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## Exome Sequencing Identifies a Novel TRPV4 Mutation in a CMT2C Family

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**Exome sequencing identifies a novel TRPV4 mutation in a CMT2C family**

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Guida Landouré contributed to patient characterization, completed some sequencing, mutagenesis and cell death assays, and drafted the manuscript.

Jeremy M. Sullivan completed cell death assays and cell calcium imaging.

Janel O. Johnson performed exome sequencing.

Clare H Munns performed cell calcium imaging.

Yijun Shi performed sequencing and cloning.

Oumarou Diallo sequenced controls.

Raphael J. Gibbs analyzed exome sequencing data.

Rachelle Gaudet performed the TRPV4 structural analyses.

Christy Ludlow contributed to patient recruitment and laryngeal evaluations.

Kenneth H. Fischbeck examined patients and interpreted genetic analysis data.

Bryan J. Traynor performed exome sequencing.

Barrington G. Burnett designed experiments and completed mutagenesis and cell death assays.

Charlotte J. Sumner directed the study, examined patients, performed cell death assays and completed the manuscript.

Authors report no disclosure. Bryan J. Traynor is a member of the editorial board of *Neurology*.

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## Introduction

Mutations in over 50 genes have been associated with Charcot-Marie-Tooth (CMT) disease. As new genes continue to be discovered, the task of sequencing each of them individually with traditional techniques becomes increasingly burdensome. In addition, these approaches have disadvantages that may reduce their sensitivity. Exome sequencing uses targeted sequence capture of exons and next-generation sequencing to evaluate the sequence of all exons simultaneously. Here, we report a family with the CMT2C phenotype in whom exome sequencing revealed a novel mutation in the *transient receptor potential vanilloid 4 (TRPV4)* gene missed by previous Sanger sequencing.<sup>1</sup>

## Methods

### Standard Protocol Approvals, Registrations, and Patient Consents

Subjects were evaluated after giving written consent to an NINDS Institutional Review Board-approved protocol. DNA extracted from peripheral blood was sequenced on a Genome Analyzer IIx platform (Illumina, CA). Sequence variants were confirmed by Sanger sequencing using newly-designed primers.

### Cellular studies

Calcium imaging and cell death assays were performed using transiently-transfected HEK293 cells as previously described.<sup>1</sup> Statistical significance was determined using two-tailed unpaired t-tests.

## Results

### *Exome sequencing reveals a novel mutation in a family with CMT2C*

We evaluated nine family members (figure, A), three of whom exhibited features consistent with the CMT2C phenotype,<sup>1,2</sup> including progressive distal limb muscle weakness and atrophy, hoarseness of voice, and stridor on exertion (Supplemental Table). Nerve conduction studies confirmed an axonal neuropathy with phrenic nerve involvement; laryngoscopy showed reductions in vocal fold opening and closing. Two individuals had scoliosis, but skeletal dysplasia was not evident on bone radiographs. One individual had bilateral sensorineural hearing loss.

Previous Sanger sequencing failed to identify *TRPV4* mutations in this family (figure, B), designated Family 3.<sup>1</sup> In the present study, we performed exome sequencing in one affected individual and, surprisingly, identified a novel sequence variant (c.557G>A) in *TRPV4*. No significant variants were identified in other CMT-causing genes. A review of the Sanger sequencing primers used to amplify this region of *TRPV4* revealed that the forward and reverse primers each contained a single nucleotide polymorphism (SNP). Further Sanger sequencing with newly-designed primers revealed the c.557G>A sequence change in all three individuals with CMT2C, but not in the six unaffected individuals (figure, B), nor in 200 controls. This nucleotide change results in an arginine to glutamine substitution at amino acid 186 (R186Q), a highly conserved residue of the TRPV4 protein (figure, C), a calcium-permeable cation channel. Like previously described neuropathy-causing TRPV4 mutations, R186 is an exposed arginine residue situated on the convex face of the TRPV4 ankyrin repeat domain (ARD) (figure, D),<sup>1,2</sup> a domain mediating regulatory interactions.<sup>3</sup>

### ***Expression of R186Q-TRPV4 causes cellular toxicity***

Neuropathy-causing TRPV4 mutations (e.g. R269C) have been shown to increase TRPV4 channel activity, leading to elevated intracellular calcium levels and cellular toxicity.<sup>1</sup> Here, we examined HEK293 cells expressing wild-type (WT), R186Q and R269C forms of TRPV4. As previously reported, cells expressing R269C-TRPV4 showed significantly increased basal calcium levels (figure, E) and cellular toxicity (figure, F; Supplemental Figure) compared to cells expressing WT-TRPV4. Cells expressing R186Q-TRPV4 showed a slight increase in calcium levels at 24 hours and significantly increased cell death at 36 hours (figure, E and F; Supplemental Figure). Both effects were completely abrogated by the TRP channel antagonist ruthenium red (figure, E and F) suggesting the R186Q mutation increases constitutive TRPV4 activity.

