CCNF mutations in amyotrophic lateral sclerosis and frontotemporal dementia

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Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are overlapping, fatal neurodegenerative disorders in which the molecular and pathogenic basis remains poorly understood. Ubiquitinated protein aggregates, of which TDP-43 is a major component, are a characteristic pathological feature of most ALS and FTD patients. Here we use genome-wide linkage analysis in a large ALS/FTD kindred to identify a novel disease locus on chromosome 16p13.3. Whole-exome sequencing identified a CCNF missense mutation at this locus. Interrogation of international cohorts identified additional novel CCNF variants in familial and sporadic ALS and FTD. Enrichment of rare protein-altering CCNF variants was evident in a large sporadic ALS replication cohort. CCNF encodes cyclin F, a component of an E3 ubiquitin–protein ligase complex (SCF<sup>CCNF</sup>). Expression of mutant CCNF in neuronal cells caused abnormal ubiquitination and accumulation of ubiquitinated proteins, including TDP-43 and a SCF<sup>CCNF</sup> F substrate. This implicates common mechanisms, linked to protein homeostasis, underlying neuronal degeneration.

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A missense mutation in CCNF in a large ALS/FTD family. Four family members with ALS or FTD (FALS10, individuals II:10, II:13, III:1 and III:15) were chosen for whole-exome sequencing. The mean read depth for these patients was $119.3 \times 10^8$, with an average of $6.01 \times 10^8$ base pairs sequenced per individual. To identify candidate mutations, exome sequence variants were annotated and filtered (Supplementary Table 2) using the following criteria: the variant was present in four affected family members, resulted in altered amino-acid sequence, and was absent from public SNP databases including dbSNP, the 1000 Genomes Project (0.001 frequency, October 2011 release), the NHLBI Exome Sequencing Project (ESP) exome variant server (6,503 sequenced human exomes), and ExAC database (0.001 frequency). Of the two variants that remained following filtering, one was located within the linked region on chromosome 16p13.3 and the other lies in a region on chromosome 22 that was excluded by linkage analysis (LOD scores $< 2$ for multiple flanking markers). The variant in the linked region lies in the CCNF gene, leads to an A to G substitution at position 1,861 of the coding DNA (c.1861A>G) and results in an amino-acid substitution of serine with glycine at codon 621 at the protein level (p.S621G). Sanger sequencing of 29 family members demonstrated segregation of the mutation in all affected family members for whom DNA was available (three ALS and one FTD). We also genotyped the offspring of other ALS patients for whom DNA was unavailable (deceased), and demonstrated segregation of the mutation in a further three patients (that is, obligate carriers, Fig. 1a,b). As such, we have shown segregation of the CCNF c.1861A>G mutation in seven ALS patients from family FALS10. The mutation was present in four at-risk family members (three <40 years, one <60 years). This variant was absent from 1,831 control individuals recruited from the same population. This variant is present in the ExAC database as a singleton (MAF = $8.629 \times 10^{-6}$). It is important to note that the ExAC database also includes other reported ALS/FTD mutations as singletons (including SOD1, FUS and GRN). Here unbiased whole-exome sequencing in family FALS10 identified two novel missense variants as candidate pathogenic mutations, one of which lies within the novel linked locus on chromosome 16p13.3 and segregated with disease.
disease onset in familial ALS cases with a CCNF mutation is 55.3 ± 8.0 (Supplementary Table 3). Detailed clinical descriptions are provided in the Supplemental Information. No clear relationship was evident between the location of mutations and clinical phenotype. Familial ALS/FTD mutations in CCNF were present in the overall cohorts from these diverse geographic populations at frequencies ranging from 0.6 to 3.3%, which is comparable to the frequency of mutations in TARDBP (which encodes TDP-43) and FUS that have been reported in familial ALS cohorts.13.

