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Supplemental information

De novo variants in *FRMD5* are associated with developmental delay, intellectual disability, ataxia, and abnormalities of eye movement

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Supplemental Data

Supplemental note: case reports

Proband 1: c.340T>C (p.Phe114Leu)

This is a 3-year-old male with global developmental delay, drug-refractory epilepsy and ataxia. He did not have any exposure to alcohol or chemical substances during the prenatal period. His biological parents were healthy and non-consanguineous.

At the age of 6 months, he presented with frequent infantile spasms and absence seizures about 5-7 times per day, and was diagnosed with West syndrome. He was treated with topiramate and other anti-epilepsy drugs, but his seizures were not well controlled and he still had 1-2 seizures per month. At the age of ~2 years, he had another form of epileptic attack, manifesting as generalized tonic-clonic seizures and status epilepticus. The 24-hour continuous video electroencephalogram (EEG) at the age of 11 months demonstrated high amplitude spikes, sharp waves, and spike-slow complex discharges in the right frontal and temporal lobes. Brain MRI at the age of 2 years was normal. The head circumference is within normal limits.

Besides drug-refractory epilepsy, he had developmental delay in motor and verbal capabilities. He did not meet gross and fine motor developmental milestones in the first year of life. He could not stand up spontaneously or with assistance at the age of 1 year old. By the age of 3, he could stand up independently but was unable to walk independently. He also had a slow, wide-based gait using a walking aid. By the age of 3 years, he could not communicate using simple words. A recent physical evaluation found strabismus, hyperactive reflexes, normal muscle strength and tone throughout, and ataxia.

Trio exome sequencing (ES) detected a *de novo* missense variant in the candidate gene *FRMD5,* NM_032892.5 c.340T>C (p.Phe114Leu) that was confirmed by Sanger sequencing.

Proband 2: c.1051A>G (p.Ser351Gly)

This is a 9-year-old male with severe global developmental delay, refractory epilepsy and microcephaly. The prenatal and birth history were unremarkable. He was born at term with a birth weight of 3,600 g. At the age of 3 months, his family members began to notice that he could not raise his head; he also had a relatively small head circumference (39.0 cm, -1 SD by the age of 3 months). He has severe motor delay, demonstrated by the inability to stand and walk independently at the age of 8 years. He had poor motor coordination, especially in hand movement, and could not hold any objects. By the age of 8 years, he could not speak any simple words nor follow simple instructions. Neurologic examination shows muscular hypotonia, ataxia and horizontal nystagmus.

At the age of 3 months, he presented with infantile spasms and was diagnosed with West syndrome. The individual's mean seizure frequency ranges from 2-3 times to 30-50 times per day. Various anti-epilepsy drugs were used but the seizures were not well-controlled. At the age of 6 years, he began to receive sodium valproate, topiramate and clonazepam. With these treatments, he still has 3-5 seizures per month. At the age of 6 years, his EEG displayed interictal high amplitude spikes and 2-3 Hz spike-wave complexes in the bilateral frontotemporal area and the bilateral frontal lobe and occipital lobe. At the age of 6 years, MRI of the brain demonstrated pachygyria in bilateral temporal lobes.

Trio ES revealed a *de novo* missense variant in *FRMD5,* NM_032892.5, c.1051A>G (p.Ser351Gly), which was confirmed by Sanger sequencing.

Proband 3: c.1053C>G (p.Ser351Arg)

This 27-year-old woman of Jewish, Spanish, and Portuguese ancestry was initially evaluated at Baylor's Parkinson's Disease Center and Movement Disorders Clinic at the age of 15 years for a life-long history of jerking movements, gait and balance difficulty and abnormal eye movements. She is the product of an uncomplicated gestation and normal spontaneous vaginal

delivery. Her mother first noted abnormal eye movements at 2 weeks of age, initially diagnosed as nystagmus. The head circumference is within normal limits. MRI of the brain at age 3 months was normal. She had delayed developmental milestones; her first word was spoken at 18 months, but she spoke in full sentences at 2 years. She started to walk independently at age 3 years. Starting at six months of age when the individual had a fever, she developed brief spasms of her arms and legs, initially diagnosed as febrile seizures. Over time, however, these progressed to nearly continuous jerky movements affecting her upper and lower limbs, trunk and face. She has experienced occasional severe spasms of her entire body, lasting up to 20-45 minutes while completely awake. In addition, she has episodic cramping and inversion of her feet during which time she is unable to walk, especially in the afternoon and evening. She was initially suspected of having myoclonic epilepsy, but her EEG has been negative. She has always had an unsteady gait associated with frequent falls. She used a walker or wheelchair part of the time and required assistance when walking up and down stairs. Her myoclonus is worse with stress, fever and when hungry or fatigued. She has major difficulty with fine motor skills: difficulty with buttons, zippers, tying shoes and writing. She needs assistance with cutting and pouring liquids but is able to feed herself. In addition to her motor symptoms she has complained of numbness in legs and arms and hands, and she has started using a heating pad for 20 minutes to help recover her feeling. She is sensitive to hot and cold weather and feels her feet are "hot" when wearing shoes. She had difficulty with reading and math and was diagnosed with dyslexia. At the time of puberty she was diagnosed with migraines.

Gabapentin, baclofen and clonazepam have partly relieved her myoclonus and pain but she still has some “cramps” in her feet with flexion of the toes, especially when inactive. Levodopa slightly improved her gait and swallowing but was discontinued because of severe mood swings. Tetrabenazine caused severe drowsiness, and deutetrabenazine caused mood swings without improvement of her myoclonus. Her gabapentin dosages was recently increased to 600 mg 3x/day, along with baclofen 60 mg/day, and clonazepam 1.5 mg/day.

