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2 BioID analysis of the cyclin F interactome reveals that ALS-variant cyclin F  
3 alters the homeostasis of paraspeckle-associated proteins

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36 ***Highlights***

37 • Previously, we identified missense mutations in *CCNF* that are linked to  
38 Amyotrophic lateral sclerosis/Frontotemporal dementia (ALS/FTD) and have shown  
39 that a single mutation in cyclin F can cause defects to major protein degradation  
40 systems in dividing cells.  
41 • Cyclin F has very few known interaction partners, many of which have roles in cell  
42 cycle progression. Accordingly, we used BioID and mass spectrometry to identify  
43 novel binding partners of cyclin F that may reveal insight into the role of cyclin F in  
44 neurodegeneration.  
45 • Mass spectrometry and bioinformatic studies demonstrate that cyclin F interacts  
46 with several RNA binding proteins. This includes the essential paraspeckle  
47 proteins, RBM14. Notably, this interaction could be validated by standard  
48 immunoprecipitations and immunoblotting. Cyclin F could also be found to interact  
49 with a series of essential proteins which form the paraspeckle complex.

50

51       • We further evaluated the effect of cyclin F(S621G) on the homeostasis of these  
52           novel interaction partners in primary neurons in response to a known paraspeckle  
53           inducer, MG132. Notably, we demonstrate significant defects in the homeostasis of  
54           RBM14 and SFPQ, but not NONO, when cyclin F carries an S621G mutation.

55       • Unlike other paraspeckle proteins, RBM14 levels have not previously been  
56           reported in the post-mortem brain and spinal cord of ALS patient post-mortem  
57           tissue. Here, we note significant defects in the homeostasis of RBM14 in the post-  
58           mortem tissue of ALS patients.

59

60       **Abstract**

61       *Background:* Previously, we identified missense mutations in *CCNF* that are causative of  
62           familial and sporadic amyotrophic lateral sclerosis (ALS) and frontotemporal dementia  
63           (FTD). *CCNF* encodes for the protein cyclin F, a substrate recognition component of the  
64           E3-ubiquitin ligase,  $SCF^{cyclin\ F}$ . We have previously shown that mutations in *CCNF* cause  
65           disruptions to overall protein homeostasis; causing a build-up of ubiquitylated proteins (1)  
66           as well as defects in autophagic machinery (2).

67

68       *Methods:* Here, we have used an unbiased proteomic screening workflow using BioID, as  
69           well as standard immunoprecipitations to identify novel interaction partners of cyclin F,  
70           identifying the interaction between cyclin F and a series of paraspeckle proteins. The  
71           homeostasis of these new cyclin F interaction partners, RBM14, NONO and SFPQ were  
72           monitored in primary neurons using immunoblotting. In addition, the homeostasis of  
73           RBM14 was compared between control and ALS/FTD patient tissue using standard IHC  
74           studies.

75

76 *Results:* Using BioID, we found over 100 putative interaction partners of cyclin F and  
77 demonstrated that cyclin F closely associates with a number of essential paraspeckle  
78 proteins, which are stress-responsive proteins that have recently been implicated in ALS  
79 pathogenesis. We further demonstrate that the turnover of these novel binding partners  
80 are defective when cyclin F carries an ALS/FTD-causing mutation. In addition the analysis  
81 of RBM14 levels in ALS patient post-mortem tissue revealed that RBM14 levels were  
82 significantly reduced in post-mortem ALS patient motor cortex and significantly reduced in  
83 the neurons of spinal cord tissue.

84

85 *Conclusion:* Overall, our data demonstrate that the dysregulation of paraspeckle  
86 components may be contributing factors to the molecular pathogenesis of ALS/FTD.

87

88 **Keywords:** **BioID, cyclin F, paraspeckles, RBM14, amyotrophic lateral sclerosis,**  
89 **frontotemporal dementia, proteomics, ubiquitylation, homeostasis.**

90

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101 **Background**

102 Amyotrophic lateral sclerosis (ALS) is typically a late-onset neurodegenerative disease  
103 characterised by the selective degeneration of upper and lower motor neurons of the  
104 cerebral cortex, brainstem and spinal cord. It is the most common form of motor neurone  
105 disease (MND) with poor prognosis and limited treatment options. A proportion of ALS  
106 patients also develop clinical or subclinical frontotemporal dementia (FTD) and  
107 pathological and genetic overlap is now recognised, indicating that they represent a  
108 spectrum of disease (3). Approximately 5-10% of ALS patients carry an autosomal  
109 dominant genetic mutation. Familial mutations have been reported in over 30 genes  
110 including *SOD1* (4, 5), *VCP* (6), *TARDBP* (7), *FUS* (8, 9), *OPTN* (10), *SQSTM1* (11),  
111 *UBQLN2* (12), *MATR3* (13) and *TBK1* (14, 15). Identification of these genes has drawn  
112 attention to protein clearance pathways, proteins that accumulate within insoluble  
113 cytoplasmic inclusions and defects in RNA processing in disease pathogenesis. Recently,  
114 we identified several novel missense mutations in *CCNF* in patients with ALS/FTD (1).  
115 *CCNF* encodes for cyclin F, a 786 amino acid protein that forms part of the multi-protein  
116 Skp1-Cul1-F-Box (SCF<sup>cyclin F</sup>) E3 ligase that is known to regulate cell cycle progression  
117 through timely ubiquitylation of substrates to regulate their homeostasis through  
118 proteasomal degradation (16).

119

120 We have previously reported that a familial ALS/FTD mutation in cyclin F (denoted cyclin  
121 F<sup>S621G</sup>) alters the ubiquitylation activity of SCF<sup>cyclin F</sup>, leading to the accumulation of  
122 ubiquitylated proteins (1). In addition, we have also shown that the activity of cyclin F may  
123 be regulated by post-translational modifications and that the loss of a phosphorylation site  
124 causes aberrant ubiquitylation activity (2). Ultimately this leads to defects in bulk

125 degradation processes and an upregulation in caspase-mediated cell death pathways  
126 (17).

127

128 Currently, there are few known interaction partners of cyclin F. These proteins are  
129 generally associated with cell-cycle function, including substrates such as ribonucleoside-  
130 diphosphate reductase subunit M2 (RRM2) (18), nucleolar and spindle-associated protein  
131 1 (NuSAP) (19), centriolar coiled-coil protein of 110 kDa (CP110) (20), cell division control  
132 protein 6 homolog (CDC6) (21), stem-loop binding protein (SLBP) (22), exonuclease 1  
133 (exo1) (23) and fizzy-related protein homolog (Fzr1) (24). In addition, known interaction  
134 partners of cyclin F include Skp1 (forming part of the ubiquitin ligase complex), b-myb (25)  
135 and CKII (26). Given that the interaction partners of cyclin F that have been reported to  
136 date are predominantly involved in cell-cycle regulation, it is not immediately obvious how  
137 cyclin F<sup>S621G</sup> might trigger neurodegeneration in non-dividing neurons. Therefore, we  
138 hypothesised that there are other interaction partners of cyclin F that may help to  
139 understand the processes that may become defective in non-dividing cells.

140

141 BiOID can be used to identify protein interaction partners and proteins in close proximity  
142 (~10 nm radius) (27) to the protein of interest using an engineered biotin-ligase, BirA\* (28,  
143 29). An advantage of using BiOID over standard immunoprecipitation (IP) methods, is the  
144 ability to identify transient, low abundance interaction partners as well as proteins that are  
145 not soluble in standard IP buffers (30). In recent years, BiOID has been used to identify  
146 novel binding partners of a number of proteins including lamin A (31) and E-cadherin (32),  
147 ZO-1 (33), TDP-43 and fragmented TDP-43 (34). In addition, BiOID has been utilized to  
148 identify substrates of β-TrCP 1 and 2 (35).

149

150 In this study, we have used BiolD followed by mass spectrometry (MS) to characterise the  
151 interactome of cyclin F. In doing so, we identified more than 100 putative interaction  
152 partners of cyclin F, including a group of RNA binding proteins that are also essential  
153 paraspeckle proteins. Previously we have demonstrated that an ALS-causing mutation in  
154 cyclin F causes defects in major protein degradation systems, thus we evaluate the  
155 dysregulation of these proteins in primary neurons and in ALS patient tissue.