Enrichment of rare protein-altering variants in sporadic ALS. To further evaluate whether rare protein-altering CCNF variants are associated with sporadic ALS, we examined an independent replication cohort of cases and controls. CCNF variants were

Table 1 | ALS and/or FTD mutations in CCNF.

| Amino-acid change | Nucleotide change | Exon | Cohort | Control samples (Sanger and exome) | Public database MAF |
|-------------------|------------------|------|--------|------------------------------------|--------------------|
| **Familial**      |                  |      |        |                                    |                    |
| p.S3G             | 7A > G           | 1    | 1/99 US FTLD-ALS | 0/1038 US controls | Absent |
|                   |                  |      |        | 0/657 AU controls                 |                    |
|                   |                  |      |        | 0/967 AU control exomes           |                    |
| p.K97R            | 290A > G         | 4    | 1/159 UK FALS  | 0/897 UK controls          | Absent |
|                   |                  |      |        | 0/967 AU control exomes           |                    |
| p.S195R           | 585T > G         | 6    | 1/30 SP FALS  | 0/967 AU control exomes       | Absent |
| p.S509P           | 1525T > C        | 13   | 1/99 IT FALS  | 0/361 CA controls           | Absent |
| p.S621G           | 1861A > G        | 16   | 1/75 AU FALS  | 0/864 AU Sanger controls    | Absent |
|                   |                  |      |        | 0/967 AU control exomes           |                    |
| p.I772T           | 2315T > C        | 17   | 1/159 UK FALS | 0/897 UK controls          | Absent |
|                   |                  |      |        | 0/967 AU control exomes           |                    |
| **Sporadic**      |                  |      |        |                                    |                    |
| p.T181I           | 542C > T         | 6    | 1/283 JA SALS | 0/514 JA controls           | Absent* |
|                   |                  |      |        | 0/967 AU control exomes           |                    |
| p.R392T           | 1175G > C        | 11   | 1/99 US FTLD | 0/1038 US controls          | Absent |
| p.T543I           | 1628C > T        | 15   | 1/283 JA SALS | 0/967 AU control exomes    | Absent |
| p.E624K           | 1870G > A        | 16   | 1/49 US SALS trios | 0/801 AU Sanger controls | Absent |
|                   |                  |      |        | 0/967 AU control exomes           |                    |

ALS, Amyotrophic lateral sclerosis; AU, Australian; CA, Canadian; ESP, Exome Sequencing Project; IT, Italian; MAF, minor allele frequency; SP, Spanish; UK, United Kingdom; US, USA. CCNF accession NM_001761. Data were mined from whole exome or genome sequence data and validated by Sanger sequencing. Public databases include dbSNP; NHLBI ESP Exome Variant Server and 1000 Genomes Project.

*Variant was present as a singleton in ExAC, frequency of 8.6 x 10^-6.
identified in 611 Australian sporadic ALS cases and 1,424 cases from the ALS Data Browser data set. For the control data set, we used rare CCNF variants from the ExAC database (MAF <0.0001). Variants were subject to analysis if they were considered functional (nonsense, missense or frameshift) and passed quality filters (described in Methods). Fisher’s exact test revealed an enrichment of rare protein-altering variants in CCNF among sporadic ALS patients (1.39%) compared with controls (0.67%), \( P = 6.58 \times 10^{-4} \).

UPS dysfunction and ubiquitination of TDP-43. CCNF (also called FBXO1) encodes the 786 amino-acid cyclin F protein (Fig. 1c). Cyclin F is a member of the cyclin protein family, but unlike most cyclins, it does not bind or activate a cyclin dependent kinase (CDK)\(^{14}\). Cyclin F is also a member of the F-box protein family characterized by an F-box motif that binds directly to SKP1, which in turn recruits CUL1 to form a SCF (SKP1-CUL1-F-box protein) E3 ubiquitin–protein ligase complex (SCF\(^{\text{Cyclin F}}\))\(^{15,16}\). E3s mediate the ubiquitination and proteasomal degradation of target proteins and are an integral component of the ubiquitin proteasome system (UPS).

