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Supplemental Data

Loss of Function of Glucocerebrosidase GBA2

Is Responsible for Motor Neuron Defects

in Hereditary Spastic Paraplegia

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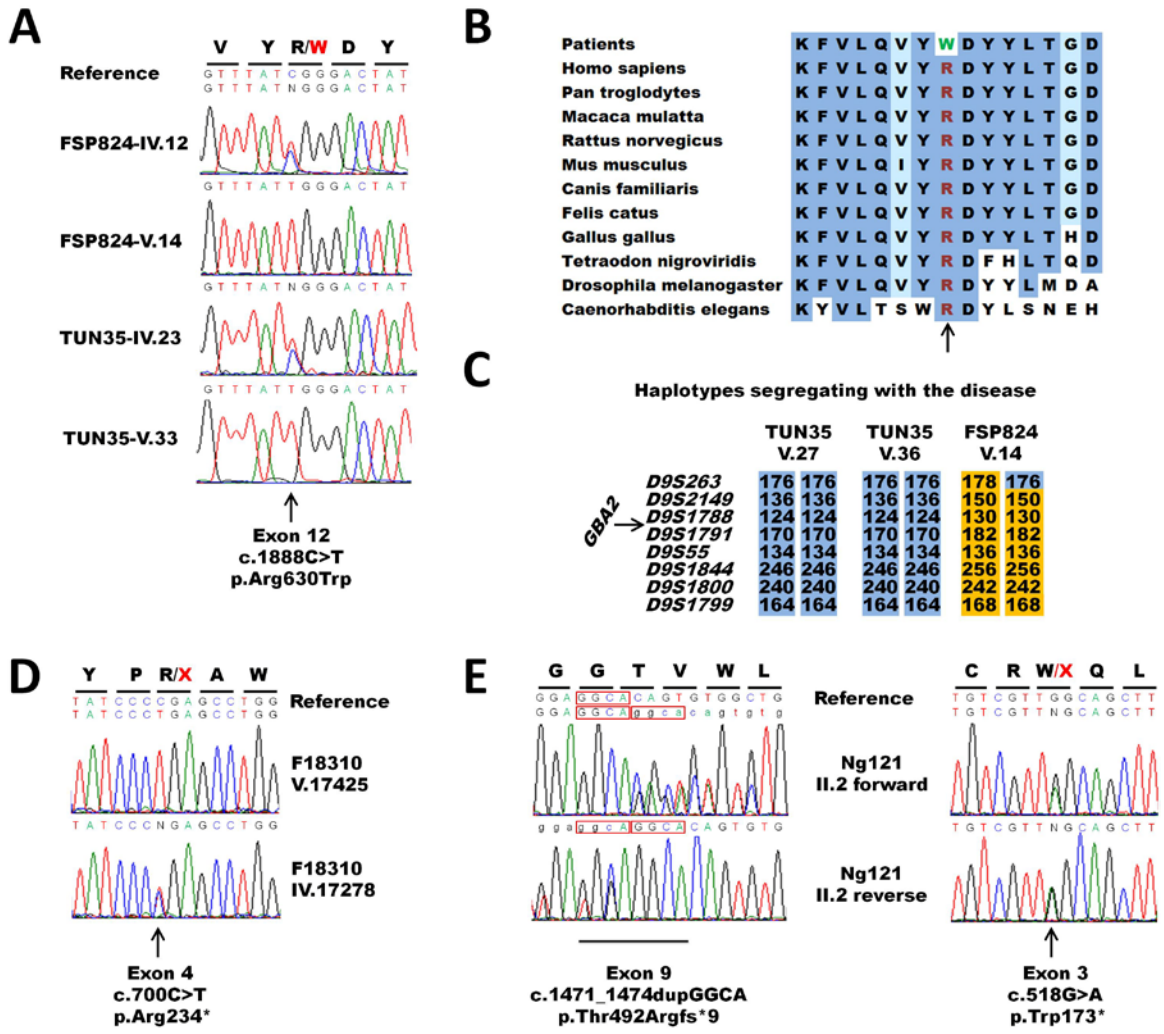

Figure S1. Mutations in *GBA2* (RefSeq NM_020944.2)

(A, D, E) Sequencing chromatograms showing the identified mutations in subjects of the TUN35 and FSP824 families (A) and in individuals of families F18310 (D) and Ng121 (E). All mutations were checked using ALAMUT 2.2 and Mutalyzer: c.518G>A (p.Trp173*), c.700C>T (p.Arg234*), c.1888C>T (p.Arg630Trp) and c.1471_1474dupGGCA (p.Thr492Argfs*9).

