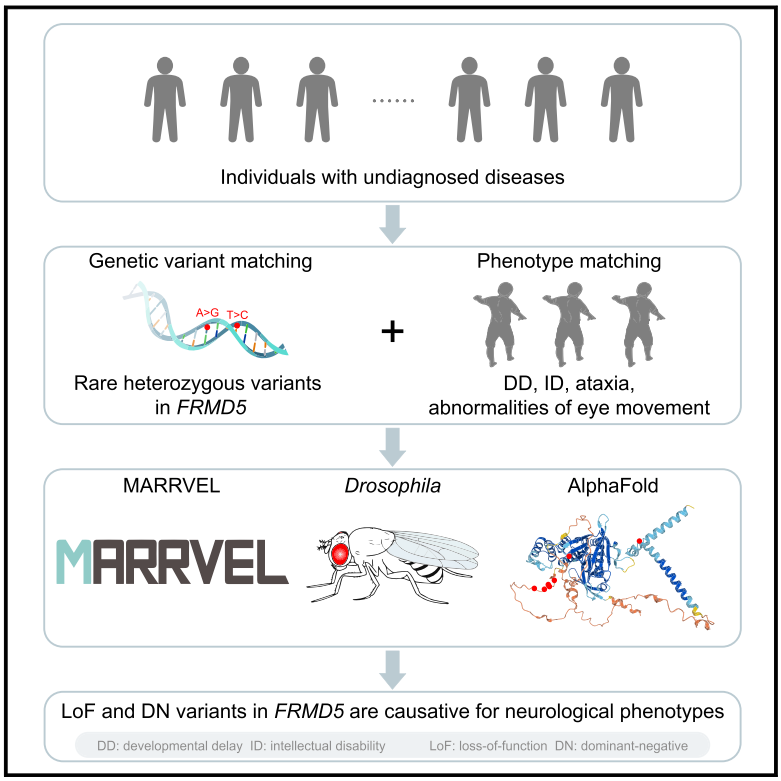
De novo variants in *FRMD5* are associated with developmental delay, intellectual disability, ataxia, and abnormalities of eye movement

Graphical abstract



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We report eight individuals with rare heterozygous variants in *FRMD5* who present with developmental delay, intellectual disability, ataxia and abnormalities of eye movement. Experimental evidence based on *Drosophila* studies and protein structure predictions indicate that these variants cause loss-of-function as well as dominant-negative effects.

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

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Summary

Proteins containing the FERM (four-point-one, ezrin, radixin, and moesin) domain link the plasma membrane with cytoskeletal structures at specific cellular locations and have been implicated in the localization of cell-membrane-associated proteins and/or phosphoinositides. FERM domain-containing protein 5 (FRMD5) localizes at cell adherens junctions and stabilizes cell-cell contacts. To date, variants in *FRMD5* have not been associated with a Mendelian disease in OMIM. Here, we describe eight probands with rare heterozygous missense variants in *FRMD5* who present with developmental delay, intellectual disability, ataxia, seizures, and abnormalities of eye movement. The variants are *de novo* in all for whom parental testing was available (six out of eight probands), and human genetic datasets suggest that *FRMD5* is intolerant to loss of function (LoF). We found that the fly ortholog of *FRMD5*, *CG5022 (dFrmd)*, is expressed in the larval and adult central nervous systems where it is present in neurons but not in glia. *dFrmd* LoF mutant flies are viable but are extremely sensitive to heat shock, which induces severe seizures. The mutants also exhibit defective responses to light. The human *FRMD5* reference (Ref) cDNA rescues the fly *dFrmd* LoF phenotypes. In contrast, all the *FRMD5* variants tested in this study (c.340T>C, c.1051A>G, c.1053C>G, c.1054T>C, c.1045A>C, and c.1637A>G) behave as partial LoF variants. In addition, our results indicate that two variants that were tested have dominant-negative effects. In summary, the evidence supports that the observed variants in *FRMD5* cause neurological symptoms in humans.

The FERM (four-point-one, ezrin, radixin, and moesin) domain is often located at the N terminus of FERM domain-containing proteins (FDCPs), linking the cytoskeletal network to the plasma membrane.[1] The FDCPs play important roles in cellular movements and migration by binding to a variety of proteins and lipids.[2] They contribute to membrane dynamics to mediate migration of the cell when responding to directional cues.[3,4] There are about 50 FDCPs in the human genome, and they participate in a variety of biological processes, such as wound healing and immune responses in health as well as cancer metastasis.[2] Fewer than 20 FDCPs have been reported to be associated with human diseases,[5] and the functions of the majority of the FDCPs remain to be discovered.

The FERM domain-containing protein 5 (*FRMD5* [MIM: 616309]) is localized to adherens junctions.[6] Previous studies have documented that knockdown of *FRMD5* promotes lung cancer cell migration and invasion.[6,7] FRMD5 inhibits migration through binding to integrin subunit beta 5 (ITGB5) and Rho-associated coiled-coil-containing protein kinase 1 (ROCK1).[7] However, other scientists showed that knockdown of *FRMD5* suppresses hepatocellular carcinoma cell (HCC) proliferation and tumorigenesis and that *FRMD5* is elevated by Wnt/β-catenin activation in human HCCs.[8] In addition, the transcriptional activity of *FRMD5* is regulated by β-catenin in colorectal cancer cells.[9] These data indicate different functional outcomes of loss of *FRMD5* in different contexts. However, variants in *FRMD5* have not been associated with a disease in the Online Mendelian Inheritance in Man (OMIM) database.[10]

We identified eight individuals with rare heterozygous missense *FRMD5* variants who present with neurodevelopmental disorders. Proper informed consent was obtained from legal guardians of the individuals. The variants are *de novo* in all the cases except for probands 7 and 8, for

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whom the variants were not detected in maternal samples, but the paternal samples are unavailable. A summary of the clinical information, including nucleotide changes, of these probands can be found in Table 1. All probands exhibit developmental delay including motor delay. All probands present with intellectual disability, except proband 1, who is too young to be diagnosed. Seven probands have ataxia. They all exhibit abnormalities of eye movement. Among them, probands 2, 4, 5, and 7 have nystagmus, whereas probands 3, 6, and 7 have opsoclonus. Proband 1 has strabismus, and proband 8 has intermittent esotropia. Nystagmus and opsoclonus are abnormal involuntary eye movements, whereas strabismus is an abnormal conjugate eye movement. Five individuals have seizures, and proband 8 has an abnormal EEG. Some individuals have refractory seizures. Three of the eight individuals have abnormal brain MRIs (probands 2, 7, and 8). Proband 2 exhibited pachygyria in bilateral temporal lobes at the age of 6 (Figures 1A and 1B). For more detailed information, other symptoms, and other potential variants not within *FRMD5*, please see the case reports in the supplemental information.

