

Spectrin mutations cause spinocerebellar ataxia type 5

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We have discovered that β -III spectrin (*SPTBN2*) mutations cause spinocerebellar ataxia type 5 (SCA5) in an 11-generation American kindred descended from President Lincoln's grandparents and two additional families. Two families have separate in-frame deletions of 39 and 15 bp, and a third family has a mutation in the actin/ARP1 binding region. β -III spectrin is highly expressed in Purkinje cells and has been shown to stabilize the glutamate transporter EAAT4 at the surface of the plasma membrane. We found marked differences in EAAT4 and GluR δ 2 by protein blot and cell fractionation in SCA5 autopsy tissue. Cell culture studies demonstrate that wild-type but not mutant β -III spectrin stabilizes EAAT4 at the plasma membrane. Spectrin mutations are a previously unknown cause of ataxia and neurodegenerative disease that affect membrane proteins involved in glutamate signaling.

The dominant spinocerebellar ataxias (SCAs) are a heterogeneous group of neurodegenerative disorders characterized by uncoordinated gait, limb and eye movements, slurred speech and swallowing difficulties. Nine of the 11 known SCA mutations are microsatellite repeat expansions¹. In 1994, we mapped SCA5 to 11q13, a centromeric region with suppressed recombination². MRI and autopsy findings show cerebellar cortical atrophy, Purkinje cell loss and thinning of the molecular layer³. Additional SCA5 families from France and Germany were reported with similar clinical and neuroradiological findings^{4,5}.

The American family used in this study has two major branches that descend from the paternal grandparents of President Abraham Lincoln (Fig. 1). Clinical evaluations and DNA collection were performed on 299 family members, including 90 affected individuals (age of onset, 4–68 years). Recombinations were used to refine the critical region to 2.99 Mb containing ~100 genes (Fig. 2a). Haplotype comparisons between families identified a 255-kb region of possible conservation between the American and French families. Although this haplotype was also found in 3/84 (3.5%) control chromo-

somes, we prioritized this region because of the possibility that this conservation resulted from a common ancestral mutation. DNA from an affected chromosome-separated cell line known to contain the American SCA5 mutation was used to construct a BAC library and clone contig of the region. We subsequently performed shotgun sequencing of BAC clones VI-C2, VI-C11 and IV-H4, which spanned the area of haplotype conservation (Fig. 2b).

We found a 39-bp deletion in exon 12 of the β -III spectrin gene (*SPTBN2*) that causes an in-frame 13-amino acid deletion (E532_M544del) within the third of 17 spectrin repeats (Figs. 2c and 3a, Supplementary Table 1 online). The mutation, which is detectable by PCR (Fig. 3a), was found in all 90 affected individuals (age at exam, 7–80 years; mean, 45 years) and 35 presymptomatic carriers (age at exam, 13–67 years; mean, 34 years).

Although the American and French families share a common haplotype, the 39-bp American deletion was not found in the French family. Similar to the American family, the French family has a short in-frame deletion in the same spectrin repeat consisting of a 15-bp deletion in exon 14 (1886_1900del; L629_R634delinsW; Figs. 2c and 3b). With the exception of the insertion of a tryptophan, this deletion does not disrupt the remainder of the ORF (Fig. 3b). The French mutation was found in all six available affected individuals and one apparently presymptomatic carrier (age 24).

In the German family, a T to C transition mutation (758T \rightarrow C) in exon 7 that causes a leucine to proline change (L253P; Figs. 2c and 3c) was found in the calponin homology domain containing the actin/ARP1 binding site. This region is highly conserved, with the Leu253 residue found in all five human β -spectrin proteins as well as in chimpanzee, mouse, rat, dog and fly (Fig. 3c). The German mutation cosegregated with the disease in 12 available affected individuals. None of the three SCA5 mutations was found on 1,000 control chromosomes.

β -III spectrin, a 2,390-amino acid protein highly expressed in Purkinje cells^{6,7}, is homologous to four other human β -spectrin