(B) Conservation of amino acid Arg (R) 630 across various species.

(C) Haplotypes of eight chromosome 9 microsatellites covering the *SPG46* candidate region in three subjects with the p.Arg630Trp homozygous mutation. Alleles are given in base pairs.

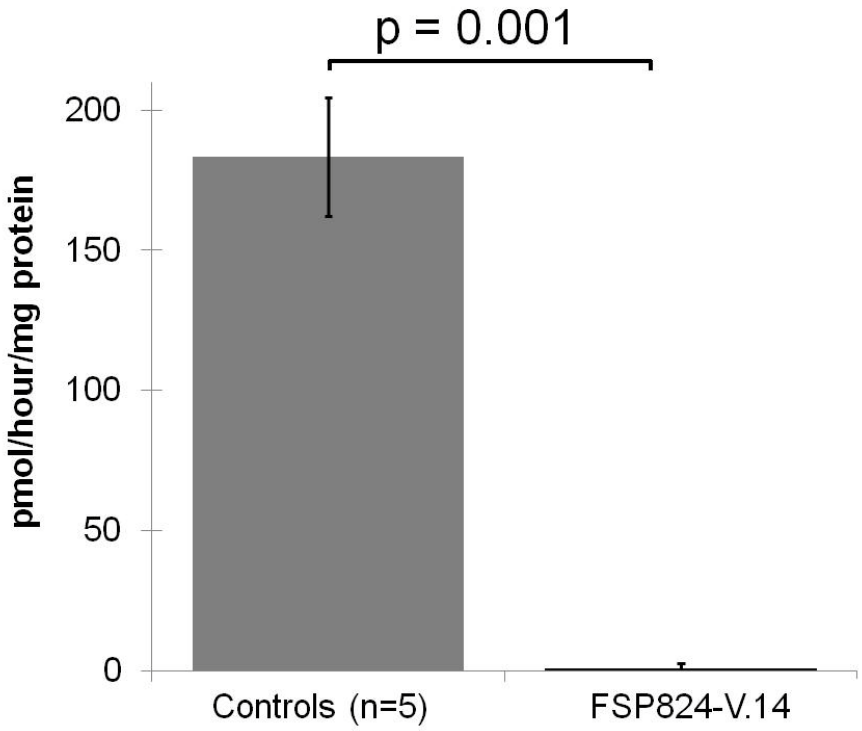

Figure S2. Glucosylceramidase Activity in Lymphoblasts

Lymphoblastoid cell lines of one SPG46 individual (FSP824-V.14, sampling at 30 years of age) and of five healthy controls (sampling at 31, 35, 52, 69 and 73 years) were obtained following transformation with the Epstein-Barr virus of mononuclear white blood cells. Lymphoblasts were maintained in RPMI 1640/L-Glutamine culture medium (PAA) supplemented with 15% fetal bovine serum, penicillin (100 UI/ml) and streptomycin (100 µg/ml) and 1% HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) buffer. Cells were harvested, pelleted and washed twice with PBS pre-cooled at 4°C before re-suspension in water with a protease inhibitor cocktail (Complete, EDTA-free, Roche). The activity of the non-lysosomal glucosylceramidase was tested using 20 µg of total protein that were preincubated with 5 mM of conduritol-β-epoxide (CBE) (Sigma) to inhibit the lysosomal glucocerebrosidase GBA1 for 1 hour at 4°C, rotating at 1000 rpm. Assays were incubated with 5 mM of the artificial 4-methylumbelliferyl-β-glucoside substrate (4-MU-β-glucoside, Sigma) in McIlvaine buffer (0.1 M citrate and 0.2 M phosphate buffer, pH 5.8) for 1 hour at 37°C rotating at 500 rpm and enzymatic reactions were stopped by the addition of 200 µl of glycine-carbonate buffer (pH 10.7). The amount of liberated 4-MU was determined with a FlexStation 3 Benchtop Multi-Mode Microplate Reader (Molecular Devices). The data were expressed as pmoles of converted substrate/ mg cell proteins/ hour. All measures were done in triplicate. Statistical comparison was done between the mean of the triplicates of the affected subject compared to the means obtained in controls.
Error bars are SEM.

