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Investigating the role of filamin C in Belgian patients with frontotemporal dementia linked to GRN deficiency in FTLD-TDP brains

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Abstract

TAR DNA-binding protein 43 (TDP-43) inclusions are pathological hallmarks of patients with frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS). Loss of TDP-43 in zebrafish engenders a severe muscle and vascular phenotype with a concomitant elevation of filamin C (FLNC) levels, an observation confirmed in the frontal cortex of FTLD-TDP patients. Here, we aimed to further assess the contribution of FLNC to frontotemporal dementia (FTD) etiology. We conducted a mutational screening of FLNC in a cohort of 529 unrelated Belgian FTD and FTD-ALS patients, and a control cohort of 920 unrelated and age-matched individuals. Additionally we performed an in-depth characterization of FLNC expression levels in FTD patients and a murine FTD model.

In total 68 missense variants were identified of which 19 (MAF < 1%) were patient-only. Gene burden analysis demonstrated a significant association between the presence of rare variants in FLNC and disease (P = 0.0349, RR = 1.46 [95% CI 1.03–2.07]). Furthermore, elevated FLNC expression levels, observed previously in FTLD-TDP patients, were mainly attributable to FTD patients with the progranulin (GRN) p.0(IVS1 + 5G > C) loss-of-function mutation. Increased FLNC levels were, to a lesser extent, also identified in a FLNC p.V831I variant carrier and in FTD patients with the p.R159H mutation in valosin-containing protein (VCP). The GRN-associated increase of FLNC was confirmed in the frontal cortex of aged Grn knockout mice starting at 16–18 months of age. Combined quantitative proteomic and bioinformatic analyses of the frontal cortex of FTD patients possessing elevated FLNC levels, identified multiple altered protein factors involved in accelerated aging, neurodegeneration and synaptogenesis.

Our findings further support the involvement of aberrant FLNC expression levels in FTD pathogenesis. Identification of increased FLNC levels in aged Grn mice and impaired pathways related to aging and neurodegeneration, implies a potential role for FLNC in mediating or accelerating the aging process.

Keywords: Filamin C, Genetics, Frontotemporal lobar degeneration, Granulin GRN, Haploinsufficiency, Proteomics

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Introduction

Frontotemporal dementia (FTD) is a progressive presenile dementia with degeneration of the frontal and anterior temporal lobes of the brain [1]. The clinical phenotype of FTD patients is heterogeneous and includes cognitive, behavioral and language impairments with a variable co-occurrence of amyotrophic lateral sclerosis (ALS) [2]. Formation of insoluble protein deposits, largely composed of ubiquitinated, hyperphosphorylated TAR DNA-binding protein 43 (TDP-43), defines one of the major pathological subtypes of frontotemporal lobar degeneration (FTLD-TDP) as well as ALS patients, introducing a clinical-pathological continuum of TDP-43 proteinopathies [3–5]. TDP-43 is a multifunctional RNA-binding protein involved in different RNA-related processes including transcription and splicing regulation [6, 7].

Over the past ten years, considerable progress has been made in unraveling the genetic basis of the FTD-ALS continuum. Today, more than 10 genes are linked to FTD-ALS disorders at variable frequencies [8, 9]. A major part of the mutation spectrum described for FTLD-TDP patients is covered by loss-of-function mutations in progranulin (GRN) that codes for a multifunctional growth factor with neurotrophic properties in the central nervous system (CNS) [10] and a hexanucleotide repeat expansion mutation in C9orf72 [11]. Less frequently, mutations in TANK-binding kinase 1 (TBK1), TAR DNA-binding protein 43 (TARDBP), valosin-containing protein (VCP), sequestosome 1 (SQSTM1) and ubiquitin 2 (UBQLN2) can lead to both familial and sporadic forms of FTD and ALS [8].

The high prevalence of TDP-43 pathology in FTD and ALS patients suggests that pathways disrupting TDP-43 integrity might be shared between patients with different clinical, pathological and genetic etiologies. In line with the molecular genetic findings, multiple pathways related to RNA-processing, protein aggregation and proteostasis are likely contributing to the multifactorial nature of FTD-ALS disorders [9]. Recently, an unexpected requirement of TDP-43 for muscle maintenance, vessel patterning and perfusion was found upon deletion of the TDP-43 homologues in zebrafish [12]. Several muscle-specific proteins were altered on proteomic analysis of this zebrafish model, underscoring the role of TDP-43 in muscle integrity. The most upregulated protein in TDP-43 knockout zebrafish and in the frontal cortex of FTLD-TDP patients, however, was filamin C (FLNC) [12]. Filamins are evolutionary conserved, multidomain actin-binding proteins involved in the organization of the cytoskeleton and plasma membrane stabilization. Besides cross-linking F-actin filaments, filamins scaffold also a wide range of signaling functions through interactions with more than 90 binding partners including intracellular signaling molecules, transmembrane receptors, and ion channels [13, 14]. The vertebrate filamin family consists of filamin A (FLNA), filamin B (FLNB) and filamin C (FLNC) which share ≈70 % homology over the entire protein sequence. Structurally, FLNC is a large homodimer of approximately 290-kDa subunits that consists of an N-terminal actin-binding domain (ABD), composed of two calponin homology domains (CH1 and CH2), followed by 24 Immunoglobulin (Ig)-like repeats [15–17]. Two splice variants have been described for FLNC which differ from each other in the presence of exon 31 encoding the hinge region between Ig-like domains 15 and 16 (Fig. 1a). FLNC expression is largely restricted to cardiac and skeletal muscles, although other non-muscular cells, including neuronal cells, express lower but detectable levels of FLNC [18–20]. In line with the expression pattern of FLNC, mutations identified in FLNC have been shown to be the underlying cause of different progressive muscular dystrophies, including distal and myofibrillar myopathy (DM and MFM, respectively), and hypertrophic cardiomyopathy (HCM) [21–23].

In the present study, we showed that rare variants (MAF < 1 %) identified in the coding region of FLNC are significantly associated with a higher risk of developing FTD. We also performed an in-depth characterization of FLNC transcript and protein levels in FTD patients with different genetic etiologies and found that elevated FLNC levels observed in the frontal cortex of FTLD-TDP patients are mainly associated with the GRN p.0(IVS1 + 5G > C) loss-of-function mutation. Validation of this novel association was obtained using a constitutive Grn knockout (Grn<sup>−/−</sup>) mouse model [24]. To appreciate the pathophysiological relevance of increased FLNC levels, we analyzed the frontal cortex of FTD patients with different genetic etiologies for significantly altered gene-specific pathways using combined proteomic and bioinformatic approaches.

