Homozygous Mutations in \textit{TBC1D23} Lead to a Non-degenerative Form of Pontocerebellar Hypoplasia

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Pontocerebellar hypoplasia (PCH) represents a group of recessive developmental disorders characterized by impaired growth of the pons and cerebellum, which frequently follows a degenerative course. Currently, there are 10 partially overlapping clinical subtypes and 13 genes known mutated in PCH. Here, we report biallelic \textit{TBC1D23} mutations in six individuals from four unrelated families manifesting a non-degenerative form of PCH. In addition to reduced volume of pons and cerebellum, affected individuals had microcephaly, psychomotor delay, and ataxia. In zebrafish, \textit{tbc1d23} morphants replicated the human phenotype showing hindbrain volume loss. \textit{TBC1D23} localized at the trans-Golgi and was regulated by the small GTPases Arl1 and Arl8, suggesting a role in trans-Golgi membrane trafficking. Altogether, this study provides a causative link between \textit{TBC1D23} mutations and PCH and suggests a less severe clinical course than other PCH subtypes.

Originally named by Brun in 1917,\textsuperscript{1} pontocerebellar hypoplasia (PCH) is a devastating neurological disorder characterized by impaired growth and/or degeneration of cerebral structures, primarily the pons and cerebellum. To date, ten different clinical subtypes of PCH have been described, the majority leading to a neurodegenerative course, manifesting with progressive intellectual and motor decline.\textsuperscript{2,3} Treatments are only palliative and the prognosis is poor, as most affected individuals die during infancy or childhood. Despite the expansion of known genes associated with PCH, most individuals remain without genetic diagnosis, suggesting that additional causes remain to be identified.

We recruited a cohort of 75 families with likely autosomal-recessive PCH, of which 53 (70.6%) documented parental consanguinity and 19 (25.3%) had two or more affected individuals. All affected members were clinically evaluated by a pediatric neurologist and geneticist, blood and/or saliva samples and skin biopsies were collected from participating individuals after obtaining proper informed consent, and DNA for whole-exome sequencing (WES) was extracted from at least one affected member of each family as described.\textsuperscript{4} The study followed the IRB guidelines and was approved by the ethical committees of UC San Diego, The Rockefeller University, and other participating institutions. In consanguineous families, we emphasized homozygous, rare (<0.1% allele frequency in our exome database of 5,000 individuals), and potentially damaging variants (Genomic Evolutionary Rate Profile [GERP] score > 4 or phastCons > 0.9).\textsuperscript{5} Likely causative mutations were identified in 47 families (62.6% of the total) (Figure 1A). Seven families (9.3%) had a likely causative variant in a gene not previously implicated in PCH. A total of 34 families (45.3%) carried mutations in genes previously associated with degenerative forms of PCH, encoding proteins involved in tRNA splicing, mRNA processing, and protein synthesis.\textsuperscript{5–8} Six families (8%) demonstrated a non-degenerative course of PCH, and among these, \textit{TBC1D23} mutations were identified as a cause for this condition. \textit{TBC1D23} has been recently linked also with autosomal-recessive intellectual disability.\textsuperscript{9} We found six affected individuals with mutations in \textit{TBC1D23} from four unrelated families from Egypt (families I and II), Turkey (family III), and Lebanon (family IV) (Figures 1B and S1).

Family I presented with two affected boys of 16 years (I-IV-1) and 2 years (I-IV-5) of age, family II presented with two non-identical girl twins of 4 years of age (II-III-1 and II-III-2), family III presented with one boy of 14 months of age (III-IV-1), and family IV presented with a girl of 6 months of age (IV-II-1) (Figure 1B). Some of these individuals manifested reduced head circumference at birth (≥−2 SD standard deviations [SD] below the mean) and all showed signs of global psychomotor deficits since early infancy, involving gross and fine motor skills, language (expressive > receptive), and social interaction due to communication impairment (Table 1). In the most recent clinical evaluation, all subjects were microcephalic.
neurological exam was remarkable for generalized weakness (6/6 affected subjects), global hypotonia (5/6), and cerebellar deficits such as uncoordinated limb movements (4/6), hyporeflexia (3/6), and impaired or no ambulation (6/6). Brainstem symptoms including dysphagia and dysarthria were present in subjects from family I. None of the six individuals manifested clinical signs of neurological deterioration and, hitherto, they are all alive. Brain MRI showed pontocerebellar hypoplasia in all subjects, along with thin corpus callosum (I-IV-5, II-III-1, II-III-2, IV-II-1) and cortical hypoplasia (I-IV-5, II-III-1, II-III-2) (Figure 1C). The radiological findings of subject III-IV-1 did not change appreciably over a 3-year interval (Figure S2), which is consistent with the non-progressive course of this form of PCH.

