A de novo EGR2 variant, c.1232A > G p.Asp411Gly, causes severe early-onset Charcot-Marie-Tooth Neuropathy Type 3 (Dejerine-Sottas Neuropathy)

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EGR2 (early growth response 2) is a crucial transcription factor for the myelination of the peripheral nervous system. Mutations in EGR2 are reported to cause a heterogenous spectrum of peripheral neuropathy with wide variation in both severity and age of onset, including demyelinating and axonal forms of Charcot-Marie Tooth (CMT) neuropathy, Dejerine-Sottas neuropathy (DSN/CMT3), and congenital hypomyelinating neuropathy (CHN/CMT4E). Here we report a sporadic de novo EGR2 variant, c.1232A > G (NM_000399.5), causing a missense p.Asp411Gly substitution and discovered through whole-exome sequencing (WES) of the proband. The resultant phenotype is severe demyelinating DSN with onset at two years of age, confirmed through nerve biopsy and electrophysiological examination. In silico analyses showed that the Asp411 residue is evolutionarily conserved, and the p.Asp411Gly variant was predicted to be deleterious by multiple in silico analyses. A luciferase-based reporter assay confirmed the reduced ability of p.Asp411Gly EGR2 to activate a PMP22 (peripheral myelin protein 22) enhancer element compared to wild-type EGR2. This study adds further support to the heterogeneity of EGR2-related peripheral neuropathies and provides strong functional evidence for the pathogenicity of the p.Asp411Gly EGR2 variant.

Charcot-Marie-Tooth (CMT) neuropathy is group of degenerative motor and sensory peripheral neuropathies which are clinically and genetically heterogenous. Pathogenic variants in over 90 genes cause CMT, and whole-exome sequencing (WES) is now an effective tool for screening known causative genes in unsolved CMT families. Pathogenic variants in the EGR2 gene (early growth response 2) cause a broad spectrum of peripheral neuropathy phenotypes. This includes two forms of severe early-onset peripheral neuropathy, Dejerine-Sottas neuropathy (DSN/CMT3)1-3 and congenital hypomyelinating neuropathy (CHN/CMT4E)4,5, as well as adult-onset demyelinating CMT1D with mild-moderate symptoms3-12 and variable-onset axonal CMT with varied symptom severity13,14.

EGR2 encodes a C2H2-type zinc-finger transcription factor that regulates the expression of genes involved in the formation and maintenance of myelin, including GJB1 (gap junction beta 1), MPZ (myelin protein zero), MBP (myelin basic protein), MAG (myelin associated glycoprotein), PRX (periaxin), and PMP22 (peripheral myelin protein 22)4,15-18. Approximately half of the reported disease-associated EGR2 variants are de novo and are associated with a severe phenotype15. Here, we report a sporadic de novo EGR2 variant in the third zinc-finger domain, c.1232A > G p.Asp411Gly, discovered through WES candidate gene screening. This variant manifested as a severe DSN phenotype with early onset at two years of age.

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**Results**

**Clinical history and neurological examination.** The proband is a 56-year-old male who was diagnosed with DSN at six years of age following a nerve biopsy (results not available). He had no family history of peripheral neuropathy, and no family consanguinity. His father and mother had normal nerve conduction studies (NCS) in their fifties. His eldest sister also had a normal neurological examination and NCS at age 25, and the proband reported that this sister has not developed symptoms of peripheral neuropathy in her fifties.

At two years of age, his mother noted that he was consistently fatigued and was repeatedly falling when attempting to walk. He also reported loss of sensation in his feet in early childhood and was never able to run. He was not a candidate for ankle-foot orthoses, and subsequently had a triple arthrodesis at age 15 to stabilise his left ankle. He was also diagnosed with scoliosis and had a Harrington rod inserted at age 16 to correct this. During his teenage years, he had multiple unexplained episodes of hemiparesis and double vision after severe migraine pain behind his eyes, and these episodes would last for approximately two weeks. He reports his mother and sister also suffered from frequent severe migraines, particularly between the ages of 30–45.