Her ES was done at Baylor Genetics in 2014, and trio ES was performed and analyzed at Invitae in 2019. The missense variant in *FRMD5,* NM_032892.5, c.1053C>G (p.Ser351Arg) (27/49 reads from the locus) was identified as a *de novo* variant. No other mutations or variants known to cause diseases were identified.

Proband 4: c.1054T>C (p.Cys352Arg)

This 17-year-old Saudi boy was evaluated at six years of age due to developmental delay and abnormal eye movements; he has had no follow-up since. He was a 3.67 kg product of a 41-week gestation delivered via Cesarean section for failure to progress, to a 42-year-old G5, P4>5, Ab0 L4>5 (G: gravida, P: para, Ab: abortions, L: living children) whose pregnancy was uncomplicated. On day of life #2 he was placed in the newborn intensive care unit because of shaking of legs and hands and abnormal movement of his eyes. He was treated with phenobarbital but this was stopped at 1.5 years of age, and these movements did not change after cessation of therapy. He had very low muscle tone early in life. He fed well (breastfed) with no problem. He received physical therapy, and parents reported that he continued to make improvement with his muscle tone and strength. He had a brain MRI that was reported to be normal although may have shown evidence of oxygen deprivation at birth. Other prior tesing was reported to have been normal (nerve conduction velocities of radialis and tibialis nerves, EEG, and biochemical testing, including plasma amino acid, urine organic acid, CSF neurotransmitter, urine oligosaccharide and lysosomal enzyme analyses). He had a stem cell transplant in China in 2011, and parents noted some improvement. Family history was remarkable for two paternal cousins (a girl and boy) with autism. Four older siblings were healthy and developmentally normal. There was no other family history of birth defects, intellectual disability or related problems. There was no family history or recurrent miscarriages. Parents are from Saudi Arabia. There is no known consanguinity.

He started crawling at 16 months, sitting up at age 2, and walking at age 5. His first word was at age 3 and was able to say sentences at 5 years. Physical examination at 6 years of age revealed weight at 98th centile, height at 61st centile and head circumference +2.05 SDs. He had vertical and possibly rotary nystagmus/flutter that was constant and did not change with closing eyelids nor with trying to fix on an object. There was a single hyperpigmented macule on the abdomen. He had central hypotonia with increased muscle tone in the right upper extremity. Gait was wide-based and ataxic. He did not have dysmorphic facial features or other differences noted on physical examination. ES was initially reported as non-diagnostic, but research re-analysis identified a *de novo* c.1054T>C (p.Cys352Arg) variant (64/147 reads from the locus) in *FRMD5* (NM_032892.5).

Proband 5: c.1054T>C (p.Cys352Arg)

The male proband was born 9 days overdue after a normal and uncomplicated pregnancy. A pendular nystagmus was evident from the first few days of life. He smiled socially at four weeks.

Global developmental delay has emerged around age 6 months. He had developmental delay at 2.5 years, and his head circumference was 48.5 cm. He was not walking, first crawling at 21 months. The proband walked unaided with an ataxic gait at 3 years. His language development was delayed, even when considering that he grew up in a bilingual household. He knew 50 words by 2.5 but was unable to formulate sentences. A marked pendular nystagmus was noted, with restricted upward gaze. The opthalmology examination was normal. All other cranial nerves were intact. He was found to be very hypotonic, but with no intention tremor or athetosis and normal reflexes. Power was 5/5 in all limbs. He was toilet trained at 6 years. At age 4 years, he had a wide vocabulary, although his words were quite unclear. His mother thought he was able to put perhaps five words together. He has some behavioral issues, in

particular anger in response to change. He can be oppositional at times. He has learning difficulties with an IQ around 70.

By the time he was 18 years old, his growth parameters were adequate, and he had no significant dysmorphism. He was walking with marked dyskinesia, intention tremor with dysmetria bilaterally and ataxia. The pendular nystagmus remains. He has hyperkinetic movements of his upper limbs, and hypermobility. Over the 16 years of follow up, it was not found to be a progressive condition.

Individual is the only child of a healthy and non-consanguineous White couple. There is no family history of any movement disorders, muscle disorders or developmental delay. His father had simple febrile convulsions as a toddler. He has a much older half-brother through his father who has dyslexia. His parents are separated, and he lives with his mother.

He was worked up for metabolic disease, chromosomal analysis, lactate determination, blood and urine amino acids and organic acids, and DNA analysis for Prader Willi Syndrome with no conclusive result except the noted genetic defect. He also had a muscle biopsy, EMG (electromyography) and nerve conduction studies to assess for any problems here. These were all normal. Two separate brain MRIs showed no abnormalities. Whole exome sequencing and whole genome sequencing have not identified a diagnosis, but research re-analysis identified a *de novo* c.1054T>C (p.Cys352Arg) variant in *FRMD5* (NM_032892.5).

Proband 6: c.1060T>C (p.Ser354Pro)

This is a 9-year-old girl with congenital opsoclonus, ataxia, hypotonia, learning difficulties, developmental delay and incontinence concerns. She is the only child to non-consanguineous parents, with no significant family history. She was born at term, following an uneventful pregnancy.

Abnormal eye movements were noted from birth by her parents, and she was diagnosed with congenital opsoclonus. She had extensive investigations into the cause for the opsoclonus

including metabolic tests, MRI brain, MIBG (a meta-iodobenzylguanidine scan), EEG and lumbar puncture, all of which were normal. She is now cared for by the visual impairment team. She also has hypermetropia requiring glasses for correction and has regular reviews by the orthoptist team.