Aberrant misfolded proteins are targeted for disposal by protein degradation pathways including the UPS and autophagic–lysosomal system, both of which are components of the complex network that maintains protein homeostasis (proteostasis). The accumulation of neuronal protein aggregates in ALS patients implicates dysfunction of the proteostasis network through inappropriate or inadequate response to aberrant proteins\(^{17}\). As a ubiquitin–protein ligase, cyclin F catalyses the transfer of ubiquitin to target proteins\(^{15}\). To investigate whether a ubiquitin–protein ligase, cyclin F catalyses the transfer of ubiquitin to target proteins15. To investigate whether the UPS reporter, GFP\(^u\), that consists of a 16 amino-acid degron (CL1, a specific substrate for the UPS), fused to the carboxyl terminus of green fluorescent protein (GFP)\(^{18}\), the degron sequence ensures rapid degradation of the GFP fusion through ubiquitin-mediated pathways, and the accumulation of this GFP reporter indicates UPS impairment. We confirmed that GFP\(^u\) signal correlates with UPS function in a motor neuron-like cell line (NSC-34) by inhibiting proteasome function using either the chemical inhibitor MG132, or expression of a mutant huntingtin exon 1 fragment containing an expanded polyQ sequence previously shown to inhibit the proteasome\(^{19}\) (Supplementary Fig. 2). Next, we co-transfected the NSC-34 cell line with GFP\(^u\) and either wild-type cyclin F or wild-type cyclin F (Supplementary Table 4). Significantly higher levels of GFP\(^u\) fluorescence were observed for cyclin F with ALS/FTD-associated variants, indicating UPS dysfunction (Fig. 2a). This effect was independent of cyclin F expression levels (Fig. 2b). To examine whether the significant accumulation of GFP\(^u\) arose from the loss of proteasome activity or occurred upstream of the proteasome, we used two separate ubiquitin-independent small peptide 20S proteasome activity assays. These demonstrated that the UPS dysfunction was not due to altered proteolysis in the proteasome (Supplementary Fig. 3), consistent instead with the dysfunction stemming from abnormal ubiquitination or transport to the proteasome, mechanisms that are mediated, in part, by cyclin F. Consistent with these observations, western blotting confirmed the presence of significantly more ubiquitinated proteins in neuronal cell lines expressing mutant cyclin F (Fig. 3a,b). Collectively our data suggest that ALS/FTD-associated variants in CCNF modify the activity of SCF\(^{\text{Cyclin F}}\) resulting in overaccumulation of ubiquitinated proteins. To specifically demonstrate this, we found higher levels of the known SCF\(^{\text{Cyclin F}}\) target, RRM2 (Fig. 3a,b), including higher levels of ubiquitinated RRM2 (Fig. 3c), in neuronal cells expressing mutant cyclin F. Notably, we also observed substantially elevated levels of ubiquitinated TDP-43 in neuronal cells expressing mutant cyclin F (Fig. 3c).

**Discussion**

We performed whole-genome linkage analysis and whole-exome sequencing in one of the largest ALS/FTD pedigrees to be described in recent years. ALS/FTD was significantly linked to a single locus encompassing CCNF and exome sequencing identified a single CCNF mutation that segregated with seven affected family members. We extended mutation discovery and rare variant analysis to ALS/FTD cohorts from diverse geographic populations, most of European ancestry. Analysis in a replication cohort showed a significant enrichment of novel and rare protein-altering CCNF variants in sporadic ALS patients. This suggests that aberrant cyclin F plays a role in both familial and sporadic ALS pathogenesis. Diagnosis of primary lateral sclerosis (PLS, an upper motor neuron disease) in a mutation carrier suggests that the spectrum of CCNF-linked motor neuron disease is wider than ALS.
Neuronal accumulations of ubiquitinated TDP-43 are a major pathological feature of almost all ALS cases, and the majority of FTD cases. However, the mechanisms responsible for TDP-43 ubiquitination are poorly understood. We demonstrated that ALS/FTD-associated variants in CCNF are responsible for abnormal increases in ubiquitination of TDP-43 and may to be responsible for wider changes in protein homeostasis. Further studies can now commence to confirm whether CCNF mutations lead to aberrant misfolded proteins and the accumulation of neuronal protein aggregates in ALS and FTD patients.

