European Journal of Human Genetics (2019) 27:747–759
https://doi.org/10.1038/s41431-018-0331-z

ARTICLE

Spatially clustering de novo variants in *CYFIP2*, encoding the cytoplasmic FMRP interacting protein 2, cause intellectual disability and seizures

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Received: 30 May 2018 / Revised: 31 October 2018 / Accepted: 7 November 2018 / Published online: 21 January 2019

Abstract

CYFIP2, encoding the evolutionary highly conserved cytoplasmic FMRP interacting protein 2, has previously been proposed as a candidate gene for intellectual disability and autism because of its important role linking FMRP-dependent transcription regulation and actin polymerization via the WAVE regulatory complex (WRC). Recently, de novo variants affecting the amino acid p.Arg87 of *CYFIP2* were reported in four individuals with epileptic encephalopathy. We here report 12 independent patients harboring a variety of de novo variants in *CYFIP2* broadening the molecular and clinical spectrum of a novel *CYFIP2*-related neurodevelopmental disorder. Using trio whole-exome or -genome sequencing, we identified 12 independent patients carrying a total of eight distinct de novo variants in *CYFIP2* with a shared phenotype of intellectual disability, seizures, and muscular hypotonia. We detected seven different missense variants, of which two occurred recurrently (p.(Arg87Cys) and p.(Ile664Met)), and a splice donor variant in the last intron for which we showed exon skipping in the transcript. The latter is expected to escape nonsense-mediated mRNA decay resulting in a truncated protein. Despite the large spacing in the primary structure, the variants spatially cluster in the tertiary structure and are all predicted to weaken the interaction with WAVE1 or NCKAP1 of the actin polymerization regulating WRC-complex. Preliminary genotype–phenotype correlation indicates a profound phenotype in p.Arg87 substitutions and a more variable phenotype in other alterations. This study evidenced a variety of de novo variants in *CYFIP2* as a novel cause of mostly severe intellectual disability with seizures and muscular hypotonia.

Introduction

Intellectual disability (ID) is a genetically heterogeneous disorder with an estimated prevalence of 2–3%, with 0.3–0.5% severely affected [1]. Epilepsy is a frequent comorbidity [2, 3], which worsens its psychosocial outcome [4]. Genetic defects are thought to be a major cause of ID and epilepsy, but the identification of causative variants is complicated by tremendous genetic heterogeneity. Next-generation sequencing techniques, such as whole-exome sequencing (WES) and whole-genome sequencing (WGS), have been shown to be outstanding tools for the identification of causative variants in a large number of established and novel disease genes, especially when a patient-parent trio is available [5–10]. However, prioritization of candidate variants and genes can still be challenging. Aside from ranking the impact of the variants themselves (truncation, substitution, etc.), characteristics like expression pattern,

Supplementary information The online version of this article (https://doi.org/10.1038/s41431-018-0331-z) contains supplementary material, which is available to authorized users.

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biological function and processes, interaction partners, and molecular networks as well as phenotypes of knockout models are often used to underpin the potential role of genes in disease pathogenesis. Several ID and epilepsy genes converge into common networks and play key roles in neurogenesis, neuronal migration and synaptic functions [1, 11]. Hence, other involved players and protein families are suitable candidates for novel ID and epilepsy disorders.

The evolutionary highly conserved cytoplasmic FMRP interacting proteins (CYFIPs) represent one such candidate protein family for ID and epilepsy. Its two members in humans, CYFIP1 (MIM 606322) and CYFIP2 (MIM 606323) (also known as PIR121), interact with the fragile X mental retardation protein (FMRP), a RNA-binding protein with an important role in translational control, the absence of which leads to fragile X syndrome (MIM 300624) [12]. Additionally, CYFIP1 and CYFIP2 were detected at the synapse [12] and are members of the canonical WAVE regulatory complex (WRC). This complex is activated by interaction of CYFIP with the small Rho GTPase Rac1 [13–16] and dominant-negative and constitutively active *RAC1* variants have been recently reported in ID (MIM 617751) [17]. The WRC is a key regulator of actin dynamics [18] and missense and/or loss-of-function variants in two non-muscle actins, *ACTB* and *ACTG1*, cause syndromic forms of ID (MIM 243310 and 614583) [19, 20]. Given their role as "link" between Rac1, the WAVE complex, and FMRP, the CYFIP proteins have already been proposed as good candidates for ID and autism [13]. However, the focus remained on the more extensively studied *CYFIP1*, which is also one of the four genes deleted in the 15q11.2 BP1–BP2 microdeletion syndrome located within the Prader-Willi/Angelman syndrome region [21]. Concerning *CYFIP2*, only recently three variants affecting the Arginine at position p.87 have been published as causative in four patients with early-onset epileptic encephalopathy [22].

Using WES or WGS in trios of an affected child and its healthy parents, we independently identified eight distinct, spatially clustering de novo variants in *CYFIP2* in a total of 12 patients delineating the genetic and clinical spectrum associated with variants in *CYFIP2*.

Methods

Subjects and high-throughput sequencing

The twelve patients underwent clinical examination at centers in Switzerland, Germany, The United Kingdom, Estonia, the USA, and France. Trio WES or WGS was either performed on a routine diagnostic basis with subsequent consent for publication or as part of a research study approved by the local ethics commissions on human research and were in keeping with international standards. A detailed clinical description of the patients is provided in Table 1 and the case reports in the supplement.

Patient 1 (P1) was part of a study including 63 unrelated patients with early-onset epileptic encephalopathy (EE) and trio WES was carried out as described elsewhere [23, 24]. P2, P4, and P5 were part of eight cases with de novo *CYFIP2* rare nonsynonymous variants identified in 2793 cases with ID/DD and seizures who underwent trio WES by the GeneDx Laboratory (Gaithersburg, MD, USA) as described elsewhere (supplement and Tanaka et al. [25]). The remaining five cases could not be included into this study due to lack of consent for publication. The variant reported in P3 was identified by trio WGS conducted at the HudsonAlpha Institute for Biotechnology (Huntsville, AL, USA) in a cohort of 437 patients with ID/DD or other rare undiagnosed conditions. Analysis was performed as described elsewhere [26]. P6 was one of 128 diagnostic Estonian patients with developmental delay (DD), dysmorphism and/or other developmental problems and the *CYFIP2* variant was detected by trio WES performed by the Genomics Platform at the Broad Institute of MIT and Harvard (details see supplemental methods). P7 and P9 underwent trio WES as part of the Deciphering Developmental Disorders (DDD) project as previously described [6]. The trio exome data of P9 were re-examined as part of a local 'solving the unsolved' project in Manchester using a previously described pipeline [27]. P8 was one of 1312 patients with ID and epilepsy that underwent trio WES at Ambry Genetics (Aliso Viejo, CA, USA) as described elsewhere [28]. WES of P10 and P11 and their parents was performed as previously described (supplement and Kremer et al. [29]). The variant in P12 was detected by trio WES using a MedExome (Roche, Madison, WI, USA) and a NextSeq500 (Illumina, San Diego, CA, USA) at the NGS platform of the Lyon University Hospital.