She did not meet her developmental milestones. As a baby she had poor head control and was noted to be hypotonic. She began to crawl at 17 months. She began to walk with the use of aids at 2 years old. She walked with an unsteady broad gait and ataxia. Her balance and gait remain to be an issue with frequent falls and poor coordination. She uses a walker and a wheelchair. Her growth parameters including her head circumference were all in normal ranges.

Regarding her fine motor ability, she continues to struggle with a pincer grip at 9 years old. She had delays in her speech and language development, only starting to speak at 18 months old. At 3 years old she could make simple 3-word sentences. At 9 years old, she has slurred and slow speech and can confuse words. She has a moderate learning disability, currently attending a mainstream school with additional help. She is awaiting an autism assessment.

As a child it was noted that she had a prominent startle response. There was a query regarding the possibility of her suffering from absence seizures on the background of staring episodes. An EEG did not illustrate any abnormal findings. There have not been any seizure episodes queried after this early event.

She is on medication for sleeping and anxiety. Her behavior is an aspect which can be problematic with self-regulation issues. She remains under the care of pediatricians for urinary incontinence and is on desmopressin.

She was reviewed by clinical genetics at 2 years old. She had a CGH array which was normal and she was enrolled into a research study for developmental delay. The trio ES revealed a *de novo* c.1060T>C (p.Ser354Pro) variant in *FRMD5* (NM_032892.5).

Proband 7: c.1045A>C (p.Ser349Arg)

This is a 16-year-old male with ataxia, congenital nystagmus, atypical absence epilepsy and mild intellectual disability. He exhibited episodic abnormal eye movements since one week of age, deviation downward with a shaking of the eyes back and forth, with occasional bilateral arm shaking as well. Apgar scores were 8 and 9 at 1 and 5 minutes. His birth weight was 7 pounds 5 ounces, and there were no neonatal complications. Laboratory studies included normal electrolytes, normal CSF, normal video EEG, normal brain MRI, and normal abdominal imaging. Ophthalmology evaluation noted nystagmus and opsoclonus and recommended urine catecholamines, which were normal. Testing for mitochondrial disorders was normal. Evaluation for autoimmune encephalitis and pediatric neurotransmitter disease was normal. He had also had extensive testing for an inborn error of metabolism, which was negative. Prior seizure-like episodes were marked by staring for several seconds, upward eye rolling, or head dropping forward and to the side for up to several minutes. He had multiple eye rolling and staring episodes. In the past he was placed on Keppra, but this was discontinued due to side effects, and he has not been on any other anticonvulsant medications. His previous EEG was unremarkable as was an MRI study, but the REEG at the age of 8 showed a mildly abnormal EEG due to the presence of Occipital Intermittent Rhythmic Delta Activity (OIRDA) but became normal 2 months later. OIRDA has been noted to occur in association with generalized epilepsy.

He started sitting up at age 12 months, walked with a walker at age 5. His first word was at 12 months, but he is still difficult to understand, and he does not read or write. His head circumference was 57.2 cm at 12 years of age (within normal limits). The brain MRI at 4 years showed tiny cystic foci in the periventricular white matter of both cerebral hemispheres. These may represent remnants of cystic periventricular leukomalacia. The ventricles are normal in size. No mass effect, acute infarct or intracranial hemorrhage was seen. There was no developmental anomaly. The pituitary gland was normal with a T1 bright spot in the neurohypophysis. The corpus callosum, brainstem and cerebellum were normal. Spectroscopy

on the same day showed mild decreases in NAA (N-acetyl-aspartate) and mild increases in choline compared to age-matched control values. These findings were said to be non-specific and possibly related to brain development.

His current symptoms include fatigue, nystagmus, developmental delays, learning problems, headaches, seizures, staring spells, aggression, mood swings, memory problems, tics, spasms, walking problems (able to perform heel and toe walking but unable to perform tandem gait, casual gait mildly ataxic, unable to stand on either foot), interrupted sleep, and daytime sleepiness.

The ES revealed a rare heterozygous missense variant in *FRMD5*, NM_032892.5, c.1045A>C (p.Ser349Arg), which was confirmed by Sanger sequencing. It also revealed a variant of uncertain significance in *MFN2* (MIM: 608507): NM_014874.3, c.175G>T (p.Asp59Tyr), associated with autosomal dominant Charcot Marie Tooth disease type 2A. The mother was negative, and the father unavailable for testing.

Proband 8: c.1637A>G (p.Tyr546Cys)

This individual is a 15.5-year-old male who was initially evaluated by a clinical geneticist at age 2 years secondary to developmental delay and spasticity. He was noted to have delays at around 8 months of age as he was unable to complete his developmental milestones compared to his half-siblings. A brain MRI obtained at 2.5 years showed delays in myelination. An extensive evaluation for metabolic disorders was performed and was reported as normal including lactate acid, ammonia, acylcarnitine profile, creatine and guanidinoacetate levels, orotic acid, plasma amino acids and urine organic acids. Chromosome studies and chromosome microarray were normal. Fragile X testing was also normal. ES at 7 years of age was only remarkable for a heterozygous variant of uncertain significance (VUS) in *KMT2C* (MIM: 606883): NM_170606.2, c.13534C>A (p.His4512Asn). Pathogenic variants in this gene have been found in individuals with Kleefstra syndrome (MIM: 617768) who do not have *EHMT1*

(MIM: 607001) variants. The clinical presentation was not suggestive for Kleefstra syndrome however. A further ES review showed a variant in *FRMD5* c.1637A>G (p.Tyr546Cys), which was confirmed by Sanger sequencing. The VUS in *KMT2C* and the *FRMD5* variant were not found in his mother, and the father was not available for testing.