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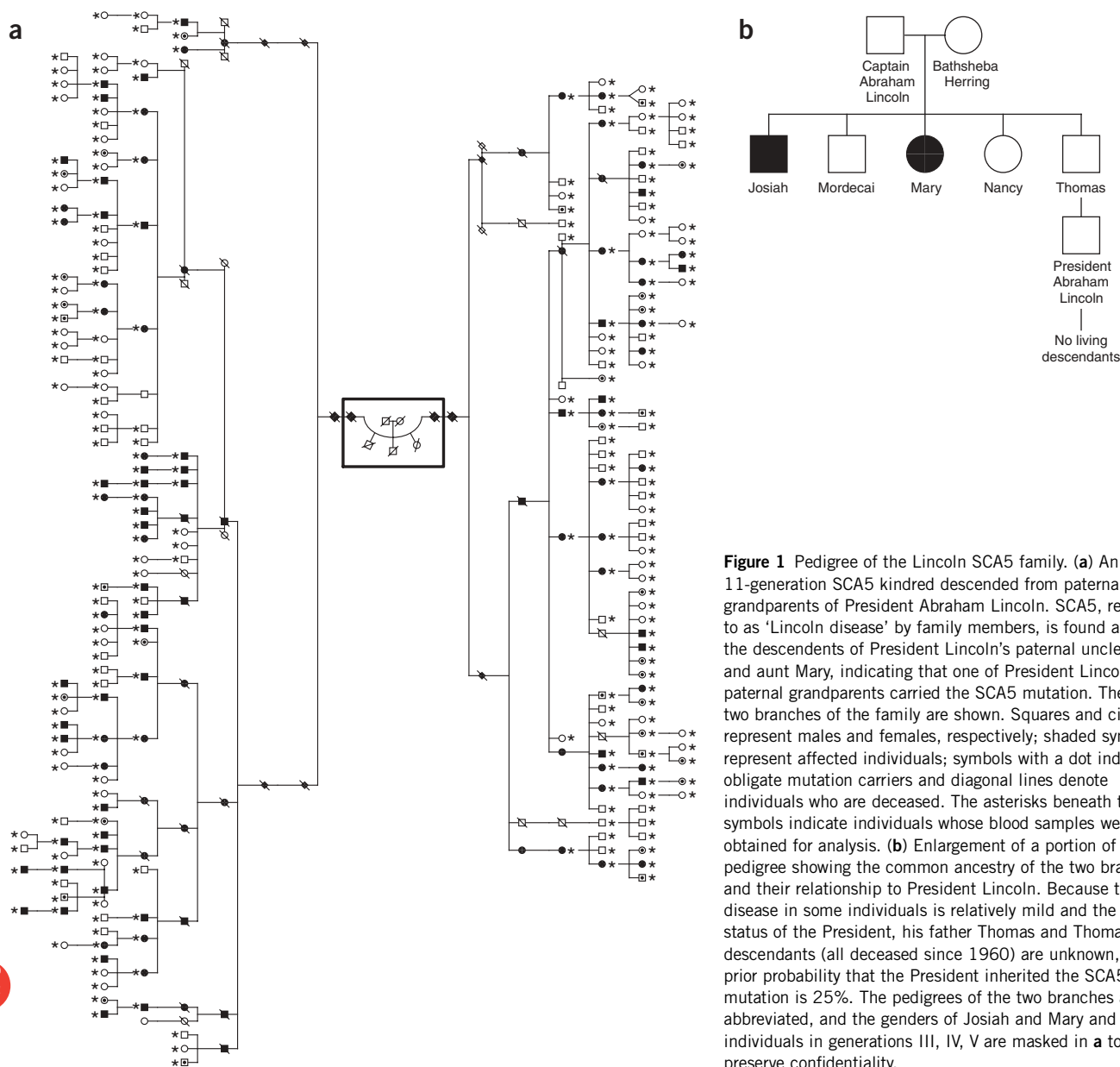


Figure 1 Pedigree of the Lincoln SCA5 family. **(a)** An 11-generation SCA5 kindred descended from paternal grandparents of President Abraham Lincoln. SCA5, referred to as 'Lincoln disease' by family members, is found among the descendants of President Lincoln's paternal uncle Josiah and aunt Mary, indicating that one of President Lincoln's paternal grandparents carried the SCA5 mutation. These two branches of the family are shown. Squares and circles represent males and females, respectively; shaded symbols represent affected individuals; symbols with a dot indicate obligate mutation carriers and diagonal lines denote individuals who are deceased. The asterisks beneath the symbols indicate individuals whose blood samples were obtained for analysis. **(b)** Enlargement of a portion of the pedigree showing the common ancestry of the two branches and their relationship to President Lincoln. Because the disease in some individuals is relatively mild and the clinical status of the President, his father Thomas and Thomas's descendants (all deceased since 1960) are unknown, the prior probability that the President inherited the SCA5 mutation is 25%. The pedigrees of the two branches are abbreviated, and the genders of Josiah and Mary and individuals in generations III, IV, V are masked in **a** to preserve confidentiality.

proteins. The functional unit of spectrin is typically a noncovalently joined tetrameric complex consisting of two α and two β spectrin subunits. β -III spectrin has been reported to be associated with Golgi and vesicle membranes⁷ and to bind to the dynactin subunit ARP1, suggesting a possible role in transport⁸. Another function of β -spectrin is the stabilization of membrane proteins⁹. Notably, β -III spectrin stabilizes the Purkinje cell-specific glutamate transporter EAAT4 (ref. 10). RT-PCR analysis showed that both normal and deleted β -III spectrin transcripts were expressed in affected cerebellar autopsy tissue (Fig. 3d). Immunohistochemistry of β -III spectrin showed staining of Purkinje cell bodies, dendrites and axons in both SCA5 and control cerebella with marked Purkinje cell loss, dendritic atrophy and significant thinning of the molecular layer in SCA5 (Fig. 3e).

We performed protein blot analysis on cerebellar autopsy tissue to investigate whether the 39-bp spectrin deletion mutation affects EAAT4. Protein levels of EAAT4 in SCA5 cerebellum extracted by radioimmunoprecipitation assay (RIPA) buffer were markedly

reduced relative to calbindin, a Purkinje cell-specific control (Fig. 4a). EAAT4 and calbindin are both highly expressed in Purkinje cells, with little or no expression in other cells within the cerebellar cortex. Notably, when using a harsher extraction buffer (8 M urea and 4% SDS), approximately equal ratios of EAAT4 to calbindin were seen in SCA5 and control (Fig. 4b), suggesting that EAAT4 solubility or distribution is affected by mutant β -III spectrin.

