Bi-allelic variants in RNF170 cause hereditary spastic paraplegia

- Supplementary Material –

Supplementary methods

Supplemental information on variant annotation methods

Family A - Genome sequencing of both affected siblings from Familiy A (A.4, A.5) was performed using Truseq PCR-free sample preparation (Illumina), followed by sequencing on a HiSeq X HD v2.5 instrument (Illumina). More than 1,684,998,067 reads with an average read length of 150bp were produced. Data was processed and analysed using the Genesis pipeline[1]. 99.77% of reads could be mapped to the UCSC human reference assembly (hg19); the average read depth was 34.75.

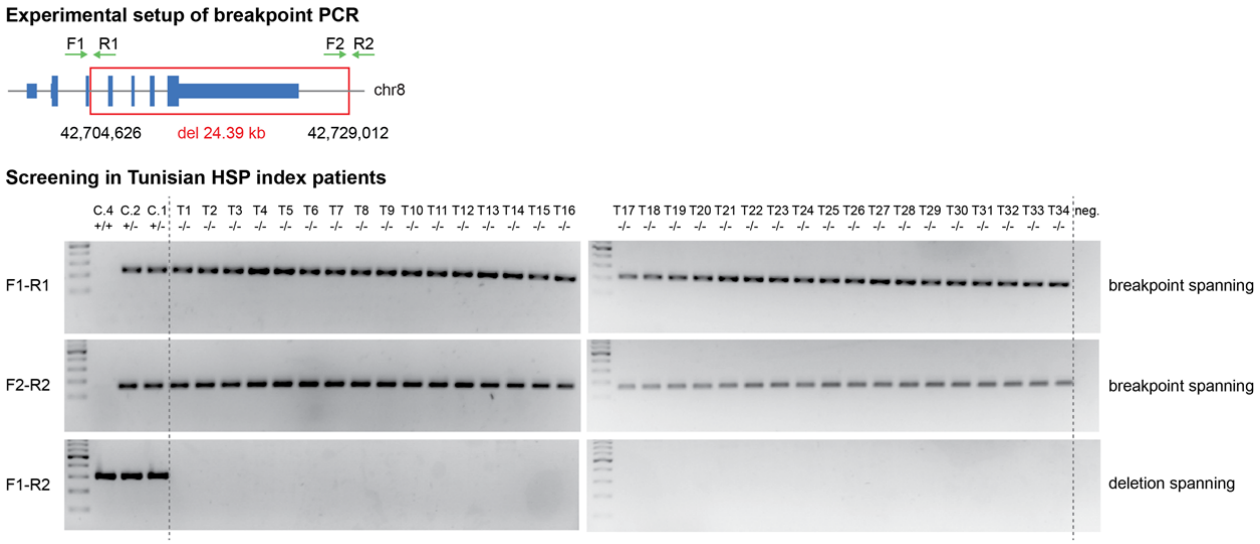
Family B - Exome sequencing of one affected sibling (B.4) was performed using the Agilent SureSelect All Exon V6 kit (Agilent) and a HiSeq 2500 (Illumina) platform. Reads were aligned to the human reference genome (UCSC hg19), with Burrows-Wheeler Aligner (BWA,V.0.7.8-r455)[2]. High quality indel and single nucleotide variant calling and annotation were performed using GATK v3.1 using standard filtering criteria (read depth >=10%, genotype quality score >=50).2 Candidate genes were prioritized by searching for homozyogus variants with a minor allele frequency < 0.1% in 1000 in-house ethnically-matched Iranian control exomes, dbSNP, 1000 Genomes and ExAC.

Family C - Trio-Exome sequencing of the index case and his parents (C.1, C.2, C.4) was performed using a Sure Select Human All Exon 60Mb V6 Kit (Agilent) for enrichment and the HiSeq4000 (Illumina) platform for sequencing. An average of 135,888,843 reads were produced per sample and aligned to the UCSC human reference assembly (hg19) with BWA v.0.5.8.1 More than 98% of the exome was covered at least 20× and the average coverage was