Throughout the text, figures, and tables, all reported variants are designated for the *CYFIP2* reference transcript NM_001291722.1, which encodes for NP_001278651.1, following the current HGVS nomenclature (varnomen.hgvs.org) with exons numbered from 1 to 32 consecutively, and NC_000005.10 was used as a genomic reference sequence for the intronic variant. All reported variants have been submitted to the Leiden Open Variation Database (www.LOVD.nl/CYFIP2, patient IDs 00180890–00180901).

RT-PCR on RNA

To confirm the predicted splice effect of the variant c.3669+1G>T detected in P2, RNA from the patient and six controls was extracted using the PAXgene System (PreAnalytiX, Hombrechtikon, Switzerland) and transcribed

Table 1 Clinical data of patients with *CYFIP2* variants

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	I1-4 from Nakashima et al., [22]
***CYFIP2* variant**[a]	c.259C>T, p.(Arg87Cys)	c.3669+1G>T, p.(Glu1174Aspfs*3)[b]	c.259C>T, p.(Arg87Cys)	c.259C>T, p.(Arg87Cys)	c.2174A>G, p.(Gln725Arg)	c.1992C>G, p.(Ile664Met)	c.1993G>A, p.(Glu665Lys)	c.259C>T, p.(Arg87Cys)	c.1992C>G, p.(Ile664Met)	c.1363G>C, p.(Ala455Pro)	c.2170G>C, p.(Asp724His)	c.322T>C, p.(Tyr108His)	c.260G>T p.(Arg87Leu) c.259C>T p.(Arg87Cys) c.260G>C p.(Arg87Pro) c.259C>T p.(Arg87Cys)
Gender	Female	Female	Male	Female	Female	Female	Male	Female	Male	Male	Male	Female	All male
Age of last investigation	6y 11m	6y 2m	9y	6y 9m	1y 7m	2y 2m	4y	9y	9y 4m	1y 4m	4y	2y 9m	3-11y
Consanguinity, ethnicity	No, Caucasian	No, Caucasian	No, Caucasian	No, Nigerian	No, Caucasian	No, Caucasian	No, Caucasian	No, Mexican	No, Caucasian	No, Caucasian	No, Caucasian	No, Caucasian	No (Japanese, Japanese, Eritrean, Malaysian)
Gestational age (weeks)	40 0/7	40 4/7	40	full term	40	39	38	40 0/7	40 6/7	39 0/7	41 2/7	40	40, 30, 38, 38
Birth HC/ length/weight (standard deviations)	−1.5/−1.7/−0.4	NA/−2.1/−0.1	NA/NA/−0.4	NA/NA/−0.8 at age 6m: −1.4/ −0.8/−1.6	−1.5/+1.0/+0.3	+1.1/+0.4/+1.1	NA/NA/+0.1	NA/NA/−1.5	NA/NA/−0.6	−0.2/0/−1.4	+0.1/0/+0.1	+0.1/−1.7/−1.2	I1:+0.8/+1.3/ +1.9 I2: −1.0/−3.5/ −2.7 I3: +1.1/NA/ +1.4 I4: −3/−1/−0.7
HC/length/ weight at last investigation (standard deviations)	−6.1/−0.2/−0.4	−3.7/−3.8/−2.8	NA/−1.8/−0.8	-3.6/−2.0/−0.57	−1.4/+0.8/+0.7	−0.4/+2.0/+0.7	−0.9/−0.8 / −0.5	−4.8/ +0.2/+0.7	at age 5y 7m −1.5/+1.0/+0.9	-1.8 / +1.9 / -0.5	-1.7 / -0.4 / -1.2	-1.9 / -1.3 / -1.5	I1: NA I2: −5.1/−1.8/ −2.4 I3: −2.6/+0.3/ +1.7 I4: −3.7/−4.7/ −4.9
Microcephaly	Yes	Yes	No	Yes	Relative microcephaly	Relative microcephaly	No	Yes	Relative microcephaly	Relative microcephaly	Crossing of centiles, P50 → P5	Borderline	No, yes, yes, yes
Morphological features	High and broad forehead, large backwards rotated ears, upslanting narrow palpebral fissures, full cheeks, short philtrum, short mouth with everted lips, microretrognathia, high narrow palate, microdontia, sparse eyebrows, abnormality of the hairline, tapered long fingers, bilateral overriding of the 2nd and 3rd toes	High and broad forehead, large backwards rotated ears, narrow down-slanting palpebral fissures, mild hypertelorism, short mouth with everted lips	Large ears, full cheeks, narrow palpebral fissures, everted lips retrognathia, long fingers	None	High bulging forehead with temporal narrowing, hypertelorism, narrow up-slanting palpebral fissures, bulbous nasal tip, large ears, short mouth with everted lips, retrognathia	High forehead with temporal narrowing, abnormality of the hairline, large backwards rotated ears, narrow upslanting palpebral fissures, hypertelorism, flat nasal bridge, high narrow palate, short open mouth with everted lips	High and broad forehead, mild hypertelorism, narrow downslanting palpebral fissures, large backwards rotated ears, long dark eye lashes, short mouth, retrognathia, deep plantar creases	Broad and high forehead, deep set eyes, flat nasal bridge, large posteriorly rotated ears, open mouth with everted lips	Hypertelorism, epicanthic folds, broad nasal tip, deep set eyes, narrow upslanting palpebral fissures, short mouths with everted lips, large ears	scarce frontal and temporal hair, long philtrum, thin upper lip, overfolded helices of both ears, pectus excavatum, tapered fingers, long toes	Low-set ears, prominent earlobes, 1 freckled cafe-au-lait spot, dorsal linear freckling complete left leg, mild funnel chest, mild displacement of mammillae, mild clinodactyly, macrostomia, diastema mediale, mild plagiocephalus, pasty back of hands, prominent lips	High and broad forehead, large backwards rotated ears, synophris, deep-set eyes, retrognathia, friable nails, tapered fingers	I1, I2: arched eyebrows and swelling of lip and gingiva I3: none I4: tented mouth
Developmental delay	Yes, global	Yes, global	Yes, global	Yes, global	Yes, global	Yes, global	Yes, global	Yes, global	Yes, global	Yes, global	Yes, global	Yes, global	Yes, all global
Age at unassisted sitting	No unassisted sitting	9m	Yes, age unknown	No unassisted sitting	No unassisted sitting	No unassisted sitting	1y	No unassisted sitting	8m	13m (unstable)	3y 11m	No unassisted sitting	No unassisted sitting in all
Age at independent walking	No walking	Never walked, was standing and cruising, then regressed (at age 4y)	No walking, can scoot	No walking	No walking	No walking yet	About 2y	No walking	2y	No independent standing or walking yet	No walking	No walking	No walking in all

Table 1 (continued)

