Title

CYFIP1 overexpression amplifies IL-6/STAT3 and IFN-gamma/STAT1 signaling: potential implications for neuroinflammation and autism spectrum disorder

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Abstract

Autism spectrum disorder (ASD) encompasses a group of neurodevelopmental disorders characterised by impaired social interaction, delayed language development, and repetitive or restrictive behaviours. While both genetic and environmental factors contribute to ASD, the specific molecular mechanisms underlying these interactions remain unclear. In a recent preprint, we showed that cytokine signaling may be dysregulated in chromosome 15q-duplication syndrome (Dup(15q)), one of the most common syndromic forms of ASD. Specifically, Dup(15q) induced pluripotent stem cells (iPSC)-derived neurons exhibit an amplified Signal Transducer and Activator of Transcription-3 (STAT3) response to Interleukin-6 (IL-6), a proinflammatory cytokine often upregulated in ASD. To identify which gene in the 15q region may be responsible for modifying cytokine signalling responses in Dup(15q), we focus on the *Cytoplasmic*

FMRP-Interacting Protein 1 (CYFIP1) gene, as increased *CYFIP1* dosage in Dup(15q) is associated with increased severity of neurobehavioral symptoms in individuals with Dup(15q) and *CYFIP1* dysregulation has been linked to altered expression of various genes involved in immunoregulatory signalling pathways. Here, we explore the effects of *CYFIP1* overexpression (*CYFIP1*-OE) on cytokine signaling, demonstrating that *CYFIP1*-OE in HEK293 cells modifies the expression and activity of several cytokine signaling-related transcription factors, including STAT3 and STAT1. Additionally, we use SH-SY5Y neuroblastoma cells to assess neuron-related phenotypes, showing that *CYFIP1*-OE alters IL-6-induced neurite outgrowth. These findings provide novel insights into how CYFIP1 may contribute to dysregulated cytokine responses in ASD, advancing our understanding of the molecular mechanisms that underlie neuroinflammatory processes in this disorder.

Keywords

Autism spectrum disorder, CYFIP1, Chromosome 15q-duplication syndrome, STAT3, IL-6, cytokine signalling, neuritogenesis, STAT1, IFN-γ

Background

Autism spectrum disorder (ASD) is a heterogeneous group of neurodevelopmental conditions in which both genetic variation and environmental exposures contribute to pathogenesis (1). Due to the highly heterogeneous nature of the disorder, both diagnosis and treatment remain challenging. Genetic factors, including both rare and common

49 variants, are implicated in ASD, with over 1,000 genes identified as potentially
50 contributing to its onset and development (2). In addition to genetic predisposition,
51 environmental risk factors, particularly maternal immune activation (MIA) and
52 neuroinflammation, have been recognised as key contributors to ASD pathogenesis (3,4).
53 Therefore, it is likely that the interaction between an individual's genetic background and
54 specific environmental exposures plays a crucial role in triggering the pathogenesis
55 underlying ASD (3). Consequently, elucidating the molecular mechanisms by which ASD-
56 associated genes can alter neurodevelopmental processes in response to environmental
57 stressors is critical for advancing our understanding of the disorder.
58
59 *Janus Kinase and Microtubule-Interacting Protein 1* (*JAKMIP1*) was previously identified
60 as a potential molecular link between syndromic forms of ASD and neuroinflammation
61 (5). *JAKMIP1* expression was commonly dysregulated in Fragile X syndrome (FXS) and
62 chromosome 15q-duplication syndrome (Dup(15q)), both genetic disorders often
63 comorbid with ASD (5). Furthermore, we discovered a role for JAKMIP1 in regulating
64 Interleukin-6 (IL-6)-induced Signal Transducer and Activator of Transcription-3 (STAT3)
65 signaling in neuronal cells, whereby deficiency of JAKMIP1 in SH-SY5Y neuroblastoma
66 cells impairs STAT3 activation and neuritogenesis in response to IL-6 stimulation (5).
67 These findings suggest that dysregulation of *JAKMIP1* in ASD may alter the ability of
68 neurons to respond to inflammatory cytokines, potentially leading to aberrations in
69 neuronal morphology.
70
71 To investigate whether *JAKMIP1* dysregulation in syndromic ASD could modify how cells
72 respond to cytokines, we previously assessed IL-6-induced STAT3 signalling in a

Dup(15q) induced pluripotent stem cell (iPSC)-derived neuronal model (6), as Dup(15q) is one of the most common forms of syndromic ASD, accounting for around 1% of ASD cases (7). Compared to a control iPSC line, we observed that *JAKMIP1* expression fluctuates in Dup(15q) iPSC and iPSC-derived cortical neurons (iNeurons), being downregulated in Dup(15q) iPSC and upregulated in iNeurons. Despite these opposing trends, we found that *STAT3* expression was consistently reduced in both the Dup(15q) iPSCs and iNeurons, indicating that Dup15q cells would likely display altered responses to IL-6. Curiously, though *STAT3* expression was reduced, IL-6-induced STAT3 activity was significantly enhanced in both Dup(15q) iPSCs and iNeurons. These findings suggest that unlike in the SH-SY5Y cells, JAKMIP1 is likely not regulating *STAT3* expression or activity in Dup(15q) and instead, another gene in the chromosome 15q region exerts stronger influence on STAT3 activity.

Several genes are encoded within the 15q region of chromosome 15, however, a gene of particular interest in relation to ASD and cytokine signalling is *Cytoplasmic FMRP-Interacting Protein 1* (*CYFIP1*), as studies have demonstrated that duplications (or even deletions) encompassing *CYFIP1* increase the severity of neurobehavioural phenotypes (8), and that *CYFIP1* expression is upregulated in the brains of individuals with Dup(15q) and ASD (9). Although CYFIP1 is most known for its roles in repressing neuronal protein translation as part of Fragile X Messenger Ribonucleoprotein (FMRP)-containing ribonucleoprotein complexes (10) and regulating actin polymerisation as a core component of the WAVE regulatory complex (WRC) (11), there is evidence to suggest that *CYFIP1* dysregulation may alter the expression of various genes involved in immunoregulatory signalling pathways (12). For example, a recent study profiling

97 transcriptomic changes in Dup(15q) stem cell-derived neurons with upregulated *CYFIP1*
98 expression suggested that increased dosage of chromosome 15q11.2 genes could alter
99 the expression gene targets of SMAD Family Member 3 (SMAD3), Nuclear Factor Kappa B
100 (NF-κB) or Interferon-Stimulated Response Elements (ISRE) (12). Moreover, another
101 study identified *CYFIP1* as a potential hub gene that may coordinate Transforming Growth
102 Factor Beta 1 (TGF-β1)/SMAD3 signalling (13). Therefore, we hypothesised that CYFIP1
103 may be responsible for the observed enhanced IL-6-induced activity in Dup(15q) iPSCs
104 and iNeurons (6).
105
106 This paper therefore explores the effects of increased *CYFIP1* expression (modelled by
107 *CYFIP1*-overexpression (OE)) on cytokine signalling. We provide the first evidence to
108 directly support a novel role for CYFIP1 in modulating cytokine signalling, demonstrating
109 that *CYFIP1*-OE in HEK293 cells alters the expression and activity of cytokine signalling-
110 related transcription factors, *STAT1* and *STAT3*. Taking this further, we show how
111 alterations in the STAT3 signalling pathway due to *CYFIP1*-OE affects IL-6-induced neurite
112 outgrowth in human SH-SY5Y neuroblastoma cells, illustrating that these findings
113 continue to be relevant in a more neuronal-relevant model.
114
115
116 **Methods**
117 **HEK293 and SH-SY5Y cell culture and treatment**
118 Human embryonic kidney (HEK)-293 cells (#CRL-1573™, *American Type Culture*
119 *Collection*) and human neuroblastoma cells (#CRL-2266™, *American Type Culture*
120 *Collection*) were thawed from frozen stocks stored in 10% dimethyl sulfoxide (#D2650-

121 5X5ML, *Sigma-Aldrich*®) at −80°C. HEK293 cells and SH-SY5Y cells were maintained in
122 10-cm diameter Petri dishes (#P7612-360EA, *Sigma-Aldrich*®) with 10 mL Dulbecco's
123 modified eagle medium/nutrient mixture F-12 with GlutaMAX (referred to henceforth as
124 DMEM/F-12) (#11524436, *Fisher Scientific*™) supplemented with 10% foetal bovine
125 serum (FBS) (#11550356, *Fisher Scientific*™), and incubated at 37°C, 5% CO_2, 95%
126 humidity. Cells were passaged at 70-80% confluency using 3 mL of pre-warmed TrypLE™
127 (#10718463, *Fisher Scientific*™) for 5 min and TrypLE™ was inactivated by dilution in an
128 equal volume of DMEM/F-12 + 10% FBS.
129
130 Cells were stimulated with 20 ng/mL IL-6 (#7270IL-025, *R&D Systems*) or 20 ng/mL
131 Interferon-γ (IFN-γ) (#285-IF-100, *R&D Systems*) diluted in DMEM/F-12 + FBS (or in
132 DMEM/F-12 supplemented with Retinoic Acid (RA) and Brain-Derived Neurotrophic
133 Factor (BDNF) for neurite tracing experiments). For all experiments using IL-6 or IFN-γ,
134 control cells were used which were treated with corresponding media not containing IL-
135 6 or IFN-γ. Cells were treated with IL-6 or IFN-γ for different amounts of time depending
136 on the purpose of the experiment. To assess STAT3 activation by Western blotting for
137 phosphorylated STAT3 (P-STAT3^{Y705}), cells were treated with IL-6 for 30 minutes. To assess
138 STAT3 or STAT1 transcriptional activity by Dual-Luciferase® Reporter (DLR™) assays, cells
139 were treated with IL-6 or IFN-γ for 18 hours post-transfection. To measure mRNA
140 expression of STAT3 or STAT1 responsive genes, cells were treated with IL-6 or IFN-γ for 4
141 hours. Finally, to assess IL-6-induced neurite outgrowth, cells were treated with IL-6 over
142 a 48-hour period of differentiation.
143
144 **HEK293 cell transfection**

