

1 **Loss-of-function in *IRF2BPL* is associated with neurological phenotypes**

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52 neurodegeneration, C3HC4 Ring finger, EAP1

53 **Abstract**

54 The *Interferon Regulatory Factor 2 Binding Protein Like (IRF2BPL)* gene encodes a member of
55 the IRF2BP family of transcriptional regulators. Currently the biological function of this gene is
56 obscure, and the gene has not been associated with a Mendelian disease. Here we describe
57 seven individuals affected with neurological symptoms who carry damaging heterozygous
58 variants in *IRF2BPL*. Five cases carrying nonsense variants in *IRF2BPL* resulting in a
59 premature stop codon display severe neurodevelopmental regression, hypotonia, progressive
60 ataxia, seizures, and a lack of coordination. Two additional individuals, both with missense
61 variants, display global developmental delay and seizures and a relatively milder phenotype
62 than those with nonsense alleles. The bioinformatics signature for *IRF2BPL* based on
63 population genomics is consistent with a gene that is intolerant to variation. We show that the
64 *IRF2BPL* ortholog in the fruit fly, called *pits* (*protein interacting with Ttk69 and Sin3A*), is broadly
65 expressed including the nervous system. Complete loss of *pits* is lethal early in development,
66 whereas partial knock-down with RNA interference in neurons leads to neurodegeneration,
67 revealing requirement for this gene in proper neuronal function and maintenance. The
68 nonsense variants in *IRF2BPL* identified in patients behave as severe loss-of-function alleles in
69 this model organism, while ectopic expression of the missense variants leads to a range of
70 phenotypes. Taken together, *IRF2BPL* and *pits* are required in the nervous system in humans
71 and flies, and their loss leads to a range of neurological phenotypes in both species.

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75 **Introduction**

76 The etiology of neurodevelopmental disorders can vary and can include prenatal
77 exposures, maternal disease, multifactorial causes and single genes. *De novo* genomic
78 contributions were recently highlighted in a large cohort studied as part of the Deciphering
79 Developmental Disorders Study, with an estimate that 42% of the cohort carried pathogenic *de*
80 *novo* mutations in the coding region of genes.¹⁻³ The phenotypic and genomic heterogeneity of
81 neurodevelopmental disorders can pose a diagnostic challenge. Several known Mendelian
82 disorders such as Rett syndrome (MIM: 312750), the neuronal ceroid lipofuscinoses (NCLs),
83 and X-linked adrenoleukodystrophy (X-ALD, MIM: 300100) display neurodevelopmental
84 regression as a common element and illustrate the range of symptoms and pathologies
85 associated with neurological symptoms. The known or proposed mechanisms of disease can
86 include altered transcriptional control (Rett syndrome)^{4,5}, accumulation of a substrate with loss
87 of neurons (juvenile infantile NCL, MIM: 204200)^{6,7}, or inflammation and demyelination (X-
88 ALD).⁸⁻¹⁰ More recently, new genes associated with severe developmental phenotypes and
89 neurodegeneration have been discovered.^{11,12} This has been largely possible due to the use of
90 high-throughput sequencing methods such as next-generation sequencing (NGS), in
91 conjunction with sequencing databases for control cohorts such as ExAC and gnomAD¹³⁻²¹,
92 variant prediction²², model organism information (e.g. MARRVEL.org)²³ and crowdsourcing
93 programs to identify additional cases, such as GeneMatcher.org.²⁴ These tools have greatly
94 promoted gene discovery and assisted in ascertaining the role of the candidate variants for
95 disease. Programs such as the Undiagnosed Diseases Network (UDN)^{25,26}, promote multi-site
96 collaboration that combines NGS and functional data that facilitate the diagnosis of rare
97 disorders.

98 Here we describe a cohort of seven cases, ascertained for neurological symptoms, who
99 share predicted pathogenic variants in *IRF2BPL*, an intronless gene at 14q24.23.²⁷ The
100 transcript is expressed in many organs, including in the central nervous system (CNS)

101 components such as the cerebellum (GTex, accessed 1/29/2018).²⁸ The IRF2BPL protein and
102 its two mammalian paralogs, IRF2BP1 and IRF2BP2, share two highly conserved domains.
103 These include a coiled-coil DNA binding domain (IRF2BP zinc finger domain) at the amino-
104 terminus and a C3HC4-type RING finger domain at the carboxy-terminus. IRF2BPL also
105 contains polyglutamine and polyalanine tracts. In between the two conserved domains is a
106 variable region that contains a potential nuclear targeting signal.²⁷ IRF2BPL also contains
107 several putative PEST (proline, glutamic acid, serine, and threonine-rich) sequences throughout
108 the protein suggesting that the IRF2BPL protein is post-translationally regulated²⁹ (Figure 1A).

109 IRF2BPL has been proposed to have a role in the initiation of puberty in female non-
110 human primates and rodents.³⁰ It acts as a transcriptional activator for Gonadotropin Releasing
111 Hormone in the CNS.³⁰ Expression of *Irf2bpl* in the hypothalamus of female rats increases
112 during puberty and site-specific reduction of *Irf2bpl* in the preoptic area disrupts the estrus
113 cycle.³¹ In addition, IRF2BPL has also recently been proposed to function as an E3 ubiquitin
114 ligase that targets β -catenin for proteasome degradation in gastric cancer.³² Despite these
115 studies, the *in vivo* function of IRF2BPL in all species remains largely undefined. Here we
116 describe a novel role for *IRF2BPL* in the functional and structural maintenance of the nervous
117 system. We provide evidence in seven patients and use functional assays in fruit flies to
118 support the findings that these variants cause dramatic changes to IRF2BPL function and that
119 IRF2BPL plays a role in both development and neuronal maintenance.

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121 **Subjects and Methods**

122 *Demographics, ascertainment, and diagnoses (Table 1 and supplementary table 1).*

123 The individuals are all unrelated and between 2.5 and 43 years of age, including one patient
124 who died at 15 years of age. Four are male, six are Caucasian, and one identified as Hispanic.

125 They were evaluated by the genetics or neurology service as part of clinical care for

126 neurological symptoms (subjects 2, 4, 5, 6, 7) or as participants (subjects 1 and 3) in the UDN.²⁶

127 In non-UDN cases, use of the GeneMatcher website²⁴ facilitated ascertainment of two of the
128 subjects (6 and 7); the UDN page for *IRF2BPL* facilitated contact with subject 5, and subjects 2
129 and 4 were ascertained through communication with the clinical lab that performed whole
130 exome sequencing (WES). WES was performed as a trio in five subjects (1, 3, 5, 6, and 7)
131 and proband-only in two (2 and 4). WES was performed with written informed consent for
132 clinical sequencing and in accordance with institutional review board procedures for research
133 sequencing for all subjects. Consent for publication and images were obtained from all
134 guardians.

135 The seven cases reported here have a constellation of neurological findings of variable
136 severity. While almost all suffered from epilepsy [Human Phenotype Ontology³³ term (HP:
137 0001250)], those with nonsense variants in *IRF2BPL* had severe, progressive
138 neurodevelopmental regression (HP: 0002376), dysarthria (HP: 0001260), spasticity (HP:
139 0001257), and symptoms of movement disorders (HP: 0100022). There was also cerebral
140 volume loss in the two oldest cases (3 and 5; HP: 0002506), and cerebellar volume loss in case
141 5 (HP:0007360). In contrast, the two cases with missense variants have a generally milder
142 course, with symptoms of epilepsy, speech delay (HP: 0000750), and hypotonia (HP: 0009062).
143 Each proband is described below, along with additional details in Table 1 and Supplementary
144 Table 1.

145 Subject #1

146 This is a 7-year-old male who met early gross and fine developmental milestones in the
147 first two years of life (Supplementary Figure 1). He had a minor speech delay, as he had his
148 first word at 15 months of age. His development was normal until 2.5 years, when clumsiness
149 and excessive falling were noted. At the age of 4 years, the patient was unable to walk without
150 someone holding his hands or using a harness or a walker (Supplemental Movie 1). During an
151 evaluation at 7 years, the patient had lost the ability to walk, was not able to grasp objects, was
152 not able to feed himself and had no expressive language. He had progressive loss of gross and

153 fine motor function, speech and self-help skills. Additionally, he had a diagnosis of dystonia,
154 choreoathetosis, and variable spasticity that primarily affected his lower extremities.

155 Previous evaluations included a normal brain magnetic resonance imaging (MRI),
156 audiology, and electromyography (EMG) at 5 years of age. He developed esotropia at the age
157 of 4 years that progressively became of a larger angle. Ophthalmic examination at the age of 5
158 years was notable for visual acuity better than 20/70 in either eye with full fields to distraction.
159 There was evidence of large angle esotropia (higher than 65) and bilateral facial palsies with
160 ophthalmoplegia sparing the eyelids and the pupils. There was a severe horizontal gaze palsy
161 but vertical gaze was also limited. Saccades were decreased in both eyes. Funduscopy
162 examination was normal and he had a normal electroretinogram with normal optical coherence
163 tomography scan of the retina and the retinal nerve fiber layer (RNFL) (measured 85 μm on the
164 RNFL, which is normal for age).

165 While his neurodevelopmental course demonstrated significant developmental
166 regression, his cognition was tested with the Peabody Picture Vocabulary Test, Fourth Edition,
167 at 6 years of age. He scored in the average range for cognition, consistent with a previous
168 score on the Verbal Ability composite of the Differential Ability Scales- Second Edition,
169 administered when he was 3 years, 9 months of age. Although he did not have clinical seizures
170 at the time, an electroencephalogram (EEG) at 6 years of age showed diffuse slowing that was
171 consistent with generalized brain dysfunction and interictal discharges from the left occipital and
172 right temporal leads, indicating a possible area of epileptogenic potential. At almost 7 years of
173 age, he had an episode of abnormal movements resembling myoclonus and was diagnosed
174 with a seizure disorder, which was controlled with levetiracetam.

175 Prior biochemical, cytogenetic and molecular evaluations are summarized in
176 Supplementary Table 2. Reanalysis of previously obtained research WES data (trio) at
177 Columbia University identified heterozygosity for a *de novo* truncating variant in *IRF2BPL*,
178 NM_024496.3, c.514G>T (p.E172X) that was Sanger confirmed. Additionally, he had a *de*

179 *novo* missense variant downstream and in *cis* to the nonsense variant in *IRF2BPL*,
180 NM_024496.3, c.584G>T (p.G195V).

181 Subject #2

182 The patient is a female who met all of her early gross developmental and speech
183 milestones. At 5-6 years of age her family began to notice progressive gait disturbance. Her
184 initial diagnosis by the neurology service was dystonia, and she was given a trial of carbidopa-
185 levodopa (Sinemet) with no improvement. Symptoms progressed, and by 8 years of age she
186 had notable lower extremity spasticity with dysarthria, drooling, dysphagia, and incontinence.
187 Examination at 9 years of age demonstrated an interactive girl with intact cognition, decreased
188 speech fluency and severe dysarthria. Coordination exam demonstrated past pointing. Gait
189 was wide-based with spastic diplegia and scissoring. She had an intrathecal baclofen pump
190 placed at 9 years of age with no improvement. She had lost the ability to stand alone or walk by
191 10 years and was unable to use her hands or speak by 11 years. She had a dysconjugate gaze
192 at 11 years. MRI spine studies were initially normal but at 10 years demonstrated marked cord
193 thinning. MRI of the brain demonstrated mild cerebellar volume loss at 8 years. The
194 cerebellum was small and the corpus callosum was 'bulky'. Her respiratory function became
195 compromised and ventilation by continuous positive airway pressure was recommended at 10
196 years. A G-tube was placed at 10 years for dysphagia and aspiration. During her last formal
197 examination at 13 years of age, her cognition was difficult to assess, as she was no longer able
198 to communicate. All symptoms continued to progress until her family chose to limit her medical
199 interventions for feeding intolerance, and she died at 15 years of age. A nonsense variant in
200 *IRF2BPL* was detected on whole exome sequencing (WES), NM_024496.3, c.562C>T
201 (p.R188X), confirmed by Sanger sequencing.