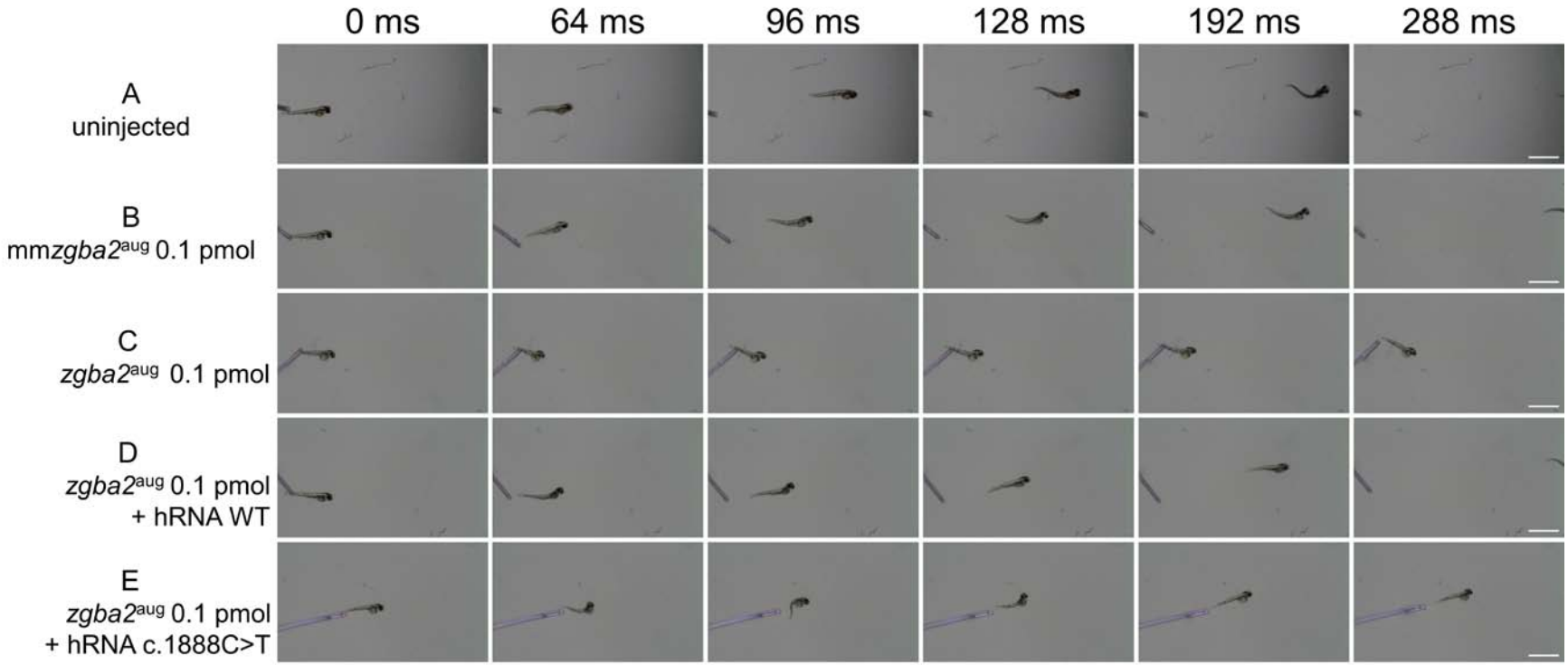

Figure S3. *zGba2* Inactivation Leads to Locomotor Defects that are Rescued by the WT *hGBA2* mRNA, but not by the Mutated c.1888 C>T mRNA

This figure shows the series of images taken at various times after the zebrafish tail was lightly touched. Uninjected (A) or mm*zGba2*^{aug} morphants (B) instantly reacted by swimming rapidly away. In contrast, larvae depleted for *zGba2* (C) showed a very weak response when touched on the tail. When AMO *zGba2*^{aug} was co-injected with h*GBA2* WT mRNA (D), morphants recovered a perfect swimming reflex, in contrast to double AMO *zGba2*^{aug} – h*GBA2* c.1888C>T mRNA injected morphants (E), which showed severe locomotor defects. Scale bar, 2 mm.

