Mutant Cyclin F Impedes COPII Vesicle-Mediated ER-Golgi Trafficking and ER-Associated Degradation, Inducing ER Stress and Golgi Fragmentation in ALS/FTD

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Abstract

Background

Mutations in the *CCNF* gene encoding cyclin F are associated with sporadic and familial amyotrophic lateral sclerosis (ALS) and frontotemporal dementia, but the underlying pathophysiological mechanisms are unknown. Proper functioning of the endoplasmic reticulum (ER) is essential for physiological cellular function.

Methods

We used human neuroblastoma SH-SY5Y and human embryonic kidney HEK293T cell lines and mouse primary neurons-overexpressing two familial ALS cyclin F mutants to examine whether mutant ALS/FTD-associated cyclin F perturbs key functions of the ER and Golgi compartments. Specific cellular assays were used to examine ER-Golgi transport (VSVG^{ts045}), the budding of vesicles from ER membranes and ER-associated degradation (ERAD). Immunocytochemistry was used to examine the morphology of the Golgi and ER-exit sites, and to detect ER stress and apoptosis. Western blotting was used to examine the content of vesicles budding from ER membranes and the interaction between Sec 31 and cyclin F. Flow cytometry was used to examine cell death.

Results

We demonstrated that mutant cyclin F inhibited protein transport from the ER to Golgi apparatus by a mechanism involving aberrant vesicle sorting from the ER. It also impeded ER-associated degradation, whereby misfolded ER proteins are ubiquitinated and degraded by the proteasome. This was associated with induction of ER stress and Golgi fragmentation, leading to apoptosis.

Conclusion

Together, these results demonstrate that ER dysfunction is a pathogenic pathway associated with ALS/FTD-variant cyclin F.

Background

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by the progressive degeneration of both upper and lower motor neurons. ALS displays genetic and pathogenic overlap with the related condition frontotemporal dementia (FTD) in up to 15% of ALS patients [1, 2]. Approximately 10% of ALS cases are familial (FALS), which are clinically indistinguishable from sporadic ALS (SALS). Mutations in genes including *SOD1*, *TARDBP*, and *FUS* are present in FALS, and we recently identified missense mutations in the *CCNF* gene, encoding cyclin F, in patients with FALS/FTD [3]. Understanding early pathogenic mechanisms is important for designing effective therapeutic targets, but they remain virtually uncharacterised for cyclin F-associated ALS/FTD.

CCNF [NM_001761.3] (also called *FBXO1*) encodes cyclin F [NP_001752.2], an orphan member of the cyclin protein family [4, 5]. Whilst cyclins are important regulators of the cell cycle [4, 6], unlike most cyclins, cyclin F does not activate cyclin-dependent kinases (CDK). However, it is the founding member of a larger family of proteins containing a F-box domain [7]. This domain binds directly to S-phase kinase-associated protein 1 (SKP1) which recruits cullin-1 (CUL1) to form the SCF (SKP1-CUL1-F-box protein) E3 ubiquitin-protein ligase complex, an integral component of the ubiquitin proteasome system (UPS) [7]. While other cyclins use the catalytic subunit of CDK to phosphorylate their substrates, cyclin F uses its F-box to promote the ubiquitination of target substrates. Also, cyclin F contains two nuclear localization signals (NLS) at the C-terminus which facilitates its cellular localization in the nucleus [4]. Cyclin F contains a PEST sequence (enriched in proline, glutamic acid, serine and threonine) which controls its self-degradation through UPS-independent mechanisms [8]. Ubiquitination has important roles in protein transport by serving as a sorting signal for protein cargo and by controlling the activity of the trafficking machinery [9] and marking proteins for destruction [10]. We previously showed that ALS/FTD-linked *CCNF* p.S621G (c.1861A > G) and p.S195R (c.585T > G) mutations disrupt the UPS and impair ubiquitin-mediated proteasomal degradation [3]. The p.S621G mutant cyclin F protein also disrupts Lys48-specific ubiquitination, leading to defects in the autophagic machinery [11, 12]. Transient overexpression of mutant S621G cyclin F in zebrafish larvae results in abnormal motor axon morphology, which correlates with motor dysfunction [13].

Defects in the proteostasis network are widely implicated in ALS/FTD [14, 15] and the endoplasmic reticulum (ER) and Golgi apparatus compartments play important roles in maintaining proteostasis. A major function of the ER is the production of secretory/transmembrane proteins and their subsequent transport to the Golgi, from where they are sorted to their final destinations. ER-Golgi transport includes multiple steps, and it is initiated when vesicles containing protein cargo bud from the ER coated in coat protein II (COPII) complex, consisting of Sec31, Sec13, Sec23, Sec24 and Sar1. After the budding of COPII vesicles at ER exit sites [16, 17], tethering, docking and fusion to target membranes at the Golgi follows [18, 19]. The ER also plays an essential function in protein folding and quality control. ER-associated degradation (ERAD) ensures that only properly folded proteins are secreted, by retro-translocation of misfolded proteins to the cytosol for degradation by the UPS [20]. Ubiquitination of ERAD substrates ensures that they are recognised by the proteasome, but accumulating evidence suggests ubiquitination also performs a regulatory role in ERAD [21].

Dysfunction to the ER induces ER stress, the accumulation of misfolded/unfolded proteins within the ER, which triggers the unfolded protein response (UPR). Whilst initially protective, the UPR triggers apoptosis if prolonged or unresolved, hence ER stress is closely linked to cell survival. Similarly, proper functioning of the Golgi apparatus is closely linked to cellular viability and perturbations in this process are associated with apoptosis [22]. The unique morphology of the Golgi, with its tethered stacks of cisternae, is required for its function. However, the Golgi apparatus can undergo disassembly and fragmentation into condensed, tubulovesicular punctate structures when its functions are compromised or during pathological conditions [23, 24].

In this study, we demonstrate that expression of mutant ALS/FTD cyclin F in neuronal cells perturbs key functions of the ER. Protein transport between the ER and Golgi apparatus was inhibited in mutant cyclin F expressing cells by a mechanism involving the formation of aberrant coat protein complex II (COPII) vesicles and/or ER-exit sites. This was accompanied by activation of ER stress, Golgi fragmentation, impairment of ERAD and induction of apoptosis. This study therefore reveals novel insights into the pathogenic mechanisms induced by cyclin F mutations in ALS/FTD, involving perturbations to both the ER and Golgi compartments.

Materials & Methods

Constructs

Expression constructs encoding wild-type (WT) and mutant S621G or S195R *CCNF* cDNA fused to C-terminal mCherry, were generated by subcloning *CCNF* cDNA into pmCherry-C1 vector (Addgene: <https://www.addgene.org/32975/>) as previously described [3]. The point mutations in *CCNF* (S621G or S195R) were introduced using a Q5® Site-Directed Mutagenesis kit, according to the manufacturer's protocol (New England Biolabs E0554) [3]. Constructs encoding WT Venus or mutant single strand deglycosylation-dependant Venus (SS-ddVenus), where the C-terminus was fused to the unstable null Hong-Kong substrate (NHK-Venus; NHK-ddVenus) [25], were a kind gift from Dr Jess E Grotzke (Department of Immunobiology, Yale University School of Medicine, New Haven, USA) [26]. The mutant vesicular stomatitis viral glycoprotein (*VSVG^{ts045}*) fused to EGFP in the pEGFP-C1 vector was a kind gift from Dr Jennifer Lippincott-Schwartz (National Institutes of Health, Bethesda, USA).