Abnormal protein homeostasis has been hypothesized to play a role in ALS pathogenesis. Convincing genetic linkage to familial ALS has also been shown for UBQLN2, which encodes ubiquitin 2, a protein that physically associates with ubiquitin ligases and pro teaseosomes to mediate protein degradation. Ubiquitin-2-positive neuronal inclusions are seen in affected motor neurons of some ALS and ALS/FTD patients, and ALS-linked mutations in ubiquitin 2 have also been shown to impair protein degradation mediated by the UPS. Several other molecules that are functionally linked with cellular protein degradation pathways have also been associated with ALS (reviewed by Ling et al.). Putative ALS-associated variants have been described in SQSTM1/p62, a ubiquitin binding protein with roles in protein degradation via the proteasome and autophagy. Interestingly, one of these SQSTM1/p62 variants and two cyclin F mutations described here, lie in a PEST sequence, a domain that is thought to act as a signal peptide for protein degradation. ALS mutations have also been described in OPTN, an autophagic adaptor protein that binds substrates targeted for degradation and delivers them to autophagosomes. OPTN-linked pathogenic mechanisms remain to be determined. A VCP mutation linked to inclusion body myopathy with Paget’s disease of the bone and frontotemporal dementia (IBMPF D) impairs ER-associated degradation of ubiquitinated proteins from the ER. VCP mutations have also been described in ALS cases but the mechanisms by which these lead to motor neuron degeneration remain unclear. There is also evidence that defects in molecules functionally related to cyclin F play a role in other neurodegenerative diseases. Mutations in the related F-box only protein 7 gene (FBXO7) cause autosomal recessive, early-onset, parkinsonian-pyramidal syndrome and lead to decreased stability of the FBXO7 protein. Similar to cyclin F, the F-box motif of FBXO7 interacts directly with Skp1 to form the SCF E3 ubiquitin–protein ligase complex. Furthermore, around 10% of early-onset Parkinson’s disease cases are caused by mutations in PARK2, which encodes Parkin, an E3-ubiquitin ligase. Parkin mutations impair degradation of its substrates, leading to accumulation of toxic products and eventually cell death.

The known ALS proteins TDP-43 and FUS are capable of assembling into stress granules in response to oxidative stress and environmental insults, a process that is accelerated by ALS-linked mutations (reviewed by Li et al.). Indeed, stress granules have been described as the crucibles of ALS pathogenesis. Stress granules play a role in messenger RNA homeostasis and form during cellular stress, presumably to halt translation of non-essential transcripts. TIA-1, a messenger RNA-binding protein, translocates from the nucleus to seed stress granule formation in the cytoplasm. It was recently demonstrated that TIA-1 knockdown in mouse spinal cord and cerebellum led to marked and consistent increase in the expression of CCNF.

Like most known ALS-linked molecules, it remains to be determined whether the functional consequences of CCNF mutations lead to a toxic gain of function or dominant-negative loss of function or haploinsufficiency. We found one CCNF frameshift variant (p.L372fs) that did not segregate with disease, suggesting that a dominant toxic gain of function may be required for mutant CCNF pathogenicity. Segregation of the mutation with disease was clearly established in the large discovery family but could not be determined for other familial mutations in the discovery cohort due to the
Control exome data from 967 neurologically healthy individuals of predominantly Western European descent obtained from the Diamantina Institute, University of Queensland (Diamantina Australian Control Collection).

Bioinformatics. Sequencing reads generated by the Illumina platform were aligned to the hg19 human genome assembly using BWA v0.6.1 (ref. 30; variants were called using SAMTools v0.11.6 (ref. 31) and annotated using ANNOVAR32. Annotated variants were compared among affected family members and controls using R v3.1.3 (http://www.R-project.org/). Filtering of variants was performed using dbSNP (releases 131, 132, 134 and 137; https://www.ncbi.nlm.nih.gov/snp/), 1000 Genomes Project (Nov 2010 release; http://www.1000genomes.org/) and the ESP exome variant server (ESP5000 data release; http://evs.gs.washington.edu/ESVS/).