145 24 hours prior to transfection, 300,000 HEK293 cells were seeded into Nunc cell-culture
146 treated 6-well plates (#10469282, *Fisher Scientific*™) in 3 mL DMEM/F-12 + 10% FBS. The
147 following day, HEK293 cells were transfected with either an RFP-T2A-CYFIP1 plasmid to
148 induce *CYFIP1*-overexpression (these cells are referred to as '*CYFIP1*-OE cells), or the
149 backbone RFP-T2A plasmid as a control (these cells are referred to as 'control cells')
150 using Lipofectamine™ LTX Reagent (#15338100, *Fisher Scientific*™) following
151 manufacturer instructions (i.e., final amount of 2.5 μg of plasmid and 5 μL of
152 Lipofectamine™ LTX per well). Media was replaced the following day. All experiments were
153 performed 72 hours post-transfection.
154
155 **SH-SY5Y cell transfection**
156 2,000,000 SH-SY5Y cells were transfected by Nucleofection™ (#V4XC-2012, *Lonza*
157 *Bioscience*) following manufacturer instructions with 5 μg of either a mixture of the RFP-
158 T2A backbone vector and pCAβ-YFP (encoding yellow fluorescent protein (YFP)) in a 1:1
159 ratio as the control condition; or a mixture of RFP-T2A-CYFIP1 and pCAβ-YFP (4:1 ratio) to
160 assess the effects of *CYFIP1*-OE on neuritogenesis. A pCAβ-YFP plasmid was co-
161 transfected to enable better visualisation of the cells for neurite tracing as the RFP
162 encoded by the *CYFIP1*-OE plasmid photo-bleaches quickly. Media was replaced the
163 following day.
164
165 **Protein extraction and Western blotting**
166 72 hours post-transfection, cells were washed with ice-cold 1x phosphate buffered
167 saline (1xPBS) (#P4417-50TAB, Sigma-Aldrich®) then collected with a cell scraper in 300
168 μL of ice-cold RIPA lysis buffer (150 mM NaCl, 50 mM Tris/Cl pH = 8.0, 0.5% Sodium

169 Deoxycholate, 0.1% SDS, 1% Triton X-100) supplemented with 1 mM PMSF, 1%
170 Phosphatase Inhibitor Cocktail 2 (#P5726-1ML, Sigma Aldrich®), 1% Phosphatase
171 Inhibitor Cocktail 3 (#P0044-1ML, Sigma Aldrich®) and 1% Complete Protease Inhibitor
172 Cocktail (#P8340-1ML, Sigma Aldrich®). Cell lysates were incubated on ice for 30
173 minutes, cleared by centrifugation at 17,000xg for 15 minutes at 4°C, then supernatants
174 collected. Bicinchoninic Acid assays (#10678484, *Thermo Scientific*) were performed to
175 estimate protein concentrations and 50 μg of total protein was diluted with Laemmli
176 buffer (final concentration of 60 mM Tris/Cl pH = 6.8, 10% Glycerol, 2% SDS, 5% 2-
177 Mercaptoethanol, 0.02% Bromophenol Blue), then boiled for 5 minutes at 95°C. 50 μg of
178 total protein for each sample was loaded into 4–15% precast polyacrylamide gels
179 (#4561085, *Bio-Rad Laboratories*) alongside a pre-stained protein ladder (#PL00001,
180 *Proteintech Group*). Electrophoresis was performed in the Mini-PROTEAN® Tetra Vertical
181 Electrophoresis Cell (#1658004, *Bio-Rad Laboratories*) with 1×SDS-PAGE running buffer
182 (2.5 mM tris-base; 19 mM glycine (#10070150, *Fisher Chemical*™); 0.01% SDS; pH = 8.3
183 in ddH_2O). Proteins were then transferred onto methanol-activated PVDF membranes
184 (#IPVH304F0, *Millipore*) by a wet transfer method in the Mini-PROTEAN® Tetra Vertical
185 Electrophoresis Cell. PVDF membranes were then blocked for at least 1 hour at room
186 temperature with 5 mL of 5% skim milk in 1x tris-buffered saline (TBS; 2 mM Tris-Base; 15
187 mM NaCl; pH = 7.6 in ddH_2O) supplemented with 0.1% Tween® 20 (#10485733, *Fisher*
188 *BioReagents*™) (1xTBS-T). After blocking, membranes were sequentially incubated with 5
189 mL of primary antibody (gentle rocking for 2-3 hours at room temperature or overnight at
190 4°C), then secondary antibody (gentle rocking for 30 minutes-1 hour at room
191 temperature). Primary and secondary antibodies were diluted in 5% skim milk in 1x TBS-
192 T (or 5% BSA in 1x TBS-T for anti-P-STAT3^{Y705}). The dilution factors and specific antibodies

193 used are listed in **Table 1**. Three 5-minute washes with 1x TBS-T were performed between
194 antibody incubations. GAPDH was used as a loading control to normalise expression of
195 target proteins. Proteins were visualised using the Odyssey® CLx Imager (*LI-COR*
196 *Biosciences*) and the Image Studio software (Version 5.2; *LI-COR Biosciences*) was used
197 to perform densitometric analyses to compare the abundance of target proteins between
198 samples.

199

Catalogue Number	Supplier	Host Species	Target	Dilution	Conjugated Fluorophore
ab156016	*Abcam*	Rabbit	CYFIP1	1:1000	
sc-47724	*Santa Cruz Biotechnology*	Mouse	GAPDH	1:1000	
13846-1-AP	*Proteintech*	Rabbit	JAKMIP1	1:1000	
9139S	*Cell Signaling Technology*	Mouse	STAT3	1:1000	
9131l	*Cell Signaling Technology*	Rabbit	P-STAT3^{Y705}	1:1000	
35519	*Invitrogen*	Goat	Mouse	1:5000	DyLight™ 680
SA5-10036	*Invitrogen*	Goat	Rabbit	1:5000	DyLight™ 800

200 ***Table : Antibodies used in this study.***

201

202 **RNA extraction, cDNA conversion and qRT-PCR**

203 72 hours post-transfection, total RNA was isolated and purified using the Direct-zol™ RNA
204 Miniprep kit following manufacturer instructions (#R2051, *Zymo Research*). 500 ng of
205 RNA was converted to cDNA using PrimeScript™ RT reagent kit (#RR037A, *Takara Bio*
206 *Europe*) following manufacturer instructions. qRT-PCR was then performed using HOT
207 FIREPol® EvaGreen® qPCR Master Mix with ROX (#01-02-00500, *Solis BioDyne*) with the
208 QuantStudio 12K Flex qPCR machine (*Thermo Fisher Scientific*™). For primer sequences,
209 see **Table 2**. mRNA expression levels were standardized against two reference genes,

210 *Glyceraldehyde-3-Phosphate Dehydrogenase* (*GAPDH*) and *RNA Polymerase II Subunit*

211 *A* (*POLR2A*) using the Pfaffl method (14) and normalised to the control cells.

212

Gene	Strand	Sequence
CYFIP1	Forward	TAC GAG ACG CTG CTG AAG CAG A
	Reverse	TCG TCC AAT CGC CAG TTC TAG G
GAPDH	Forward	TCC TCT GAC TTC AAC AGC GAC
	Reverse	GCT GTA GCC AAA TTC GTT GTC A
NF-KB	Forward	GCA GCA CTA CTT CTT GAC CAC C
	Reverse	TCT GCT CCT GAG CAT TGA CGT C
POLR2A	Forward	CCA TCA AGA GAG TCC AGT TCG
	Reverse	ACC CTC CGT CAC AGA CAT TC
SOCS1	Forward	TTC GCC CTT AGC GTG AAG ATG G
	Reverse	TAG TGC TCC AGC AGC TCG AAG A
SOCS3	Forward	CCA AGG ACG GAG ACT TCG ATT C
	Reverse	GGG AAA CTT GCT GTG GGT GA
STAT1	Forward	ATG GCA GTC TGG CGG CTG AAT T
	Reverse	CCA AAC CAG GCT GGC ACA ATT G
STAT3	Forward	GGG AAG AAT CAC GCC TTC TAC
	Reverse	ATC TGC TGCT TCT CCG TCA C
STAT5B	Forward	GCC ACT GTT CTC TGG GAC AAT G
	Reverse	ACA CGA GGT TCT CCT TGG TCA G

Table 2: Primers used in this study.