Materials and methods

Belgian FTD and control cohorts

The FTD patients and control cohorts were ascertained in the framework of the Belgian Neurology (BELNEU) consortium, a multicenter collaboration of dementia expertise centers located in Belgium, covering Flanders, Wallonia and Brussels [25]. Molecular genetic screening of FLNC was performed on 529 FTD patients with an average age at onset of 63.8 ± 10.3 years (45.5 % women). Index patients were evaluated and diagnosed according to standard protocols including detailed neurological examination, neuropsychological testing, neuroimaging, biochemical analyses, and electroencephalography (EEG) [26]. Clinical diagnosis of FTD was reached according to the international Lund and Manchester group criteria for FTD [1] and the international consensus criteria by Rascovsky et al. [27] for behavioral variant FTD (bvFTD).
Post mortem neuropathological diagnosis of FTLD was available in 33 patients (6.2 %), including 3 FTD-ALS patients, 1 patient with mixed dementia and 25 FTD patients. A positive family history, i.e. at least one first degree relative with a FTD-ALS spectrum disease, was recorded for 30.4 % of the FTD cohort, while 33.8 % had a sporadic form of the disease. Familial history was unknown for 35.2 % of the patients included in this cohort. FTD patients included in this cohort had previously been screened for mutations in known FTD and ALS genes including MAPT, GRN, C9orf72, VCP, CHMP2B, TARDBP, FUS, and SQSTM1 [9], which revealed 5 MAPT mutations (0.9 %), 28 GRN mutations (5.3 %), 35 C9orf72 repeat expansion mutations (6.6 %), 6 VCP mutations (1.1 %), 1 CHMP2B mutation (0.2 %), 2 TARDBP mutations (0.4 %), 3 FUS mutations (0.6 %), and 10 SQSTM1 mutations (1.9 %).

The control cohort consisted of 920 unrelated and age-matched individuals, primarily community-dwelling volunteers or spouses of patients, with an average age at inclusion of 68.0 ± 13.2 years (60.8 % women). Subjective memory complaints, neurologic or psychiatric antecedents and a familial history of neurodegeneration were ruled out by means of an interview. Cognitive screening was performed using the Mini-Mental State Examination (MMSE; cut-off score ≥26) [28] or the Montreal Cognitive Assessment (MoCA) test (cut-off score ≥25) [29]. The spouses of patients were examined at the Memory Clinic at Middelheim or Hoge Beuken hospitals of the Hospital Network Antwerp, Belgium, and at the Memory Clinic of the hospital Gasthuisberg of the University Hospitals of Leuven, Belgium.

All research participants or their legal guardian provided written informed consent for participation in genetic and clinical studies. Clinical study protocols and informed consent forms for patient ascertainment were approved by the local medical ethics committees of the collaborating neurological centers in Belgium. Genetic study protocols and informed consent forms were approved by the ethics committees of the University Hospital of Antwerp and the University of Antwerp, Belgium.

**FLNC sequencing**

The coding sequence of FLNC, including flanking intron-exon boundaries, was screened in the Belgian FTD and control cohorts using a massive parallel sequencing approach and a customized Multiplex Amplification of Specific Targets for Resequencing (MASTR) assay (Multiplicom; www.multiplicom.com). Exons 46-48 of FLNC were not covered in the MASTR assay due to more than 98 % sequence identity with the FLNC pseudogene (pseFLNC), located 53.6 kb downstream of the functional FLNC gene [30]. Primers for multiplex PCR were designed using the mPCR primer design tool (Multiplicom) [31]. Specific target regions were amplified using multiplex PCR, and equimolar pooled amplicons were purified using Agencourt AMPureXP beads (Beckman Coulter). Individual barcodes (Illumina Nextera XT) were incorporated.
in a universal PCR step prior to sample pooling. Bridge amplification and sequencing of barcoded samples was performed using an Illumina MiSeq platform, with the Illumina v2 reagent kit.

Alignment and mapping of the reads against the human genome reference sequence hg19 were performed with the Burrows-Wheeler Aligner [32]. Variant calling and annotation were performed using GATK (version 2.2) in combination with GenomeComb software [33, 34]. Identified variants were independently validated using direct Sanger sequencing on genomic DNA. FLNC codon numbering was based on GenBank Accession Number NM_001458.4 and amino acid substitutions are numbered according to GenPept Accession Number NP_001449.3 (http://www.ncbi.nlm.nih.gov/).

The potential pathogenicity of patient-only coding variants were predicted using SIFT (http://sift.jcvi.org/) [35], PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) [36] and SNAP2 (https://www.rostlab.org/services/SNAP/) [37] prediction software tools.

Human and murine brain tissue

Human frontal cortex (BA10) was snap frozen in liquid nitrogen upon autopsy and stored at −80 °C for subsequent mRNA and protein analyses. FLNC expression levels were analyzed in brain tissue from 23 dementia patients including 1 FLNC p.V831I carrier, 7 carriers of a GRN loss-of-function mutation [10], 3 carriers of the VCP p.R159H mutation [38], 3 carriers of a C9orf72 repeat expansion [11], 4 patients with FTLD-TDP brain pathology but no mutation in any of the known causal FTD genes, and 1 patient with a mixed Alzheimer’s disease (AD) and FTLD-TDP brain pathology. In addition, we analyzed brain expression levels in 2 patients with AD, one patient with dementia with Lewy Bodies (LBD) and one Down syndrome patient, as well as in twelve age-matched control persons. A detailed overview of the patients with autopsy and neuropathological examination is provided in Table 1. A partial overlap in patients is present with samples included in the FLNC expression studies of Schmid et al. 2012 [26]; i.e. control individuals (n = 12), 5 FTD

| DR number | Gender | Age at onset (years) | Age at death (years) | Family History | Clinical diagnosis | Mutation | Pathological diagnosis |
|-----------|--------|----------------------|----------------------|---------------|--------------------|----------|------------------------|
| DR287.1   | F      | 65                   | 71                   | F             | FTD                | GRN - p.A89Vfs*41 | FTLD-TDP type A         |
| DR2.3     | F      | 63                   | 71                   | F             | FTD                | GRN - p.0(IVS1 + 5G > C) | FTLD-TDP type A         |
| DR8.1     | F      | 62                   | 68                   | F             | FTD                | GRN - p.0(IVS1 + 5G > C) | FTLD-TDP type A         |
| DR25.5    | M      | 70                   | 73                   | F             | FTD                | GRN - p.0(IVS1 + 5G > C) | FTLD-TDP type A         |
| DR27.1    | F      | 58                   | 63                   | F             | FTD                | GRN - p.0(IVS1 + 5G > C) | FTLD-TDP               |
| DR28.1    | M      | 56                   | 62                   | F             | FTD                | GRN - p.0(IVS1 + 5G > C) | FTLD-TDP type A         |
| DR25.1    | F      | 69                   | 75                   | F             | FTD                | GRN - p.0(IVS1 + 5G > C) | FTLD-TDP type A         |
| DR40.1    | F      | 44                   | 56                   | F             | FTD                | VCP - p.R159H   | FTLD-TDP type D         |
| DR40.7    | M      | 49                   | 57                   | F             | FTD                | VCP - p.R159H   | FTLD-TDP               |
| DR7.4     | M      | 63                   | 68                   | F             | FTD                | VCP - p.R159H   | FTLD-U                 |
| ADR1      | M      | 52                   | 62                   | F             | FTD                | FLNC - p.V831I | Pick's disease           |
| DR439.1   | M      | 54                   | 69                   | F             | FTD                | C9orf72 - G4C2 expansion | FTLD-TDP type B          |
| DR29.1    | F      | 50                   | 55                   | F             | FTD                | C9orf72 - G4C2 expansion | FTLD-UPS               |
| DR14.1    | M      | 56                   | 60                   | F             | FTD                | C9orf72 - G4C2 expansion | FTLD-TDP type B          |
| DR386.1   | M      | 72                   | 83                   | S             | MXD                | unknown         | FTLD-TDP and AD         |
| DR189.1   | M      | 47                   | 50                   | F             | FTD                | unknown         | FTLD-TDP type B         |
| DR87.1    | F      | 79                   | 88                   | S             | FTD                | unknown         | FTLD-TDP               |
| DR864.1   | M      | 59                   | 62                   | S             | FTD - ALS | unknown         | FTLD-TDP type B         |
| DR102.1   | F      | 72                   | 79                   | S             | FTD                | unknown         | FTLD-TDP               |
| DR246.1   | M      | 62                   | 72                   | F             | DLB                | unknown         | LBD                     |
| DR865.1   | M      | 75                   | 86                   | U             | AD                 | unknown         | AD (III-IV Braak)        |
| DR39.1    | F      | 61                   | 75                   | F             | AD                 | APP - c.369C>G  | AD-CAA                  |
| D51.1     | -      | -                    | -                    | S             | Down Syndrome     | 47XX,+21 or 47XY,+21 | AD-CAA                  |