All six affected individuals carried mutations predicted to result in altered splicing, occurring at or near canonical splice sites (Figure 1D; Table 1), so we used genome build hg19 and transcript GenBank: NM_001199198.2 (transcript 1) to annotate splicing effects. We found that both TBC1D23 transcripts were differently expressed in human tissues, with transcript 1 primarily expressed in the fetal and adult brain and spinal cord (Figure 2A). To study potential effects of the variants on splicing, we used RT-PCR to amplify annotated transcripts from fibroblasts of families II and III, who carried mutations c.1687+2T>A and c.1687+1G>A, respectively (primers are available upon request).
Table 1. Description of Clinical Findings of Individuals with TBC1D23 Mutations

<table>
<thead>
<tr>
<th></th>
<th>I-IV-1</th>
<th>I-IV-5</th>
<th>II-III-1</th>
<th>II-III-2</th>
<th>III-VI-1</th>
<th>IV-II-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutation (genomic hg19) chr3:g.100029386G&gt;A</td>
<td>chr3:g.100029386G&gt;A</td>
<td>chr3:g.100035033T&gt;A</td>
<td>chr3:g.100035033T&gt;A</td>
<td>chr3:g.100035033T&gt;A</td>
<td>chr3:g.100014144A&gt;G</td>
<td>chr3:g.100014144A&gt;G</td>
</tr>
<tr>
<td>Mutation (cDNA) (NM_001199198.2) c.1553G&gt;A</td>
<td>c.1553G&gt;A</td>
<td>c.1687+2T&gt;A</td>
<td>c.1687+2T&gt;A</td>
<td>c.1687+1G&gt;A</td>
<td>c.726–2A&gt;G</td>
<td></td>
</tr>
<tr>
<td>Gestational age</td>
<td>40</td>
<td>38</td>
<td>37</td>
<td>37</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Weight at birth (kg)</td>
<td>3 (−0.90 SD)</td>
<td>2.8 (−1.22 SD)</td>
<td>1.6 (−3.47 SD)</td>
<td>1.5 (−3.66 SD)</td>
<td>3.8 (+0.49 SD)</td>
<td>8.8 at age 2 years (−3 SD)</td>
</tr>
<tr>
<td>Length at birth (cm)</td>
<td>50 (−0.06 SD)</td>
<td>48 (−0.81 SD)</td>
<td>48 (−0.65 SD)</td>
<td>47 (−1.10 SD)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>HC at birth (cm)</td>
<td>32.2 (−1.62 SD)</td>
<td>32 (−1.71 SD)</td>
<td>32 (−1.90 SD)</td>
<td>31.5 (−2.24 SD)</td>
<td>42 at 14 months (−3.84 SD)</td>
<td>41.2 at 2 years (−4.4 SD)</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>delayed since infancy, but first seen at 16 years of age</td>
<td>2 years</td>
<td>delayed since infancy, but first seen at 4 years of age</td>
<td>delayed since infancy, but first seen at 4 years of age</td>
<td>14 months</td>
<td>6 months</td>
</tr>
<tr>
<td>Weight (kg), age at last examination</td>
<td>38, 16 years (−2.65 SD)</td>
<td>14, 6 years (−2.96 SD)</td>
<td>14.5, 4 years (−0.79 SD)</td>
<td>11.5, 4 years (−2.62 SD)</td>
<td>N/A</td>
<td>11.9, 7.5 years (−4.11 SD)</td>
</tr>
<tr>
<td>Height (cm), age at last examination</td>
<td>143, 16 years (−3.56 SD)</td>
<td>103, 6 years (−2.44 SD)</td>
<td>103.4 years (+0.50 SD)</td>
<td>97, 4 years (−0.89 SD)</td>
<td>N/A</td>
<td>98, 7.5 years (−4.89 SD)</td>
</tr>
<tr>
<td>HC (cm), age at last examination</td>
<td>48, 16 years (−4.77 SD)</td>
<td>44.5, 6 years (−5.27 SD)</td>
<td>43.5, 4 years (−3.96 SD)</td>
<td>41.5, 4 years (−5.25 SD)</td>
<td>50, 16 years (−3.4 SD)</td>
<td>42, 7.5 years (−7.77 SD)</td>
</tr>
</tbody>
</table>

