Mutations in the Novel Mitochondrial Protein REEP1 Cause Hereditary Spastic Paraplegia Type 31

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Hereditary spastic paraplegia (HSP) comprises a group of clinically and genetically heterogeneous diseases that affect the upper motor neurons and their axonal projections. For the novel *SPG31* locus on chromosome 2p12, we identified six different mutations in the receptor expression–enhancing protein 1 gene (*REEP1*). *REEP1* mutations occurred in 6.5% of the patients with HSP in our sample, making it the third-most common HSP gene. We show that REEP1 is widely expressed and localizes to mitochondria, which underlines the importance of mitochondrial function in neurodegenerative disease.

In hereditary spastic paraplegia (HSP), the degeneration of corticospinal tract axons leads to progressive lower-limb spastic paralysis. Traditionally, HSP types have been divided into pure and complicated forms, which are characterized by additional symptoms such as mental retardation, epilepsy, cerebellar ataxia, or optic atrophy.[1] Genetic studies have revealed as many as 31 different chromosomal HSP loci. Five genes have been identified for autosomal dominant subtypes.[2] Mutations in the genes *spastin* (*SPG4*) and *atlastin* (*SPG3A*) account for up to 50% of all HSP cases. Mutations in *KIF5A* (MIM 602821), *HSP60* (MIM 118190), and *NIPA1* (MIM 608145) each occur in <1% of HSP cases.[3,4]

Elsewhere, we performed a genomewide linkage study and identified a "pure" HSP locus at chromosome 2p12 (*SPG31*).[5] Two families, DUK2036 and DUK2299, yielded a combined two-point LOD score of 4.7 at marker *D2S2951*. Fine-mapping and haplotype analysis narrowed the locus to ~8.8 Mb between *D2S139* and *D2S2181* (fig. 1*A*). We chose nine candidate genes (*CTNNA2, SUCLG1, TGOLN2, MATA2A, VAMP8, VAMP5, IMMT, VPS24,* and *REEP1* [MIM 609139]) on the basis of emerging pathways for spastic paraplegia and conserved protein domains contained in proteins that cause neurodegenerative diseases.[6] Of those genes, we sequenced all exons, including 40 bp of flanking intronic and UTR sequences. We identified a single base-pair deletion, c.507delC, in family DUK2299 and a splice-site mutation, c.182-2A→G, in family DUK2036 in the receptor expression–enhancing protein 1 gene (*REEP1*), also known as "*C2orf23*" (fig. 1*B* and 1*C*). These sequence changes resulted in frameshifts leading to altered stop codons. The mutations cosegregated with the disorder in the linked pedigrees (fig. 2). REEP1 (GenBank accession number NP_075063) belongs to a novel protein family that was identified only recently.[7] Its subcellular location and molecular function are largely unknown. We screened for additional REEP1 mutations in a sample of 90 independent HSP-affected families of European descent, neither selected for pure HSP phenotype nor tested for mutations in other HSP genes. All individuals were studied under internal review board–approved procedures. We identified four more mutations that led to significant sequence changes in *REEP1*: one missense mutation (c.59C→A; Ala20Glu), one deletion (c.526delG; Gly176fs), and two 3′ UTR changes (c.606+43G→T and c.606+50G→A) (fig. 1*B* and 1*C* and table 1). All mutations cosegregated with the HSP phenotype in the available pedigrees and were undetected in 365 control individuals of European descent (730 chromosomes) (fig. 2).

The splice-site mutation in linked family DUK2036 disrupted the canonical 5′ acceptor splicing signal "AG" of exon 4 (fig. 1*B* and 1*C*). REEP1 was not expressed in peripheral-blood cells, and neuronal tissue was not available from affected family members (fig. 3*K*). Thus, we could not demonstrate the aberrant mRNA (GenBank accession number NM_022912), but simulation of the resulting splice efficiency with NNSPLICE revealed a reduction from 99% efficiency of the wild-type AG allele to 0% of the mutant GG allele. Missplicing of exon 4 will result in a frameshift followed by a premature stop codon. The two mutations in the 3′ UTR presented unusual changes for a Mendelian disease. However, both mutations altered the sequence of a predicted highly conserved binding site for the microRNA (miRNA) gene *miR-140* (fig. 1*D* and 1*E*). miRNAs constitute a new large class of small noncoding RNA genes that target protein-coding genes for posttranscriptional repression[8] (see the MicroRNA Registry). The c.606+43G→T mutation in *REEP1* disrupted a G:U wobble base pair, and the c.606+50G→A change replaced a G:U wobble base pair with an A:U Watson-Crick pairing. It has been shown that