202 Subject #3

203 This is a 20-year-old male who met gross and fine motor milestones early in life. Overall
204 development through the first several years was felt to be normal, although some balance

205 problems with walking may have occurred around 2 years. However, he started stumbling and
206 falling at school at approximately 5-6 years. He developed ataxia and began drooling. A
207 neurological evaluation at age 7 years found nystagmus, hyperactive reflexes, bilateral Babinski
208 signs, and dysmetria. Worse leg weakness and more falls were reported at 8 years. By age 10
209 his symptoms had progressively worsened, and he had begun experiencing 1-3 myoclonic jerks
210 daily, feeding difficulties, facial weakness, and had lost his speech and ability to walk. By age
211 13 he was G-tube dependent (from worsening dysphagia) and had slowed saccadic eye
212 movements and severe contractures of the hands and feet. He could not hold objects. He is
213 wheelchair dependent with extremely limited range of motion of all joints. Spastic quadriplegia is
214 managed with a baclofen pump. He has a roving gaze and no visual attention.

215 Previous evaluations have included MRI of the brain that was normal at age 7 years,
216 but had diffuse cerebral atrophy with *ex vacuo* dilatation of the lateral ventricles but no
217 evidence of white matter disease at 13 years. At 20 years of age, brain MRI demonstrated
218 severe cerebral volume loss of the bilateral hemispheres, patchy periventricular subcortical
219 white matter signal hyperintensity on fluid-attenuated inversion recovery, and marked thinning
220 of the corpus callosum (Figure 2A). Neuropsychological evaluation with the Vineland
221 Adaptive Behavior Scales (3rd edition) at age 20 years indicated continued global and
222 significant delays across all aspects of the patient's functioning. A *de novo* nonsense variant
223 in *IRF2BPL* was identified on WES, NM_024496.3, c.562C>T (p.R188X) and Sanger
224 confirmed.

225 Subject #4

226 This is a 16-year-old female with global developmental, hypotonia, and speech delay.
227 She had prenatal exposure to alcohol and controlled substances. She was diagnosed with
228 subclinical epilepsy, due to staring episodes and abnormal EEGs, wide-based gait with high-
229 arched feet at 6 years. Cognitive testing was completed at this age (Bracken Basics
230 Concepts III Receptive and School Readiness Composite; Conner's Parent Rating Scale;

231 Child Behavior Checklist) and resulted in a diagnosis of ADHD and intellectual functioning at
232 approximately the 1-3 year old level. Since 12 years old, there has been a decline in gross
233 and fine motor abilities, speech and self-help skills with a recent diagnosis of catatonia and
234 lower extremity dystonia in the past year. This was initially attributed to sexual abuse, but has
235 been persistent since she was removed from the abusive environment. She had a normal
236 brain MRI at 6 and 13 years of age. At 15, a thin corpus callosum, still within normal limits,
237 was observed on brain MRI. A swallow study at 15 years of age revealed silent aspiration
238 with all tested consistencies. She now is gastrostomy tube dependent, and requires
239 assistance with all activities of daily living. She is largely wheelchair dependent, though able
240 to walk with assistance for short periods. She has progressive macular degeneration that
241 was stable upon her latest examination at 15 years.

242 Biochemical and cytogenetic testing were completed and are summarized in
243 Supplementary Table 2. She had exome sequencing in 2016 that revealed a mutation in
244 *BEST1* that explains her retinal symptoms (Macular dystrophy, vitelliform, 2, VMD2 [MIM:
245 153700]). A truncating variant in *IRF2BPL*, NM_024496.3, c.379C>T (p.Q127X) was also
246 reported and was Sanger confirmed.

247 Subject #5

248 This is a 43-year-old male who had psychomotor retardation with hypotonia. He had
249 febrile seizures between 2 and 4 years of age, was diagnosed with epilepsy at 10 years, and
250 developed refractory myoclonic epilepsy by 12 years of age. His neurodevelopmental course
251 showed regression, and at the age of 15 years he had ataxia, spastic rigidity, dystonia and
252 dyskinesia, and a diagnosis of spastic-athetoid cerebral palsy. Progressive cognitive
253 deterioration was noticed, and communication became difficult. At 28 years, he could barely
254 walk independently and could not feed himself. Together with the refractory seizures, the
255 progressive motor problems quickly led him to be wheelchair-bound. At 29 years, he had a
256 vagal nerve stimulator placed to control his seizures, and an intrathecal baclofen pump was

257 placed at 30 years to treat his spasticity. Dystonic attacks involving the axial and
258 appendicular musculature became more frequent. He was treated for recurrent aspiration
259 pneumonia and spontaneous pneumothorax. At the age of 35, his epilepsy was under
260 control, but his dystonia became generalized (limbs, axial, facial with tongue protrusion) and
261 worsened gradually, leading to a completely bedridden state. Treatment with botulin toxin
262 gave only limited benefit. At 42 he had a right-sided pallidotomy, also with limited and
263 transient beneficial effect. Brain MRI at 34 showed global atrophy (cerebral, cerebellar and
264 brainstem), thinning of corpus callosum, and no white matter or cortical lesions (Figure 2B).
265 MR-spectroscopy showed normal findings.

266 Biochemical, cytogenetic and molecular analyses are summarized in Supplementary
267 Table 2. Trio WES at Ghent University Hospital revealed a *de novo* heterozygous nonsense
268 mutation (NM_024496.3, c.376C>T (p.Q126X)) in *IRF2BPL*, resulting in a premature stop
269 codon, which was Sanger confirmed.

270 Subject #6

271 This is an 11-year-old male with gross motor and speech delays. He rolled over at 14
272 months and walked at 21 months. At approximately 14 months he received a diagnosis of
273 generalized myoclonic epilepsy that was difficult to control. He had complete regression of
274 speech at 2 years and remains nonverbal, though his receptive language skills appear to be
275 appropriate. He was diagnosed with autism spectrum disorder (ASD) at 3.5 years. He has not
276 had additional regression in developmental skills.

277 Evaluations are summarized in Supplementary Table 2 and include a normal brain MRI
278 at 7 years of age. Trio WES detected a *de novo* missense variant in the candidate gene
279 *IRF2BPL*, NM_024496.3, c.1115C>G (p.P372R) that was confirmed by Sanger sequencing.

280 Subject #7

281 The patient is a female who was diagnosed with infantile spasms at age 6 months.
282 Seizures subsided with combination antiepileptics at approximately 9 months. Anti-seizure

283 medications were discontinued thereafter with no recurrence of episodes. Brain MRI and MR
284 spectroscopy were both normal. She met gross motor milestones early in life, though on last
285 assessment at 17 months, she continued to be non-verbal and hypotonic. Providers noted
286 deceleration of developmental progress with onset of infantile spasms at 6 months of age,
287 though the patient has not had frank regression. Facial examination revealed subtle
288 dysmorphic facial features including epicanthal folds, mild telecanthus, and almond-shaped
289 eyes with round facies. Developmental assessment using the Bayley Scales of Infant and
290 Toddler Development, 3rd edition (Bayley-3) at 15 months revealed cognitive, expressive and
291 receptive language, and fine and gross motor functioning below levels expected for age (<
292 1st percentile). There was no family history of seizures or developmental delay. A
293 chromosomal microarray revealed a 400 kb interstitial duplication at 7q31.31 of uncertain
294 clinical significance. Additional tests are listed in Supplementary Table 2. A *de novo*
295 missense variant was detected on *IRF2BPL*, NM_024496.3, c.1254G>C, (p.K418N) on trio
296 WES on 42/87 of the reads.

297

298 **Methods**

299 Exome sequencing: Subjects had WES performed on a clinical or research basis. Exome data
300 are summarized in Supplementary Table 3. Across the performing labs, the minimum average
301 depth of coverage was 100X across assays, and minimum proportion of the target at >10X
302 coverage was 95%.

303

304 Exome reanalysis (subject 1): FASTQ files were obtained from the relevant source with parental
305 consent. Alignment and variant calling have been previously described.³⁴ Novel genotypes
306 were filtered for quality and control observations in public database controls. We highlighted
307 variants in known OMIM genes or mouse essential genes, loss-of-function (LoF) variants that
308 are in genes with known pathogenic LoF variants or reported as haploinsufficient by ClinGen³⁵,

309 and LoF intolerant by high probability of LoF intolerance (pLI) score (>0.9).²⁰ Conservation of
310 the variant site is reported with the Genomic Evolutionary Rate Profiling (GERP) score.³⁶
311
312 Generation of fly stocks: All fly strains used in this study were generated in house or obtained
313 from the Bloomington Drosophila Stock Center (BDSC) and cultured at room temperature
314 unless otherwise noted. The *pits*^{MI02926-TG4.1} allele was generated by genetic conversion of the
315 MiMIC^{37,38} (Minos Mediated Integration Cassette) insertion line, *y*¹ *w*^{*} *Mi{MIC}CG11138*^{MI02926} via
316 recombination-mediated cassette exchange (RMCE) as described³⁹⁻⁴¹. The recessive lethality
317 associated with the *pits*^{MI02926-TG4.1} allele was rescued using an 80 Kb P[acman] duplication
318 (*w*[1118]; *Dp*(1;3)DC256, *PBac*{*y*+*mDint2*} *w*[+*mC*]=DC256}VK33)⁴² as well as by a 20 kb
319 genomic rescue construct (see below). Expression pattern of *pits* was determined by crossing
320 *pits*^{MI02926-TG4.1} to *UAS-mCD8-eGFP* (BDSC_32184). In addition, the
321 *y*¹ *w*^{*} *Mi{MIC}CG11138*^{MI02926} line was converted to a protein trap line (*pits*::GFP) by injection of
322 a construct that could either produce a splice acceptor (SA)-T2A-GAL4-polyA mutant allele or a
323 SA-eGFP-splice donor (SD) protein trap allele depending on the inserted direction. The
324 successful generation of the *pits*::GFP allele was confirmed by both PCR of genomic DNA and
325 anti-GFP immunohistochemistry/Western blotting of fly tissue.