To gather information on human *FRMD5* and the potential impact of the variants, we used the Model organism Aggregated Resources for Rare Variant ExpLoration (MARRVEL) tool,[11] which gathers information from multiple sources including Genome Aggregation Database (gnomAD[12]), OMIM, Database of Genomic Variants (DGV[13]), etc. *FRMD5* has a probability of loss-of-function (LoF) intolerance (pLI) score of 1.00 based on gnomAD,[12] suggesting that *FRMD5* may be a haploinsufficient gene and that loss of a single copy of the gene may cause the observed phenotypes. *FRMD5* has a missense *Z* score of 1.98, suggesting that *FRMD5* missense variants may not be tolerable.[14] However, there are few heterozygous LoF variants, and individuals with deletions that uncover *FRMD5* locus are observed in control[12,13] and disease datasets.[15,16] Together, these human population genetic data suggest that haploinsufficient or dominant-negative variants of *FRMD5* may create phenotypes.

Seven different missense variants in *FRMD5* (GenBank: NM_032892.5) were identified among the eight probands (c.1054T>C is shared by probands 4 and 5), and none of the variants are found in gnomAD. All the variants are predicted to be deleterious based on combined annotation dependent depletion (CADD) scores above 20 (Table 1).[17] Interestingly, five of the seven FRMD5 variants (p.Ser349-Arg, p.Ser351Gly, p.Ser351Arg, p.Cys352Arg, and p.Ser354-Pro) are clustered within very few amino acids (aa 349–354) in the FERM-adjacent (FA) domain, suggesting a hotspot region for *FRMD5*. Since DECIPHER[15] has not annotated any hotspot region for *FRMD5*, we queried MutScore,[18] a pathogenicity predictor, for region-specific constraint/missense variant analysis. There is no significant clustering for pathogenic or benign variants detected by MutScore (Figure S1). The MutLand plot from MutScore for *FRMD5* domains shows relatively higher scores for the FERM domain region (aa 21–354), suggesting that missense variants in this region are more likely to be pathogenic (Figure S1). The aa 530–555 region in the C terminus of the protein displays intermediate scores, while the aa 349–354 region does not show higher scores than the FERM domain region (Figure S1). In summary, little information is available about the aa 349–354 region of the FA domain.

Besides, there are two variants that map outside the aa 349–354 region: p.Phe114Leu maps to the FERM-middle (M) domain, whereas p.Tyr546Cys maps to the C terminus of the protein. There are two variants that are not confirmed to be *de novo*: p.Ser349Arg (maps to aa 349–354) and p.Tyr546Cys. Although p.Tyr546Cys maps to an uncharacterized region of the protein, the *in silico* data suggest that p.Tyr546Cys is deleterious, whereas the other variants observed in gnomAD with high frequencies are mostly predicted to be benign/tolerated (Table S1).

Drosophila dFrmd is an ortholog of *FRMD5*

To investigate the function of *FRMD5 in vivo*, we utilized *Drosophila* as the model organism.[19] The *Drosophila* RNAi Screening Center (DRSC) Integrative Ortholog Prediction Tool (DIOPT)[20] predicts one fly gene, *CG5022* (hereafter referred to as *dFrmd*), as the ortholog of both human *FRMD5* and *FRMD3*. The DIOPT score between *FRMD5* and *dFrmd* is 12 out of 16, suggesting a high level of homology between the two genes. The overall similarity and identity between FRMD5 and dFrmd are 47% and 33%, respectively (Figures 2A and S2), and the two proteins show similar domain topology, including the well-conserved FERM domain (Figure 2B). Taken together, these data indicate that fly *dFrmd* is orthologous to *FRMD5* in humans.

To study *FRMD5* in flies, we generated the fly reagents listed in Figure 2C and Table S2. These include a *CRISPR-Mediated Integration Cassette* (*CRIMIC*) allele of the *dFrmd* ($dFrmd^{CRIMIC\text{-}TG4}$),[21] which has a *Splice Acceptor (SA)-T2A-GAL4-polyA* cassette inserted in the first intron of the gene (Figure 2C). The $dFrmd^{CRIMIC\text{-}TG4}$ is likely a null allele, as it creates a truncated *dFrmd* mRNA (Figure 2D), and our real-time PCR data show that the $dFrmd^{CRIMIC\text{-}TG4}$ reduced the *dFrmd* mRNA levels to less than 1% (Figure 2E). This $dFrmd^{CRIMIC\text{-}TG4}$ allele also leads to the expression of *GAL4* under the endogenous gene-regulatory elements (Figure 2D) and allows us to assess the expression pattern of *dFrmd*, to explore LoF phenotypes, and to test the rescue ability of fly and human cDNAs.[21–23]

dFrmd is expressed primarily in neurons of the fly CNS

We first determined the expression pattern of *dFrmd* by crossing the $dFrmd^{CRIMIC\text{-}TG4}$ allele to *UAS-mCherry.NLS* (nuclear-localized mCherry fluorescent protein). The mCherry expression is obviously enriched in the larval central nervous system (CNS) (Figures 3A–3C). In both larval CNS and adult brain, mCherry (*dFrmd*) co-localizes with some Elav (pan-neuronal nuclear marker)-positive cells, but no obvious overlap was observed between mCherry and Repo (pan-glial nuclear marker) (Figures 3C