Dual-luciferase reporter assays

50,000 HEK293 cells were seeded into 24-well plates and incubated overnight. The following day, cells were transfected with 2 μL of STAT3 Cignal Reporter plasmid (#336841, GeneGlobe ID: CCS-9028L, *Qiagen*) or 2 μL of STAT1 (GAS) Cignal Reporter plasmid (#336841, GeneGlobe ID: CCS-009L, *Qiagen*) using Lipofectamine™ LTX as previously described. Four hours post-transfection, cells were treated with DMEM/F-12 +10% FBS with or without IL-6- or IFN-γ and incubated overnight. 18 hours later, Firefly and Renilla luciferase activities were measured using the Dual-Luciferase® Reporter (DLR™) Assay System (#E1960, *Promega*) following manufacturer instructions.

224 Luminescence was measured in white-bottomed LUMITRAC™ 96-well plates (#655074,
225 *Greiner Bio-One*) using the PHERAstar FS microplate reader (*BMG LABTECH*).
226
227 **SH-SY5Y differentiation and neurite outgrowth analysis**
228 Sterile 13-mm-diameter glass coverslips (#631-1578, *VWR®*) were placed into 24-well
229 plates and coated with 300 μL of 20 μg/mL poly-D-Lysine (PDL; #354210, *Corning®*) by
230 overnight incubation at 37°C. 24 hours post-transfection, 30,000 SH-SY5Y cells were
231 seeded onto PDL-coated coverslips in DMEM/F-12 + 10% FBS. The following day, media
232 was replaced with 20 ng/mL BDNF and 10 μM RA in DMEM/F-12 (without FBS). 48 hours
233 later, SH-SY5Y cells were fixed with 4% paraformaldehyde (PFA), and nuclei were
234 counterstained with 4',6-diamidino-2-phenylindole (DAPI) (#D9542, *Sigma-Aldrich®*) for
235 15 minutes. Coverslips were mounted onto glass microscope slides with ProLong™
236 Diamond Antifade Mountant (#15468070, *Fisher Scientific™*) and slides were imaged on
237 the inverted DMi8 widefield microscope (*Leica Microsystems*).
238
239 All image analysis was performed manually using the *FIJI* software (*ImageJ*; Version 2.9.0;
240 (15)). Prior to image analysis, a scale bar was used to set a pixel to μm ratio to each image,
241 after which the "Segmented Line" tool was used to measure the distance from the edge
242 of the nucleus to the furthest end of each neurite. The length of the longest neurite
243 produced by a single cell (referred to as longest neurite length (LNL); a measure of neurite
244 extension) and the sum length of all neurites produced by a single cell (referred to as total
245 neurite length (TNL); a measure of neuritogenesis) were measured.
246
247 **Statistical analysis**

Statistical analyses were performed using GraphPad Prism (GraphPad Prism Version 9.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com). Unpaired Student's t-Tests were used to assess statistical differences in measurements between two groups (e.g., *CYFIP1*-OE against control). When investigating the effects of and interactions between two independent variables (e.g., the effect of both *CYFIP1*-OE and IL-6 treatment on STAT3 transcriptional activity), two-way analysis of variance (ANOVA) were performed. Appropriate post hoc tests were also performed to account for multiple testing. Data are expressed as mean values ± standard error of the mean (SEM). P-values < 0.05 were considered statistically significant.

Results

Modelling CYFIP1-overexpression

To model increased *CYFIP1* expression, we utilised plasmid vectors encoding an RFP-T2A-CYFIP1 construct (to induce *CYFIP1*-OE) or RFP-T2A as a control, which we transfected into HEK293 cells, referred to as *CYFIP1*-OE and control cells respectively (**Figures 1A and 1B**). We then confirmed increased *CYFIP1* mRNA expression using qRT-PCR (**Figure 1C**) and protein expression using Western blotting (**Figures 1D and E**).

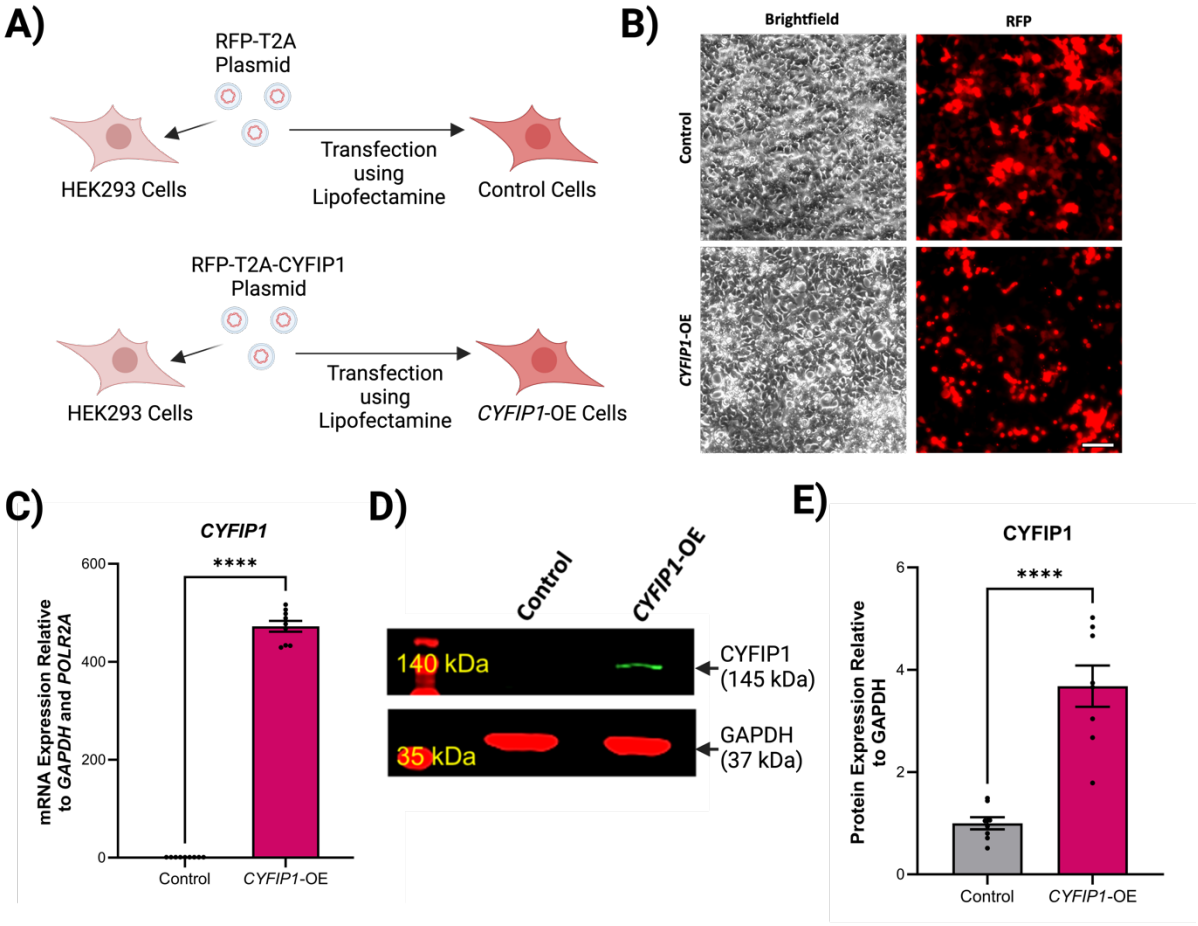


Figure 1 | Modelling CYFIP1 overexpression in HEK293 cells. A) *HEK293 cells were transfected with either RFP-T2A plasmid as a control (referred to as Control), or RFP-T2A-CYFIP1 to induce CYFIP1-overexpression (OE) (referred to as CYFIP1-OE). Created with BioRender.com.* ***B)*** *Control and CYFIP1-OE cells were imaged 72 hours post-transfection live on the Leica DMi8 widefield microscope. Scale bar = 100 μm.* ***C)*** *Relative mRNA expression of CYFIP1 in Control and CYFIP1-OE cells 72 hours post-transfection measured by qRT-PCR.* ***D)*** *Increased CYFIP1 protein expression in CYFIP1-OE cells 72 hours post-transfection confirmed via Western blotting.* ***E)*** *Densitometric analysis of total CYFIP1 protein expression relative to the GAPDH loading control. All values are normalised to the Control cells. For all experiments, values are presented as mean ± SEM of N = 3 independent experiments, 2-3 technical replicates each. Statistical significance against Control was determined using Unpaired Student's T-Tests; ****P < 0.0001.*

280

281 ***CYFIP1*-OE leads to reduced JAKMIP1 expression**

282 It has been previously demonstrated that *JAKMIP1* expression is reduced following

283 *CYFIP1*-OE in human neuroblastoma cells (5). Therefore, to confirm whether this is the

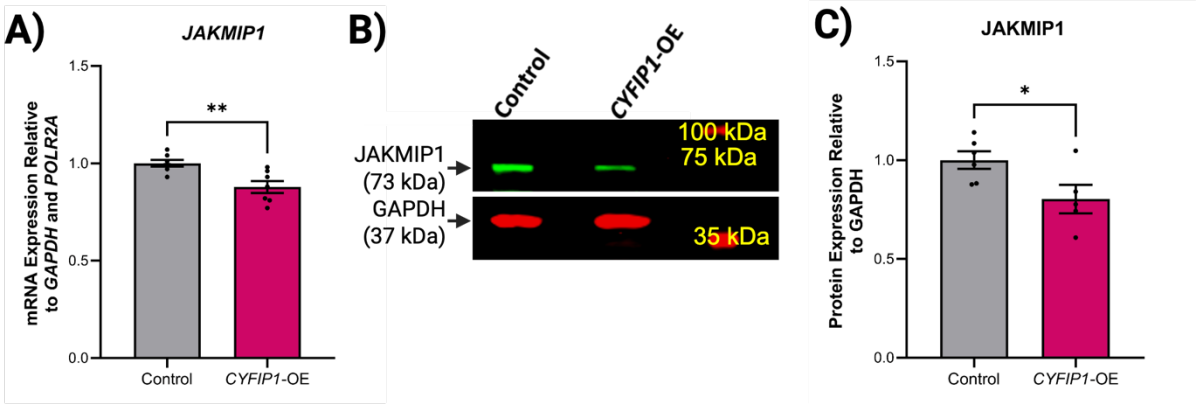
284 case in HEK293 cells, we measured *JAKMIP1* expression in *CYFIP1*-OE HEK293 cells by

285 qRT-PCR and Western blotting. Agreeing with Nishimura et al., we found that *JAKMIP1*

286 mRNA (**Figure 2A**) and protein expression (**Figure 2B and C**) is significantly reduced in

287 *CYFIP1*-OE HEK293 cells.