On examination at the age of 31, he presented with muscle wasting of the hands and feet, as well as pes cavus, hammer toes, corns, and callouses. He walked with a high steppage gait. He had mildly weak hip flexion, and mildly weak ankle dorsiflexion (3/5). He had absent reflexes in the knees and ankles, with distal loss of vibration sense to the knees. There was loss of pain sensation to above the ankles and decreased joint position sense in the toes. He had a tremor of the hands, with weakness of wrist and finger extension. NCS conducted when the patient was 36 years old (Table 1) showed a severe demyelinating motor and sensory neuropathy.

Autonomic testing conducted at this time showed no postural hypotension, normal cardiovascular reflexes, and normal sudomotor reflexes in both upper and lower limbs indicating no significant autonomic neuropathy. An audiogram showed mild bilateral hearing loss above 1500 Hz, and he also reported occasional tinnitus. Right and left ear brainstem auditory evoked potential (BAEP) waveforms were poorly formed with a bilateral delay in wave V. Right and left full and half field visual evoked potentials (VEP) showed delayed P100 waveforms. An MRI of the brain showed multiple subcortical and periventricular white matter hyperintensities.

On re-examination at age 52, NCS of the left median sensory, left median motor, and left peroneal motor nerves showed no response. He also reported difficulty opposing his thumb and fingers, and a progressive loss of sensation in his right hand. On examination, there was slight curling of the fingers and wasting of the intrinsic hand muscle. Dorsiflexion in his fingers was mildly weak (grade 3/5), as was his finger abduction (grade 3/5). An additional NCS conducted 12 months later additionally showed no response of the left ulnar sensory nerve, and a severely reduced latency of 19.5 m/s in the left ulnar motor nerve. Four years later at the age of 56, his ability to oppose his thumb and fingers had worsened, and he reported that he was unable to write, or use a knife and fork. He additionally had allodynia below his knees. Another brain MRI was conducted at this time (Fig. 1),

| Nerve         | Stimulation site | Recording site | Latency (m/s) | Amplitude (mV) |
|---------------|-----------------|----------------|---------------|----------------|
| Motor         |                 |                |               |                |
| R. median     | Wrist           | APB            | 29.2 (N < 6.2) | 1.2 (N > 3)   |
| R. common peroneal | Ankle       | EDB            | No response   |                |
| R. tibial     | Ankle           | AH             | 21.9 (N < 4.6) | 0.5 (N > 3)   |
| Sensory       |                 |                |               |                |
| R. sural      | Calf            | Ankle          | No response   |                |
| R. median     | Digit 2         | Wrist          | No response   |                |

Table 1. Nerve conduction study conducted demonstrated a severe motor and sensory demyelinating neuropathy. Normal values are indicated in brackets next to the value obtained. APB: abductor pollicis brevis; AH: abductor hallucis; POP FOS: popliteal fossa.

Figure 1. Axial (a,b) and Sagittal (c) T2 weighted images demonstrated multiple white matter hyperintensities, predominantly periventricular in location (red arrows). There were no contrast enhancing lesions suggesting active disease.

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both known to cause FMH. ATP1A2 or SCN1A genes, which are FMH-affected eldest sister. Additionally, the proband had no variants in the is likely pathogenic. c.6692G CACNA1A can also cause an FMH phenotype in addition to an inherited neuropathy, segregation of the amino acid substitution p.Asp411Gly. Unreported peripheral neuropathy phenotype. These results confirmed that the proband is heterozygous for a previously reported in variant databases including NCBI dbSNP20, 1000 Genomes21, gnomAD22, and ExAC23. Three additional variants were also considered given their low minor allele frequency (MAF <0.1%) (CACNA1A, c.6692G > A, NM_023035.2; BAG3 c.494C > T; NM_004281.3; and ITPR1 c.6692A > G, NM_001168272.1). Sanger sequencing confirmed the heterozygous EGR2 c.1232A > G variant in the proband (Fig. 2a), which was absent in the unaffected mother, father, and sister (Fig. 2b). The variants CACNA1A c.6692G > A (NM_023035.2), BAG3 c.494C > T (NM_004281.3), and ITPR1 c.6692A > G (NM_001168272.1) did not segregate with the peripheral neuropathy phenotype. These results confirmed that the proband is heterozygous for a previously unreported de novo variant in the third zinc-finger domain of EGR2 [chr10: 62,813,406T > C (hg38)], leading to the amino acid substitution p.Asp411Gly. Due to the additional phenotype of hemiplegic migraine in our proband, as well as migraine in his mother and eldest sister, variant filtering of WES was also conducted for genes related to familial hemiplegic migraine (FMH). As variants in CACNA1A can also cause an FMH phenotype in addition to an inherited neuropathy, segregation of the CACNA1A c.6692G > A variant was conducted. However, it was present in the mother and absent in the FMH-affected eldest sister. Additionally, the proband had no variants in the ATP1A2 or SCN1A genes, which are both known to cause FMH.