**Psychomotor Development**

<table>
<thead>
<tr>
<th></th>
<th>I-IV-1</th>
<th>I-IV-5</th>
<th>II-III-1</th>
<th>II-III-2</th>
<th>III-VI-1</th>
<th>IV-II-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gross motor</td>
<td>delayed; can walk alone</td>
<td>delayed; sits only</td>
<td>delayed; can walk alone</td>
<td>delayed; walks supported</td>
<td>delayed</td>
<td>delayed</td>
</tr>
<tr>
<td>Fine motor</td>
<td>delayed</td>
<td>delayed</td>
<td>delayed</td>
<td>delayed</td>
<td>absent</td>
<td>delayed</td>
</tr>
<tr>
<td>Language</td>
<td>delayed</td>
<td>delayed</td>
<td>delayed</td>
<td>delayed</td>
<td>absent</td>
<td>delayed (babbling at 7 years of age)</td>
</tr>
<tr>
<td>Social</td>
<td>delayed</td>
<td>delayed</td>
<td>delayed</td>
<td>delayed</td>
<td>absent</td>
<td>delayed</td>
</tr>
<tr>
<td>Regression of acquired milestones</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

**Neurological Findings**

<table>
<thead>
<tr>
<th></th>
<th>I-IV-1</th>
<th>I-IV-5</th>
<th>II-III-1</th>
<th>II-III-2</th>
<th>III-VI-1</th>
<th>IV-II-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brainstem findings</td>
<td>dysarthria, no history of apnea, hearing deficit, dizziness, or dysphagia</td>
<td>minimal dysphagia, no history of apnea, hearing deficit, or dizziness.</td>
<td>–</td>
<td>–</td>
<td>N/A</td>
<td>–</td>
</tr>
<tr>
<td>Cerebellar deficits</td>
<td>truncal and appendicular ataxia</td>
<td>truncal and appendicular ataxia</td>
<td>truncal and appendicular ataxia</td>
<td>truncal and appendicular ataxia</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Muscle strength (scale 0–5 in upper and lower extremities)</td>
<td>grade 4/5</td>
<td>grade 4/5</td>
<td>grade 4/5</td>
<td>grade 4/5</td>
<td>grade 5/5</td>
<td>grade 3/5</td>
</tr>
<tr>
<td>Muscle tone</td>
<td>hypotonia</td>
<td>hypotonia</td>
<td>hypotonia</td>
<td>hypotonia</td>
<td>normal muscle tone</td>
<td>hypotonia; reduced muscle tone at 2 years, but developed spasticity especially in lower limbs at 7 years.</td>
</tr>
</tbody>
</table>

(Continued on next page)
Affected individuals had shorter transcripts relative to control individuals (Figure 2B), and sequencing of the amplified PCR products confirmed that shorter transcripts had skipped exon 16 (Figure 2C), leading to a shift in the reading frame and truncated protein (p.His534Trpfs*36) (Figures 1D and 2D). Fibroblasts from families I and IV were not available to assess splicing. Family IV carried a variant in a canonical splice site (c.726+2A>G) and family I carried a missense mutation at the last base of exon 14 (c.1553G>A), both of which were also expected to compromise splicing. Cellular localization of endogenous TBC1D23 was examined in control, carrier, and affected individuals’ fibroblasts using specific antibodies. In control fibroblasts, TBC1D23 overlapped with the trans-Golgi marker TGN46 and showed signal adjacent to the cis-Golgi marker GM130 (Figure 2E). This trans-Golgi staining pattern of TBC1D23 was absent in cells from affected individuals, whereas cells from carriers showed reduced staining intensity. Despite the loss of detectable TBC1D23 in affected subjects (Figures 2D and 2E), there was no obvious alteration in the relative positions of the cis/trans-Golgi markers or the ribbon-like structure of the Golgi (Figure 2E).