326 All transgenic constructs were generated by Gateway (Thermo Fisher) cloning into the
327 pUASg-HA.attB plasmid.⁴³ The human *IRF2BPL* cDNA clone³⁰ was made to match the
328 NM_024496.3 transcript. Flanking Gateway *attB* sites were added by PCR of the template
329 cDNA clone, and then shuttled to the pDONR223 by BP clonase II (Thermo Fisher). Variants
330 were generated by Q5 site directed mutagenesis (NEB), fully sequenced (Sanger) and finally
331 cloned into pUASg-HA.attB via LR clonase II (Thermo Fisher). All expression constructs were
332 inserted into the VK37 (*PBac*{*y*+*attP*}VK00037) docking site by ϕ C31 mediated
333 transgenesis.⁴⁴ The 20Kb genomic rescue (GR) line was generated by inserting the P[acman]
334 clone CH322-141N09 (BACPAC Resources)⁴⁵ into the VK37 docking site. The *UAS-pits-RC*

335 flies were generated by obtaining the *pits* cDNA (RE41430, RC isoform) clone from the
336 Drosophila Genomics Resource Center (DGRC) and performing Gateway (Thermo Fisher)
337 cloning into pUASg-HA.attB. The *pits* RNAi line ($P\{KK108903\}VIE-260B$) was obtained from
338 Vienna Drosophila Resource Center (VDRC). The GAL4 lines used in this study from BDSC
339 are: $nSyb-GAL4$ ($y^1 w^+$; $P\{w^{+m}=nSyb-GAL4.S\}3$) BDSC_51635, $Rh1-GAL4$ ($P\{ry^{+7.2}=rh1-$
340 $GAL4\}3$, ry^{506}) BDSC_8691, $Act-GAL4/CyO$ ($y^1 w^+$; $P\{w^{+mC}=Act5C-GAL4\}25FO1/CyO$, y^+)
341 BDSC_4414.

342

343 *Drosophila* behavioral assays: To perform the bang sensitivity assay⁴⁶, flies were anesthetized
344 no sooner than 24 hours prior to testing with CO₂ and housed individually. At the time of testing,
345 flies were transferred to a clean vial without food and vortexed at maximum speed for 15
346 seconds. The time required for flies to recover in an upright position was measured. Typically
347 25 flies were tested per data point. The climbing assay⁴⁷ was performed in a similar manner but
348 at the time of testing flies were transferred to a fresh vial and given 1 minute to habituate before
349 tapped to the bottom of the vial three times and examined for negative geotaxis (climbing)
350 response to reach the 7 cm mark on the vial. Flies were given a maximum of 30 seconds to
351 reach the top.

352

353 Histological and ultrastructural analysis of the fly retina: 45-day-old flies were dissected in ice
354 cold fixative (2% PFA / 2.5% glutaraldehyde / 0.1 M sodium cacodylate). Heads were fixed,
355 dehydrated and embedded in Embed-812 resin (Electron Microscopy Sciences) as described
356 previously.⁴⁸ For histological sections (200 nm thick) were stained with toluidine blue and
357 imaged with a Zeiss microscope (Axio Imager-Z2) equipped with an AxioCam MRm digital
358 camera. For transmission electron microscopy (TEM), sections (50 nm thick) were stained with
359 1% uranyl acetate and 2.5% lead citrate. TEM images were obtained using a transmission

360 electron microscope (model 1010, JEOL). Images were processed with Photoshop (Adobe) and
361 45 day old toluidine blue images were color matched to the 5 day old images for clarity.

362

363 Overexpression of human *IRF2BPL* in *Drosophila*: We overexpressed reference and variant
364 *IRF2BPL* cDNAs in flies by crossing the *UAS-IRF2BPL* males to virgin female flies from a
365 ubiquitous (*Act-GAL4*) driver stock. The progeny of these crosses were cultured at various
366 temperatures (18°C, 22°C, 25°C, and 29°C) to express the human proteins at different levels
367 (lowest expression at 18°C and highest expression at 29°C).^{38,49} Expected Mendelian ratios of
368 flies was assessed. For temperatures $\geq 22^\circ\text{C}$ over 100 flies were assessed for each cross. For
369 18°C crosses, over 50 flies were assessed from each group.

370 For additional methods please see supplemental material.

371

372 **Results**

373 **Summary of Clinical Findings**

374 The subjects described here are between 2.5 and 43 years of age, including one who
375 died at 15 years of age. Comprehensive clinical information is included in Supplementary Table
376 1 and Supplementary Table 2.

377 All seven individuals have monoallelic variants in *IRF2BPL* (Table 1 and Supplementary
378 Table 1). The nonsense variants identified are: p.Q126X, p.Q127X, p.E172X, and p.R188X
379 (found in 2 individuals). There was remarkable similarity in the clinical course of the five cases
380 that had nonsense variants. These include an unremarkable course in their initial development,
381 followed by loss of developmental milestones and development of a seizure disorder at a
382 variable age, with 2 years being the youngest. Additional findings included a movement disorder
383 with dystonia and choreoathetosis, and cerebellar signs such as ataxia, dysarthria, dysmetria
384 and dysdiadochokinesia. Three of the probands had had anomalies of eye movements but had
385 normal retinæ and optic nerves. Head circumference appeared to remain appropriate for age

386 in those with available measurements. Brain MRI was normal early in life, but cerebral and
387 cerebellar atrophy was noted in the two oldest cases. Cognitive status was difficult to ascertain,
388 particularly as verbal skills were lost. However, cognitive abilities appeared to remain intact over
389 a short follow up period in one case and to deteriorate in an older individual. One case was
390 deceased at 15 years of age when the family elected to limit care after continued decline. The
391 variants were *de novo* in all for whom parental testing was available (three out of five cases).

392 Missense variants (p.P372R and p.K418N) were also reported in *IRF2BPL* for two
393 individuals who had a variable phenotype of global developmental delay and seizures. The
394 older case (subject 6) had a diagnosis of ASD. Despite the frequent finding of seizures,
395 antiepileptic drugs were successfully weaned in subject 7, without recurrence over a short
396 follow up period. In both missense cases, the variants were *de novo*. Neither case has
397 developed a progressive loss of milestones, abnormal eye movements, or a movement disorder
398 at 2 or 10 years of age.

399

400 ***IRF2BPL* de novo variants are deleterious based on bioinformatics data**

401 *IRF2BPL* is a gene that is highly intolerant to variation with an Residual Variation
402 Intolerance Score (RVIS)⁵⁰ of 9.3% and a pLI score of 0.97, with no observed LoF variants
403 included in the calculation.²⁰ The only LoF variants present in ExAC and gnomAD are
404 frameshifts, which either do not pass quality filtering or appear to be artifactual calls in repetitive
405 regions using the browser -visualization tool.²⁰ *IRF2BPL* is also constrained to missense
406 variants ($z = 4.73$).²⁰ All variants found in the subjects were absent from the gnomAD and ExAC
407 databases. The Combined Annotation Dependent Depletion (CADD)⁵¹ score for all of the
408 variants was > 34 , which places them among the variants predicted to be most deleterious. The
409 four nonsense variants introduce a premature termination codon either downstream (p.E172,
410 p.R188) or at the end (p.Q126, p.Q127) of the poly-glutamine tract and upstream of the first
411 PEST sequence (Figure 1A), whereas the missense variants are within the variable region of

412 the protein. We used the DOMINO tool to assess the likelihood of monoallelic variants in
413 *IRF2BPL* to cause a Mendelian disease. *IRF2BPL* had a DOMINO score of 0.962 out of 1
414 predicting that monoallelic variants would very likely cause disease (Figure 1B).⁵²

415

416 ***IRF2BPL* variants are severe loss-of-function mutations based on functional assays in** 417 **flies**

418 To validate the functional consequences of these variants, we utilized *Drosophila*
419 *melanogaster* as a model organism. Experiments in fruit flies have previously provided
420 experimental support in identifying causal variants for human disease^{19,53-55} and these
421 approaches have been an integral part of the UDN.^{13,14,16,21,56} The fly ortholog of *IRF2BPL* is a
422 poorly characterized gene, *CG11138*. This gene was studied in the context of epigenetic
423 regulation through biochemical methodologies during embryogenesis, and was named *pits*
424 (*protein interacting with Ttk69 and Sin3A*).⁵⁷ Although the overall identity (30%) and similarity
425 (36%) between *IRF2BPL* and *Pits* may not seem high, the architecture of the protein is very
426 similar and the sequences of the annotated domains show high conservation (79% identity for
427 the zinc finger domain, 76% identity for the C3HC4 RING domain) (Figure 3A). The *Pits* protein
428 also has a DIOPT⁵⁸ score of 12/15 suggesting that it is likely to be a true ortholog of *IRF2BPL*.
429 Two other human paralogs of *IRF2BPL* share a similar high DIOPT score (12/15 for *IRF2BP1*;
430 11/15 for *IRF2BP2*). These data indicate that *pits* is the sole fly gene that is orthologous to the
431 three *IRF2BP* family genes in humans.

432 To generate a *pits* mutant fly, we used a MiMIC insertion in an intron of *pits*, named
433 *Mi{MIC}CG11138^{Mi02926}* (Figure 3B).^{37,38} MiMICs are engineered transposable elements that
434 contain inverted *attP* sites derived from phage Φ C31 flanking a swappable cassette. This
435 allows replacement of the content of the *MiMIC* insertion using Recombination-Mediated
436 Cassette Exchange (RMCE) by expressing the Φ C31 integrase to swap the *MiMIC* cassette
437 with a *SA-T2A-GAL4-polyA* (T2A-GAL4) cassette.³⁹⁻⁴¹ This insertion results in a truncated *pits*

438 transcript due to the polyA signal. During translation this short transcript produces a short
439 protein that is truncated at the T2A site yet allows re-initiation of translation to produce the
440 GAL4 protein. The GAL4 protein is expressed in a proper spatial and temporal fashion, i.e.
441 those of the endogenous gene³⁸ (Figure 3B). This allows rescue of the *T2A-GAL4* induced
442 allele, typically a null allele, with a UAS-(fly)cDNA for about 70% of the genes tested.⁴⁰

443 The *pits* gene is on the X chromosome, and *pits*^{MI02926-TG4.1} males are hemizygous lethal,
444 as they fail to survive past the first instar larval stage, and most die as embryos. Lethality can
445 be rescued to viable adults by introduction of an 80 Kb or a 20 Kb P[acman] genomic BAC
446 rescue (GR) construct, the latter only carrying the *pits* gene^{42,44} (Figure 3C and 3D). Hence, *pits*
447 is an essential gene, and expression of the gene in the proper genomic context fully rescues the
448 LoF of *pits*.

449 We attempted to rescue lethality observed in *pits*^{MI02926-TG4.1} flies by overexpression of
450 UAS-*pits* or UAS-*IRF2BPL* and failed to obtain viable flies. Intriguingly, we were also unable to
451 obtain viable heterozygous female flies that contain both the *pits*^{MI02926-TG4.1} allele and UAS-*pits*
452 or UAS-*IRF2BP* (Figure 3D). These data show that overexpression of the fly or human
453 IRF2BPL in the cells that endogenously express Pits is toxic to the fly. Indeed, expression of
454 the UAS-*IRF2BPL* under the control of a ubiquitous driver (*Act-GAL4*) also causes lethality
455 (Figure 3E).