The development was his main area of concern and significant for moderate to severe global delay. He started crawling at age 2.5 years, walked at 4 years and was late developing overall. He had toe walking due to spasticity. He had limited speech and was only able to say a few words. Attends a special education program and receives ABA (applied behavior analysis) therapy secondary to a diagnosis of autism. Family history was noncontributory. His mother had two sons and a daughter from a previous marriage, who are healthy. There no one else in the family with similar symptoms.

At age 11 years he was evaluated by neurology due to a movement disorder with frequent grimacing, neck extension, hand wringing, self-hugging, and anxiety with strangers. He also had bruxism. He has been followed by gastroenterology and nutrition secondary to suboptimal growth. He had appropriate growth early in infancy, but length and weight starting lagging at 8-10 months of age despite adequate nutrition. He eats well and has no constipation although has regurgitation and rumination behaviors. He is followed by the renal service because of horseshoe kidneys and mild hydronephrosis. At 10 years of age his EEG was abnormal due to the presence of rare spike activity in the right central region. This finding indicates the presence of a focal potentially epileptogenic process in this region. No electrical seizures recorded.

His last physical exam was done at 13.5 years. His height was 134.3 cm (Z= -3.05) and weight 29.8 kg (Z= -2.78), all below normal parameters according to CDC growth curves. His head circumference was 53.4 cm (26th percentile using Nelhaus growth chart). He has no dysmorphic features. His neurological exam was normal for cranial nerves and deep tendon reflexes. He displayed several repetitive movements and behaviors including shaking of the

head, neck extension, self-hugging, wringing of the hands and some hand flapping. He also had bruxism. The gait stance was unusual with slight flexion of the knees. Genitourinary exam was normal with Tanner stage II. The skin was very dry and thick on his hands and dry in the inner aspect of his feet. The rest of the exam was unremarkable.

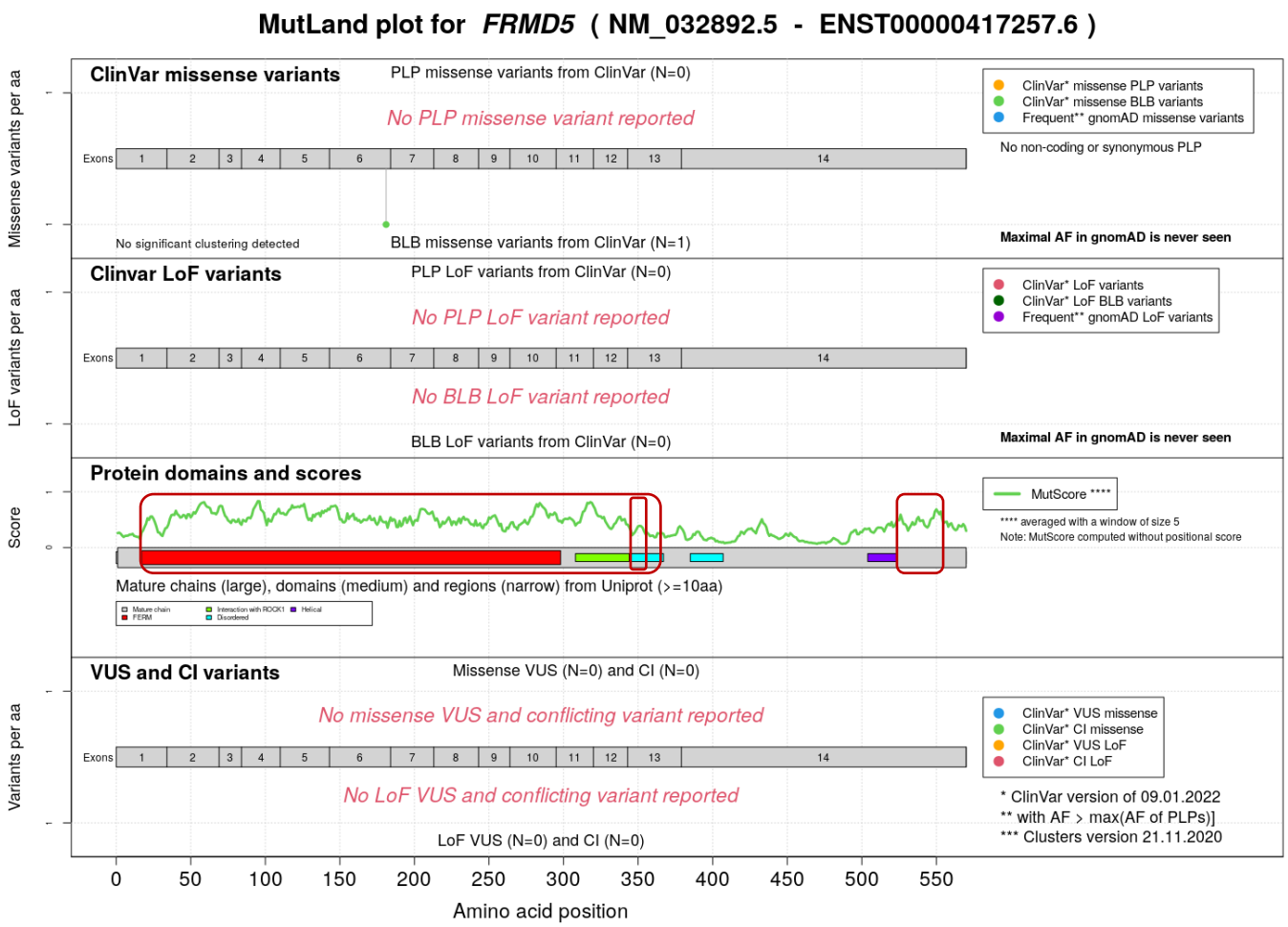


Figure S1. Visual presentation of MutScore prediction for *FRMD5*

The MutLand graphical output for *FRMD5*. No pathogenic and likely pathogenic (PLP) variants were detected. One variant, c.542C>T (p.Thr181Met), is shown as a benign and likely benign (BLB) variant. For more information of this variant please see Table S1. The predicted scores are averaged with a window size of 5. The regions of the protein mentioned in the text are highlighted with red boxes. Among them, the FERM domain region (AA 21-354) shows high scores, suggesting that missense variants in this region are more likely to be pathogenic. The AA 530-555 region in the C-terminus of the protein shows intermediate scores. For more information of p.Tyr546Cys variant please see Table S1. The clustering region (AA 349-354) does not show higher scores. No variants of uncertain significance (VUS) and conflicting interpretation (CI) variants were reported before July, 2022.