TBC1D23 belongs to a family of Tre2-Bub2-Cdc16 (TBC) domain-containing Rab-specific GTPase-activating proteins (TBC/RabGAPs) that regulate membrane trafficking by inactivating Rabs.10-12 Most TBC/RabGAPs contain two catalytic residues, Arg and Gln, to stimulate the hydrolysis of GTP in Rab proteins.13,14 TBC1D23 falls in the category of unconventional TBC/RabGAPs since it lacks the catalytic Arg-Gln residues and it might, a priori, work through a different mechanism to induce GTP hydrolysis14 or it might have a Rab-independent function. When compared to TBC1D20, which acts on Rab1, none of the 55 Rabs tested showed robust activation of GTP hydrolysis in the presence of purified TBC1D2315 (Figure S3). This raised the possibility that TBC1D23 is a Rab-binding protein, or effector, rather than a Rab regulator and it might target to the Golgi via this means. However, two lines of evidence argue against this. First, a region in TBC1D23 (469–570 aa), C-terminal to the TBC1 and Rhodanese domains, is responsible for its targeting to the trans-Golgi (Figures 3A and 3B). Second, TBC1D23 remains associated with trans-Golgi membranes when Rabs are depleted, with just a subset (Rab1a/b, Rab2a/b, Rab6a/b, Rab7a, Rab14a/b) giving rise to altered TBC1D23 localization due to their effects on Golgi structure or trafficking to and from the Golgi (Figure S3). This suggested a Rab-independent targeting mechanism.

Like Rabs, Ras superfamily GTPases of the Arl and Arf group are known to be involved in recruitment of cytosolic proteins to membrane surfaces. Strikingly, depletion of Arl1, but not ArfRP1 or other Arfs or Arls, resulted in the complete loss of TBC1D23 from the trans-Golgi (Figure 3C). Conversely, knocking down Arl8 resulted in elevated staining for TBC1D23 at the trans-Golgi (Figure 3C). These findings connect TBC1D23 to an
Arl1-dependent trafficking process at the trans-Golgi16–19 and to Arl8 function in the endosome-lysosome system.20–22 Thus far, these two Arls have not been associated with human disease and the impact of their interaction with TBC1D23 on brain development requires additional studies.

To investigate the role of TBC1D23 in brain development, we designed a zebrafish model of disease. A single tbc1d23 ortholog (GenBank: NM_200487) encodes a protein with 77% identity with the human TBC1D23 amino acid sequence. 

transcript 1 is predominantly expressed in the central nervous system.

(A) RT-PCR of both TBC1D23 transcripts in different human tissues reveals that transcript 1 is predominantly expressed in the central nervous system.

(B) RT-PCR from control (C), unaffected parents (F, father; M, mother), and affected subjects (1 and 2 from family II, 1 from family III). Parents (carriers) showed splicing defect in the mutated allele, represented by the lack of exon 16 in both transcripts. Homozygous individuals manifested splicing defect in both alleles.

(C) Sequencing of PCR bands confirms the absence of exon 16 in both mutant transcripts.

(D) Western blot shows that TBC1D23 is absent in affected individuals and decreased in carriers compared to control.

(E) Representative immunofluorescence images of fibroblasts from control, carrier, and affected. In control and carrier fibroblasts, TBC1D23 (rabbit 17002; Proteintech, 1:1,000) is located toward the trans-Golgi network as it overlaps with TGN46 (sheep; AbD Serotec; 1:1,000) and not with GM130 (mouse clone 35; BD; 1:1,000). In agreement with western blot results, cells from the affected individual did not show signal except non-specific staining of the centrosome. The enlarged region shows details of TBC1D23 with the different Golgi markers. Scale bars represent 10 μm.

Arl1-dependent trafficking process at the trans-Golgi16–19 and to Arl8 function in the endosome-lysosome system.20–22 Thus far, these two Arls have not been associated with human disease and the impact of their interaction with TBC1D23 on brain development requires additional studies.