456 The above data show that the lethality caused by overexpression of the reference
457 *IRF2BPL* gene can be used as a functional assay to test if the variants are functional (toxic) as
458 well. To modulate the levels of expression we ubiquitously expressed the variants with *Act-*
459 *GAL4* as the UAS-cDNA expressed using this driver exhibits temperature dependence, with
460 significantly greater expression at higher temperatures.^{38,49} We conducted these experiments at
461 18°C, 22°C, 25°C and 29°C. Expression of the reference *IRF2BPL* cDNA consistently causes
462 lethality at all temperatures tested, whereas the three nonsense variants (p.E172X, p.Q127X,
463 and p.R188X) consistently produced viable animals at all temperatures tested (Figure 3E). This

464 provides evidence that the truncated proteins are not toxic and are very likely LoF alleles.
465 Interestingly, the missense variant p.K418N is lethal when expressed at higher temperatures,
466 whereas the flies are viable at lower temperatures, indicating that it retains some toxic function
467 and hence behaves as a partial LoF allele. The p.P372R variant still remained lethal upon
468 ubiquitous expression, even at lower temperatures indicating its toxic function is similar to the
469 reference protein at least in this assay. We confirmed that the UAS-driven human cDNA in flies
470 expresses IRF2BPL at relatively similar levels by making HA-tagged constructs for the
471 reference and p.E172X truncation and performing Western blot using an anti-HA antibody, as
472 commercially available antibodies for IRF2BPL recognize epitopes downstream of the
473 premature termination codon, in the C-terminus of IRF2BPL. UAS expression of these
474 constructs in neurons by *nSyb* (neuronal Synaptobrevin)-*GAL4* was relatively similar
475 (Supplemental Figure 2A). Additionally, we confirmed that the untagged reference and two
476 missense variants were expressed at similar levels using a commercial antibody against
477 IRF2BPL and performing Western blot, suggesting that the reduced toxicity of p.K418N is not
478 due to destabilization of the protein (Supplemental Figure 2B).

479

480 ***pits* is expressed and required in the CNS**

481 To examine the endogenous levels of *pits* as well as its subcellular localization, we
482 generated a GFP protein trap line of *pits*. We integrated a protein trap (splice acceptor (SA)-
483 linker-eGFP-linker-splice donor (SD)) cassette into the *pits*^{M102926} via RMCE. Although the SA-
484 eGFP-SD functions as an artificial exon in the middle of the *pits* gene (Supplemental Figure
485 3A), *pits::GFP* flies are homozygous viable and do not display any obvious phenotype indicating
486 that the internal GFP tag is not deleterious. This is in concert with our previous findings that
487 most (75%) proteins tolerate the presence of an internal GFP incorporated by the SA-eGFP-SD
488 MiMIC insertions.³⁸ The GFP fusion proteins also reflect the localization of the endogenous
489 proteins.^{37,38} We first confirmed the presence of a single protein of the appropriate size on SDS-

490 PAGE in males (Figure 4A). As shown in Figure 4B and 4C, the tagged protein is widely
491 expressed in the brain. In the third instar larval brain the protein is widely expressed and is
492 enriched in the mushroom body (MB, Figure 4B, yellow arrow). In the adult brain Pits::GFP is
493 localized in most neurons, including the cell bodies and nuclei (co-staining with a pan-neuronal
494 nuclear marker, Elav) of many neurons as well as their axons (Figure 4C). This is in agreement
495 with a previous study showing *pits* is expressed in the nucleus.⁵⁹ Pits::GFP does not show
496 expression in the glia of the adult CNS upon pan-glial staining with Repo (Supplemental Figure
497 3B). Additionally, we also determined the expression of *pits* using the GAL4 reporter from
498 *pits*^{MI02926-TG4.1} allele crossed to a UAS-membrane bound GFP (Supplemental Figure 3C) which
499 allow detection of cells that express the gene of interest at low levels⁴⁰ We also generated z-
500 stacks confocal image movies of *pits*::GFP co-stained with Elav (Supplemental Movie 2) and
501 Repo (Supplemental Movie 3). These data reveal that *pits* is highly expressed in the MB, the
502 learning and memory center of the flies⁶⁰ as well as in the antennal mechanosensory and motor
503 center, which are required for balance and hearing and motor coordination, a cerebellar insect
504 equivalent.⁶¹ Again, no expression was observed in glia from these analyses.

505 Due to the lethality observed in *pits*^{MI02926-TG4.1} flies, we determined the function of *pits* in
506 the brain through gene knock-down studies using RNA interference (RNAi).⁶² Ubiquitous
507 knock-down of *pits* using *Act-GAL4* resulted in semi-lethality (Figure 5A). This RNAi has
508 specificity to *pits* as it consistently reduces the endogenous Pits protein level to ~50% of control
509 RNAi when assessed via Western blot (Figure 5B). To determine if partial knock-down of *pits*
510 results in neurological defects, we expressed the *pits* RNAi with the *nSyb-GAL4* driver. Young
511 flies (~5 days post-eclosion) displayed normal climbing (Figure 5C) and mechanical stress
512 tolerance (bang sensitivity) (Figure 5D). However, aged flies that were 30 and 45 days post-
513 eclosion displayed progressive abnormalities in climbing, and became bang sensitive at 30
514 days post-eclosion when compared to controls (Figure 5C and 5D).

515 To determine if an age-dependent deterioration in neural morphology can be observed,
516 we reduced *pits* expression in photoreceptors of the fly eye using rhodopsin (*Rh1*)-*GAL4*. The
517 fly retina is a well-characterized system to explore neurodegeneration in *Drosophila* due to the
518 highly stereotypical organization of neurons (photoreceptors) and glia cells (pigment cells, cone
519 cells) in this tissue^{19,63}. Indeed, *Pits::GFP* shows a robust nuclear signal in the photoreceptors
520 of the fly retina when co-stained with *Elav* (Supplemental Figure 4). Flies were raised in a 12 hr
521 light/dark cycle for 45 days, and histological examination of retina sections stained with toluidine
522 blue was performed. We observed a severe disorganization of the ommatidia (units of
523 photoreceptors) and notable rhabdomere (light-sensing organelle) loss (Figure 6A). This loss
524 was not observed in young flies (5 days post-eclosion), nor in aged control RNAi flies. To
525 examine ultrastructure, we performed transmission electron microscopy (TEM) of the same
526 tissue and observed numerous abnormalities in the ultrastructure of the *Rh1-GAL4>pits-RNAi*
527 retina compared to age matched *Rh1-GAL4>control-RNAi* eyes (Figure 6B and 6C,
528 Supplemental Figure 5 and Supplemental Figure 6): 1) a significant decrease in intact
529 rhabdomeres (Figure 6D); 2) a significant increase in the presence of tubulovesicular like
530 structures (TVS), associated with some neurodegenerative models⁶⁴ (Figure 6C, red arrow);
531 and 3) the abnormal presence of neuronal lipid droplets in photoreceptors (Figure 6C, yellow
532 arrow, Supplemental Figure 7A), often associated with mutants that cause high reactive oxygen
533 species (ROS) accumulation.⁶⁵ However, we did not detect obvious changes in mitochondrial
534 morphology or the number of mitochondria per photoreceptor (Supplemental Figure 7B). In
535 summary, *pits* is required for proper maintenance of neuronal function and structure in flies.

536

537 **Discussion**

538 We present seven subjects with rare heterozygous variants in *IRF2BPL*, a gene that has
539 not previously been associated with disease in humans. The cases with nonsense variants
540 exhibited a remarkably similar, progressive course of neurological regression that eventually led

541 to severe disability. In contrast, the missense variants observed in two subjects are associated
542 with milder neurological symptoms such as seizures, developmental delay, and ASD.
543 Interestingly, the nonsense variants in this cohort cluster in or just downstream of the
544 polyglutamine tract within the variable region, while the missense variants map further
545 downstream (Figure 1A). The bioinformatics signature of the gene suggests that *IRF2BPL* is
546 highly intolerant to variation, and that the variants reported in the cohort are among the most
547 deleterious. The DOMINO score was suggestive of dominant inheritance. RNA sequencing
548 from a blood sample for subject 1 (c.514G>T (p.E172X) revealed expression of the transcript
549 (data not shown). The lack of nonsense-mediated decay of *IRF2BPL* transcript with a nonsense
550 mutation is consistent with the fact that this is a single exon gene.⁶⁶ Western blotting on subject
551 samples with nonsense variants could not be pursued due to lack of a commercially-available
552 antibody that recognizes an epitope upstream of the premature truncation.

553 To date, a role for *IRF2BPL* in humans is limited to an association with developmental
554 phenotypes. For example, *IRF2BPL* has been identified in the top 1,000 genes that are
555 significantly lacking in functional coding variation in non-ASD samples and are enriched for *de*
556 *novo* LoF mutations identified in ASD cases.⁶⁷ Other large scale sequencing studies have
557 identified *de novo* variants in *IRF2BPL* in ASD (2 individuals) and major developmental
558 disorders (2 individuals).^{1,3} Intriguingly the ASD variants include a missense in the conserved
559 DNA binding domain (p.F30L) and a frameshift near the end of the protein (p.A701fs*65). The
560 developmental disorder cohort also has a missense (p.R391C) and frameshift that similarly
561 terminates near the end of the protein (p.L713Pfs*54).^{1,3} The missense variants in these cases
562 compared to our two cases indicate that the full spectrum of *IRF2BPL* related phenotypes is still
563 developing. The p.A701fs*65 frameshift, in particular, suggests that truncations near the end of
564 the protein may cause variable phenotypes. Another possibility is the presence of mosaicism in
565 these large cohort studies. Our findings offer additional evidence that variants in *IRF2BPL* are
566 implicated in neurological symptoms, and additionally extend the phenotype into neurological

567 regression. Furthermore, our model organism experiments using fruit flies supports an
568 important role for *IRF2BPL* in embryologic development as well as neuronal maintenance.
569 *IRF2BPL* is well-conserved and the fly gene, *pits*, is expressed widely in the nervous system
570 during development and in adulthood. The *pits*^{MI02926-TG4.1} LoF mutant fails to survive past early
571 larval stages and mostly die as embryos. Although we were not able to rescue the *pits*^{MI02926-}
572 ^{TG4.1} allele with either the human or fly cDNA, the *pits*^{MI02926-TG4} is rescued with a genomic rescue
573 construct specific to *pits*, showing that this chromosome does not carry other lethal or second
574 site mutations. Interestingly, ubiquitous overexpression of *IRF2BPL* or *pits* in flies is toxic,
575 suggesting that the gene is highly dosage sensitive. Many genes implicated in
576 neurodegenerative disorders cause overexpression phenotypes in *Drosophila*.^{68,69} In our
577 experiments, however, overexpression of the nonsense variants was not toxic. These results
578 suggest that the mechanism of disease may be through loss of protein function and
579 haploinsufficiency of *IRF2BPL*. Overexpression of the missense variants showed a range of
580 effects. While overexpression of p.K418N was only lethal at higher temperatures (i.e., higher
581 levels of expression), p.P372R was lethal at any temperature. These results suggest that some
582 of the proteins expressed with the missense variants retained a function that was toxic when
583 overexpressed, or perhaps suggest a gain of function mechanism. Understanding the precise
584 molecular function of Pits/IRF2BPL will allow one to design experiments to test this possibility.
585 In summary, either excess or loss of *pits* or *IRF2BPL* is highly detrimental to survival. Given that
586 expression at low levels (*Act-GAL4* at 18°C) is still toxic suggests that the level of expression of
587 the gene product is highly regulated *in vivo* through mechanisms such as ubiquitination through
588 the PEST domain.