Table 1. Clinical features of affected individuals with *FRMD5* variants

Proband	1	2	3	4	5	6	7	8
Exome sequencing (ES)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
FRMD5 variant (GenBank: NM_032892.5)	c.340T>C	c.1051A>G	c.1053C>G	c.1054T>C	c.1054T>C	c.1060T>C	c.1045A>C	c.1637A>G
Protein change	p.Phe114Leu	p.Ser351Gly	p.Ser351Arg	p.Cys352Arg	p.Cys352Arg	p.Ser354Pro	p.Ser349Arg	p.Tyr546Cys
CADD	29.1	23.3	22.6	23.6	23.6	27.8	23.4	26.3
Current age (years)	3	8	27	17	18	9	16	15.5
Inheritance	*de novo*	*de novo*	*de novo*	*de novo*	*de novo*	*de novo*	no paternal sample	no paternal sample
Age of onset	6 months	3 months	neonatal period	neonatal period	neonatal period	neonatal period	neonatal period	8 months
Sex	M	M	F	M	M	F	M	M
Motor delay	+	+++	+	+	++	+	+	++
Developmental delay	+	+++	+	+	++	+	+	++
Intellectual disability	N/A	+++	+ (borderline)	+	++	+	+	+
Seizures	++	+++	+	+	–	–	+	abnormal EEG
Ataxia	+	+	+	++	+	+++	+	–
Hypotonia	–	+	–	+	+	+	N/A	+
Spasticity	+	+	–	+	–	–	N/A	+
Abnormality of the eye	strabismus	nystagmus	opsoclonus	ocular vertical nystagmus/flutter	nystagmus, intermittent flutter with poor fixation, mild myopia, delayed visual maturation	opsoclonus, hypermetropia, visually impaired	nystagmus and opsoclonus	intermittent esotropia
Brain MRI	normal	pachygyria in bilateral temporal lobes	normal	normal	normal	normal	cystic foci in the periventricular white matter	delays in myelination
Additional features	feeding difficulties	feeding difficulties, severe constipation	myoclonus and dystonia; difficulty with fine motor skills; dyslexia; migraines	N/A	learning disability, behavioral problems, dystonia, dyskinetic and spasm-like movements and postures	moderate learning disability, urinary incontinence, behavior and self-regulation concerns, poor sleep, anxiety	learning problems, fatigue, headaches, interrupted sleep	ASD, renal anomalies

We report eight individuals with rare heterozygous variants in *FRMD5* who present with developmental delay, intellectual disability, ataxia, and abnormalities of eye movement. Experimental evidence based on *Drosophila* studies and protein structure predictions indicate that these variants cause loss-of-function as well as dominant-negative effects. CADD, combined annotation-dependent depletion; M, male; F, female; N/A, not available; EEG, electroencephalography; MRI, magnetic resonance imaging; ASD, autism spectrum disorder; –, +, ++, and +++, none, mild, moderate, and severe.

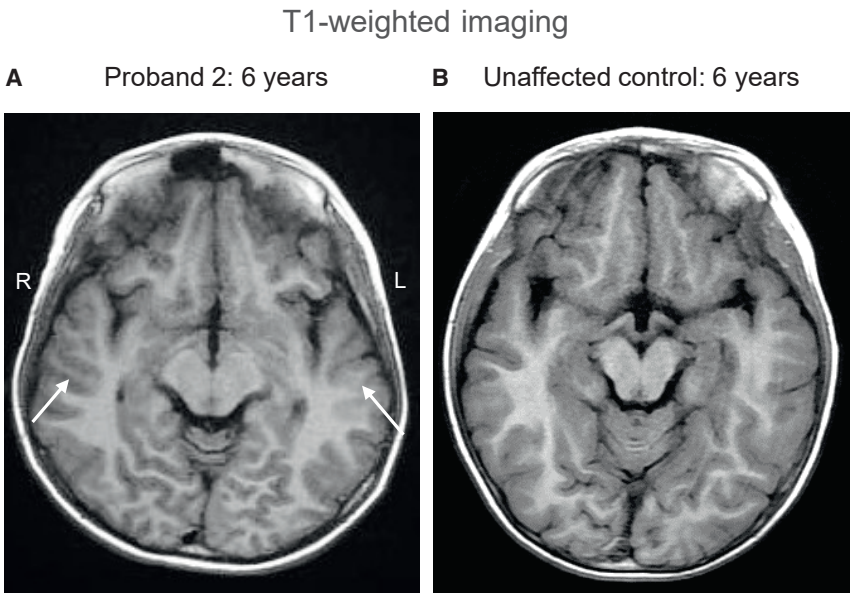

Figure 1. Brain MRI of proband 2
(A and B) Axial T1-weighted image from proband 2 at 6 years shows pachygyria in bilateral temporal lobes (A, white arrows), when compared to the unaffected control (B) of the same age and sex.

and 3D), indicating that *dFrmd* is mainly expressed in a subset of neurons, consistent with single-cell sequencing data.[24,25] To reveal the projections of *dFrmd*-expressing neurons, we used the $dFrmd^{CRIMIC\text{-}TG4}$ allele to drive *UAS-mCD8::RFP* (a membrane-bound red fluorescent protein). As shown in Figure 3E, RFP (*dFrmd*) labels neuropils of the central brain and ventral nerve cord in the larval CNS (Figure 3E). In the adult brain, RFP signals are observed in the optic lobes, antennal lobes, and mushroom bodies as well as other brain regions (Figure 3F). Altogether, these data show that *dFrmd* is specifically expressed in the neurons, in agreement with the prominent expression of *FRMD5* in the human CNS.[26]

Loss of *dFrmd* in flies causes heat-induced seizures and is rescued by the human *FRMD5* reference, but the variants rescue poorly

To explore the role of FRMD5 in the nervous system, we assessed phenotypes associated with *dFrmd* loss in flies. We generated *dFrmd* LoF mutant flies by crossing $dFrmd^{CRIMIC\text{-}TG4}$ to a deficiency (Df) line lacking *dFrmd*. The $dFrmd^{CRIMIC\text{-}TG4}$/Df mutants are viable and fertile and do not show obvious morphological abnormalities. Given that the probands exhibit seizures, we induced seizure-like behaviors in flies by mechanical stimulation (bang sensitivity assay)[27] or exposure to 42°C (heat shock assay).[28,29] The $dFrmd^{CRIMIC\text{-}TG4}$/Df mutants do not show obvious bang sensitivity but are very sensitive to heat shock, which induces severe seizures (Figures 4A and 4B). The mutant flies cannot climb properly and display wing fluttering, leg twitching, and abdominal muscle contractions (Video S1), and loss of *dFrmd* causes a slow recovery after heat shock (Figures S3A and S3B and Video S2). The heat-induced seizures are rescued by a genomic rescue (GR) construct that carries a copy of the *dFrmd* locus (Figures 2C, 4A, and S3A), indicating that the loss of *dFrmd* is the cause of the heat-sensitivity phenotype.

