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(Article begins on next page)

1 **Intellectual Disability and Behavioral Deficits Linked to *CYFIP1* Missense Variants**

2 **Disrupting Actin Polymerization**

3

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26

27 **Keywords:** *CYFIP1*, Autism Spectrum Disorder, Actin remodeling, *Drosophila*, Social deficits,
28 Motor impairment.

29

30 **Short running title:** *CYFIP1* SNVs Affect Actin Polymerization and Behavior

31

1 **Abstract**

2 *Background:* 15q11.2 Deletions and duplications have been linked to autism spectrum disorder
3 (ASD), schizophrenia, and intellectual disability (ID). Recent evidence suggests that dysfunctional
4 *Cytoplasmic FMR1 Interacting Protein 1 (CYFIP1)* contributes to the clinical phenotypes observed
5 in individuals with 15q11.2 deletion/duplication syndrome. *CYFIP1* plays crucial roles in neuronal
6 development and brain connectivity, promoting actin polymerization and regulating local protein
7 synthesis. However, the impact of single nucleotide variants in *CYFIP1* to neurodevelopmental
8 disorders is limited.

9 *Methods:* Here, we report a family with two probands exhibiting ID, ASD, spastic tetraparesis, and
10 brain morphology defects carrying biallelic missense point mutations in the *CYFIP1* gene. We
11 used skin fibroblasts from one of the proband, parents, and typically developing individuals to
12 investigate the effect of the variants on the functionality of *CYFIP1*. In addition, we generated
13 *Drosophila* knock-in mutants to address the effect of the variants *in vivo* and gain insight into the
14 molecular mechanism underlying the clinical phenotype.

15 *Results:* Our study revealed that the two missense variants are in protein domains responsible
16 for maintaining the interaction within the wave regulatory complex (WRC). Molecular and cellular
17 analyses in skin fibroblasts from one proband showed deficits in actin polymerization. The fly
18 model for these mutations exhibited abnormal brain morphology and F-actin loss and
19 recapitulated the core behavioral symptoms, such as deficits in social interaction and motor
20 coordination.

21 *Conclusions:* Our findings suggest that the two *CYFIP1* variants contribute to the clinical
22 phenotype observed in the proband that reflects deficits in actin-mediated brain development
23 processes.

24

1 **Introduction**

2 The 15q11.2 (BP1-BP2) has emerged as a susceptibility locus for neuropsychiatric disorders,
3 including intellectual disabilities and neurobehavioral disturbances (1-6). Copy number variants
4 (CNV) in the 15q11.2 region have been identified in patients with autism spectrum disorders
5 (ASD), schizophrenia (SCZ), neurodevelopmental delay, intellectual disability (ID) and epilepsy
6 (1, 5, 7-17). Although, four genes are present within the human BP1-BP2 region (*TUBGCP5*,
7 *CYFIP1*, *NIPA2*, and *NIPA1*), increasing evidence points to the relevance of *Cytoplasmic FMR1*
8 *Interacting Protein 1* (*CYFIP1*). Due to its crucial role in synaptic development and functionality
9 (18, 19), neuronal connectivity (19, 20), brain wiring (21, 22) and GABAergic signaling (23, 24),
10 dysregulation of *CYFIP1* has been suggested to contribute to the clinical phenotype observed in
11 patients with 15q11.2 variations. *CYFIP1* CNVs have been associated with ASDs, SCZ, epilepsy,
12 and cognitive deficits (2, 8, 10, 11, 15, 17, 25-33). Single nucleotide variants (SNV) in coding
13 regions affecting the protein sequence have been reported in ASD cases (34-36) and in an
14 individual with congenital heart disease and learning disability (37). In addition, SNVs in the
15 noncoding regions of the *CYFIP1* gene have been detected in large-scale whole genome
16 sequencing (WGS) studies in cohorts affected by ASD (38-41). Finally, GWAS-ATLAS reveals
17 *CYFIP1* association with neurological and metabolic disorders (42). However, the precise role of
18 *CYFIP1* in those clinically relevant cases remains elusive.

19 *CYFIP1* plays a role in neurodevelopment by linking FMRP-dependent local protein synthesis
20 with actin cytoskeleton remodeling through Rac1 small GTPase signaling (18, 43). When bound
21 to Rac1, *CYFIP1* is part of the Wave Regulatory Complex (WRC), containing *WAVE1/2/3*, *ABI1/2*,
22 *NCKAP1* and *HPSC300* (44-46), which regulates the actin nucleation activity of the Arp2/3
23 complex. Under basal conditions, *CYFIP1* inhibits the Arp2/3 complex, while upon binding of
24 Rac1-GTP, *CYFIP1* conformational change in the WRC allows activation of the Arp2/3 complex
25 and actin polymerization (45-53). Fine-tuned actin cytoskeleton dynamics plays a crucial role in

1 neurodevelopmental processes such as dendritic spine morphology (54), axonal guidance and
2 branching (55), and synapse functionality (56, 57).

3 This study aimed to understand how SNVs in *CYFIP1* could contribute to neurodevelopmental
4 deficits. We report a family case with two probands carrying biallelic missense variants in the
5 *CYFIP1* gene, one variant inherited from each parent. Individuals are affected by severe ID and
6 ASD, together with epileptic encephalopathy, spastic tetraparesis, and microcephaly. Parents do
7 not show the clinical phenotype reported for the probands. Using available skin fibroblasts from
8 one of the probands, parents and typical developing individuals, and *Drosophila* CRISPR knock-
9 in (KI) models for the two missense variants, we investigated the impact of these SNVs on the
10 functionality of CYFIP1 and their contribution to the clinical phenotype of the proband.

11

1 **Methods and Materials**

2 **Ethics statements.** Human skin fibroblasts from Proband 1 and their parents were received from
3 clinical collaborators and coauthors of this study. All procedures performed in this study were in
4 accordance with the ethical standards of the French Institute Cochin research committee and with
5 the Declaration of Helsinki of 1964 and its subsequent amendments. Written informed consent to
6 the molecular diagnosis of genetic diseases was obtained from parents.

7

8 **Human fibroblast cell lines.** Fibroblasts from healthy volunteers (typically developing individuals
9 (TDI) were obtained from: 1) purchased from Coriell Institute Cell Repositories (Camden, NJ); 2)
10 UZ/KU Leuven Biobank (Dr. Hilde Van Esch, Belgium) and previously described (58, 59) (n = 4,
11 age range 22-33 years and n = 3 age 42-57). Human fibroblasts from father (I-1), mother (I-2) and
12 proband 1 (II-2) were obtained from the Institut Cochin, Université de Paris (France). See
13 Supplemental Information for culture conditions.

14

15 **Live imaging and analysis of actin cytoskeleton remodeling.** Cells on glass bottom plates
16 (MatTek) were transfected with CMV-Lifeact-EGFP (60) using Lipofectamine 2000 transfection
17 reagent (Invitrogen, Thermo Fisher Scientific) in Opti-MEM (Gibco, Thermo Fisher Scientific).
18 After 6 h, the medium was replaced and 24 h cells were imaged. Time-lapse imaging was
19 performed for 2 min/cell, on a Nikon A1R Eclipse Ti for fast and high-resolution acquisition. Argon
20 laser at 5% and 60x objective (UPlanSApo, NA 1.2, water immersion) were used. A chamber
21 around the microscope kept the sample at a constant temperature of 37 °C, CO₂ level and
22 humidity. Kymographs were generated from pixel-wide lines drawn orthogonally to the cell edge
23 in regions where lamellar protrusions of fibroblasts were actively protruding, using FIJI (61).