**In silico analyses show that p.Asp411Gly EGR2 affects a highly conserved residue and that it is likely pathogenic.** Amino acid sequence alignment demonstrates that the p.Asp411Gly variant occurs at a highly conserved residue in EGR2 with surrounding amino-acid residues being conserved between orthologues in different vertebrate species (Fig. 3). Multiple in silico analysis techniques were utilised to predict the conservation of the Asp411 amino-acid residue and pathogenicity of the p.Asp411Gly variant. GERP24, phastCons25, and PhyloP26 scores all provided further support that this amino-acid residue is highly evolutionarily constrained (Table 2). The functional effect of the p.Asp411Gly variant was predicted to be damaging by Polyphen227, PROVEAN28, SIFT29, and MutationTaster223 (Table 2).

p.Asp411Gly EGR2 has reduced transcriptional regulatory activity compared to wild-type EGR2 in cultured schwann cells. To determine the relevant functional consequences of the p.Asp411Gly EGR2 variant, the ability of wild-type and p.Asp411Gly EGR2 to induce activity of a previously reported EGR2 response element at the PMP22 locus was tested in a Schwann cell line.11 A construct expressing wild-type or p.Asp411Gly EGR2 was transfected into cultured rat Schwann (S16) cells along with a construct harbouring an EGR2 response element upstream of a minimal promoter25 and a firefly luciferase (Fluc) reporter gene. To control

**Figure 2.** (a) Sequencing traces of the variant c.1232A > G EGR2 in the affected proband (II:2) and the wild-type sequence in the unaffected father (I:1). Genbank sequence NM_000399.3 was used as a reference for the EGR2 coding sequence. (b) Pedigree of the two-generation kindred and associated genotypes. Solid square denotes the affected male, an open square denotes an unaffected male, and an open circle denotes an unaffected female. An asterisk indicates individuals sent for Sanger sequencing of the EGR2 c.1232A > G variant.
for cell viability and transfection efficiency, a pCMV-Renilla luciferase (Rluc) construct was also transfected. The fold induction of Fluc, measured as the ratio of Fluc activity to Rluc activity, was employed to test the ability of EGR2 (wild-type or p.Asp411Gly) to activate the PMP22 enhancer. These assays demonstrated that wild-type EGR2 was able to increase expression of Fluc (~110 fold increase over the empty vector; Fig. 4) consistent with previous reports that the PMP22 enhancer includes an EGR2 response element. In contrast, p.Asp411Gly EGR2 induced Fluc activity at a significantly lower level (~40 fold increase over the empty vector $p = 2.1 \times 10^{-13}$ compared to wild-type; Fig. 4) indicating that the p.Asp411Gly variant reduces the capacity for EGR2 to appropriately activate the PMP22 enhancer. These results reveal a functional effect of p.Asp411Gly EGR2 and support the pathogenicity of this variant.