AD Alzheimer’s disease, APP amyloid precursor protein, CAA cerebral amyloid angiopathy, DBL dementia with Lewy bodies, UPS ubiquitin proteasome, system, DLBD diffuse Lewy body disease, F familial, S sporadic, U unknown, MXD mixed dementia, VCP valosin-containing protein

Samples labeled with * were previously analyzed in FLNC expression studies published by Schmid et al. 2012 [26]
patients (2 GRN p.0[IVS1 + 5G > C] carriers, 2 C9orf72 repeat expansion carriers, 1 FTLD-TDP patient without known genetic cause, 2 AD patients and 1 LBD patient (FTD patient samples used in both studies are indicated with a "symbol a" in Table 1).

Human FLNC expression profiles obtained from FTD patients were validated using a progranulin knockout (Grn−/−) mouse model for FTD [24]. Mouse whole brain tissue was harvested at different ages, weighed and cut midsagittal according to a standard protocol [39]. Right hemispheres were snap frozen in liquid nitrogen and stored at −80 °C for subsequent mRNA and protein analysis. Left hemispheres were fixed in 2 % paraformaldehyde (PFA) for 18–20 h and prepared for paraffin embedding.

**RNA extraction for semi-quantitative real-time PCR**

Semi-quantitative real-time PCR (qRT-PCR) was performed to quantify expression levels of FLNC in the human frontal cortex and mouse right hemisphere. Total RNA was isolated from crunched frozen brain tissue using the RiboPure™ RNA Purification kit followed by a DNase treatment with the TURBO™ DNase kit (both Ambion, Life Technologies). The RNA integrity values (RIN) of patient and control samples ranged between 5 and 8.4. First-strand cDNA was synthesized utilizing the SuperScript® III First-Strand Synthesis System (Life Technologies) with random hexamer primers. qRT-PCR reactions were performed using the Fast SYBR® Green mix and run on an ABI ViiA™ 7 Real-Time PCR System (both Applied Biosystems, Life Technologies). Each sample was measured in duplicate and at least two independent experiments were performed. Quantification of mRNA levels was achieved with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-Actin (ACTB) as internal control genes. Normalization of the housekeeping genes was performed using geometric averaging of the expression levels, as described by Vandesompele et al. [40]. Primer pairs were designed using Primer Express software (Life Technologies). Primer sequences are available upon request.

**Immunoblotting**

Protein extractions of human and murine brains were prepared in modified radioimmuno-precipitation (RIPA) buffer [150 mM NaCl, 0.5 % sodium deoxycholate, 1 % NP-40, 50 mM Tris–HCl; pH 8.0] supplemented with 1 % sodium dodecyl sulfate (SDS), as described previously [41]. Buffers were supplemented with protease and phosphatase inhibitors (1x Complete Protease inhibitor cocktail and 1x PhosSTOP, both Roche). Protein concentrations were determined with a Pierce™ bichinchoninic acid (BCA) colorimetric protein assay kit (Thermo Scientific). Equal amounts of protein were loaded and separated on 3–8 % NuPAGE® Tris-Acetate or 4–12 % NuPAGE® Bis-Tris gels (Life Technologies) and electrotransferred onto a polyvinylidene difluoride membrane (PVDF, Hybond P; Amersham Biosciences). After protein transfer, membranes were blocked in 5 % skimmed milk in phosphate-buffered saline (PBS) containing 0.1 % Tween® 20 (Merck). Membranes were probed with a range of primary antibodies listed in Additional file 1: Table S1. Immunodetection was performed with host-specific secondary antibodies conjugated with horseradish peroxidase (HRP) and the ECL-plus chemiluminescent detection system (Thermo Scientific). Western blot results were visualized using the ImageQuant™ LAS4000 digital imaging system and quantified with ImageQuant™ TL software (GE Healthcare Life Sciences). Quantitative data were normalized to GAPDH expression levels, as described earlier [39].

**Cell culture**

Human cervical carcinoma cells (HeLa; CCL-2; ATCC) were grown in Minimum Essential Medium Eagle (MEM, Life Technologies) supplemented with 10 % fetal calf serum (FCS, Life Technologies) and 500 U/500 µg penicillin/streptavidin. Human neuroblastoma (KELLY) cells (Sigma) were grown in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies) supplemented with 10 % FCS, 2 mM L-Glutamine and 500 U/500 µg penicillin/streptavidin. Cells were propagated in a humidified incubator at 37 °C and 5 % CO₂.

**RNA interference**

GRN expression was silenced in HeLa and KELLY cells using three independent gene-specific siRNAs (Ambion, Life Technologies). A non-targeting control siRNA with scrambled sequences (Qiagen) and a negative control (only transfection reagent) were included for each siRNA experiment (sequences are listed in Additional file 1: Table S2). Cells were transiently transfected with 20 nM of gene-specific or scrambled siRNA using siLentFect™ transfection reagent (Bio-Rad) according to the manufacturer's recommendations. Cellular RNA and protein extractions were prepared 48 h post transfection according to standard protocols [41]. Briefly, cells were washed twice in ice-cold PBS and lysed in RIPA 1 % SDS buffer supplemented with protease and phosphatase inhibitors (1x Complete Protease inhibitor cocktail and 1x PhosSTOP, both Roche). Lysates were sonicated on ice, clarified by centrifugation at 14,000 rpm and supernatant was used for subsequent immunoblotting experiments.

**Sample preparation for iTRAQ labeling and mass spectrometry analysis**

Human frontal cortex of 5 patients, including 2 FTD carriers of GRN p.0[IVS1 + 5G > C] (DR8.1 and DR2.3), 2 FTD carriers of VCP p.R159H (DR40.1 and DR40.7), 1
p.V831I carrier (ADR1) (Table 1), and of 3 control individuals, were extracted using the Qproteome Cell Compartment kit (Qiagen). Proteins extracted from the cytoplasmic compartment using Qproteome were used for subsequent mass spectrometry analyses. Following acetone precipitation of the cytoplasmic protein extracts, protein lysate concentrations were measured using the RC DCT™ protein assay (Bio-Rad Laboratories Inc.). A total protein concentration of 100 μg from each sample was reduced with tris(2-carboxyethyl)phosphine (TCEP) and cysteine residues were blocked using the cysteine-alkylation reagent methyl methanethiosulfonate (MMTS). Next, samples were digested with sequencing-grade trypsin (Promega) and peptides were used for isobaric iTRAQ-8plex (isobaric mass-tag labeling for relative and absolute quantification; iTRAQ [42]) mass tag labeling, i.e. 113, 114, 115, 116, 117, 118, 119 or 121 labels, according to the manufacturer’s protocol (Applied Biosystems). Controls and patients samples were labeled for 1 h at room temperature with isobaric reagents as follows: 3 control individuals (labels 113-115); 2 GRN p.0(IVS1 + 5G > C) carriers (labels 117-118), 2 VCP p.R159H carriers (labels 119-121) and a FLNC p.V831I carrier (label 116). Following labeling, the tagged peptides were pooled in equal volume ratios into one sample mix.