SH-SY5Y and HEK293T cell lines

Undifferentiated human neuroblastoma SH-SY5Y and human embryonic kidney HEK293T cell lines (ATCC) were cultured for 24h to 80% confluence at 37°C in a humidified atmosphere of 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM; Gibco), supplemented with 10% (v/v) heat-inactivated foetal bovine serum (Gibco). Authentication of cell line identities was confirmed via short tandem repeat profiling (Garvan Institute, Sydney). Cells were transfected for 48h or 72h with plasmids encoding pmCherry-C1 empty vector (EV), WT or mCherry-tagged mutant S621G or S195R *CCNF*, or co-transfected with plasmids encoding EV, mCherry-tagged WT or mutant *CCNF* with plasmids encoding GFP-tagged *VSVG^{ts045}*, or Venus/ddVenus using Lipofectamine™ 2000 (Invitrogen), following the manufacturer's instructions. Neuronal SH-SY5Y cells were used for immunocytochemistry studies, whereas HEK293T cells were used primarily for Western blotting studies because of their high transfection efficiency.

Mouse primary cortical neurons

Primary neurons were harvested from the cortex of C57BL/6 mouse embryos at embryonic day 16–18. The culture of primary neurons was performed as described previously [27]. Briefly, cortical tissue was dissected, cut into pieces under sterile conditions in Hanks' Balanced Salt solution (Gibco) and digested in 10 units/ml papain (Sigma) in 0.2 mg/ml L-cysteine, 1 mM CaCl₂ and 0.5 mM EDTA (pH 8) in DMEM

(Gibco) for 10 min at 37°C. Cells were subsequently dissociated, resuspended in plating medium (Neurobasal medium [Gibco] supplemented with 10% (v/v) heat-inactivated foetal bovine serum [Gibco], 2% (v/v) B-27 supplement [Gibco], 1% (v/v) GlutaMAX™ [Gibco] and 100 µg/ml penicillin-streptomycin) and seeded for 12h on 15 mm glass coverslips previously coated overnight with 0.1 mg/ml poly-D-lysine (Sigma). Cells were then incubated in neuronal medium (Neurobasal medium [Gibco] supplemented with 2% (v/v) B-27 supplement [Gibco], 1% (v/v) GlutaMAX™ [Gibco] and 100 µg/ml penicillin-streptomycin) at 37°C in a humidified atmosphere of 5% CO₂. Half of the medium was changed every three days. After 5 days *in vitro*, neurons were transfected with constructs encoding EV, WT or mutant S621G or S195R *CCNF* using Lipofectamine™ 2000 (Invitrogen) following the manufacturer's instructions. Primary neurons were then incubated for 48h before fixation in 4% paraformaldehyde (PFA) in 0.1M PBS.

Immunocytochemistry

SH-SY5Y and HEK293T cells grown on 13 mm coverslips were washed in 0.1 M PBS (pH 7.2) and fixed in 4% PFA in PBS for 10 min. After 3 washes in PBS, cells were permeabilised in 0.1% (v/v) Triton X-100 in PBS for 5 min and the non-specific background staining was blocked, using 3% (w/v) bovine serum albumin (BSA) in PBS for 45 min at room temperature with gentle rocking. Cells were then incubated overnight at 4°C with primary antibodies diluted in 1% (w/v) BSA in PBS: polyclonal rabbit anti-calnexin (1:100; Abcam 22595) or monoclonal mouse anti-CHOP (1:50; Santa Cruz sc7351), anti-XBP1 M-186 (1:25; Santa Cruz sc-7160), anti-FLAG (1:500; Sigma F3165), anti-Sec31A (1:100; BD Biosciences 612351), or anti-GM130 (1:50; BD Transduction 610823). After rinsing, cells were incubated for 1h at room temperature with gentle rocking and the appropriate secondary antibodies (diluted 1:250 in PBS) coupled to either Alexa 647 (Life Technology), Alexa 594 (Molecular Probes) or Alexa 488 (Life Technology). Cells were then washed as above and treated with 0.5 µg/ml Hoechst 33342 reagent (Sigma). After 3 washes in PBS, coverslips were mounted onto slides in fluorescent mounting medium (Dako) and cells were photographed with 20x/na = 0.8, 40x/na = 1.3, 63x/na = 1.4 or 100x/na = 1.46 objectives on a Zeiss LSM 880 inverted confocal laser-scanning microscope, equipped with a LSM-TPMT camera (Zeiss). Additional low-resolution images were acquired with 20x/na = 0.5, 40x/na = 0.75, 63x/na = 1.4 or 100x/na = 1.46 objectives on an AxioImager Z2 fluorescent microscope (Zeiss) equipped with a monochrome AxioCamHRm digital CCD camera (Zeiss).

VSVG^{ts045} transport assay

SH-SY5Y cells co-transfected with constructs encoding cyclin F or VSVG^{ts045} were incubated overnight at 40°C to accumulate VSVG^{ts045} in the ER. Cycloheximide (Sigma) diluted 20 µg/ml in DMEM was then added and the cells were incubated at 32°C for 30 min to allow VSVG^{ts045} to traffic to the Golgi. After one wash in PBS, samples were fixed in 4% PFA in 0.1M PBS (pH 7.2) for 10 min and processed for immunocytochemistry as described above. At least 20 cells expressing both cyclin F and VSVG^{ts045} were photographed in each group. Mander's coefficient was calculated for each cell to determine the degree of colocalisation (where 0 indicated no colocalisation and 1 indicated total colocalisation) of VSVG^{ts045} or

cyclin F with either calnexin or GM130, using the JaCoP plugin [28] in ImageJ (<http://rsbweb.nih.gov/ij/index.html>). All experiments were performed in triplicate.

Immunoprecipitation

HEK293T cells transfected for 48h with constructs encoding cyclin F were removed by scraping in 400 μ l of non-denaturing lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% (v/v) NP40 pH 7.4, 1% protease and 1% phosphatase inhibitor cocktail [Roche]) for 30 min, followed by sonication for 10s on ice and centrifugation at 14,000 g for 10 min at 4°C. The concentration of protein in each lysate was calculated using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Cell lysate (500 μ g of proteins) was incubated with either 2 μ g of rabbit polyclonal anti-FLAG antibody (Sigma F7425) or 2 μ g of mouse monoclonal anti-ubiquitin antibody (SantaCruz sc-8017) for 1h at 4°C in a rotary shaker. Protein G/A Dynabeads (Thermo Fisher Scientific 10003D) were washed 3 times in lysis buffer and then incubated with each sample for 2h at 4°C in a rotary shaker. The beads along with protein complexes were separated by placing the tubes in a magnetic rack. After rinsing in lysis buffer, the beads were mixed with 30 μ l of Laemmli buffer (BioRad 161–0747) and boiled at 95°C for 5 min before Western blotting was performed. Supernatants (10 μ g of both input and flow-through) were mixed with Laemmli Buffer and NuPage sample reducing agent (Novex NP0009) and boiled at 95°C for 5 min.

In vitro budding assay

A modified *in vitro* assay was used to analyse ER vesicle budding [23, 29]. Briefly, HEK293T cells co-transfected with constructs encoding cyclin F and VSVG^{ts045} were incubated overnight at 40°C to accumulate VSVG^{ts045} in the ER. Cells were then washed in PBS, resuspended in 5/90 buffer (50 mM HEPES and 90 mM potassium acetate in demineralized water) and incubated with rat liver cytosol (ThermoFisher) and an energy regenerating system (50 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase and 1 mM ATP) at 32°C for 30 min. Identical samples were incubated at 4°C to monitor non-specific ER fragmentation. The cells were removed by low-speed centrifugation at 4,000 g for 1 min at 4°C, followed by 15,000 g for 1 min, and budded vesicles were recovered by centrifugation at 100,000 g for 1h at 4°C from the resulting supernatant. The levels of VSVG^{ts045} cargo in the budded vesicle fractions were quantified by Western blotting using anti-VSVG (1:1000; Sigma V4888) and anti-COPII (Sec23; 1:500; Pierce, Rockford, IL, PA1-069A) antibodies. The relative intensity of VSVG and COPII/Sec23 to β -actin was normalised to untreated cells.