Decreased EAAT4 transcript levels have been previously reported in SCA1 transgenic mice before Purkinje cell loss^{11,12}, suggesting that loss or dysfunction of EAAT4 may be a common downstream molecular change. To determine if the extractability differences of EAAT4 in SCA5 are a nonspecific change caused by Purkinje cell degeneration, we examined EAAT4 extractability in SCA1 transgenic mice with significant Purkinje cell loss (Fig. 4c,d). Consistent with previous reports, we found reduced levels of EAAT4 by protein blot, and in contrast to SCA5, EAAT4 levels were similarly reduced in RIPA and urea extracts. EAAT4 immunostaining of remaining Purkinje cells in

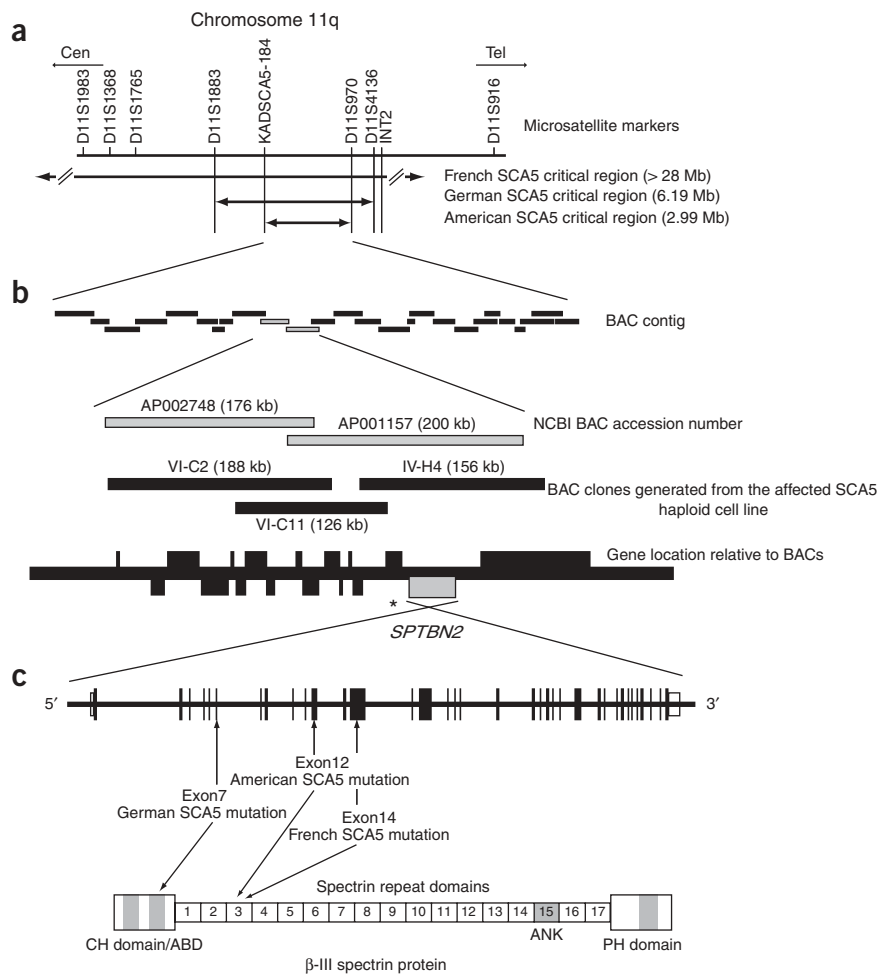


Figure 2 Mapping and cloning SCA5 mutations. **(a)** Critical regions defined by recombination events in the three SCA5 families are indicated by black arrows. The boundaries of the French critical region are not defined because no recombination events were found among affected family members. Markers defining recombination events, along with other published markers are shown. **(b)** BAC map spanning SCA5 region. Enlarged BACs span a 255-kb region of haplotype conservation between the American and French families and contain 11 new polymorphic STR markers and 8 SNPs. Black blocks below schematic of SCA5-specific BAC clones represent approximate size and location of genes. The gray box represents *SPTBN2*. **(c)** Illustration of *SPTBN2* gene (top) and protein structure (bottom). Size and location of 3' and 5' UTR (open rectangles) and exons (filled rectangles) are shown (top). The calponin homology (CH)-actin binding domain (ABD), ankyrin binding domain (ANK) and pleckstrin homology domain (PH) are shaded in gray. * indicates that the direction of *SPTBN2* transcription relative to chromosome 11q is reversed.

typically restricted to movements in a fixed small area (less than 1 μm ; **Fig. 4g**, **Table 1**, **Supplementary Video 1**). To further investigate the physiological relevance of the interaction between EAAT4 and wild-type β -III spectrin, we transfected EAAT4 with β -III spectrin and followed the trafficking of EAAT4. Consistent with previous biochemical studies¹⁰, coexpression of wild-type β -III spectrin stabilized EAAT4, with only 5% of diffraction spots moving at or near the mem-

brane and none showing large lateral movements ($>4 \mu\text{m}$; **Fig. 4h**, **Table 1**, **Supplementary Video 2**). However, in the presence of mutant β -III spectrin with the 39-bp deletion, the stabilization of EAAT4 was lost, and the transporter was highly motile, making many lateral movements over 4 μm (**Fig. 4i**, **Table 1**, **Supplementary Video 3**). To confirm the specific interaction between EAAT4 and β -III spectrin, we transfected β -III spectrin with EAAT3, another glutamate transporter also expressed in Purkinje cells. Neither wild-type (**Table 1**; **Supplementary Videos 4,5**) nor mutant β -III spectrin (data not shown) had any substantial effect on EAAT3 stability. The lack of an effect on EAAT3 does not exclude the possibility that mutant β -III spectrin affects other membrane proteins. These studies, however, provide evidence that mutant β -III spectrin can disrupt the stability of EAAT4, and because altered expression of EAAT4 on the membrane is known to increase susceptibility of Purkinje cells to injury and degeneration, it therefore may contribute to Purkinje cell degeneration in SCA5 (ref. 13).