589 There are compelling parallels in phenotypes between a partial Pits reduction in flies
590 and symptoms observed in patients, which may suggest evolutionary conservation in neuronal
591 mechanisms. 1) Almost all patients had seizures or EEG abnormalities, and a reduction of Pits
592 in neurons leads to a bang-sensitive phenotype in flies. Bang-sensitivity is associated with

593 seizure-like paralysis that has phenotypic as well as genetic parallels with human epilepsy.^{70,71}
594 2) The five patients carrying nonsense variants in *IRF2BPL* displayed progressive motor
595 dysfunction that manifested after early childhood. Similarly, neuronal reduction of Pits in flies
596 caused a progressive decline in climbing ability that was not observed in young flies. 3) The two
597 oldest patients, who had nonsense variants, had evidence of cerebral atrophy in adulthood.
598 Correspondingly, we found that a reduction in pits expression in photoreceptors leads to a slow
599 and age-dependent loss of neuronal integrity. 4) The cerebellar symptoms and cerebellar
600 atrophy may correspond to a requirement for Pits expression in the antennal mechanosensory
601 and motor center. These neurons are required for balance, auditory and motor coordination, a
602 cerebellar insect equivalent⁶¹ and Pits is abundantly expressed in these cells. These results
603 support the notion that *IRF2BPL* / Pits have fundamental roles in central nervous system
604 development and maintenance.

605 *IRF2BPL* has similarity to its mammalian paralogs *IRF2BP1* and *IRF2BP2* and has been
606 shown to interact with *IRF2* (Interferon Regulatory Factor 2).^{72,73} However, flies lack an obvious
607 homolog to *IRF2*, indicating that *pits* and *IRF2BPL* may have additional conserved functions
608 that are currently unknown. Recently, a study in *Drosophila* indicated that the fly homologue,
609 Pits, regulates transcription during early embryogenesis by interacting with a histone
610 deacetylase Sin3A (*SIN3A* and *SIN3B* in human) and a co-repressor Tramtrack 69 (no
611 identified human ortholog).⁵⁷ Consistent with previous studies in flies and rats, our data suggest
612 both Pits and *IRF2BPL* are found predominantly in the nucleus.^{59,74} We showed robust
613 expression in the nucleus of photoreceptors and numerous neurons, but also observed
614 Pits::GFP in axons and cell bodies, and little to no staining in the dendrites of the mushroom
615 body. This could indicate that Pits may have a non-nuclear role in specific subcellular
616 compartments in neurons. Finally, through neuron-specific knock-down experiments in flies, we
617 demonstrate that *pits* is important for function and/or neuronal maintenance over time.

618 We observed different phenotypes within the cohort based on the type of variant. All four
619 nonsense variants truncate the IRF2BPL protein upstream of the putative nuclear localization
620 signal, the conserved C' terminal RING domain, and multiple putative PEST sequences.
621 Although little is known about IRF2BPL/Pits, most studies implicate its expression and function
622 in the nucleus.^{30,57,59,74,75} However, the RING domain of IRF2BPL has recently been shown to
623 act as an E3-ligase ubiquitinating β -catenin and suppressing Wnt signalling in gastric cancer.³² It
624 is currently unclear whether this pathway could be altered in IRF2BPL-associated disease.
625 Finally, the predicted PEST sequences suggest that IRF2BPL is highly regulated and support
626 our data that overexpression of the protein can be detrimental. Toxicity was observed by
627 overexpression of fly or human cDNA constructs in all cells (*Act-GAL4*) or within the cell-types
628 in which pits is endogenously expressed (*pits*^{*MI02926-TG4.1*}). While *IRF2BPL* ubiquitous expression
629 or overexpression within the cell types that pits is endogenously expressed caused lethality, we
630 did not observe lethality or any remarkable phenotypes by overexpression of IRF2BPL or the
631 variants when expressed specifically in neurons (*nSyb-GAL4*) (data not shown). Therefore,
632 increased Pits/IRF2BPL protein expression may be detrimental to only certain cells. Our
633 functional assays of nonsense and missense variants support haploinsufficiency and tight
634 control of protein expression and/or turn-over. Future studies will determine IRF2BPL targets
635 for ubiquitination and binding partners that mediate neurological phenotypes.

636 In summary, we have implicated dominant *de novo* variation in *IRF2BPL* to a new
637 neurological disorder in humans. We observed that patients with nonsense variants in this
638 single exon gene suffer from a progressive and devastating neurological regression, and
639 individuals with rare missense variants also show a spectrum of neurological phenotypes. The
640 bioinformatics signature supports a deleterious effect in *IRF2BPL* at the gene and variant level.
641 We provide functional analysis in flies to support a loss-of-function model of *IRF2BPL*-
642 associated disease. Future studies examining the mechanism of early death in *pits* mutant flies,
643 as well as molecular mechanisms of neurodegeneration in *pits* knockdown animals, will likely

644 shed light on conserved pathways essential for neurological development and provide guidance

645 to design an effective therapeutic or preventive strategy.

646

647 **Conflicts of Interest**

648 The Department of Molecular and Human Genetics at Baylor College of Medicine receives
649 revenue from clinical genetic testing conducted by Baylor Genetics. David Goldstein is a
650 founder of and holds equity in Pairnomix and Praxis, serves as a consultant to AstraZeneca,
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652

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708

709 Web Resources:

710 GeneMatcher, <http://www.genematcher.org/>

711 OMIM, <http://www.omim.org/>

712 Undiagnosed Diseases Network, <https://undiagnosed.hms.harvard.edu>

713 UDN *IRF2BPL* page, <https://undiagnosed.hms.harvard.edu/genes/irf2bpl/>

714 MARRVEL, <http://www.marrvel.org/>

715 ExAC, <http://exac.broadinstitute.org/>

716 gnomAD, <http://gnomad.broadinstitute.org/>

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951 **Figure Legends**

952 **Figure 1: IRF2BPL protein structure and gene constraint.**

953 (A) IRF2BPL is 796 amino acids (AA) long and contains two highly conserved domains (IRF2BP
954 zinc finger and C3HC4 RING) and the N- and C- termini. Within the variable region are multiple
955 polyalanine and PEST sequences and a 25 AA polyglutamine tract (AA 103 to 127). All four
956 nonsense variants occur early in the transcript before predicted PEST sequences, and the two
957 missense variants (highlighted in orange) occur in the middle of the protein.

958 (B) *IRF2BPL* is highly constrained based on the lack of LoF variants in ExAC²⁰ resulting in a
959 high probability of LoF intolerance (pLI) score and missense constraint z-score. Predictions
960 based on the DOMINO algorithm indicate that variation in IRF2BPL is likely to lead to a
961 dominantly inherited disease.

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977 **Figure 2: Progressive cerebral atrophy in patients with *IRF2BPL* nonsense truncations.**

978 (A) Brain MRI for subject 3 at 7, 13, and 20 years (top row, axial FLAIR; bottom row, sagittal
979 T1). Brain MRI was normal at 7 years. However, at age 13, there was severe diffuse cerebral
980 atrophy with *ex-vacuo* dilatation of the lateral ventricles. There may be slightly increased white
981 matter signal in the peritrigonal region, but otherwise the white matter appears intact and does
982 not suggest a leukodystrophy. The cerebellum has only minimal atrophy. There is mild atrophy
983 of the basal ganglia and brainstem (not shown). At age 20 years, there is further atrophy,
984 including severe volume loss in the bilateral cerebral hemispheres, further thinning of the
985 corpus callosum, mild worsening of the increased white matter signal in the peritrigonal region,
986 further atrophy of the cerebellum and brainstem.

987 (B) MRI images of subject 5 at 34 years depicting global cerebral and cerebellar atrophy,
988 thinning of corpus callosum and brainstem without focal brain lesions (axial T2-weighted and
989 sagittal T1-weighted). Images were taken on a 1.5T Siemens.

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1002 **Figure 3: The IRF2BPL homolog, Pits, is highly conserved, and human disease variants**
1003 **display dramatic loss-of-function**

1004 (A) The two annotated domains (IRF2BP zinc finger and C3HC4 ring) for IRF2BPL (as well as
1005 IRF2BP1 and IRF2BP2) display very high conservation with the fly homolog Pits.

1006 (B). The *pits*^{MI02926-TG4.1} allele was generated by genetic conversion of *y¹ w^{*}*
1007 *Mi{MIC}CG11138*^{MI02926} by recombination-mediated cassette exchange (RMCE) *in vivo*. The
1008 resulting mutant incorporates a SA-T2A-GAL4 that acts as an artificial exon resulting in early
1009 truncation of the *pits* transcript and cellular replacement with expression of GAL4 under the
1010 endogenous *pits* regulatory elements.

1011 (C) Genomic location of genomic rescue (GR) constructs inserted on chromosome 2 (VK37) of
1012 the fly. Note that the 20 Kb rescue line is specific to only the *pits* gene.

1013 (D) Reintroduction of either GR construct (Figure 3C) rescues lethality for *pits*^{MI02926-TG4.1} flies but
1014 rescue is not observed by overexpression of the fly or human cDNA. Female *pits*^{MI02926-TG4.1}/*FM7*
1015 virgins are crossed to males of either GR or UAS lines, and progeny are examined for males
1016 containing *pits*^{MI02926-TG4.1} and the rescue construct (minimum progeny examined n=91).

1017 Examination of female flies heterozygous for the presence of *pits*^{MI02926-TG4.1} and the rescue
1018 construct reveals a lack of toxicity in female *pits*^{MI02926-TG4.1} /+; *UAS-IRF2BPL-E172X*/+ flies
1019 indicating loss-of-function.

1020 (E) The ubiquitous expression of *UAS-IRF2BPL* or variants with *Act-GAL4* reveals that all
1021 nonsense variants are strong loss-of-function mutations and the p.K418N causes partial loss of
1022 function.

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1028 **Figure 4: The *IRF2BPL* homolog, *pits*, is expressed in both the developing and adult CNS**
1029 **and is present in the nucleus of a wide subset of neurons.**

1030 (A) Male fly heads were lysed and run on SDS-PAGE to determine the presence of Pits::GFP.

1031 (B) Pits is widely expressed in 3rd instar larvae, assessed by immunostaining of homozygous
1032 *pits*::GFP animals and viewed by confocal microscopy (z-stack - max projection). Note the
1033 enrichment in the mushroom body (yellow arrow).

1034 (C) Single slice confocal images of the adult CNS show *pits* expression in neurons (co-localized
1035 with Elav). Notably *pits* is expressed in the cell bodies of the adult mushroom body (top left
1036 panel), is enriched in the central complex (yellow arrow) and is not present in the dendrites of
1037 the mushroom body (bottom left panel). Scale bar is 50 μ m.

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1054 **Figure 5: Neuronal knock-down of *pits* leads to progressive behavioral deficits.**

1055 (A) Ubiquitous knock-down of *pits* results in semi-lethality, as shown by lower than expected
1056 genotypic ratios of survival into adulthood. Flies were compared to *Act-GAL4>control-RNAi* and
1057 *y w Act-GAL4/+*.

1058 (B) The *pits* RNAi can partially knock-down ~50-60% of the *pits* isoforms consistently observed
1059 in female flies.

1060 (C) Pan-neuronal knock-down of *pits* (*nSyb-GAL4>pits-RNAi*) leads to a bang-sensitive
1061 paralytic phenotype in aged animals that is not observed in control flies or young animals.
1062 Multiple cohorts of flies were anesthetized and single housed 24 hours prior to testing with 15
1063 seconds of vortexing in an empty vial. Statistical analyses were with one-way ANOVA followed
1064 by Tukeys post-hoc test. Results are means \pm SEM (* $p < 0.05$; NS, not significant)

1065 (D) Pan-neuronal knock-down of *pits* using RNAi leads to progressive climbing deficits that are
1066 only observed in aged flies. Singly housed flies (similar to above) were given 1 minute to
1067 habituate to an empty vial before being tapped 3 times. Flies were given 30 seconds to cross
1068 the 7 cm mark on the vial. Flies that failed to cross the line were given a score of 30 (only seen
1069 at day 45). One-way ANOVA followed by Tukeys post-hoc test. Data are mean \pm SEM (* $p < 0.05$,
1070 ** $p < 0.01$; NS, not significant).