Table S1. *SPG46* Exon Capture and Next-Generation Sequencing in Subjects of Family TUN35

	Individuals	
	TUN35-V.33	**TUN35-V.32**
Total number of reads	195,811	558,763
Average length (bp)	311	389
Mapped reads	177,574 (91%)	555,255 (99%)
Unique reads overlapping enriched regions	106,419 (60%)	340,753 (61%)
Number of enriched regions	1,727	1,727
Regions with matching unique reads	1,705 (99%)	1,725 (100%)
Regions covered entirely	1,203 (70%)	1,658 (96%)
Regions with an effective coverage over 10X	1,026 (59%)	1,690 (98%)
Effective coverage on enriched region	14X	76X

Sequencing of all exons of the *SPG46* linkage interval was performed with the use of a custom sequence capture array (Roche NimbleGen, Madison, WI) focusing on exons (n=1,727 from 183 genes) and 1 kb of untranslated regions (UTR) on chromosome 9 between positions 27,536,543 bp and 75,455,094 bp that was used to hybridize shotgun fragment libraries obtained from DNA of both selected subjects. Massively parallel sequencing was performed on this enriched library using the Roche 454 GS FLX sequencer (Roche) with Titanium reagents. Sequence data were then aligned with the hg18 version of the human genome as a reference (Ensembl database) at the Genoscope facility and analyzed using in-house software.

A mean of 377,287 sequencing reads was obtained with an average length of 350 bp, of which 95% of all reads could be mapped, 60% of them overlapping the enriched regions. From the total of 1,727 enriched regions, 83% were covered entirely, and 78% of targeted bases were covered at least 10-fold. The mean coverage of enriched regions was 45-fold. Variant filtering excluded those that had fewer than three reads or accounted for 25% or less of all reads.

Table S2. Primers Used for *GBA2* Sanger Sequencing

Name	Oligonucleotide sequence
GBA2-EX1F	CCGTCCAGGACCTACAGAGA
GBA2-EX1R	GACAAGAAGCCCAACTGCTT
GBA2-EX1altF	CTGTGCGTAAAAAGGGGAAC
GBA2-EX1altR	CGCCTACCCCTCCTATATCC
GBA2-EX2-3F	GAGGAAGAAAGGTTTGAAAGCA
GBA2-EX2-3R	CAGGAAGCATCCCGAGAGTA
GBA2-EX4F	GTAGTGGAGAAGGGAGAGAAAGC
GBA2-EX4R	AGATATGGGGTTTCACGTGTTAG
GBA2-EX5F	AGGTTGGAGCCATGAACATC
GBA2-EX5R	AGCTCCCCAGCTCCTCTTAC
GBA2-EX6F	CACGAGTCACGGTAAGGAAAG
GBA2-EX6R	GCTTTGGCCTGAGAGAAACA
GBA2-EX7F	GATGGTGGGAAGAGAGGTTG
GBA2-EX7R	CTCTGCCAAGCTGAGATCCT
GBA2-EX8F	GTAGGCATTGCTGGAGCTGT
GBA2-EX8R	CCTCCCAAGATGGAAAGGAT
GBA2-EX9F	CCGGTATTGGATGACAGGTG
GBA2-EX9R	CCCCTCTGTAGCTTGTCCTC
GBA2-EX10F	GGTTAGGATGAGGGGGTGTT
GBA2-EX10R	CTGGGGGAGAGACACAGAAG
GBA2-EX11F	TATGCTTCCTTTGCCCTCAT
GBA2-EX11R	GGTTCAGGTCCTTCCAATCA
GBA2-EX11seqF	GCTCTGGCCACTCTCAGGGA
GBA2-EX12-13F	GCACCTGTGAAAAGGAGGAA
GBA2-EX12-13R	CTGATCAGGGACATGGGTTT
GBA2-EX14-15F	GAGGCTATGCAGACCAGACC
GBA2-EX14-15R	TGGACGTTCAGCTCAAAGATAG
GBA2-EX16F	GACTCCAGGCCGCTATTACA
GBA2-EX16R	CCCAGCAAGTGGAGGAGATA
GBA2-EX17F	GGCTTCTGTCTGTGGGTCAT
GBA2-EX17R	GTCAGGGAACAGGTGAGGAA

All coding exons of *GBA2*, including flanking splicing sites and at least 50 bp of intronic sequence on each side, were PCR-amplified using specific primers in a final volume of 10 µl with 0.4 unit of Dream Taq (Fermentas), 20 ng of genomic DNA in 1/5 Solution Q (Qiagen), at an annealing temperature going down regularly from 65 to 55°C between two PCR cycles. Sequencing reactions were performed using BigDye chemistry according to the manufacturer's protocol (Applied Biosystems). The sequence products were run on an ABI-3730 sequencer, and electrophoretic profiles were compared with the hg19 reference sequence (NM_020944.2) using Seqscape 2.6 (Applied Biosystems) and BioEdit Sequence Alignment Editor (Ibis Biosciences, Carlsbad, CA). The effects of variants and mutations were analyzed using ALAMUT 2.2 (Interactive Biosoftware, Rouen, France).