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159 **METHODS**

160 *Plasmids and Cloning*

161 Expression constructs encoding wild type and S621G *CCNF* cDNA fused to an N-terminal  
162 mCherry fluorophore were used as described previously (1). Wild type and S621G *CCNF*  
163 cDNA fused to a C-terminal Flag-tag was also cloned into a pcDNA 3.1 vector. BirA\* alone  
164 or BirA\* in frame with cyclin F was cloned into pcDNA5/FRT/TO. Constructs encoding  
165 RBM14-HA were cloned into a pcDNA3.1 vector.

166

167 *Cell culture*

168 Human Embryonic Kidney Cells (HEK293) and HEK293 Flp-In T-Rex cells were grown  
169 and maintained in Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich)  
170 supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich).  
171 Plated cells were grown and maintained in a humidified incubator held at a constant  
172 temperature of 37°C, with 5% CO<sub>2</sub>. All cell lines were tested for mycoplasma prior to  
173 experimental work using the MycoAlert Mycoplasma Detection Kit (Lonza).

174

175 In order to generate stably-transfected cell lines, HEK293 Flp-In T-Rex cells (Thermo)  
176 were double-transfected using constructs encoding Flp-recombinase (pOG44) as well as  
177 constructs encoding BirA<sup>\*</sup>-cyclin F using Lipofectamine 2000 (Thermo) according to the  
178 manufacturer's instructions. After 48 hours, cells were selected with 100 µg/mL  
179 Hygromycin (InvivoGen) and 15 µg/mL Blasticidin (InvivoGen). In order to ensure the cells  
180 were stably-transfected and that transgene expression could be induced, tetracycline  
181 (Sigma-Aldrich) was added to cell culture media at a final concentration of 0.1µg/mL for  
182 18-24 hours. Tetracycline-dependent gene expression was monitored using standard  
183 immunoblotting procedures.

184

185 *Primary cell culture*

186 Primary mouse cortical neurons were cultured as previously described (36). Briefly, brains  
187 were obtained from embryos on embryonic day 16.5. Cerebral hemispheres were sub-  
188 dissected, digested in trypsin at 37°C and homogenized using fire-polished glass pipettes  
189 into single cell suspension. Cells were seeded out at 5 million cells per 10 cm dish in  
190 medium containing 10% FBS/high glucose DMEM (Life Technologies). Medium was  
191 changed 2 hours post seeding and cells were subsequently maintained in Neurobasal  
192 medium supplemented with Glutamax and B27 supplement (Life Technologies).

193

194 *Proximity-labelling in live HEK293 Flp-In T-Rex cells*

195 Stably transfected HEK293 Flp-In T-Rex cells were grown and maintained in DMEM  
196 supplemented with 10% FBS and 100 µg/mL Hygromycin (InvivoGen) and 15 µg/mL  
197 Blasticidin (InvivoGen). Once cells reached 70% confluency, expression of BirA<sup>\*</sup> or BirA<sup>\*</sup>-  
198 cyclin F (wild-type and CCNF variants) was induced by adding 0.1 µg/mL of tetracycline  
199 (Sigma-Aldrich) to cell culture media. In order to biotinylate proteins in proximity to the

200 transgene, 50  $\mu$ M of biotin (Sigma-Aldrich) was simultaneously added to the culture  
201 media. After 18-24 hours, cells were washed with PBS and harvested into ice-cold PBS.  
202 Harvested cells were washed twice with ice-cold PBS and centrifuged at 2000xg for 10  
203 minutes at 4°C. Washed cell pellets were snap frozen at -80°C until further use.

204

205 *Total cell lysis*

206 For total cell lysis, frozen cell pellets were first defrosted on ice and resuspended in ice-  
207 cold modified RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1mM EDTA, 1mM  
208 EGTA, 0.1% SDS, 0.5% Sodium deoxycholate, pH 7.4) containing appropriate amounts of  
209 protease and phosphatase inhibitor cocktails (Roche). Cells were incubated in RIPA buffer  
210 for 15 minutes on ice with intermittent vortexing before probe sonication using a Sonic  
211 Ruptor 250 at 50% power and pulser settings set to 30%. Lysates were subject to a total  
212 of 10 pulses each before centrifugation at 14,000xg for 20 minutes at 4°C. The  
213 supernatant containing cellular proteins was aliquoted and stored at -80°C until further  
214 analysis.

215

216 *Biotin pull-downs*

217 Cleared lysates containing biotinylated proteins in modified RIPA buffer were incubated  
218 with 30  $\mu$ L of pre-washed streptavidin-coated magnetic beads (Thermo Fisher) for 3 hours  
219 at 4°C whilst rotating. In order to isolate biotinylated proteins from the complex mixture, a  
220 magnetic rack was used to isolate magnetic beads. Isolated magnetic beads were washed  
221 5 times in modified RIPA buffer. Captured biotinylated proteins were eluted by  
222 resuspension in Laemmli sample buffer (BioRad), containing NuPAGE Sample Reducing  
223 Agent (Invitrogen) and were boiled at 95°C for 10 minutes. The eluents were prepared for  
224 1D SDS-PAGE as described below.

225 *Immunoprecipitations*

226 HEK293 cells were transfected with constructs encoding mCherry-cyclin F, Flag-cyclin F  
227 or RBM14-HA using Lipofectamine 2000 according to the manufacturer's instructions.  
228 Transfected cells were harvested after 24 hours and cell pellets were resuspended in NP-  
229 40 lysis buffer (1 % (v/v) Nonidet P-40 in Tris-buffered saline (TBS), 2 mM EDTA,  
230 cOmplete protease inhibitor cocktail and phosSTOP (Roche)). The resuspended cells  
231 were vortexed, then probe sonicated (10 seconds, Setting 3, Branson Sonifier 450). The  
232 cell lysates were centrifuged at 14, 000×g for 30 minutes to remove cell debris. For  
233 immunoprecipitations, approximately 500 µg of cellular protein was incubated with 1 µg of  
234 flag antibody or 20 µL of RFP-Trap®\_MA (Chromotek). The magnetic beads were  
235 collected using a magnet and washed three times in NP-40 lysis buffer. For western blot  
236 analysis, beads were resuspended in 1x Loading buffer (BioRad) containing 1x reducing  
237 reagent (NuPage) and boiled at 95°C for 10 minutes.

238

239

240 *SDS PAGE and Immunoblotting*

241 Equal amounts of protein were separated on a 4-12% Bis-Tris SDS PAGE gel. Proteins  
242 were transferred onto a nitrocellulose membrane using a Bio-Rad Trans-blot Turbo semi-  
243 dry transfer cell (1.3 A, 25 V, 7 mins). The membranes were blocked in 3% skim milk  
244 powder in PBST for half an hour prior to incubation with primary antibody overnight at 4°C  
245 or 1 hour at RT. Primary antibodies used in this study were: rabbit polyclonal anti-cyclin F  
246 (1:300; cat# sc-952, Santa Cruz Biotechnology), mouse monoclonal anti-mCherry (1:300;  
247 cat# 632543, Clonetech), mouse monoclonal anti-β-actin (Abcam, dilution- 1:12,000,  
248 catalogue #ab6276-101), mouse monoclonal anti-GAPDH (Proteintech, dilution-  
249 1:10,000), mouse monoclonal anti-α-tubulin (Sigma-Aldrich, dilution- 1:1000, catalogue

250 #T5168), rabbit polyclonal anti-RBM14 (Sigma-Aldrich, dilution- 1:1000, catalogue  
251 #HPA006628), mouse monoclonal anti-PSPC1 (Santa Cruz, dilution- 1:500, catalogue  
252 #sc-374367), mouse monoclonal anti-PSF (Santa Cruz, dilution- 1:1000, catalogue #sc-  
253 101137), rabbit polyclonal anti-Matrin 3 (Proteintech, dilution- 1:1000, catalogue #12202-  
254 2-AP).

255

256 After incubation with primary antibodies, the membranes were washed in PBS-T three  
257 times for 10 minutes before fluorescently labelled IRDye 800CW Goat Anti-Rabbit IgG  
258 Secondary Antibody (1:15,000; LI-COR) or fluorescently labelled IRDye® 680RD Goat  
259 anti-Mouse IgG Secondary Antibody (1:15,00; LI-COR) secondary antibodies was added  
260 for 30 minutes at RT. Immunoblots were imaged using a Li-Cor Odyssey imaging system  
261 at the appropriate wavelength.