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	I1-4 from Nakashima et al., [22]
Age at first words	No speech	18m	No speech	No speech	No speech	No speech	18m	No speech	NA	No speech, invariable vocalizing	No speech	No speech	No speech in all
Speech development	Only non-verbal	A few words, a few signed words only	Only non-verbal	Only non-verbal	None	Only non-verbal	Mild delay only	Only non-verbal	4 words with meaning at 19 months, 20 words with meaning at 3y 5m, fluent sentences at 6y	Only non-verbal	Only non-verbal	Only non-verbal	
Intellectual disability[c]	Profound	Profound	Profound	Profound	Too young to assess	Severe to profound (too young to specify)	Mild to moderate	Profound	Moderate	Moderate to severe (too young to specify)	Profound	Profound	Severe - profound in all
Other abnormal behavior	Frequent laughing spells and screaming when waking up	Stereotypical writhing movements, frequent laughing spells at night	No	No	No	No	Autistic features, ADHD	NR	ADHD, ASD, recurrent stereotypes when excited, flaps arms behind head, repetitive questioning, hypermotoric/ active behavior	Unusual frequent oral explorations	NR	No	NA
Seizures (age of onset)	Yes (11m)	Yes (4y), staring episodes (unclear if epileptic) <1y	Yes (5w)	Yes (2–3m)	Yes (7–8m focal impaired awareness seizures; 11m epileptic spasms)	Yes (1y 2m)	Yes (3y)	Yes (9m)	Febrile seizure at 6m	Yes (6.5m)	Yes (4m)	Yes (8m)	Yes for all (onset 1.5m–6m)
Seizure type	Atypical absences, tonic, myoclonic, hyperkinetic seizures, status epilepticus	Absences, generalized tonic or tonic-clonic and gelastic seizures	Epileptic spasms	Generalized tonic, atypical absence, and myoclonic seizures	Epileptic spasms, focal seizures with impaired awareness	Focal impaired awareness seizures with non-motor onset, status epilepticus	Febrile seizures, absences, focal seizures	Epileptic spasms, tonic seizures, atypical absences, generalized tonic clonic seizures, status epilepticus,	Febrile seizure at 6m, otherwise no seizure activity	West-syndrome	Atypical absences, polytopic myoclonia	Clonic and hypermotoric (pedaling) seizures	I1, I4: West syndrome I2, I3: Ohtahara syndrome I1: epileptic spasms, tonic I2: focal, tonic, epileptic spasms I3: focal, myoclonic, epileptic spasms I4: generalized tonic clonic
Current seizure frequency (treatment)	Multiple short absence seizures per day and ca. 4 tonic seizures per day (VPA, TPM)	Seizure-free since age 4y 11m (CBD oil)	Seizure-free since age 5y (LCM)	2x/day on average, very variable (TPM, LEV, CLZ)	Occasional seizures (VGB, ZNS, LCM, CLB)	Seizure-free since age 1y 9m (no treatment)	Seizure-free since age 6y (no treatment)	Tonic seizures 1-2x/day for 10-15s, usually in mornings (CLZ, LEV, VPA)	None (no treatment)	Seizure-free since age 9m (VPA)	Multiple short absences per day, no myoclonia (Pyridoxine hydrochloride)	Max. 1x/month, continuous eye lid myoclonia (LEV, TPM, VGB)	I1-3: Intractable I4: seizure-free since age 3y (VPA)
EEG findings	Focal and generalized sharp-slow wave activity, generalized epileptiform activity, short episodes of small amplitude alpha-/ theta activity with behavioral arrest (atypical absence?)	Intermittent slowing	Normal	Multifocal and generalized interictal epileptiform discharges	Multifocal epileptiform discharges	Disturbed and slower main part, probably epileptic activity from central part of both hemispheres during sleep	Anterior epileptiform activity	Hypsarrhythmia, slow spike and wave discharges, multifocal spike and wave discharges, bitemporal sharp and slow wave discharges	Not performed	Hypsarrhythmia, reduced multifocal spikes and waves	Right-accentuated hemi-hypsarrhythmia-like activity (responsive to Pyridoxine), subclinical intermittend rhythmic delta activity posterior	Encephalopathic pattern, multifocal spike wave activity	I1: hypsarrhythmia, slow spike-wave I2: suppression-burst, hypsarrhythmia, multifocal epileptic discharges I3: suppression-burst, hypsarrhythmia I4: suppression-burst, hypsarrhythmia

Table 1 (continued)

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	I1-4 from Nakashima et al., [22]
MRI anomalies	Dysmorphic hemispheres, frontal lobe hypoplasia with simplified gyration	Periventricular leukomalacia, white matter volume loss	Normal at age 5w 5d	Mild diffuse hypomyelination and atrophy, abnormal thickening of the medullary cavity of the sphenoid bone	Mild diffuse cerebellar parenchymal volume loss, symmetric hyperintense T2 signal abnormality within the central tegmental tracts	Normal at the age of 7m and 13m	Normal at age 3y 5m	Moderate diffuse atrophy, abnormal signal in the thalami and putamen bilaterally which suggests gliosis	Not performed	Marked subarachnoid spaces at age 6.5m	No structural lesions, wide liquor space (at age 7m and 1y 9m)	Mild bilateral frontal lobe hypoplasia, abnormal signal of the basal ganglia, hypoplasia of the chiasma and retrochiasmatic region	I1: cerebral atrophy I2: mild cerebral and cerebellar atrophy I3: various anomalies I4: normal
Muscular hypo-/ hypertonia	Truncal hypotonia, hypertonicity of lower limbs	Truncal hypotonia, mild hypertonicity of lower limbs	Truncal hypotonia	Truncal hypotonia, limb hypertonicity	Severe generalized hypotonia	Hypotonia	Truncal hypotonia	Truncal hypotonia, limb spasticity	Generalized hypotonia	Generalized hypotonia	Axial hypotonia	Axial hypotonia	I1: no I2: hypotonia and spasticity I3, I4: hypotonia
Other neurological issues	Hypermotoric behavior	Mild ptosis	Dystonia	Dystonia	No	Brisk tendon reflexes	NR	Hyporeflexia, muscle weakness	NR	No	Discrete distal dyskinesia	Persistent non-epileptic eye lid myoclonia and abnormal ocular movement since age 2d	I2: Babinski sign and hyperactive deep tendon reflexes I3: weak deep tendon reflexes, primitive reflexes I4: hyperactive deep tendon reflex with clonus
Other features	Hyperopia, optical nerve atrophy, alternating divergent strabismus, difficulty falling asleep (treated successfully with Melatonin), very slow hair and teeth growth	Constipation, frequent awakenings at night with "laughing spells", frequent episodes of low muscle tone in the mornings	Hyperlaxity of joints, requires G-tube, exotropia, sleep disturbances, kidney stones, recurrent ear infections, muscle biopsy showed signs of mitochondrial disorder	Osteopenic bones, requires G-tube, cortical vision loss, sensory exotropia, asthma, laryngomalacia, benign neutropenia, microcystosis, OSAS, on CPAP	Reflux, dysphagia, requires G-tube, retinal pigmentary changes, vision impairment, hypermetropia, bilateral glaucomatous optic atrophy, secundum ASD/ PFO, cough, requires cough assist, type 1 laryngeal cleft, neutropenia, absent otoacoustic emissions and acoustic reflexes in the left ear	None	Constipation, strabismus	Stridor, frequent respiratory infections, progressive thoracolumbar scoliosis, dysphagia, requires G-tube, cortical visual impairment, sleep disturbances	Umbilical hernia repair at 1y, intermittent strabismus (resolved at age 5y), frequent urinary tract infections, eczema, sleep disturbances treated with Melatonin	Pectus excavatum, OAE -screening not normal, icterus prolongatus	Can eat pureed food, hypersalivation, recurrent pulmonary infections, coxa antetorta and subluxation left hip, strabismus divergens alternans, myopia, bilateral cryptorchism, orchidopexia	Major feeding difficulties, requires G-tube, optical nerve hypoplasia, severe asthma, pulmonary infections	

