C allele segregating on the disease haplotype (Fig. 2 and Supplementary Fig. 2b)—showed only the T allele when we sequenced their lymphoblast and/or brain *PGRN* cDNA (Fig. 3b). In contrast, an unaffected relative was heterozygous for rs5848 in both genomic and cDNA sequences. These data support a complete absence of mutant *PGRN* mRNA transcript, most likely due to read-through of intron 0. Nuclear retention signals remaining on the unspliced transcript will prevent it from leaving the nucleus, marking it for nuclear degradation<sup>17</sup>. Western blot analysis of lymphoblast cell protein extracts from probands DR8 III-28 and DR27 III-4 supported these data and demonstrated a reduction of *PGRN* protein levels (Fig. 3c). Together, these data demonstrate that the DR8 founder haplotype carries a *PGRN* null allele that does not produce protein. Robust *PGRN* immunoreactivity was observed in a subset of cortical neurons in patient DR8 III-28 (Supplementary Fig. 3a). However, despite using a panel of antibodies that recognize all regions of the *PGRN* protein, the neuronal inclusions were negative for *PGRN*.

In addition to the IVS0+5G>C mutation in the eight probands of the Belgian FTDU-17 founder family, we identified three other *PGRN* mutations in three familial patients of the Belgian FTD series by genomic sequencing of all 13 exons and flanking intronic regions of *PGRN* (Table 1 and Fig. 1b). In one FTD patient, with an onset age of 62 yr and a sister suffering from dementia who died at 64, we identified in exon 1 a G>A transition that destroyed the native Kozak sequence surrounding the Met1 translation initiation codon (c.3G>A). We could not verify the effect of the Met1 mutation on transcript or protein expression because we had no RNA source available for this FTD patient who died without autopsy at age 72 yr. However, supportive data were obtained for a US patient who carried a different Met1 mutation (c.2T>C) showing a substantial reduction in expression levels of the mutant transcript allele<sup>8</sup>. In the two other familial patients, different frameshift mutations predicted carboxy-terminal-truncated proteins, Pro127ArgfsX2 and Ala237TrpfsX4, resulting from a dinucleotide deletion in exon 4 and a four-nucleotide insertion affecting the intron 7 splice donor site and predicting exon 7 skipping (Table 1 and Fig. 1b). Both patients are still alive but no lymphoblasts were available for *PGRN* cDNA or protein verification. However, in a separate study it was shown that *PGRN* frameshift mutations also produce null alleles through nonsense-mediated decay of mutant mRNA transcripts, lowering *PGRN* protein levels<sup>8</sup>. Together, our *PGRN* mutation data explained 10.7% (11 out of 103) of the genetic aetiology of FTD and 25.6% (11 out of 43) of familial FTD in the Belgian patient series. In the same group, *MAPT* mutation frequencies were 2.9% (3 out of 103) overall and 7% (3 out of 43) in familial FTD, indicating that *PGRN* mutations are an approximately 3.5 times more frequent cause of FTD in Belgian patients. The contribution of *PGRN* mutations to the genetic aetiology of FTD might be higher because the methods that we used would not have detected whole-gene deletions or out-frame exonic deletions/duplications resulting in loss of functional *PGRN*, neither did we systematically examine the *PGRN* 5′ regulatory region for mutations affecting *PGRN* transcriptional activity. In the Belgian



**Figure 1** | FTDU-17 candidate region and *PGRN* mutations. **a**, Location of *PGRN* in the FTDU-17 candidate region of chromosome 17q21. The red bar represents the minimal linked region based on a centromeric recombinant in family 1083 (ref. 1) and a telomeric recombinant in family UBC17 (ref. 2). Genetic sizes are according to the Marshfield gender-averaged linkage map; physical sizes according to human reference sequence NCBI build 35.

**b**, Schematic presentation of *PGRN* mutations in Dutch FTDU-17 family 1083, Belgian FTDU-17 founder family DR8, and in unrelated Belgian FTD patients (Table 1). The predicted changes are shown for the largest *PGRN* transcript (GenBank accession number NM\_002087.2). In the transcripts, regions encoding normal protein sequence are coloured green, whereas mutant regions are red.