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1080 **Figure 6: Knock-down of pits in the photoreceptor leads to degenerative phenotypes in**
1081 **aged animals.**

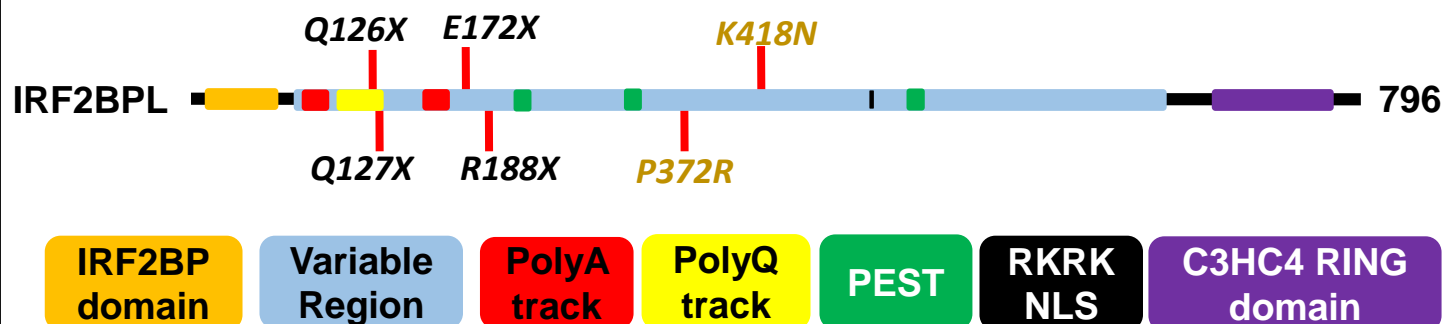
1082 (A) Toluidine blue staining of 5- and 45-day-old retina from *Rh1-GAL4>luciferase-RNAi* and
1083 *Rh1-GAL4>pits-RNAi* flies revealing disorganization of the ommatidia and photoreceptor loss.

1084 Scale bar is 5 μ m.

1085 (B to C) TEM images showing the photoreceptors and the ommatidia (B, scale bar is 2 μ m) and
1086 photoreceptors (C, scale bar is 1 μ m) section. The red arrow indicates the presence of
1087 tubulovesicular like structures (TVS), and the yellow arrow indicates neuronal lipid droplets (C).

1088 Further TEM images are available in Supplemental Figures 5 and 6. Quantification of
1089 rhabdomere loss (D) and TVS structures (E) on a minimum of n=27 randomized sections from
1090 the retina from three animals per genotype. Statistical analyses were with unpaired two-tailed t-
1091 tests. Results are mean \pm SEM (**p<0.01, ****p<0.0001).

A. IRF2BPL Protein Domains and Nonsense and Missense Variants



B. IRF2BPL tolerance to change and predicted inheritance

Probability of LoF intolerance (pLI)			Missense Constraint			DOMINO	
Expected	Observed	pLI score	Expected	Observed	z score	Score	Inheritance prediction
10.5	0	0.97	384.8	195	4.73	0.962	Very likely dominant

A. Subject 3 MRI measurements

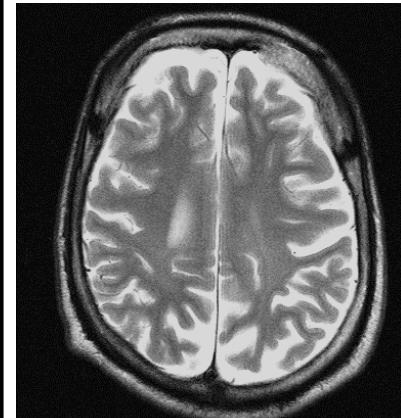
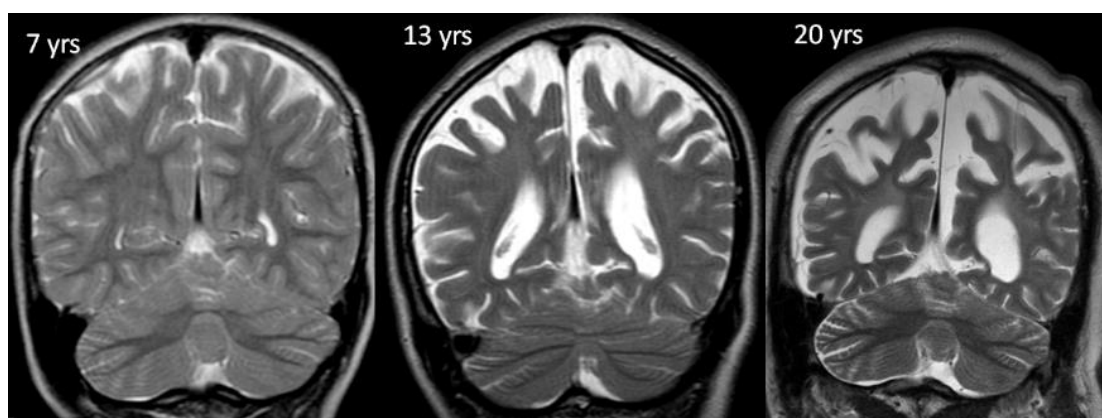
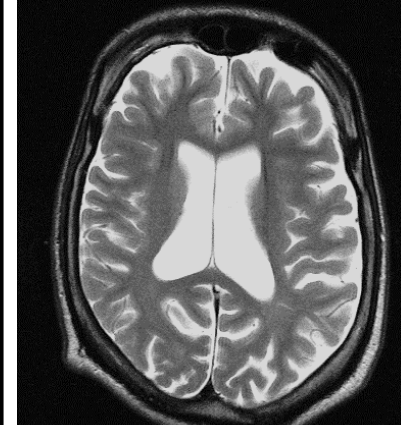
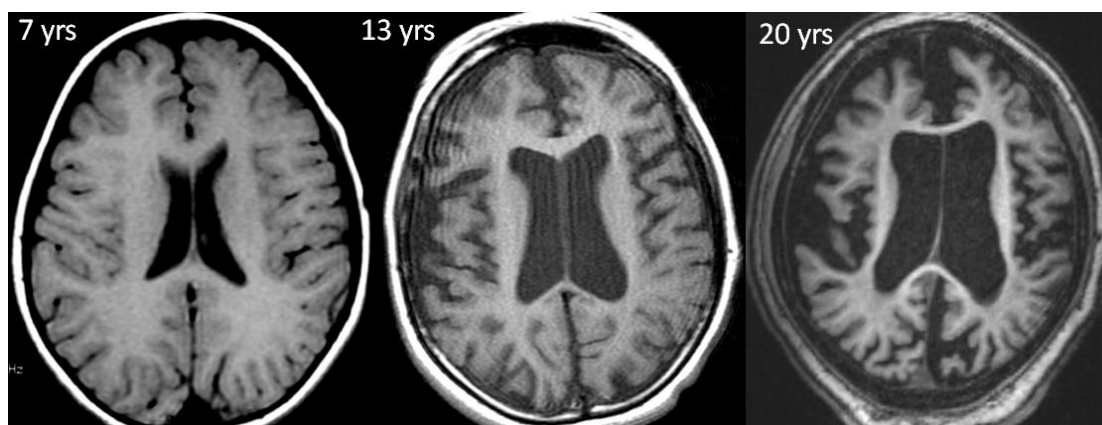
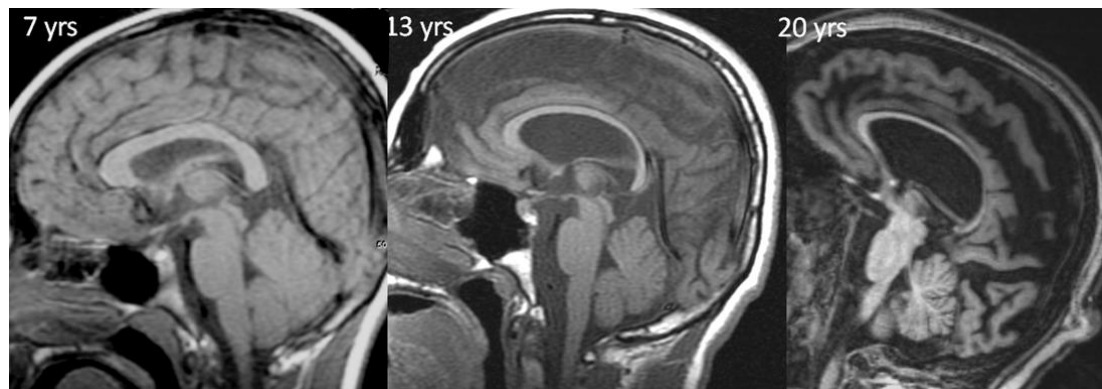
B. Subject 5 MRI measurements

7 yo (2005)

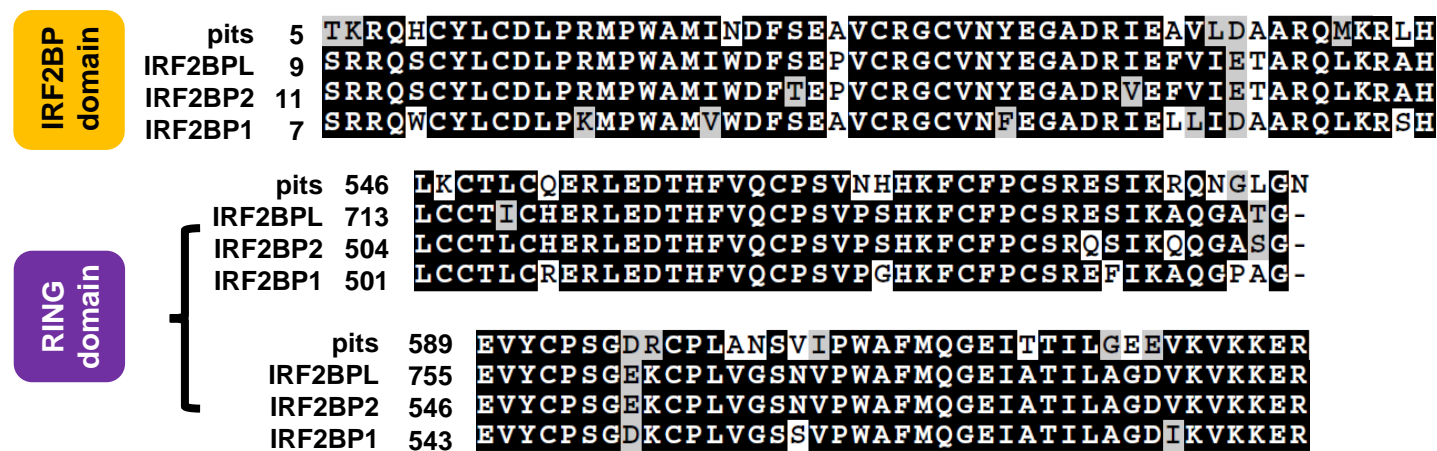
13 yo (2011)

20 yo (2017)