Western blotting

HEK293T cells transfected with constructs encoding cyclin F for 48h were washed in PBS, lysed in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% (w/v) SDS, 1% protease inhibitor cocktail (Sigma) and 1% phosphatase inhibitor cocktail (Sigma). The supernatant was then cleared by centrifugation at 12,000 g for 10 min at 4°C. The total amount of protein in each sample was quantified using a Pierce BCA Protein Assay Kit (Thermo Fisher Sci.), following the manufacturer's instructions. Proteins in the medium fraction were concentrated using Amicon Ultra (0.5 ml) columns (Merck). Proteins (10–20 μ g) were separated on

7.5%, 4–15% or 4–20% gels (BioRad) and either stained with a Silver Stain kit (Thermo Fisher Sci.) or transferred onto nitrocellulose membranes according to the manufacturer's instructions (BioRad). Blots were pre-incubated in blocking solution containing 5% (w/v) skim milk in Tris-buffered saline, followed by incubation overnight at 4°C in primary antibodies diluted in blocking solution: polyclonal rabbit anti-cyclin F (1:500; Santa Cruz sc952), anti-ubiquitin (1:1000; Abcam ab7780), anti-VSVG (1:1000; Sigma V4888), anti-COPII/Sec23 (1:500; Pierce, Rockford, IL, PA1-069A), or monoclonal mouse anti-Sec31A (1:500; BD Biosciences 612351) or anti- β -actin (1:4000; Sigma A5441 [AC-15]) antibodies. After rinsing, blots were incubated in peroxidase-conjugated secondary antibodies (1:2000; Millipore) for one hour at room temperature or processed using fluorescent antibodies (Li-Cor Biosciences) following the manufacturer's instructions. Immunoreactivity was revealed using the Clarity™ ECL Western Blotting Substrate (BioRad) and images were obtained either with the BioRad ChemiDoc MP system using Image Lab™ software (BioRad) or with Odyssey® CLx and Image Studio software (Li-Cor Biosciences). The intensity of each band relative to β -actin was quantified using ImageJ.

ERAD assay

SH-SY5Y cells co-transfected with constructs encoding cyclin F and either NHK-Venus, NHK-ddVenus or SS-ddVenus for 48h, were fixed in 4% PFA in 0.1M PBS, pH 7.2, and mounted as above. Images were acquired using an Axio Imager Z2 fluorescent microscope at 20x/na = 0.8 magnification. At least 100 cells expressing cyclin F and Venus/ddVenus were scored as the percentage of NHK-Venus, NHK-ddVenus or SS-Venus fluorescent cells from three different experiments.

Quantitative analysis of cells

The percentage of cells expressing cyclin F with Golgi fragmentation was quantified from 10–30 primary neurons per group and from at least 50 SH-SY5Y or 100 HEK293T cells per group from $n = 3$ independent experiments. Only cells where the Golgi structure was clearly visible were analysed. The Golgi was considered fragmented when at least 5 fragments were clearly visible. The area covered by the Golgi fragments was calculated using ImageJ.

The percentage of cells displaying nuclear immunoreactivity to CHOP or XBP1 was quantified from 30 + primary neurons per group and at least 100 + HEK293T cells per group expressing cyclin F from $n \geq 3$ independent experiments. All analyses were performed blind.

Quantitative analysis of apoptotic nuclei

Apoptotic nuclei were defined as condensed when they were under 5 μm in diameter or fragmented (multiple condensed Hoechst-positive structures in one cell) [30]. The percentage of apoptotic cells was quantified from 10–30 primary neurons per group or from at least 100 HEK293T cells per group expressing cyclin F from $n \geq 3$ independent experiments. Cells undergoing cell division were excluded from analysis.

Sytox Blue staining

HEK293T transfected with cyclin F constructs for 72h were harvested by adding trypsin for 1 min at room temperature. The cells were then collected in PBS, centrifuged at 1,200 rpm for 5 min and resuspended in 200 μ l of buffer containing 10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4. The cell suspension was treated with 1 μ M Sytox Blue nucleic acid stain (Invitrogen) for 10 min at room temperature in the dark. The cell suspension was then analysed for SYTOX blue positive cells after gating for cells positive for mCherry fluorescence, using a BD FACS Canto™ II flow cytometer (BD Biosciences).

Statistics

Data are presented as mean value $\pm$ standard error of the mean (SEM). Statistical comparisons between group means were performed using GraphPad Prism 6 software (Graph Pad software, Inc.). One-way or two-way ANOVA followed by *post hoc* Tukey test for multiple comparisons was used when justified. The significance threshold was set at $p = 0.05$.

Results

ALS/FTD mutant Cyclin F inhibits ER-Golgi transport

To probe cellular disease mechanisms induced by mutant cyclin F, we first expressed familial ALS/FTD-associated mutant cyclin F (Fig. 1A) [3], tagged with mCherry, in HEK293T cells. Western blotting of cellular lysates prepared from cells expressing mutant cyclin F demonstrated no significant difference ($p > 0.05$) between the expression levels of wild-type (WT) and mutant cyclin F (Fig. 1B, C).

Protein trafficking defects have been previously detected in ALS but have not been described before in association with mutant cyclin F [23, 31]. Hence, we first examined if mutant cyclin F inhibits ER-Golgi trafficking. We used a temperature sensitive mutant of vesicular stomatitis viral glycoprotein (VSVG^{ts045}) [32, 33, 23, 34], to examine trafficking of VSVG^{ts045} from the ER to Golgi in cells co-expressing cyclin F and VSVG^{ts045} (Fig. 1D). VSVG^{ts045} has been widely used as a reporter of membrane trafficking for canonical ER-to-Golgi transport [35, 36]. The transport of VSVG^{ts045} was examined by immunocytochemistry using anti-calnexin (Fig. 1E) or anti-GM130 (Fig. 1G) antibodies to label the ER and Golgi compartments, respectively. Analysis using Mander's coefficient, where 0 indicated no co-localisation and 1, high co-localisation, demonstrated that VSVG^{ts045} co-localised significantly more with calnexin in mutant cyclin F^{S621G} expressing cells (0.6 ± 0.03), after incubation at the permissive temperature (32°C), compared to control cells expressing either cyclin F^{WT} (0.3 ± 0.03) or empty vector (EV) (expressing mCherry alone) (0.06 ± 0.009) (Fig. 1F). These data therefore indicate that more VSVG^{ts045} was retained in the ER in mutant cyclin F^{S621G} expressing cells compared to controls. Additional control cells from each group were incubated only at 40°C, confirming that VSVG misfolds and is retained within the ER at 40°C (Fig. 1F). Similarly, in cells expressing mutant cyclin F^{S621G}, significantly less VSVG^{ts045} colocalised with GM130 (0.2 ± 0.02) compared to control cells expressing EV (0.8 ± 0.02) or cyclin F^{WT} (0.6 ± 0.03) (Fig. 1H), where VSVG^{ts045} extensively colocalised with GM130, indicating efficient ER-Golgi transport. Hence, VSVG^{ts045} is retained in the ER and less is transported to the Golgi

apparatus in cells expressing mutant cyclin F compared to control cells, indicating that mutant cyclin F perturbs ER-Golgi transport.

ALS/FTD mutant cyclin F interferes with the budding of COPII vesicles from the ER

ER-Golgi trafficking includes multiple steps, consisting of COPII-dependent vesicle budding from the ER, tethering and docking to target membranes, and finally SNARE protein-dependent vesicle fusion [18, 19]. Newly synthesized proteins are exported from the ER via coat protein complex II (COPII)-coated vesicles, which form in specialized zones within the ER, termed ER exit sites (ERES) [16, 18, 19]. The first stage of ER-Golgi transport is therefore the formation of COPII vesicles from the ER membranes.

First, to provide evidence that VSVG^{ts045} is transported from ER to Golgi in COPII vesicles, we repeated the experiment above (Fig. 1), but performed immunocytochemistry for Sec31A (rather than calnexin or GM130), a marker of the newly budded ER-derived vesicles at ER exit sites (Fig. 2A). Neuronal SH-SY5Y cells expressing GFP-tagged VSVG^{ts045} were incubated overnight at 40°C to accumulate misfolded VSVG^{ts045} followed by 30 min incubation at 32°C. Following immunocytochemistry, VSVG^{ts045} was found to be associated with Sec31-positive budding vesicles (Fig. 2B), consistent with its packaging into COPII vesicles to be transported from the ER.

