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Supplemental Data

Mutations in *NKX6-2* Cause Progressive Spastic Ataxia and Hypomyelination

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Figure S1

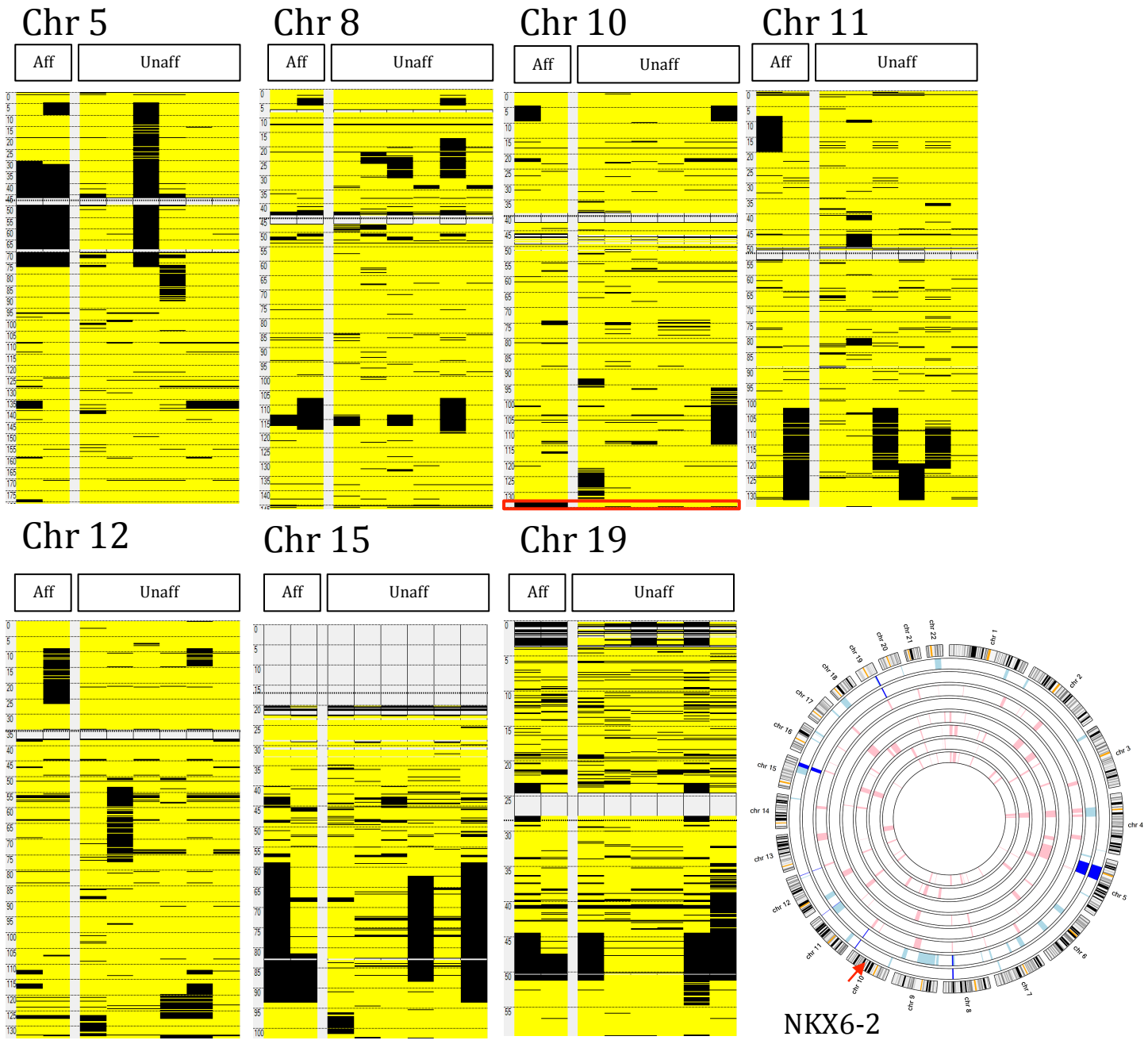
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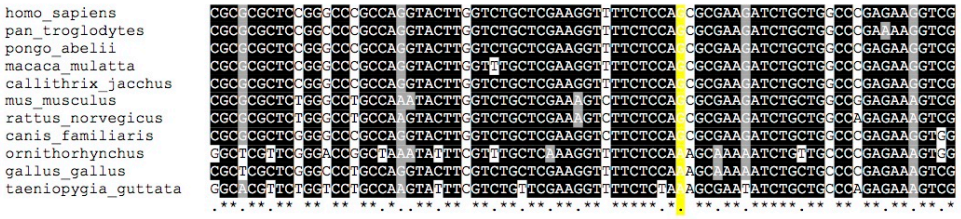
Figure S2