**Figure 2 | Segregation of the *PGRN* splice donor site mutation IVS0+5G>C in Belgian founder family DR8.** Segregation in the 17q21-linked branch DR8 is shown for microsatellite markers flanking the founder haplotype (Fig. 1), the *PGRN* IVS0+5G>C mutation, the *PGRN* 3' UTS SNP rs5848 used in cDNA analyses (Fig. 3b), and the *MAPT* H1-H2 inversion haplotype analysed in the chromosomal fluorescence *in situ* hybridization (FISH; Supplementary Fig. 1e, f). Black bars represent the disease haplotype of

patients and obligate carriers. In generation I of DR8, the disease haplotype was arbitrarily assigned to individual I-1. Numbers within each diamond are unaffected at-risk individuals that were included in the genotyping. The arrowhead identifies the proband; the asterisk indicates that a pathological diagnosis of FTDU was available for proband III-28 (Supplementary Table 2 and Supplementary Fig. 3a). Filled symbols represent patients; open symbols unaffected relatives.

founder family the 16 patients carrying the IVS0+5G>C mutation had onset ages varying between 45 and 70 yr (mean onset age  $63.4 \pm 6.8$  yr; mean age at death  $68.3 \pm 4.4$  yr). There were also four obligate carriers in generation II of family DR8 (Fig. 2) who died without symptoms of dementia: one died at a young age (II-1, 41 yr), two at ages within the onset range (II-8 at 44 yr and II-9 at 54 yr) and one at 81 yr (II-3)<sup>3</sup>. These highly variable onset ages and potential incomplete penetrance of the disease indicated that modifying factors are modulating onset age and as such contribute to a more complex genetic aetiology for FTDU-17. A first analysis of the apolipoprotein E gene (*APOE*) indicated that the *APOE* genotype has no effect on onset age (data not shown). Also of interest is that many of the FTDU-17 patients in the Belgian founder family had symptoms of non-fluent aphasia as a prominent feature of their disease (Supplementary Table 1)<sup>3</sup>.

Our mutation data for *PGRN* explained linkage of FTDU-17 in Dutch family 1083 (ref. 1) and the Belgian founder family DR8 (ref. 3). Although studies of nonsense and frameshift mutant transcripts indicated that they were probably degraded by nonsense-mediated mRNA decay (Supplementary Fig. 2c)<sup>8</sup>, it could not be fully excluded that undetected low amounts of truncated proteins exerted their pathogenic effect through a dominant-negative or gain-of-function

mechanism. However, identification of a loss-of-allele mutation in intron 0, IVS0+5G>C, provided convincing evidence that the pathogenic mechanism in FTDU-17 is indeed a loss of functional *PGRN* (haploinsufficiency). The IVS0+5G>C mutation either prevents splicing out of the first intron, intron 0, causing nuclear retention and degradation of the mutant transcript, or the mutant allele is never transcribed. Either way, the mutant allele is non-functional and the final result is a reduction in *PGRN* protein.

*PGRN* is a member of a family of cysteine-rich polypeptides with growth modulatory activity and a role in several physiological and pathological processes. In brain, *PGRN* is widely expressed in neurons and glia cells but its actual functions are not very well understood<sup>18</sup>. *PGRN* has been implicated in the development of male-specific differentiation of the hypothalamus, and it is highly expressed in glioblastomas<sup>19,20</sup>. Loss of functional *PGRN* in FTDU-17 and potentially also in other neurodegenerative brain diseases<sup>21,22</sup> supports a role for *PGRN* in neuronal survival. However, although partial deficiency of *PGRN* causes FTDU-17, overexpression of *PGRN* has been linked to the progression of many different cancers, indicating that *PGRN* dosage is important in these diseases. Nevertheless, the identification of a role for *PGRN* in neurodegeneration opens new avenues for treatment, as *PGRN* has been shown to