Figure S2. Protein sequence alignment of *Homo sapiens* FRMD5 and *Drosophila* dFrmd

Alignment of FRMD5 and dFrmd. Among the seven variants identified, three variants labeled in green color are at conserved amino acids (p.Ser351Gly, p.Ser351Arg, and p.Tyr546Cys). Protein isoforms for alignment: *Homo sapiens* FRMD5: NP_116281.2; *Drosophila* dFrmd: NP_609384.1. Similarity: 47%; Identity: 33%. Symbols in the protein alignment: identical (|), similar (:), different (.), absent (–). Data are from DIOPT.

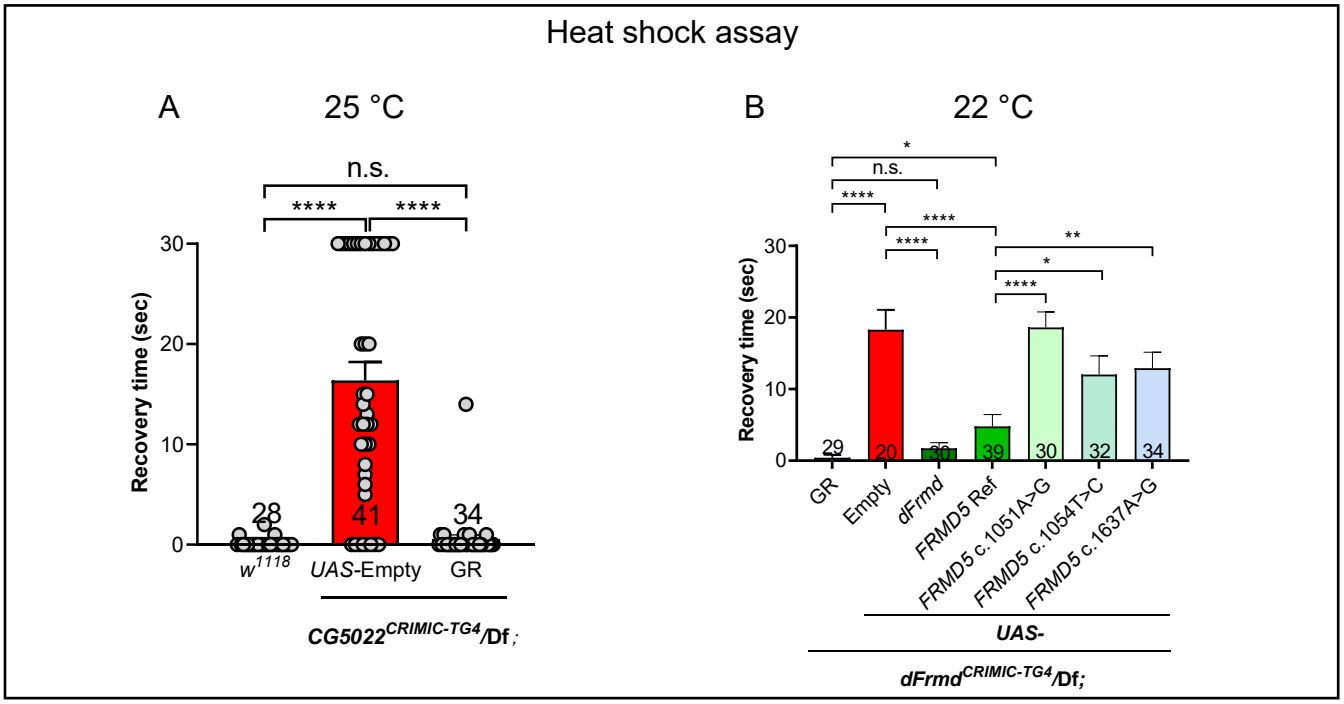


Figure S3. Loss of *dFrmd* in flies causes slow recovery after heat shock, and is rescued by *FRMD5* reference but less so by the variants

(**A**) *dFrmd* LoF mutant flies show slow recovery from seizures when compared to controls (w^{1118}/w^{1118}) after 42 °C water bath for 30 seconds. The phenotype can be fully rescued using a genomic rescue (GR) line. Flies were raised at 25 °C and tested at 14-15 days. Data are represented as mean + SEM. Unpaired t tests. ****P < 0.0001; n.s., no significance.

(**B**) The slow recovery after heat shock can be significantly rescued by fly *dFrmd* WT or human *FRMD5* Ref, but the human *FRMD5* variants have significantly reduced rescue abilities when compared to the Ref. Flies were raised at 22 °C and tested at 14-15 days. Data are represented as mean + SEM. Unpaired t tests. *P < 0.05; **P < 0.01; ****P < 0.0001; n.s., no significance.