Next, we attempted to rescue the heat-induced seizures of *dFrmd* LoF mutants by expressing human *FRMD5* reference (Ref) or variant cDNAs. We generated the *UAS-dFrmd* wild-type (WT) and *UAS-FRMD5* transgenic fly lines and crossed them into the $dFrmd^{CRIMIC\text{-}TG4}$/Df background. Both the *UAS-dFrmd* WT and UAS-*FRMD5* Ref cDNA transgenes fully rescued the phenotype of *dFrmd* LoF mutants, at 25°C (Figure 4A) and 22°C (Figure 4B). In contrast, the three tested *FRMD5* variants exhibit significantly reduced rescue abilities when compared to the Ref (Figures 4A, 4B, and S3B), indicating that the tested *FRMD5* variants (c.1051A>G, c.1054T>C, and c.1637A>G) are partial LoF variants.

Loss of *dFrmd* in flies causes specific ERG defects

We also explored if FRMD5 affects synaptic transmission or phototransduction. We performed electroretinogram (ERG) recordings to assess the ability of the photoreceptors (PRs) to capture and transduce light signals and to assess if the PRs communicate properly with postsynaptic cells.[30,31] ERG recordings of the *dFrmd* LoF mutants did not show obvious defects at 10 days post-eclosion (Figure 4C) but started to show reduced On transients at 20–21 days (Figure 4D), and obviously decreased On and Off transients were observed in the *dFrmd* LoF mutants on day 30 (Figure 4E) when compared to the GR rescued flies. These data indicate that dFrmd is required to maintain proper synaptic transmission between the presynaptic photoreceptors and the postsynaptic lamina cells in an age-dependent manner. The On transient phenotype is fully rescued by expression of the *FRMD5* Ref cDNA, but the two variants that were tested (c.1051A>G and c.1637A>G) show significantly reduced rescue abilities (Figures 4F and 4G), again indicating that they are partial LoF variants.

Ectopic expression of human *FRMD5* Ref is toxic, whereas the variants are less toxic

To further investigate the nature of the *FRMD5* variants, we performed ectopic expression assays by expressing *UAS-FRMD5* cDNAs using different GAL4 drivers at different temperatures, as the GAL4 expression increases with temperature.[32,33] Interestingly, ubiquitous expression of *FRMD5* Ref using *daughterless-GAL4* (*da-GAL4*) causes semi-lethality at 18°C and full lethality at 22°C and 25°C

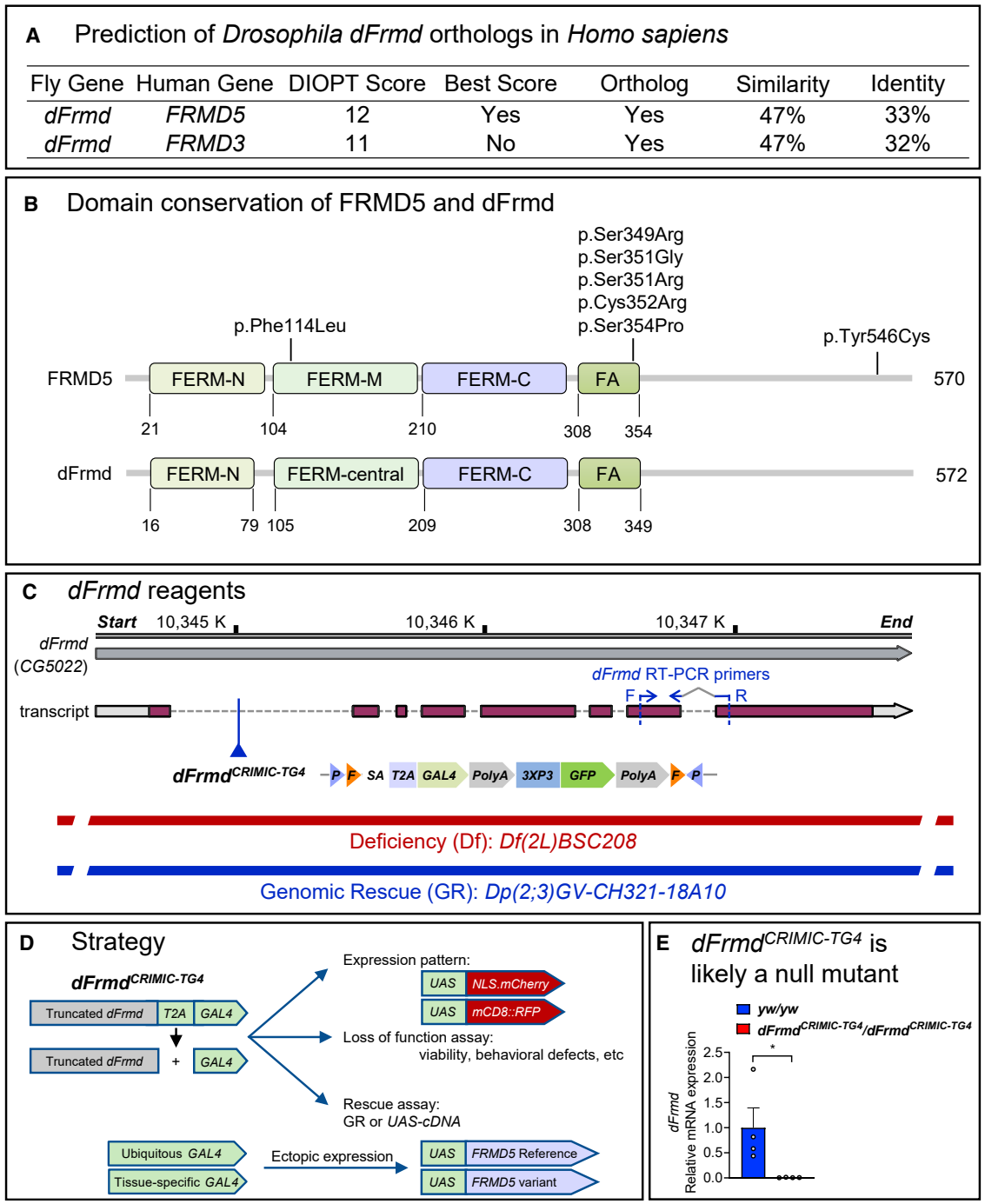

Figure 2. *CG5022* (*dFrmd*) is the *FRMD5* ortholog in fly

(A) *FRMD5* and *FRMD3* share the same fly ortholog, *dFrmd*. Data were obtained from DIOPT (DRSC Integrative Ortholog Prediction Tool).