**Table 1 | *PGRN* mutations identified in Belgian FTD patients**

Patient/family	Pathology†	Onset age in years	Genome‡	Mutation* Predicted RNAs	Predicted protein	Location
DR8	FTDU	63.8 (n = 5)	g.96241G>C (IVS0+5G>C)	-	p.0	IVS 0
DR2	Alive	66.3 (n = 4)	g.96241G>C (IVS0+5G>C)	-	p.0	IVS 0
DR25	FTDU	69.5 (n = 2)	g.96241G>C (IVS0+5G>C)	-	p.0	IVS 0
DR26	Alive	65	g.96241G>C (IVS0+5G>C)	-	p.0	IVS 0
DR27	FTDU	58	g.96241G>C (IVS0+5G>C)	-	p.0	IVS 0
DR28	FTDU	57	g.96241G>C (IVS0+5G>C)	-	p.0	IVS 0
DR31	FTDU	66	g.96241G>C (IVS0+5G>C)	-	p.0	IVS 0
DR119	Alive	45	g.96241G>C (IVS0+5G>C)	-	p.0	IVS 0
DR118	Died without autopsy	62	g.100069G>A	c.3G>A	p.Met1?¶	EX 1
DR120	Alive	56	g.101160_101161delCT	c.380_381delCT	p.Pro127ArgfsX2	EX 4
DR91	Alive	67	g.102065_102066insCTGA	c.709_835del	p.Ala237TrpfsX4	IVS 7

\* Positions of the different *PGRN* mutations are depicted in Fig. 1. Sequencing all 13 exons of *PGRN* in 190 control individuals did not identify these or other nonsense or frameshift mutations. Sequencing of exon 0 in 246 additional control individuals showed that the IVS0+5G>C mutation was absent.

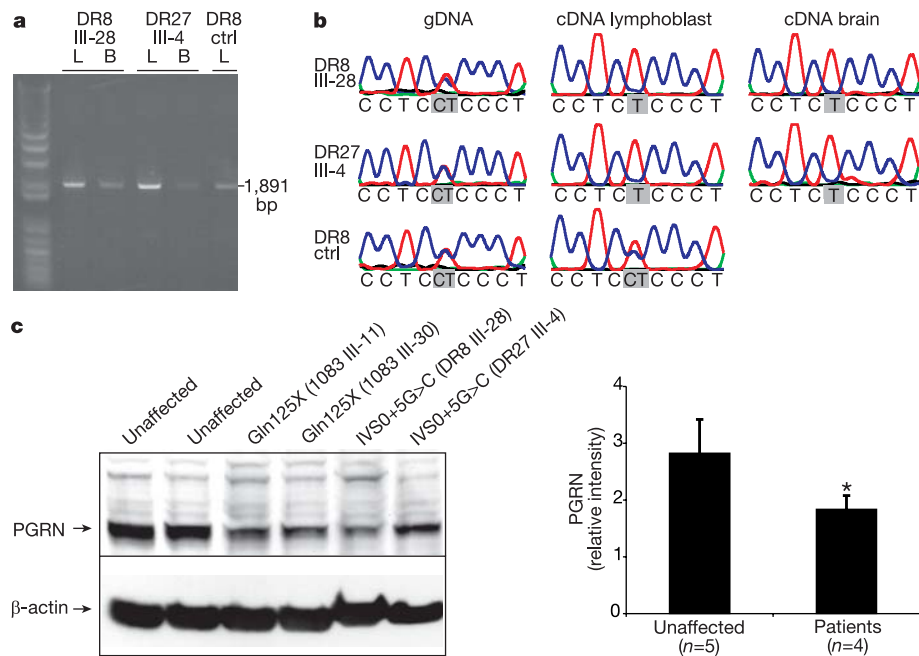
† FTDU is an autopsied brain pathology diagnosis.

‡ Numbering relative to the reverse complement of GenBank accession number AC003043 and starting at nucleotide 1.

§ Numbering according to the largest *PGRN* transcript (GenBank accession number NM\_002087.2) and starting at translation initiation codon.

|| Numbering according to the largest *PGRN* isoform (GenPept accession number NP\_002078.1).

¶ Mutation in Met1 translation initiation codon.



**Figure 3 | Transcript and protein analyses of the *PGRN* IVS0+5G>C mutation.** **a**, Whole-length *PGRN* PCR amplicons obtained from first-strand cDNA prepared from lymphoblast (L) or brain (B) of probands DR8 III-28 and DR27 III-4 (Fig. 2 and Supplementary Fig. 2b), and an unaffected relative in DR8 showing the expected transcript length of 1,891 bp. **b**, Sequence of SNP rs5848 located in the 3' UTS of *PGRN* in gDNA and cDNA prepared from lymphoblasts or brain of probands DR8 III-28 and DR27 III-4, and an unaffected relative in DR8 heterozygous for SNP rs5848. Amplicons were prepared from first-strand cDNA using PCR primers in

exon 5–6 and 3' UTS amplifying all known *PGRN* transcripts. **c**, Analysis of wild-type *PGRN* protein normalized to  $\beta$ -actin from lymphoblasts of patients DR8 III-28 (Fig. 2), DR27 III-4 (Supplementary Fig. 2b) and unaffected relatives, and of patients of Dutch family 1083 segregating the Gln125X mutation (Supplementary Fig. 2a). Normalized *PGRN* staining in lymphoblasts of IVS0+5G>C and Gln125X carriers was reduced by 30–35% compared to unaffected relatives. Arrows indicate the wild-type *PGRN* (~85 kDa) and  $\beta$ -actin. Error bars represent s.e.m.; asterisk,  $P = 0.028$  ( $t$ -test).