1 **Fly stocks and genetics.** Flies were maintained on standard cornmeal fly food at 25 ° C, 60 %
2 humidity, in a 12-h light/dark cycle. Wild type cantonized *w¹¹¹⁸* (23, 62) flies were used as control.
3 All transgenic lines tested were initially backcrossed into the *w¹¹¹⁸* background. Because the two
4 variants are present in a boy, male flies 5-7 days after eclosion were used for all experiments.
5 Behavioral experiments were performed between ZT1-ZT4 (Zeitgeber Time). Detailed information
6 on CRISPR/Cas9 generation of the *Drosophila* Cyfip-KI mutants, immunofluorescence,
7 mushroom body morphometric measurement and behavioral experiments are provided in
8 Supplemental Information.

9

10 **Statistics.** Analyses were performed using GraphPad Prism software (v.9). The Shapiro-Wilk test
11 for normal distribution of the data was used. Statistical tests are listed in the respective figure
12 legends. *p*-values < 0.05 were considered significant. The results are represented as mean ±
13 standard error of the mean (S.E.M.).

14

15 See Supplemental Information for description of the following methods: exome sequencing,
16 description and in silico analysis of the sequence variants, variant validation, structural variant
17 modeling, RNA isolation and qPCR, western blotting, surface sensing of translation (SUnSET)
18 assay, immunofluorescence, and immunoprecipitation of the CYFIP1 complex.

19

1 **Results**

2 **Exome sequencing and genetic analyses reveal potentially pathogenic missense variants** 3 **in *CYFIP1***

4 We identified a non-consanguineous family with two individuals (II-2 and II-3) affected by a severe
5 neurodevelopmental disorder (Figure 1A). Both individuals show developmental delay,
6 microcephaly (with head circumference (HC) below 2 standard deviations), inability to walk
7 without assistance, and absence of speech, consistent with a severe form of intellectual disability
8 (ID). ID was classified in both probands as severe or profound (according to DSM-5 criteria (63)).
9 The two probands exhibit autistic symptoms characterized by a “pseudo-Angelman” phenotype
10 (paroxysmal laughter and happy demeanor) and severe motor deficits. Proband 1 shows
11 hyperactivity and repetitive self-mutilating behavior (head-banging). Frequent generalized non-
12 motor seizures with onset between 3-6 years of age have been detected in both probands. Brain
13 FLAIR magnetic resonance imaging reported white matter hyperintensities in the thalamus with
14 moderate ventriculomegaly in proband 1. Table 1 reports a detailed summary of the clinical
15 characteristics of the two probands. Clinical evaluations report that neither of the parents (I-1 and
16 I-2) has clinical phenotypes. The family also has one unaffected sibling (II-1).

17 We performed Whole Exome Sequencing (WES) analysis on proband 1 (II-2) and his parents (I-
18 1 and I-2). In total 26'060 to 26'216 single nucleotide polymorphisms (SNPs) and 902 to 906 small
19 deletions/insertions (1-10 bp) were identified across the exomes of this family. After testing for *de*
20 *novo*, recessive and X-linked inheritance and filtering the variants for moderate/high pathogenicity
21 score impact (PolyPhen-2, SIFT, CADD and REVEL scores), we identified 14 potential pathogenic
22 genetic variants (Table 2). Pathogenic variants that were not detected in both probands and that
23 were not involved in syndromic disorders that could explain the clinical phenotype were excluded.
24 We narrowed the list down to two biallelic variants in the *Cytoplasmic FMRP interacting protein 1*
25 (*CYFIP1*) gene, potentially involved in the neurodevelopmental disorder of the probands. The

1 variant p.(Ile476Val), inherited from the father (15/152142 alleles in gnomAD v.3.0) was reported
2 tolerated, disease-causing, and “possibly damaging” by the pathogenicity prediction tools. The
3 variant p.(Pro742Leu), inherited from the mother (6/152210 alleles) was predicted to be tolerated
4 and benign by *in silico* analysis. Both missense variants were also detected in the second proband
5 and were confirmed by direct Sanger sequencing. Genetic analysis showed that the probands
6 were compound heterozygotes, while the parents and the sibling are heterozygotes for a single
7 variant (Figure 1A-B). Considering these findings and the prominent roles of *CYFIP1* in
8 neurodevelopment, we focused our attention on understanding the role of the two *CYFIP1* SNVs.

9 We utilized the available crystal structure of the human WRC (45, 64) to map the two *CYFIP1*
10 variants (Figure 1C-D). The p.(Ile476Val) variant is situated in the globular N-domain, in proximity
11 to the RAC1 binding site A (Figure 1C)(45). The isoleucine residue is embedded within a complex
12 set of secondary structural elements, specifically α -helices (Figure 1D, lower insets). Our
13 hypothesis suggests that the substitution of valine for isoleucine at position 476 could potentially
14 destabilize the structure of *CYFIP1*, due to the smaller size and lower hydrophobicity of valine
15 compared to isoleucine, resulting in a decrease in hydrophobicity. This would lead to a subtle
16 disruption in the contacts between the two secondary structural elements of the proteins and
17 hinder interaction with the amino acids Leu605 and Phe608 (Figure 1D, lower insets). This is
18 further supported by the protein stability analysis, which estimates a $\Delta\Delta G$ value of $+ 1.28 \pm 0.036$
19 kcal/mol for the Ile476Val variant.

20 The p.(Pro742Leu) variant is located in the central domain, that confers structural flexibility to
21 *CYFIP1* (Figure 1C), and contributes to the binding with the translation factor eIF4E and to the
22 interactions with WAVE1 and NCKAP1 in the WRC (45, 47, 48, 51, 64-66). A butterfly-like motion
23 has been associated with the ability of *CYFIP1* to switch from the WAVE complex towards the
24 eIF4E complex (43, 65), suggesting that the decrease in flexibility associated with the Pro742Leu
25 mutation could affect this switching ability (Figure 1D, upper insets). This is consistent with the

1 protein stability analysis, which revealed that this variation has a slightly destabilizing effect on
2 the 3D structure of CYFIP1 with a $\Delta\Delta G$ value of $+ 0.72 \pm 0.023$ kcal/mol (Figure 1D, upper insets).
3 Importantly, both variants affect amino acid regions that are highly conserved between taxa, from
4 humans to fruit flies (Figure 1E), underlying the relevance of these domains.

5

6 **Bi-allelic *CYFIP1* variants underlie a complex disorder affecting actin remodeling**

7 To determine the effect of the missense variants in *CYFIP1* at the functional level, we used the
8 available skin fibroblasts from proband 1, the two parents and age-matched typical developing
9 individuals (TDI). No defective levels of *CYFIP1* mRNA (Figure 2A) and protein levels (Figure 2B)
10 were observed in proband 1, neither differences in expression for the other genes deleted in the
11 BP1-BP2 deletion syndrome (*NIPA1*, *NIPA2*, and *TUBGCP5*), nor the Angelman syndrome-
12 associated gene *UBE3A* (Figure S1A).

