**Supporting Protocols**

Each individual DNA, both SSC and NINDS, was diluted with TE to approximate working concentrations of 20 ng/µL, heated at 45° for 30 minutes and vortexed thoroughly twice during this soak to break up clumps of genomic DNA, and then quantitated fluorometrically using PicoGreen dye (Invitrogen, Life Technologies, Carlsbad, CA). 1 µL of working individual sample was added to 24 µL TE, and 200x stock PicoGreen was diluted to 1x working concentration and 25 µL of this were added to the sample dilution for a total volume of 50 µL. This was done 3 times for each sample to ensure accurate estimation. The sample mix was quantified on a Biotek Synergy HT fluorometer (BioTek US, Winooski, VT). After quantification, DNAs were pooled 8 individuals at a time by case/control status, 500 ng/individual, such that all pooled samples were 8 cases or 8 controls for a total of 4 µg input DNA. Pooling was optimized prior to beginning the data collection phase of the project. 8 individuals per pool was initially modeled theoretically as the optimal screening size based on the parameters of the library and the microemulsion platform protocol, i.e. the largest pool size for which a **minor allele frequency (MAF**) 3 standard deviations below the mean (**mean MAF** for a single heterozygous variant in a pool of 8 individuals is 6.25%, 3 SD below that is 2.45% based on library size and microemulsion parameters) was still discernible above background error rate in sequencing (the **MAF** threshold for calling was later raised slightly to 3.5% upon viewing initial test high-throughput sequence data). The detection thresholds first modeled theoretically on the microemulsion platform and library parameters were subsequently confirmed by running test lanes using eight samples that had previously had one gene sequenced by Sanger (to give true negatives) and run on an Illumina 1M genotyping array (to give true positives). Using the results from these experiments we were able to generate receiver operating characteristic (ROC) curves to determine the ideal thresholds. With these results, we estimated a sensitivity of 94.7% and a specificity of 99.99%. Since there are 200,000 base pairs being analyzed in each individual in Raindance run, even a small decrease in specificity would generate numerous false positives which would exponentially drive up Sanger confirmation costs and required effort. This was the limiting factor in running more samples per lane; 8 per lane was the most cost-effective pooling strategy that still provided sufficient coverage and specificity to confidently distinguish true and false positives at a reasonable cutoff. MAF of 3.5% was the optimal cutoff for maximizing the sensitivity-specificity trade-off. 8 individuals per lane was found to represent the best balance of cost versus depth of data and of sensitivity versus specificity.

Pooled DNA samples were sheared on a Covaris S2 to an approximate size range of 3 kb using the specific Covaris 3kb shear tubes (Covaris, Inc., Woburn, MA) and protocol (duty cycle: 20%, intensity: 0.1, cycles/burst: 1000), then cleaned up on Qiagen Min-Elute columns (Qiagen GmbH, Hilden, Germany) with minor modifications to the standard protocol (10 µL 3M NaC2H3O2 was added to the 5:1 PB:sample mix to facilitate proper pH-driven DNA binding), the dry spin after the PE wash was 2 minutes, not 1, and elution was with with 9.0 µL of elution buffer (not 11) to generate a final volume around 7.7 µL, the input volume of DNA for the RDT1000. Successful shears were determined with the DNA 7500 protocol on an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) according to what a normally distributed curve centered around 3 kb should look like (a smaller amount of product, 0.2 µL, was added to more marker, 5.8 µL, to conserve DNA).
 The sheared genomic DNA pool was combined with RainDance microemulsion PCR master mix prepared according to the protocol. The microemulsion droplet merges were run with a standard protocol on the RDT1000 machine (Raindance Technologies, Lexington, MA). All merges were at least 85% efficient (85% of PCR master mix droplets merged successfully 1:1 with a library primer pair droplet, considered the threshold for "very good" by RainDance Tech); if not, new DNA pools were sheared and the merge was redone to at least 85% efficiency. Successful merges had excess oil pipetted from the bottom according to RainDance protocol and were PCR'd under the following conditions:

2 minutes at 94°C, 55 cycles of (30 seconds at 94°C, 30 seconds at 54°C and 60 seconds at 68°), followed by 10 minutes at 68°and a hold at 4°C.