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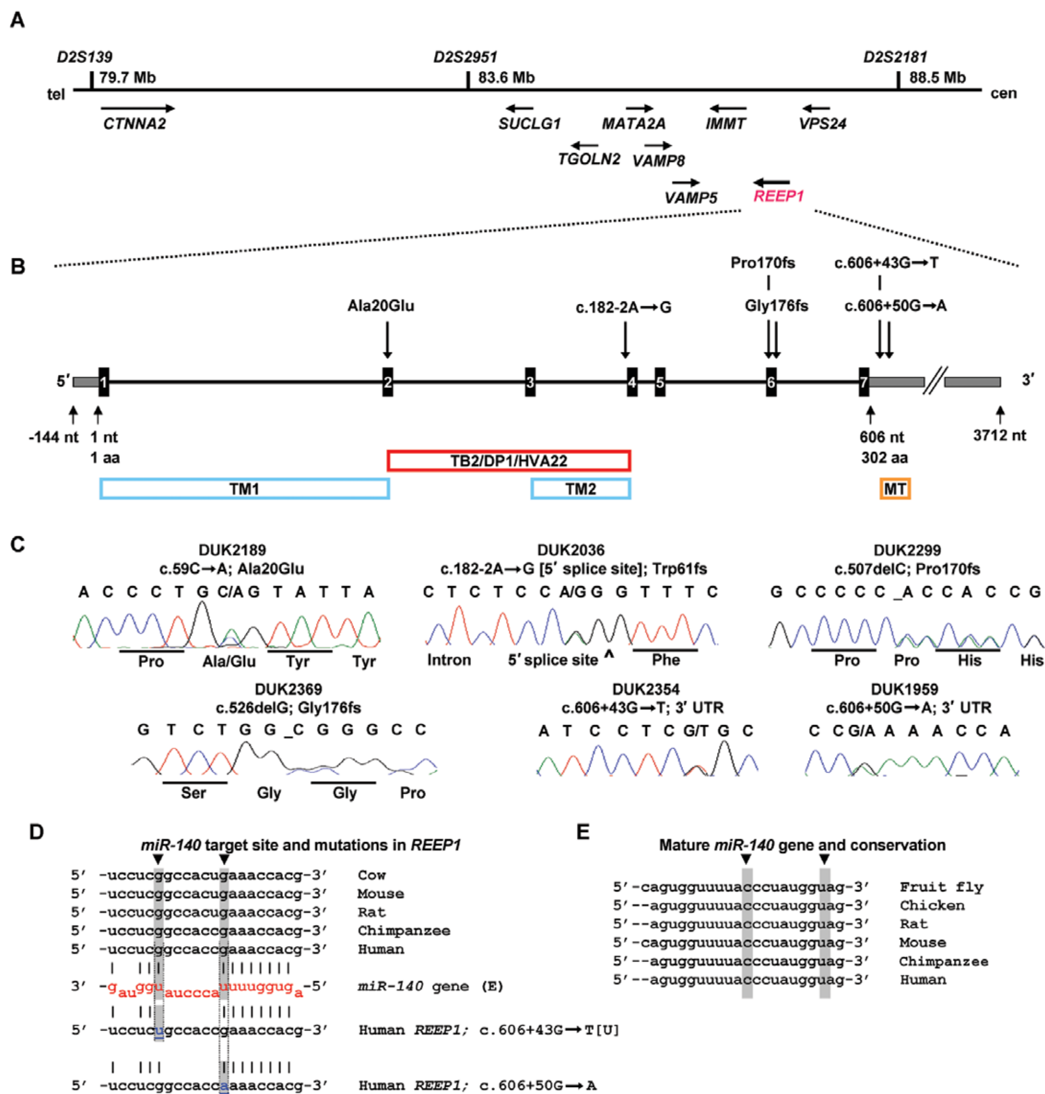

Figure 1. Overview of the *SPG31* locus and identified genetic variation in *REEP1*. *A,* Within the minimal chromosomal *SPG31* region, we screened several genes by direct sequencing analysis. *B, REEP1* consists of seven exons (*black boxes*) and contains two predicted transmembrane domains (TM1 and TM2) and a "deleted in polyposis" domain (TB2/DP1/HVA22). The 3′ UTR comprises a conserved miRNA target site for *miR-140* (MT). *C,* Sequencing traces of the identified unique mutations in six families with uncomplicated HSP. Sequencing analysis was performed using an ABI3730, following standard procedures. PCR primers are available from the authors on request. *D,* The *miR-140* target site in the 3′ UTR is highly conserved. Two mutations within that site change nucleotides that provide G:U wobble base pairing: c.606+43G→U and c.606+50G→A (*gray shading, blue letters,* and *arrows*). *E,* The mature *miR-140* gene is also highly conserved, including the two residues that would bind to the mutated nucleotides in the target site (*gray shading* and *arrows*). cen = centromere; tel = telomere.