13 *CYFIP1* coordinates cytoskeletal actin remodeling through interactions with the WRC (18, 20, 67)
14 and protein synthesis through interactions with FMRP and eIF4E (18, 19, 33, 43, 68). Impairments
15 in these molecular mechanisms are notably implicated in ASD pathogenesis (34, 68-74). We
16 performed surface sensing of translation (SUnSET) (75) to measure relative protein synthesis
17 rates in human fibroblasts (58). No differences in puromycin incorporation were observed
18 between fibroblasts from the proband and/or parents and TDI individuals, suggesting that these
19 variants do not affect overall protein synthesis levels (Figure S1B). In contrast, using phalloidin-
20 TRITC immunofluorescence to detect filamentous (F) actin (Figure 3A), we found that fibroblasts
21 from the proband 1 displayed a reduction in phalloidin intensity and therefore, F-actin levels
22 (Figure 3B). In addition, an increase in the aspect ratio (AR: major axis/minor axis, Figure 3C) in
23 the proband fibroblasts, indicated a subtle and yet significant change in cell morphology from a
24 typical spindle-like to a more stellate-shaped cell phenotype (Figure 3D).

1 F-actin remodeling is crucial for lamellar protrusion and cell motility (76). We performed live
2 imaging of actin lamellar protrusion dynamics in TDI and proband 1 fibroblasts after transfection
3 with Lifeact-GFP (60) to allow visualization of F-actin dynamics (77). Kymograph analysis of the
4 lamellar protrusions (Figure 3E) revealed a significant reduction in protrusion length, number, and
5 velocity (Figure 3F-H) in proband 1 cells, confirming the actin polymerization defects observed in
6 the presence of bi-allelic *CYFIP1* variants. Next, we investigated whether the two missense
7 variants affect the interaction of CYFIP1 within the WRC. We coprecipitated the CYFIP1-WRC
8 complex from TDI (Figure 3I-K, lane 1-3) and proband 1 (Figure 3I, lane 4-6) fibroblasts and
9 assessed the levels of CYFIP1 interactors with known involvement in Arp2/3 activation and actin
10 remodeling (44, 49, 50, 64, 78, 79). Notably, a significant reduction of CYFIP1 interaction with
11 NCKAP1 and WAVE1 in the proband 1 compared to TDI fibroblasts was observed (Figure 3I-K).
12 Collectively, these results suggest that the biallelic missense variants in the cells of proband 1
13 possibly impair the WRC functionality in actin polymerization due to the reduced CYFIP1 stability
14 inside the complex leading to the deficits in cytoskeleton actin dynamics.

15

16 ***CYFIP* SNVs recapitulate human deficits in actin polymerization in *Drosophila***

17 We utilized the model organism *Drosophila melanogaster* to investigate the effects of bi-allelic
18 *CYFIP1* missense variants on ID/ASD pathologies. Notably, *Drosophila* has been extensively
19 used to model specific aspects of human pathologies (80-82). The *Drosophila* genome contains
20 a single homolog (*Cyfp*) (83) of the two vertebrate genes, *CYFIP1* and *CYFIP2*, with high
21 homology (DIOPT score 15/18, 65% amino acid identity and 80% similarity). Alignment of the
22 human *CYFIP1* and the *Drosophila* *Cyfp* protein sequences revealed that the SNVs-containing
23 regions are conserved in the two organisms (Figure 4A), therefore, using the *scarless* CRISPR-
24 Cas9 gene-editing approach (Figure 4B), we generated knock-in (KI) fly lines for the human

1 missense variants, the p.(Ile476Val), p.(Ile471Val) in the fly genome and the p.(Pro742Leu)
2 variant, p.(Pro760Leu) in the fly genome.

3 While the *Cytip* homozygous null mutants are lethal at the pupa stage (83). The homozygous KI
4 animals (*Cytip*^{I471V}/*Cytip*^{I471V} and *Cytip*^{P760L}/*Cytip*^{P760L}) and the compound heterozygous genotype
5 (*Cytip*^{I471V}/*Cytip*^{P760L}) were viable, fertile, and did not display visible morphological phenotypes. No
6 differences in *Cytip* mRNA levels were detected in mutant flies (Figure S2A), consistent with the
7 previous observation in human skin fibroblasts (Figure 2A). Therefore, we asked if the actin
8 polymerization deficits observed upon the presence of the two variants were recapitulated within
9 the KI fly model system. We performed biochemical fractionation to measure the F- and globular
10 (G) actin amounts in the control and *Cytip* mutant heads. F-actin appeared to be significantly
11 reduced compared to G-actin in *Cytip*^{I471V}/*Cytip*^{P760L} flies in comparison to control and parental
12 lines, as shown by the reduced F-/G- actin ratio (Figure 4C). These results show that flies carrying
13 both *Cytip* variants, exhibit actin cytoskeleton deficits, like the molecular phenotypes observed in
14 the human cells.

15

16 **CYFIP SNVs affect neuronal morphology in *Drosophila***

17 CYFIP1 has been shown to regulate dendrite morphogenesis and axonal growth, pathfinding, and
18 branching (67, 84). To determine whether the missense *CYFIP1* variants affect neuronal
19 morphology in the fly model, we analyzed the structure of a well-characterized subset of neurons
20 called small lateral ventral neurons (s-LNVs). Their stereotyped axonal projection pattern (Figure
21 4D-E) allows studies of axonal arborization phenotypes (85, 86). Changes in s-LNVs axonal 3D
22 arborization were analyzed in *Cytip* mutant flies and control flies (Figure 4D-F), as previously
23 described (87). 3D spread analysis of the dorsal s-LNVs termini (Figure 4G) showed a decreased
24 in the spread of the arborizations in flies carrying the two missense variants, compared to control

1 flies and to flies heterozygous for the single missense variant. In addition, the axonal volume
2 occupied by the dorsal axonal projections was reduced in the *Cytip*^{I471V/P760L} and *Cytip*^{P760L/+}
3 mutants compared to the control flies (Figure 4H), indicating a morphological deficit. To further
4 investigate the brain morphology in the different *Cytip-KI* mutant flies, we conducted morphology
5 analyses of the mushroom bodies (MB) and the ellipsoid body (EB), high brain structures involved
6 in behaviors, from learning and memory, to social behavior and motor skills (88, 89) (Figure S2B-
7 C). No gross morphological defects were observed in mutant flies, however, a detailed
8 morphometric analysis (89) of the different structures revealed consistent subtle differences in
9 neuroanatomical organization. We quantified the widths and lengths of α - and β -lobes and surface
10 areas of the ellipsoid body (Figure S2D-F), uncovering significant differences in α - and β -lobe
11 lengths between control and *Cytip*^{I471V/Cytip}^{P760L} flies (Figure S2D). In addition, the α -lobe length
12 in heterozygous flies for the single and the double variants was significantly increased, while the
13 β -lobe length was increased in *Cytip*^{I471V/Cytip}^{P760L} and in *Cytip*^{P760L/+} flies (Figure S2D). No
14 changes in the α - and β -lobes width was observed (Figure S2E). Finally, an increase in the
15 ellipsoid surface relative to the control was observed in heterozygous flies for the two single
16 variants and in biallelic variant flies (Figure S2F). In summary, we showed that biallelic *Cytip*
17 missense variants in flies induces axonal lobe extension defects, suggesting that the biallelic
18 presence of the *CYFIP1* variants negatively impacts the neuronal and brain morphology in flies.

19

20 **CYFIP SNVs showed ASD-like behaviors in *Drosophila***

21 *CYFIP1* heterozygosity in animal models induced cognitive impairments (15), ASD- and SCZ-like
22 behaviors (22, 62, 90) including social and motor deficits (21-23, 90, 91).