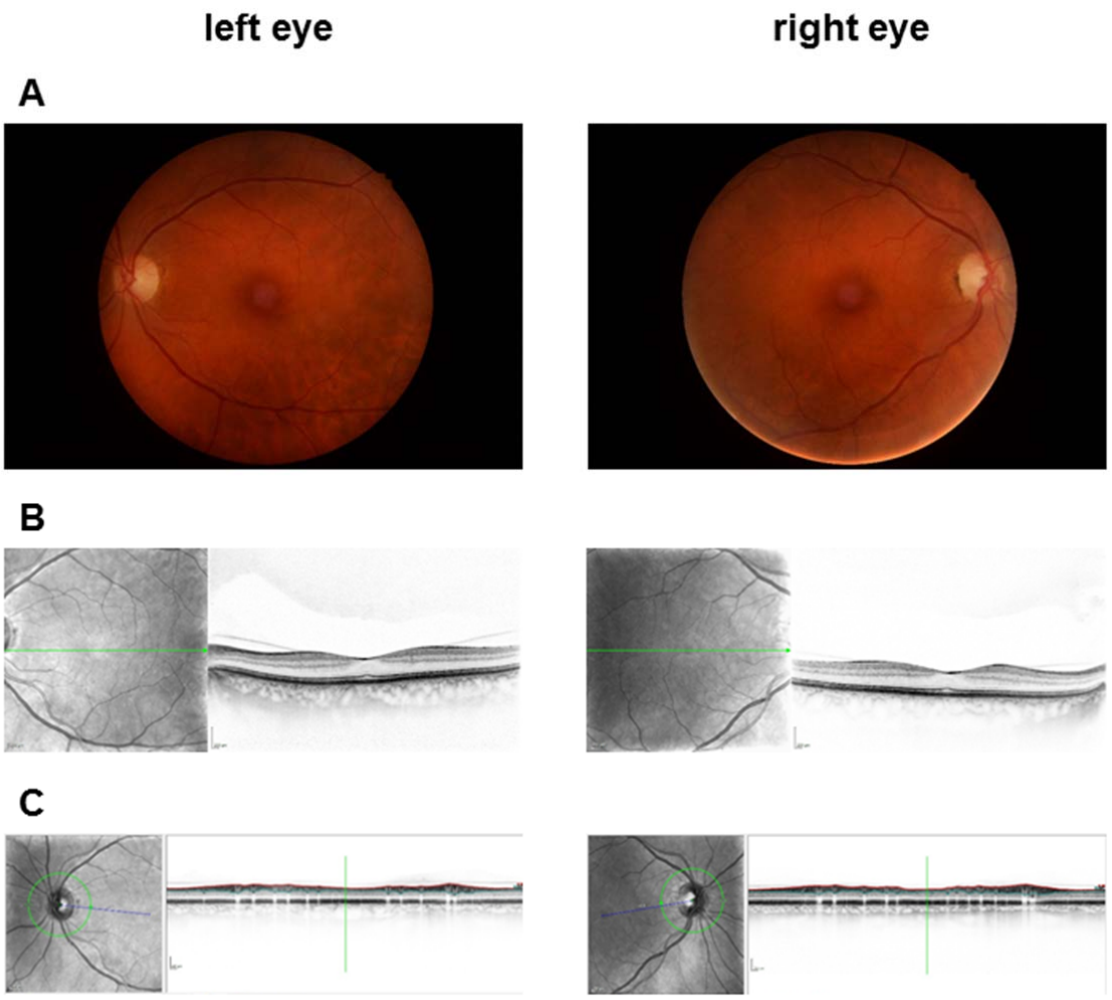
more than 126×. Single-nucleotide variants (SNVs) and small insertions and deletions were detected with SAMtools v.0.1.7. Copy number variations (CNVs) were detected with ExomeDepth[3] and Pindel[4]. Variant prioritization was performed based on an autosomal recessive (MAF <0.1%) and autosomal dominant (*de novo* variants, MAF <0.01%) inheritance model.

Family D – Duo exome sequencing of both affected siblings (D.3, D.4) was done using an xGen Exome Research Panel v1.0 for targeted enrichment and a HighSeq 4000 sequening platform (Illumina). The sequence reads were aligned to the reference genome, hg19, using BWA MEM and underwent duplicate removal (Picard v2.5.0), indel realignment and base quality realignment (GATK) and variant calling (HaplotypeCaller) as recommended in the GATK Best Practices[5]. The detected variants were annotated by ANNOVAR[6]. Variants were filtered to consider only homozygous SNVs and short Indels due to their higher probability of contribution to the disease in this consanguineous family.