Here we report a previously unknown mutational mechanism for spinocerebellar ataxia, identifying three separate mutations in the β -III spectrin gene (*SPTBN2*) responsible for SCA5. The American and French families have similar but separate in-frame deletions within the third spectrin repeat and are likely to disrupt the highly ordered triple- α helical structure of the repeat, changing the overall shape of the tetrameric α - β -spectrin complex. Although it is possible that some feature of the shared haplotype between the American and French families led to similar microdeletions, it

SCA5 showed a consistent thinning of the dendritic arbor and darker staining of the cell body (**Fig. 4e**), whereas SCA1 transgenic animals showed uniform but lighter staining (**Fig. 4f**). These results indicate that the redistribution of EAAT4 in SCA5 is not caused by Purkinje cell degeneration and that EAAT4 is probably altered by different mechanisms in SCA1 and SCA5.

To further examine EAAT4 and to determine if mutant spectrin also causes changes in other membrane-bound Purkinje cell proteins, we performed subcellular fractionations of cerebellar tissue and subsequent protein blot analyses (**Supplementary Figure 1** online). In contrast to control, the synaptic membrane proteins EAAT4 and GluR δ 2 were not enriched in the synaptosomal fractions in SCA5 tissue, suggesting that mutant β -III spectrin affects the cellular localization of these proteins.

To further characterize the physiological effects of mutant β -III spectrin on EAAT4, we performed a series of controlled cell culture experiments. We transfected HEK293 cells with eGFP-EAAT4 and used total internal reflection fluorescence (TIRF) microscopy to follow the lateral movement of the glutamate transporter on the cell's membrane. The glutamate transporters normally alternated within seconds between two main states: periods of rapid movement on the cell's membrane and restricted motion within a submicrometer area (**Fig. 4g-i**, **Supplementary Videos 1-5** online). When EAAT4 was expressed along with an empty control vector, almost 40% of the EAAT4 diffraction spots were actively moving at or near the plasma membrane ($\sim 4 \mu\text{m}$), whereas the slow-moving diffraction spots were