23 Male flies were tested for competition for food (aggression paradigm), a well-established social
24 behavior assay (23) (Figure 5A). *Cytip*^{I471V/+}, *Cytip*^{P760L/+} and *Cytip*^{I471V/Cytip}^{P760L} flies showed

1 fewer social events than controls, indicating that the *Cytip* variants contribute to the aggression
2 phenotype and their combination exacerbate this phenotype (Figure 5B and Video S1). Second,
3 we assessed fly social group behavior using the Social Space Arena paradigm (Figure 5C).
4 Analyzing the overall social space distribution between each fly, we observed that most control
5 flies (> 40%) spend time at a close distance (< 0.5 cm). In contrast, *Cytip-KI* mutants exhibit a
6 different distribution in the arena (Figure 5D). Notably, *Cytip^{I471V}/Cytip^{P760L}*, *Cytip^{I471V/+}* and
7 *Cytip^{P760L/+}* flies distribute in the arena at farther distance from each other compared to control
8 flies. In addition, the social preference index as in (92, 93) revealed a decreased index for the
9 *Cytip* variants (Figure 5E). Overall, our analyses suggest that the *Cytip* missense variants give
10 susceptibility to social interaction defects strengthening the role of CYFIP in regulating social
11 interactions (23).

12 Additionally, we assessed motor skills in the flies carrying the missense variants. We evaluated
13 motor reflex as the ability to climb after sudden stimuli (namely, negative geotaxis behavior)
14 (Figure 5F) (94). The number of flies above a 6 cm distance 9 seconds after the startle input was
15 recorded (Figure 5G). The number of *Cytip^{I471V}/Cytip^{P760L}* flies above the target line compared to
16 control and flies with the missense variant in heterozygosity was significantly reduced. No
17 difference was observed between controls and *Cytip^{I471V/+}* and *Cytip^{P760L/+}* (Figure 5G and Video
18 S2). The reduced climbing behavior of *Cytip^{I471V}/Cytip^{P760L}* flies might be related to deficits in motor
19 reflexes or reflect a general locomotion deficit. To tease these apart, we assessed the total
20 locomotion (activity paradigm) (Figure 5H-I). Activity analysis revealed a reduced number of total
21 beam crossings in the bi-allelic mutants *Cytip^{I471V}/Cytip^{P760L}* compared to control, to *Cytip^{I471V/+}* and
22 *Cytip^{P760L/+}* flies, reflecting reduction in locomotion in the presence of both missense variants
23 (Figure 5I). These results confirm that the bi-allelic presence of the missense variants induces
24 motor deficits, including motor reflex deficits and hypo-activity in *Drosophila*.

25

1 **Discussion**

2 Previous reports have identified SNVs in *CYFIP1* in individuals with neurodevelopmental
3 disorders (NDD) or affected by ASD and learning disabilities (34-41), with no clinical evaluation
4 or investigation of the pathogenicity. Here, we report a complete clinical, molecular, and functional
5 characterization of two rare biallelic missense variants in the *CYFIP1* gene, predicted to be
6 deleterious and pathogenic. The two probands are affected by severe ID, motor deficits, repetitive
7 behavior, and social deficits. Notably, alterations in pathways regulated by CYFIP1 lead to cellular
8 (*i.e.*, spine structure and neurotransmission) and molecular (*i.e.*, actin dynamics and protein
9 synthesis) (18, 19, 23, 24, 33, 43, 68, 91, 95) defects possibly contributing to the severe
10 phenotype. *CYFIP1* haploinsufficiency in animal models revealed deficits in brain connectivity
11 abnormalities and ASD/SCZ- like behavioral phenotypes, *i.e.*, social interaction, repetitive
12 behaviors, learning, and sensory-motor processing (15, 21-23, 90).

13 The two variants cluster with domains relevant for the CYFIP1 function. In *silico* structural
14 modeling analysis revealed that the amino acid substitutions could impair the flexibility, the
15 motions of the secondary structure, and the network of hydrophobic interactions, possibly
16 affecting binding to eIF4E or to WRC (45, 47, 48, 51, 65, 96). Protein synthesis was not affected
17 by the presence of the missense variants, while a general reduction of actin cytoskeleton
18 dynamics and polymerization was observed. These findings are supported by the protein stability
19 analysis and immunoprecipitation experiments, indicating that the identified SNVs destabilize the
20 CYFIP1 structure inside the WRC, with the Pro742Leu and Ile476Val mutations acting inter- and
21 intra-molecularly, respectively, and impairing the interaction between CYFIP1 and its binding
22 partners, NCKAP1 and WAVE1. This disruption is likely to lead to WRC function defects that
23 might explain the observed phenotypes in the actin cytoskeleton and lamellar behavior in Proband
24 1 fibroblasts. Notably, NCKAP1 and WAVE family protein, including WAVE1, are known to
25 regulate the activity of Arp2/3 complex-mediated actin assembly (45, 50, 52, 64, 97-100),

1 necessary to promote cell shape, motility, and functionality. In line with our findings, F-actin
2 polymerization is reduced upon disruption of the CYFIP1-NCKAP1 interaction in dendritic spines
3 (18) and actin remodeling, migration and lamellipodia formation are impaired after NCKAP1
4 abrogation (49, 101). In addition, lamellipodia protrusion length deficits, reduced F-actin at leading
5 edges and deficits in dorsal ruffles formation and actin elongation have been observed upon
6 WAVE1 deletion (97, 102-104).

7 Dysregulation in actin polymerization causes neuronal and glial developmental abnormalities
8 (105), leading to ASD and neurological disorder, including seizures (106-109). Fine-tuned
9 cytoskeleton function regulates proper spine morphology, with spine abnormalities likely to confer
10 susceptibility to epilepsy due to disruptions to the excitatory/inhibitory (E/I) balance of neuronal
11 circuitries (110). E/I balance and GABAergic signaling dysregulations have been described in
12 animal models for *CYFIP1* haploinsufficiency, from flies to mice (23, 24). Interestingly, epileptic
13 encephalopathy, and intellectual disability have also been reported in patients with missense
14 variants in the *CYFIP1*-homologous gene, *CYFIP2* (106, 111, 112). *Drosophila Cyfip* null mutant
15 exhibit impaired axonal growth, guidance, and branching (20, 83). *CYFIP1* deficiency has been
16 shown to reduce axonal growth (84) and to regulate axonal outgrowth (67). We observed that the
17 *CYFIP1* variants affect mushroom bodies and ellipsoid body morphology and s-LNVs neurons
18 axonal branching in *Drosophila*. Interestingly, *Rho*, *Cdc42* and *Rac1* over-expression in these
19 neurons in *Drosophila* induces axonal branch overgrowth (87), a phenotype opposite than the
20 one observed in the *Cyfip-KI* bi-allelic mutants. We speculate that the reduced axonal projection
21 and volume that we observed in flies harboring the bi-allelic missense variants might be related
22 to deficits in actin polymerization. However, other mechanisms – such as guidance cues, neuronal
23 activity, and neurotransmitter release (55, 113, 114), not depending on actin remodeling - might
24 be involved in the observed cellular phenotype.

1 Social and motor coordination deficits are hallmarks of ASD and NDDs (115) and reported within
2 the 15q11.2 deletion (7, 13). These phenotypes have also been described in animal models for
3 *CYFIP1* haploinsufficiency with brain connectivity abnormalities and motor coordination deficits
4 (21-23, 90). The observed behavioral deficits in social interaction and motor skills in flies harboring
5 the *CYFIP1* missense variants, suggest that the combination of both Cyfip I471V and Cyfip P760L
6 contribute to the clinical phenotypes of the two probands. Furthermore, in the social domains,
7 social deficits have also been observed in the presence of single missense variants in
8 heterozygosity. Although mutants carrying the single or both missense mutations show a similar
9 pattern for certain cellular and behavioral phenotypes, the variability in the data make us cautious
10 to conclude on the specific contribution of each variant while the presence of both variants result
11 in a clear more severe phenotype. Consistently, it was shown that the combination of rare and
12 recessive biallelic mutations contribute to the pathogenicity of NDDs and ASD (116-120).