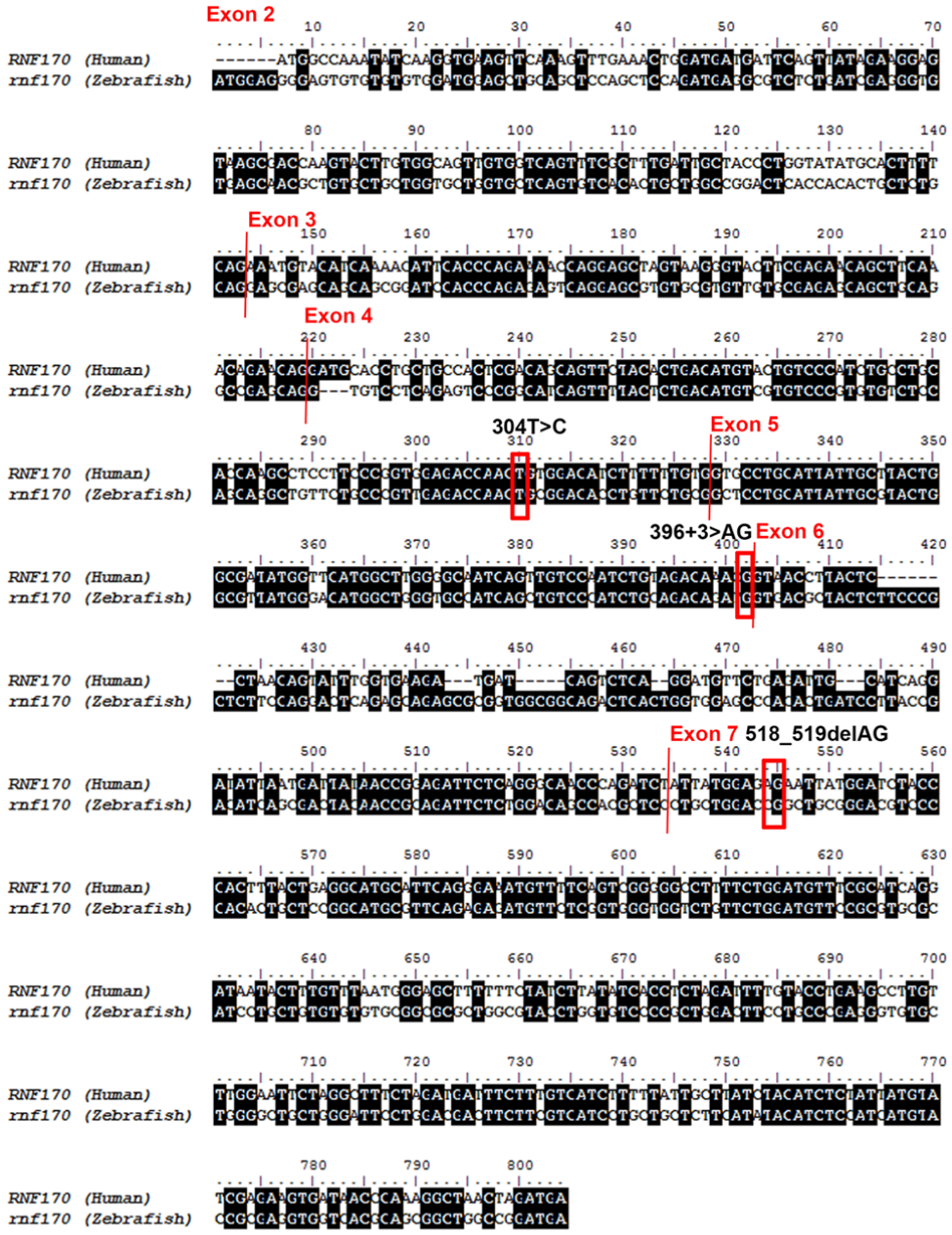
Supplementary Figures



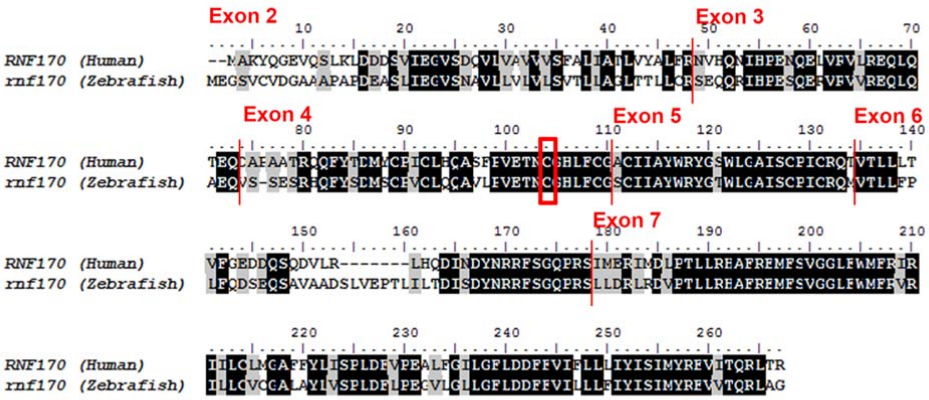
Supplementary Figure 1: Screening for the chr8:g .42,704,626_42,729,012delinsTTTTGGT mutation in 34 Tunisian HSP index patients of unknown genetic etiology.



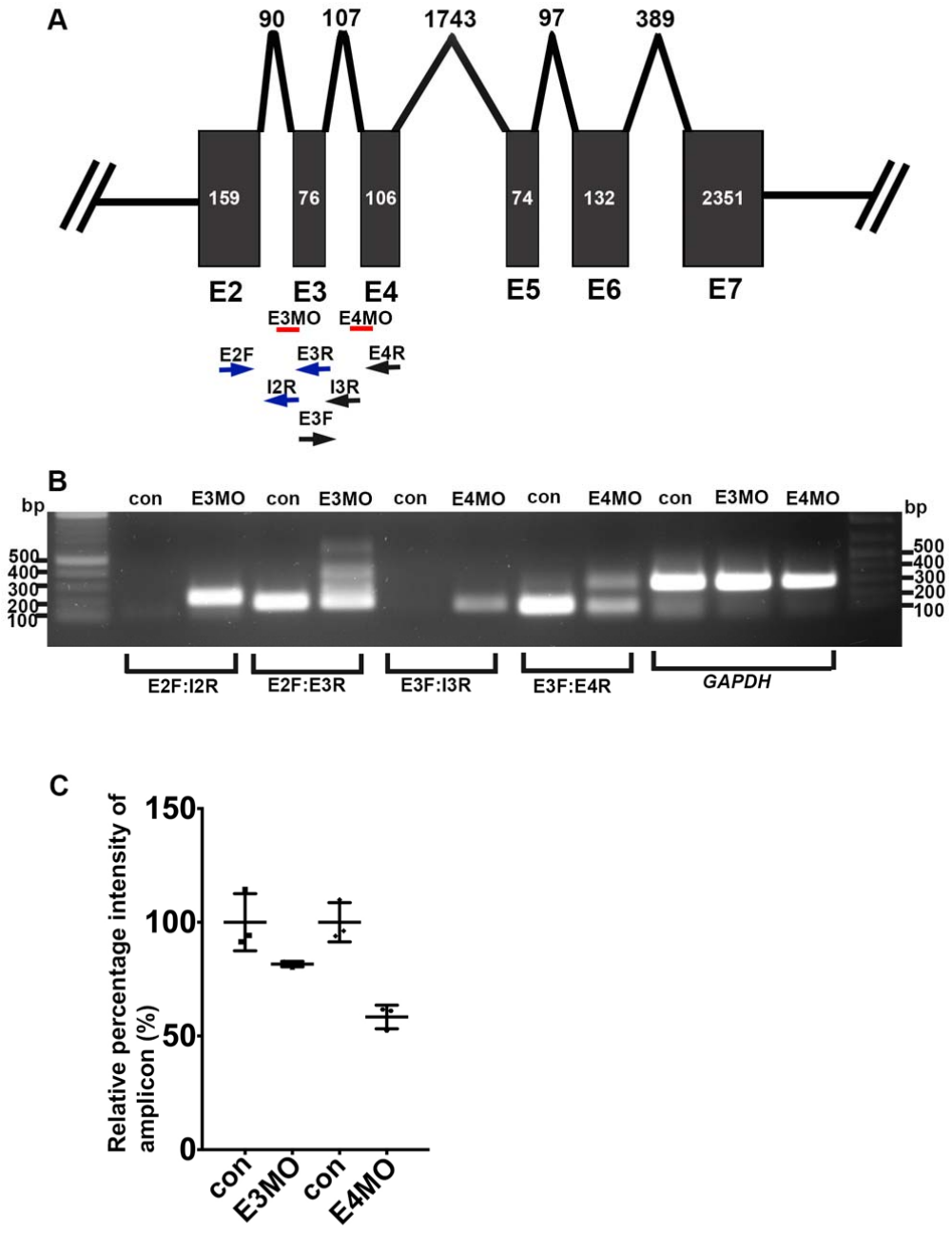
Supplementary Figure 2: Fundoscopy and optical coherence tomography (OCT) of patient A.4. Color fundus photography shows mild paleness of the optic nerve head in the temporal sector on both eyes; OCT reveals temporal optic nerve atrophy on both eyes, but otherwise unremarkable retinal structures, in particular no chorioretinal atrophy.