We next used a modified *in vitro* reconstituted assay (Xu and Hay, 2004) to directly assess whether VSVG^{ts045}-GFP was associated with membranes that form following ER retention, which includes those from COPII vesicles, the ER-Golgi intermediate compartment (ERGIC) and intra/post Golgi carriers (Fig. 2B). This assay therefore aims to reconstitute ER vesicle budding using rat liver cytosol (as a source of soluble COPII) and an ATP regeneration system. ER-derived COPII vesicles are released into the extracellular buffer during incubation [29, 37] and semi-intact cells are used as a source of ER, given that the integrity of the ER is preserved under these conditions [29, 38, 39]. Semi-intact HEK293T cells co-expressing cyclin F and VSVG^{ts045} for 24h were incubated at 40°C overnight to retain VSVG^{ts045} in the ER, then incubated at 32°C to allow VSVG^{ts045} to be incorporated into COPII vesicles. The light membranes, including ER-derived vesicles, were then recovered by cellular fractionation (Fig. 2A).

VSVG^{ts045} and Sec23 expression were first examined in control cell lysates sampled before the budding reaction. No significant difference in VSVG and Sec23 levels was detected between all groups (untreated cells or cells expressing EV, WT or mutant cyclin F) (Fig. 2C-E). This indicates that differences in protein expression do not account for the results of the budding assay.

VSVG^{ts045} was then quantitated in the budded vesicular preparations by Western blotting using an anti-VSVG antibody (Fig. 2F). Significantly less VSVG^{ts045} was present in the preparations derived from cells expressing mutant cyclin F^{S621G} compared to UT cells (4.9-fold) and cells expressing mCherry only (EV) (3.8-fold, Fig. 2G), consistent with impairment in protein transport from the ER to Golgi (Fig. 1). There was a trend towards less VSVG^{ts045} in preparations derived from cells expressing mutant cyclin F^{S195R}, but

this was not statistically significant. The presence of COPII was also assessed in the vesicular fractions using anti-Sec23 antibodies (Fig. 2F). Interestingly, significantly less Sec23 was also associated with vesicles obtained from cells expressing mutant cyclin F^{S621G} or mutant cyclin F^{S195R} compared to UT (cyclin F^{S621G}: 2.3-fold; cyclin F^{S195R}: 2.3-fold) and EV cells (cyclin F^{S621G}: 1.9-fold; cyclin F^{S195R}: 1.9-fold) (Fig. 2H). These results reveal that less COPII and vesicular cargo (VSVG^{ts045}) are present in ER-derived vesicular preparations from cells expressing mutant cyclin F compared to controls. This further implies that vesicular budding between the ER and Golgi is defective in cells expressing mutant cyclin F.

ER-derived exit sites are perturbed in cells expressing ALS/FTD mutant cyclin F

We next examined the first step of ER-Golgi trafficking by analysing Sec31-positive clusters of ER exit sites in HEK 293T cells expressing mutant cyclin F by airyscan microscopy (Fig. 3A-C). ER-exit site clusters were identified as hollow and spherical structures staining positive for Sec31, with a diameter ranging from 60 to 350 nm. The mean diameter of these clusters was significantly decreased (1.2-fold, $p < 0.05$) in cells expressing ALS/FTD mutant cyclin F compared to UT cells or those expressing either EV or WT cyclin F (Fig. 3B). Significantly more small ER-exit site clusters and conversely, significantly fewer large ER-exit site clusters were present in cells expressing mutant cyclin F compared to UT, EV or WT cyclin F cells (Fig. 3C). These results suggest that the first step of ER-Golgi trafficking, vesicle budding from the ER, was perturbed in cells expressing ALS/FTD-mutant cyclin F.

It has been previously established that ubiquitination of Sec31 regulates the size of COPII coats, allowing for the trafficking of large cargo [40], and we previously demonstrated that mutant cyclin F impairs ubiquitination [12]. This raises the possibility that aberrant ubiquitination of Sec31 in cells expressing ALS/FTD mutant cyclin F impacts the size of COPII vesicles, perturbing the transport of large cargoes. The ubiquitination of Sec31 was therefore examined by immunoprecipitation (IP) in cells co-expressing FLAG-tagged Sec31 with either EV or cyclin F proteins (Fig. 3D). IP of cell lysates was performed using an anti-FLAG antibody, and Western blotting for ubiquitin was performed. The ubiquitination of Sec31 was significantly decreased in cells expressing mutant cyclin F^{S621G} compared to those expressing mCherry alone (EV, 5-fold) or cyclin F^{WT} (8-fold) (Fig. 3E). These findings were not due to differences in the expression levels of Sec31 between populations (Fig. 3F, G). Hence, the ubiquitination of Sec31 was significantly decreased in cells expressing mutant cyclin F.

To further examine the transport of large cargoes, bulk protein secretion was examined in the conditioned medium from cells expressing cyclin F (Fig. 3E). Silver staining revealed no significant difference in the levels of secreted proteins overall in the media. However, when proteins over 100 kDa were specifically examined, significantly less total protein of this size was detected in the conditioned medium from mutant cyclin F expressing cells compared to the other groups (Fig. 3G). These results therefore imply that bulk secretion of large proteins is impaired in cells expressing mutant cyclin F, providing further evidence that mutant cyclin F perturbs protein trafficking between the ER and Golgi compartments.

ALS/FTD mutant cyclin F impedes ER-associated degradation

The results described above demonstrate that one important function of the ER, the transport of secretory and transmembrane proteins from the ER to the Golgi apparatus, is perturbed by mutant cyclin F. ERAD is another important quality control function of the ER that monitors the fidelity of protein folding, and those proteins that fail to fold or assemble properly are degraded [41, 42]. Furthermore, ERAD is a complex, multistep process, that is regulated by ubiquitination, which is defective in cells expressing mutant cyclin F. Hence, we next examined whether ERAD is defective in cyclin F-associated ALS/FTD. ERAD begins with the recognition and targeting of substrates, followed by ubiquitination, retro-translocation and proteasomal degradation. Here we used a substrate with an ER-targeted signal sequence (K^b -SS) fused to a mutant version of Venus, ddVenus (deglycosylation-dependant Venus), in which Asp is substituted to Asn at position 82 [26]. This mutation results in glycosylation and a sharp reduction in fluorescence, which is restored to wildtype levels when Asn is converted back to Asp. Removal of oligosaccharides by endogenous peptide:N'glycanase (PNGase) in the cytosol results in deamidation of glycosylated Asn, converting it to Asp which restores fluorescence [26]. Given that ERAD involves two stages, entry of substrate into the ER followed by its retro-translocation to the cytoplasm, both glycosylation of ddVenus in the ER and its deglycosylation in the cytosol are required for fluorescence (Fig. 4A, B). Hence the accumulation of ddVenus fluorescence indicates specific impairment of ERAD. In addition, a second ERAD substrate was used, fluorescent ddVenus fused to the null Hong Kong genetic variant of $\alpha 1$ -antitrypsin (NHK-ddVenus), which misfolds terminally in the ER and is also degraded specifically by ERAD [25, 26, 43]. In cells expressing empty vector, cyclin F^{WT} or mutant cyclin F^{S621G}, ERAD was probed using substrates NHK-Venus (a control for transfection efficiency), NHK-ddVenus or SS-ddVenus (Fig. 4C-E). Quantification demonstrated that significantly more fluorescent cells were present in mutant cyclin F expressing populations compared to cyclin F^{WT} (NHK-ddVenus: 1.7-fold; SS-ddVenus: 1.5-fold,) or mCherry only (EV) populations (NHK-ddVenus: 33.1-fold; SS-ddVenus: 33.8-fold) (Fig. 3D-F). Hence, expression of mutant cyclin F significantly impairs ERAD compared to WT and controls.