Labeled peptides were separated and resolved in two dimensions, first by strong cation exchange (SCX) chromatography followed by nano-reversed phase (RP, C18) chromatography on a 2D nano liquid chromatography (LC) system (Ultimate 3000 RSL, Dionex, Thermo Scientific). The nano-LC system was connected online to a Thermo Fisher Q Exactive™ Plus Orbitrap Mass Spectrometer (MS) system (Thermo Scientific).

Data-dependent acquisition (DDA) was obtained with a full MS scan and a subsequent data dependent MS2 (ddMS2) scan. The full MS scan had a resolving power of 70,000 and a scan range from 350 to 1500 m/z. The top 10 precursor ions were selected for ddMS2 higher-energy collisional dissociation (HCD) fragmentation with a resolution of 17,500.

Data analysis, including identification and quantification of labeled peptides, was performed using the Proteome Discoverer software (v2.0, Thermo Scientific). Protein identification was performed using the most recently updated human UniProt/SwissProt database. Raw peptide identification was done using workflow settings for the Sequest HT search engine including a 10 ppm precursor mass tolerance, 0.02 Da fragment mass tolerance, and tolerance of maximum two missed trypsin cleavage sites. Only peptide spectrum matches (PSM) that contained all eight reporter ion channels were considered for quantification and reporter intensities were exported for further analysis.

Bioinformatic analyses of mass spectrometry data
Proteomic datasets generated from the Proteome Discoverer v2.0 software were analyzed using a multidimensional bioinformatic approach. The primary ratiometric iTRAQ data was processed and normalized by performing a log2 transformation on the signal ratios retrieved from the quantitative MS. Proteins demonstrating significant deviation limits outside plus or minus 2 standard deviations (SD; 95 % confidence limits) from the mean protein expression levels (based on normalized background analysis) were used for further analysis.

To facilitate the specific separation of complex datasets, and more specifically the significantly-regulated proteins, originating from either the FLNC, GRN or VCP mutation carriers (compared to control individuals), we employed the novel Venn diagram platform, VennPlex [43]. Our primary analyses were focused on significantly-regulated protein subsets containing proteins common to FLNC, GRN, and VCP carriers (FTD-common), but also protein subsets specific and unique to FLNC, GRN or VCP carriers. Classical functional annotation of these protein subsets was performed with Gene Ontology annotation (GO; http://geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes pathway analysis (KEGG; http://www.genome.jp/kegg/) using the NIH DAVID Bioinformatics Resources 6.7 suite [44]. For both GO term annotation (biological process only) and KEGG pathway analyses we employed a cut-off of at least two proteins needing to be present to fully populate a particular GO term group or KEGG pathway with a probability of enrichment value of P ≤ 0.05. A hybrid score was employed to generate a single index for a specifically enriched GO term group or KEGG pathway. Hybrid scores are generated by the multiplication of the negative log10 of enrichment probability with the enrichment factor and the number of proteins populating the specific GO term group or KEGG pathway.

Biomedical natural language processing (NLP) text analysis was performed on the common and unique significantly-regulated protein datasets using Textrous! [45, 46]. Textrous! utilizes NLP techniques, including latent semantic indexing (LSI), sentence splitting, word tokenization, parts-of-speech tagging, and noun-phrase chunking, to mine the MEDLINE-database (NCBI, http://www.ncbi.nlm.nih.gov/), PubMed Central articles (NCBI, http://www.ncbi.nlm.nih.gov/), the Online Mendelian Inheritance in Man catalog (OMIM®, http://www.omim.org/), and the Mammalian Phenotype Browser obtained from The Jackson Laboratory (http://www.informatics.jax.org/searches/MP_form.shtml). Textrous! has the ability to generate output data even with very small input datasets for the selection, ranking, clustering, and visualization of English words obtained from the input user data. Significant data word clouds were created from Textrous!-based semantic noun and
noun-phrase outputs using the web-based application Wordle (http://www.wordle.net/) [45]. The text size of the word clouds is directly proportional to the input word frequency. Analyses of word frequencies from Textrous! noun-phrase outputs were made using WriteWords (http://www.writewords.org.uk/word_count.asp).

LSI analysis was performed using GenelIndexer (Computable Genomix), as described previously [47, 48]. In brief, GenelIndexer correlates the strength of association, using LSI, between specific proteins in a dataset with user-defined interrogation text terms. GenelIndexer employs a comprehensive human or murine scientific text database of $\geq 2 \times 10^6$ scientific abstracts to perform this text-protein correlation analysis. The possible LSI cosine similarity correlation scores for a gene to be associated with an input interrogation term range from 0 to 1, with the stronger correlation scores approaching 1. A correlation score of $\geq 0.1$ indicates at least an implicit correlation, between the specific gene and the user-defined input interrogation term.

**Statistical analyses**

We determined rare, low frequency and frequent variants as genetic variants with a minor allele frequency (MAF) below 1 %; between 1-5 % and above 5 %, respectively. Rare variant gene burden analysis was performed by collapsing rare alleles and comparing the overall frequency of rare variant alleles between patients and control groups using chi-squared ($\chi^2$) statistics. For two controls, more than one FLNC variant was identified in the CDS and these variants were counted as one mutated allele as no relatives were available for phase determination of the variants. The odds ratio (OR) and 95 % confidence intervals (CI) were calculated as well. A two-sided p-value $P < 0.05$ was considered as significant.

Expression studies were performed in duplicate and repeated at least two times with results reported as mean ± standard deviation (SD). $P$ values for description of statistical significance of differences were calculated by Mann–Whitney U testing using the GraphPad Prism 5 Software. Values were considered to be significant if $*P < 0.05$, $**P < 0.01$ or $***P < 0.001$.

**Results**

**Identification of rare FLNC variants in Belgian FTD patients**

We sequenced the coding sequence (CDS) of FLNC in the Belgian FTD ($n = 529$) and control ($n = 920$) cohorts and identified a total of 68 different genetic variants that affected the coding sequence of FLNC. We observed 19 missense variants (MAF <1 %) in 21 patients, which were absent from control individuals (Fig. 1b, Table 2). The clinical diagnosis of the FLNC carriers was predominantly FTD, except for the 2 patients with p.D710N and p.T2025I, who were diagnosed with FTD-ALS and corticobasal syndrome (CBS) (Table 2). Eight carriers had a positive family history of disease, but their families lacked information preventing co-segregation analysis with disease. The patient-only variants were found across the actin binding domain and different Immunoglobulin (lg)-like domains of FLNC without indications of clustering in specific functional domains (Fig. 1b, Table 2). Comparative genomics analysis indicated that the majority of FLNC variants predicted substitution of evolutionary conserved amino acid residues in FLNC (Additional file 2: Figure S1). For 5 FLNC variant carriers with FTD, another genetic causal or risk variant had previously been identified (Table 2), 2 with a C9orf72 repeat expansion, 2 with a GRN loss-of-function mutation and 1 with the TREM2 p.R47H risk allele (Table 2).