ASD atrial septal defect, *CBD* cannabidiol, *CLB* clobazam, *CLZ* clonazepam, *CPAP* continuous pressure, *d* days, *EEG* electroencephalography, *G-tube* gastrostomy tube, *HC* head circumference, *LCM* lacosamide, *LEV* levetiracetam; *m* months, *MRI* magnetic resonance imaging, *NA* not available, *NR* not reported, *OAE* otoacoustic emissions, *OSAS* obstructive sleep apnea syndrome, *PFO* persistent foramen ovale, *TPM* topiramate, *VGB* vigabatrin, *VPA* valproate, *w* weeks, *y* years, *ZNS* zonisamide

[a]c. position according to reference sequence NM_001291722.1 and p.position according to NP_001278651.1

[b]NC_000005.10(NM_001291722.1):c.3669+1G>T

[c]classification according to DSM-V

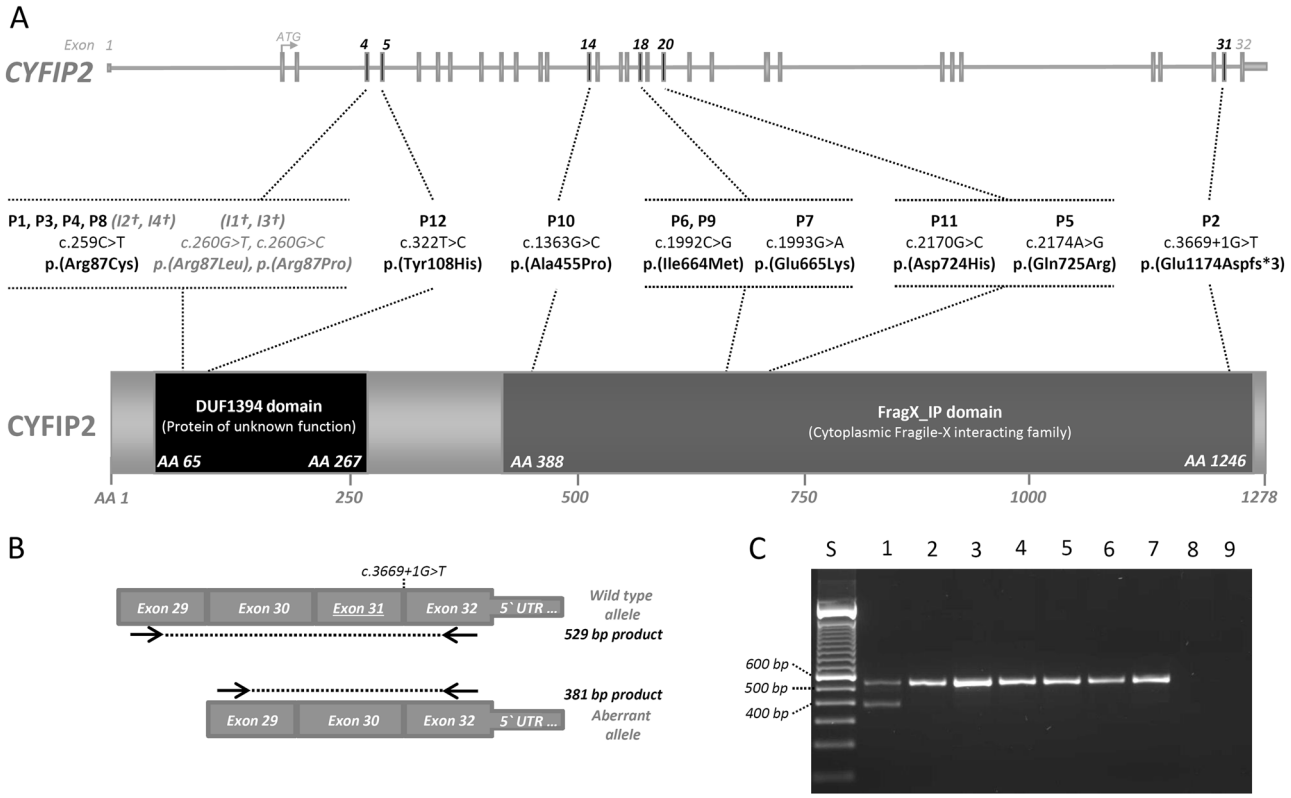

Fig. 1 Gene and protein structure of CYFIP2 with the eight variants identified in twelve patients. **a** Schematic drawing of the *CYFIP2* gene as well as CYFIP2 protein with its conserved domains. The position of the eight variants identified in our twelve patients (indicated in black) as well as the recently published variants (Nakashima et al. [22], indicated in grey and with †) are depicted. All variants occurred *de novo* and have been detected in heterozygous state (variant nomenclature and gene structure according to NM_001291722.1 with exons numbered from 1 to 32 consecutively and NC_000005.10 for intronic sequences, protein structure according to NP_001278651.1 and NCBI's conserved domain database [51]). **b**, **c** Splice variant c.3669+1G>T affecting the conserved splice donor site after exon 31 induces skipping of exon 31 in P2. RT-PCR on RNA from peripheral blood leukocytes with primers located in the exons 29 and 32 (indicated as arrows) resulted in an additional aberrant product of 381 bp in the patient (lane 1), whereas the amplification in six controls (lanes 2–7) resulted in the expected 529 bp fragment only (S, size standard; lane 8, genomic DNA control; lane 9, no template control). Exon skipping in the patient was verified by the sequencing of amplified products (Supplemental Figure S1) and is predicted to result in a frameshift followed by a premature stop codon after 3 altered amino acids (NM_001291722.1:r.3522_3669del, p.(Glu1174Aspfs*3))

reversely using Superscript III (ThermoFisher Scientific, Waltham, MA, USA). RT-PCR was performed by using specific primers located in the exons 29 and 32 of *CYFIP2* and products were analyzed by agarose gel electrophoresis. Exon skipping in the patient was verified by Sanger sequencing of amplified products.

Variant modelling

The effect of the variants was investigated on the basis of the crystal structure of the WAVE regulatory complex (PDB: 3P8C [14], 4N78 [30]). This complex was crystallized with CYFIP1, which was replaced by CYFIP2 (88% sequence identity to CYFIP1) in our model using Modeller 9.16 [31]. Chimera [32] was used for structure analysis and visualization. The effect of the variants on the CYFIP2-WAVE1 binding affinity were assessed using BindProfX [33].