Table S3. Clinical Description of SPG46 Affected Individuals

Family	Ng121			F18310		FSP824 (SR59)	TUN35				
Mutation(s)	c.518G>A, p.Trp173* + c.1471_1474dupGGCA, p.Thr492Argfs*9 (heterozygous)			c.700C>T, p.Arg234* (homozygous)		c.1888C>T, p.Arg630Trp (homozygous)	c.1888C>T, p.Arg630Trp (homozygous)				
Consanguinity / Origin	No / Belgium			Yes / Turkey		Yes / Portugal	Yes / Tunisia				
Individual No. (sex)	II.1 (F)	II.2 (F)	II.3 (M)	V.16484 (F)	V.17425 (F)	V.14 (F)	V.27 (M)	V.26 (F)	V.32 (F)	V.33 (F)	V.36 (F)
Age at last examination (years)	63	61	58	40	22	30	30	32	35	31	32
Age at death (years)	63	61	72	NA	NA	NA	NA	NA	NA	NA	NA
Age at onset (years)	7	7	7	1	Infancy	16	2	2	2	6	10
Disease duration (years)	56	54	65	39	~20	14	28	30	33	25	22
Disability scale[a]	7	7	6	2	4	3	2	3	4	3	3
LL spasticity	Severe	Severe	Severe	Severe	Moderate	Severe	Severe	Moderate	Severe	Severe	Mild
LL reflexes	Very brisk	Very brisk	Very brisk	Very brisk	Very brisk	Very brisk	Very brisk	Very brisk	Very brisk	Very brisk	Very brisk
LL distal weakness	Moderate	Moderate	Moderate	Mild	Moderate	Moderate	Moderate	Moderate	Moderate	Mild	Moderate
LL distal amyotrophy	+	+	+	-	-	-	-	-	-	-	-
Extensor plantar reflexes	+	+	+	+	+	+	+	+	+	+	+
UL spasticity	Severe	Severe	Moderate	Mild	Mild	Moderate	Mild	Mild	Mild	Moderate	Mild
UL reflexes	Very brisk	Very brisk	Very brisk	Very brisk	Very brisk	Very brisk	Brisk	Very brisk	Brisk	Brisk	Brisk
UL distal weakness	Moderate	Moderate	Moderate	-	Reduced fine motor skills	Mild	Mild	-	-	-	-
UL distal amyotrophy	+	+	+	-	-	-	-	-	-	-	-
Cognitive decline	Severe	Very severe	Moderate	Mild / Moderate	Moderate	Very mild, frontal type (MMSE 22, total IQ 65, verbal IQ 72, performance IQ 72)	Mild	Mild	Mild	Mild	Mild
Cerebellar ataxia and dysarthria	+	+	+	+	+	+	+	+	+	+	+
Decreased vibration sense	+	+	+	+	+	-	+	-	-	-	-
Pseudo bulbar dysarthria	+	+	+	-	+	+	+	+	-	+	-

Pes cavus	-	-	-	-	-	-	+	+	+	+	-
Scoliosis	+	+	+	-	-	-	+	-	-	+	-
Urinary symptoms	-	+	+	+	+	Mild	+	+	-	-	-
Genital symptoms and hormonal exploration	-	Small amount of pubic hair	Hypogonadism, hypogenitalism	-	NE	-	Hypogonadism, infertility (see Table S4)	NE	NE	NE	NE
Cataract	+	+	+	+	+	+	+	+	+	+	+
Other signs	Kyphosis, hearing loss, anemia	Kyphosis, hearing loss, anemia	Hyperkyphosis, hearing loss, anemia	Diabetes mellitus, anemia	Diabetes mellitus, obesity, cleft lip and palate	Mild facial and pronounced cervical dystonia with rectrocollis	Facial dyskinesia, bilateral action hand tremor	-	Depression	Strabismus	-
Sural nerve biopsy	Collateral sprouting, onion bulbs	Bands of Büngner, collateral sprouting, onion bulbs	Bands of Büngner, collateral sprouting, onion bulbs	ND	ND	ND	ND	ND	ND	ND	ND
ENMG	Axonal polyneuropathy	Axonal polyneuropathy	Axonal sensorimotor neuropathy	Axonal demyelinating sensorimotor neuropathy	Axonal-demyelinating sensorimotor neuropathy	Normal	Normal	ND	ND	ND	ND
Brain MRI	ND (brain CT: discrete cerebellar atrophy)	TCC, cerebellar and cerebral atrophy, mammillary body atrophy and mesencephalon atrophy (hummingbird/colibri sign), periventricular WMH	TCC, cerebellar and cerebral atrophy, mammillary body atrophy and mesencephalon atrophy (hummingbird/ colibri sign)	TCC, cerebellar atrophy	ND	Very mild TCC, slight cerebral atrophy	TCC, cerebellar and cerebral atrophy	ND	TCC, cerebellar and cerebral atrophy	ND	ND