(B) Protein domains are conserved between FRMD5 and dFrmd.

(C) Genomic structure of *dFrmd* locus and reagents used in this study. Real-time PCR primers to detect the *dFrmd* mRNA levels are also labeled.

(D) Strategy to study *FRMD5* in flies. Using $dFrmd^{CRIMIC\text{-}TG4}$, we determined the expression pattern and the loss-of-function (LoF) phenotypes and performed rescue assays. We also ectopically expressed *FRMD5* reference (Ref) and variants using different GAL4 drivers to assess their effects *in vivo*.

(E) Real-time PCR data show that $dFrmd^{CRIMIC\text{-}TG4}$ is a severe LoF or null mutant. Relative *dFrmd* mRNA expression levels in $dFrmd^{CRIMIC\text{-}TG4}$ mutant larvae decrease to <1% when compared to controls (*yw/yw*). Each dot represents an independent sample that contains 3–5 larvae. Data are represented as mean + SEM. Unpaired t tests. *$p < 0.05$.

(Figure S4A). Furthermore, the expression of *FRMD5* Ref using a wing-specific *nubbin-GAL4* (*nub-GAL4*) causes semi-lethality at 18°C and 22°C and full lethality at 25°C (Figures 5A and 5B), and the surviving flies show wing defects (Figure 5C). These data indicate that overexpression of *FRMD5* Ref is toxic in a dose-dependent manner. Similarly,

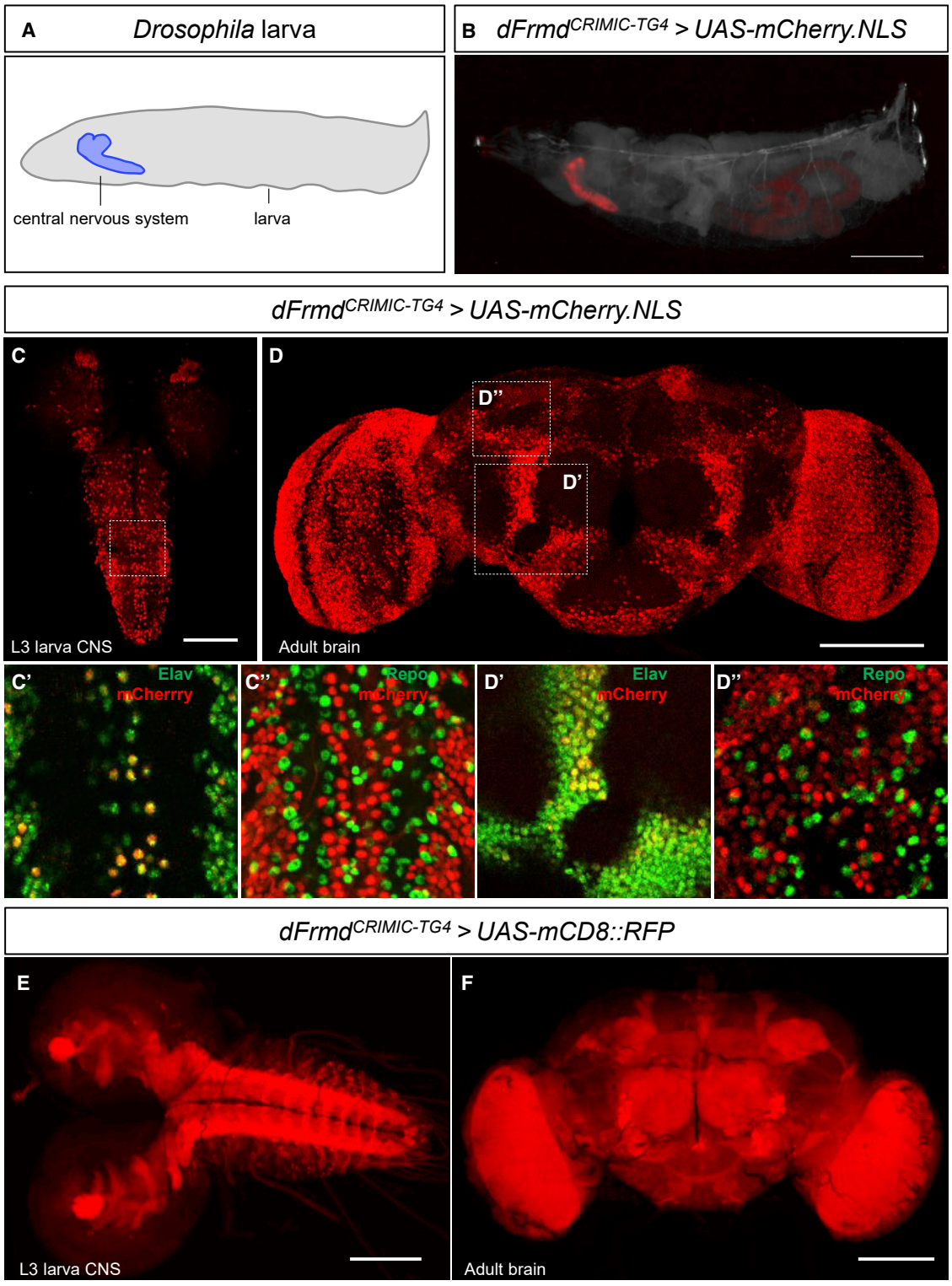

Figure 3. ***dFrmd*** **is expressed in neurons in the CNS**

(A) Schematic of the whole *Drosophila* larva highlighting the CNS.