13 In conclusion, we reported a rare case of biallelic missense variants in the *CYFIP1* gene in two
14 individuals with NDD. Using the fly model, we investigated their impact in the absence of the
15 confounding effects of genetic background gaining insights on the pathogenicity underlying the
16 relevance of *CYFIP1* in NDDs. Variants in the *CYFIP1* gene can represent susceptibility factors
17 for variable cognitive, neurological, and psychiatric disorders and may result in severe NDDs such
18 as those observed in the probands. Future work on KI mouse models and brain organoids will
19 complement our study to further clarify the functional consequences of *CYFIP1* variants in
20 mammalian brain development.

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3

4 **Figure Legends**

5 **Figure 1: Identifications of two missense variants in human *CYFIP1*.**

6 (A) Pedigree chart showing segregation of rare pathogenic *CYFIP1* variants c.1426A > G;
7 p.(Ile476Val) and c.2225C > T; p.(Pro742Leu) in the family. Unfilled shapes denote typically
8 developing individuals (TDI), while filled shapes denote probands; squares are males; circles are
9 females. (B) Electropherograms of Sanger sequencing of genomic DNA of exons 14 and 20 of
10 the *CYFIP1* from the family members (I-1, I-2, II-2 and II-3). The variants are highlighted in yellow.
11 For exon 20 the overlapping peaks have been detected as “N” (an ambiguous, or unknown base)
12 from the automatic detection software and interpreted as “T”. (C) Schematic representation of the
13 *CYFIP1* protein (NP_001274739.1). The functional domains are color coded: in magenta and
14 orange (Rac1-binding sites), green (WAVE1-VCA), blue (Abi2-HSPC300) and purple (eIF4E),
15 Arf1 (grey) respectively (45, 46, 51, 53, 65, 66). The putative FMRP binding region is shown in
16 light blue (18, 43, 121). In light yellow the putative NCKAP1 binding regions (45, 47, 52, 66).
17 Asterisks indicate missense variants, in black, the previously identified in individuals with ASD
18 and learning disabilities (34-37) and in red the new identified variants in this work. (D) Structure
19 of the WAVE regulatory complex (WRC) indicating the sites of *CYFIP1* variants. *CYFIP* is shown
20 in red. Amino acid residues affected by missense variants are enlarged in circles. Next to each
21 substitution the $\Delta\Delta G$ value is reported. (E) Alignment of human *CYFIP1* protein
22 (NP_001274739.1) against the mouse (NP_001158133.1), zebrafish (NP_997924.1) and
23 *Drosophila* (NP_650447.1) homologs showing the conservation of sequences where the
24 missense mutations are located. The variants identified in the probands are marked in light blue.

25

26 **Figure 2: Characterization of the *CYFIP1* missense variants.**

1 (A) Quantification of *CYFIP1* mRNA levels by real-time qPCR in the affected individual (proband
2 1, p.[(Ile476Val)];[(Pro742Leu)]) compared to TDI (3 lines, 22-33 years old and 1 line, 50 years
3 old) and parents (the father, p.[(Ile476Val)];[(=)]) and the mother p. [(=)]; [(Pro742Leu)])
4 fibroblasts. TDI n = 12, Proband 1 n = 3, Father n = 3, Mother n = 3, technical replicate. One-Way
5 ANOVA, n.s. not significant. Data are represented as mean \pm S.E.M. (B) Left, representative
6 Western blot showing *CYFIP1* in fibroblasts from the proband 1, parents and TDI (4 lines 22-33
7 years old and 2 lines 50-57 years old). Right, bar plots showing the quantification of *CYFIP1* levels
8 normalized over Vinculin, α -Tubulin, and total protein content. TDI n = 31, Proband 1 n = 6, Father
9 n = 7, Mother n = 7, technical replicates. One-Way ANOVA. n.s. not significant. Data are
10 represented as mean \pm S.E.M.

11

12 **Figure 3: Impact of human *CYFIP1* missense variants on actin polymerization.**

13 (A) Representative images of primary fibroblasts from Proband 1, parental and TDI lines stained
14 with phalloidin-TRITC to detect F-actin and DAPI for nuclei (Scale bar = 55 μ m). Quantification
15 TRITC fluorescent intensity (B) and aspect ratio (C-D) in the proband 1, parents and TDI (4 lines
16 22-33 years old and 3 lines 43-57 years old), fibroblasts. (C) Schematic representation of the
17 Aspect Ratio parameter. Created with BioRender.com. Dots represent the quantification of a
18 technical replicate performed on cells at different passage (TDI n = 26, Proband 1 = 5, Mother =
19 5, Father = 5, 50-60 cells for each line). One-Way ANOVA followed by Sidak's multiple
20 comparison test; exact significant *p*-values are reported in the figure. Data are represented as
21 mean \pm S.E.M. (E) Representative kymographs at the level of the lamellar protrusions extracted
22 from the fibroblasts from the Proband 1 and TDI (2 lines of 22 years). Quantification of length (F)
23 number (G) and velocity (H) of lamellar protrusions. Dots represent kymographs from TDI (2 lines,
24 n = 131 and Proband 1 n = 82, from 18-20 cells per genotype recorded and analyzed) (scale bar
25 = 1 μ m). (F and H) unpaired Student's t-test. (G) Mann Whitney test, exact significant *p*-values
26 are reported in the figure. Data are represented as mean \pm S.E.M. (I-J) Representative Western

1 blot showing the CYFIP1 immunoprecipitated complex from TDI (2 lines) and Proband 1
2 fibroblasts protein extracts (lanes 1-3 and 4-6, respectively, Input) and the detection of NCKAP1
3 (I) and WAVE1 (J). (K) Quantification of NCKAP1 and WAVE1 in the CYFIP1 immunoprecipitated
4 complex. The protein levels were normalized to the immunoprecipitated CYFIP1 levels. Dots
5 represent the quantification of a technical replicate performed on cells at different passages.
6 NCKAP1 (TDI n = 16, Proband 1 n = 9), WAVE1 (TDI n = 8, Proband 1 n = 5). Multiple t-test
7 corrected for multiple comparisons using Holm-Sidak method, exact significant *p*-values are
8 reported in the figure. Data are represented as mean ± S.E.M.

9

10 **Figure 4: Modelling the human *CYFIP1* missense variants in *Drosophila*.**

11 (A) Alignment of the protein sequences of the human CYFIP1 with the fly CYFIP showing the
12 similarity in the region affected by the missense variants. The variants identified in the probands
13 are marked in light blue. (B) Generation of *Cytip*^{I471V} and *Cytip*^{P760L} alleles using the *scarless*
14 genome editing approach. (C) Upper panel: representative Western blots for F-actin and G-actin
15 levels from *Drosophila* whole brain. Lanes belongs to the same blot, n = 5-6 (pool of 20 fly heads)
16 in control and *Cytip* KI flies. Lower panel: Quantification of the F/G actin ratio normalized to control
17 flies. One-way ANOVA followed by Sidak's multiple comparisons test, exact significant *p*-values
18 are reported in the figure. Data are represented as mean ± S.E.M. (D-H) 3D structure analysis of
19 the s-LNvs axonal arborization in control and *Cytip* KI mutant flies. (D) Schematic representation
20 of an hemibrain of *Drosophila* with highlighted the location and structure of the s-LNvs axonal
21 dorsal projections. Left insets show a schematic magnification of the s-LNv dorsal projections and
22 a max intensity confocal projection s-LNvs dorsal projections stained with PDF antibody. (E)
23 Representative 3D reconstructions of the s-LNv dorsal projections showing the local z- and y axis.
24 (F) Representative confocal max intensity projections of the s-LNv dorsal projections in control
25 and *Cytip* mutant flies (upper panels, scale bar = 10 μm) and their 3D reconstructions (lower
26 panels) (z-axis in blue-to-red color scale), 1 pixel = 0.06 μm and z-step size = 1 μm (see

1 Supplemental Materials and Methods for details). (G - H) Quantification of the 3D spread (G) and
2 the axonal 3D volume (H) covered by the axonal arborization. n = 24-29 axonal projections from
3 14-15 brains per genotype. One-way ANOVA followed by Sidak's multiple comparisons test: exact
4 significant *p*-values are reported in the figure. Data are represented as mean ± S.E.M.