ALS/FTD cyclin F mutants induce ER stress

The impairment of two important ER functions, protein trafficking to the Golgi and ERAD, implies that mutant cyclin F perturbs the overall homeostasis of the ER. To investigate this possibility further, we next examined whether cyclin F induces ER stress using immunocytochemistry following established methods [44–46] (Fig. 5). XBP-1 and CHOP are both transcription factors that translocate to the nucleus when activated during ER stress, and CHOP becomes activated only when the UPR becomes pro-apoptotic [47]. Nuclear immunoreactivity to XBP-1 and pro-apoptotic CHOP therefore indicate activation of the UPR (Fig. 5A). Expression of mutant cyclin F significantly increased the proportion of cells with nuclear XBP1 immunoreactivity, compared to cells expressing WT (cyclin F^{S621G}: 2-fold; cyclin F^{S195R}: 2.2-fold) or mCherry only (EV) (cyclin F^{S621G}: 5.9-fold; cyclin F^{S195R}: 6.3-fold) (Fig. 5B, C). Similarly, significantly more cells with nuclear immunoreactivity to CHOP were observed in populations expressing mutant cyclin F

compared to cells expressing WT (S621G: 1.7-fold; S195R: 1.9-fold) or EV (cyclin F^{S621G}: 5.9-fold; cyclin F^{S195R}: 6.6-fold; Fig. 5D, E). To note, compared to mCherry, expression of WT cyclin F resulted in significantly more cells with nuclear immunoreactivity to CHOP, but not to XBP1. These results indicate that ALS/FTD-associated mutations in cyclin F induce ER stress in neuronal cells.

To confirm the findings obtained in cell lines, ER stress was next examined in primary cortical neurons expressing cyclin F by immunocytochemistry, where nuclear reactivity to CHOP indicated activation of the UPR (Fig. 5F). Quantification demonstrated that significantly more primary neurons expressing mutant cyclin F displayed nuclear immunoreactivity to CHOP (S621G: 1.7-fold; S195R: 2.2-fold) compared to WT or mCherry (EV) cells (Fig. 5G). Hence, ALS/FTD-associated mutations in cyclin F activate ER stress in primary cortical neurons, confirming the results obtained in cell lines.

ALS/FTD mutant cyclin F induces fragmentation of the Golgi

Fragmentation of the Golgi apparatus results following induction of cellular stress, including ER and oxidative stress [48, 49], and when ER-Golgi trafficking is impaired [23, 50–52]. Hence, perturbation of ER-Golgi transport and induction of ER stress by mutant cyclin F implies that Golgi fragmentation is also induced. Therefore, we next assessed whether the Golgi apparatus was fragmented in cells expressing mutant cyclin F (Fig. 6). The morphology of the Golgi was examined by immunocytochemistry using an anti-GM130 antibody. In SH-SY5Y (Fig. 6A) and HEK293T (**Supplementary Fig. 2A**) cells expressing EV or Cyclin F^{WT}, the Golgi displayed its typical morphology of continuous stacked membranous vesicles. However, in mutant cyclin F^{S621G} and cyclin F^{S195R} expressing HEK293T cells and SH-SY5Y cells, the Golgi apparatus was fragmented, displaying multiple disconnected elements or tubular-vesicular clusters [53]. Quantification revealed that the percentage of cells displaying fragmented Golgi was significantly increased in cellular populations expressing mutant cyclin F^{S621G} (HEK293T: 1.5-fold; SH-SY5Y: 2-fold) or cyclin F^{S195R} (HEK293T: 1.6-fold; SH-SY5Y: 1.9-fold), compared to WT, EV or UT cells (**Supplementary Fig. 2B** and Fig. 6B). We also examined Golgi fragmentation in the GM130-immunostained HEK293 cells prepared for the VSVG^{ts045} assay (Fig. 1). Quantification revealed that significantly more mutant cyclin F^{S621G} cells displayed fragmented Golgi compared to WT (2.6-fold) or EV cells (13.2-fold change, **Supplementary Fig. 3A**) to a similar degree in each case ($57.3 \pm 8.6\%$, compared to $64.6 \pm 1\%$ in Fig. 6). Hence Golgi fragmentation was present in cells in which VSVG^{ts045} secretion was impaired, confirming that inhibition of ER-Golgi secretion correlates with fragmentation of the Golgi.

To further confirm these results and to provide a more unbiased quantification method, the surface area covered by the Golgi fragments was next assessed. Golgi stacks can be dispersed (mini-stacks) or completely disassembled, hence the surface area covered by the Golgi also indicates fragmentation [48]. Quantification of this area revealed a significant increase in the area covered by fragmented Golgi in cells expressing cyclin F^{S621G} (1.9-fold) and cyclin F^{S195R} (2.2-fold) mutants compared to cyclin F^{WT} expressing cells and other controls (Fig. 6C). Similarly, we also examined the area covered by Golgi

fragments in the GM130-immunostained HEK293 cells prepared for the VSVG^{ts045} assay (Fig. 1). Quantification revealed that a significant increase in the area covered by fragmented Golgi was present in cells expressing cyclin F^{S621G} compared to WT (2.6-fold) or EV cells (13.5-fold, **Supplementary Fig. 3b**) again to a similar degree (57.4%, compared to 64.7% in Fig. 6). Hence inhibition of ER-Golgi secretion correlates with fragmentation of the Golgi. Together these results demonstrate that mutant cyclin F induces Golgi fragmentation in neuronal cells.

To further confirm the above results, we next examined mouse cortical primary neurons expressing mutant cyclin F and controls for Golgi fragmentation. Immunocytochemistry of primary neurons expressing cyclin F was first performed using an anti-GM130 antibody to examine the morphology of the Golgi apparatus (Fig. 6D). Significantly more neurons expressing mutant cyclin F displayed Golgi fragmentation compared to those expressing WT (cyclin F^{S621G}: 2.2-fold; cyclin F^{S195R}: 2.9-fold) and mCherry (EV) (cyclin F^{S621G}: 6.3-fold; cyclin F^{S195R}: 8.1-fold) (Fig. 6E). Hence, ALS/FTD-associated mutations in cyclin F induce Golgi fragmentation in primary cortical neurons, confirming the results obtained in cell lines.

ALS/FTD mutant cyclin F induces cell death

ER stress induces apoptosis when prolonged or severe, and Golgi fragmentation is also associated with apoptosis [54, 55]. Hence cellular death was next analysed by flow cytometry following Sytox Blue staining, in SH-SY5Y cells expressing cyclin F (Fig. 7A, B). Quantitative analysis demonstrated significantly more dead cells in populations expressing mutant cyclin F^{S621G} compared to UT cells (3.7-fold) and those expressing cyclin F^{WT} (1.4-fold) or mCherry (3.8-fold), indicating that mutant cyclin F^{S621G} expression induces cell death.

This was next examined in primary neurons, where the presence of condensed nuclear morphology indicated the induction of apoptosis, as previous [44, 56]. Examination of the nuclei and quantitative analysis of primary neurons expressing cyclin F demonstrated that the percentage of neurons undergoing apoptosis was significantly increased in populations expressing mutant cyclin F compared to those expressing cyclin F^{WT} (cyclin F^{S621G}: 1.9-fold; cyclin F^{S195R}: 1.8-fold) or mCherry (cyclin F^{S621G}: 7.9-fold; cyclin F^{S195R}: 7.3-fold) (Fig. 7C, D). Hence, these data confirm that ALS/FTD-cyclin F mutants induce apoptosis.

Discussion

This study demonstrates that ALS/FTD-associated mutations in cyclin F perturb ER homeostasis by inhibiting two key functions; ERAD and transport of proteins from the ER to the Golgi apparatus. Consistent with these observations, Golgi fragmentation, ER stress, and induction of apoptosis was also detected in cell lines and mouse cortical primary neurons expressing mutant cyclin F. Together these data imply that dysfunction to the ER is an important process in neuronal degeneration induced by mutant cyclin F, thus providing novel insights into *CCNF*-associated ALS/FTD.