262

#### 263 *In-gel trypsin digestion*

264 Equal amounts of protein were loaded and separated on a 4-15% SDS-PAGE gel  
265 (BioRad). The resulting gel was briefly incubated in fixing solution (50% methanol, 10%  
266 acetic acid) and proteins were stained with Coomassie blue R250 until protein bands were  
267 visible. The gel was then left to destain overnight in Destain solution (25% methanol).  
268 After destaining, protein bands were excised from gels into 5 fractions. Gel fractions were  
269 then cut into smaller pieces (~1 mm<sup>2</sup>) and further destained with 50% methanol/50 mM  
270 ammonium bicarbonate (pH 8). Gel pieces were then washed and dehydrated in 50%  
271 acetonitrile (ACN)/50 mM ammonium bicarbonate for 10 minutes, then incubated with  
272 100% ACN until gel pieces were completely dehydrated. ACN was removed, and gel  
273 pieces were dried under vacuum centrifugation before being incubated with 10 mM  
274 dithiothreitol (DTT) in 50 mM ammonium bicarbonate (AmBiC) for 40 minutes at 37°C.

275 Excess DTT was removed before gel pieces were incubated with 25 mM iodoacetamide  
276 (IAA) in 50 mM ammonium bicarbonate for 40 minutes at room temperature in the dark.  
277 Gel pieces were then washed twice with 50% ACN/50 mM ammonium bicarbonate for 10  
278 minutes each time before the supernatant was removed and gel pieces were incubated in  
279 100% (v/v) ACN to dehydrate gel pieces as described earlier. Excess ACN was removed  
280 and gel pieces were left to dry.

281

282 Gel pieces were incubated with trypsin (12.5 ng/μl; proteomics grade, Sigma-Aldrich)  
283 diluted in 50 mM ammonium bicarbonate and incubated overnight at 37°C. After  
284 incubation, the supernatant was transferred into fresh tubes and acidified with formic acid  
285 (FA). The gel pieces were incubated in 50% ACN, 2% FA. Supernatants containing tryptic  
286 peptides were pooled and lyophilised. For desalting, peptides were resuspended in 0.1%  
287 FA and desalted using pre-washed and equilibrated C18 OMIX tips (Agilent). Once  
288 desalted, samples were again lyophilised and stored at -80°C until MS analysis.

289

290 Prior to mass spectrometry, lyophilised peptides were resuspended in 0.1% FA and bath  
291 sonicated for 20 minutes. The resuspended peptides were then centrifuged at 14, 000×g  
292 for 15 minutes to remove any insoluble debris, and the clarified peptides were analysed by  
293 LC-MS/MS. The peptide fractions were separated on an Ultimate 3000 nanoLC (Thermo  
294 Fisher Scientific) fitted with the Acclaim PepMap RSLC column (Thermo Fisher Scientific),  
295 making use of a 60 minutes gradient (2–95% v/v acetonitrile, 0.1% v/v formic acid)  
296 running at a flow rate of 300 nl/minute. Peptides eluted from the nano LC column were  
297 subsequently ionized into the Q Exactive™ Plus mass spectrometer (Thermo Fisher  
298 Scientific). The electrospray source was fitted with an emitter tip 10μm (New Objective,  
299 Woburn, MA) and maintained at 1.5 kV electrospray voltage. The temperature of the

300 capillary was set to 250°C. Precursor ions were selected for MS/MS fragmentation using a  
301 data-dependent “Top 10” method operating in FT-FT acquisition mode with HCD  
302 fragmentation. FT-MS analysis on the Q Exactive™ Plus was carried out at 70,000  
303 resolution and an AGC target of  $1 \times 10^6$  ions in full MS. MS/MS scans were carried out at  
304 17,500 resolution with an AGC target of  $2 \times 10^4$  ions. Maximum injection times were set to  
305 30 and 50 milliseconds respectively. The ion selection threshold for triggering MS/MS  
306 fragmentation was set to 25,000 counts and an isolation width of 2.0 Da was used to  
307 perform HCD fragmentation with normalised collision energy of 27.

308

309 *Bioinformatics and statistics*

310 The raw files were searched using Proteome Discoverer 2.4 software (Thermo Fisher  
311 Scientific) incorporating the Sequest search algorithm employing the *Homo sapiens*  
312 Uniprot FASTA databases. Peptide identifications were determined taking into account a  
313 20-ppm precursor ion tolerance and 0.1 Da MS/MS fragment ion tolerance for FT-MS and  
314 HCD fragmentation respectively. Peptide modifications were also considered whereby  
315 cysteine carbamidomethylation was considered a static modification. Variable  
316 modifications included methionine oxidation, asparagine and glutamine deamidation,  
317 lysine biotinylation, and acetylated N-terminal residues. Trypsin was set as the enzyme of  
318 use, allowing for three missed cleavages at the most. Data was also processed using a  
319 label-free quantitation (LFQ) workflow employing the Minora Feature node, making use of  
320 a Protein FDR validator node which estimates the false discovery rates at the protein level  
321 as well as a percolator node to estimate the FDR at the PSM level. Results were adjusted  
322 so that the final global FDR was less than 1% at the protein and peptide level. A *q*-value  
323 of 0.01 was required to validate protein identifications.

324

325 Statistical analyses were typically conducted using GraphPad Prism 8.2.1 software or  
326 Ingenuity Pathway Analysis (IPA). In GraphPad Prism, statistical analyses involved the  
327 use of a paired t-test. Comparisons were considered significant if the *p*-values were less  
328 than 0.05.

329

330 Statistically significant protein functions were identified using Ingenuity Pathway Analysis  
331 (IPA). Here a Right-Tailed Fisher's Exact Test was used. Results were considered  
332 statistically significant if the *p*-value was less than 0.05.

333

334 *Adeno-associated viruses (AAV)*

335 Vectors encoding full length human WT cyclin F or cyclin F carrying the S621G mutation  
336 (n-terminal V5-tagged) was cloned into a rAAV vector under the human synapsin  
337 promoter using the plasmid pAAV-hSyn-EGFP (gift from Bryan Roth; Addgene, #50465)  
338 as backbone and removing EGFP. The same vector with EGFP expression was used as  
339 control. Packaging of rAAV9 vectors were performed as previously described (37) using  
340 the capsid AAV9.PHP.B (38). Briefly, HEK293T cells in 15cm dishes were each  
341 transfected with 12.5 $\mu$ g of vector plasmid containing gene of interest, 25 $\mu$ g of pF $\Delta$ 6 and  
342 12.5 $\mu$ g of AAV rep-cap using PEI-max in IMDM (Sigma-Aldrich). Cells were harvested 48  
343 hours post-transfection by scraping and centrifuged at 350 $\times$ g for 30 minutes. The cell  
344 supernatant was subjected to polyethylene glycol (PEG) precipitation and cell pellet was  
345 further lysed using a freeze-thaw cycle and combined with the PEG mixture. After lysis  
346 with sodium deoxycholate and 3 rounds of freeze/thaw cycles, the supernatant was  
347 collected for purification in an OptiSeal tube (Beckman-Coulter) containing iodixanol  
348 layers (15%, 25%, 40%, 54%; Sigma-Aldrich). Purified virus was collected using a 19G  
349 syringe, inserted just below the 405 gradient and during dialyzed and concentrated using

350 Amicon Ultra-15 CFU with 100 kDa cutoff filter (Millipore). The virus was sterile filtered  
351 through a Spin-X 0.22  $\mu$ m centrifuge filter (Corning).

352

353 *AAV transduction into primary neurons*

354 For AAV transduction, cortical neurons were transduced with WT CCNF, CCNF S621G or  
355 EGFP AAV at MOI of 5000 on DIV 3. At 10 days *in vitro* (DIV), cells were treated with 0.2  
356  $\mu$ M of proteasome inhibitor, MG132, for 12 hours.