 After successful merge and PCR, the samples were cleaned up according to RainDance protocol with RDT droplet destabilizer to break the emulsion, except that samples were mixed with pipetting instead of vortexing, to eliminate any leaking around the caps. The heavier hydrophobic phase of oil and droplet destabilizer was removed from the tube by pipetting from the bottom. Remaining aqueous PCR product phase was purified on Qiagen MinElute columns as described above, with elution in 17 µL of EB resulting in 16 µL of product. Eluted product was run on an Agilent Bioanalyzer according to DNA1000 protocol to ascertain successful PCR.
 After PCR cleanup, product was brought to a volume of 19 µL with the addition of low TE, and 2.5 µL of New England BioLabs blunting buffer and 2.5 µL of 1 mM dNTPs were added, along with 1 µL of NEB Blunting Enzyme (New England BioLabs, Ipswich, MA). This reaction mix was incubated at 22° for 15 minutes to blunt, 70° for 5 minutes to inactivate the enzyme, and subsequently held at 4°. Concatenation was performed by adding 25 µL of NEB Quick Ligase buffer and 5 µL of NEB Quick Ligase, mixing thoroughly by pipetting up and down, and transferring to a thermal cycler holding at 22° for at least 16 hours. After at least 24 hours, an additional 3 µL of Quick Ligase was added, the samples were pipetted to mix again, and incubated at 37° for 1 hour, then held at 4°.
 Concatenated samples were processed according to Illumina multiplexed library preparation protocol using Illumina reagents (Illumina, Inc., San Diego, CA), with minor modifications. Concatenated DNA was brought to 100 µL with TE buffer, and sheared on a Covaris S2 (duty cycle: 10%, intensity: 5, cycles/burst: 200) with Covaris snap-cap glass shear tubes to a mean size of 200 bp. Cleanup was on Qiagen Qiaquick columns according to Qiagen protocol, with the addition of 10 µL 3M NaC2H3O2 to enhance binding and a dry spin of 2 minutes after PE wash instead of 1. Elution was with 62 µL of EB for a total volume of 60 µL.
 30 µL of the eluted sheared product were processed for end repair according to Illumina protocol, saving 30 µL in case of failure. Cleanup was on Qiaquick columns with the modifications described above and elution with 33 µL of EB to yield 32 µL. The product was 3' adenylated according to Illumina protocol and cleaned up on a MinElute column as described above and eluted with 11.4 µL EB for 10 µL product. This product was adapter-ligated according to standard protocol, cleaned up on a Qiaquick column as described above, and eluted with 62 µL of EB to yield 60 µL of adapter-ligated DNA fragments.
 The adapter-ligated DNA fragments were size selected on Invitrogen agarose e-gels (Invitrogen, Life Technologies, Carlsbad, CA) as follows: two wells of 20 µL dH2O, one well of 20 µL Invitrogen 50 bp ladder diluted to 20 ng/µL, one well of 20 µL dH2O, three wells of 20 µL sample, one well of 20 µL dH2O, one well of 20 µL Invitrogen 50 bp ladder, two wells of 20 µL dH2O. Run time was for 12 minutes according to the E-gel 1-2% program for agarose gels. Bands were cut about 20 bp wide and centered at 250 bp, where the bulk of the fragments ran out, rather than at 400 bp as recommended in Illumina literature. 20 bp-thick slices were also cut above and below the original site and saved, in case of failure/loss of sample. Gel slices were cleaned up using Qiagen Gel Extraction protocols on a MinElute column using 480 µL of QG and 160 µL of isopropanol for each gel slice, and eluting with 25 µL EB for 24 µL product.
 Size-selected DNA fragments were enriched using Illumina primers InPE 1.0, InPE 2.0, and a selected Illumina barcode index out of 12 available. Barcode indices were selected to be paired two to a lane (one case pool and one control pool) and a system was used so that no two paired barcodes would have any nucleotide overlap, to maximize data extraction. Barcode pairs were 1-2, 3-9, 4-7, 5-11, 6-12, and 8-10. PCR was performed according to standard Illumina multiplexing protocol with 22 µL of product from above, 1 µL of InPE 1.0, 1 µL of InPE 2.0, and 1 µL of selected barcode. Amplified samples were then cleaned up on MinElute columns as described above and eluted with 41 µL of EB for a total of 40 µL. This product was then re-purified on an E-gel under the 1-2% program for 12 minutes, with its matched pair for sequencing, because this avoided wasted gel space, and because post-amplification/barcoding, there was no risk of cross-contamination (the samples were to be subsequently cluster-generated and run on the same lane). E-gels were loaded as follows: one well of 20 µL Invitrogen 100 bp ladder diluted to 20 ng/µL, one well of 20 µL dH2O, two wells of 20 µL case sample, one well of 20 µL dH2O, one well of 20 µL Invitrogen 100 bp ladder, one well of 20 µL dH2O, two wells of 20 µL control sample, well of 20 µL dH2O, one well of 20 µL Invitrogen 100 bp ladder. The final gel purification step removed primer dimer or any other non-product fragments from the amplified fragments. These slices were cleaned up on Gel Extraction MinElute columns as described above and eluted with 17 µL of EB for a final volume of 16 µL. 1 µL of this product was run on an Agilent 2100 Bioanalyzer according to DNA1000 protocol to quantitate final concentration. Case and control samples were pooled in a 1:1 equimolar ratio and submitted for sequencing on an Illumina Genome Analyzer IIx to be run under single-end 75 bp sequencing conditions, with a phi X control in one lane of the flowcell for standardized comparison.