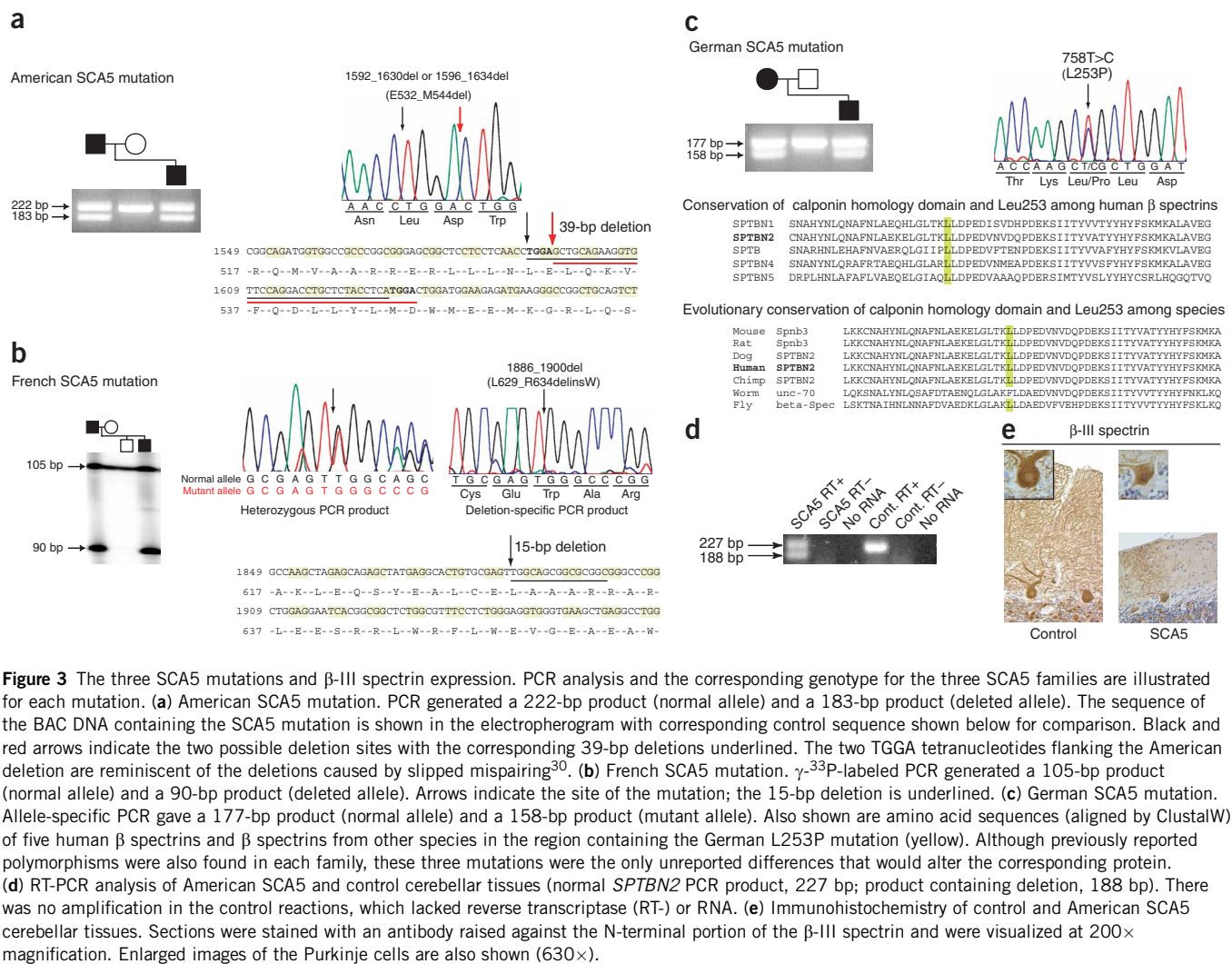


Figure 3 The three SCA5 mutations and β -III spectrin expression. PCR analysis and the corresponding genotype for the three SCA5 families are illustrated for each mutation. (a) American SCA5 mutation. PCR generated a 222-bp product (normal allele) and a 183-bp product (deleted allele). The sequence of the BAC DNA containing the SCA5 mutation is shown in the electropherogram with corresponding control sequence shown below for comparison. Black and red arrows indicate the two possible deletion sites with the corresponding 39-bp deletions underlined. The two TGGG tetranucleotides flanking the American deletion are reminiscent of the deletions caused by slipped mispairing³⁰. (b) French SCA5 mutation. γ -³³P-labeled PCR generated a 105-bp product (normal allele) and a 90-bp product (deleted allele). Arrows indicate the site of the mutation; the 15-bp deletion is underlined. (c) German SCA5 mutation. Allele-specific PCR gave a 177-bp product (normal allele) and a 158-bp product (mutant allele). Also shown are amino acid sequences (aligned by ClustalW) of five human β spectrins and β spectrins from other species in the region containing the German L253P mutation (yellow). Although previously reported polymorphisms were also found in each family, these three mutations were the only unreported differences that would alter the corresponding protein. (d) RT-PCR analysis of American SCA5 and control cerebellar tissues (normal *SPTBN2* PCR product, 227 bp; product containing deletion, 188 bp). There was no amplification in the control reactions, which lacked reverse transcriptase (RT-) or RNA. (e) Immunohistochemistry of control and American SCA5 cerebellar tissues. Sections were stained with an antibody raised against the N-terminal portion of the β -III spectrin and were visualized at 200 \times magnification. Enlarged images of the Purkinje cells are also shown (630 \times).

seems more likely that the shared haplotypes are coincidental, as this haplotype is found on 3.5% of control chromosomes. The German family has a missense mutation in the calponin homology domain, which may disrupt the ability of spectrin to bind to the actin cytoskeleton and similarly affect the stabilization of membrane proteins or cause alterations in transport by disrupting binding to ARP1 and the dynein motor complex⁸.

The cell fractionation studies suggest that mutant β -III spectrin (with a 39-bp deletion) affects localization of the synaptosomal proteins EAAT4 and GluR δ 2. Notably, transcript levels of EAAT4 are downregulated in SCA1 transgenic mice^{11,12}. Further evidence for the possible role of EAAT4 in ataxia comes from intracisternal antisense knockdown experiments in rats that resulted in progressive ataxia¹⁴. In addition, mutations in GluR δ 2 cause ataxia in both *lurcher* and *hotfoot* mice^{15,16}. Loss of EAAT4 and GluR δ 2 at the plasma membrane in SCA5 could lead to glutamate signaling abnormalities that, over time, could cause Purkinje cell death in SCA5.

The reported interaction of spectrin with the dynactin-dynein motor complex suggests that SCA5 mutations could affect protein trafficking, as in other neurodegenerative diseases. These disorders include a dominantly inherited motor neuron disease caused by mutations in p150^{Glu}, a subunit of dynactin (*DCTN1*)¹⁷, and a

motor neuronopathy caused by missense mutations in the mouse dynein heavy chain gene (*Dync1h1*)¹⁸. In Huntington disease, alterations of the huntingtin-HAP1-p150^{Glu} complex induce transport deficits and loss of neurotrophic support contributing to neuronal toxicity¹⁹, and axonal transport defects are found in individuals with Alzheimer disease and in mouse models²⁰.

Identifying additional mutations in *SPTBN2* that cause ataxia in families having unknown mutations will provide further insight into the functions of β -III spectrin and the molecular mechanisms of neurodegenerative diseases. Specifically, it will be of interest to determine if mutations in *SPTBN2* also cause SCA20, a clinically distinct form of ataxia with a critical region including *SPTBN2* (ref. 21). It will also be important to determine if mutations in *SPTBN5* or *SPTBN1*, which map to the SCA11 and SCA25 critical regions, respectively, also cause ataxia^{22,23}. Consistent with the possibility that the β -spectrins may have additional roles in disease, dominantly inherited mutations in a β -spectrin homologue cause an uncoordinated phenotype (*unc-70*) in *Caenorhabditis elegans*²⁴, and recessive mutations in the mouse spectrin β -4 gene (*Spnb4*), an orthologue of human beta-IV spectrin (*SPTBN4*), cause a progressive ataxia with hindlimb paralysis, deafness and tremor in quivering mice (also known as *qv*)⁹.