5

6 **Figure 5: Effects of the human *CYFIP1* missense variants on *Drosophila* social and motor**
7 **behaviors.**

8 (A-B) Control and *Cyfi*p mutant flies were analyzed for the total number of social interactions in a
9 competition for food assay. (A) Schematic representation of the behavioral protocol and setup for
10 the competition for food assay. (B) Quantification of the total number of social interaction events.
11 n > 10 (pool of 8 male flies each, from 3-5 independent experiments). One-way ANOVA followed
12 by Sidak's multiple comparisons test; exact significant *p*-values are reported in the figure. Data
13 are represented as mean ± S.E.M. (C-E) Social space behavior. (C) Representative images of
14 the social space arena in control and *Cyfi*p mutant flies. Different distances among flies are
15 depicted in circles: in red, a close distance (0-0.5 cm) and in blue (1.0 – 1.5 cm) (scale bar = 1
16 cm). (D) Distance to the closest flies indicated as percentage distribution of flies in the indicated
17 ranges of distances, 0 - 0.5 cm, 0.5 - 1.0 cm, 1.0 -1.5 cm and > 1.5 cm bins. n > 8 arenas of 29-
18 30 flies each per genotype. Kolmogorov–Smirnov test was assessed on the total distribution of
19 the distances to the closest fly, exact significant *p*-values are reported in the figure. Data are
20 represented as mean ± S.E.M. (E) Social preference (social space index) based on the distribution
21 of flies over a 1 cm maximum distance. n > 8 arenas per genotype. One sample t- test compared
22 to equal preference (0), exact significant *p*-values are reported in the figure. Data are represented
23 as mean ± S.E.M. (F-G) Rapid Iterative Negative Geotaxis climbing assay (RING) in control and
24 *Cyfi*p KI mutants. (F) Schematic representation of the climbing assay (negative geotaxis)
25 behavioral protocol. (G) Quantification of the number of flies (in percentage) that climbed the 6
26 cm distance in 9 seconds after sudden stimulus. n > 11 (groups of 17-20 flies each). One-way

1 ANOVA followed by Sidak's multiple comparisons test; exact significant p -values are reported in
2 the figure. Data are represented as mean \pm S.E.M. (H-I) Locomotor activity in control and *Cyfi*
3 KI mutants measured with the *Drosophila* Activity Monitoring System (DAMs, Trikinetics). (H)
4 Schematic representation of the experimental setup composed of an activity monitoring tube for
5 individual fly activity recording. The monitoring tubes are in an incubator with control light dark
6 cycle and automatically recorded. Created with BioRender.com. (I) Quantification of the total
7 activity in control and *Cyfi* KI mutants. Activity (beam crossings) over 24 hr. $n = 70$ -100 flies per
8 genotype, from > 3 independent experiments. One-way ANOVA followed by Sidak's multiple
9 comparisons test: exact significant p -values are reported in the figure. Data are represented as
10 mean \pm S.E.M.
11

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13

KEY RESOURCES TABLE

Resource Type	Specific Reagent or Resource	Source or Reference	Identifiers	Additional Information
Add additional rows as needed for each resource type	Include species and sex when applicable.	Include name of manufacturer, company, repository, individual, or research lab. Include PMID or DOI for references; use "this paper" if new.	Include catalog numbers, stock numbers, database IDs or accession numbers, and/or RRIDs. RRIDs are highly encouraged; search for RRIDs at https://scicrunch.org/resources .	Include any additional information or notes if necessary.

Table 1. Clinical features of individuals with recessive *CYFIP1* missense variants.

	Individual 1 (II-2) – Proband 1	Individual 2 (II-3)
CYFIP1 variant	p.(Ile476Val); p.(Pro746Val)	p.(Ile476Val); p.(Pro746Val)
Inheritance	p.(Ile476Val) paternal p.(Pro746Val) maternal	p.(Ile476Val) paternal p.(Pro746Val) maternal
Sex	Male	Male
Gestational age (weeks)	39 (amenorrhea)	40 (amenorrhea)
Birth HC/length/weight standard deviations	32,5cm (-2 SD) /50 cm (M)/3315gr (M)	32 cm (-2 SD)/ 48 (-1,5 SD)/ 2630gr (-1,5 SD)
HC/length/weight at last investigation standard deviations	52 cm (-3SD)/ 157 cm (-3SD)/ 43,5 Kgs (-2,5SD) at 17,5 years	48 cm (-3SD)/ 120,5 cm (M)/ 23,4kgs (-1SD) at 9 years
Microcephaly	Yes	Yes
Morphological features	Large mouth, thick lips, hypertelorism, low hairline	Large mouth, thick lips, hypertelorism, low hairline
Developmental delay	Severe developmental delay can sit up, walk with assistance	Severe developmental delay can sit up, walk with assistance
Age at unassisted sitting	4 years	1 year
Age at independent walking	No independent walking	No independent walking
Age at first words	No words	No words
Age at last evaluation	17 years and 8 months old	13 years et 4 months old
Speech development	Non-verbal	Non-verbal
Intellectual disability	Profound	Profound
Abnormal behavior	Paroxysmal bursts of laughter/ pseudo-Angelman behavior (happy demeanor) Hyperactivity Repetitive behavior (head banging)	Unmotivated laughter/ pseudo-Angeln behavior (happy demeanor)
Seizure (age of onset)	Yes (3 years)	Yes (6 years)
Epilepsy syndrome	Yes	Yes
Seizure type/ medicament	Absence seizures / ZONISAMIDE and valproate	Absence seizures / valproate
Current seizure frequency	Several times a week	Stabilized epilepsy
EEG findings	Paroxysm	Slow track
Cerebral MRI findings	FLAIR hyper signal in the thalamus, moderate ventriculomegaly, normal spectroscopy	Normal
Muscular hypo-/hypertonia	Spasticity of the lower limbs, pyramidal syndrome	Trunk ataxia
Other neurological issues	Autistic symptoms	Autistic symptoms
Other features	Scoliosis (arthrodesis), strabismus	Valgus flat feet (arthrodesis)

Abbreviations: HC: head circumference; EEG: electroencephalogram; MRI: magnetic resonance imaging.

Table 2. Detection of the SNVs by Sanger sequencing in the proband 1 and prediction of pathogenicity.