### Discussion

The spectrum of peripheral neuropathy associated with disease-causing EGR2 variants ranges from severe early onset DSN and CHN, to later onset demyelinating CMT1 and axonal CMT2. Using WES and functional testing we have determined that a de novo missense EGR2 variant, c.1232A > G p.Asp411Gly, causes a severe and early onset DSN phenotype by reducing the capacity for EGR2 to function as a transcription factor. This variant can be classified as ‘pathogenic’ according to guidelines determined by the American College of Medical Genetics and Genomics (PS2, PS3, PM1, PM2, PP2, PP3)33. This pathogenic variant affects the third zinc-finger domain of EGR2, which is highly conserved among species and is a DNA-binding motif crucial for its role as a transcription factor in the regulation of myelin genes34. All reported pathogenic variants are within these three zinc-finger regions except for p.Ile268Asn, which causes recessive CHN and is within an inhibitory domain (Table 3)4.

Functional testing conducted here with luciferase-based reporter assays demonstrated that p.Asp411Gly EGR2 activates transcription less effectively than wild-type EGR2, similar to what has been previously reported for pathogenic EGR2 variants located in the zinc-finger domains13,15,34,35. EGR2 function is crucial for myelination of the peripheral nervous system, and Krox20$^{-/-}$ (murine EGR2 orthologue) mice demonstrated arrested

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**Table 2.** Multiple in silico analyses support the pathogenicity of the EGR2 p.Asp411Gly variant. $^a$GERP scores above +2 are considered evolutionarily constrained (−11 to +6). $^b$phastCons scores approaching 1 are predicted to be highly conserved (0.00 to 1.00). $^c$phyloP values that are positive are predicted to be evolutionarily conserved (−14 to +6). $^d$Polyphen2 scores closer to 1 are predicted to be damaging to protein function (0.00, benign to 0.999, damaging). $^e$Provean scores describe the effect of the protein variation as deleterious or neutral (−13 to 4). $^f$SIFT scores predict the functional impact of a variant (0.00, tolerated to 1.00, damaging). $^g$MutationAssessor describes the predicted functional impact of variant (predicted functional: high or medium to predicted non-functional: low or neutral). $^h$MutationTaster2 predicts the functional impact of a variant: disease causing (probably deleterious), disease causing automatic (previously reported as deleterious), polymorphism (predicted to be non-pathogenic), polymorphism automatic (previously reported as non-pathogenic).
differentiation of Schwann cells at an early stage. Interestingly, heterozygous Krox20<sup>+/−</sup> mice are phenotypically normal, yet all reported pathogenic EGR2 variants in the zinc-finger domains cause peripheral neuropathy in a heterozygous state (Table 3). This is likely explained by the finding that in addition to pathogenic EGR2 variants resulting in a decrease or loss of EGR2 function, it has been shown that pathogenic EGR2 variants in all three zinc-finger domains (p.Arg359Trp, and p.Ser382Arg, p.Asp383Tyr, and p.Arg409Trp) result in the dominant-negative inhibition of wild-type EGR2<sup>15,18</sup> and SOX10<sup>18</sup>. These results are consistent with the reduction in transcriptional activation we observed in our luciferase assays with p.Asp411Gly EGR2.

The severity and onset of peripheral neuropathy caused by EGR2 variants is highly heterogeneous, and it is yet to be determined why this broad phenotypic variability exists. This variation is exemplified by the p.Arg359Trp variant, which causes demyelinating CMT1D<sup>3</sup>, DSN<sup>5,36</sup>, and CHN<sup>5</sup>. This is also true for different variants of the amino acid adjacent to the p.Asp411Gly variant we have reported here, where p.Glu412Gly causes both demyelinating CMT1D<sup>12</sup> and axonal CMT1<sup>4</sup>, and p.Glu412Lys causes DSN<sup>35</sup>. Given these observations, it is possible that additional genetic factors exist which modify the phenotype of EGR2 variant, such as those recently reported for CMT1A<sup>37</sup>.