Subsequent to next-generation sequencing, reads were run through a data analysis pipeline on a high performance cluster (HPC). They were then aligned to the human genome (NCBI build 36, hg18) using the aligner BWA, followed by conversion to SAM format and generation of a Pileup file using SAMtools. The Pileup file was then analyzed and annotated, using an in-house script, to identify variants with a haploid coverage greater than 80, minor allele frequency (MAF) greater than 3.5% and a PHRED-like score (generated from the quality scores in the initial reads) greater than 20. The MAF and Phred-like score cutoffs were determined empirically as described above. Annotation was to all possible isoforms to ensure that any deleterious variants were detected.

Subsequent to alignment and annotation, Sanger sequencing using individual pairs of the stock Raindance library primers (or rarely, custom designed primers for difficult regions) was necessary to deconvolute the 8-individual pools and determine which individual possessed each mutation of interest. The same lymphoblastoid DNA stocks were used for Sanger confirmations. The thresholds for filtering to confirmations of interest, defined as rare and potentially deleterious, were allele frequencies ≤ 2%, and an effect of either missense change, nonsense change, splice site disruption, and start or stop codon disruption. Confirmations of variants were not attempted in the final stages of the experiment if successful confirmation of the same variant in earlier case and control samples indicated that the variant was not rare and exclusive, and thus not of interest to us.

**Identity verification**

While it was impossible to determine individual genotypes from within a pool, a large enough sampling of variants from the genotype data enabled a broad prediction of what the collective 8-individual allele frequencies should look like across a broad range of SNPs. The relationships between known SNP genotypes (SNPs common to all genotyping platforms and falling within Raindance amplicons), combined into 8-individual allele frequencies, and predicted pool allele frequencies from sequence data were graphically plotted in R. The R2 values were used to determine which pools appeared to be correctly identified, which were clearly misidentified, and which were of ambiguous identity. Pools with R2 greater than or equal to 0.8 were considered to be verified, pools with R2 less than or equal to 0.**57** were considered to be falsely identified, and pools with R2 intermediate between these were considered probable verifications but not certain. Pools in the latter two categories had their allele frequencies from Illumina data plotted against the Raindance prediction data from all other pools. This process salvaged 31 misidentified pools, with 15 pools demonstrating ambiguous or unresolved identity issues. Samples in these pools were excluded from any further analysis.