ER-Golgi transport is a vital gateway to the endomembrane system because one third of all proteins transit through the ER-Golgi compartments before reaching their final cellular locations [18, 57]. Our results imply that ALS/FTD-associated p.S621G and p.S195R mutations in cyclin F perturb the first stage of ER-Golgi transport: the budding of COPII vesicles from the ER. The COPII coat is composed of five separate proteins, including Sec23 and Sec31, and its assembly is essential for the formation of transport vesicles on the cytosolic face of the ER membrane. Curvature of the ER membrane, concentration of cargo and vesicular release then results. Defective COPII vesicles are known to inhibit secretion [40] and here we detected several abnormalities in COPII in mutant cyclin F cells. Less COPII and less vesicular cargo were present in ER-derived membranes obtained from mutant cyclin F expressing cells compared to controls. Furthermore, the Sec31-clusters (representing one or more ER-exit sites) were smaller in these cells, implying that either fewer COPII vesicles were formed overall or that the same number of COPII vesicles were present, but each contained less Sec31. Typical COPII vesicles are 60–70 nm in diameter, and it was not possible to resolve individual COPII vesicles or ER-exit sites using the methods used here. Furthermore, whilst Sec23 levels correlate with the load of protein cargo [40], here the levels of Sec23 were comparable to cargo load, suggesting that the defect is related to aberrant COPII vesicles themselves rather than the incorporation of protein cargo. Hence, these results imply that there is a defect in the formation of COPII vesicles and/or ER-exit sites in mutant cyclin F expressing cells.

COPII vesicles are normally too small to transport some secreted macromolecules, including procollagen fibrils, pre-chylomicrons, and pre- very low-density lipoproteins (VLDLs). However, ubiquitination of Sec31 by the CUL3-KLHL12 complex allows for the generation of large vesicles to facilitate the transport of these bulky cargo, thus controlling the traffic of COPII vesicles [40, 58]. Calcium-dependant control of CUL3-KLHL12 also ubiquitinates lysine residues on Sec31 [40, 59]. Similarly, deubiquitylation of Sec31 by USP8 antagonizes the formation of large procollagen-containing carriers [60]. The exact site of Sec31 ubiquitination may not be crucial for regulation of COPII vesicle trafficking, but it is a signal to recruit other effectors to assemble the coat or to regulate its catalytic activity. We previously demonstrated that mutant cyclin F^{S621G} dysregulates ubiquitination at Lys48, resulting in disruption of biological networks responsive for cellular survival and maintenance [11, 13]. Here, we demonstrate that in cells expressing mutant cyclin F, ubiquitination of Sec31 was reduced and bulk secretion of large proteins (> 100kDa) was inhibited, although the secretion of proteins smaller than this was unchanged. This together with inhibition of ER-Golgi trafficking in these cells implies that aberrant ubiquitination of Sec31 impairs the formation of COPII vesicles, forming smaller ER-exit site clusters and inhibiting the transport of large cargoes, which would ultimately impact neuronal functions. Future studies are therefore warranted to confirm this possibility and to identify the specific large cargoes whose secretion is inhibited by mutant cyclin F. Mutations in the genes encoding Sec23A [61] or Sar1B [62] lead to the accumulation of large proteins such as collagen, or lipid particles into the ER [61] which causes Skull-Lenticular-Sutural Dysplasia [61] and Chylomicron Retention Disease [62]. This highlights the importance of the integrity of COPII vesicles in the regulation of protein secretion.

Collagen forms the major structural component of the extracellular matrix and it is the most abundant cargo for COPII vesicles [60]. This is not surprising given that collagen constitutes up to 30% of the total protein mass of a typical organism. It was previously believed that fibrillar collagens are absent in the brain [63]. However, neurons are now known to express collagen [64, 65], and an increasing number of studies highlight key roles for collagen in both the PNS and CNS [66]. Whilst this area remains poorly understood, it is clear that expression and deposition of collagen in the nervous system is highly dynamic and tightly controlled, and is implicated in regulation of axonal outgrowth and synaptic differentiation [67]. Furthermore, some types of collagen are regulated in response to stress and are dysregulated in several neurological conditions, including Alzheimer's diseases [66]. regulated in conditions. COL6

Ubiquitination also regulates ERAD because poly-ubiquitin chains on ERAD substrates are remodelled at several stages during ERAD [68, 69]. Interestingly, we also detected impairment of ERAD in cells expressing mutant cyclin F. ERAD normally removes terminally misfolded proteins from the ER lumen or membrane, targeting them for degradation to membrane-embedded E3 ligase complexes, where they undergo ubiquitination on their cytosolically exposed protein domains. Hence, misfolded proteins in the ER would not be degraded efficiently in cyclin F-associated ALS/FTD, leading to their accumulation in the ER. This would further induce ER stress and disrupt ER homeostasis. These mechanisms may all combine, thus exacerbating ER stress and further perturbing ER homeostasis. Consistent with activation of pro-apoptotic CHOP during ER stress, we also demonstrate that expression of ALS/FTD cyclin F mutants induces neuronal death. This finding is also consistent with apoptotic death observed in the transient fish models overexpressing S621G cyclin F [13].

Impairment of ER-Golgi and other forms of transport has been previously described in ALS [23, 70–73], in cells expressing mutant SOD1 [23], mutant TDP-43 [23], mutant FUS [23] or mutant ubiquilin2 [31], and these events were previously linked to ER stress [23]. ER stress has now been widely implicated in ALS and mutations in *SOD1* [44], *TARDBP* [74], *FUS* [75], *OPTN* [76–78], *VCP* [79], *UBQLN2* [31], *VAPB* [80, 81] and *C9orf72* [82] all induce ER stress. Previously it has been established that mutant forms of SOD1 and TDP-43 induce ER stress from the cytoplasm [70, 71, 83], or from the cytoplasmic face of the ER [23] respectively, by inhibiting ER-Golgi transport [23]. Preliminary experiments examining whether cyclin F is present in the ER were inconclusive, raising the question of how ER stress is induced in ALS/FTD. As ALS mutant cyclin F mislocalizes to the cytoplasm where it promotes cytoplasmic aggregation of TDP-43 [84], it is possible that mutant cyclin F induces ER stress from the cytoplasm by inhibiting ER-Golgi transport. However, we cannot rule out the possibility that mutant cyclin F may directly induce ER stress from the ER itself, although mutant cyclin F aberrantly ubiquitinates Sec 31, which is also localised in the cytoplasm.

Golgi fragmentation is also a well-described event in ALS [24, 53, 85] where the Golgi apparatus undergoes morphological changes, resulting in disruption of its characteristic ribbon-like structure [85, 86]. Fragmentation of the Golgi apparatus has been described in sporadic ALS patients [87] and before disease onset in SOD1^{G93A} mice [88], prior to neuromuscular denervation and axon retraction [89]. It is also a feature of other neurodegenerative diseases [85] but it has not been previously described for mutant cyclin F. In this study, we demonstrate that ALS/FTD-associated mutations in cyclin F trigger

Golgi fragmentation. The proper organization of the Golgi depends on efficient bidirectional ER-Golgi vesicle transport [86, 90] and both membrane flow and cargo load influence its structure and function. Hence, blocking the export of cargo-containing ER carriers [91, 92] or depleting cargo receptors [93] results in Golgi fragmentation and some of the tubulo-vesicular Golgi clusters can further fuse with the ER, increasing ER stress [94]. In addition, inhibition of intra-Golgi trafficking or vesicle transport from the Golgi to the plasma membrane can result in Golgi fragmentation, if prolonged [50–52]. Hence, in this study, it is possible that the inhibition of ER-Golgi transport by mutant cyclin F triggers Golgi fragmentation and ER stress. However, there are many possible cellular stressors than induce Golgi fragmentation so the directionality of these links cannot be conclusively established.