In the head at 48 hpf (Figures 4A and 4B), suggesting a role in brain development. To test whether knockdown tbc1d23 in zebrafish replicates the human phenotype, we knocked down tbc1d23 using a translation blocking morpholino targeting the ATG start codon (tbc1d23-ATG MO; 5'-CTTCCCCTACAGCATCCGCCATTGC-3') and a splice blocking morpholino targeting intron 4 to exon 5 (tbc1d23-splice MO; 5'-GCAGTCTCTGCAAAAGGCAATGC-3'). In contrast to scramble MO, both ATG and splice MO-injected embryos (3 ng each) had reduced brain and eye size and manifested curved tails at 48 hpf (more severe in ATG MO embryos), and this phenotype was partially rescued with injection of zebrafish tbc1d23 AD
Figure 3. Trans-Golgi Location of TBC1D23 Depends on the C-Terminal Region and Is Regulated by Arl1 and Arl8

(A and B) HeLa cells were transfected using Mirus LT1 (Mirus LCC) with full-length GFP-tagged TBC1D23 (A) or with deletion constructs outlined in the schematic (B). After 20 hr of the transfection, cells were fixed with 3% PFA (wt/vol) for 15 min, permeabilized with 0.1% Triton X-100 (vol/vol) for 7 min, and then stained for TGN46 using a standard protocol. TBC1D23 was visualized using GFP. As illustrated, the region between amino acids 469 and 570 is responsible for the targeting of TBC1D23 to the trans-Golgi.

(C) HeLa cells were transfected using Oligofectamine (Life Technologies) with a specific library of siRNA duplexes targeting Arfs and Arls (Dharmacon) for 72 hr (a subset of this library is shown). Cells were then fixed and stained as mentioned previously with antibodies against TBC1D23, TGN46, and GM130. The depletion of Arl1 caused complete loss of TBC1D23 from the trans-Golgi, whereas knocking down Arl8 increased TBC1D23 staining at the trans-Golgi. Depletion of ArfRP1, which controls Arl1 targeting to the Golgi, did not alter TBC1D23 expression or localization. Western blot demonstrates efficient knockdown of targeted Rabs by siRNA duplexes and shows the resulting expression of TBC1D23. In this case, HeLa cells were transfected with siRNA duplexes to Arl1, ArfRP1, and Arl8 or non-specific control for 72 hr and transfected with expressing EGFP-tagged Arl1, ArfRP1, and Arl8 20 hr before collection for western blot. Antibodies against EGFP (raised against full-length GFP in sheep) and Tubulin (mouse DM1A; Sigma-Aldrich) were used.

Scale bars represent 10 µm.
Figure 4. A Zebrafish Model Reproduces the Human Phenotype
(A) RT-PCR shows expression of zebrafish tbc1d23 at different developmental stages relative to control mobk13.23
(B) In situ RNA hybridization in AB embryos at 48 hr post-fertilization (hpf) with sense (negative control) and antisense (signal) probes for tbc1d23. The expression of tbc1d23 occurs primarily in the head, pointing out its potential role in the development of cerebral structures.
(C) Representative images of fishes injected with scramble, ATG, and splicing morpholinos (MO, all 3 ng) and rescued with zTbc1d23 mRNA (150–200 ng). Red arrows show reduced size of cerebellum and brainstem along with enlarged IV ventricle in ATG and splicing MO fishes relative to scramble MO and rescued fishes.
(D) Quantification of morphometric parameters (9–11/condition). Reduced head and eye size and length of both ATG and splicing MO fish was partially rescued with zTbc1d23 mRNA (n = 10/condition).

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mRNA (Figures 4C and 4D). These findings were corroborated in \textit{Tg(HuC:Kaede)} transgenic zebrafish line, which expresses the fluorescent protein Kaede in neurons. The ATG MO injected \textit{Tg(HuC:Kaede)} zebrafish showed reduced signal in the neural axis and manifested altered morphology of forebrain, brainstem, and cerebellum relative to scramble MO embryos (Figure 4E). Altogether, \textit{tbc1d23} disruption in zebrafish replicates the human phenotype by impairing brain growth and development.