357

358 *Immunohistochemistry and microscopy*

359 Post-mortem paraffin-embedded cervical spinal cord sections from ALS patients (n=4) and  
360 controls (n=3) were obtained from the New South Wales Brain Bank Network. For  
361 immunohistochemical staining, tissue sections were heated at 70°C for 30 minutes,  
362 deparaffinized with xylene and rehydrated with a descending series of ethanol washes. To  
363 retrieve antigens, sections were boiled for 20 minutes in low pH buffer (pH 6.1; Dako, CA,  
364 USA). Endogenous peroxidase activity and non-specific binding were blocked by  
365 incubation with 3% hydrogen peroxide in methanol for 15 minutes followed by 5% normal  
366 goat serum (Vector Laboratories, CA, USA) with 0.1% TWEEN-20 in PBS for 1 hour.  
367 Sections were incubated at 4°C overnight with primary antibody rabbit anti-RBM14 (1:100,  
368 Sigma-Aldrich) and then at room temperature for 1 hour with biotinylated goat anti-rabbit  
369 IgG (Vector Laboratories). The avidin-biotin complex detection system (Vector  
370 Laboratories) with 3,3'-diaminobenzide as chromogen (Dako) was used to detect the  
371 immunoreactive signal. Nuclei were counterstained with hematoxylin before sections were  
372 dehydrated with an increasing series of ethanol washes followed by xylene. Sections were  
373 coverslipped using Di-N-Butyle Phthalate in xylene (DPX, Dako).

374

375 Tissue sections were visualized using the ZEISS Axio Imager 2 microscope and analysed  
376 using Fiji Image J. To quantify RBM14 neuronal nuclei staining, each image was first  
377 deconvoluted with the Image J 'H DAB' Deconvolution Macro (39). A region of interest  
378 (ROI) was drawn around the neuron nucleus and pixel intensity was scored using the IHC  
379 Profiler plugin categorizing overall RBM14 staining in the ROI as either high positive,  
380 positive, low positive or negative (40). Only neurons with a clear nucleus were included.  
381 The number of neurons with RBM14 expression in the high positive and positive zone or  
382 low positive and negative zone were plotted and analysed with Fishers' exact test  
383 (significance set to 0.0166667 after Bonferroni correction).

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400 **RESULTS**

401 *BioID identifies known and novel protein interaction partners of cyclin F*

402 In order to identify transient and low abundance interaction partners of cyclin F, we used a  
403 proximity-based biotinylation method known as BioID (31). Here, we first cloned cyclin F in  
404 frame with a modified biotin ligase (denoted BirA\*-cyclin F<sup>WT</sup>). In addition, we cloned a  
405 mutant of cyclin F<sup>LP/AA</sup> (which has previously been reported to stabilize the interaction  
406 between cyclin F and transiently interacting proteins) in frame with BirA\*, generating  
407 BirA\*-Cyclin F<sup>LP/AA</sup> (Figure 1a). In order to tightly control the expression of the fusion  
408 protein in cultured cells, we generated stably transfected HEK293 T-Rex Flp-In cell lines  
409 (Thermo). The T-Rex Flp-In system was selected as it ensures that only a single copy of  
410 the transgene is placed within the exact same insertion site in the host genome, whilst the  
411 expression of cyclin F, a cell cycle regulator, is controlled in a tetracycline-dependent  
412 manner.

413

414 To conduct the BioID experiments, transgene expression was firstly induced in HEK293  
415 Flp-In T-Rex cells using tetracycline. This is followed by addition of biotin to cell culture  
416 media for 24 hours. The resulting cells were lysed in harsh lysis buffer, before the  
417 biotinylated proteins were isolated using streptavidin-coated beads. These resulting  
418 proteins are then analysed by immunoblotting and mass spectrometry (Figure 1B). To  
419 begin BioID experiments that identify binding partners of cyclin F, we first confirmed that  
420 there was no leakage in either transgene expression or biotin-labelling, whilst initiation of  
421 cyclin F-BirA\* expression and the addition of biotin leads to the biotinylation of proteins  
422 (Figure 1C). To identify proteins in proximity to cyclin F, cells expressing either cyclin F-  
423 BirA\* or BirA\* alone were expressed in HEK293 Flp-In T-Rex cells. Here, we induced  
424 expression of the transgene with tetracycline, added biotin, and after 24 hours cells were

425 harvested. Biotinylated proteins were enriched using Streptavidin Magnetic Beads  
426 (Thermo) and prepared for immunoblotting and subsequent proteomic analysis. Notably,  
427 immunoblotting revealed that the biotinylation profile was greater in BirA\* only expressing  
428 cells (Figure 1D), which correlated with higher expression levels of BirA\* compared to  
429 BirA\*-cyclin F. Thus, prior to MS analysis we adjusted the input of isolated biotinylated  
430 proteins accordingly (Supplementary Figure 1).

431

432 We carried out an in-gel trypsin digestion of the biotinylated proteins followed by liquid  
433 chromatography-mass spectrometry (LC-MS/MS). In total, 918 proteins were identified,  
434 with 163 proteins found in cyclin F-BirA\* and cyclin F-BirA\* (LP/AA) combined, but not  
435 when biotin ligase was expressed alone (Figure 2A). The list of protein identifications was  
436 further filtered such that proteins were considered interaction partners of cyclin F if they: *i.*  
437 increased at least two-fold when comparing BirA\*-cyclin F to BirA\* and *ii.* were present in  
438 at least 2 out of 3 biological replicates of BirA\*-cyclin F expressing cells. The final list of  
439 high-confidence interactors of cyclin F yielded 119 proteins (presented in Supplementary  
440 Table 1). Within this list, we identified cyclin F and RING-box protein 1 (Rbx1). Rbx1 and  
441 cyclin F are both essential units of the Skp1-Cul1-Fbx<sup>(cyclin F)</sup> E3 ubiquitin ligase complex,  
442 confirming that the BiolD assay has biotinylated proteins within close proximity as  
443 expected. Casein kinase II (CKII), another previously identified binding-partner of cyclin F  
444 (26, 41), was also found in this list.

445

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449

450 *Bioinformatic pathway analysis identifies known and novel functions of cyclin F*

451 Next, Ingenuity Pathway Analysis (IPA) analysis was used to assign statistically significant  
452 molecular functions to proteins associated with cyclin F (Figure 2B). Consistent with  
453 known function of cyclin F, IPA unveiled a statistical enrichment of interaction partners  
454 with roles in ‘Cell Cycle Progression’ ( $p<3.51E-04$ ) (Figure 2C), as well as ‘DNA  
455 Replication, Recombination and Repair’ ( $p<1.59E-02$ ). Several novel molecular functions  
456 were also identified, one of which involves ‘RNA Post-Transcriptional Modification’ with  
457 specific functions of ‘Processing of mRNA’ ( $p=4.92E-07$ ), ‘Splicing of mRNA’ ( $p=1.41E-06$ ),  
458 ‘Processing of rRNA’ ( $p=1.82E-04$ ), ‘Unwinding of mRNA’ ( $p=1.56E-02$ ), ‘Annealing of  
459 hnRNA’ ( $p=1.56E-02$ ), ‘Processing of RNA’ ( $p=1.90E-09$ ) and ‘Splicing of RNA’ ( $p=2.70E-07$ ).  
460

461

462 We also used IPA to analyse the 111 proteins that were uniquely associated with cyclin  
463  $F^{LP/AA}$ , as this protein list includes stabilized interaction partners. In this list, we found a  
464 series of proteins which had roles in ‘RNA Damage and Repair’ ( $p=4.27E-10$ ), ‘Processing  
465 of RNA’ ( $p=1.38E-08$ ), ‘Processing of rRNA’ ( $p=7.98E-08$ ), ‘Splicing of RNA’ ( $1.83E-04$ ).  
466 Within the list of proteins with roles known to be involved in RNA metabolism, there were  
467 also a series of RNA binding proteins including TAF15, EWS and RBM14; proteins also  
468 known to form paraspeckles (Figure 3). Notably, IPA predicted changes in the  
469 homeostasis of these proteins to affect the expression of RNA, prompting further  
470 investigation into the relationship between cyclin F and these interaction partners.

471

472 *Cyclin F is closely associated with paraspeckle proteins*

473 Given that RBM14 is essential for building subnuclear paraspeckles (42), we validate the  
474 interaction between cyclin F and RBM14 using standard immunoprecipitation and

475 immunoblotting (Figure 4A). Here we noted that cyclin F(WT)-flag and cyclin F(S621G)-  
476 flag could co-immunoprecipitate with endogenous RBM14, with no effect from the  
477 mutation. In addition, both overexpressed cyclin F(WT)-flag and cyclin F(S621G)-flag  
478 could co-immunoprecipitate with RBM14-HA. Conversely, RBM14-HA could co-  
479 immunoprecipitate with both cyclin F(WT)-flag and cyclin F(S621G)-flag (Figure 4B).