Conclusion

In summary, this study identifies novel cellular mechanisms triggered by ALS/FTD-associated mutant cyclin F. In Fig. 8, we provide one hypothetical model to illustrate the cellular events triggered by mutant cyclin F, based on the findings of this study. However, it is also possible that ER stress or Golgi fragmentation is the upstream trigger, which would subsequently trigger impairment of ER-Golgi transport and impede ERAD given that these events are all closely related. Alternatively, it is possible that the cellular events detected in this study result from a combination of these defects. Further studies are therefore required to probe the directionality of these links.

Abbreviations

ALS	
Amyotrophic lateral sclerosis	
BSA	
Bovine serum albumin	
CUL1	
cullin-1	
COPII	
Coat protein complex II	
ER	
Endoplasmic reticulum	
ERAD	
Endoplasmic reticulum associated degradation	
ERES	
ER exit sites	
EV	
Empty vector	
FALS	
Familial ALS	

FTD
Frontotemporal dementia
NLS
Nuclear localization signals
PFA
Paraformaldehyde
SALS
Sporadic ALS
SKP1
S-phase kinase-associated protein 1
SEM
standard error of the mean
UPR
Unfolded protein response
UPS
Ubiquitin proteasome system
UT
untransfected
VSVG
vesicular stomatitis viral glycoprotein
WT
Wild-type

Declarations

Ethical Approval

All husbandry and experimental procedures were performed in compliance with the Animal Ethics Committee, Macquarie University, NSW, Australia (ARA 2017/030) and the Internal Biosafety Committee, Macquarie University (NLRD 5201401007 and 5974-52019597412350).

Consent to participate

Not applicable

Consent for publication

All authors discussed results, provided feedback, and approved the final manuscript.

Availability of data and material

The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing financial interests.

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Author contributions

J.D.A: conceived and directed the project. A.M.G.R., V.S. and J.D.A.: designed the experiments. A.M.G.R. and V.S.: performed the VSVG assay; A.M.G.R. analysed the results; A.M.G.R.: performed and analysed budding assay with the help of M.B.; A.M.G.R.: performed and analysed immunoprecipitation experiments with the help of S.L.R. and A.L.; A.M.G.R.: performed silver stain of bulk proteins with the help of M.V., C.J. and S.S.; A.M.G.R.: performed mouse neuronal primary cultures, transfection and immunohistochemistry with the help of A.K. A.M.G.R: performed and analysed ER stress, ERAD, Golgi fragmentation experiments. A.M.G.R. and V.S.: performed and analysed cell death experiments. A.M.G.R. and J.D.A: wrote the manuscript. A.M.G.R, L.O., K.L.W., S.Y., I.P.B., R.S.C., J.D.A.: revised the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

ALS/FTD mutant cyclin F interferes with ER to Golgi trafficking. (A) Schematic diagram illustrating domain structure of cyclin F showing the locations of the p.S621G mutation in the PEST domain and of the p.S195R mutation between the F-box and the cyclin domain. (B) Western blotting of cyclin F in lysates prepared from untransfected mouse HEK293 cells (UT) or cells expressing wild-type (WT) or mutant cyclin F in pmCherryC1 vector (EV: empty vector). (C) Relative intensity of cyclin F to β -Actin in B. Mean $\pm$

SEM, symbols represent 2 independent experiments, one-way ANOVA, ns, non-significant. (D) Experimental paradigm: cells expressing VSVGts045 for 24h were incubated at 40°C overnight to misfold VSVGts045, leading to its accumulation in the ER. When cells are placed at the permissive temperature (32°C), VSVGts045 refolds and transits to the Golgi apparatus within 30 min. (E) Immunocytochemistry of Calnexin in SH-SY5Y cells co-expressing GFP-tagged VSVGts045 (VSVG) and mCherry-tagged empty vector (EV), wild-type (WT) or cyclin FS621G, after incubation at 40°C and 32°C. Scale bar = 5 µm. (F) The degree of co-localisation of VSVGts045 with Calnexin was quantified using Mander's coefficient. (G) Immunocytochemistry of GM130 in undifferentiated SH-SY5Y cells co-expressing GFP-tagged VSVGts045 and mCherry-tagged EV or WT or cyclin FS621G, after incubation at 40°C and 32°C. Scale bar = 5 µm. (H) The degree of colocalisation of VSVGts045 with GM130 was quantified using Mander's coefficient. (F, H) Graphs represent mean ± SEM. Symbols represent 3 independent experiments, two-way ANOVA (factors "temperature" and "genotype") followed by a post-hoc Tukey test for multiple comparisons; ns, non-significant, ***p<0.001 vs cells expressing UT and EV, ###p<0.001 vs cells expressing cyclin FWT.

Figure 2

ALS/FTD mutant cyclin F perturbs the budding of COPII vesicles. (A) Immunocytochemistry of Sec31 in SH-SY5Y cells expressing GFP-tagged VSVGts045 (VSVG), after incubation at 40°C and 32°C. Scale bar = 5 μm. (B) Schematic diagram depicting the experimental procedure used for the cell-free vesicle budding reactions. Briefly, VSVGts045 was trapped in the ER by incubating HEK293T cells overnight at 40°C and ER-vesicle budding was reconstituted in vitro in perforated cells expressing EV or cyclin F by incubating

cells at 32°C with rat liver cytosol and an ATP regenerating system. Budded vesicles were collected by centrifugation. (C) Western blotting of cyclin F, VSVGts045 and Sec23 in lysates from UT or cells expressing EV, cyclin FWT or mutant, before ER-derived vesicles were isolated. β -Actin was used as a loading control for protein expression. (D) Relative intensity of VSVGts045 to β -Actin (normalized to UT) in C. (E) Relative intensity of Sec23 to β -Actin (normalized to UT) in C is similar between all groups ($p>0.05$). (F) Budded vesicle preparations were subjected to Western blotting using anti-VSVG and anti-Sec23 antibodies. β -Actin was used as a loading control for protein transfer and expression. UT, untransfected cells, EV, empty vector expressing mCherry only. (G) Relative intensity of VSVGts045 in blots in F normalised to β -Actin and control untransfected cells (UT). (H) Relative intensity of Sec23 in blots in F normalised to β -Actin and control untransfected cells (UT). (D, E, G, H) Graphs represent mean $\pm$ SEM. Symbols represent 3 independent experiments, one-way ANOVA followed by a post-hoc Tukey test for multiple comparisons, ns, non-significant, * $p<0.05$ and ** $p<0.01$ vs cells expressing UT and EV.

Figure 3

ALS/FTD mutant cyclin F perturbs ER-exit sites and interferes with protein secretion. (A) Fluorescent confocal airyscan microscopy images of HEK293 cells following immunocytochemistry for Sec31 and Hoechst staining in untransfected cells (UT) or cells expressing mCherry only (EV), mCherry-tagged WT or mutant Cyclin FS621G for 48h. Left panel: scale bar = 5 μ m. Right panel: Higher magnification images acquired with Airyscan of the area delimited by white squares on images on left panel. Scale bar = 0.5

μm . Each dot represents Sec31-positive ER exit sites. (B) Diameter of ER-exit site clusters calculated from images obtained in A (right panel). For EV, WT and Cyclin FS621G: symbols represent ERES cluster diameter calculated from $n=30$ cells from 3 independent experiments, $***p<0.001$, one-way ANOVA. (C) Proportion of HEK293T cells with ER-exit site diameters below 90 nm; between 90 and 120 nm; and over 120 nm. Mean $\pm$ SEM; symbols represent $n=3$ independent experiments, $**p<0.01$ difference vs UT, EV and WT, one-way ANOVA, followed by post-hoc Tukey test. ER exit-site diameter was quantified from 500+ ERES per group from HEK293T cells from 3 independent experiments. The total number of ER-exit sites quantified in each group was UT: $n=1,827$; EV: $n=2,114$; WT: $n=1,722$; S621G: $n=1,746$. (D) Immunoprecipitation (I.P.) of FLAG-tagged Sec31 using an anti-FLAG antibody in lysates prepared from cells co-expressing Sec31 and either EV or cyclin F. FLAG-tagged Sec31 was not expressed in untreated samples (UT). Western blotting was performed using anti-FLAG, anti-Sec31 and anti-ubiquitin antibodies. Left panel: input (10 μg of proteins). Right panel: I.P. (E) Relative intensity of ubiquitin to Sec31 of blots in A. (F) Western blotting using an anti-Sec31 antibody in lysates prepared from HEK293T cells expressing empty vector (EV), wild-type (WT) or mutant cyclin FS621G. β -Actin was used as a loading control for protein expression. (G) Relative intensity of Sec31 to β -Actin (normalized to UT) in C is similar between all groups ($p>0.05$). (H) Silver stained SDS-PAGE gel of total proteins secreted into the conditioned medium of untransfected (UT) cells, or cells expressing empty vector (EV) or cyclin F (72 h post-transfection). (I) Relative intensity of total protein bands in the conditioned medium in E. (J) Relative intensity of protein bands > 100 kDa in the conditioned medium in E. (B, C, E, G, I, J) The graph represents mean $\pm$ SEM, one-way ANOVA. Symbols represent $n = 2-4$ independent experiments; one-way ANOVA followed by a post-hoc Tukey test for multiple comparisons; ns, non-significant, $*p<0.05$ and $**p<0.01$ vs UT, EV, $\#p<0.05$ and $###p<0.001$ vs WT.