(B) Expression pattern of *dFrmd* in whole third instar (L3) larva of the indicated genotype. Note that mCherry (*dFrmd*) is mainly expressed in the larval CNS. Scale bar, 1 mm.

(C and D) Expression pattern of *dFrmd* in the L3 larval CNS (C) and adult brain (D) is visualized using $dFrmd^{CRIMIC\text{-}TG4}$ allele-driven expression of *UAS-mCherry.NLS* co-stained with markers for neurons (Elav) or glia (Repo). Single-layer confocal images from the dashed squares indicate that mCherry is co-localized with Elav (C′, D′) but not Repo (C″, D″). Scale bars, 100 μm.

(E and F) The $dFrmd^{CRIMIC\text{-}TG4}$ allele-driven expression of *UAS-mCD8::RFP* (membrane-bound RFP) confirmed the broad expression of *dFrmd* in L3 larval CNS (E) and the adult brain (F). Scale bars, 100 μm.

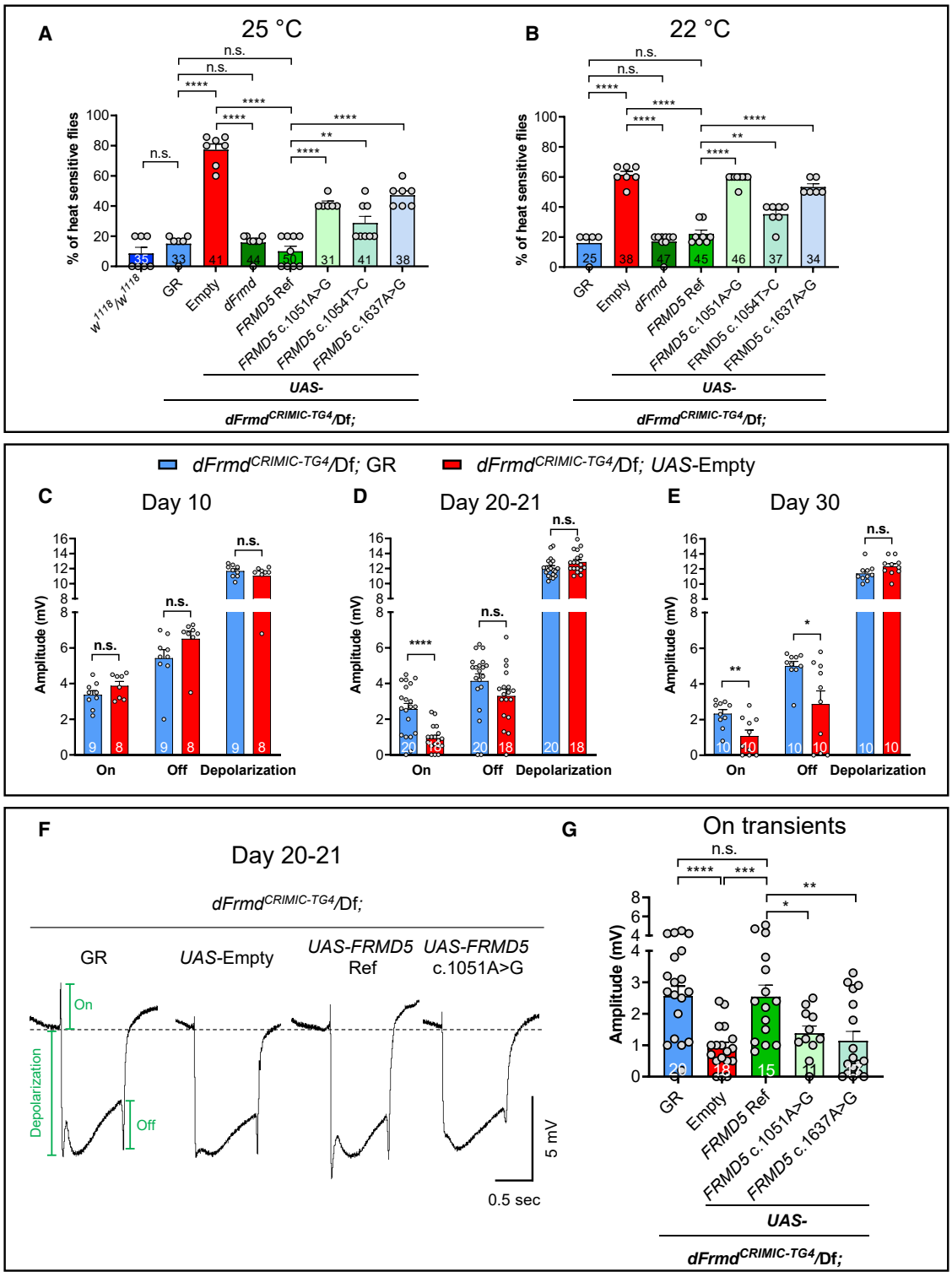

Figure 4. Loss of *dFrmd* in flies causes heat-induced seizures and specific ERG defects and is rescued by *FRMD5* reference but less so by the variants

(A and B) *dFrmd* LoF mutants exhibit heat-induced seizures. The percentage of *dFrmd* LoF mutant flies with seizures is significantly higher than controls (w^{1118}/w^{1118}) after exposure to a 42°C water bath for 30 s. The phenotype can be fully rescued with a genomic rescue (GR). The heat-induced seizures 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 at 25°C (A) and 22°C (B). Flies were raised at 25°C (A) or 22°C (B) and tested at 14–15 days. Each dot represents an independent test of 5–8 flies.

(C–E) *dFrmd* LoF mutants show age-dependent ERG defects. The mutants do not show any ERG defect at day 10 (C), show decreased On transients at 20–21 days (D), and show decreased On and Off transients at day 30 (E). Flies were raised at 22°C.