288



289

290 ***Figure 2 | CYFIP1-OE leads to reduced JAKMIP1 mRNA and protein expression. A)***

291 *Relative mRNA expression of JAKMIP1 in Control and CYFIP1-OE cells measured by qRT-*

292 *PCR.* ***B)*** *Western blotting of JAKMIP1 expression in Control and CYFIP1-OE cells.* ***C)***

293 *Densitometric analysis of total JAKMIP1 protein expression relative to the GAPDH loading*

294 *control. For all experiments, values are presented as mean ± SEM of N = 3 independent*

295 *experiments, 2-3 technical replicates each. Statistical significance against Control was*

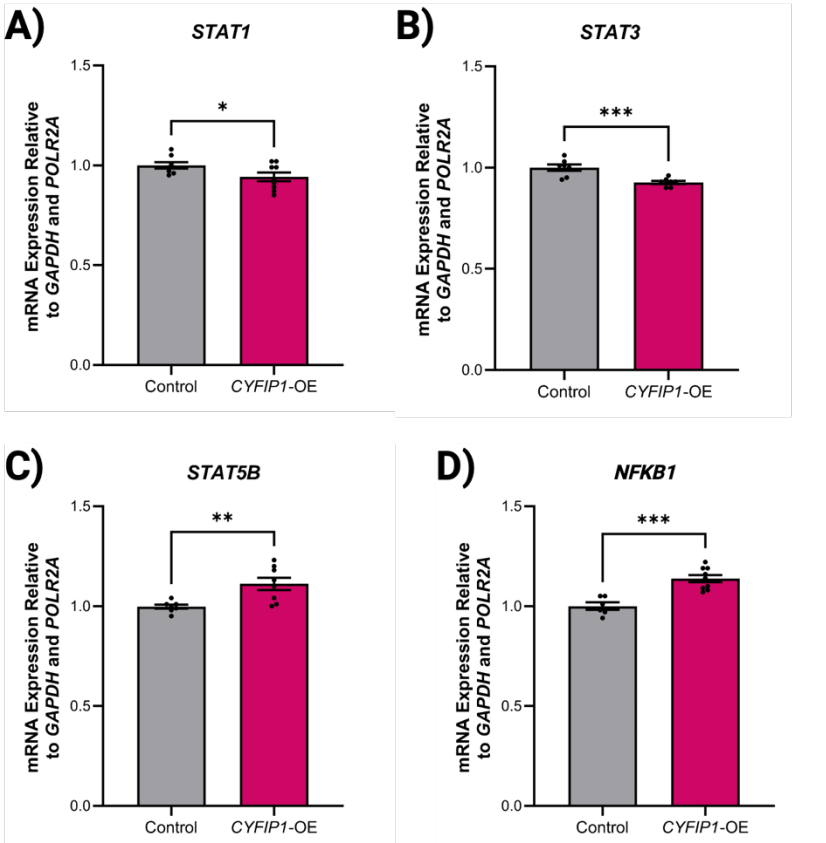
296 *determined using Unpaired Student's T-Tests; *P < 0.05; **P < 0.01.*

297

298 ***CYFIP1*-OE alters the expression of several cytokine signalling-related transcription**
299 **factors**

300 We have previously demonstrated that Dup(15q) iPSCs and iNeurons with increased
301 *CYFIP1* expression show reduced *STAT3* expression (6). Furthermore, we have
302 demonstrated that *JAKMIP1*-knockout SH-SY5Y cells also show reduced *STAT3*
303 expression and that JAKMIP1 deficiency may alter other cytokine signalling pathways too
304 (6). Therefore, to determine whether *CYFIP1*-OE may also lead to altered expression of
305 cytokine signalling-related transcription factors, qRT-PCR was performed to measure
306 mRNA expression of *STAT1*, *STAT3*, *STAT5B* and *NFKB1*. We observed that the expression
307 of *STAT1* (**Figure 3A**) and *STAT3* (**Figure 3B**) were reduced in *CYFIP1*-OE HEK293 cells,
308 whereas the expression of *STAT5B* (**Figure 5C**) and *NFKB1* (**Figure 5D**) were increased.
309 This suggests that CYFIP1 may also modulate the expression of cytokine signalling-
310 related transcription factors, likely via modulation of *JAKMIP1*.

311



312

313 ***Figure 3 | CYFIP1-OE alters the expression of other cytokine signal-regulated***

314 ***transcription factors.*** *Relative mRNA expression of* ***A)*** *STAT1,* ***B)*** *STAT3,* ***C)*** *STAT5B or* ***D)***

315 *NFKB1 in Control and CYFIP1-OE cells measured by qRT-PCR. For all experiments, values*

316 *are presented as mean ± SEM of N = 3 independent experiments, 2-3 technical replicates*

317 *each. Statistical significance against Control was determined using Unpaired Student's*

318 *T-Tests; *P < 0.05; **P <0.01; ****P < 0.0001.*

319

320 ***CYFIP1*-OE enhances IL-6-induced STAT3 activity**

321 To investigate whether reduced *STAT3* expression in *CYFIP1*-OE HEK293 cells leads to

322 altered cytokine-induced STAT3 responses we first performed Western blotting to assess

323 IL-6-induced STAT3^{Y705} phosphorylation (representative of STAT3 activation) (**Figure 4A**).

324 Despite the reduction in STAT3 expression (**Figure 4B**), *CYFIP1*-OE cells still show strong

325 responses to IL-6, demonstrating no significant difference in IL-6-stimulated STAT3^{Y705}

326 phosphorylation levels compared to control cells, both at the 'whole-cell level' (when
327 STAT3^{Y705} phosphorylation levels are normalised to GAPDH) (**Figure 4C**) and upon closer
328 examination of the phosphorylation kinetics of STAT3 itself (when STAT3^{Y750}
329 phosphorylation levels are normalised to total STAT3 protein levels) (**Figure 4D**). This
330 suggests that although *CYFIP1*-OE cells show reduced STAT3 expression, STAT3
331 activation following IL-6 stimulation is unaltered.
332
333 STAT3 activity is modulated by various post-translational modifications in addition to
334 phosphorylation at the Y705 residue. Therefore, despite observing no change in STAT3^{Y705}
335 phosphorylation following *CYFIP1*-OE, we examined whether *CYFIP1*-OE cells show
336 altered IL-6-induced transcriptional activity by performing a DLR™ assay. This assay
337 places the control of a Firefly luciferase under a STAT3 response element, meaning that
338 increased Firefly luminescence is indicative of increased STAT3 transcriptional activity.
339 Using this DLR™ assay, we found that *CYFIP1*-OE cells show increased Firefly
340 luminescence following IL-6 stimulation than control cells (**Figure 4E**), suggesting that
341 IL-6-induced STAT3 transcriptional activity is enhanced in *CYFIP1*-OE cells regardless of
342 the reduced STAT3 expression.
343
344 To validate the DLR™ assay results, qRT-PCR was used to measure the mRNA expression
345 of STAT3-responsive genes in *CYFIP1*-OE cells following IL-6 stimulation. Somewhat
346 contradictory to the DLR™ assay, levels of *STAT3* mRNA in IL-6-treated *CYFIP1*-OE cells
347 were not higher than those in untreated control cells (**Figure 4F**). However, considering
348 that *CYFIP1*-OE cells already show reduced *STAT3* mRNA expression at baseline, this is
349 unsurprising. Therefore, after normalising the levels of IL-6-stimulated *STAT3* expression

350 to unstimulated *STAT3* expression, *CYFIP1*-OE cells show an enhancement in IL-6-
351 induced *STAT3* mRNA expression compared to control cells (**Figure 4G**). The expression
352 of *SOCS3* shows a similar phenomenon whereby IL-6-stimulated *CYFIP1*-OE cells
353 appear to show lower *SOCS3* mRNA levels compared to IL-6-treated control cells (**Figure**
354 **4H**), however the fold change in *SOCS3* expression relative to unstimulated cells shows
355 that IL-6-induced *SOCS3* expression is magnified in *CYFIP1*-OE cells (**Figure 4I**). Taken
356 together, these findings complement the DLR™ assay results, suggesting that *CYFIP1*-OE
357 cells show enhanced STAT3 transcriptional activity following IL-6 stimulation.
358