G:U wobble base pairing has an inhibitory effect on miRNA-mediated repression of translation.[9] Thus, both detected mutations would foster suppressive miRNA-mediated effects on translation, leading to less available REEP1 protein. Both affected nucleotides are highly conserved in the *REEP1* 3′ UTR as well as in *miR-140* of different species (fig. 1*D* and 1*E*). In addition, it has been shown that *miR-140* is expressed in the cortex of rat and monkey as well as in cultured primary cortical neurons from rat.[10,11] Thus, we suggest that the identified sequence variants will affect the amount of translated *REEP1* in patients with HSP.

The figure is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Figure 2. Different mutations identified in six unrelated HSP-affected families: DUK2369, DUK2354, DUK1959, DUK2036, DUK2189, and DUK2299. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Three of the four coding changes led to alternative stop codons. It is likely that the resulting mRNA will be targeted for nonsense-mediated decay, resulting in haploinsufficiency of the mutant allele. Interestingly, both miRNA target-site mutations disrupted G:U base pairing and are therefore likely to lead to less translated protein. We suggest that loss of function and haploinsufficiency are the mechanisms of action in REEP1-related spastic paraplegia.

Table 1. Clinical and Genetic Features of the Identified Families with *REEP1* Mutations

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

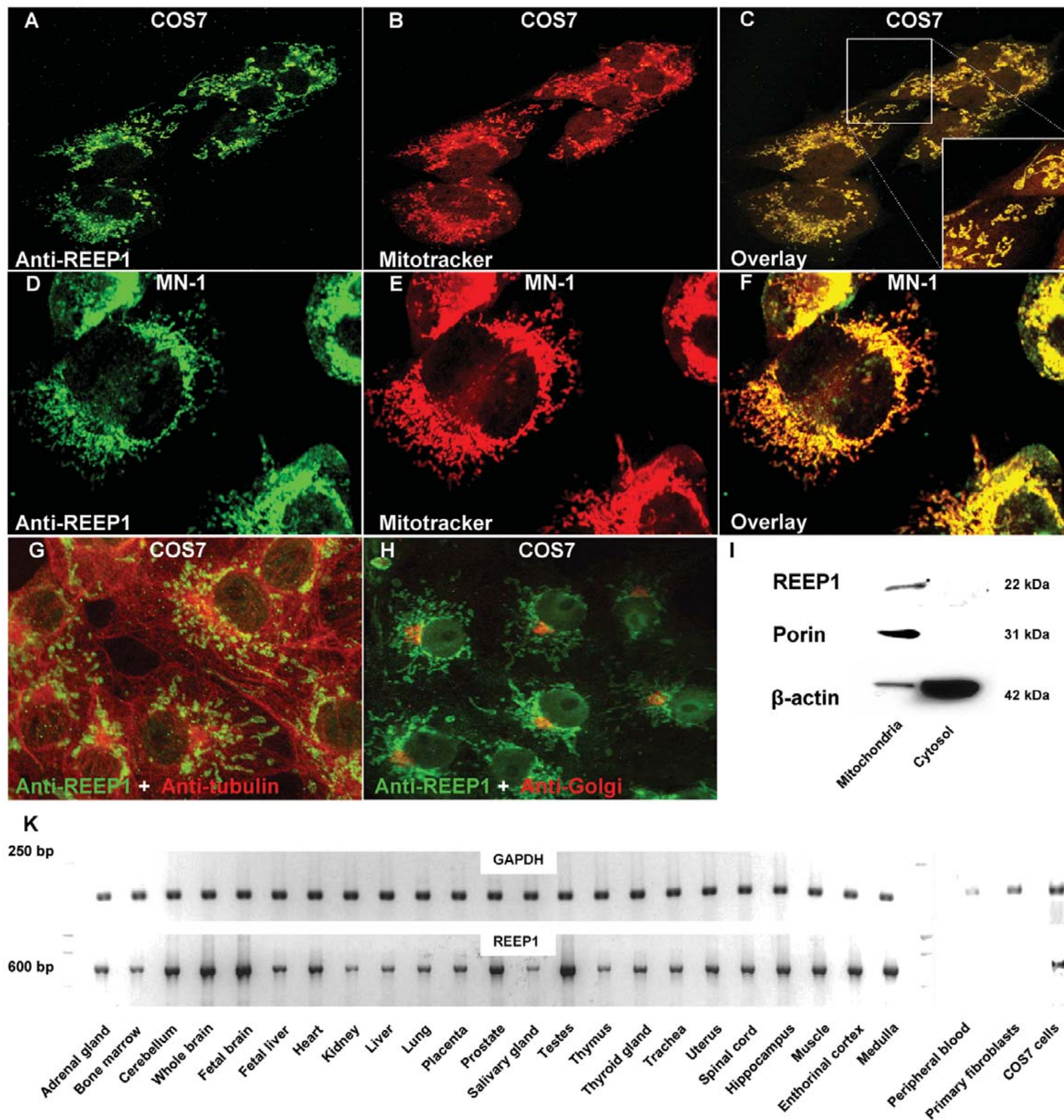

Figure 3. REEP1 localized to mitochondria. *A–F,* Immunohistochemistry with two different REEP1 antibodies revealed colocalization with the marker Mitotracker Red. COS7 (kidney) and MN-1 (motor neuron) cells were maintained in Dulbecco's modified Eagle medium with 10% fetal bovine serum and were incubated at 37°C supplemented with 5% CO_2. Cells were observed by confocal laser scan microscopy (Visitech model VT-Infinity). *G* and *H,* No colocalization was observed with microtubules and Golgi. *I,* COS7 cells were homogenized and mitochondria were separated from the cytosolic fraction by centrifugation. The REEP1 antibodies were probed against COS7 cell lysate on a western blot and showed a band of the expected size in the mitochondrial fraction. The blot was reprobed with porin, which constitutes a mitochondrial membrane protein, and β-actin. Note that the cytosolic fraction was highly enriched, as shown by the β-actin bands. *J,* RT-PCR on a series of tissues revealed ubiquitous expression. REEP1 was not expressed in peripheral blood and fibroblasts derived from a skin biopsy.

The figure is available in its entirety in the online edition of *The American Journal of Human Genetics.*

Figure 4. Antibody design for REEP1. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics.*

As shown by Saito et al.[7] and extended in the present study, *REEP1* is expressed in various nonneuronal and neuronal tissues, including spinal cord (fig. 3*K*). This follows the now-common finding of almost ubiquitous tissue expression for a number of genes that cause distinct neurodegenerative phenotypes. Saito et al. showed that REEP1 has a weak promoting effect on the expression of G protein–coupled odorant-receptor proteins at the cell surface.