480

481 Next we questioned whether cyclin F could also bind other essential components of the  
482 paraspeckle complex, NONO and SFPQ. Indeed, mCherry-cyclin F was found to  
483 immunoprecipitate with these essential paraspeckle components in addition to RBM14  
484 (Figure 4C). In all cases, both cyclin F<sup>WT</sup> and cyclin F<sup>S621G</sup> were able to co-  
485 immunoprecipitate with these paraspeckle components. Together the data validated the  
486 mass spectrometry data and further demonstrated that cyclin F interacted with protein  
487 components of the paraspeckle complex.

488

489 *Cyclin F<sup>S621G</sup> causes disruption of paraspeckle homeostasis in primary neurons*  
490 Previously it has been established that proteasome inhibitor, MG132 is able to initiate  
491 paraspeckle assembly and lead to elongation of the paraspeckle structure. During this  
492 time, paraspeckle protein levels remain largely consistent (43). To assess the effect of  
493 cyclin F<sup>S621G</sup> on paraspeckle regulation in response to MG132, we overexpressed cyclin  
494 F<sup>WT</sup>, cyclin F<sup>S621G</sup> or an empty vector in primary mouse cortical neurons by AAV infection,  
495 then collected protein lysates following treatment with 0.2  $\mu$ M of MG132 or a vehicle  
496 control (Figure 5A). Notably we observed that, in response to MG132 treatment, there was  
497 a significant increase in RBM14 of 1.54 fold (Figure 5B) as well as a significant increase in  
498 SFPQ levels of 1.21 fold-change (Figure 5C) in cyclin F<sup>S621G</sup> overexpressing cells  
499 compared to the wild-type control. There was no significant difference in the expression of

500 NONO in response to MG132 treatment (Figure 5D) suggesting that mutant cyclin F may  
501 lead to a disruption in the homeostasis of some essential paraspeckle components.

502

503 *RBM14 homeostasis is dysregulated in the motor cortex and spinal cord of ALS patients*  
504 RBM14 homeostasis has not previously been reported in patient postmortem tissue. To  
505 determine whether RBM14 levels are dysregulated in post-mortem ALS patient tissue, we  
506 measured RBM14 levels in motor cortex and spinal cord neurons via semi-quantification  
507 of immunohistochemical labeling. Since RBM14 is a known nuclear protein, we  
508 specifically compared RBM14 expression in neuronal nuclei from control and ALS patients  
509 (Table 2). In control spinal cord neurons, RBM14 showed either primarily nuclear staining  
510 or staining in both nuclei and cytoplasm. In comparison, in ALS patient tissues, there was  
511 a significant reduction of nuclear RBM14 in spinal cord neurons (Figure 6).

512

## 513 **DISCUSSION**

514 In this study, we have identified novel protein interactors of cyclin F using BiID coupled  
515 with mass spectrometry. We found that cyclin F was closely associated with paraspeckle  
516 proteins including RBM14, NONO, and SFPQ. Furthermore, we demonstrate that the  
517 homeostasis of RBM14 and SFPQ, essential components of the paraspeckle complex, is  
518 influenced by cyclin F and becomes defective when cyclin F carries an S621G mutation  
519 linked to ALS/FTD. Finally, we show yet another paraspeckle protein, RBM14, may be  
520 involved in ALS pathogenesis through the dysregulation of protein levels in post-mortem  
521 motor cortex and spinal cord of ALS patients.

522

523 We previously reported the identification of disease-causing variants in CCNF in familial  
524 and sporadic ALS/FTD patients and have reported defects in major protein degradation

systems in cells overexpressing cyclin F(S621G) (1). Given these known deficits, it is logical to predict that the role of cyclin F in ALS pathogenesis may be associated with defective protein degradation pathways. Given that many interaction partners of cyclin F were unknown, we undertook unbiased proteomic screening to identify interaction partners cyclin F. To take advantage of the T-Rex and Flp-In systems (allowing controlled copy number integration at the exact same site) we performed the BiOID assay in HEK293 cells, and in doing so, identified more than 100 high-confidence interaction partners. We acknowledge that many of these may not be relevant to motor neurons as their biological function is related to activities such as cell division. However, we did identify several proteins involved in RNA processing pathways that are likely to be relevant to ALS/FTD.

The BiOID assay identified a close association of cyclin F with a group of paraspeckle proteins. Further work revealed that an ALS-causing mutation in cyclin F leads to the defective homeostasis of essential paraspeckle proteins, RBM14 and SFPQ. Paraspeckles are a class of subnuclear bodies that form within the interchromatin space of mammalian cells (44). These RNA-protein structures form as RNA binding proteins interact with the long non-coding RNA (lncRNA), *NEAT1* (45). Alterations to paraspeckle assembly and function has important implications in the context of neurodegeneration as paraspeckles have a clear role in controlling gene expression (44). In particular, paraspeckles are known to regulate multiple cellular processes such as cell stress responses, cellular differentiation and viral infections (44). Therefore, disruption to paraspeckle assembly or function results in inability to rapidly transcribe stress-responsive proteins required for maintaining cellular viability. Notably, the formation of paraspeckles, and the dysregulation of this process, is emerging as a biological marker of ALS. For example, the assembly of paraspeckle proteins around *NEAT1\_2* has been reported in spinal motor neurons of early-stage ALS patients (46). In addition, compromised

551 paraspeckle formation has been identified in cell and animal models of FUSopathies, with  
552 mislocalised FUS resulting in neuronal inclusions of paraspeckle components (47). In both  
553 studies, the increased levels of paraspeckle assembly may represent a downstream,  
554 protective cellular response to stress. We now report a different type of possible  
555 involvement of paraspeckles in ALS pathogenesis. We show that RBM14 homeostasis is  
556 dysregulated in post-mortem brain and spinal cord of ALS patients. RBM14 has been  
557 shown to connect key paraspeckle subcomplexes, a function which requires the presence  
558 of its prion-like domain (42). Thus, the dysregulation of RBM14 (and potentially other  
559 paraspeckle proteins), a core paraspeckle protein, may impair paraspeckle  
560 assembly/function and leave motor neurons vulnerable to cellular stress and therefore  
561 more susceptible to neurodegeneration. Importantly, in this study, we have shown that  
562 RBM14 dysregulation occurs in the brain and spinal cord of patients, regardless of the  
563 presence of *CCNF* mutation, suggesting that the dysregulation of RBM14 homeostasis  
564 may be one contributing step in the multi-stage pathogenesis of ALS.

565 Of the more than 30 genes (and their corresponding protein products) that are now linked  
566 to ALS, two broad functional categories have emerged; protein-degradation pathways  
567 (indirectly because of the presence of abnormal protein aggregates, and directly through  
568 regulators of protein degradation such as cyclin F and ubiquilin-2) and RNA processing.  
569 However, the link between these two distinct groups of proteins remain poorly understood.  
570 Perhaps the strongest association to date is represented by TDP-43 and to a lesser extent  
571 FUS, which are both major constituents of intraneuronal aggregates, and their core  
572 function being associated with RNA processing (48). We now present a new and different  
573 linkage, demonstrating that cyclin F influences the homeostasis of key paraspeckle  
574 components. Future studies should look to identify the cellular processes, such as RNA  
575 processing in response to stress stimuli, that may become dysregulated due to the

576 reduction of RBM14 in the nucleus of affected motor neurons, as this may provide deeper  
577 insight into the underlying causes of ALS/FTD. Notably, this study also identifies RBM14  
578 dysregulation in sporadic cases of ALS. Together the data further link the dysregulation of  
579 the ubiquitin-proteasome system and RNA processing to the pathogenesis of ALS/FTD.