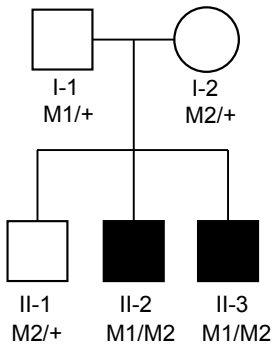
Gene variant	Genomic position	Mode of transmission	Dbsnp	PolyPhen-2	SIFT	CADD phred	Revel	GnomAD v3	SPiP prediction	Reason for exclusion
<i>CLCN3</i> (c.2031G>A p.Val677=)	chr4:g.170628299G>A	<i>De novo</i>	Not reported	Benign	Tolerated			Not reported		Considered benign by all predicting bioinformatic softwares; Not present in proband 2
<i>SFI1</i> (c.1323G>A p.Leu441=)	chr22:g.31985435G>A	<i>De novo</i>	rs1479552764	Benign	Benign			Not reported		Considered benign by all predicting bioinformatic softwares; Not present in proband 2
<i>POLA1</i> (c.617C>T p.Thr206Met)	chrX:g.24734570C>T	X-linked	rs200356660	Benign	Tolerated	7.66	Benign	5/112242	69.33% risk of altering the consensus splice site	Considered benign/tolerated by SIFT, CADD, PolyPhen2, REVEL; Not present in proband 2
<i>MAGEB2</i> (c.47G>A p.Arg16His)	chrX:g.30236744G>A	X-linked	rs151181148	Possibly damaging	Tolerated	10.29	Benign	33/112089		Considered benign/tolerated by SIFT, CADD, REVEL; Not present in proband 2
<i>SMC1A</i> (c.861G>A p.Lys287=)	chrX:g.53439197C>T	X-linked	rs782543093	Benign	Benign			13/111573		Considered benign by all predicting bioinformatic softwares;
<i>UXT</i> (c.264C>G p.Asn88Lys)	chrX:g.47516991G>C	X-linked	Not reported	Probably damaging	Damaging	24.1	Uncertain	Not reported	30.67% risk of creation of a new splice site	Gene involved in syndromic disorder that cannot explain the probands phenotype (124)
<i>IFIH1</i> (c.1230T>G p.Ile410Met)	chr2:g.163138952A>C	Recessive	Not reported	Probably damaging	Damaging	22.70		Not reported		Inherited from the unaffected father; Not present in proband 2
<i>IFIH1</i> (c.949C>T p.Gln317*)	chr2:g.163144791G>A	Recessive	rs74162079			37.00		5/151872		Inherited from the unaffected mother; Loss of function of the gene is related to syndromic disorders that cannot explain the probands phenotype (125)

<i>TTN</i> (c.89846C>T p.Thr29949Ile)	chr2:g.179417781G>A	recessive	rs1284316750	Possibly damaging	Damaging	22.5	Uncertain	1/247238		Not present in proband 2
<i>TTN</i> (c.55378A>G p.Thr18460Al a)	chr2:g.179466439T>C	recessive	rs727503600	Probably damaging	Tolerated	24.00	Uncertain	17/152066		Gene involved in syndromic disorder that cannot explain the probands phenotype (126)
<i>CYFIP1</i> (c.1426A>G p.Ile476Val)	chr15:g.22954276A>G	recessive	rs148341871	Possibly damaging	Tolerated	22.70	Benign	15/152142		//
<i>CYFIP1</i> (c.2225C>T p.Pro742Leu)	chr15:g.22962505C>T	recessive	rs139576657	Benign	Tolerated	23.40	Benign	6/152210		//
<i>SCAF1</i> (c.436A>C p.S146R)	chr19:g.50150045A>C	recessive	Not reported	Benign	Damaging	23.8	Benign	Not reported		Not present in proband 2
<i>SCAF1</i> (c.3181G>A p.A1061T)	chr19:g.50156827G>A	recessive	rs183980772	Benign	Damaging	22.8	Benign	42/152020		Not present in proband 2

Selected variants have been narrowed down using consecutive filters based on different models of inheritance (*De novo*, X-linked and recessive) and damage prediction scores (PolyPhen-2 (102), SIFT (127), CADD (128), Revel (129) and SPIP (130)). All the variants listed have been checked for their presence in the second affected individual (II-3).

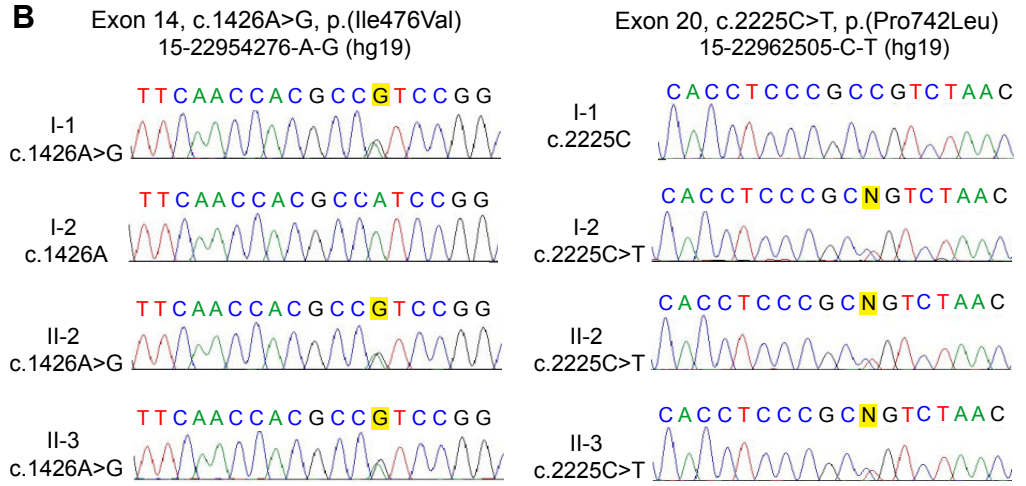
Figure 1.