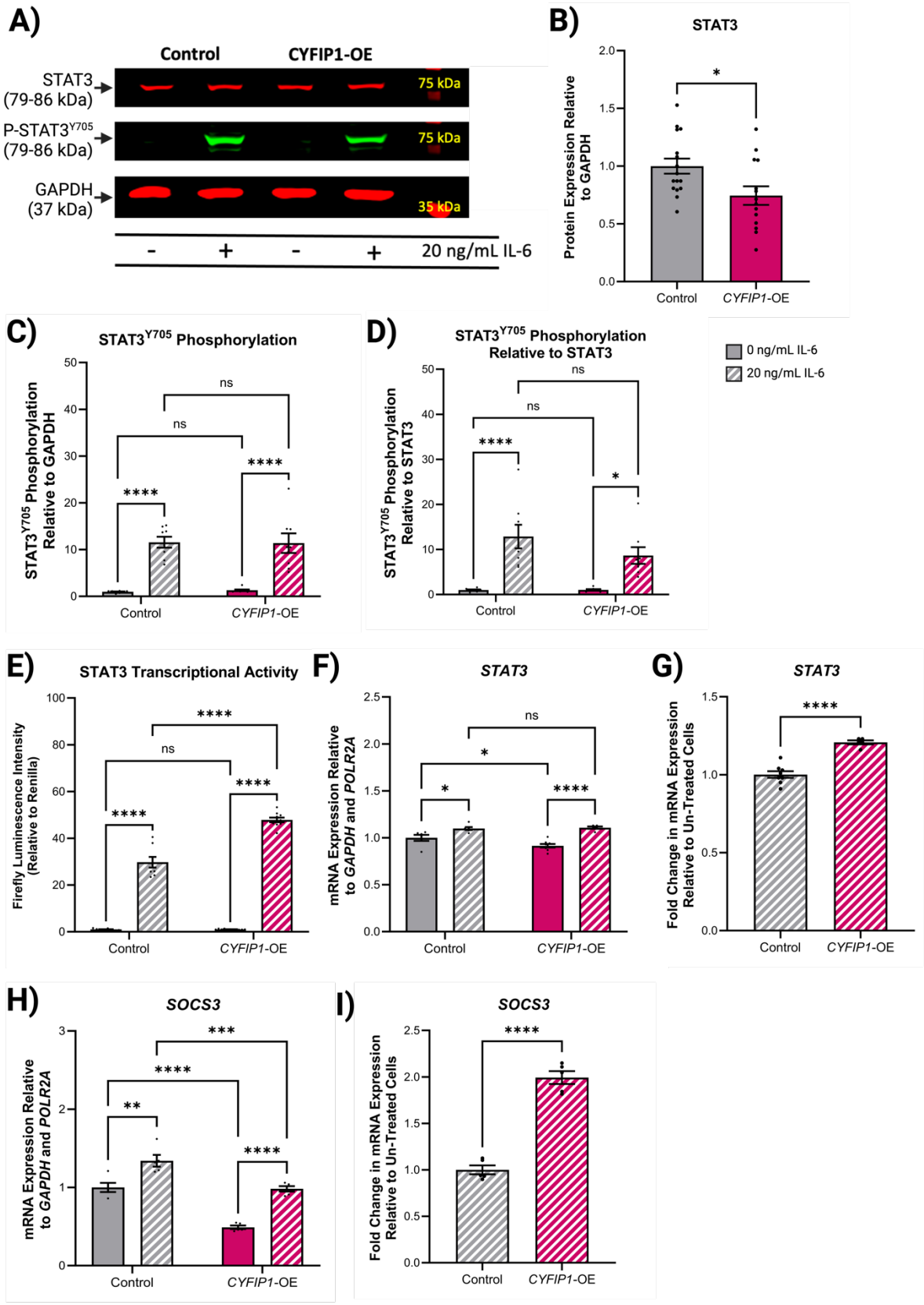


Figure 4 | CYFIP1-OE enhances STAT3 transcriptional activity but not STAT3 activation. A) *Western blotting of STAT3 expression and phosphorylation (P-STAT3^{Y705}) in Control and CYFIP1-OE cells following 30-minute treatment with 0 ng/mL IL-6 or 20 ng/mL*

363 *IL-6. Densitometric analysis of **B)** total STAT3 protein expression relative to the GAPDH*
364 *loading control, **C)** P-STAT3 relative to the GAPDH loading control or **D)** P-STAT3 relative*
365 *to total STAT3 protein expression. **E)** Total transcriptional activity at a STAT3-regulated*
366 *promoter in Control and CYFIP1-OE cells stimulated with or without 20 ng/mL IL-6 for 18*
367 *hours post-transfection with Cignal Reporter plasmids. Firefly luminescence intensity is*
368 *expressed as a ratio to Renilla luminescence intensity to adjust for transfection efficiency.*
369 ***F)** Relative mRNA expression of STAT3 in Control and CYFIP1-OE cells treated with 0*
370 *ng/mL or 20 ng/mL IL-6 for 4 hours, measured by qRT-PCR. **G)** Fold change in STAT3 mRNA*
371 *expression following IL-6 treatment relative to untreated cells in part F). Values presented*
372 *as mean ± SEM of N = 3 independent experiments, 3 technical replicates each. Statistical*
373 *significance against Control was determined using Unpaired Student's T-Tests; ****P <*
374 *0.0001. **H)** Relative mRNA expression of SOCS3 in Control and CYFIP1-OE cells treated*
375 *with 0 ng/mL or 20 ng/mL IL-6 for 4 hours, measured by qRT-PCR. **I)** Fold change in SOCS3*
376 *mRNA expression following IL-6 treatment relative to untreated cells in part H). For all*
377 *experiments, values are presented as mean ± SEM of N = 3 independent experiments, 3-*
378 *4 technical replicates each. Statistical significance against Control was determined*
379 *using Unpaired Student's T-Tests when comparing two groups, or two-way ANOVAs with*
380 *Tukey's Honest Significant Difference tests when comparing multiple groups; ns – P >*
381 *0.05; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.*
382
383 **_CYFIP1_-OE enhances IL-6-induced neuritogenesis**
384 To understand how this enhanced STAT3 response to IL-6 in *CYFIP1*-OE cells observed at
385 a molecular level affects a broader IL-6-stimulated cellular process, we differentiated
386 *CYFIP1*-OE SH-SY5Y cells in the presence of IL-6 and measured the neurite lengths

387 produced (**Figure 5A**), as we have previously shown that IL-6 induces neurite outgrowth
388 in SH-SY5Y cells (6). As expected, control and *CYFIP1*-OE cells differentiated in the
389 presence of IL-6 exhibit longer LNL (**Figure 5B**) and TNL (**Figure 5C**) measurements than
390 cells differentiated without IL-6. *CYFIP1*-OE cells appear to have enhanced neurite
391 outgrowth responses to IL-6, as although *CYFIP1*-OE cells produce shorter neurites
392 compared to the control as baseline, the LNL (**Figure 5B**) and TNL (**Figure 5C**)
393 measurements are no longer significantly different between the two populations when
394 differentiated with IL-6. These data appear to agree with the suggestion that *CYFIP1*-OE
395 leads to enhanced STAT3 responses to IL-6, and that this enhancement in IL-6-induced
396 STAT3 activity leads to increased IL-6-stimulated neurite extension and production.
397