Our patient had findings on brain MRI and evoked potentials that may be consistent with multiple sclerosis, however it was determined that he did not fulfill diagnostic criteria. White matter lesions have been reported in some patients with CMT<sup>38–41</sup>, and the proband had no contrast enhancing lesions suggesting active demyelinating disease in both brain MRIs conducted. In CMTX1, which is caused by pathogenic variants in GJB1, white matter lesions were reported in 7.5% of a large cohort<sup>42</sup>. Given that EGR2 mediates transcription of GJB1, it is possible that the reduced efficiency of p.Asp411Gly EGR2 in activating transcription resulting in a phenotype similar to that seen in some CMTX1 patients. Additionally, the FMH phenotype may be a chance association, or due to another genetic cause that has not been identified, as there were no variants in hemiplegic migraine genes that segregated with the phenotype. White matter lesions have also been reported in patients with migraines and patients with sporadic hemiplegic migraine<sup>43–46</sup>, further complicating the phenotype seen in our proband.

In this study we report a novel de novo EGR2 variant, c.1232A>G p.Asp411Gly, as the cause of severe, early-onset CMT3 (DSN). We provide robust functional data to support the deleterious functional effect of this mutation. The phenotype is complicated by clinical features of white matter lesions and FMH which may be a chance association, although it would be important to consider the possibility of central nervous system involvement in future cases of EGR2-related neuropathies.

**Methods**

**Subjects.** The proband and three family members were recruited and informed consent was obtained for this study using protocols approved by the Sydney Local Health District Human Ethics Research Committee (SLHD HERC). All experiments were performed in accordance with relevant guidelines and regulations from SLHD HREC. Genomic DNA was extracted from peripheral blood using the PureGene Kit (Qiagen) following manufacturer's instructions.

**Whole-exome sequencing (WES).** WES was performed on genomic DNA (2.5 μg) in the proband and was outsourced to Macrogen (South Korea) as previously described<sup>19</sup>.
Bioinformatic analysis. Evolutionary conservation of the EGR2 D11 amino acid residue was conducted using GERP (http://mendel.stanford.edu/sidowlab/downloads/gerp/index.html), phastCons and PhyloP (http://compgen.cshl.edu/phastweb/runtool.php). The predicted functional effect of the EGR2 p.Asp411Gly mutation was assessed using Polyphen2 (http://genetics.bwh.harvard.edu/pph2/), PROVEAN (http://provean.jcvi.org/seq_submit.php), SIFT (https://sift.bii.a-star.edu.sg/www/SIFT_seq_submit2.html), MutationAssessor (http://mutationassessor.org/r3/), and MutationTaster2 (http://www.mutationtaster.org/).

Sanger sequencing. All primers and PCR conditions are available upon request. PCR amplicons were sent to Garvan Molecular Genetics, Garvan Institute (Sydney, Australia) for Sanger sequencing using BigDye Terminator cycle sequencing protocols and visualised using Sequencher 2.3 software (Gene Codes Corporation).

Haplotype analysis. PCR amplicons from microsatellite markers (D1S347, D1S249, D2S2228, D2S140, D16S519) were sent for fragment analysis at Garvan Molecular Genetics, Garvan Institute (Sydney, Australia), and visualised using GeneMarker (SoftGenetics). Microsatellite marker size was determined using GS600 size standard.

Luciferase reporter gene expression constructs. Mutagenic primers were designed to model the p.Asp411Gly EGR2 mutation in an expression construct containing the mouse EGR2 open-reading frame directed by the CMV promoter (gift of John Svaren, University of Wisconsin). Site-directed mutagenesis was performed using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) and the manufacturer’s instructions. Mutant constructs were verified by Sanger sequencing (University of Michigan Medical School DNA Sequencing Core).

Cell culture, transfection, and luciferase assays. Cultured rat Schwann cells (S16) were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% (v/v) fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco), 50 U/mL penicillin, and 50 g/mL streptomycin. For luciferase assays, ~1 × 10^6 S16 cells were plated in each well of a 96-well untreated cell culture plate and grown overnight at 37 °C with 5% CO₂. After 24 h, the cells were transfected with the experimental constructs using Lipofectamine 2000 (Invitrogen) diluted 1:100 in OptiMem (Life Technologies). Cells in each well received 200 ng of an expression construct: either empty pEIB (expression vector containing a Fluc reporter gene with no genomic insert) or the pEIB-Forward vector with a PMP22 enhancer upstream of a Fluc reporter gene. S16 cells were co-transfected with 100 ng per well of wild-type or mutant EGR2 constructs in addition to the PMP22 enhancer construct. For an internal control for transfection efficiency, 2 ng of a pCMV-Rluc construct were transfected into cells in all wells. Each experimental construct was diluted in OptiMem and incubated with an equal volume of Lipofectamine 2000 in OptiMem for