580

581 **CONCLUSIONS**

582 This study employed an unbiased proteomic screening assay which revealed that cyclin F  
583 interacts with several core proteins of the paraspeckle complex. Using  
584 immunoprecipitation, we confirmed the interaction between cyclin F and three paraspeckle  
585 proteins; RBM14, NONO and SFPQ. Notably, we demonstrate that the pathogenic cyclin  
586 F<sup>S621G</sup> variant disrupts the homeostasis of these proteins and their responsiveness to a  
587 stressor that stimulates paraspeckle formation. Finally, we report for the first time that  
588 RBM14 levels are dysregulated in brain and spinal cord of ALS patients relative to healthy  
589 patient controls. Collectively, these data suggest that cyclin F may influence stress  
590 responses through modulation of the paraspeckle complex, and that disruption in  
591 paraspeckle homeostasis may contribute to the molecular pathogenesis of ALS/FTD.

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598 **Abbreviations**

|     |          |  |
|-----|----------|--|
| 599 | AAV      | Adeno-associated viruses                         |
| 600 | ACN      | Acetonitrile                                     |
| 601 | AGC      | Automatic gain control                           |
| 602 | ALS      | Amyotrophic Lateral Sclerosis                    |
| 603 | AmBic    | Ammonium bicarbonate                             |
| 604 | BCA      | Bicinchoninic acid assay                         |
| 605 | BiOID    | Proximity dependent <i>Biotin Identification</i> |
| 606 | Bis-Tris | 1,3-bis(tris(hydroxymethyl)methylamino)propane   |
| 607 | BSA      | Bovine serum albumin                             |
| 608 | DIV      | Days <i>in vitro</i>                             |
| 609 | DMEM     | Dulbecco's Modified Eagle Medium                 |
| 610 | DMSO     | Dimethyl sulfoxide                               |
| 611 | DNA      | Deoxyribonucleic acid                            |
| 612 | DTT      | Dithiothreitol                                   |
| 613 | EDTA     | Ethylenediaminetetraacetic acid                  |
| 614 | EGFP     | Enhanced green fluorescent protein               |
| 615 | FA       | Formic acid                                      |
| 616 | FT-MS    | Fourier transform-mass spectrometry              |
| 617 | FBS      | Fetal bovine serum                               |
| 618 | FDR      | False discovery rate                             |
| 619 | FTD      | Frontotemporal Dementia                          |
| 620 | HCD      | Higher energy collision dissociation             |
| 621 | MS       | Mass spectrometry                                |
| 622 | IAA      | Iodoacetamide                                    |

|     |          |  |
|-----|----------|--|
| 623 | IgG      | Immunoglobulin G   |
| 624 | IMDM     | Iscove's Modified Dulbecco's Medium                        |
| 625 | IP       | Immunoprecipitation  |
| 626 | IPA      | Ingenuity Pathway Analysis                                 |
| 627 | LFQ      | Label-free quantitation                                    |
| 628 | MND      | Motor neurone disease                                      |
| 629 | MOI      | Multiplicity of Infection                                  |
| 630 | nanoESI  | Nanoelectrospray ionization                                |
| 631 | NHEJ     | Non-Homologous End Joining                                 |
| 632 | NP-40    | Nonidet P-40   |
| 633 | PBS      | Phosphate buffered saline                                  |
| 634 | PBST     | Phosphate buffered saline containing Tween-20              |
| 635 | PEG      | Polyethylene glycol  |
| 636 | PEI      | Polyethylenimine   |
| 637 | PSM      | Peptide-spectrum match                                     |
| 638 | RIPA     | Radioimmunoprecipitation assay buffer                      |
| 639 | ROI      | Region of interest   |
| 640 | SCF      | Skp1-Cul1-F-Box  |
| 641 | SDS      | Sodium dodecyl sulphate                                    |
| 642 | SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| 643 | UPS      | Ubiquitin-proteasome system                                |
| 644 |          |  |
| 645 |          |  |
| 646 |          |  |
| 647 |          |  |

648 **DECLARATIONS**

649 ***Ethics approval and consent to participate***

650 International, national, and/or institutional guidelines for the care and use of animals were  
651 followed. Ethics approval was also obtained for the use of human tissue.

652 ***Consent for publication***

653 Not applicable

654 ***Availability of data and material***

655 The mass spectrometry proteomics data have been deposited to the ProteomeXchange  
656 Consortium via the PRIDE partner repository with the dataset identifier PXD014163 and  
657 10.6019/PXD014163.

658 ***Competing interests***

659 The authors declare that they have no competing interests.

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665 ***Authors' Contributions***

666 R.C. and A.L. and S.L.R. conceptualized the project. S.L.R. conducted the BiOID studies,  
667 MS analysis, follow-up biochemical studies and wrote the manuscript. F.C. assisted with  
668 MS sample runs. A.D. assisted with stable cell line generation. S.Y. and N.G. generated  
669 lysates from patient tissue and conducted IHC studies using patient tissue. C.G.A. and  
670 Y.D.K. conducted primary neuron transduction and drug treatment. M.P.M., I.B., A.L.,

671 R.C., J.M.D., M.M., L.M.I., B.S. assisted with editing the manuscript. All authors read and  
672 approved the final manuscript.

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674

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678

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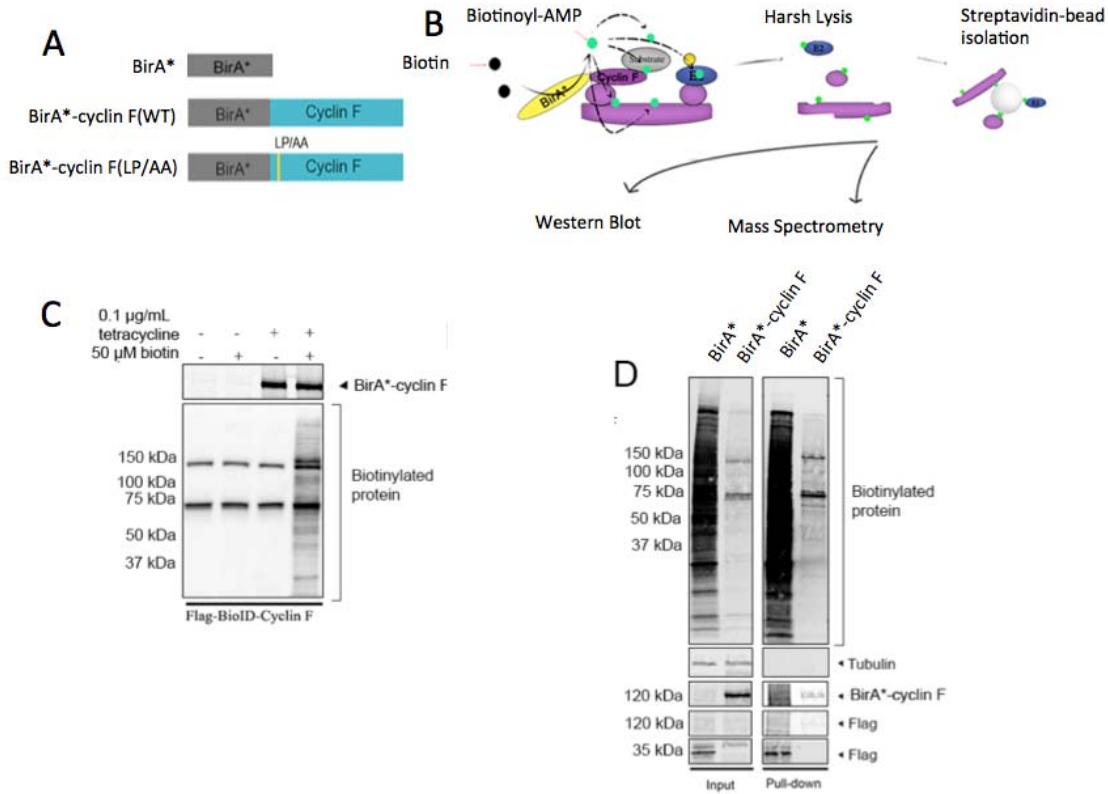
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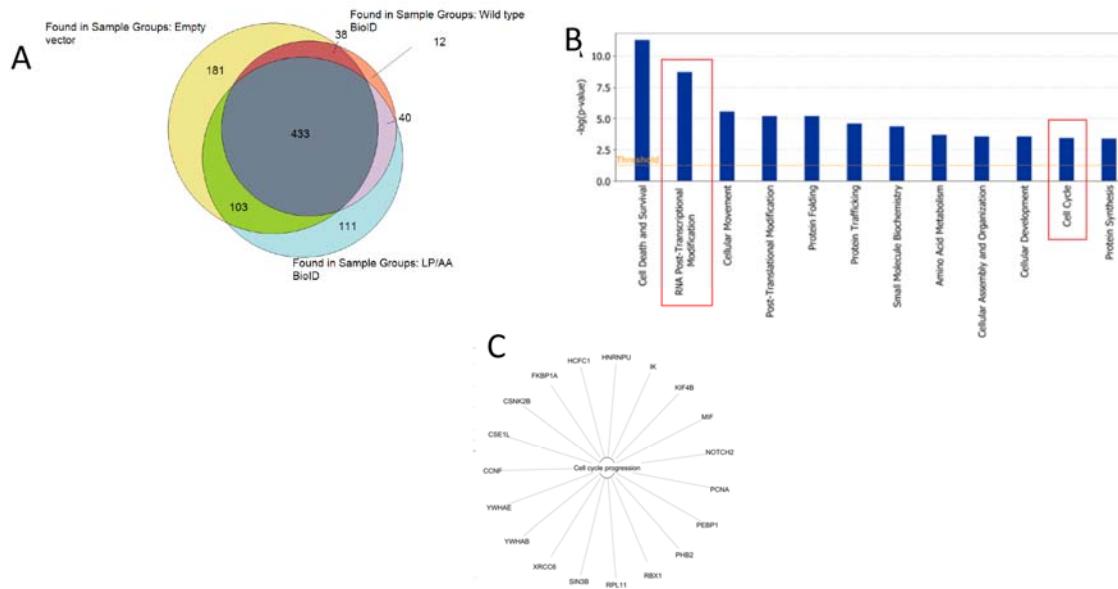
Table 1. Details of patient tissue