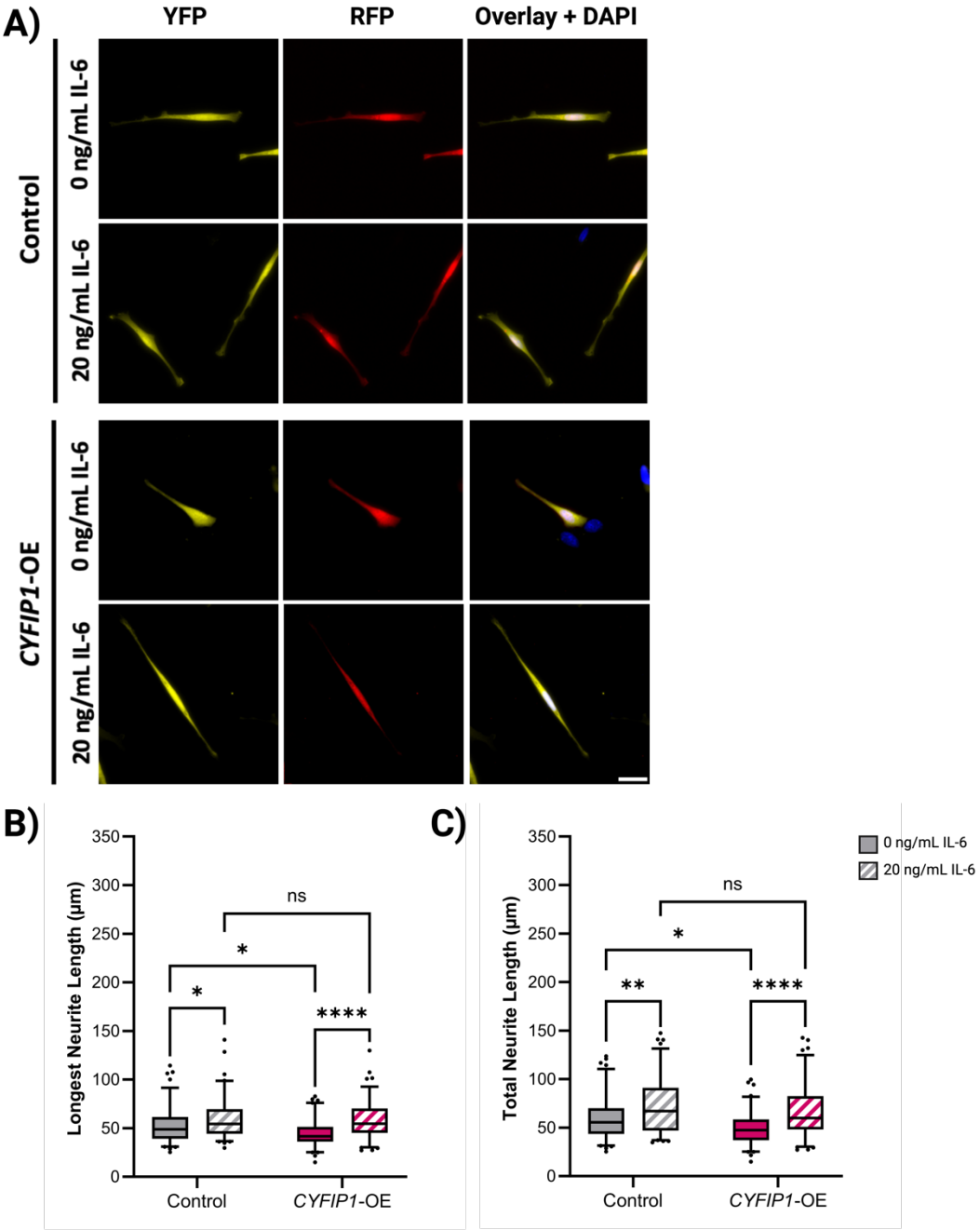


Figure 5 | CYFIP1-OE enhances IL-6-induced neuritogenesis. *SH-SY5Y cells were Nucleofected with either the RFP-T2A backbone vector and pCAβ-YFP as the control condition; or RFP-T2A-CYFIP1 and pCAβ-YFP to assess the effects of CYFIP1-OE on IL-6-influenced neuritogenesis. Cells were differentiated for two days with 20 ng/mL BDNF and 10 µM RA under serum starvation with or without 20 ng/mL IL-6, fixed with 4% PFA and stained with DAPI.* ***A)*** *Fluorescence micrographs of differentiated SH-SY5Y cells.*

405 *Images captured using an upright Leica DM4000 B LED microscope. Scale bar = 50 μm.*
406 *Neurite tracing analysis was performed using the FIJI software.* ***B)*** *The length of the*
407 *longest neurite produced by the cell was measured for all conditions.* ***C)*** *The sum length*
408 *of all neurites produced per cell is presented for each condition. For all experiments,*
409 *values are presented as mean ± SEM of N = 4 independent experiments, 3 technical*
410 *replicates each. Statistical significance against Control was determined using two-way*
411 *ANOVAs with Tukey's Honest Significant Difference tests; ns – P > 0.05; *P < 0.05; **P <*
412 *0.01; ****P < 0.0001.*
413
414 ***CYFIP1*-OE enhances IFN-γ-induced STAT1 transcriptional activity**
415 Having determined that *CYFIP1*-OE alters the expression of several cytokine signalling-
416 related transcription factors, and that *CYFIP1*-OE enhances IL-6-induced STAT3 activity,
417 we aimed to investigate whether *CYFIP1*-OE alters the activity of another cytokine
418 signalling-related transcription factor. STAT1 was chosen to follow up with because
419 similar to *STAT3* expression, the expression of *STAT1* was also reduced in *CYFIP1*-OE
420 HEK293 cells. A DLR™ assay which placed the control of a Firefly luciferase under a GAS
421 element (the response element STAT1 binds to initiate transcription) was performed. We
422 found that *CYFIP1*-OE HEK293 cells show increased Firefly luminescence following IFN-
423 γ stimulation compared to control cells (**Figure 6A**), suggesting that IFN-γ-induced STAT1
424 transcriptional activity is enhanced in *CYFIP1*-OE cells, again regardless of the reduced
425 *STAT1* expression.
426
427 As validation of the DLR™ assay, qRT-PCR was performed to measure the expression of
428 STAT1-response genes following IFN-g stimulation. *CYFIP1*-OE cells show increased IFN-

429 γ-induced *STAT1* expression (**Figure 6B**), suggesting an enhanced transcription response

430 to IFN-γ stimulation. Additionally, although not significantly different at baseline, *CYFIP1*-

431 OE cells show increased *SOCS1* expression following IFN-γ treatment (**Figure 6C**), again

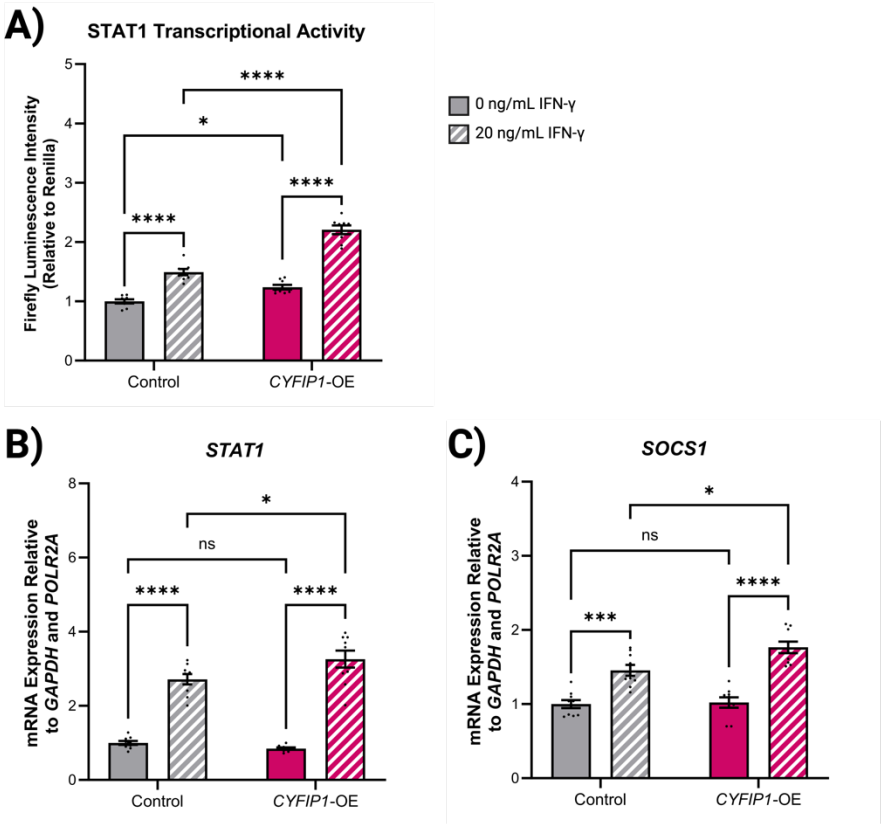
432 suggesting an enhanced STAT1 transcriptional response to IFN-γ stimulation. This also

433 suggests that the observed alterations in IL-6/STAT3 signalling are not limited to STAT3

434 and that CYFIP1 may play a role in modulating a larger range of cytokine signalling

435 pathways.

436



437

438 ***Figure 6 | CYFIP1-OE leads to enhanced STAT1 transcriptional activity following IFN-***

439 ***γtreatment. A)*** *Total transcriptional activity at a STAT1-regulated promoter in Control and*

440 *CYFIP1-OE cells stimulated with or without 20 ng/mL IFN-γfor 18 hours post-transfection*

441 *with Cignal Reporter plasmids. Firefly luminescence intensity is expressed as a ratio to*

442 *Renilla luminescence intensity to adjust for transfection efficiency.* ***B)*** *Relative mRNA*

443 *expression of STAT1 in Control and CYFIP1-OE cells treated with 0 ng/mL or 20 ng/mL IFN-*
444 *γ for 4 hours, measured by qRT-PCR.* ***C)*** *Relative mRNA expression of SOCS1 in Control*
445 *and CYFIP1-OE cells treated with 0 ng/mL or 20 ng/mL IFN-γ for 4 hours, measured by*
446 *qRT-PCR. For all experiments, values are presented as mean ± SEM of N = 4 independent*
447 *experiments, 3 technical replicates each. Statistical significance against Control was*
448 *determined using two-way ANOVAs with Tukey's Honest Significant Difference tests; ns –*
449 *P > 0.05; *P < 0.05; ***P < 0.001; ****P < 0.0001.*
450
451
452 **Discussion**
453 **A novel role for CYFIP1 in modulating IL-6/STAT3 signalling independent from**
454 **JAKMIP1**
455 Nishimura *et al*. (5) first linked *JAKMIP1* to ASD and Dup(15q), reporting that *JAKMIP1*
456 expression was altered in Dup(15q) and that overexpression of the chromosome 15q
457 gene *CYFIP1* correspondingly resulted in *JAKMIP1* downregulation. We expanded upon
458 this in our previous work, identifying JAKMIP1 as a modulator of IL-6 cytokine signaling in
459 SH-SY5Y cells, and demonstrating that Dup(15q) iPSCs and cortical neurons with
460 elevated *CYFIP1* dosage and expression exhibit enhanced IL-6-induced STAT3 activity (6).
461 However, given that the magnified IL-6 signaling observed in these Dup(15q) cells did not
462 correlate with *JAKMIP1* expression levels, we hypothesised that another gene within the
463 15q region, likely *CYFIP1*, might be driving this increased responsiveness to IL-6.
464
465 To test this hypothesis, we induced *CYFIP1* overexpression in HEK293 cells and
466 discovered that this resulted in the altered expression of several cytokine signaling-