Results

Genetic findings

Through our trio WES study, utilizing GeneMatcher [34], Decipher Database [35], and personal communications, we identified 12 patients with eight distinct de novo *CYFIP2* variants (seven missense and one splice site variant): c.259C>T p.(Arg87Cys) in P1, P3, P4, and P8; c.322T>C p.(Tyr108His) in P12; c.1363G>C p.(Ala455Pro) in P10; c.1992C>G p.(Ile664Met) in P6 and P9; c.1993G>A p.(Glu665Lys) in P7; c.2170G>C p.(Asp724His) in P11; c.2174A>G p.(Gln725Arg) in P5; and c.3669+1G>T p.(?) in P2 (Fig. 1, Table 1, for alternative nomenclature see Supplemental Table S1). Notably, all the variants are absent from GnomAD [36] and all except p.(Tyr108His) are predicted unanimously to have a deleterious effect on protein

function by automated online prediction tools (missense variant predictions: SIFT, PolyPhen2, MutationTaster; splice variant predictions: MaxEnt, NNSplice, Human Splicing Finder, GeneSplicer, SpliceSiteFinder-like; all predictions provided by Alamut Visual Version 2.10 July 2017 (Interactive Biosoftware, Rouen, France)). Furthermore, the *CYFIP2* gene is indicated to be intolerant to both, missense ($z = 6.15$) and loss-of-function (LoF) (probability of LoF intolerance pLI = 1.00) variants in ExAC [36].

The variant c.3669+1G>T is predicted to affect the conserved splice donor site in the last intron of *CYFIP2*, and RT-PCR performed using RNA from peripheral blood leukocytes of P2 confirmed skipping of exon 31 (NM_001291722.1:r.3522_3669del) (Fig. 1 and Supplemental Figure S1). Lack of exon 31, which is the second to last exon, is predicted to result in a frameshift followed by a premature stop codon (p.(Glu1174Aspfs*3)). Accordingly, this aberrant transcript is not expected to be subject to nonsense-mediated mRNA decay [37].

Variant modelling

The missense variants p.(Arg87Cys) and p.(Tyr108His) are located in the DUF1394 subdomain of CYFIP2, whereas the remaining missense variants (p.(Ala455Pro), p.(Ile664Met), p.(Glu665Lys), p.(Asp724His), and p.(Gln725Arg)) are located in the FragX_IP subdomain (Fig. 1). In the three-dimensional fold of CYFIP2, both the subdomains are tightly interlocked, placing Arg87 in close spatial proximity to Ile664, Glu665, Asp724, and Gln725. Moreover, these variants are located at the CYFIP2-WAVE1 interface (Fig. 2a, b). As noted by Nakashima et al. [22], the Arg87 sidechain forms tight interactions with two glutamate residues and intermolecular interactions to Tyr151 of WAVE1. Since these interactions are lost in the p.(Arg87Cys) variant, this exchange is predicted to cause structural instability around the variant site thereby leading to aberrant WAVE1 activation [22].

In contrast to Arg87, residues Ile664, Glu665, Asp724, and Gln725 form weaker intramolecular interactions suggesting that the variants found at these positions are not expected to significantly affect the stability of CYFIP2 itself. Nevertheless, all the five residues form direct interactions with WAVE1, which are predicted to be disturbed by these variants (Fig. 2a). A closer inspection reveals that interactions are formed with α6- and C-helices of WAVE1 (Fig. 2b). The C-helix (residues 531-543) is a part of the WAVE1 VCA-region and was shown to be critical for activation of the Arp2/3 complex [38]. The tight interaction between helices α6 and C plays an important role for stabilizing the VCA-region in an inactive conformation. Thus, these five missense variants are predicted to weaken the CYFIP2–WAVE1 interface at this functionally important site favoring release of the VCA-region leading to WAVE1 activation. To quantify the effect of the variants we calculated the changes in the free energy of the CYFIP2–WAVE1 interaction for each of the WAVE1 interface variants (free energy change in kcal/mol): p.(Arg87Cys) 1.75; p.(Ile664Met) 1.20; p.(Glu665Lys) 1.32; p.(Asp724His) 2.08; and p.(Gln725Arg) 1.09. Here we report changes in CYFIP2–WAVE1 affinity instead of CYFIP2 stability, which explains the difference in free energy reported in this manuscript for p.(Arg87Cys) (1.75 kcal/mol) compared with that reported by Nakashima et al. (~4.5 kcal/mol) [22].

Tyr108 is located close to the CYFIP2–WAVE1 interface and the adjacent residue Ile107 forms interactions with Tyr140 in the meander region of WAVE1 (Fig. 2c), which cooperatively stabilizes the VCA-element. These interactions are critically affected by alterations of the adjacent residues. For example, phosphorylation of Thr138 (WAVE1) by Cdk5 contributes to WRC activation and leads to altered cellular actin dynamics [14]. Thus, it appears feasible that replacement of Tyr108 (CYFIP2) by a charged histidine also perturbs the CYFIP–WAVE interaction at this functionally important site. Consequently, the variant p.(Tyr108His) may also lead to increased WAVE activation.

The splice variant c.3669+1G>T likely results in a C-terminally truncated CYFIP2 protein (p.(Glu1174Aspfs*3)). The C-terminus (red part illustrated in Fig. 2a) forms numerous contacts to NCKAP1, another component of the WRC, that are lost in the altered protein. A loss of interactions with NCKAP1 is also expected for the p.(Ala455Pro) missense variant, which is located in the CYFIP2-NCKAP1 interface (Fig. 2d). The calculated free energy change of 1.21 kcal/mol is in the same range as that observed for the variants in the CYFIP2-WAVE1 interface. Since Ala455 is located in an α-helix, an Ala455Pro exchange is additionally expected to disturb the structure of CYFIP2 itself, thus enhancing the damaging effect of this variant.

Clinical findings

Phenotype data are summarized in Table 1 and more detailed patient reports are provided in the supplement. The patients shared a phenotype of ID/DD (12/12) and epilepsy (11/12) except for P9, which was only reported to have had a single febrile seizure at the age of 6 months. In the majority of the cases (8/11), seizure onset occurred within the first year of life (range 5 weeks–4 years). In six patients seizures were intractable, whereas five children were reported to be currently seizure-free. All patients showed generalized or truncal hypotonia, in four cases combined with limb spasticity. Dysphagia with dependency on gastric tube feeding was present in five of 12 patients. Nine of 12 patients had visual impairment and/or strabismus. Eight