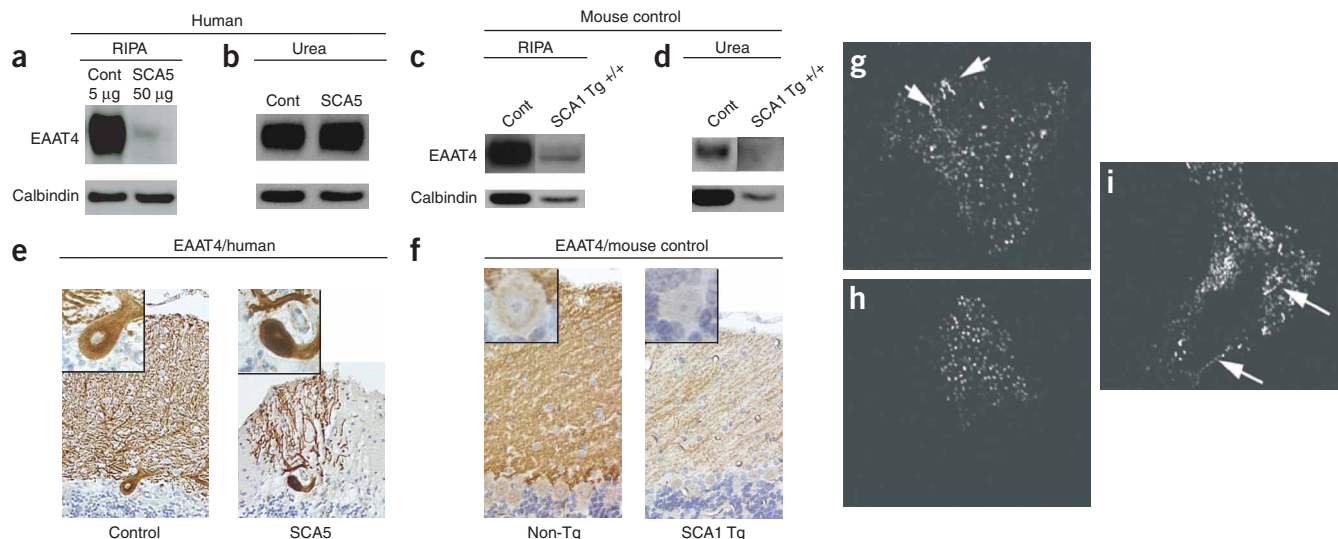


Figure 4 Protein blots, immunohistochemistry and TIRF microscopy demonstrate effects of mutant β -III spectrin on EAAT4. (**a–d**) Immunoblots comparing lysates extracted with RIPA buffer (**a,c**) or 8 M urea and 4% SDS (**b,d**). When possible, samples were normalized for Purkinje cell loss with calbindin. Markedly less EAAT4 relative to the calbindin control was extracted from human SCA5 cerebella compared with control tissue in the RIPA extracts (**a**), but similar levels of EAAT4 were found in the harsher 8 M urea, 4% SDS buffer (**b**). As a control, we examined mouse extracts from homozygous 12-week-old SCA1 B05 mice (Tg +/+) but did not observe similar increases in EAAT4 in the urea versus the RIPA extracts (**c,d**). (**e,f**) EAAT4 immunohistochemistry of American SCA5 (**e**), murine SCA1 (**f**) and corresponding human and murine controls. Sections were stained with EAAT4 antibody and visualized at 200 \times magnification. Enlarged images of the Purkinje cells are also depicted (630 \times). Darker EAAT4 staining was observed in the SCA5 Purkinje cell bodies (representative sample) but not in Purkinje cells from SCA1 transgenic mice or controls. (**g–i**) Fast lateral trafficking of EAAT4 was modulated by β -III spectrin interaction, as shown by superimposed time-lapse images. (**g**) A superimposed image shows the total lateral movement of EAAT4 when expressed with an empty vector in HEK293 cells (arrows). (**h**) When EAAT4 and wild-type β -III spectrin were cotransfected, no lateral fast movement was seen. (**i**) When EAAT4 and mutant β -III spectrin containing the 39-bp SCA5 deletion were cotransfected, fast movement was seen again (arrows; see **Supplementary Videos 1–5**).

The current estimate of 28 dominant ataxia loci provides an opportunity to use human genetics to define the fundamental causes and common molecular pathways underlying this group of neurodegenerative diseases¹. Downregulation of both β -III spectrin and EAAT4 transcripts found by microarray analysis in two mouse ataxia models, SCA1 transgenic (H. Orr, personal communication) and staggerer mice²⁵, suggests the convergence of pathogenic mechanisms triggered by distinct mutations. The identification of SCA5 mutations in a gene encoding a well-known cytoskeletal protein will allow testing of specific hypotheses of disease pathogenesis involving destabilization of membrane proteins, glutamate dysregulation and vesicle trafficking deficits, which will provide insight into the down-

stream molecular mechanisms common to SCA5 and other neurodegenerative diseases.

The history of ataxia in the Lincoln family raises the question of whether President Abraham Lincoln carried the SCA5 mutation. Historical descriptions suggest that the President had an uneven gait—an early sign of ataxia. William Russell, a reporter for the *London Times*, wrote of Lincoln in 1861, “Soon afterwards there entered, with a shambling, loose, irregular, almost unsteady gait, a tall, lank, lean man...” The identification of the SCA5 mutation makes it possible to unequivocally determine if President Lincoln carried the mutation using preserved artifacts containing his DNA. In 1991, the identification of a gene underlying Marfan syndrome sparked debate on the testing of President Lincoln’s DNA to determine whether his tall stature could have resulted from that disease²⁶. Unlike for Marfan syndrome, the Lincoln family history indicates President Lincoln was at risk of developing SCA5. Determining President Lincoln’s status relative to SCA5 would be of historical interest and would increase public awareness of ataxia and neurodegenerative disease.

Table 1 Mutant β -III spectrin alters lateral trafficking of glutamate transporters

Construct	Total diffraction spots analyzed	Percentage of diffraction spots not moving
eGFP-EAAT4 + empty vector	685	62.0 \pm 8.7
eGFP-EAAT4 + wild-type β -III spectrin	122	94.2 \pm 9.7
eGFP-EAAT4 + mutant β -III spectrin	375	67.5 \pm 4.4
eGFP-EAAT3 + empty vector	547	61.0 \pm 11.2
eGFP-EAAT3 + wild-type β -III spectrin	337	58.7 \pm 5.4

TIRF microscopy of HEK293 cells was performed and digital movies of the imaged cells were evaluated using Metamorph. Each diffraction spot was analyzed separately. For each condition, three to six different experiments were recorded from different dishes and different days. Results are given as mean \pm s.d.