467 related transcription factors. In particular, *STAT1* and *STAT3* expression were reduced,
468 whereas *STAT5B* and *NFKB1* expression were raised, suggesting that CYFIP1 may
469 potentially exert broad cellular functions that impact the signaling networks of multiple
470 cytokines. Taking this forward, we measured IL-6-induced activation and activity of
471 STAT3. Consistent with the Dup(15q) iPSCs and neurons from our previous work, we
472 observed that despite the reduced *STAT3* expression, IL-6-induced STAT3 responses were
473 enhanced in *CYFIP1*-OE HEK293 cells. Furthermore, *CYFIP1* overexpression also
474 potentiated IL-6-triggered neuritogenesis in SH-SY5Y cells. As such, this study provides
475 the first evidence that CYFIP1 may play a role in regulating IL-6/STAT3 signalling in both
476 HEK293 and SH-SY5Y cells, and its upregulation could be responsible for the enhanced
477 responsiveness to IL-6 exhibited by Dup(15q) iPSCs and neurons.
478
479 **How does CYFIP1 influence STAT1 and STAT3 activity?**
480 The mechanisms underlying CYFIP1-mediated regulation of *STAT1* and *STAT3* expression
481 and activity remain unclear. Regarding STAT3, reduced *STAT3* expression in *JAKMIP1*-
482 deficient cells (6) may suggest that JAKMIP1, rather than CYFIP1, is responsible for
483 regulating *STAT3* expression. This could occur through its interactions with various RNA-
484 binding proteins (RBPs) or splicing factors (6). However, it is important to note that
485 *CYFIP1* overexpression enhances IL-6-induced STAT3 transcriptional activity, whereas
486 *JAKMIP1*-knockout impairs it (6). Thus, whilst JAKMIP1 could be responsible for
487 controlling *STAT3* expression, when considering STAT3 activity, our results indicate that it
488 is more likely that CYFIP1 is responsible by a mechanism independent of JAKMIP1.
489

490 Interestingly, despite *CYFIP1* overexpression leading to increased STAT3 transcriptional
491 activity following IL-6 treatment, we observed no significant difference in the levels
492 $STAT3^{Y705}$ phosphorylation in *CYFIP1*-OE cells, suggesting that CYFIP1 does not alter
493 STAT3 phosphorylation kinetics, and thus STAT3 activation. One reason for this
494 discrepancy could be because of the influence of other post-translational modifications
495 of STAT3. For example, $STAT3^{K685}$ acetylation may be necessary for IL-6-induced STAT3-
496 responsive gene expression (16). Rather than modulating STAT3 activation through
497 phosphorylation, perhaps CYFIP1 may somehow control STAT3 activation through
498 acetylation, however, the exact mechanism by which CYFIP1 could regulate acetylation
499 is unclear.
500
501 In terms of *STAT1* expression and activity, it would not be unreasonable to suggest that
502 these changes in STAT1 activity may be mediated by the changes in *JAKMIP1* expression.
503 JAKMIP1 was previously reported to inhibit Interferon-α (IFN-α) signalling (17).
504 Importantly, both IFN-α and IFN-γ signalling cascades involve the phosphorylation and
505 activation of STAT1. It is therefore likely that CYFIP1-induced downregulation of *JAKMIP1*
506 may reduce the inhibiting influence of JAKMIP1 on IFN signalling, thereby enhancing the
507 IFN response. Furthermore, STAT1 activity is also known to be altered by acetylation,
508 similar to STAT3 (18). If CYFIP1-mediated regulation of STAT3 is dependent on post-
509 translational modifications, the same mechanism(s) could be responsible for CYFIP1
510 regulation both STAT3 and STAT1 activity. Of course, all of these possibilities would
511 require further validation.
512
513 **Relevance to ASD and neurodevelopment**

514 Cytokine signalling plays crucial roles in neurodevelopment beyond immune responses,
515 with STAT3 and STAT1 signalling in particular regulating several key processes. For
516 example, STAT3 signalling has been demonstrated to control the switch between
517 neurogenesis and gliogenesis, promoting both neural progenitor cell self-renewal and
518 astrocyte differentiation (19). Similarly, STAT1 signalling has been shown to be involved
519 in neurogenesis, and promoting neurite outgrowth (20). Given how crucial STAT3 and
520 STAT1 signalling are for neurodevelopment, it is unsurprising that alterations in STAT3 and
521 STAT1 signalling are associated with NDDs such as ASD (21,22). MIA, where an immune
522 response is triggered by infection of exposure to immunogenic material during
523 pregnancy, is associated with increased incidence of ASD (23). Both IL-6 and IFN-γ are
524 pro-inflammatory cytokines that have been implicated in the pathogenesis of ASD, with
525 IL-6 being a key player in MIA-induced ASD-like behaviours (23,24). Considering that
526 *CYFIP1*-OE enhances STAT3 and STAT1 responses to IL-6 and IFN-γ respectively suggests
527 that CYFIP1 may play a pivotal role in amplifying the inflammatory signals during MIA,
528 potentially exacerbating the effects of immune activation on foetal brain development.
529
530 Amplification of both the IL-6/STAT3 and IFN-γ/STAT1 signalling pathways by CYFIP1
531 provides a potential molecular mechanism that explains the overlap between immune
532 activation and ASD, particularly in cases where no obvious inflammatory trigger is
533 identified. Elevated cytokine levels are frequently reported in individuals with ASD (25),
534 and our results suggest that *CYFIP1* overexpression could act as a molecular
535 convergence point, increasing the sensitivity of the developing brain to immune signals
536 even under baseline inflammatory conditions. This could help to explain cases of ASD

537 where immune activation is not readily apparent but inflammation-induced
538 neurodevelopmental disruption still occurs.
539
540
541 **Conclusion**
542 In summary, this study identifies a novel role for CYFIP1 in regulating the expression of
543 cytokine signalling-related transcription factors, as well as enhancing their activity
544 following cytokine stimulation. By amplifying these inflammatory pathways, CYFIP1
545 could play a central role in exacerbating the neuroinflammatory environment, leading to
546 altered neurodevelopment and increased susceptibility to ASD. Further research is
547 required to understand the mechanism by which CYFIP1 modulates these cytokine
548 signalling pathways and to investigate this in the context of ASD with MIA.
549
550
551 **List of abbreviations**
552 ASD – Autism spectrum disorder
553 Dup(15q) – Chromosome 15q-duplication syndrome
554 CYFIP1 – Cytoplasmic FMR1-intercating protein 1
555 iPSC – Induced pluripotent stem cell
556 IL-6 – Interleukin-6
557 STAT3 – Signal transducer and activator of transcription 3
558 OE – Overexpression
559 MIA – Maternal immune activation
560 JAKMIP1 – Janus kinase and microtubule-interacting protein 1

561 FXS – Fragile X syndrome

562 iNeurons – iPSC-derived neurons

563 FMRP – Fragile X messenger ribonuclear protein

564 WRC – WAVE regulatory complex

565 SMAD3 – SMAD family member 3

566 NF-kB – Nuclear factor of κB

567 ISRE – Interferon response element

568 TGF-β1 – Transforming growth factor β1

569 GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

570 DLR™ – Dual-luciferase reporter™

571 SOCS3 – Suppressor of cytokine signalling 3

572 LNL – Longest neurite length

573 TNL – Total neurite length

574 IFN-γ - Interferon-γ

575 RBP – RNA-binding protein

576

577

578 **Declarations**

579 **Ethics approval and consent to participate:**

580 Not applicable.

581

582 **Consent for publication**

583 Not applicable.

584

585 **Availability of data and materials**

586 The datasets used and/or analysed during the current study are available from the
587 corresponding author on reasonable request.

588

589 **Competing interests**

590 The authors declare that they have no competing interests

591

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595

596 **Author's contributions**

597 E-R.M. conceived and designed the research, performed the laboratory research,
598 generated the data and figures, and contributed to manuscript writing and editing. J.G.M.
599 provided critical input and contributed to manuscript editing. M.A.R. provided critical
600 input and reviewed the manuscript. A.O-A. conceived and designed the research,
601 provided critical input, reviewed the manuscript, and secured funding. All authors
602 reviewed and approved the final manuscript.