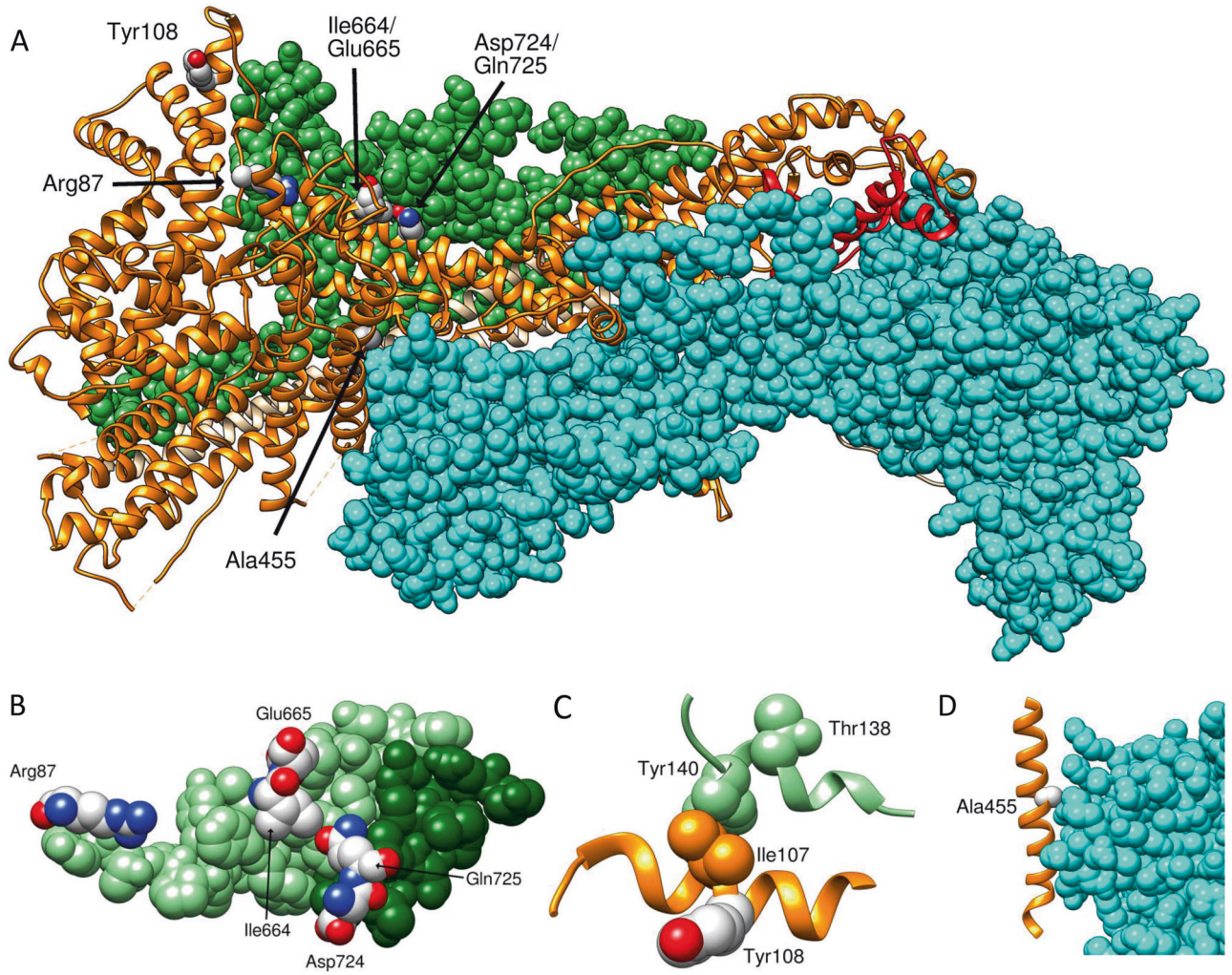

Fig. 2 **a** Structure of the WAVE regulatory complex (WRC) indicating the sites of variants. CYFIP is shown in orange and the C-terminus, which is absent in the p.(Glu1174Aspfs*3) splice variant, is highlighted in red. The C-terminus forms tight interactions to the NCKAP1 protein (shown in cyan space-filled presentation). Residues affected by missense variants are shown in space-filled presentation and colored by atom types. Residues Arg87, Ile664, Glu665, Asp724 and Gln725 interact with WAVE1 (shown in green space-filled presentation) and Ala455 interacts with NCKAP1. Tyr108 is located in close vicinity to the CYFIP-WAVE1 interface. **b** Close-up view on the CYFIP2-WAVE1 interaction site disturbed by most of the variants observed. WAVE1 residues 151-173 comprising helix α6 (important for VCA domain stabilization) and residues 531-543 corresponding to the C-helix (part of VCA domain) are shown in light green and dark green, respectively. The interacting CYFIP residues Arg87, Ile664, Glu665, Asp724, and Gln725 are shown in space-filled presentation and colored by atom types. **c** Close-up view of the CYFIP2-WAVE1 interface in the vicinity of Tyr108. Residues 133-142 of WAVE1 are shown in green and residues 100-114 of CYFIP2 are shown in orange (ribbon representation). Key residues discussed in text are shown in space-filled presentation and Tyr108 is colored by atom type. **d** Close-up view on the CYFIP2-NCKAP1 interface in the vicinity of Ala455. NCKAP1 is shown in cyan and residues 444-469 of CYFIP2 are shown as orange ribbon with Ala455 in space-filled presentation

patients developed absolute or relative microcephaly and in two patients the centile of head circumference decreased markedly. Common morphological features shared among patients in this cohort included long, tapered fingers, high forehead, narrow, mildly up-slanting palpebral fissures, apparent hypertelorism, bulbous nasal tip, full cheeks, everted lip vermillion, and retrognathia (Fig. 3). Brain MRI was performed in 11 of the patients and showed unspecific structural anomalies in six patients including cerebellar, white matter or diffuse atrophy in four patients, dysmorphic hemispheres and frontal lobe hypoplasia in one patient, and hypoplasia of the frontal lobe, the chiasma and retrochiasmatic region in one patient. Three patients had normal cerebral MRI scans at the age of 5 weeks (P3), 7 months and 1 year 1 month (P6), and 3 years 5 months (P7), respectively. EEG was available for all 11 patients with recurrent seizures and showed variable findings including focal, multifocal and generalized epileptiform discharges, and hypsarrhythmia. Developmental milestones were absent or delayed in all 12 patients. Only 6/12 patients achieved unassisted sitting and 2/12 were able to walk. Nine of 12 patients did not develop any verbal communication and one