Figure 4

ALS/FTD mutant cyclin F S621G impedes ERAD. (A) Fluorescent ERAD substrates used to monitor ERAD. Wild-type Venus and mutant ddVenus C-terminal, in which Asn was substituted for Asp at position 82 (SS-ddVenus), were fused to a well-known ERAD substrate, the unstable null Hong Kong variant of alpha-1-antitrypsin (NHK-Venus; NHK-ddVenus)^{32,33}. The SS-ddVenus construct contains an ER-targeted signal sequence (K^b-SS) fused to dd-Venus. (B) The system is based on glycosylation and deglycosylation of Venus protein. Removal of a glycan by the cytosolic enzyme Peptide:N-glycanase (PNGase) results in deamidation of the glycosylated Asn, converting it to an Asp residue. The fluorescence results from protein that both reaches the ER and also retrotranslocates to the cytosol. Both glycosylation and deglycosylation (ER entry and exit) are required for fluorescence³³. (C) Representative fluorescent microscopy images of SH-SY5Y cells expressing empty vector (EV) and cyclin F with ERAD substrates (arrows). NHK fused to Venus was used as a control for cell transfection. Scale bar = 10 μ m. (D-F) The proportion of cells co-expressing CcNF with NHK-Venus (D), NHK-ddVenus (E) or SS-ddVenus (F) relative to the total number of mCherry-fluorescent cells was quantified. Graphs represent median

mean $\pm$ SEM. Symbols represent three independent experiments, *** p <0.001 difference vs EV; # p <0.05, ### p <0.001 difference vs WT, one-way ANOVA followed by a post-hoc Tukey test for multiple comparisons.

Figure 5

ALS/FTD mutant cyclin F induces ER stress (A) Schematic diagram representing the UPR pathway. Misfolded proteins in the ER accumulate, induce ER stress and activate the UPR. UPR modulators PERK

and IRE1 are activated by dimerization followed by phosphorylation, inducing activation of the UPR transcription factor XBP1, resulting in production of its spliced form (sXBP1). Under long-term ER stress, the UPR pro-apoptotic pathway is triggered, leading to the activation of the transcription factor CHOP and expression of apoptotic UPR genes⁴⁵. PERK, protein kinase R-like endoplasmic reticulum kinase; CHOP, C/EBP-homologous protein; IRE1 α , inositol-requiring protein 1 α ; XBP1, X-box binding protein 1. (B) Representative fluorescence microscopy images of HEK293 cells expressing mCherry-tagged cyclin F, following immunocytochemistry for XBP1s and Hoechst staining in cells expressing EV, WT or mutant cyclin F. Arrows: cells with nuclear XBP1 immunoreactivity. Scale bar = 10 μ m. (C) Proportion of cells with nuclear XBP1s immunoreactivity was quantified. (D) Representative fluorescence microscopy images of HEK293T cells expressing mCherry-tagged cyclin F, following immunocytochemistry for CHOP and Hoechst staining in cells expressing EV, WT or mutant cyclin F. Arrows: cells with nuclear CHOP immunoreactivity. Scale bar = 10 μ m. (E) Proportion of cells with nuclear CHOP immunoreactivity was quantified. (F) Representative fluorescence microscopy images of mouse primary cortical neurons expressing mCherry-tagged cyclin F, following immunocytochemistry for CHOP and Hoechst staining in neurons expressing EV, WT or mutant cyclin F. Arrows: neurons with nuclear CHOP immunoreactivity. Scale bar = 10 μ m. (G) The proportion of neurons with nuclear CHOP immunoreactivity was quantified. (C, E, G) Graphs represent mean $\pm$ SEM. Symbols represent 3-6 independent experiments, (C, E) n = 50+ cells per group, (G) n = 10-30 neurons per group; one-way ANOVA followed by a post-hoc Tukey test for multiple comparisons; ns, non-significant, ***p<0.001 vs UT and EV; #p<0.05, ##p<0.01 and ###p<0.001 vs cells expressing cyclin FWT. Nuclei are defined with a white outline.

Figure 6

ALS/FTD mutant cyclin F induces Golgi fragmentation (A) Fluorescent confocal microscopy images of EV or cyclin F, following immunocytochemistry for GM130 and Hoechst staining, of SH-SY5Y cells expressing EV or cyclin F. Arrows: Golgi fragments. Scale bar = 10 μ m (B) The proportion of cells with fragmented Golgi in A was quantified. (C) The area covered by Golgi fragments was measured in each cell. (D) Fluorescent confocal microscopy images of empty vector (EV) or cyclin F, GM130 and Hoechst in

mouse primary cortical neurons expressing EV or cyclin F. Arrows: Golgi fragments. The dashed white line delimits the outline of the neuron. Scale bar = 5 μ m. (E) The proportion of primary neurons with fragmented Golgi was quantified. (B, C, E) Graphs represent mean $\pm$ SEM. Symbols represent 3 independent experiments, (B, C) n = 50+ cells per group from, (E) n = 10-30 neurons per group; one-way ANOVA followed by a post-hoc Tukey test for multiple comparisons; ns, non-significant, **p<0.01 and ***p<0.001 vs UT and EV; #p<0.05 and ##p<0.01 vs cells expressing cyclin FWT.

Figure 7

ALS/FTD mutant cyclin F induces cellular death (A) Flow cytometry analysis of SH-SY5Y cells expressing mCherry-tagged cyclin F after staining with Sytox Blue, a marker for dead cells. UT, untransfected, EV, empty vector, WT, wild-type. (B) Quantification of Sytox Blue positive cells. (C) Representative fluorescent microscopy images of mouse cortical primary neurons expressing cyclin F, stained with Hoechst. Condensed nuclei (arrows), indicative of neuronal death, are present in cells expressing mutant cyclin F. Scale bar = 10 μ m. (D) The proportion of cells with condensed nucleus fragments was quantified. (B, D) Graphs represent mean $\pm$ SEM. Circles represent n = 3-4 independent experiments, one-way ANOVA, followed by a post-hoc Tukey test for multiple comparisons, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.001$ compared to UT and EV; # $p < 0.05$ and ## $p < 0.01$ compared to cyclin FWT.

- Pathological events in ALS**
- ① Atypical ER exit sites
 - ② ER to Golgi transport defect
 - ③ ERAD dysfunction
 - ④ Golgi fragmentation
 - ⑤ ER stress

Figure 8

Schematic diagram based on the findings of this study, illustrating a possible mechanism to explain of how mutant cyclin F impairs ER homeostasis in neuronal cells, leading to apoptotic cell death in ALS. Misfolded cyclin F triggers the formation of abnormal ER-derived vesicles (1), which inhibits ER-Golgi transport (2), ERAD and proteasomal function (3), triggering Golgi fragmentation (4), ER stress (5) and apoptotic cell death.

Supplementary Files

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