Sequence conservation based multiple alignments on 37 Eutherian mammals provided by Ensembl[1]. The figure shows a constrained region surrounding the p.Leu163Val mutation in NKX6-2 homeodomain.



Supplementary Methods

Whole Exome sequencing

Family 1 and 2

In family 1 exome Exome sequencing and alignments were carried out at Macrogen (Macrogen Inc.Seoul, South Korea). Exome capture was performed using SureSelect Human All Exon V4 enrichment kit (Agilent Technologies, Santa Clara, CA, USA) and sequenced on an Illumina HiSeq4000 instrument (Illumina, San Diego, CA,USA) generating 100bp paired-end reads. Reads were aligned using BWA [2] to the hg19 human genome reference. Base quality recalibration, realignment and variant calling was done using GATK HaplotypeCaller-based pipeline [3-6]. Called variants were annotated in-house using Annovar [7] and filtered for rare homozygous and compound heterozygous variants using custom R scripts. Only variants with MAF of < 0.01 in 1000 Genomes Project [8], NHLBI GO Exome Sequencing [9], and ExAC [10], were included. A mean coverage of 80x was obtained with 93.4% of based covered at more than 20x. Similarly, in family 2 Exome sequencing was performed using SureSelect Human All Exon enrichment kit (Agilent Technologies, Santa Clara, CA, USA). Reads were aligned using Novoalign (Novocraft, Selangor, Malaysia) to hg19 genome reference and processed with Dindel[11]. In family 3, Exome capture was performed on the index using TruSeq Exome Enrichment kit (Illumina) following the manufacturer's protocol. Sample was prepared as an Illumina sequencing library, and in the second step, the sequencing libraries were enriched for the desired target using the Illumina Exome Enrichment protocol. The captured libraries were sequenced using Illumina HiSeq 2000 Sequencer. The reads were mapped against UCSC hg19 (http://genome.ucsc.edu/) by BWA (http://bio-bwa.sourceforge.net/). The SNPs and Indels were detected by

SAMTOOLS (http://samtools.sourceforge.net/). Variants from WES were filtered such that only novel (or very low frequency <0.1%), coding/splicing, homozygous variants that are within the autozygome of the affected individual and are predicted to be pathogenic were considered as likely causal variants [12; 13].

Haplotype analysis

Samples from family 1 and 2 were re-aligned using Novoalign (Novocraft, Selangor, Malaysia) to the hg19 genomic reference and variants were called using GATK HaplotypeCaller-based pipeline[3-5]. Shared regions of homozygosity were identified using HomozygosityMapper [14]

Comparative modeling

The Ensembl database [1] was used to identify homeodomain region with NKX6-2 and to obtain multispecies alignments. SWISS-MODEL [15] was used to identify known structures with a high sequence similarity to NKX6-2 and for PDB model generation. 3a01.2.A PDB model was selected as a template showing the highest sequence similarity (56.25%). DUET [16] was used to predict c.C487G mutation effect on protein stability.

Western blotting

Fibroblast samples from affected individuals and healthy control were obtained with consents. After reaching 90% confluence, the cultured cells were harvested and lysed in ice-cold lysis RIPA buffer (50 mM Tris-HCl (pH8.0), 150 mM NaCl, 0.5% DOC, 0.1% Triton x100, 0.1% Sodium Dodecyl Sulfate and SIGMAFAST Protease Inhibitor Cocktail) for 30 minutes. After that the lysate was centrifuged at 16000g at 4°C. The supernatant was transferred to a new tube and the protein concentration was measured using BCA protein assay kit (Pierce) according to manufacturer's protocol.