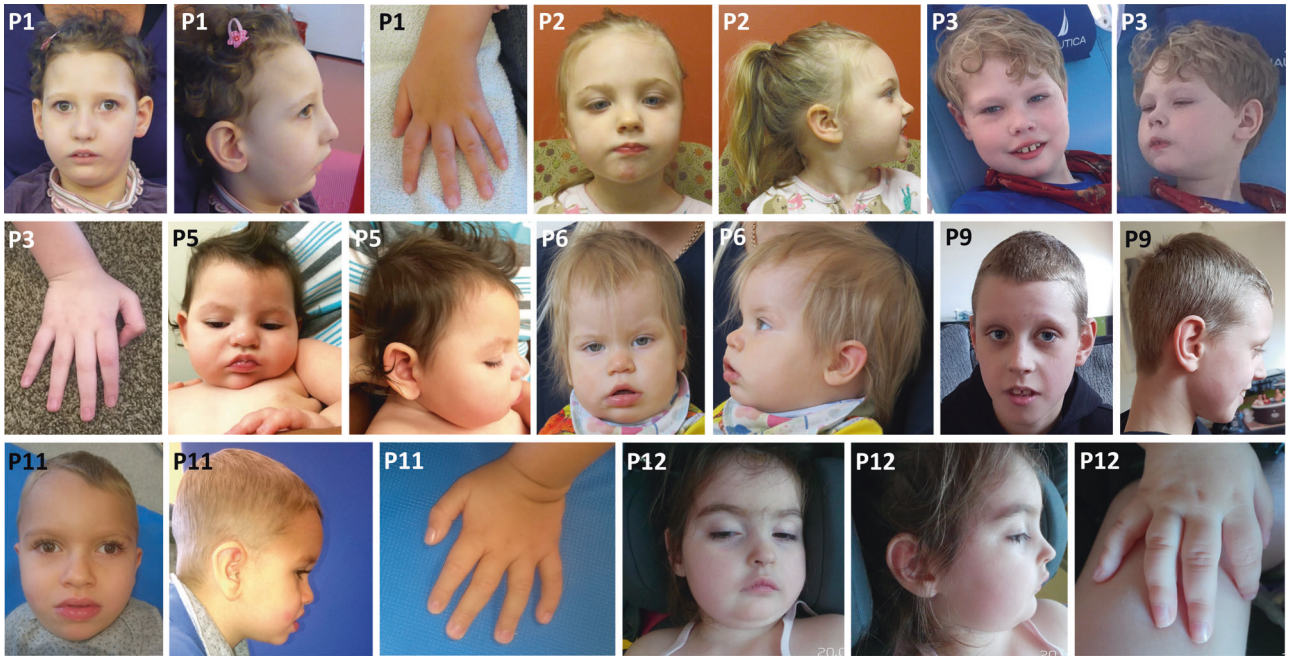

Fig. 3 Facial appearances of eight patients (P1, P2, P3, P5, P6, P9, P11, and P12) with de novo variants in *CYFIP2* and hands of four patients (P1, P3, P11, and P12)

spoke only a few words at the age of 6 years. Psychomotor regression was reported in one patient (P2), who was standing and cruising and then regressed at the age of 4 years after a single tonic-clonic seizure event. All patients are alive and their age at last evaluation ranged from 1 year 4 months to 9 years.

Discussion

In this study we report 12 patients harboring eight de novo variants in *CYFIP2* presenting with mostly severe but variable ID/DD, muscular hypotonia and, in all except one, a history of epilepsy with onset <4 years. Other commonly shared features included secondary microcephaly, limb spasticity, dysphagia, long tapering fingers, and similar facial traits (Table 1, Fig. 3).

Our findings are in line with those recently reported by Nakashima et al. [22], who identified concurrently three de novo variants in patients with epileptic encephalopathy, all affecting the Arginine at position 87 in CYFIP2. All four of their patients had early-onset epileptic encephalopathy classified as Ohtahara or West syndrome, whereas we report a broader clinical spectrum ranging from a similarly severe phenotype including intractable epilepsy and profound ID from mild to moderate cognitive impairment (P7 & P9) without epilepsy (P9) (Table 1). The p.(Arg87Cys) variant recurred in four cases of this study, and was also reported twice in the previous cohort [22]. All six patients carrying this recurrent variant, as well as two further patients [22] with other substitutions of the p.Arg87 had profound ID, early-onset epilepsy and hypotonia. Seizures were intractable in six of these eight patients while two are currently seizure-free. Six of these eight patients had secondary microcephaly. MRI findings ranged from unremarkable to hypomyelination and atrophy. All four patients reported here carrying the p.(Arg87Cys) variant showed sleep disturbances, while this feature was not addressed by the previous report. Of note, this variant was also reported as a candidate variant in a patient with West syndrome who in addition harbored further de novo variants including a variant classified as "likely pathogenic" in the Kabuki syndrome (MIM 147920)-associated *KMT2D* gene [39]. We identified a further recurrent variant, p.(Ile664Met), present in two of our patients (P6 and P9) and associated with a more variable phenotype consisting of either severe developmental and epileptic encephalopathy or moderate non-epileptic ID, respectively. Therefore, currently available data indicate a profound phenotype in p.Arg87 substitutions, but a more variable phenotype in other variants.

The *CYFIP2* gene is located in the chromosomal region 5q33.3 and encodes the cytoplasmic FMRP interacting protein 2 (CYFIP2). Only large segmental chromosomal aneusomies involving this region have been clinically described in two patients with ID and seizures, with a vast number of genes affected and unclear deletion borders in one patient [40, 41]. With regards to single gene defects, the

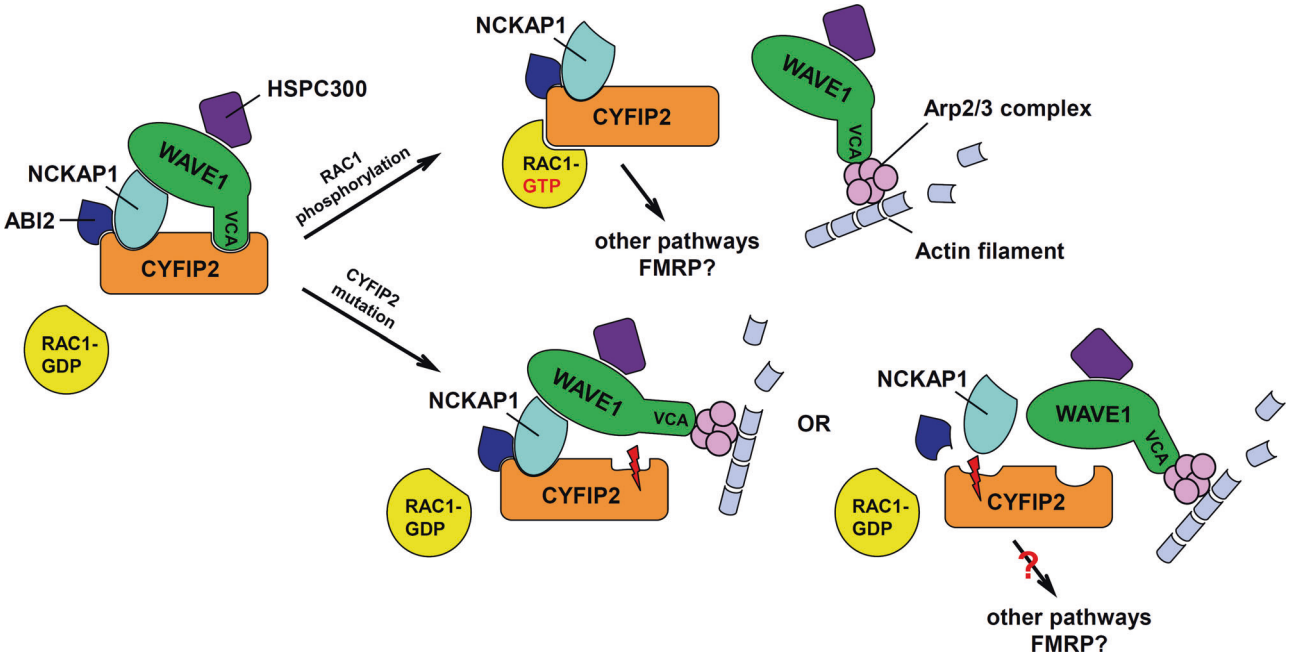

Fig. 4 Schematic structural organization of the WAVE regulatory complex (WRC) with its members WAVE1, CYFIP2, NCKAP1, ABI2, and HSPC300 in inactivated condition (left part) and after activation of WAVE function by RAC1-GTP binding (upper right part) or aberrant WAVE activation by CYFIP2 mutation as suggested by our data (lower right part)

study by Nakashima et al. [22] and this study are the first reports to establish defects in *CYFIP2* as the underlying cause of severe neurodevelopmental disorders. Since neither elsewhere nor in our series small copy number or truncating variants triggering nonsense-mediated mRNA decay have been observed, haploinsufficiency seems unlikely as disease mechanism in the observed severe phenotype.