## Discussion

CMT2C and forms of distal spinal muscular atrophy are caused by dominant missense mutations in *TRPV4*.<sup>1,2,4-6</sup> *TRPV4* mutations also cause forms of autosomal dominant skeletal dysplasia.<sup>7</sup> Here, we report a novel *TRPV4* mutation in a family with the CMT2C phenotype and no evidence of skeletal dysplasia. The localization of the R186Q substitution to the convex face of the TRPV4 ARD, a face on which skeletal dysplasia-causing mutations have not been described, provides further evidence that this region is of particular importance to TRPV4 function in the peripheral nervous system. Importantly, this mutation was not initially detected by traditional Sanger sequencing, but was identified by exome sequencing, a technology being used increasingly to detect mutations in Mendelian diseases. The initial Sanger sequencing primers used to amplify the relevant *TRPV4* genomic region overlie known SNPs that may be in linkage disequilibrium with the mutation, likely leading to monoallelic amplification of the WT allele.

Exome sequencing technology uses multiple sequence alignments of the same gene fragment, thus avoiding the false negatives that can be encountered during Sanger sequencing.

The identification of the R186Q mutation in *TRPV4* by exome sequencing both highlights the importance of the ARD in TRPV4 function in the peripheral nervous system and emphasizes the advantages of rapidly emerging sequencing technologies.

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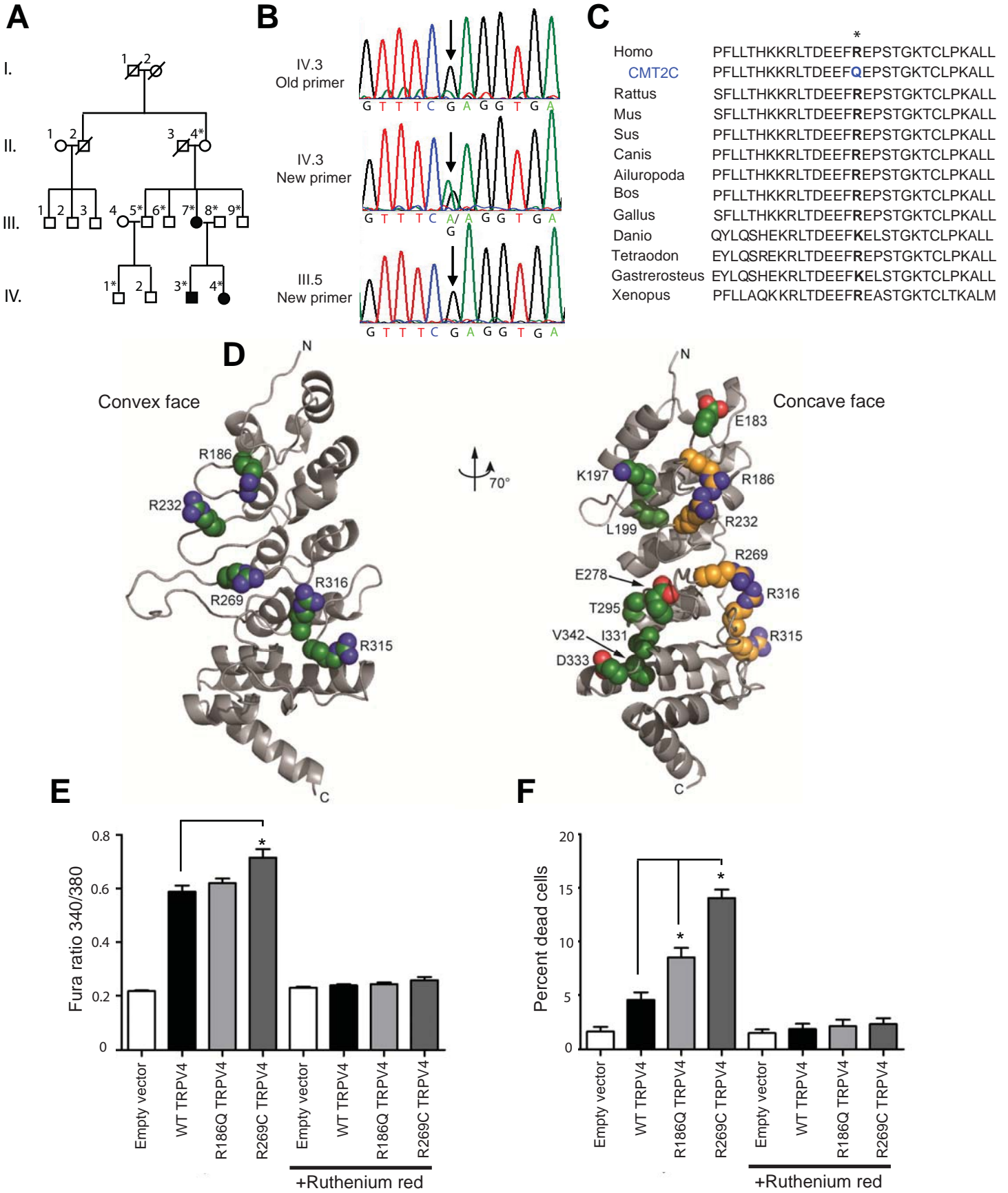
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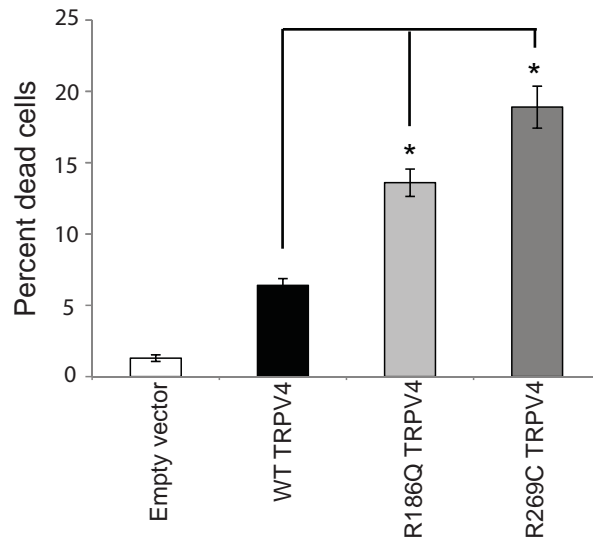
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**Figure legend**