Equal amounts of proteins (12 ug per lane) from affected individuals and healthy controls were separated by SDS/PAGE using Bis-Tris gradient gels (4–12% NuPAGE; Invitrogen) and transferred by electrophoresis onto Immobilon-P transfer membranes (Millipore). After being blocked with fat-free milk, membranes were immunoblotted with the respective rabbit anti-NKX 6-2 (Abcam ab179532; 1:1000) antibodies at 4 °C overnight. Blots were then exposed to horseradish peroxidase-conjugated goat anti-rabbit IgG (17210, Bio-Rad Laboratories, 1:5,000) for 1h at room temperature. Blots were developed using ECL-Prime (GE Healthcare), visualized via a ChemiDoc Touch Imaging System, and analysed using Image Lab 5.2 software (Bio-Rad Laboratories). For the quantifications, the signal intensity of each of the Kv1 bands was normalized to the signal intensity of the corresponding reversible Ponceau staining as a reliable loading control[17]. The NKX6-2 level were expressed as a percentage of the control sample.

Gene Co-expression Network Analyses

We generated a Gene Co-expression Network (GCN) using expression data generated by the UK Brain Expression Consortium[18; 19] and assayed with the Affymetrix Exon 1.0 ST Array in intralobular white matter originating from 83 neuropathologically-normal individuals. In total, 19152 transcripts were used by the weighted gene co-expression network analysis (WGCNA) R package[20]. In brief, after outlier removal, a "signed" GCN was constructed by creating a signed Topological Overlap Measure (TOM) matrix based on Pearson correlation. Gene modules were created by hierarchical clustering based on a 1-TOM dissimilarity matrix. The results of the initial hierarchical clustering were post-processed using the k-means clustering search method with 30 iterations.

After this, gene modules were functionally annotated with gProfileR[21] R package using Gene Ontology (GO) database without Electronic Inferred Annotations (EIA) and accounting for multiple testing with gSCS. We consider all associations with corrected P-values of less than 0.05 as significant.

Given that *NKX6-2* is known to be a transcription factor we also used an Algorithm for the Reconstruction of Gene Regulatory Networks in a Mammalian Cellular Context[22] (ARACNe-AP) to identify its most probable target genes in human white matter. The algorithm was used in its bootstrapping mode with 100 bootstraps. The resulting regulon (consisting of the predicted target genes) was functionally annotated with gProfileR R package using Gene Ontology (GO) database without Electronic Inferred Annotations (EIA) and accounting for multiple testing with gSCS.

Identification of phenotypically similar known Mendelian disorders

We used the web-resource Phenomizer (http://compbio.charite.de/phenomizer/ accessed on 10th. March 2017) to identify known Mendelian disorders with a high degree of phenotypic similarity to the affected individuals with homozygous deleterious variants in *NKX6-2*. This analysis was performed using a set of curated Human Phenotype Ontology terms, which were representative of the core clinical features of this patient group. The terms used were as follows: Abnormality of the cerebral white matter (HP:0002500); Brisk reflexes (HP:0001348); Dystonia (HP:0001332); CNS hypomyelination (HP:0003429); Cerebellar ataxia associated with quadrupedal gait (HP:0009878); Cerebellar atrophy (HP:0001272); Lower limb spasticity (HP:0002061) and Nystagmus (HP:0000639). Semantic similarity scoring was performed with no assumptions made regarding the mode of inheritance and using the Resnik (not symmetric) method. All Mendelian disorders with a corrected p-value of <0.001 (Benjamini Hochberg procedure) were retained.

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A team of world-class researchers working towards a better understanding of the genetic basis in neurological disorders

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SUPPLEMENTARY MATERIALS LEGEND

Table S1. Haplotype analysis data in family 1 and 2.

Figure S1. Homozygozity mapping in family 3 data.

Figure S2. Sequence conservation of the homeobox domain.

Supplementary Methods

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