METHODS

Human subjects. All participating subjects and control individuals referred to this study signed an informed consent form as approved by the Human Subjects Committee at the University of Minnesota or by the participating institutions. Unrelated control DNA samples were obtained from the Centre d’Etude du Polymorphisme Humain (CEPH) panel and from healthy North Americans ($n = 500$). DNA was extracted from peripheral venous blood using the Puregene kit (Gentra Systems).

Generation of chromosome-separated cell lines. Mouse/human hybrid cell lines haploid for the affected or normal copy of chromosome 11 were generated

at GMP Genetics by fusing mouse E2 cells with human lymphoblastoid cells from an affected American family member, as previously described²⁷. In brief, lymphoblast cells from an affected individual were electrofused to mouse E2 cells, and HAT plus geneticin was used to select against unfused E2 and lymphoblast cells, respectively. The surviving colonies were expanded, and clones containing only a single copy of the affected or normal chromosome 11 were selected by typing microsatellite markers that spanned the SCA5 region.

Screening of microsatellite repeat markers in the SCA5 region. A panel of 445 new di-, tri-, tetra- and pentanucleotide repeat markers were used to refine the SCA5 region and search for haplotype conservation between the families. Microsatellite repeat markers were amplified by PCR using a γ -³²P-labeled primer. Products were separated on 4% denaturing polyacrylamide gels and visualized by autoradiography. Genotyping of the single affected chromosome allowed the affected haplotype to be directly and unambiguously defined while excluding repeat-expansion mutations in nonpolymorphic markers. The E2 mouse DNA was used as a negative control to confirm that the amplified product was specific to human but not mouse DNA. All polymorphic markers were subsequently used to determine the affected haplotypes for each of the SCA5 families.

Construction of BAC libraries from an affected SCA5 haploid cell line and shotgun DNA sequencing. An incomplete *Hind*III digestion was performed on DNA from the haploid cell line containing the affected chromosome 11 and introduced into the pIndigoBAC-5 vector (Epicentre), which was then used to prepare a BAC library of approximately 352,000 recombinant clones. The BAC libraries were screened by PCR using microsatellite markers that we developed, and positive BAC clones were subsequently isolated by hybridization. Lark Technologies performed the shotgun sequencing and assembly. In brief, shotgun libraries were constructed for three BACs (VI-C2, VI-C11, IV-H4), which spanned the region of haplotype conservation between the American and French SCA5 families, by subcloning the fragmented DNA into the pUC57 vector. Sequencing reactions of the three shotgun libraries were performed and subsequently analyzed on ABI3730xl DNA sequencers. The sequence data was assembled using the Phred-Phrap-Consed software²⁸ and was compared to known BAC sequences using software at UCSC Genome Bioinformatics and the National Center for Biotechnology Information (BLAST).

SPTBN2 gene sequencing in SCA5 families and mutation screening in controls. Genomic DNA of French and German SCA5 affected individuals was used to amplify *SPTBN2* exons by PCR, and the resulting products were sequenced. After the American and French mutations were identified, family members and 1,000 control chromosomes were screened for these deletion mutations by PCR. PCR was performed by attaching γ -³²P to the 5' end of each forward primer. The resulting products were separated on 4% denaturing polyacrylamide gels and visualized by autoradiography. Allele-specific PCR analysis was used to screen for the German missense mutation. Two forward primers, one containing an altered nucleotide (C) at its 3' end and the other containing a 19-bp tail at its 5' end, were used in a single reaction to amplify both the mutant (shorter product) and normal (longer product) alleles, respectively. The resulting products were separated on 4% agarose gels and visualized by ethidium bromide. PCR was subsequently performed on 1,000 unrelated control chromosomes to screen for the German mutation. The PCR primer sequences and conditions used for *SPTBN2* sequencing and mutation screening are shown in **Supplementary Table 2**.

RT-PCR analysis. RNA was harvested from ~100 mg of cerebellar autopsy tissue from an American SCA5 affected individual and a control individual using TRIzol (Invitrogen). First-strand synthesis was performed using the Invitrogen SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen) and a primer specific to *SPTBN2* exon 14. PCR primers flanking the American SCA5 deletion region were located in exons 12 and 13, respectively. The products were separated on a 2% agarose gel and visualized with ethidium bromide. The primers and conditions for RT-PCR analysis of the American SCA5 deletion are shown in **Supplementary Table 2**.

Immunohistochemistry. The autopsy tissue from an American SCA5 family member and a control individual without neurological disease and brains from control and SCA1 B05 transgenic mice were embedded in paraffin, and 5- μ m sections were prepared. These sections were incubated in 0.3% H₂O₂ for 30 min to bleach endogenous peroxidase activity and then heated by a steamer in 10 mM citrate buffer at pH 6.0. Sections were blocked in 5% normal serum derived from animals in which the secondary antibodies had been made. Slides were incubated at 4 °C overnight with β -III spectrin or EAAT4 antibodies (Santa Cruz Biotechnology) diluted at 1:500 or 1:100, respectively. Positive staining was visualized by the avidin-biotin-peroxidase complex method (Vector) with diaminobenzidine as the chromogen and counterstained with hematoxylin.