In addition to patient-only variants, we identified another 15 missense FLNC variants that were present in both FTD patients and control individuals. Except for p.R1241C and p.R1567Q (MAF >1 % and >5 %), the other 13 were rare (MAF <1 %) (Additional file 1: Table S3a and b). We also identified 34 missense variants present only in control individuals (MAF <1 %) (Additional file 1: Table S4). Three different prediction software programs, i.e. SIFT, Polyphen-2 and SNAP, were applied to estimate the predicted pathogenicity of variants identified in FTD patients and/or controls. However, the vast majority of FLNC variants found in patients and/or controls were predicted to have non-neutral effects (Additional file 1: Table S5a-c).

Two control individuals were carrying 2 and 3 different missense variants in FLNC. Interestingly, the p.A2430V variant found in one control individual was previously described in patients with hypertrophic cardiomyopathy (HCM) [21].

**Rare variant gene burden analysis in the Belgian FTD cohort**

We performed a gene burden analysis collapsing all rare variants with an MAF <1 % across the whole protein and comparing the overall frequency of rare variant alleles between patients and controls using $\chi^2$ statistics. Considering all rare variants observed in patients, we obtained an overall cumulative frequency of 11.3 % (60/529) compared to 7.9 % in control individuals (73/920). The increased frequency in patients was significant with an odds ratio of 1.46 (chi-squared ($\chi^2$) test, $P = 0.0349$; 95 % confidence interval (CI) = 1.03–2.07).

**Increase of FLNC in FTLD-TDP patients is strongly linked to GRN haploinsufficiency**

In the initial report, published by Schmid et al. [12], the expression levels of both the short and long isoforms of human FLNC were found to be elevated in the frontal cortex of FTLD-TDP patients, compared to neurologically healthy control individuals and AD patients [12]. We
analyzed FLNC expression levels in the frontal cortex (BA10) of FTLD-TDP patients carrying a pathological mutation in VCP, GRN or C9orf72 (Table 1), and of FTLD-TDP patients without a known genetic cause (nonmutation FTLD-TDP patients). Compared to transcript levels measured in the frontal cortex of control individuals and of AD or DLB patients, both the short and long isoform of FLNC were elevated up to 8.9 and 7.2 times in GRN p.0(IVS1 + 5G > C) carriers (Fig. 2a). The long isoform of FLNC was also increased in non-mutation FTLD-TDP patients (Fig. 2a). No altered expression patterns were observed in patients with the VCP p.R159H or C9orf72 repeat expansion mutation.

Western blot analysis, using an antibody raised against the carboxyl terminus of human FLNC confirmed the elevated expression of FLNC in GRN p.0(IVS1 + 5G > C) carriers (Fig. 2a). The long isoform of FLNC was also increased in non-mutation FTLD-TDP patients (Fig. 2a). No altered expression patterns were observed in patients with the VCP p.R159H or C9orf72 repeat expansion mutation.

Table 2 Rare FLNC missense variations identified in Belgian FTD patients

| Patient | Variant | Functional domain | Gender | Clinical diagnosis | Sub-diagnosis | Family History | Age at onset (years) | dbSNP | Mutation in known gene |
|---------|---------|-------------------|--------|-------------------|---------------|----------------|---------------------|-------|------------------------|
| DR554.1 | p.R81C  | CH1               | F      | FTD               | bvFTD         | F              | 56                  | -     | GRN p.W304              |
| DR1221.1| p.E309K | Ig 1              | M      | FTD               | PNFA          | S              | 45                  | -     |                        |
| DR1222.1| p.E323V | Ig 1              | M      | FTD               | bvFTD         | S              | 71                  | -     |                        |
| DR659.1 | p.K524R | Ig 3              | M      | FTD               | bvFTD         | F              | 38                  | -     | C9orf72 G>C exp.       |
| DR654.1 | p.D710N | Ig 5              | F      | FTD-ALS           | FTD - ALS     | F              | 69                  | rs370035829 C9orf72 G>C exp. |
| DR1223.1| p.A806T | Ig 6              | F      | FTD               | PSP           | F              | 63                  | -     |                        |
| AD1     | p.V831I | Ig 6              | M      | FTD               | bvFTD         | F              | 52                  | -     |                        |
| DR1224.1| p.V1047L| Ig 8              | F      | FTD               | -             | S              | 57                  | -     |                        |
| AD1485.1| p.P1163R| Ig 10             | M      | FTD               | bvFTD         | F              | 76                  | -     |                        |
| AD1430.1| p.V1335M| Ig 11             | M      | FTD               | bvFTD         | S              | 47                  | rs368220468 |                        |
| DR1225.1| p.L1364F| Ig 12             | F      | FTD               | PSP           | U              | 83                  | -     |                        |
| AD1788.1| p.R1370Q| Ig 12             | M      | FTD               | -             | S              | 68                  | -     |                        |
| DR867.1 | p.A1551T| Ig 14             | M      | FTD               | bvFTD         | F              | 77                  | -     |                        |
| DR559.1 | p.E1571K| Ig 14             | F      | FTD               | bvFTD         | F              | 60                  | -     | TREM2 p.R47H            |
| DR1226.1| p.E1571K| Ig 14             | M      | FTD               | SD            | S              | 75                  | -     |                        |
| DR355.1 | p.R1758W| Linker Ig 15–16   | F      | FTD               | bvFTD         | S              | 75                  | rs369187211 |                        |
| DR825.1 | p.V2014A| Ig 18             | F      | FTD               | PNFA          | S              | 66                  | -     |                        |
| AD1462.1| p.T2025I| Ig 18             | F      | FTD               | PPA + EPS     | S              | 62                  | -     | GRN p.0(IVS1 + 5G > C) |
| DR1227.1| p.T2025I| Ig 18             | F      | CBS               | CBS           | U              | 70                  | -     |                        |
| DR868.1 | p.R2318Q| Ig 21             | M      | FTD               | PNFA          | S              | 71                  | -     |                        |
| AD1321.1| p.S2461N| Ig 22             | F      | FTD               | -             | U              | 63                  | -     |                        |

bvFTD behavioral variant frontotemporal dementia, CBD corticobasal degeneration, CBS corticobasal syndrome, dbSNP Single Nucleotide Polymorphism database, EPS extrapyramidal syndromes, exp. expansion, F familial, GRN progranulin, PNFA progressive nonfluent aphasia, PPA primary progressive aphasia, PSP progressive supranuclear palsy, S sporadic, TREM2 Triggering receptor expressed on myeloid cells 2, U family history undocumented

The FLNC low frequency variant p.R1241C (Additional file 1: Table S3b). Interestingly, analysis of the FLNC p.V831I carrier showed an increase in FLNC levels compared to control individuals (Fig. 2b). Absence of elevated FLNC levels in brains of AD patients was in line with the transcript data and the previous data published by Schmid et al. [12] (Fig. 2a and Additional file 2: Figure S2a). As the strongest alterations in FLNC expression were observed in FTD patients with GRN haploinsufficiency, we verified whether reduced GRN levels in vitro could confirm the FLNC increase seen in patients. No significant changes, however, could be observed in FLNC expression levels upon GRN knockdown in both HeLa or KELLY cells (Additional file 2: Figure S3a-c).