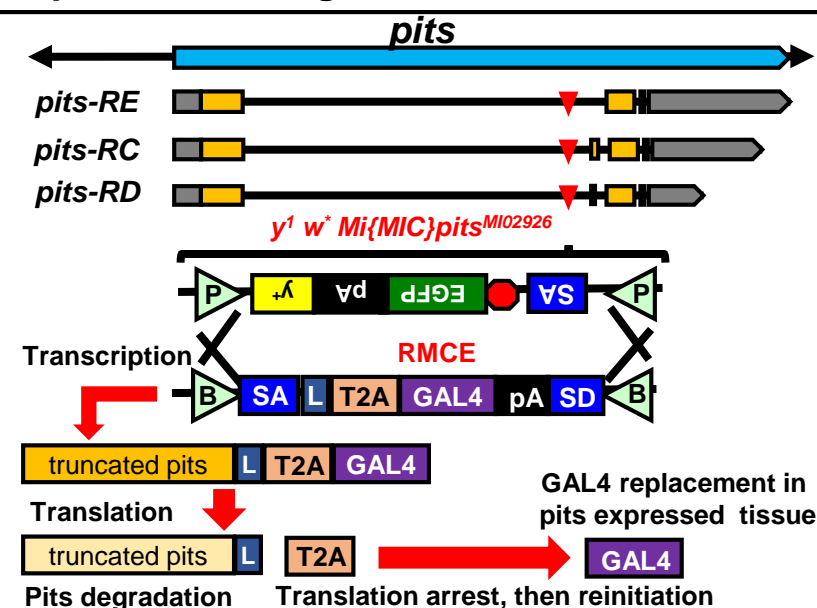
34 yo (2009)



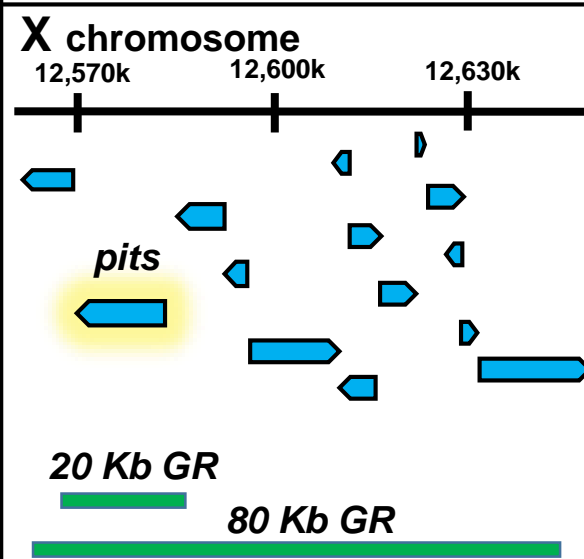
A. pits domain conservation of IRF2BP proteins



B. *pits*^{MI02926-TG4.1} generation



C. Genomic Rescue lines



D. IRF2BPL fails to rescue *pits*^{MI02926-TG4.1}

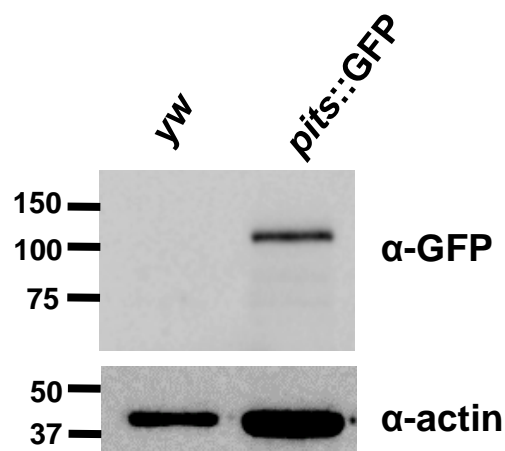
		<i>pits</i> ^{MI02926-TG4.1}	
		Hemi ♂	
		Lethal	
	80 Kb GR	Viable	
	20 Kb GR	Viable	Het ♀
UAS	<i>pits-RC</i>	Lethal	Lethal
	IRF2BPL	Lethal	Lethal
	IRF2BPL-E172X	Lethal	Viable

E. IRF2BPL nonsense variants lose toxic function

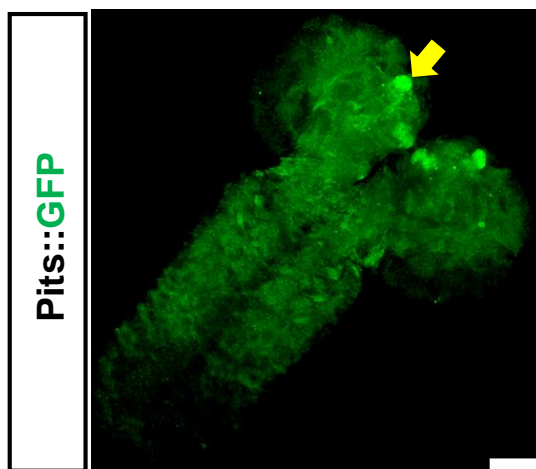
Act-GAL4 mediated transgene expression

	18°C	22°C	25°C	29°C
IRF2BPL	Lethal	Lethal	Lethal	Lethal
Reference	Lethal	Lethal	Lethal	Lethal
E172X	Viable	Viable	Viable	Viable
Q127X	Viable	Viable	Viable	Viable
R188X	Viable	Viable	Viable	Viable
P372R	Lethal	Lethal	Lethal	Lethal
K418N	Viable	Viable	Lethal	Lethal

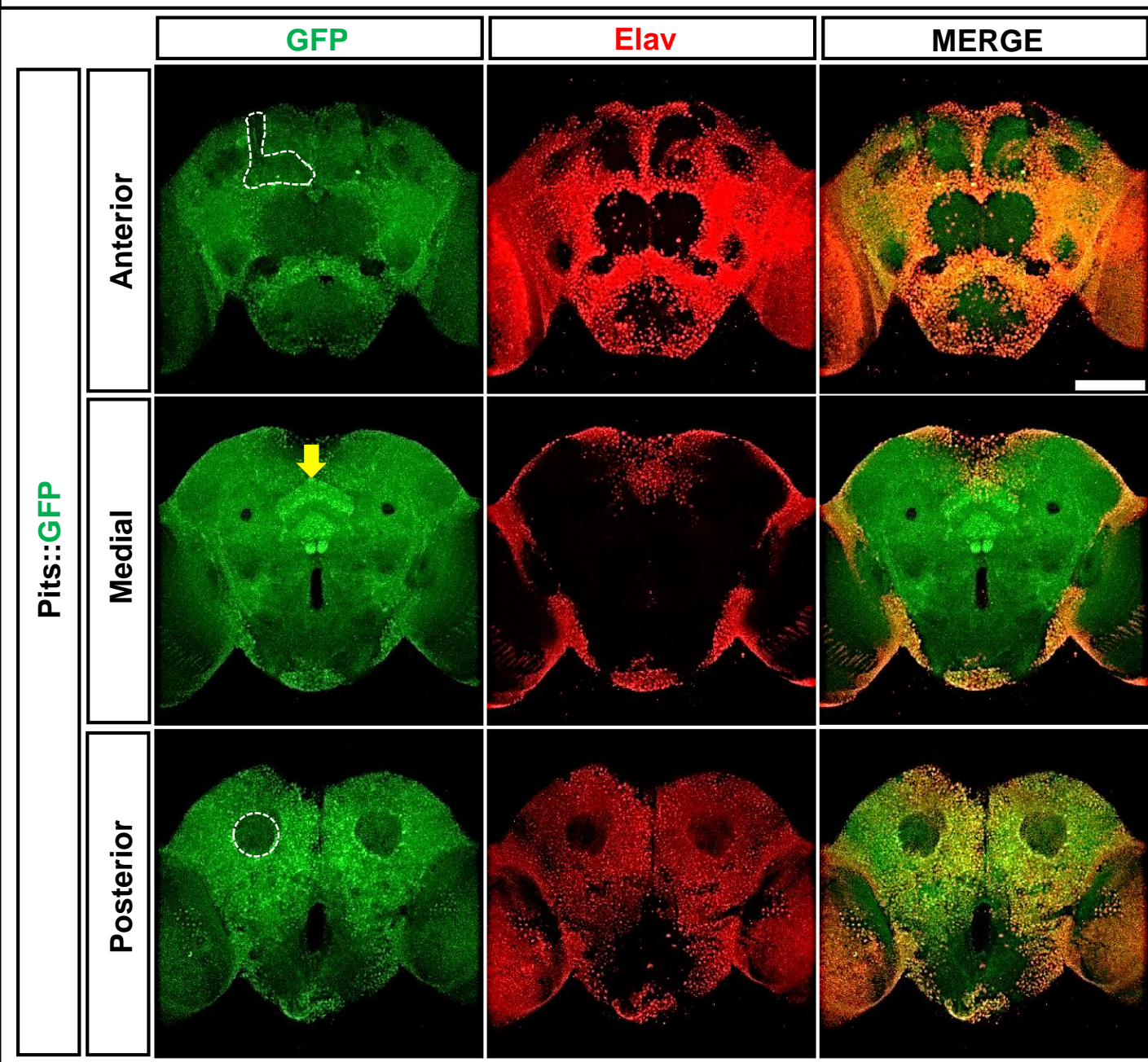
A. Pits::GFP protein is expressed



B. Pits::GFP is expressed in larval CNS



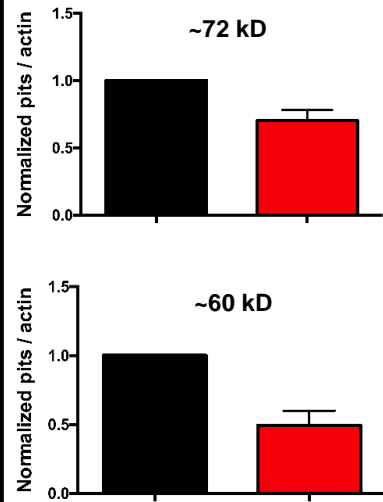
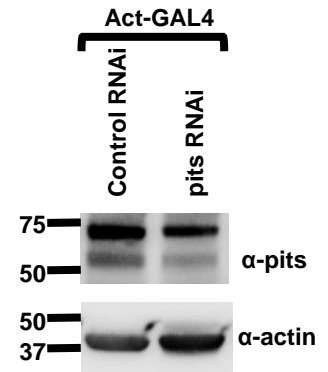
C. Pits::GFP is expressed in adult brain neurons (nucleus, cell bodies, axons)



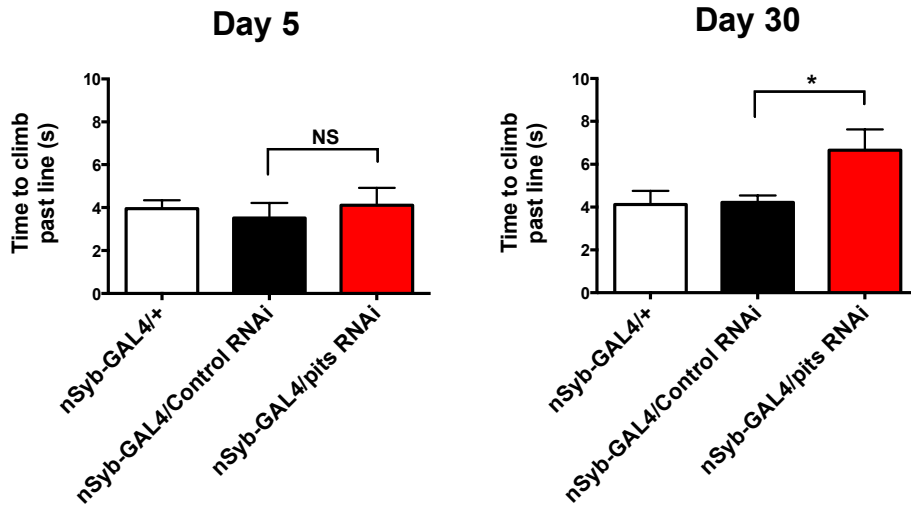
A. Ubiquitous pits knock-down is semi-lethal