stimulate other growth factors such as vascular endothelial growth factor (VEGF)<sup>23</sup> associated with adult-onset progressive motor neuron degeneration in mice<sup>24</sup>.

## METHODS

**Patients.** Belgian patients had pure FTD and were diagnosed using a standard protocol and established clinical criteria<sup>9,25</sup>. In the series of 103 patients, ten patients had a definite diagnosis of FTLD, two of dementia lacking distinctive histopathology (DLDH) and one of Pick's disease. Previous mutation analyses identified a *MAPT* mutation in three patients, Gly273Arg, Ser305Ser and Arg406Trp, and one presenilin 1 (*PSEN1*) mutation, Gly183Val, in the patient with Pick's pathology<sup>26</sup>. In the whole sample, mean onset age was  $63.8 \pm 9.1$  yr (range 40–90 yr); there were 50 females and 53 males and 43 patients had a positive family history with at least one first-degree relative affected. The FTD series included eight probands sharing the same haplotype at 17q21, indicative of a common founder<sup>3</sup>. The local medical ethical committee of the University of Antwerp approved the research protocols for clinical, genetic and neuropathological studies.

***PGRN* gene sequencing.** The sequence of non-coding exon 0 and coding exons 1–12 was determined in the 103 Belgian FTD patients and 190 neurologically healthy control individuals (mean age  $52.4 \pm 13.3$  yr, range 37–85 yr). For the *PGRN* exon 0 fragment containing the IVS0+5G>C mutation, 246 additional control individuals (mean age  $67.0 \pm 12.8$  yr, range 40–92 yr) were sequenced. Total genomic DNA was prepared from peripheral blood according to standard procedures. Standard 20- $\mu$ l polymerase chain reaction (PCR) amplifications on genomic DNA were performed to amplify exons including exon–intron boundaries with primers designed using Primer 3 (ref. 27) (Supplementary Table 2). Amplification products were purified with 1 U Antarctic phosphatase (New England Biolabs) and 1 U exonuclease I (New England Biolabs) and sequenced in both directions using the BigDye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems) on an ABI3730 automated sequencer (Applied Biosystems). Sequences were analysed with the Software Package NovoSNP<sup>28</sup>.

***PGRN* mRNA and protein analyses.** Epstein–Barr virus (EBV)-transformed lymphoblasts were cultured and mRNA was isolated using the Chemagic mRNA Direct Kit (Chemagen). Frontal brain tissue from the patients was homogenized

and total RNA was extracted using the RiboPure Kit (Ambion). First-strand cDNA was synthesized starting from mRNA or total RNA with random hexamer primers using the SuperScript III First-Strand Synthesis System for RT–PCR kit (Invitrogen). PCR was performed on both lymphoblast and brain cDNA using primers amplifying the complete coding region of the *PGRN* transcript and primers amplifying part of the transcript containing exon 5–6 to 3' UTS. Primer sequences are summarized in Supplementary Table 2. The resulting PCR products were sequenced to detect aberrant transcripts and to determine the number of transcribed alleles based on the presence of SNP rs5848.

Lymphoblasts were collected by centrifugation at 250 g and lysed in homogenization buffer. Samples were sonicated, cleared at 20,000 g and protein aliquots (40  $\mu$ g) separated on a 4–12% Bis-Tris Nupage gel (Invitrogen) and were electroblotted to Hybond P polyvinylidene difluoride membrane (Amersham Biosciences). Membranes were immunoblotted with anti-*PGRN* antibodies (acroganin N-19 and S-15) and detected with secondary antibody and ECL plus chemiluminescent detection system (Amersham Biosciences) with bands quantified on Kodak Imaging Station 440 (Eastman Kodak). Quantitative data were normalized to the signal obtained for  $\beta$ -actin (clone AC-15; Sigma).

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**Supplementary Information** is linked to the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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