Besides its anchoring and crosslinking functions, FLNC is known to scaffold a wide range of signaling pathways through interactions with signal transduction molecules, receptors and ion channels. FLNC is phosphorylated by protein kinase B-α at serine 2213 (pSer2213) and harbors also a potential cAMP-dependent protein kinase A (PKA) phosphorylation site at serine 2113 (pSer2113) [49]. Western blot analysis of the FLNC phosphorylation status at these reported residues demonstrated a considerable
increase in pSer\textsuperscript{2113} levels in GRN loss-of-function mutation carriers. In contrast to total FLNC levels, VCP mutation carriers also showed a strong increased phosphorylation of FLNC at position pSer\textsuperscript{2113}. Surprisingly, the FLNC p.V831I showed a lower degree of pSer\textsuperscript{2113} levels compared to the low frequency variant (p.R1241C) carrier (Fig. 2b). Upregulation of phosphorylation of FLNC pSer\textsuperscript{2213} could only be detected in GRN p.0(IVS1 + 5G > C) carriers and was not present in controls or other FTD-related mutation carriers (Fig. 2b).

**Elevated Flnc expression levels are confirmed in a progranulin knockout mouse model**

We aimed at validating the altered transcript profile present in FTLD-TDP patients with GRN haploinsufficiency in progranulin (Grn) knockout mice [24]. We analyzed expression levels of both long and short isoforms of mouse Flnc in heterozygous Grn\textsuperscript{+/−} and homozygous Grn\textsuperscript{−/−} transgenic mice compared to wild-type (Wt) animals at 3 months, 9 months, 16–18 months and 24 months of age. Consistent with the data obtained for FTLD-TDP patients with GRN haploinsufficiency, transcript levels of both Flnc isoforms increased significantly in an age-dependent manner in Grn\textsuperscript{−/−} brains starting from an age of 16–18 months and increasing to a fivefold at 24 months of age (P < 0.0001) (Fig. 3a). No changes in Flnc isoform expression were observed between heterozygous Grn\textsuperscript{+/−} and Wt mice up to an age of 24 months despite a small age-related tendency to increase in relative values. Utilizing a mouse Flnc-specific antibody, Flnc protein levels
were found to progressively increase with age and the strongest increase was detected at an age of 21 months in Grn<sup>−/−</sup> mice which is comparable to the transcript data (Fig. 3b).

Further in line with the human data is that FlnC levels remained unchanged in two mouse models of AD, including Tg2576 mice overexpressing the Swedish APP variant p.KM670/671NL (Taconic Farms Inc.) or T8B7 mice expressing the PSEN1 p.G384A mutation (Additional file 2: Figure S2b) [50]. Due to the high sequence homology between FlnC and other members of the filamin family, we also investigated the expression levels of filamin A (Flna, ≈72 % homology) and filamin B (Flnb, ≈70 % homology) in our Grn<sup>−/−</sup> mouse model. No significant alterations were identified for both Flna and Flnb transcripts at end-stage Grn<sup>−/−</sup> mice compared to Wt mice (Additional file 2: Figure S2c-d), suggesting that the identified alterations are FlnC specific. Expression of other FTD-related genes, e.g. Tdp-43, Vcp and C9orf72 in Grn<sup>−/−</sup> mice remained unaltered as well (Additional file 2: Figure S2e-g).

Proteomic investigation of frontal cortex of FTD patients with increased FLNC levels using iTRAQ

To investigate the post-genomic sequelae of the human FLNC phenotype we performed an exploratory quantitative proteomic study of the frontal cortex (BA10) from patients carrying the FLNC p.V831I variant, the GRN p.0(IVS1 + 5G > C) mutation and VCP p.R159H mutation compared to control individuals. C9orf72 repeat expansion carriers were not included due to the absence of a
detectable FLNC increase. We found that 294, 326 and 337 proteins were significantly and differentially regulated for FLNC (Additional file 1: Table S6), GRN (Additional file 1: Table S7) and VCP (Additional file 1: Table S8) patients, respectively. Venn diagram separation of these significant protein datasets demonstrated the presence of a core set of 71 common and coherently regulated (with respect to elevated or reduced expression) proteins across all genomic FTD etiologies (Fig. 4a, Additional file 1: Table S9). We validated phosphoglycerate mutase 2 (PGAM2) and syntaxin binding protein 2 (STXBP2) via Western blot analysis (Fig. 4b), which closely paralleled the quantitative mass spectrometry data (exemplary iTRAQ reporter ion analysis for both proteins is represented in Additional file 2: Figure S4a,b). We also explored the nature of the proteins unique to each FTD paradigm, i.e. specific to the FLNC (p.V831I), GRN (p.0[IVS1 + 5G > C]) or VCP (p.R159H) patients (Additional file 1: Table S10-12).

**Differential bioinformatic interpretation of quantitative proteomic analyses**

To create a gestalt biomedical appreciation of the differential expression datasets, we employed *Textrous!* natural language processing (NLP) based analysis to generate a focussed semantic interpretation of the common FTD dataset and the FLNC-, GRN- and VCP-unique datasets. Using the *Textrous!* individual processing module we found for the FTD-common protein dataset the strongest correlations between the words ‘dendrites,’ ‘growth-associated,’ cytoskeleton,’ ‘polymerization,’ and ‘synapses’ and following proteins: growth associated protein 43/neuromodulin (GAP43), glial fibrillary acidic protein (GFAP), brain acid soluble protein 1 (BASP1), neuroplastin (NPTN) and neuronal pentraxin-1 (NPTX1) (Fig. 5a). To effectively condense the terms involved in the individual processing matrix we extracted all the semantically-associated nouns and noun-phrases from this output to generate a higher-order word cloud (Fig. 5b, Additional file 1: Table S13a), reinforcing the strong cytoskeletal/dendritic/synaptic focus of the FTD-common dataset.