A family with CMT2C has a novel mutation in *TRPV4*. **(A)** Pedigree of the family showing vertical transmission of the disease (white=unaffected, black=affected, \*DNA collected). **(B)** Chromatographs showing the c.557G>A mutation initially missed by Sanger sequencing and later identified by exome sequencing in an affected individual. This sequence variant was not apparent using the original primers (top; the “A” variant peak is comparable to the background noise), but became clearly visible when primers that do not contain SNPs were used to amplify the genomic DNA (middle). The sequence variant is not present in unaffected individuals (bottom). **(C)** The R186 residue in TRPV4 is highly conserved across a wide range of species. **(D)** Like previously described neuropathy-causing TRPV4 mutations, the R186 residue is situated on the convex face of the TRPV4 ARD. In contrast, those skeletal dysplasia mutations occurring in the TRPV4 ARD localize to its concave face (at left: green=neuropathy mutations; at right: yellow=neuropathy mutations, green=skeletal dysplasia mutations). **(E)** Basal calcium levels are modestly increased in HEK293 cells expressing R186Q-TRPV4 and significantly increased in cells expressing R269C-TRPV4, compared to cells expressing WT-TRPV4 for 24 hours. The TRP channel antagonist ruthenium red completely blocks this calcium influx (\* $p=0.05$ ,  $n=8$  per group). **(F)** Approximately 10 % of cells expressing R186Q-TRPV4 and 15 % of cells expressing R269C-TRPV4 compared to approximately 5 % of cell expressing WT-TRPV4 were dead at 36 hours post-transfection (\* $p=0.01$ ,  $n=3$  per group). Treatment with ruthenium red completely blocked cellular toxicity. Cells were incubated with 2  $\mu\text{M}$  calcein AM and 4  $\mu\text{M}$  EthD-1 for 30 minutes at room temperature and the percentage of dead cells calculated using a Zeiss AxioImager Z1 microscope and AxioVision 4.6 software.





**Supplemental Figure.** Cells expressing mutant R186Q TRPV4 show increased rates of cell death. In cell death assay#2 (see Methods), approximately 14% of cells expressing R186Q TRPV4 and 19% of cells expressing R269C TRPV4 compared to approximately 6% of cell expressing WT TRPV4 were dead at 36 hours (\*p=0.01, n=6 per group).