ENMG: electroneuromyography; F: female; IQ: intelligence quotient; LL: lower limbs; M: male; MMSE: Mini-Mental State Examination; MRI: magnetic resonance imaging; NA: not applicable; NE: not examined; ND: not done; TCC: thin corpus callosum; UL: upper limbs; WMH: white matter hyperintensities; +: present; - absent.

[a] Disability was assessed on a 7-point scale in which 1 indicates minimal disability (slight stiffness of the legs); 2, mild disability (unable to run, but full autonomy); 3, moderate disability in walking (reduced perimeter, frequent falls); 4, severe disability (unilateral assistance required to walk); 5, bilateral assistance required to walk; 6, wheelchair bound; and 7, bedridden.

Table S4. Sperm Count, Motility and Morphology in Subject TUN35-V.27

	TUN35-V.27	Control conditions
Sperm volume (ml)	4.3 ± 1.7	2 - 6
Spermatozoid concentration (million/ml)	3.875 ± 1.575	> 20
Spermatozoid normal mobility (%)	3.5 ± 0.5	> 40
Normal morphology (%)	11.5 ± 1.5	> 30

The results are mean ± SEM derived from two independent spermograms.

Sperm was analyzed from two different semen samples of subject TUN35-V.27 collected within five months of each other. Semen samples were obtained by masturbation after 3-5 days of abstinence. Sperm concentration, viability, motility and morphology in semen were determined using standard methods.

Heads of spermatozoids were small, elongated and thin. Acrosomes were abnormal as well as the midpiece. Spermatozoids also presented a severe reduction in velocity. Morphological and behavioral spermatozoid abnormalities were accompanied by severe necrospermia.

Table S5. Comparison of Axonal Architecture between *zGba2* Morphants at 48 hpf

A. Axonal Morphology

Morphants	Normal axonal morphology (%)	Abnormal axonal morphology (%)	Number of counted axons
mm*zGba2*^{aug}	56	2	58
zGba2^{aug}	38	20	58
zGba2^{aug} + hRNA WT	47	1	48
zGba2^{aug} + hRNA mutated	28	16	44

B. Statistical Evaluation

Statistical comparison	p value	Conclusion
mm*zGba2*^{aug} vs *zGba2*^{aug}	< 0.00001	Invalidating z*Gba2* has effects on axonal branching
mm*zGba2*^{aug} vs *zGba2*^{aug} + hRNA WT	0.87	No phenotypic differences between zebrafishes injected with the mismatch AMO or *zGba2* morphant with the WT human mRNA
mm*zGba2*^{aug} vs *zGba2*^{aug} + hRNA mutated	< 0.00001	Mutant mRNA does not rescue the phenotype, due to the *zGba2*^{aug} AMO
zGba2^{aug} vs *zGba2*^{aug} + hRNA WT	< 0.00001	WT mRNA rescues the knock-down phenotype
zGba2^{aug} + hRNA WT vs *zGba2*^{aug} + hRNA mutated	< 0.00001	WT and mutant hRNA do not have similar effects on motoneuron morphology

Axonal morphology was observed with the Znp-1 staining and manually evaluated by distinguishing between normal-looking axons and abnormal-looking (i.e. shorter or over-branched) axons. Statistical analyses were performed by comparing the proportion of normal and abnormal axons between the control-injected population and injected embryos using the Chi square test. Counts were done in at least 10 different zebrafishes in all conditions.
AMO: antisense morpholino oligonucleotide; hpf: hours postfertilization; h: human; vs: versus; WT: wild type.