We next focussed on a similar NLP-based interpretation of the FLNC-specific protein dataset. Repeating the *Textrous!* NLP analysis for the FLNC p.V831I variant (Fig. 6a), as with the FTD-common dataset, we found strong correlations between the words ‘neuronal-Wiskott–Aldrich Syndrome protein (N-wasp),’ ‘cytoskeleton,’ ‘neurites,’ ‘microtubules’ and ‘filopodia’ and following proteins: CAP-GLY domain containing linker protein 1 (CLIP1), Wiskott-Aldrich syndrome protein family member 3 (WASF3), Myc box-dependent-interacting protein 1 (BIN1) and microtubule-associated protein 4 (MAP4). Interestingly, and in contrast to the FTD-common dataset interpretation, a strong neurodegenerative focus was also present in the FLNC dataset interpretation, i.e. semantic association with multiple disease-related words including ‘aggregations,’ ‘oxidation,’ ‘tangles,’ ‘tauopathies,’ ‘neurodegenerative,’ ‘inclusion’ and ‘frontotemporal’. This dual functionality of the FLNC-unique dataset, i.e. cytoskeleton/neurodegeneration was also strongly evident from the extracted higher-order word cloud (Fig. 6b, Additional file 1: Table S13b). Hence the FLNC proteomic phenotype appears closely focused on neuroskeletal mechanisms tightly linked with neurodegenerative activity.
Using an identical NLP-based informatic approach for the GRN- (Additional file 2: Figure S5a, b) and VCP-unique (Additional file 2: Figure S6a, b) datasets we found that the GRN dataset was focussed upon ‘transporter’, ‘mitochondrial’, ‘oxidation’ and ‘cellular motility’ activity while in contrast the VCP-unique dataset was more focussed upon neuronal ‘synaptic’, ‘vesicle’, ‘transmission’ and ‘plasticity’ functions (Additional file 1: Table...
S13c-d). A general overview of the top 10 highest frequency words for each ‘higher-order’ word cloud, both FTD-common and the individual unique datasets, are outlined together in Additional file 1: Table S13a-d.

Overall, it is evident that for the FLNC-, GRN- and VCP-specific paradigms a specific and idiosyncratic post-genomic FTD-related functionality is present. As we found that the FLNC-unique protein dataset was strongly...

Fig. 6 Representation of the FLNC p.V831I unique protein dataset using Textrous! and word clouds. (a) Analysis of the FLNC p.V831I unique protein dataset using Textrous! natural language processing (NLP). Strongest correlations between words (vertical) and proteins (horizontal) are presented in a Textrous! heat map. Teal-colored blocks indicate strongly associated gene-word interactions in an intensity-sensitive manner; grey blocks indicate no significant interaction (b) Word clouds obtained from Wordle (http://www.wordle.net/) analyzing the Textrous! output data unique for the FLNC p.V831I variant
associated with a neurodegenerative/oxidative damage signature we attempted to independently quantify this using an orthogonal informatic approach to Textnous. Hence we sought to evaluate the semantic correlation between user-defined words associated with neurodegeneration, dementia and aging and proteins from the FLNC-, GRN- or VCP-specific datasets. This was performed using the LSI-based GenIndexer application. Through summation of the total cosine similarity scores for the input interrogator word terms and taking into account the relative sizes of input datasets, we found that multiple factors in the FLNC-specific protein dataset (Fig. 7) demonstrated the strongest semantic correlations to multiple terms associated with FTD (Additional file 1: Table S14-16). Therefore it appears that, for the FLNC-FTD patients, both protein and bioinformatic associations demonstrate a relatively unique and profound connection to FTLD pathology.

Discussion

Investigating the pathogenicity of TDP-43 deletion in zebrafish by Schmid et al. [12] demonstrated a potential role for muscle-specific proteins in the disease mechanism underlying FTD-ALS disorders, with FLNC being the most up-regulated protein. A potential link between the loss of TDP-43 in zebrafish, and the concomitant altered expression of FLNC, and the human disease was provided by the observation of elevated FLNC levels in the frontal cortex of FTLD-TDP patients. To further explore the potential involvement of FLNC in FTLD pathogenesis, we consequently screened the Belgian FTD patient and control cohorts for the presence of FLNC variants associated with FTD and determined the genetic etiology underlying the elevated FLNC expression levels in FTLD-TDP patients as observed by Schmid et al. [12]. Molecular genetic screening of the coding sequence of FLNC resulted in the identification of 68 missense variants, of which 19 were missense variants identified in 21 FTD patients only. All variants reported in this study are rare (MAF <1 %), except the p.R1241C and p.R1567Q missense variants which were low frequency (1 % < MAF <5 %) and frequent (MAF >5 %) variants, respectively. When considering all rare variants, indifferent if they were present or not in control individuals, FTD patients demonstrated a significant enrichment of rare variants (cumulative variant frequency of 11.3 %) when compared to control individuals (cumulative variant frequency of 7.9 %) (P = 0.034; OR = 1.46, 95 % CI = 1.03–2.07).

Previously, mutations in FLNC were described as an underlying cause of two distinct types of myopathy, known as myofibrillar and distal myopathy (MFM and DM) [17]. These myopathies are characterized by a progressive weakening of either proximal (MFM) or distal (DM) skeletal muscles combined with severe muscle defects [22, 23]. The disease spectrum has recently been broadened by the identification of novel genetic alterations in FLNC in patients with hypertrophic cardiomyopathy (HCM) [21]. Patients with HCM have marked sarcomeric abnormalities in cardiac muscles which cause an increased incidence of sudden cardiac death [51].

Based on the currently available data, we can suggest that the missense variants detected in FLNC are specific to the FTD phenotype based on a number of reasons. First, none of the FLNC variants identified in the Belgian FTD cohort were previously reported as a causal factor underlying any form of (cardio)myopathy. One exception is p.A2430V which was identified in one Belgian control individual, and had previously been reported in a patient with HCM [21]. In this latter study, however, the p.A2430V variation was classified as neutral by the SIFT prediction software tool and considered a potential rare polymorphism in FLNC, which is supported by our observation of the p.A2430V variant in a Belgian control individual. Second, the FLNC carriers with FTD in our study did not show overt clinical signs of any form of myopathy. Considering that the average onset age of most FTD patients is older than of myopathy patients further strengthens our interpretation that the FLNC variants we observed are specific for FTD. Third, the missense variations in FTD are affecting evolutionary conserved regions across the actin binding domain (ABD) and the 24 Immunoglobulin (Ig)-like domains (Fig. 1b, Additional file 2: Figure S1). Although the pathogenicity of the FLNC variations remains speculative, p.V831I and p.R1241C showed alterations in the expression of the FLNC protein itself or its phosphorylation state. A number of FLNC variations - p.R81C, p.K524R, p.D710N, p.R1241C, and p.T2025I - were identified in
FTD patients who carried a causal mutation in another FTD gene i.e. FLNC p.R1241C in a VCP p.R159H carrier, FLNC p.R81C and p.T2025I in GRN loss-of-function mutation carriers [10], and FLNC p.K524R and p.D710N in C9orf72 repeat expansion carriers [11] (Table 2, Additional file 1: Table S3b). Co-occurrence of FLNC variants with causal GRN or C9orf72 mutations is not surprising since they are the most frequent mutations found in FTD patients in Belgium. Also, FLNC p.E1571K was identified in a FTD patient carrying the TREM2 p.R47H risk allele [52, 53]. Whether the presence of these FLNC missense variations may influence the pathological effect of causal mutations in C9orf72 or GRN, or exert an additional pathological effect remains to be investigated. Overall, our genetic data obtained suggested that the role of FLNC variations might not be restricted to skeletal or cardiac muscle disorders, but might also be modifying the neurodegenerative processes in FTD patients. This implies that variations found in the same or in different functional domains of FLNC can be associated with distinct disease phenotypes involving miscellaneous tissues. This can be potentially explained by scaffolding functions of FLNC where different variants in certain protein domains could have differential effects on downstream signaling pathways or modulate distinct forms of FLNC protein-protein interactions.