Sequence reads generated by MiSeq were mapped to the hg19 human genome assembly using BWA30. GATK34–36 was applied to mapped reads for realigning and recalibration of base quality scores and variant calling. ANNOVAR32 was used for annotation of variants.

Quality filters for rare variant enrichment analysis include read depth ≥ 10 in >75% of all samples and genotype quality > 20.

Conservation of cyclin F orthologues was examined by aligning sequences from a variety of species (Entrez protein database; http://ncbi.nlm.nih.gov) using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Plasmids and cloning. Expression constructs comprising wild type and mutant CCNF cDNA fused with an N-terminal mCherry were developed using pmCherry-C1-CCNF (Addgene, https://www.addgene.org/32975/) and Q5 Site-Directed Mutagenesis kit (NEB) according to the manufacturer’s protocol. All constructs were verified by DNA sequencing.

Antibodies. The following primary antibodies were used in this study: rabbit polyclonal anti-cyclin F (cat # sc-952, Santa Cruz Biotechnology), mouse monoclonal anti-TDP-43 antibody (cat # H00023435-M01, Abnova), rabbit polyclonal anti-ubiquitin (Dako), mouse monoclonal anti-RRM2 (cat # H00079240-B01, Abnova), rabbit polyclonal anti-cyclin F (cat # sc-8030, Santa Cruz Biotechnology), polyclonal anti-ubiquitin (Dako), and rabbit polyclonal anti-RRM2 (cat # sc-572, Santa Cruz Biotechnology).

Cell lines. Mouse NSC-34 (neuroblastoma/motor neuron-enriched primary spinal cord hybrid) cells were provided by Prof Neil Cashman, University of Toronto. Mouse neuroblastoma Neuro-2a cells, and human neuroblastoma SH-SY5Y cells were from the ATCC repository (ATCC Product Nos. CCL-131 and CRL-2266).

Confocal microscopy. Confocal fluorescence imaging was performed using a Leica DM6000 upright laser-scanning confocal microscope with Leica application suite advanced fluorescence software. Images were acquired with a × 63 (1.4 numerical aperture) oil-immersion objective. Images were acquired using sequential mode to avoid crosstalk between two dyes. Immunohistochemistry imaging was performed with a Zeiss Axio Imager 2 with ZEN pro program, using a × 40 objective.

Cell culture and transfection for UPS assays. NSC-34 cells were maintained in DMEM (Sigma Aldrich) containing 100 U ml⁻¹ penicillin, 100 mg ml⁻¹ streptomycin, and 10% (v/v) heat-inactivated fetal bovine serum (Sigma, Aldrich). Cells were maintained in a humidified 37 °C incubator with 5% CO₂. For the UPS assay and Enzo proteasome assay, NSC-34 cells were plated at a density of 50,000 cells per well in six-well plate. For the Abcam proteasome assay, NSC-34 cells were plated at a density of 2,000 cells per well in 96-well plate well. Transfections were carried out using Lipofectamine LTX (Life Technologies) according to manufacturer’s protocol. Transfections included 2.5 μg DNA for the UPS assay, 5 μg DNA for the Enzo proteasome assay or 0.1 μg DNA for the Abcam proteasome assay.

Ubiquitinated protein immunoprecipitation. Co-transfected Neuro-2a cells were lysed and total protein was extracted with sonication (10 s, Setting 3, Branson Sonifier 450) in extraction buffer (1% (v/v) Nonidet P-40, 50 mM Tris-HCl, pH 7.5, 130 mM NaCl) with protease inhibitor cocktail. Cellular debris was pelleted at 18,000g (30 min at 4 °C). Protein concentration was estimated using the BCA Protein Assay Reagent (Pierce Biotechnology). Typically, 3 μg of anti-ubiquitin (Dako) per 500 μg of protein extract was used for immunoprecipitations. Protein A/G magnetic beads (Pierce Biotechnology) were used to capture the antibody-protein complex. Immunoprecipitates were washed with TBS + 1% (v/v) NP-40 (3 × 10 min) to remove non-specifically bound proteins, and then resuspended in 1 × LDS buffer with 50 mM DTT, and heated at 95 °C for 10 min.