Both members of the highly conserved CYFIP protein family in humans, CYFIP1 and CYFIP2, interact with FMRP and are similarly co-localized with ribosomes and FMRP and were also detected at the synapse of mouse brain [12, 42]. However, despite their high homology it was shown that CYFIP1 interacts exclusively with FMRP, while CYFIP2 also interacts with the FMRP-related proteins FXR1P/2P [12]. Interestingly, it has been shown that regardless of unchanged RNA levels, the protein levels of CYFIP2 are increased in the blood of Fragile X patients [43]. This is in line with the fact that *Cyfip2* mRNA (but not *Cyfip1* mRNA) is ranked as a FMRP target in mouse brain [44] and the conception that FMRP acts as a suppressor of CYFIP2 translation by sequestration of its mRNA.

CYFIP1/2, along with the WAVE proteins (also known as WASF proteins), NCKAP1 (NAP1 or HEM1 in hematopoietic cells), ABI2 (or its paralogous proteins, ABI1 or NESH), and HSPC300 (BRK1), are members of the canonical WAVE regulatory complex (WRC), a key regulator of actin dynamics (Fig. 4) [13, 14, 16]. By the interaction of Rac1-GTP with CYFIP, the intrinsically inactivated complex is split into one subcomplex including CYFIP1/2, which is now capable to interact with other proteins, and another subcomplex including WAVE, which interacts with Arp2/3 triggering actin polymerization [13–16]. Here, the Arp2/3 complex is activated by binding the conserved VCA (verprolin homology, central and acidic regions) domain of WAVE, which is sequestrated in the inactivated complex by CYFIP, inhibiting WAVE activity [14, 15]. Actin filaments are important cytoskeletal structures in neurons with critical dynamics and especially the Arp2/3 complex is essential at multiple stages of neural development [45, 46].

Accordingly, in vitro and in vivo animal models of CYFIP2 (or its orthologs) showed phenotypes consistent with its discussed role as FMRP interactor and component of a key regulator of actin cytoskeleton, such as abnormalities of dendritic spines and axons [12, 13, 42, 47–50]. Of note, while homozygous null mice were lethal, indicating the necessity of CYFIP2 in early development, a heterozygous knock-out mouse showed abnormal behavior and cortical dendritic spines similar to that observed in *Fmr1*-null mice [47]. Given these findings and the high pLI score of 1.0 for *CYFIP2*, we assume that haploinsufficiency in humans may also cause a developmental phenotype. However, the apparently milder phenotype in the heterozygous *Cyfip2* knock-out mouse model would be in line with the assumption that the observed severe human *CYFIP2* phenotype is not caused by haploinsufficiency.

Despite the large spacing in the primary structure, the residues affected by the missense variants p.(Arg87Cys), p.(Ile664Met), p.(Glu665Lys), p.(Asp724His), and p.(Gln725Arg) in our patients are located in close spatial proximity at the CYFIP2–WAVE1 interface, where they are predicted to impair binding of the WAVE VCA-region (Fig. 2b), therefore leading to increased WAVE activation and consecutively to increased Arp2/3 mediated actin polymerization. A similar effect is also expected for the p.(Tyr108His) variant, although caused by a slightly different structural mechanism. Of note, an enhanced WAVE net activity was already suggested after loss of *Cyfip2* in a heterozygous mouse model [47] or *dCyfip* in *Drosophila* [49]. Increased WAVE activity was also suggested for the p.Arg87 variants observed by Nakashima et al. [22] supported by their finding of speckled co-localization of filamentous actin and mutant CYFIP2 as well as significantly increased aberrant filamentous actin accumulation in B16F1 cells transfected wildtype or mutant *CYFIP2*. However, they could not detect abnormal WAVE1 binding of the Arg87 mutants by co-immunoprecipitation in transfected HEK293T cells, but showed a consistently weaker interaction with the VCA domain in a GST pull-down assay.

The splice variant identified in P2 is predicted to result in a truncated protein (p.(Glu1174Aspfs*3)) due to exon skipping of the second to last exon. This transcript likely escapes nonsense-mediated decay. Based on our structural modelling the missing C-terminus is expected to lead to a loss of numerous contacts with NCKAP1 (also known as NAP1) within the WRC (Fig. 2) and therefore also to a disruption of the WRC and consecutive WAVE activation. A similar WRC disruption caused by loss of interactions with NCKAP1 is also expected for the p.(Ala455Pro) missense variant, which may be enhanced by decreased stability of CYFIP2.

Therefore, we speculate that the mechanism of aberrant WAVE activation caused by the different variants could be explained by activation of the Arp2/3-binding VCA domain either by (1) increased dissociation of the WRC and/or (2) disturbed interactions at the CYFIP2–WAVE1 interface favoring an exposed VCA-helix (Fig. 4). Given the variant type, the finding of unchanged CYFIP2 protein levels in a cell line of a p.(Arg87Leu) patient [22] and the results of our structural modelling, we assume that the detected variants act as partial loss-of-function variants in terms of their ability to stabilize the WRC and/or the inactive conformation of the VCA region, but eventually result in gain of function considering WAVE activation.

In conclusion, we provide evidence that a variety of spatially clustered de novo variants in *CYFIP2* are causative for a new neurodevelopmental disorder characterized by intellectual disability and seizures. Our findings suggest that all identified de novo variants lead to increased WAVE activity by weakening the interaction with other components of the WAVE regulatory complex, a key regulator of the actin cytoskeleton.

Acknowledgements The authors are grateful to the participating individuals and their families. We thank Christian Söldner and Michel Albert for their support with preparation of figures. AR, LA, and BP were supported by the University of Zurich research priority program radiz (rare disease initiative Zurich). AR was further supported by the Swiss National Science Foundation (SNSF) grant 320030_179547. The Broad Center for Mendelian Genomics (UM1 HG008900) is funded by the National Human Genome Research Institute with supplemental funding provided by the National Heart, Lung, and Blood Institute under the Trans-Omics for Precision Medicine (TOPMed) program and the National Eye Institute. Support also provided by NIH T32 GM007748 (M.H.W.). The DDD Study (Cambridge South REC approval 10/H0305/83 and the Republic of Ireland REC GEN/284/12) presents independent research commissioned by the Health Innovation Challenge Fund (grant number HICF-1009-003), a parallel funding partnership between the Wellcome and the Department of Health, and the Wellcome Sanger Institute (grant number WT098051). The research team acknowledges the support of the National Institute for Health Research, through the Comprehensive Clinical Research Network. This study makes use of DECIPHER (http://decipher.sanger.ac.uk), which is funded by the Wellcome. The views expressed in this publication are those of the author(s) and not necessarily those of the Wellcome or the Department of Health. AB was supported by the Forschungskredit UZH and the Josef Huwyler Ruth Bernet-Engeli Stiftung.

Compliance with ethical standards

Conflict of interest Kirsty McWalter, Megan T. Cho, Maria J. Guillen Sacoto, and Kristin G. Monaghan are employees of GeneDx, Inc., a wholly owned subsidiary of OPKO Health, Inc. The remaining authors declare that they have no conflict of interest.

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