**Stratification correction methods**

Single Nucleotide Polymorphism (SNP) Genotyping:Genomic DNA was extracted from peripheral blood (Simons Simplex Collection) or lymphoblastoid cell lines (NINDS controls) using standard protocols. SSC subjects were genotyped using the Illumina Human1M-Duo v1, Human 1M-Duo v3, or HumanOmni2.5 BeadChips, according to the standard Illumina protocol. NINDS samples were genotyped using the Illumina HumanOmniExpress12v1. All genotyping was performed at the Keck Biotechnology Resource Laboratory at Yale University School of Medicine.

SNP Quality Control:

The following PLINK commands were used to check gender, Mendelian inconsistencies, and cryptic relatedness :

• plink --bfile <Samplefile> --check-sex

• plink --bfile <Samplefile> –-mendel

• plink --bfile <Samplefile> --extract <Hapmap\_LD.prune.in> --mind 0.05 --geno 0.1 --maf 0.01 --hwe-all --make-bed --out <Samplefile.indep>

• plink --bfile <Samplefile.indep> --genome --min 0.05 --out <Sample.IBD.Result>

Where ‘Hapmap\_LD.prune.in’ is a pre-defined list of 129,932 independent SNPs to ensure consistency of results across samples of different sizes. This SNP list was derived from 120 Hapmap individuals with 1Mv1 Illumina data using the command:

• plink --bfile Hapmapfile indep-pairwise 50 5 0.2 --out Hapmap\_LD.prune.in

When cryptic relatedness was detected, the sample with the lower genotypic call rate was excluded. In addition, if the relationship between two samples did not match that recorded by the clinician, both samples were excluded.

Population Outlier Exclusions: After removing individuals with cryptic relatedness and other identity issues described above, Golden Helix SNP and Variation Suite v7.5.4 (SVS; Golden Helix, Inc.) was used to perform a genotype principal component analysis (PCA) among all cases and controls, using 8,210 consensus SNPs not found to be in high linkage disequilibrium. Based on a visualization of a scree plot, Eigenvalues of the first three principal components (found to correspond to 32%, 23%, and 11% of the variation) were plotted against one another and the inter-quartile range (IQR) distance around the median of the study population cluster was calculated. An outlier threshold which included all NINDS samples was selected, and SSC samples that were 6 or more IQRs from the third quartile were excluded (for more detail see <http://doc.goldenhelix.com/SVS/latest/data_quality.html#multidimensional-outlier-detection>). 50 SSC individuals were more distant from the center than were the furthest outlying NINDS controls; since the NINDS controls were defined as a Caucasian cohort, this boundary was used as the cutoff. Therefore, the final SSC set consisted of 1030 individuals. To summarize, of 1332 probands, 1266 were successfully run on the Raindance; 1234 yielded usable sequence; 1148 were not found to be ineligible or ancillary during concurrent Simons sample evaluation; 67 were excluded for insufficient genotype data for PCA; 50 were excluded for being population outliers after PCA, and 1 was excluded for relatedness to another proband via PLINK, giving a final sample of 1030. Of 1015 NINDS controls, 953 were successfully genotyped; 11 were flagged for various QC issues by PLINK, leaving 942 as the final sample.

**Counting**

We only analyzed rare variants (with frequency in our sequence data of less than 2%, and of less than 1% in all populations in the Exome Variant Server and SeattleSNP databases) seen either in cases or controls exclusively. Additionally, to offer the most conservative interpretation of the data possible, we also performed analyses in which only singleton mutations were counted. Any variant which turned out to be a cell line artifact and was not present in whole blood upon segregation analysis was removed from the analysis. While this was only applicable to cases, and not to controls since whole blood DNA and family samples were not available, it likely did not introduce a significant degree bias towards controls, since only **4 of the 165** cell-line confirmed final rare and exclusive variants in cases dropped out in whole blood and were removed from the analysis (of the remaining 161, 159 were inherited and 2 were de novo). All p-values reported were obtained from the 1-tailed Fisher exact test. Bonferroni correction was used to control for the 10 genes tested. Three variants were seen once in cases and once in controls in the combined *CNTNAP2* data set, and were removed from the combined data analyses (see Supplemental Table 2).