[7] Thus, REEP1 might have a role in trafficking of odorant receptors and other molecules through cellular compartments such as the endoplasmic reticulum and Golgi apparatus. The yeast homologue of REEP1, Yop1P, is involved in Rab-mediated vesicle transport, a pathway that has recently been implicated in axonal neuropathy type CMT2B, which is caused by mutations in RAB7.[12,13] *In silico* analysis of REEP1 predicted two transmembrane domains and the conserved protein domain TB2/DP1/HVA22 (fig. 1*B*). As shown by Chen et al., the plant homologues of *HVA22* are stress-induced genes—a characteristic known from heat-shock proteins.[14] Heat-shock proteins play a fundamental role as chaperones to promote and maintain correct protein folding, especially in cell compartments rich in reactive oxygen species, such as mitochondria. Mutations in the mitochondrial heat-shock protein 60 have been shown to cause spastic paraplegia type SPG13.[4] It has also been suggested that the spastic paraplegia gene *paraplegin* (*SPG7* [MIM 602781]) fulfills chaperonelike activities in mitochondria.[15]

We designed two specific polyclonal antibodies that targeted the C terminal of REEP1 (fig. 4). Staining of COS7 and MN-1 cells with those antibodies revealed that endogenous REEP1 colocalized with mitochondria (fig. 3*A*–3*F*). REEP1 was also present in the mitochondrial but not the cytosolic cellular fraction on an immunoblot (fig. 3*I*). Given the predicted transmembrane domains (figs. 1*B* and 5), we suggest that REEP1 is a novel mitochondrial membrane protein.

Although the results of Saito et al. suggested localization of REEP1 to the secretory pathway,[7] we did not detect colocalization of REEP1 with the Golgi (fig. 1*H*). It is conceivable that REEP1 has different cellular functions and that a fraction of the protein, which was not detectable with the available antibodies, indeed fulfills functions in the endoplasmic reticulum and Golgi. In support of this hypothesis, the ENSEMBL human genome database lists two alternative isoforms of REEP1 that might represent such differential functionality. However, we were not able to unequivocally reproduce the existence of this second isoform.

The figure is available in its entirety in the online edition of *The American Journal of Human Genetics.*

Figure 5. REEP1 is highly conserved throughout different species. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics.*

Although the specific function of REEP1 in mitochondria has not been elucidated, this finding contributes further to the evidence that mitochondrial integrity takes center stage in HSP and related neurodegenerative diseases.[6]

In summary, we have identified the gene for the *SPG31* locus, *REEP1,* which accounted, in our sample, for 6.5% of all HSP cases. We have demonstrated that REEP1 is localized to mitochondria, and, derived from its conserved protein-domain structure, REEP1 might be involved in chaperonelike activities.

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Web Resources

Accession numbers and URLs for data presented herein are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for the REEP1 protein [accession number NP_075063] and REEP1 mRNA [accession number NM_022912])

MicroRNA Registry, http://microrna.sanger.ac.uk/

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for *KIF5A, HSP60, NIPA1, REEP1,* and *SPG7*)

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