(legend continued on next page)

overexpression of *dFrmd* WT using *da-GAL4* causes semi-lethality at 25°C (Figure S4A). Moreover, *nub-GAL4*-induced *dFrmd* WT expression causes wing defects at 25°C (Figures 5A and 5C). Six *FRMD5* variants (c.340T>C, c.1045A>C, c.1051A>G, c.1053C>G, c.1054T>C, and c.1637A>G) were tested, and they all showed decreased toxicity when compared to the Ref (Figures 5A, 5B, S4A, and S4B). When the Ref causes a toxic phenotype and the variants are less toxic, the variants are classified as LoF alleles.[22,34–40] In contrast, gain-of-function variants often cause more severe phenotypes in ectopic expression assays.[41] Hence, all assays that we carried out argue that the *FRMD5* variants are partial LoF variants. It's worth noting that the ectopic expression assays indicate that the c.1051A>G is the most severe variant, consistent with the human phenotype since proband 2 with the variant exhibits the most severe symptoms. Also, for the c.1637A>G that could not be confirmed to be *de novo*, the rescue assay and ectopic expression assays consistently show that it is a partial LoF allele.

FRMD5 variants disrupt the function of FRMD5 in a dominant-negative manner

A previous study showed that FRMD5 interacts with ROCK1 via the FA domain and inhibits the ROCK1 kinase activity.[7] Hence, FRMD5 regulates actin-based cytoskeletal remodeling by modulating the kinase activity of ROCK1.[7] Since the seven variants are missense, and five are clustered in the FA domain, these variants may disrupt FRMD5 function in a dominant-negative manner. To address if the variants are dominant negative, we expressed one copy of *FRMD5* Ref together with one copy of the *FRMD5* variants in the *dFrmd* LoF background ($dFrmd^{CRIMIC\text{-}TG4}$/Df). If a variant is a LoF variant, the expression of the *FRMD5* Ref should suppress the phenotype of the *dFrmd* LoF mutant, whereas the presence of a dominant-negative variant should reduce the rescue ability of the Ref.[42] As shown in Figure 5D, expression of the *FRMD5* Ref decreases the heat sensitivity from ~80% to ~20%. In contrast, co-expression of the Ref with either c.1051A>G or c.1054T>C causes an intermediate phenotype (Figure 5D). These data suggest that the tested variants impair the rescue ability of *FRMD5* Ref. These data indicate that the variants act in a dominant-negative manner.

Since the structure of the protein is fundamental for its function, we explored if the *FRMD5* variants lead to significant conformational changes in the protein when compared to the Ref. We predicted the three-dimensional structure of FRMD5 for the variants using the AlphaFold Protein Structure Database.[43,44] The variants in the FA domain are clustered in a loop (Figure S5A). Modeling based on AlphaFold did not show any obvious structural differences between the Ref and the variants, not only for the variants in the FA domain, but also for the two other variants (Figures S5B and S5D). Moreover, based on the ectopic expression assays, there are no significant functional differences between the variants clustering in the FA domain and the two variants that do not map to the FA domain (Figures 5B and S4B). These data suggest that all these variants may have dominant-negative effects.

Among the ~50 FDCPs, there are eight proteins with their names containing "FRMD" in human (FRMD1, 3, 4A, 4B, 5, 6, 7, and 8),[2,45] and two genes encoding FRMD4A and FRMD7 have been associated with human diseases. A homozygous frameshift mutation of the *FRMD4A* (MIM: 616305) in multiple affected individuals in a family is associated with severe neurologic symptoms, which include microcephaly and intellectual disability (MIM: 616819).[46] FRMD4A is a scaffolding protein that regulates epithelial cell polarity by connecting the small GTPase ADP-ribosylation factor 6 (ARF6) and the par-3 family cell polarity regulator (PARD3).[47] Suppression of *PARD3* (MIM: 606745) expression disrupts the polarity distribution of human neural progenitor cells.[48] Interestingly, the ankyrin repeat and LEM domain containing 2 (ANKLE2)-PAR complex pathway is conserved from flies to humans, and previous work showed that bi-allelic mutations in *ANKLE2* (MIM: 616062) are associated with microcephaly in humans.[49] Moreover, loss of *Ankle2* leads to loss of neuroblasts and disrupted asymmetric cell division of neuroblasts and causes microcephaly.[50]

Mutations in *FRMD7* (MIM: 300628) cause X-linked idiopathic congenital nystagmus (MIM: 310700).[51] FRMD7 is shown to activate GTPase RAC1 signaling *in vitro*[52] and co-localizes with actin in the growth cones of differentiated NEURO2A cells.[53] Knockdown of *FRMD7* during neuronal differentiation leads to disrupted actin cytoskeleton and results in altered neurite outgrowth.[53] However, little is known about the function of FRMD7 in animal models. Interestingly, the roundabout guidance receptor 1 (ROBO1), another protein localized to growth cones of neurons, controls axonal guidance in the *Drosophila* CNS,[54] and human individuals who are homozygous for LoF variants of *ROBO1* (MIM: 602430) exhibit nystagmus.[55]

It is striking that five of the seven *FRMD5* variants are clustered within very few amino acids (aa 349–354) in the FA domain. Although our knowledge about the structure and function of the FA domain is limited, a previous study showed that the FA domain of FRMD5 is required for FRMD5-ROCK1 interaction, and FRMD5 regulates actin-based cytoskeletal rearrangements by inhibiting the ROCK1 kinase activity.[7] Our data based on ERGs suggest that *dFrmd* is required to maintain proper synaptic

(F and G) The decreased On transients at 20–21 days can be rescued by human *FRMD5* Ref but not the variants. Representative ERG curves are shown in (F), and the quantitative data are shown in (G). Green annotations show the amplitude measurement of On/Off transients and depolarization. Flies were raised at 22°C.

For (A)–(E) and (G), total fly numbers are shown in the columns. Data are represented as mean + SEM. Unpaired t tests. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; n.s., no significance.