A Ubiquitous *FRMD5* expression reduces viability

da-GAL4 > UAS-

Expression levels	18 °C	22 °C	25 °C
dFrmd	Viable	Viable	Semi-lethal
FRMD5 Ref	Semi-lethal	Lethal	Lethal
FRMD5 c.340T>C	Viable	Lethal	Lethal
FRMD5 c.1051A>G	Viable	Viable	Viable
FRMD5 c.1053C>G	Viable	Lethal	Lethal
FRMD5 c.1054T>C	Viable	Lethal	Lethal
FRMD5 c.1045A>C	Viable	Lethal	Lethal
FRMD5 c.1637A>G	Viable	Lethal	Lethal

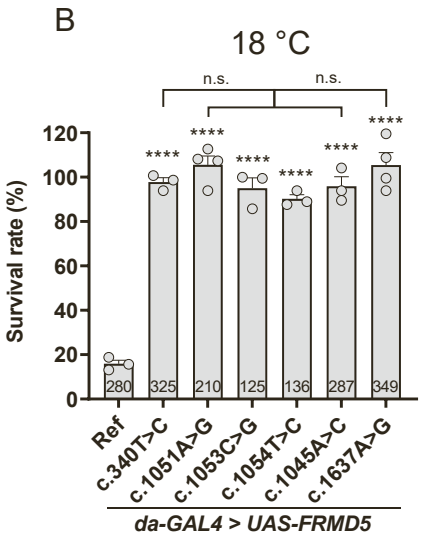
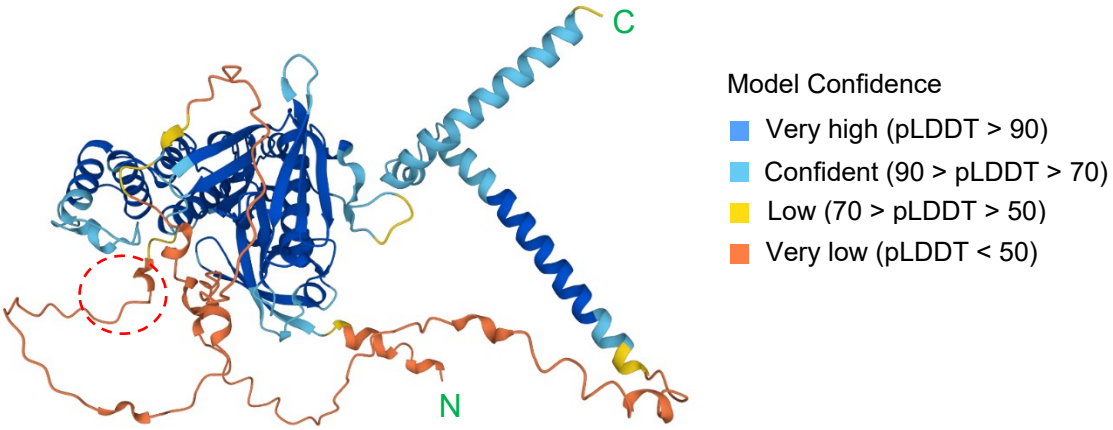


Figure S4. Ectopic expression of human *FRMD5* Ref is toxic, but the human *FRMD5* variants are less toxic

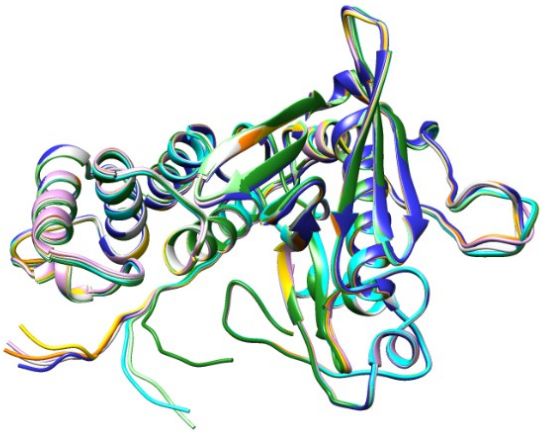
(**A**) Table summarizing the lethality phenotype of ubiquitous expression of *dFrmd*, *FRMD5* Ref and variants using *da-GAL4* at different temperatures. Note that the *FRMD5* Ref causes a more severe phenotype than the variants at 18 °C. The variants in red color could not be confirmed to be *de novo*. (**B**) Quantitative data at 18 °C are shown. The survival rate is calculated when compared to *da-GAL4>UAS-Empty*. Each dot represents an independent cross. Total fly numbers are shown in the columns. Data are represented as mean + SEM. Unpaired t tests. ****$P < 0.0001$; n.s., no significance.

A



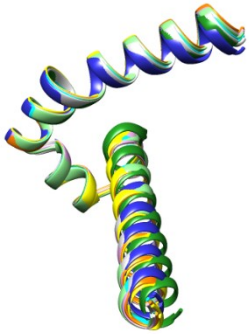
B

FERM domain (AA 21-354)



C

The AA 495-564 region



D

FRMD5	RMSD score (Å)	
	FERM domain (AA 21-354)	The AA 495-564 region
Ref	0	0
p.Phe114Leu	3.18	1.26
p.Ser351Gly	2.69	0.71
p.Ser351Arg	0.47	0.92
p.Cys352Arg	2.52	0.49
p.Ser354Pro	1.83	0.39
p.Ser349Arg	0.40	0.76
p.Tyr546Cys	0.62	1.06

Figure S5. Structural characterization of FRMD5 variants

(A-C) Predicted structures of FRMD5 reference (A) and variants (B-C) based on AlphaFold top predicted models. (A) The model confidence is shown in different colors. AlphaFold produces a per-residue confidence score (pLDDT) between 0 and 100. Some regions below 50 pLDDT may be unstructured in isolation. Note that FERM domain (AA 21-343) and the AA 495-564 region are confident (pLDDT > 70), while the other regions show low/very low confidence. The clustering region (AA 349-354) is highlighted in red dashed circle. The “N” and “C” label N-terminus and C-terminus of the protein, respectively. Structural alignment of FRMD5 variants with Ref in the FERM region (B) and the AA 495-564 region (C) was performed via UCSF Chimera[1]. (D) Root-mean-square deviation (RMSD) score for each variant compared to Ref is calculated using VMD software[2]. Two protein structures with an RMSD score of less than 3 Å would generally be considered to have no significant conformational changes[3].