| Case                    | Tissue type                  | Age<br>(y) | Gender | PMI (hr) | Disease |
|-------------------------|------------------------------|------------|--------|----------|---------|
|                         |                              |            |        |          | onset   |
| Control                 | Motor cortex                 | 37         | Male   | 24       | N/A     |
| Control                 | Spinal cord and motor cortex | 61         | Male   | 30       | N/A     |
| Control                 | Spinal cord and motor cortex | 80         | Male   | 12       | N/A     |
| Control                 | Spinal cord                  | 75         | Male   | 34       | N/A     |
| SALS                    | Motor cortex                 | 81         | Male   | 70       | 80      |
| SALS                    | Motor cortex                 | 84         | Male   | 23       | 74      |
| FALS (C9orf72)          | Motor cortex                 | 60         | Male   | 99       | 58      |
| FALS (Unknown mutation) | Spinal cord and motor cortex | 54         | Female | 16       | 53      |
| SALS (C9orf72)          | Spinal cord                  | 65         | Female | 18       | 65      |
| SALS                    | Spinal cord                  | 71         | Male   | 12.5     | 70      |
| FALS (C9orf72)          | Spinal cord                  | 75         | Male   | 74       | 21.5    |

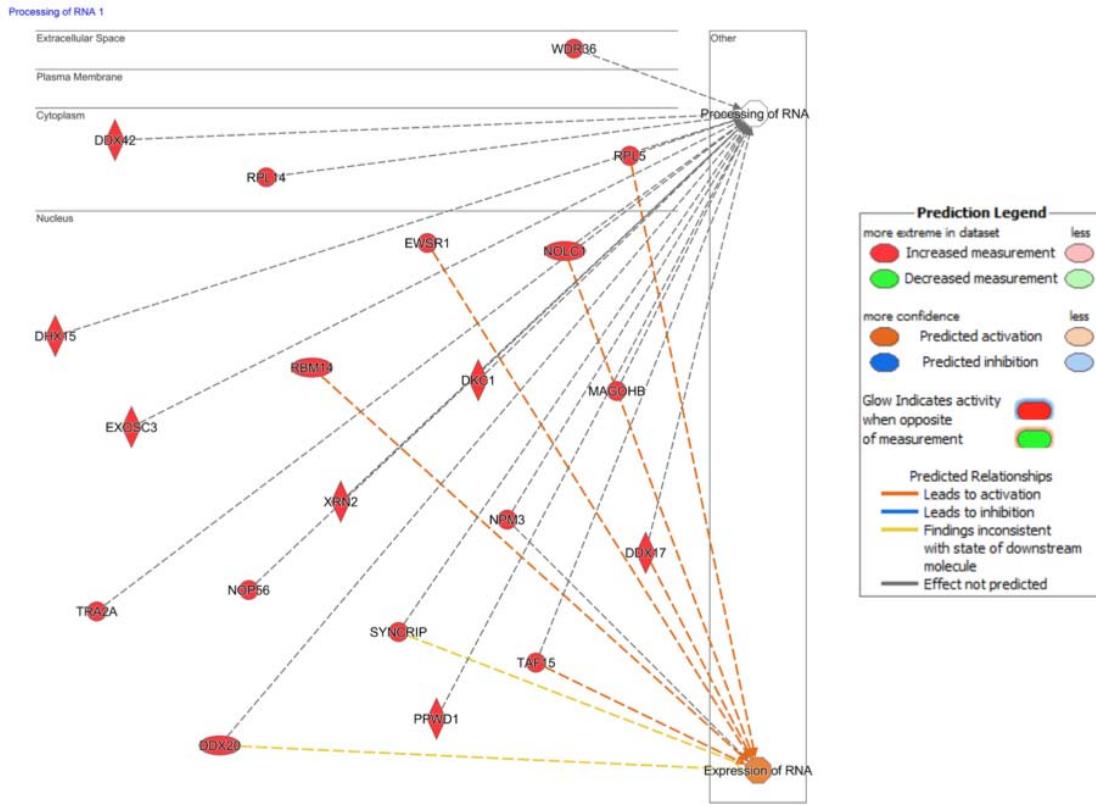




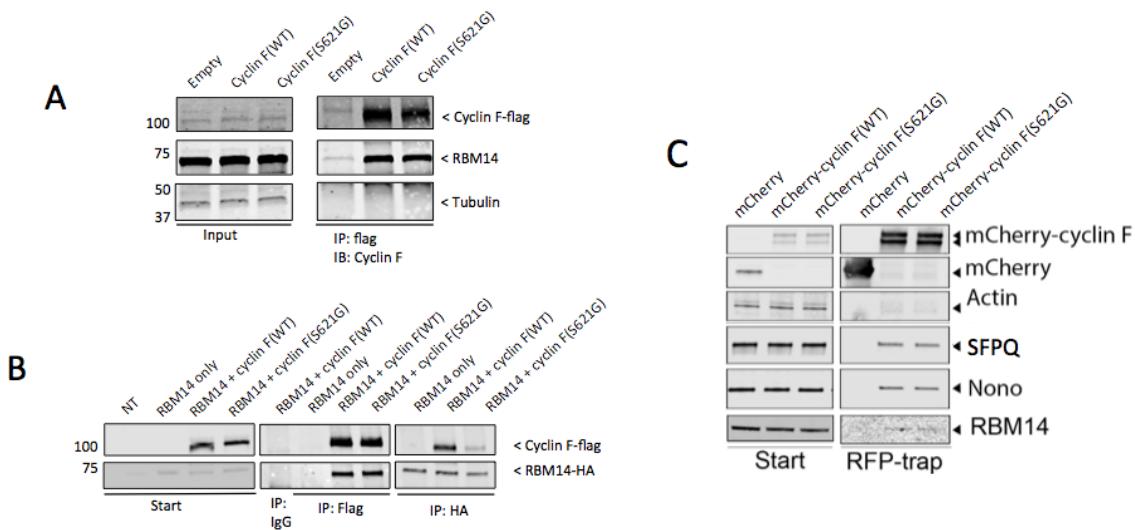
**Figure 1.** Identifying cyclin F interaction partners using BioID. **A.** BirA\* alone, BirA\*-cyclin F(WT) and BirA\*-cyclin F(LP/AA) were stably transfected into Flp-In T-Rex HEK293 cells. **B.** Schematic showing process of biotinylation by BirA\*, isolation of biotinylated proteins and identification by western blotting or mass spectrometry. **C.** Stably transfected HEK293 Flp-In cells were treated with tetracycline to induce gene expression. Addition of biotin led to the biotinylation of proteins in proximity to cyclin F, as detected by immunoblotting with fluorescently-tagged streptavidin (LiCor). **D.** The biotinylation profile of BirA\* alone or cyclin F-BirA\* before and after streptavidin-coated bead pull-downs.