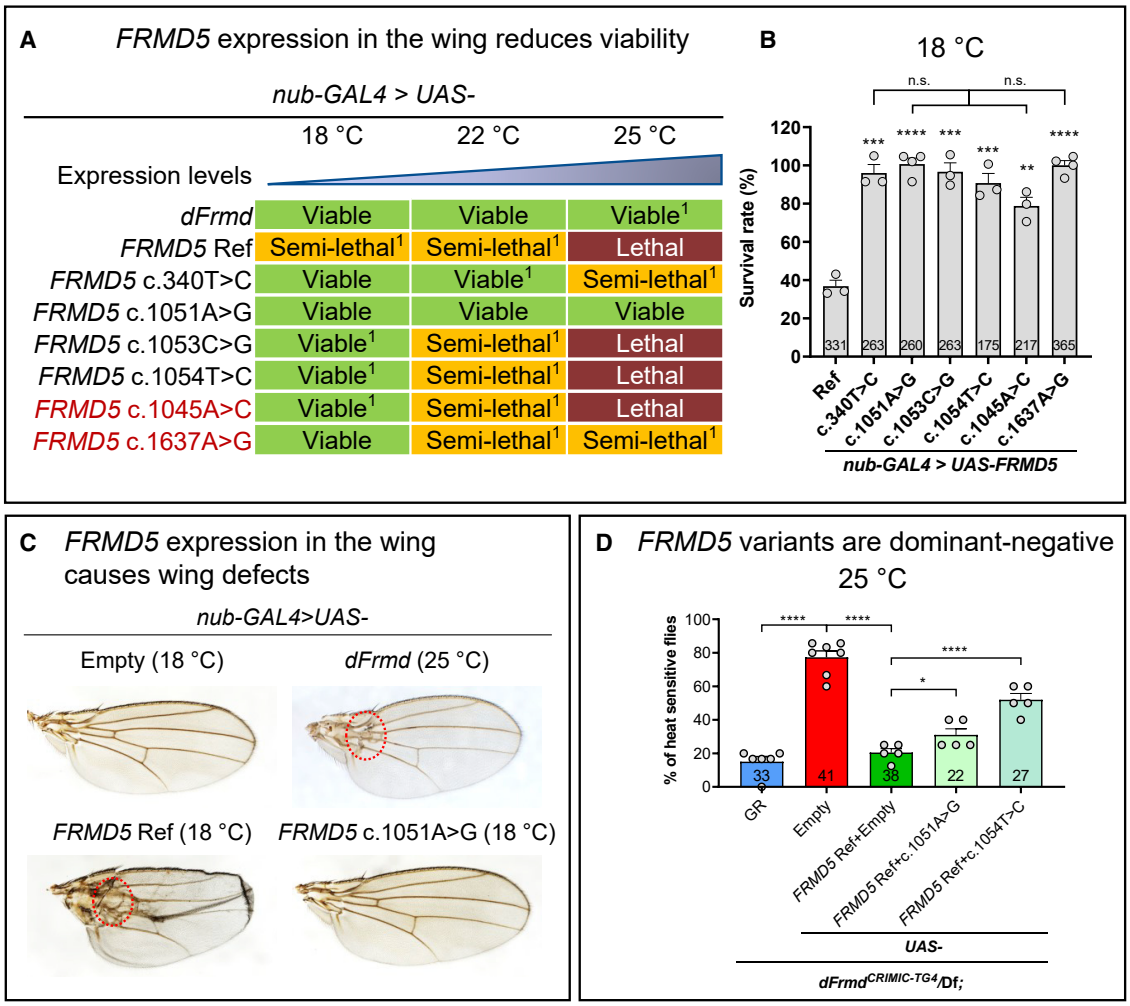

Figure 5. ***FRMD5*** **variants are less toxic, and some have dominant-negative effects**

(A) Summary of the lethality phenotype of wing-specific expression of *dFrmd*, *FRMD5* Ref, and variants at different temperatures. Note that the *FRMD5* Ref causes a more severe phenotype than the variants at 18°C. Some of the surviving flies exhibit wing defects and are noted as "[1]". The variants in red could not be confirmed to be *de novo*.

(B) Quantitative data at 18°C are shown. The survival rate is calculated when compared to *nub-GAL4>UAS*-Empty. Each dot represents an independent cross.

(C) Wing-specific overexpression of *dFrmd* and *FRMD5* Ref causes similar vein loss and blistery wing phenotypes. The defects are highlighted in red dashed circles.

(D) The heat shock assays for flies with $dFrmd^{CRIMIC\text{-}TG4}$ allele-driven expression of *FRMD5* cDNAs in the *dFrmd* LoF ($dFrmd^{CRIMIC\text{-}TG4}$/Df) background. The percentage of *dFrmd* LoF mutant flies with seizures was ~80% after exposure to a 42°C water bath for 30 s. The phenotype can be significantly rescued by human *FRMD5* Ref, but the tested *FRMD5* variants significantly reduced the rescue ability of *FRMD5* Ref. Flies were raised at 25°C and tested at 14–15 days. Each dot represents an independent test of 5–8 female flies.

For (B) and (D), total fly numbers are shown in the columns. Data are represented as mean + SEM. Unpaired t tests. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; n.s., no significance.

transmission. Further studies examining the precise biological mechanisms will lead to a better understanding of the disease pathogenesis.

Data and code availability

All reagents developed in this study are available upon request. Some of the variants were submitted to ClinVar (GenBank: NM_032892.5): c.1053C>G, SCV002564145.1; c.1054T>C, SCV002564146.1; c.1045A>C, SCV002564147.1; c.1637A>G, SCV002564148.1. The exome datasets supporting this study have not been deposited in a public repository due to privacy and ethical/legal issues.

Supplemental information

Supplemental information can be found online at https://doi.org/10.1016/j.ajhg.2022.09.005.

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Declaration of interests

The Department of Molecular and Human Genetics at Baylor College of Medicine receives revenue from clinical genetic testing completed at Baylor Genetics Laboratories. M.L. is a salaried employee and shareholder of Invitae Corp.

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Web resources

AlphaFold, https://alphafold.ebi.ac.uk/
CADD, https://cadd.gs.washington.edu/
DECIPHER, www.deciphergenomics.org
DGV, http://dgv.tcag.ca/dgv/app/home
DIOPT, https://www.flyrnai.org/diopt
gnomAD, http://gnomad.broadinstitute.org/
MARRVEL, http://www.marrvel.org/
MutScore, https://mutscore-wgt7hvakhq-ew.a.run.app/
OMIM, http://www.omim.org/
UCSF Chimera, https://www.rbvi.ucsf.edu/chimera/

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