Table S1: *In silico* characteristics of several *FRMD5* variants

***FRMD5* Variant (GRCh37)**	**Protein Change**	**Allele Frequency gnomAD**	**Number of Homozygotes gnomAD**	**MetaSVM**	**SIFT**	**POLYPHEN**	**REVEL Score**
15:44166583 C>T	p.Val405Met	0.0036	14	Tolerated	Tolerated	Benign	0.264
15:44165368 C>T	p.Gly511Asp	0.0035	5	Tolerated	Tolerated	Benign	0.209
15:44184197 C>T	p.Arg237Lys	0.0029	7	Tolerated	Tolerated	Benign	0.44
15:44166402 C>A	p.Ser465Ile	0.0016	4	Tolerated	Tolerated	Benign	0.249
15:44198035 G>A	p.Thr181Met	0.0014	6	Tolerated	Deleterious	Possibly Damaging	0.227
15:44166570 C>T	p.Arg409Gln	0.0008	1	Tolerated	Tolerated	Benign	0.188
15:44166159 T>C	p.Tyr546Cys	0	0	Damaging	Deleterious	Possibly Damaging	0.642

Table S2. Publicly available fly lines used in this study

Fly line	**Genotype**	**Source**
dFrmd$^{CRIMIC\text{-}TG4}$	*y^1 w^*; TI{GFP[3xP3.cLa]=CRIMIC.TG4.0}CG5022[CR00705-TG4.0]/SM6a*	BDSC #78994
Df	*w^{1118}; Df(2L)BSC208/CyO*	BDSC #9635
GR	*w^{1118}; Dp(2;3)GV-CH321-18A10, PBac{y[+mDint2] w[+mC]=GV-CH321-18A10}VK00031/TM3, Sb^1*	BDSC #89734
da-GAL4	*w^*; P{w[+mW.hs]=GAL4-da.G32}UH1, Sb^1/TM6B, Tb^1*	BDSC #55851
nub-GAL4	*w^*; P{w[nub.PK]=nub-GAL4.K}2*	BDSC #86108
UAS-mCherry.NLS	*w^*; P{w[+mC]=UAS-mCherry.NLS}3*	BDSC #38424
UAS-mCD8::RFP	*w^*; P{y[+t7.7] w[+mC]=10XUAS-IVS-mCD8::RFP}attP40*	BDSC #32219

Table S3: Primers used in this study.

Name	Species	Forward primer (5'-3')	Reverse primer (5'-3')	Assay
dFrmd	*Drosophila*	CTTCTCCTGGGGCACCAAAT	CTCGGCAAGCTGCTATATCTTAT	RT-PCR
rp49	*Drosophila*	TGTCCTTCCAGCTTCAAGATGACCATC	CTTGGGCTTGCGCATTTGTG	
FRMD5 c.340T>C (p.Phe114Leu)	*Homo sapiens*	TATTTAGTCCTCCTGCAGATC	CCTGGTTATTTCTTCTTTCAG	Mutagenesis
FRMD5 c.1045A>C *(*p.Ser349Arg*)*	*Homo sapiens*	GATGGTTCCCCGCCGGAGCTG	CCTGCTCTGTGTATTTCCGG	
FRMD5 c.1053C>G (p.Ser351Arg)	*Homo sapiens*	CCAGCCGGAGGTGTCCCTCCA	GAACCATCCCTGCTCTGTGTATTTCC	
FRMD5 c.1051A>G (p.Ser351Gly*)*	*Homo sapiens*	TCCCAGCCGGGGCTGTCCCTC	ACCATCCCTGCTCTGTGTATTTCCGG	
FRMD5 c.1054T>C (p.Cys352Arg)	*Homo sapiens*	CAGCCGGAGCCGTCCCTCCAT	GGAACCATCCCTGCTCTG	
FRMD5 c.1637A>G (p.Tyr546Cys)	*Homo sapiens*	CAATTCCACTGTCAATACTTTTGTCC	TTCAAACTCGGGGGTCTG	

Supplemental Material and Methods

Recruitment and sequencing of individuals

Six individuals were recruited through Maternal and Child Health Hospital of Hunan Province (proband 1 and 2) and Baylor Genetics Laboratories (proband 3, 4, 7 and 8). Proband 5 and 6 with severe undiagnosed developmental disorders were previously recruited by the Deciphering Developmental Disorders (DDD) Study[4], with the following information: Proband 5 (ID: 269740), https://www.deciphergenomics.org/ddd/research-variant/0dc53f15c5751a9b7d075050d10b1c96; Proband 6 (ID: 303400), https://www.deciphergenomics.org/ddd/research-variant/83738a9cbba51ea2fbb55855c05fd180. The procedures were followed in accordance with the ethical standards of the respective institutions. Proper informed consent was obtained from legal guardians of affected individuals. The DDD study has UK Research Ethics Committee approval (10/H0305/83, granted by the Cambridge South REC, and GEN/284/12 granted by the Republic of Ireland REC).