Besides the moderate increase of FLNC levels in the frontal cortex of the FLNC p.V831I carrier, the GRN p.0(IVS1 + 5G > C) loss-of-function and to a lower extent the VCP p.R159H mutation showed a marked up-regulation of total FLNC levels. The potential link between GRN and FLNC was further supported by the strong increase in mouse Flna levels observed in the frontal cortex of Grn+/− mice between 16-18 months and 24 months of age. Hence it seems that in this FTD paradigm a strong aging-dependent triggering process may be in evidence.

Interestingly, the VCP p.R159H seemed to have a more pronounced effect on the phosphorylation state of pSer2113 while this was more the case for pSer2213 in GRN loss-of-function patients, suggesting that alternative pathways are affected in these patients. Increased phosphorylation at pSer2113 and pSer2213 of FLNC, might hamper the interaction of FLNC with other signaling proteins or its ability to modulate downstream signal transduction [49]. Further research, however, is required to determine the downstream functional discrepancies between elevated levels of total FLNC and both phosphorylation states of FLNC.

How elevated FLNC levels in the brain can influence the development of TDP-43 proteinopathies remains unknown. Studies performed in cellular and animal models, however, have demonstrated that a precise stoichiometry of FLNC together with its associated signaling/binding proteins is of critical importance for muscle function and maintenance [54]. Furthermore, it has been suggested that a certain threshold level of filamins may be essential for cell viability [14, 55]. In non-muscle cells, filamins are reported to colocalize along stress fibers implicating that they might play a role in maintaining focal adhesion complexes and stabilization of cytoskeletal features [56]. As no changes in both Flna and Flnb could be observed, the elevated levels of FLNC identified both in human and in mouse could hamper neuronal cellular integrity or axonal/dendritic plasticity in the brain to cope with additional stressors or alterations during degenerative and aging processes. This posits is in line with the Flnc expression patterns that we measured in the brains of Grn+/− mice. As mentioned previously, our expression data obtained from aged Grn+/− mice demonstrated a strong increase of Flnc in brain between 16-18 months and 24 months. At an age of 23.5 months, the highest Flnc levels in mice also correlate with an 17.5-fold higher relative risk of dying for Grn+/− mice compared to Grn+/− mice [24]. This suggests that elevated Flnc levels might play an important role in mediating or accelerating the pathological aging process.

A potential role for accelerated aging and neurodegeneration, due to increased FLNC levels, is further supported by the identification of several protein factors in all differential datasets obtained from our exploratory quantitative proteomic analyses on the frontal cortex of a selected number of FTD patients with different genetic etiologies. For example, low levels of neurogranin show a clear correlation with cognitive deficits and aging [57]. The key role played by neuroplastins in synapse formation and its association with impaired intellectual ability might be indicative for the role of FLNC [58]. Furthermore, we identified also increased PGAM2 expression levels in the FLNC- and GRN-unique datasets as an additional musclespecific protein that shows altered expression levels in the brain. Interestingly, PGAM2 overexpression has previously been associated with an altered energy metabolism and reduced stress resistance due to a decreased respiration capacity in mitochondria and increased production of reactive oxygen species in the heart of Pgam2 overexpression mice [59]. Abnormal energy metabolism has recently been linked to inflammation [60]. Furthermore, additional proteins which were consistently altered in both the FLNC-, GRN- and VCP-unique datasets, e.g. GFAP [61], NPTN [62], DBI [63], PRDX6 [64], MARCKS [65] and BSN [66], are closely associated with cognitive function, dementia and pathological aging. (Additional file 1: Table S6-8). Altered expression levels in these proteins could explain the observed accelerated aging of Grn+/− mice and concomitant increase in Flna, but clearly further research into this aging-neurodegenerative nexus in FTD is necessary.
Compared to the GRN- and VCP-unique datasets, the FLNC-unique protein dataset showed the strongest correlation to frontotemporal dementia, neurodegeneration and aging (Figs. 6 and 7). The bioinformatic analyses of all differential datasets provided evidence that the FLNC-unique protein dataset showed the greatest similarity to the core functions of the FTD-common protein dataset with respect to the modulation of neuronal structure or cytoskeletal dynamics (Figs. 5 and 6). Further support for the strong and specific connection between the core FTD functionality and the FLNC-specific dataset was provided by the application of standard bioinformatic investigations with GO Term and KEGG pathway analyses (Additional file 1: Table S17-20). The results of our exploratory proteomic analyses of frontal cortex of FTD patients are encouraging the involvement of FLNC in FTD disease pathways, however, further experiments are required to confirm these observations and should be extended on potential brain material of additional FTD patients with concomitant elevated FLNC levels.

Hence we found four common significantly populated GO terms between the FTD-common and FLNC-specific dataset, i.e. oxidative phosphorylation, generation of precursor metabolites and energy, cellular protein complex assembly and protein polymerization. Only one common GO term could be found between the GRN-unique (transmembrane transport) or VCP-unique (ribonucleotide metabolic process) datasets and the FTD-common dataset. At the KEGG pathway level, four common pathways were identified between the FTD-common and the FLNC-unique dataset: oxidative phosphorylation, Parkinson’s disease, Alzheimer’s disease, pathogenic Escherichia coli infection. No common pathways were found between the GRN- or VCP-unique datasets and the FTD-common dataset. In both cases the bioinformatic interpretation of the FLNC-unique dataset reveals a strong neurometabolic, neuronal architectural and neurodegenerative functional bias.

Conclusions
The data presented here provide further support that FLNC, a muscle-specific protein, could be a potential novel player in FTD pathogenesis. More specifically, we report a significant association between rare variants in FLNC and FTD. We have demonstrated that elevated FLNC levels in the frontal cortex of FTD patients are mainly associated with GRN haploinsufficiency and to a lower extent to the FLNC p.V831I and VCP p.R159H mutation. The GRN-associated increase of FLNC was confirmed in the frontal cortex of aged Grn knockout mice. Moreover, proteomic analysis of FTD patients with increased FLNC levels points towards downstream alterations in pathways involved in aging, neurodegeneration and synaptogenesis, suggesting that FLNC levels might have a potential role in mediating or accelerating the aging process. However, the exact mode of action of increased FLNC levels in the brain of FTD patients is currently unknown and requires further investigation.

Additional files

Additional file 1: Supplementary tables.  (DOC 1000 kb)  
Additional file 2: Supplementary figures.  (DOC 2024 kb)

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
JJ: literature search, Figures, study design, genetic data collection, data analysis, data interpretation, writing. SP: study design, genetic data collection, data analysis, data interpretation. GR: study design, data analysis, data interpretation. SVM: patient sample collection, clinical data collection, data analysis, data interpretation. JVDZ: patient data collection, genetic data collection, data analysis, data interpretation. RC: study design, genetic data analysis, genetic data interpretation. SE, PS, AI, MV, RV, PC and PPDD: patient samples collection, clinical data collection, data analysis, data interpretation. AS and JM: patient samples collection, clinical data collection, neuropsychology data collection, data analysis, data interpretation, writing. JBS and BS: expression data collection, data analysis, data interpretation. LB, CM and IC: genetic data collection, data analysis, CR: study design, SM, CH and MC: literature search, study design, data interpretation, writing, study supervision. CVB: literature search, Figures, study design, genetic data collection, genealogy data collection, data analysis, data interpretation, writing, study supervision. All authors read and approved the final manuscript.

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