UPS reporter assay and 20S proteasome activity assay. For the UPS reporter assay, NSC-34 cells were cultured in six-well plates for 24 h, followed by co-transfection with a UPS-specific degron GFP and cyclin F constructs. The firefly reporter contains a CL1 sequence which signals ubiquitination and degradation by the proteasome13. The resulting GFP signal is a reporter for rate of protein degradation in the cell. Cells were collected 48 h post transfection by
trypsin and resuspended in PBS (Sigma-Aldrich). In order to show the sensitivity of GFP® construct, cells were either transfected with GFP® for 24 h followed by MG132 (Calbiochem) treatment at various doses for a further 24 h or co-transfected with GFP® and mCherry with or without MG132, or lastly co-transfected with GFP® and either Httex125Q or Httex146Q and incubated for 48 h. The fluorescent intensity GFP® in collected cells was analysed using flow cytometry Becton Dickinson LSR II. At least 50,000 cells per treatment were collected. Data were gated on mCherry-positive cells with an excitation at 541 nm and an emission at 575 nm. The geometric mean of the GFP® signal from this population was collected with an excitation at 488 nm and an emission at 520 nm. Data were from three independent experiments.

The proteasomal enzymatic activity of cyclin F was measured using either a 20S proteasome assay kit from Enzo Life Sciences or Abcam following the manufacturer’s instructions. For the Enzo Life Sciences proteasome activity kit, NSC-34 cells were transfected with wild-type pmCherry-C1-CCNF or mutant pmCherry-C1-CCNF in six-well plates and protein extract was generated by freeze–thaw lysis 48 h post-transfection. Protein concentration was measured by BCA protein assay (Thermo Scientific) and equal amount of protein was used in the assay. The proteasome activity was measured by hydrolysis of a fluorogenic peptide substrate Suc–Leu–Leu–Val–Tyr–R110 peptide substrate Suc–Leu–Leu–Val–Tyr–AMC (AMC: 7-amino-4-methylcoumarin). The substrate is cleaved by the 20S proteasome and the release of free AMC fluorescence is used as an indication of proteolytic activity. The fluorescent signal was measured by FLUOstar OPTIMA fluorescence plate reader (BMG Labtech) with an excitation at 360 nm and an emission at 460 nm. The data were collected at 2-min interval for 50 min. For the Abcam proteasome activity kit, NSC-34 cells were transfected with wild-type pmCherry-C1-CCNF or mutant pmCherry-C1-CCNF in 96-well plates for 48 h. A proteasome substrate Leu–Leu–Val–Tyr–R110 was added directly to the cells and incubated at 37 °C for 1 h. The substrate is cleaved by the 20S proteasome and the fluorescent signal generated from the cleavage is used as an indication of proteolytic activity. The fluorescent signal was measured by FLUOstar OPTIMA fluorescence plate reader (BMG Labtech) with an excitation at 490 nm and an emission at 525 nm.

Statistical analysis of in vitro assays. All statistical analyses were performed using GraphPad Prism Software. Two-tail unpaired Student’s t-tests (P<0.05) were used for grouped GFP® data (wild-type, cyclin F mutation (familial and sporadic)). One-way analysis of variance with Dunnett’s multiple comparison tests were used for comparison of GFP® levels in the presence of cyclin F variants, with wild type. Statistical analyses of other in vitro assays were performed using two-tailed unpaired Student’s t-tests (P<0.05). All data are expressed as means ± s.e.m. The data met the assumptions of each specific statistical test. Variance was similar between groups. Sample size was chosen based on results from pilot studies.

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