| Domain | Variant | Phenotype | Reference |
|--------|---------|-----------|-----------|
| NA1 repressor binding site | p.Ile268Asn | CHN | Warner et al., 19984 |
| Zinc-finger 1 | p.Arg355Gly | CMT | Nakamura et al., 201246 |
| | p.Asp355Val | CMT | Bellone et al., 19994 |
| | p.Asp355Gly | CMT | Wang et al., 201647 |
| | p.Arg359Gln | CMT | Mikešová et al., 20013 |
| | p.Arg359Trp | CHN | Timmerman et al., 19991 |
| | p.Arg381Cys | CMT | Britani et al., 201011 |
| | p.Arg381Leu | CMT | Wang et al., 201647 |
| | p.Arg381His | CMT | Pareyson et al., 20001 |
| | p.Asp383Tyr | DSN | Numakura et al., 2003 |
| Zinc-finger 2 | p.Ser382Arg+p.Asp383Tyr | CHN | Warner et al., 19984 |
| | p.Thr387Asn | CMT | Shiga et al., 201210 |
| | p.Arg409Trp | CMT | Warner et al., 19984 |
| | p.Arg409Gln | CMT | Leonardi et al., 2019 |
| | p.Asp411Gly | DSN | This study |
| | p.Glu412Gly | CMT | Saka Brzózková et al., 2012 |
| | p.Glu412Lys | CMT | Tozza et al., 201914 |

Table 3. Pathogenic EGR2 variants are clinically heterogeneous, and there is no apparent association between the affected EGR2 domain and the resultant phenotype.
20 min at room temperature before being applied to cells. Cells were incubated with transfection reagents for 4 h at 37 °C with 5% CO₂, at which point the transfection reagents were removed and replaced with standard growth medium (above) and allowed to grow for 48 h at 37 °C with 5% CO₂.

After 48 h, growth medium was removed, and cells were washed with 1X PBS. The cells were then lysed for 1 h at room temperature in 1X Passive Lysis Buffer (Promega), and 10 uL of lysate from each well was transferred into a white polystyrene 96-well plate (Corning). A Dual Luciferase Assay was performed using a GloMax Multi-Detection System and a Dual Luciferase Reporter 1000 Assay System kit (Promega) to determine Fluc and RLuc activities.

The ratio of Fluc to RLuc activity in each well was calculated, and ratios from individual wells were normalized relative to the average Fluc to RLuc ratio from the empty pEIB vector. The mean normalized ratio is a readout for the fold induction of Fluc by each experimental construct, and this is shown with standard deviation in the figure. Each variant was tested with two independently generated constructs (A and B in the figure) to rule out expression changes due to variation in the expression construct backbone, and each construct was tested in at least 24 individual experiments. Statistical significance was assessed using a two-tailed Student’s T-test.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Received: 2 October 2019; Accepted: 3 December 2019;
Published online: 18 December 2019

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Acknowledgements
This work was supported by the Australian National Health and Medical Research Council project grant (APP1046690) awarded to M.L.K. and G.A.N. A.A. was supported by the National Institute of Neurological Disorders and Stroke (NS073748). We wish to acknowledge A/Prof. Alastair Corbett for conducting the nerve conduction study. We thank Prof John Saren (University of Wisconsin) for the wild-type EGR2 expression construct. We thank the family members participating in this study.

Author contributions
B.G., N.G. and M.E. carried out the experiments. G.N. and K.K. collected patient data. A.A. and M.K. supervised the project. M.K. conceived the original idea. B.G. wrote the manuscript with input from all authors.

Competing interests
The authors declare no competing interests.

Additional information
**Supplementary information** is available for this paper at [https://doi.org/10.1038/s41598-019-55875-4](https://doi.org/10.1038/s41598-019-55875-4).

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