603

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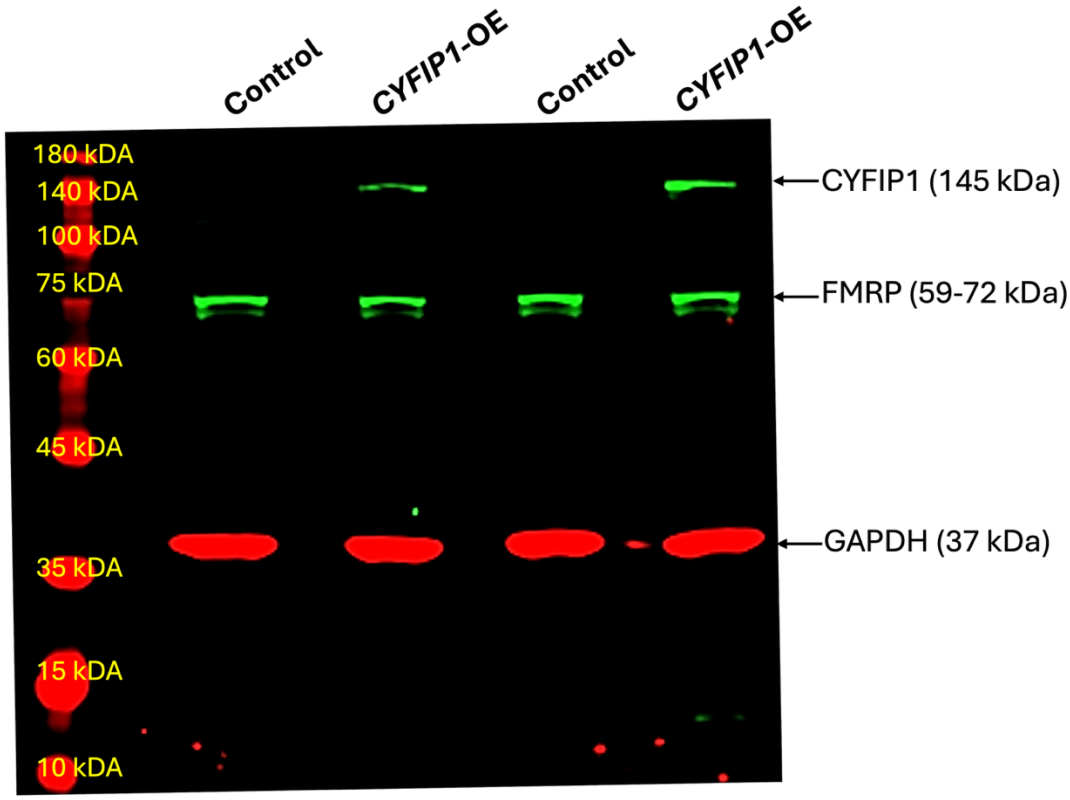
698 **Supplementary Files**

699



700

701 ***Supplementary Figure 1 | Modelling CYFIP1 overexpression in HEK293 cells.***

702 *Increased CYFIP1 protein expression in CYFIP1-OE cells 72 hours post-transfection*

703 *confirmed via Western blotting. Membrane probed with: Rabbit Anti-CYFIP1*

704 *(#ab156016, Abcam), Rabbit Anti-FMRP (#13755-1-AP, Proteintech), Mouse Anti-GAPDH*

705 *(#sc-47724, Santa Cruz Biotechnology), Goat Anti-Mouse DyLight™-680 (#35519,*

706 *Invitrogen) and Goat Anti-Rabbit DyLight™-800 (#SA5-10036, Invitrogen).*

707

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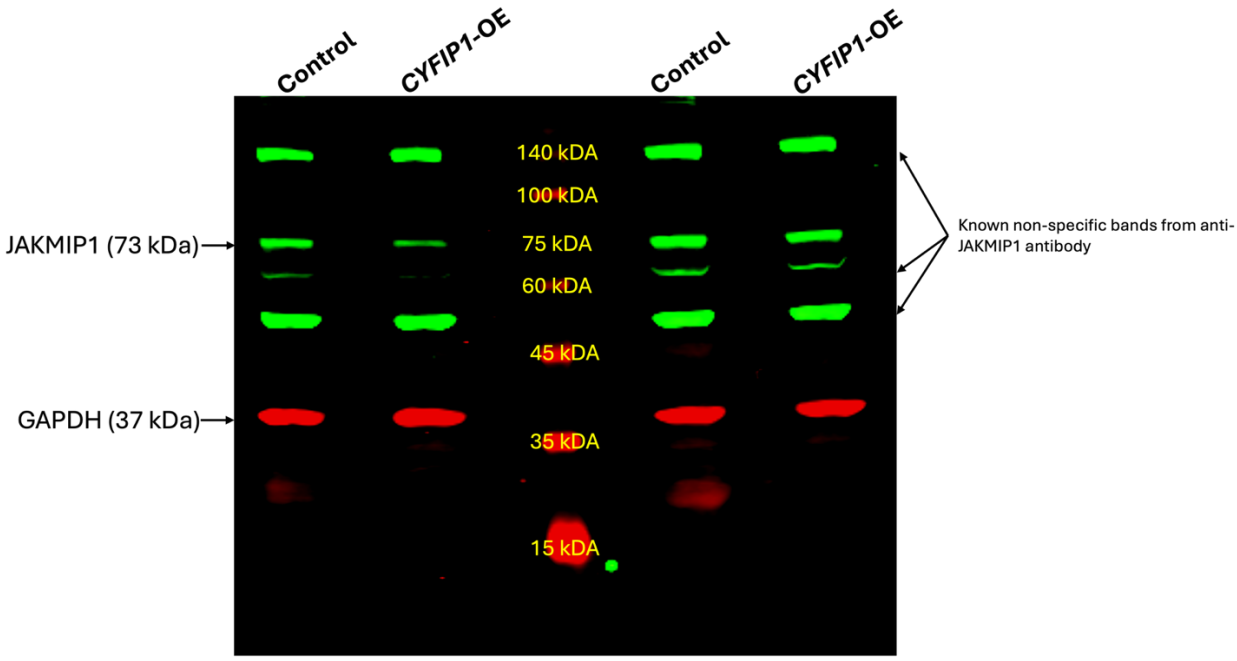
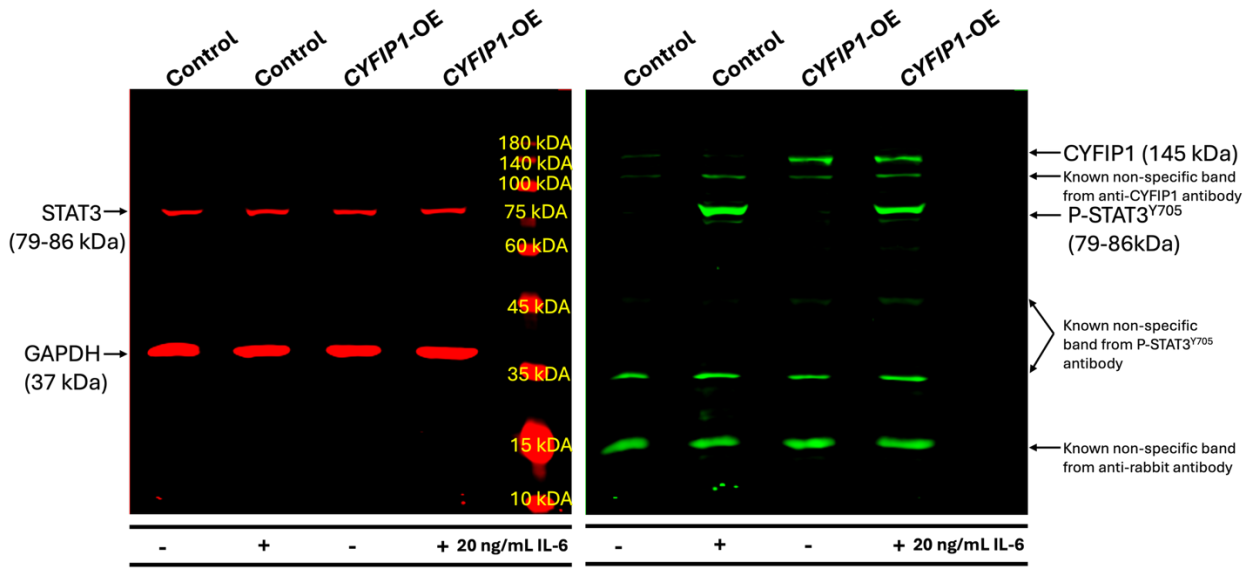


Supplementary Figure 2* | *CYFIP1-OE leads to reduced JAKMIP1 protein expression. *Western blotting of JAKMIP1 expression in Control and CYFIP1-OE cells. Membrane probed with: Rabbit Anti-JAKMIP1 (#13846-1-AP, Proteintech), Mouse Anti-GAPDH (#sc-47724, Santa Cruz Biotechnology), Goat Anti-Mouse DyLight™-680 (#35519, Invitrogen) and Goat Anti-Rabbit DyLight™-800 (#SA5-10036, Invitrogen). Known non-specific bands produced are indicated on the blot.*



Supplementary Figure 3 | CYFIP1-OE has no effect on STAT3 activation. *Western blotting of STAT3 expression and phosphorylation (P-STAT3^{Y705}) in Control and CYFIP1-OE cells following 30-minute treatment with 0 ng/mL IL-6 or 20 ng/mL IL-6. Membrane probed with: Mouse Anti-STAT3 (#9139S, Cell Signaling Technology), Rabbit Anti-P-STAT3^{Y705} (#9131l, Cell Signaling Technology), Mouse Anti-GAPDH (#sc-47724, Santa Cruz Biotechnology), Goat Anti-Mouse DyLight™-680 (#35519, Invitrogen) and Goat Anti-Rabbit DyLight™-800 (#SA5-10036, Invitrogen). Known non-specific bands are indicated on the blot.*