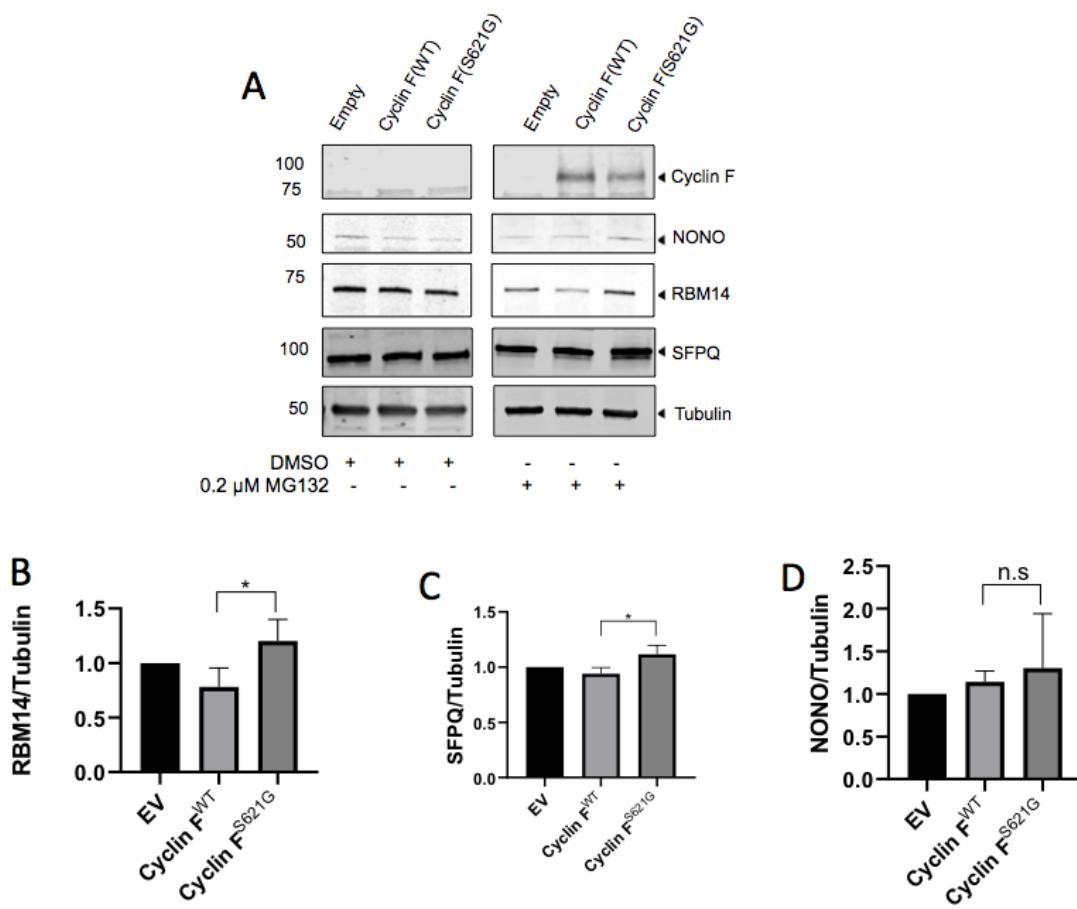
**Figure 2.** **A.** Proteomic analysis identified common and unique proteins biotinylated by BirA\* alone, BirA\*-cyclin F(WT) and BirA\*-cyclin F(LP/AA). **B.** Ingenuity Pathway analysis (IPA) of protein interaction partners showing top twelve statistically enriched molecular processes for cyclin F interaction partners. **C.** Proteins interaction partners involved in cell cycle progression.



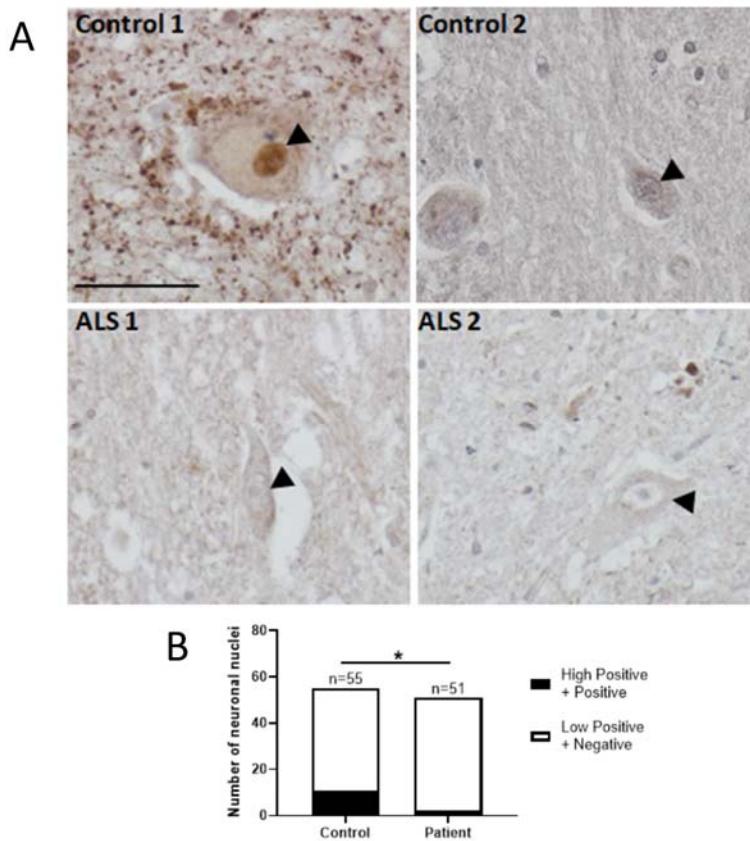
**Figure 3.** Protein interaction partners involved in RNA processing and expression along with subcellular localisation.



**Figure 4.** Cyclin F interacts with paraspeckle proteins. **A.** Flag-tagged cyclin F(WT), cyclin F(S621G) or an empty vector control were transfected into HEK293 cells. Anti-flag antibody was used to immunoprecipitate flag-tagged proteins. Eluates were evaluated by immunoblotting with the antibodies specified. **B.** Flag-tagged cyclin F(WT) or cyclin F(S621G) were co-transfected alongside RBM14-HA in HEK293 cells. Anti-flag or anti-HA antibody was used to immunoprecipitate flag-tagged or HA-tagged proteins as specified. Eluates were evaluated by immunoblotting with the antibodies specified. **C.** mCherry-cyclin F(WT) or mCherry-cyclin F(S621G) were transfected into HEK293 cells. An RFP-trap was incubated with lysates to immunoprecipitate mCherry-tagged proteins. Eluates were evaluated by immunoblotting using the antibodies specified.



**Figure 5.** Cyclin F<sup>S621G</sup> causes defective turnover of paraspeckle components in primary neurons. **A.** Primary neurons were transduced using constructs encoding cyclin F<sup>WT</sup>, cyclin F<sup>S621G</sup> or an empty vector control. Transduced neurons were treated with 0.2  $\mu$ M MG132 or a vehicle control for 24 hours before cells were lysed in RIPA buffer and analysed by immunoblotting using the antibodies as indicated. **B.** Densitometry of RBM14 upon MG132 treatment normalised to Tubulin (n=4, \*: $p<0.05$ ). **C.** Densitometry of SFPQ upon MG132 treatment normalised to Tubulin (n=4, \*: $p<0.05$ ). **D.** Densitometry of NONO upon MG132 treatment normalised to Tubulin (n=4, n.s; not significant).



**Figure 6.** RBM14 is reduced in the brain and spinal cord of ALS patients. **A.** Representative images of RBM14 immunohistochemical staining in control (n=3) and ALS patient (n=4) spinal cord tissues. Arrowheads indicate RBM14 staining in neuronal nuclei. Scale bar=50  $\mu$ m. **B.** Semi-quantification of RBM14 IHC staining showed a significant reduction of RBM14 in neuronal nuclei from ALS patients compared to controls. A total number of 55 control neurons and 51 patient neurons were analysed using Image J IHC Profiler Plugin (Fisher's exact test, \*:  $p<0.05$ ).