		25° C	29° C
		Act-GAL4	<i>yw</i>
Control RNAi	Viable		Viable
<i>Pits RNAi</i>	Semi-lethal (<50% viability)		Semi-lethal (<20% viability)

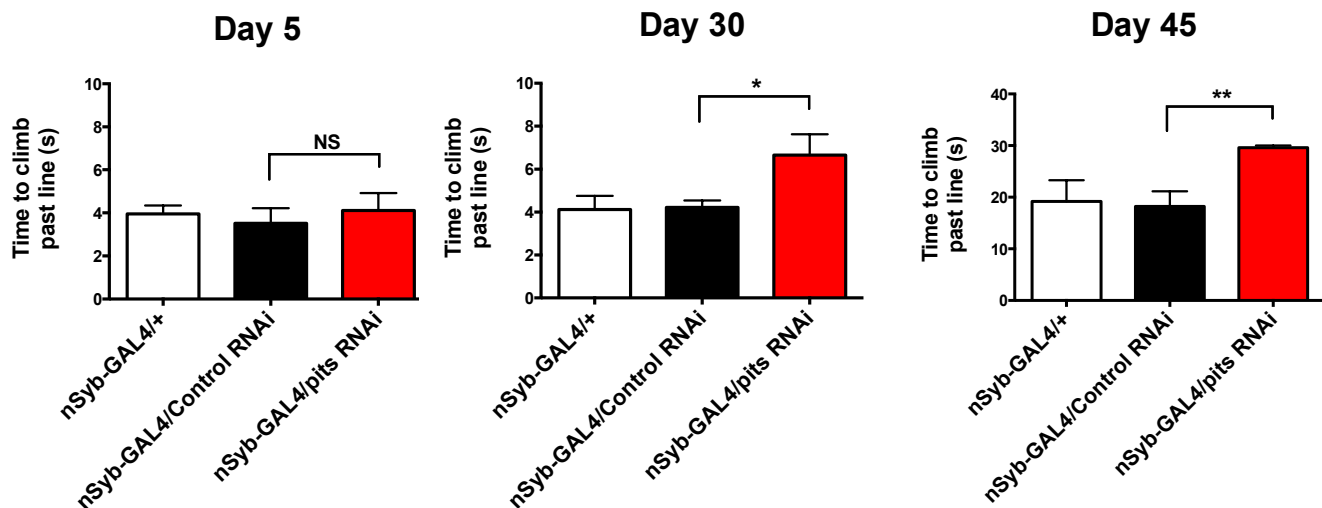
B. pits-RNAi knock-down efficiency



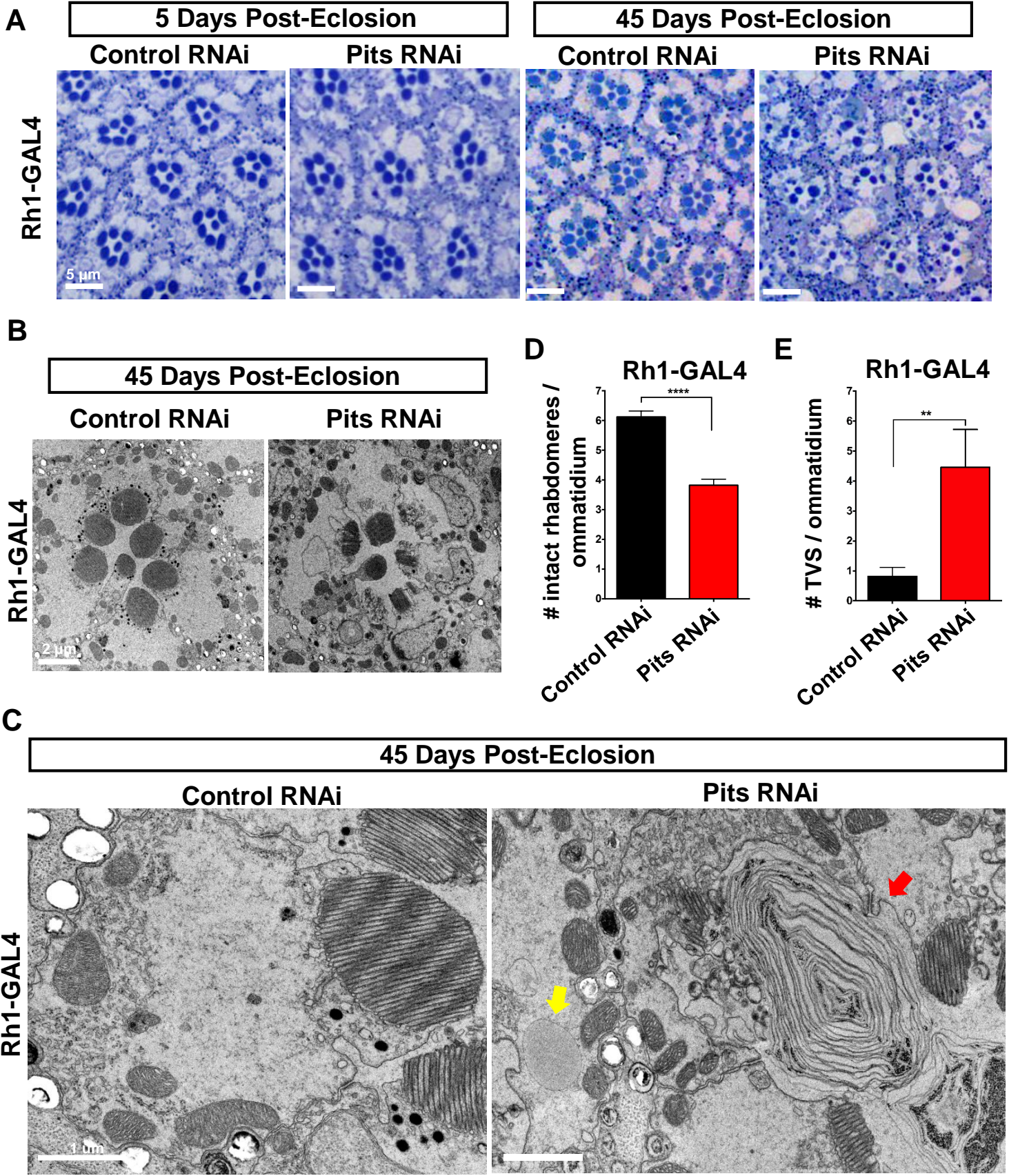
C. Neuronal knock-down of pits causes progressive bang-sensitivity defects



D. Neuronal knock-down of pits causes progressive climbing defects



Knock-down of pits in the photoreceptors causes neurodegeneration in aged flies



	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7
IRF2BPL variant	c.584G>T (p.G195V) AND c.514G>T (p.E172X) (complex rearrangement Sanger confirmed <i>de novo</i>)	c.562C>T p.R188X Sanger confirmed No parents available for testing	c.562C>T p.R188X Sanger confirmed <i>de novo</i>	c.379C>T p.Q127X Sanger confirmed, no parents available for testing	c.376C>T p.Q126X Sanger confirmed <i>de novo</i>	c.1115C>G p.P372R Sanger confirmed <i>de novo</i>	c.1254G>C p.K418N No Sanger confirmation, present in 42/87 reads <i>de novo</i>
Gender	M	F	M	F	M	M	F
Current age	7 years	Deceased at 15 years	20 years	15 years	43 years	10 years	2.5 years
Growth for age at most recent visit (Z-score)*	6 yr 6 mo Wt 20.1kg (-0.42) Lt 116.8cm (-0.67) HC-51.4cm -0.17)	12 years Wt 45 kg (0.5) Ht-168 cm (2)	20 years Wt 66 kg (-0.43) H 141 cm (-5.0) HC 59 cm (2.4)	15 years Wt 46.9kg (-0.5) Ht 162.4cm (0) HC-52cm (-2)	NA	11 yr 2 mo Wt 32.6kg (-0.62) Ht 125cm (-2.81) HC 51cm (-1.13)	2 yr 1 mo Wt 13.30kg (0.5) Ht 86.30cm (-1) HC 49.40cm (1)
Developmental delays preceding regression	Present	None	None	Present	NA	NA	NA
Age of onset of motor regression	2.5-3 years	7 years	5 years	Age at onset unknown	5-10 years	3-4 years	No regression
Current speech and language skills	No speech at 6.5 years of age	Lack of speech since 12 years	Lack of speech since 10 years	NA	NA	Has few words, utilizes augmentive communication device	Echolalia, sign language
Current gross motor skills	Wheelchair-bound since 5.5 years	In a wheelchair since 9 years of age	Non-ambulatory since 11 years	Unsteady gait, clumsy	NA	Normal stance and gait, tandem and reciprocal.	Walking easily up and down stairs, starting to run
Current oromotor skills	Mild-moderate oropharyngeal dysphagia, silent aspiration & tube feeds	Dysphagia at 10 years	Progressive dysphagia since 10 years of age	Silent aspiration at 15 years	NA	Normal	Normal
Seizures	Diagnosed with seizures at 6 years	'Staring spells'	Myoclonus at 9-10 years	Seizures at 13 years	Febrile, photosensitive and myoclonic epilepsy	Myoclonic, atonic and absence seizures at 14 years	Infantile spasms at 6 months
Movement abnormalities	Ataxia, dystonia, choreoathetosis	Dystonia, no ataxia	Ataxic gait at 6 years	Lower extremity dystonia.at 15	Ataxia, choreoathetosis,	None	None

				years	generalized dystonia		
Other neurological findings	Spasticity, cerebellar signs	NA	Spasticity, cerebellar signs, positive Romberg sign by 7 years	NA	Hyperreflexia	None	None
EEG	Abnormal at 5 years, with diffuse slowing, interictal discharges from left occipital and right temporal lobes	EEG with mild background slowing at 9 and 13 years; normal EEGs at 9 and 14 years	EEG normal at 13 years	Abnormal since 6.5 years	Abnormal at 31 years	Abnormal with generalized spike and wave	Abnormal at 6 months
Brain MRI	Normal at 5 years	'Bulky' corpus callosum, mild cerebellar volume loss, large left middle cranial fossa arachnoid cyst at 8 years. Marked cord thinning on spine MRI at 10 years	Normal at 7 years; at 13 years, diffuse cerebral atrophy and at 20 years cerebral atrophy	Normal at 6 & 13 years.	Cerebral, cerebellar, brainstem and corpus callosum atrophy at 26 years	Probable Rathke's cyst at 16 months, otherwise normal.	Normal at 6 months and at 2 years

*Z-score per Centers for Disease Control growth charts in case of weight (Wt) and height/length (Ht/Lt), or Nellhaus growth charts for head circumference (HC).

NA, not available