DNA sequencing methods

The exome sequencing (ES) were conducted on a clinical or research basis. For proband 1 and 2, Chigene performed trio ES followed by Sanger sequencing confirmation as previously described[5]. Baylor Genetics Laboratories performed ES and Sanger sequencing of probands 3, 4, 7 and 8, and the detailed sequencing and analysis protocols were published previously[6,7]. For proband 3, trio ES was performed and analyzed at Invitae. Briefly, genomic DNA obtained from the submitted sample was enriched for coding exons and adjacent splice junctions, generally exons +/- 10 base pairs, using a hybridization-based protocol. These regions were sequenced using Illumina technology to an average of ≥ 50x depth with minimum call depth of ≥ 20x. Reads were aligned to a reference sequence (GRCh37) and variants were identified using a custom-developed analysis tool. Identified variants were filtered and ranked using a proprietary algorithm, which considers known gene-phenotype associations, molecular variant

characteristics, zygosity, and population frequency, in the context of the individual's reported clinical presentation. This process is supported by an expertly curated gene-phenotype knowledgebase, as previously described[8]. Variants that may explain some or all of the individual's provided clinical indication were reviewed, interpreted, and reported according to ACMG guidelines[9]. Proband 5 and 6 with severe undiagnosed developmental disorders were recruited by the Deciphering Developmental Disorders (DDD) Study, and their samples were collected at the Wellcome Sanger Institute, where trio exome sequencing were applied to investigate the genetic causes of abnormal development. Likely diagnostic results are being reported to clinical team and their functional *de novo* mutations were confirmed in a public DDD research track in DECIPHER[4].

Fly stocks and genotypes

All flies used in this study were raised and maintained in plastic vials with standard cornmeal and molasses medium at room temperature, unless otherwise noted. Publicly available fly lines obtained from the Bloomington Drosophila Stock Center (BDSC) are listed in Table S2.

The *UAS-cDNA* lines were generated as described[10]. The *FRMD5* cDNA clone corresponding to GenBank transcript NM_032892.5, encoding isoform 2 (the longest isoform) is defined as the reference here. *FRMD5* variants were generated by Q5 site-directed mutagenesis (NEB). The fly *dFrmd* cDNA was obtained from DGRC GEO14235 (DGRC Stock 1658937; RRID: DGRC_1658937) and is defined as the wild type (WT). Primers for mutagenesis are listed in Table S3. All the human and fly cDNAs were cloned into pGW-UAS-HA.attB plasmid transgenic vector[11], and the pGW-UAS-Empty vector was used as the empty control[12]. The vectors were inserted into the VK33 (BDSC #24872) docking site by φC31 mediated transgenesis system[13].

Drosophila Behavioral Assays

Fly behavioral assays were performed as previously described[14]. For the heat shock assay, flies were transferred to an empty vial and submerged in a 42 °C water bath for 30 seconds.

The percentage of flies that are unable to keep an upright position was quantified. The time for flies to recover to a freely moving status was also measured. Flies that require more than 30 seconds to recover were recorded as 30 seconds.

ERG recording

ERG recordings were performed as previously described[15]. Briefly, flies were immobilized on a glass slide with glue. The recording electrode was placed on the corneal surface of the eye, and the reference electrode was inserted in the thorax. Flies were exposed to a series of light flashes for ERG recordings. For detailed methods of ERG recordings see Dolph et al., 2011[16].

Immunostaining

Immunostaining of fly larval and adult brains were performed as described[14]. Briefly, the samples were dissected in PBS and fixed with 4% Paraformaldehyde (PFA) in phosphate buffered saline (PBS) followed by blocking in PBS containing 0.2%Triton-X100 (0.2% PBST) with 5% normal goat serum. Primary antibodies used: rat anti-Elav deposited to the DSHB by Rubin G.M. (DSHB, 7E8A10; 1:500); mouse anti-Repo, deposited to the DSHB by Goodman C. (DSHB, 8D12; 1:50). Secondary antibodies used: goat anti-rat Alexa Fluor 647 (Jackson ImmunoResearch, 112-605-003; 1:500); goat anti-mouse Alexa Fluor 488 (Jackson ImmunoResearch, 115-545-062; 1:500). Samples were thoroughly washed with 0.2% PBST and mounted on a glass slide using Fluoromount-G (Southernbiotech, 0100-20). Samples were imaged with a confocal microscope (Leica SP8X), and images were processed using ImageJ.

Real-time (RT)-PCR

RT-PCR was performed as previously described[17] with several modifications. Total RNA was extracted by TRIzol Reagent (Thermo Fisher) using a standard protocol. Complementary DNA was made from 500-1000 ng of total RNA using All-In-One 5X RT MasterMix (abm #G592). RT-PCR reactions were performed with iTaq Universal SYBR Green Master Mix (BioRad

#1725120) and a BioRad C1000 Touch Cycler. *rp49* was used as an internal control gene. Primers were listed in Table S3.

Statistical Analysis

Statistical analysis was performed using GraphPad software (GraphPad Prism v9.0; GraphPad Software, USA). Data were analyzed by two-tailed unpaired t tests. Data are represented as mean + SEM, and n.s. (no significance) indicates $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

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(https://www.deciphergenomics.org), which is funded by Wellcome [grant number 223718/Z/21/Z]. See Nature PMID: 25533962 or www.ddduk.org/access.html for full acknowledgement. This study makes use of data generated by the DECIPHER community. A full list of centres who contributed to the generation of the data is available from https://deciphergenomics.org/about/stats and via email from contact@deciphergenomics.org. Funding for the DECIPHER project was provided by Wellcome. Those who carried out the original analysis and collection of the Data bear no responsibility for the further analysis or interpretation of the data. We thank AlphaFold for structural predictions. The molecular graphics and analyses were performed with UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311.

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