This study enhances the genetic diagnosis and expands the phenotypic spectrum of PCH. In contrast to most subtypes, individuals with \textit{TBC1D23}-associated PCH did not show clinical neurological deterioration and MRI findings did not worsen over time. None of the affected individuals have died so far and the oldest are currently 16 years old (I-IV-1 and III-IV-1). This distinctive clinical course is therefore highly valuable for family counseling and prognosis since most individuals with other PCH subtypes show progressive worsening and typically succumb during infancy or early childhood.\textsuperscript{2} \textit{TBC1D23} individuals shared some neurological manifestations with other forms of PCH, such as psychomotor impairment, microcephaly, brainstem deficits, and ataxia.\textsuperscript{3} In addition to severe volume loss of pons and cerebellum, \textit{TBC1D23} individuals manifested hypoplasia of cortex and of corpus callosum as seen in other forms of PCH\textsuperscript{3} (Figure 1C). At the systemic level, the most common findings in all affected subjects were recurrent respiratory infections and even sepsis (Table 1). This could relate to the essential role of the brainstem to swallowing function and not a degenerative disorder.32 Another non-degenerative form of PCH not included in the current classification is \textit{VLDLR}-associated PCH (MIM: 192977). Along with apoE receptor 2 (ApoER2), VLDLR serves as a Reelin receptor to regulate microtubule function in migrating neurons.35 Individuals with damaging mutations in \textit{VLDLR} manifest non-progressive cerebellar hypoplasia with flattened pons and cortical dysplasia.36,37 The majority of PCH subtypes, however, are associated with disruption of protein synthesis, for example by altering RNA processing (i.e., \textit{TSEN} genes, \textit{RARS2}, \textit{VRK1}, \textit{TOE1}, \textit{CLP1})\textsuperscript{6,8,38–40} or GTP-dependent protein synthesis (i.e., \textit{AMPD2}),\textsuperscript{41} functions that are critical for brain development and considered causative of degeneration when disrupted.\textsuperscript{2,7} In PCH, how mutations in genes that regulate protein synthesis cause neurodegeneration whereas genes involved in trafficking and signaling impairs primarily brain development, and why all these genes preferentially involve the hindbrain are questions that remain unsolved. Whether there are key molecular pathways involved in hindbrain formation where these genes converge and lead to a pontocerebellar phenotype is a matter that needs further investigation.

**p < 0.05; **p < 0.01, ***p < 0.001. Scale bars in (B) and (C) represent 200 μm, and in (E) represents 100 μm.
Accession Numbers
The accession number for the TBC1D23 sequence reported in this paper is Genbank: NM_001199198.2.

Supplemental Data
Supplemental Data include three figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2017.07.015.

Acknowledgments
The authors thank all families for participation in this study. We thank Tessa van Dijk for coordinating data gathering from family 4572. Thanks to the Rockefeller and UCSD Microscopy Cores (P30 NS047101) for imaging support. I.M.-V. was sponsored by Pilot Grant awarded by the Center for Basic and Translational Research on Disorders of the Digestive System at The Rockefeller University through the generosity of the Leona M. and Harry B. Helmsley Charitable Trust. We thank the Broad Institute (U54HG003067 to E. Lander and UM1HG008900 to D. MacArthur) and the Yale Center for Mendelian Disorders (U54HG006504 to R. Lifton and M. Gunel). This work was supported by NIH grants P01HD070494, 1R01NS089001, R01NS0848453, R01NS052455, and UL1TR001866 from the National Center for Advancing Translational Sciences (NCATS), National Institutes of Health (NIH) Clinical and Translational Science Award (CTSA) program, the Simons Foundation Autism Research Initiative (275275), Howard Hughes Medical Institute (to J.G.G.), Qatar National Research Foundation NPRP 6-1463-3-351 (to T.B.-O. and J.G.G.), NIH grant K99NS089943 (to A.G.-G.), American Academy of Neurology Clinical Research Training Scholarship 2017-205 (to I.M.-V.), Joshua Deeth Foundation (to F.B.), and a Wellcome Trust Senior Investigator Award 097769/Z/11/Z (to F.A.B.).

Received: April 1, 2017
Accepted: July 17, 2017
Published: August 17, 2017

Web Resources
ExAC Browser, http://exac.broadinstitute.org/
GATK, https://www.broadinstitute.org/gatk/
GME Variome, http://igm.ucsd.edu/gme
Mutation Assessor, http://mutation assessor.org/
PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/
PROVEAN, http://provean.jcvi.org
SIFT, http://sift.bii.a-star.edu.sg/
UCSC Genome Browser, http://genome.ucsc.edu

References