Immunoblot analysis. Cerebellar tissue from an SCA5 American family member, human control, mouse control and SCA1 B05 transgenic mice were used for protein blot analysis. Tissue was extracted with a Polytron homogenizer in RIPA lysis buffer (1 \times PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μ g ml⁻¹ PMSF, 50 Kallikrein inhibitor units (KIU) ml⁻¹ aprotinin, 1 mM sodium orthovanadate). To ensure the efficiency of protein extraction, the same cerebellar tissues were reextracted in a stronger lysis buffer containing 8 M urea, 4% SDS, 0.125 M Tris-HCl (pH 6.8), 12 mM EDTA, 3% β -mercaptoethanol and 1 \times protease inhibitors (Complete, Roche). To determine if EAAT4 was decreased in amount beyond that expected owing to Purkinje cell loss, the amount of protein loaded was normalized relative to the Purkinje cell-specific protein calbindin. After solubilization, samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane and incubated at 4 °C overnight with EAAT4 or calbindin (Sigma-Aldrich) antibodies diluted at 1:200 or 1:6,000, respectively. The immunoblot was visualized with horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (Amersham Biosciences).

Subcellular fractionation. Subcellular fractionation analysis was performed as described elsewhere²⁹ with slight modifications. Briefly, cerebellar tissues (500 mg each) from American SCA5 and control autopsy brains were re-suspended by Polytron homogenization in 5 ml of buffered sucrose (0.32 M sucrose, 5 mM Tris (pH 7.5), 0.5 mM CaCl₂, 1 mM MgCl₂ and 1 \times protease inhibitors (Complete, Roche)). Tissue was sheared by passage through an 18-gauge needle repeatedly, and the lysate was pelleted at 500g for 10 min (P1 fraction). The supernatant (S1) was separated into two 0.5-ml aliquots, and all aliquots were centrifuged at 10,500g for 15 min. For one of the aliquots, the supernatant (S2) and pellet (P2) were isolated. For the other aliquot, the pellets from the 10,500g spin (P2) were resuspended and hypotonically lysed by the addition of 50 μ l of ice-cold H₂O (with 1 \times protease inhibitors) and passage through an 18-gauge needle 10 times. This mixture was then centrifuged at 25,000g for 20 min, generating LS1 (supernatant) and LP1 (pellet) fractions. All pelletable fractions (P1, P2 and LP1) were resuspended in a lysis buffer containing 8 M urea, 4% SDS, 0.125 M Tris-HCl (pH 6.8), 12 mM EDTA, 3% β -mercaptoethanol and 1 \times protease inhibitors. All resulting fractions were then analyzed by SDS-PAGE and protein blotting using antibodies against the following proteins: EAAT4 (1:200), GluR δ 2 (1:1,000, BD Biosciences) and clathrin light chain (1:1,000, Synaptic Systems).

Cloning of EAAT4 and β -III spectrin constructs, cell culture and transfection. Standard techniques were used in the construction of the β -III spectrin control and deletion constructs and the EAAT4-GFP construct. Briefly, a full-length *SPTBN2* pBluescript cDNA clone (KIAA0302, Kazusa DNA Research Institute) was re-cloned into the mammalian expression vector pcDNA3.1 (Invitrogen) and modified by PCR using overlapping primer sets (set 1: SPA39-1f and SPA39-1r; set 2: SPA39-2f and SPA39-2r). The American family deletion was created by generating separate PCR products (SPA39 primer sets 1 and 2) followed by a third PCR reaction (primers SPA39-1f and SPA39-2r) to generate the 39-bp deletion mutation (SP- Δ 39) found in the American kindred. These PCR products were then subcloned using *Bsm*BI and *Age*I digestion. Subsequently, a myc tag was introduced into both the wild-type (SP-WT) and mutant constructs immediately downstream of the ATG start codon by PCR (using myc-f1 and myc-r primers followed by myc-f2 and myc-r primers). PCR products were then subcloned using *Kpn*I and *Pml*II digestion. Sequencing was

performed to verify the integrity of the tag, and the entire cDNA and coding errors were fixed using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). The primer sequences and PCR conditions to generate the β -III spectrin constructs are shown in **Supplementary Table 2**.

The EAAT4-GFP construct was generated using primers containing the appropriate restriction enzyme recognition sites and an overlap extension PCR-based strategy. Resulting EAAT4 PCR products were cloned into the eukaryotic expression vector pEGFP-C2 (Clontech), and coding regions were confirmed by sequencing. HEK293 cells were transfected (0.5 μ g per dish) using FuGene 6 (Roche) using standard protocols. Cells were plated directly on glass-bottom culture dishes (MatTek) and imaged 24 h after transfection.

TIRF microscopy and analysis. Light from an ion laser was introduced into an inverted epifluorescence microscope (IX81, Olympus), and the light was focused at the back focal plane of a TIRFM objective lens (PlanApo 60 \times /1.45NA, Olympus). The transfected cells on the glass coverslip were maintained at 37 °C using a temperature controller (Harvard apparatus) and were maintained at pH 7.4 by 10 mM HEPES. Images were collected by an EM-charge-coupled device camera (Olympus) operated with Metamorph 6.3 (Universal Imaging). Time-lapse images were acquired every 450 ms. Analysis, including tracking (the single projection of different images) and area calculations, were performed using Metamorph. Each diffraction spot was filtered twice (high-pass filter >3 pixels and low-pass filter <30 pixels). EAAT4 lateral movement images were superimposed to a single image to measure the total area of the transporter movement, and total trafficking distance of diffraction spots was calculated using the Metamorph tracking module.

URLS. The University of California, Santa Cruz Genome Bioinformatics website is <http://genome.ucsc.edu/>. The National Center for Biotechnology Information database is available at <http://www.ncbi.nlm.nih.gov/>. The multiple sequence alignment program ClustalW is available at <http://clustalw.genome.jp/>.

Accession numbers. GenBank: *Homo sapiens SPTBN2* mRNA, AB008567 and NM_006946.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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