A

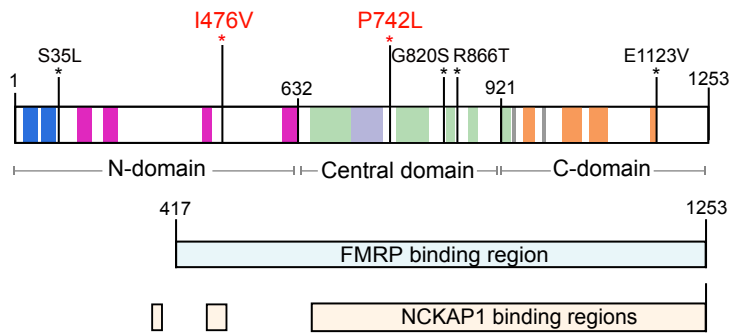


M1: *CYFIP1*: chr15:c.1426A>G
M2: *CYFIP1*: chr15:c.2225C>T

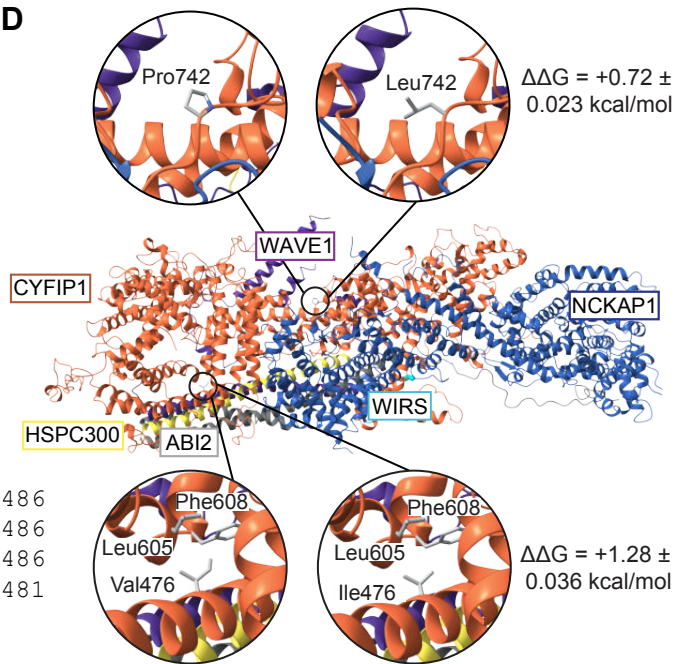
B



C



D



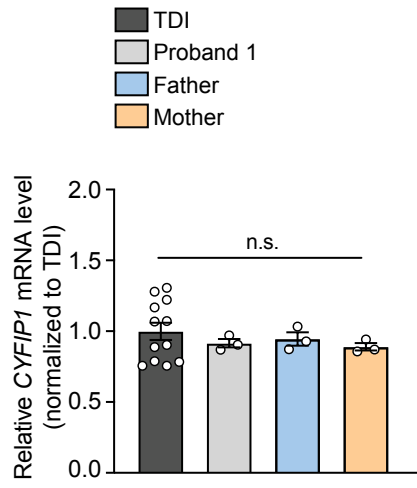
E

			p.I476		
<i>H. sapiens</i>	CYFIP1	469	ESVFNHAI	RHTVYAALQD	486
<i>M. musculus</i>	Cyfp1	469	ESVFNHAI	RHTVYAALQD	486
<i>D. rerio</i>	cyfp1	469	ESVFNHAI	RHTIYSALQD	486
<i>D. melanogaster</i>	CYFIP	464	ETVLCEAI	RNNIYSELQD	481

			p.P742		
<i>H. sapiens</i>	CYFIP1	736	GATIH-L	PSPNRYETLLK	752
<i>M. musculus</i>	Cyfp1	736	GATIH-L	PSPNRYETLLK	752
<i>D. rerio</i>	cyfp1	736	GANIS-W	PSSNRYETLLK	752
<i>D. melanogaster</i>	CYFIP	753	GFNFQSY	PRNNRYETLLK	770

Figure 2.

A



B

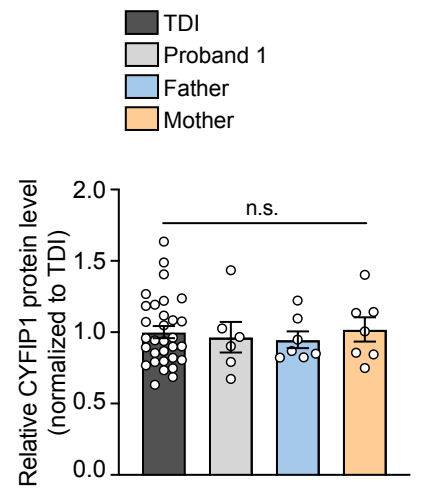
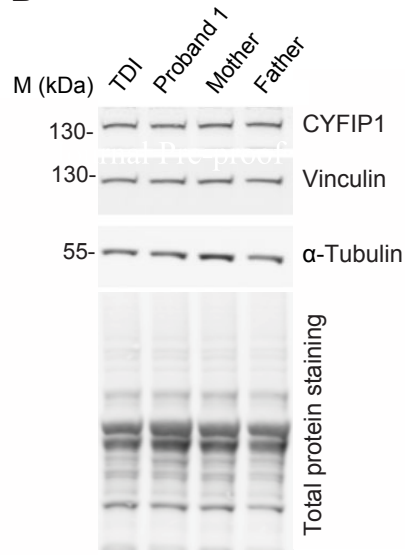


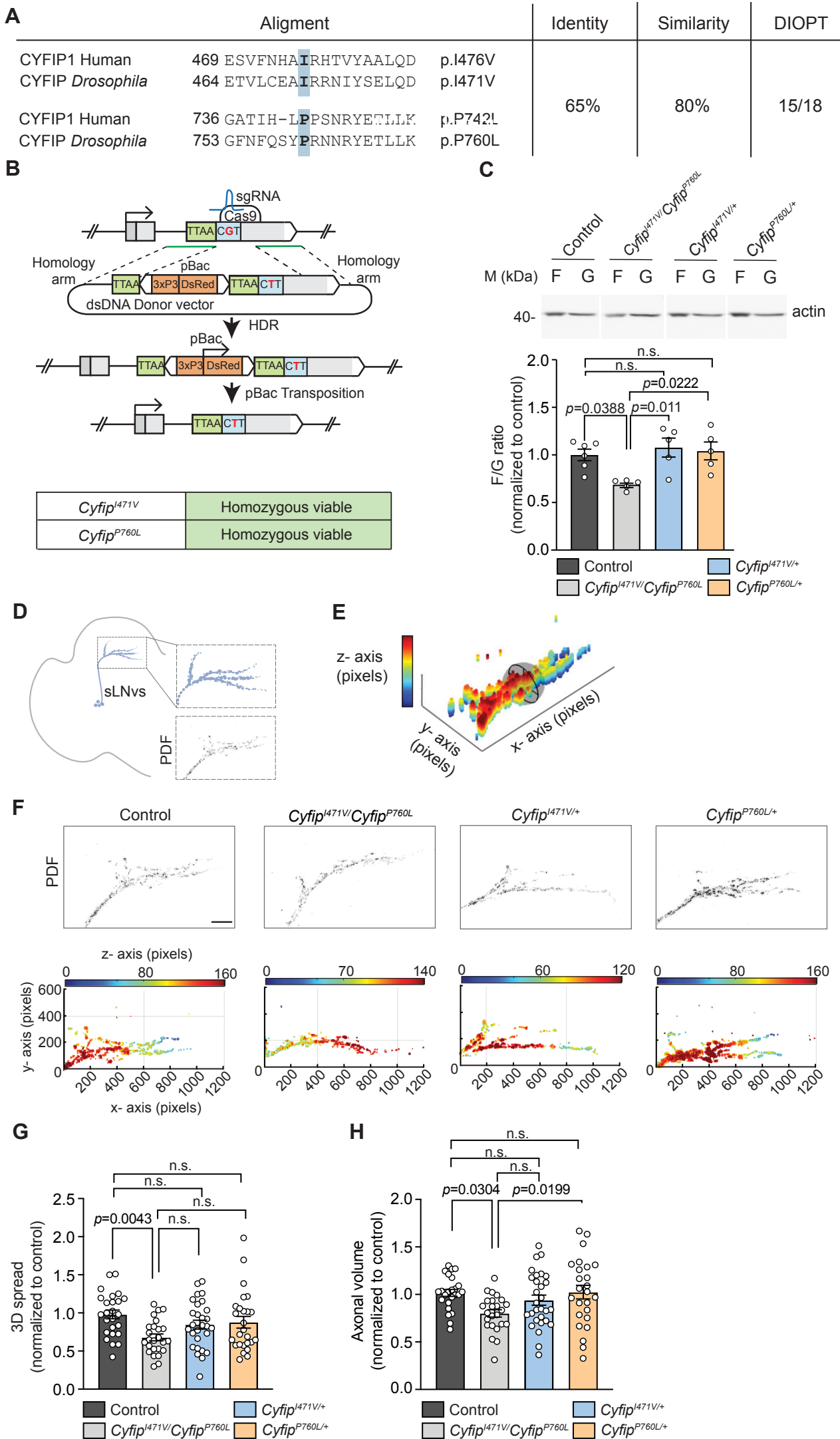
Figure 4.

Figure 5.