Supplementary Figure 3: Alignment of zebrafish *rnf170* coding region with human *RNF170*. Black highlighted nucleotides indicate regions of homology, red bars mark exon boundaries, and red boxes indicate patient mutations.

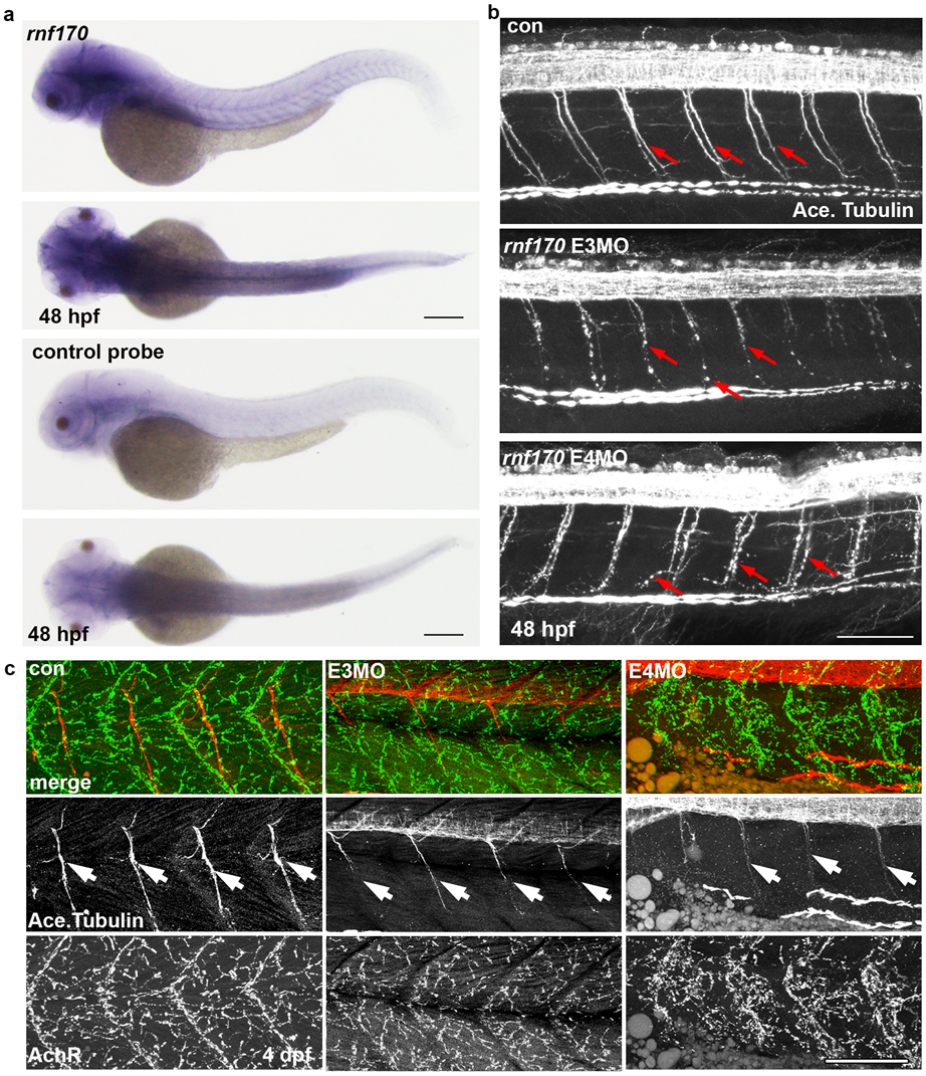


Supplementary Figure 4: Alignment of zebrafish Rnf170 protein with human RNF170. Black highlighted amino acids indicate regions of homology, red bars mark exon boundaries, and a red box indicates the patient missense mutation p.Cys102Arg.

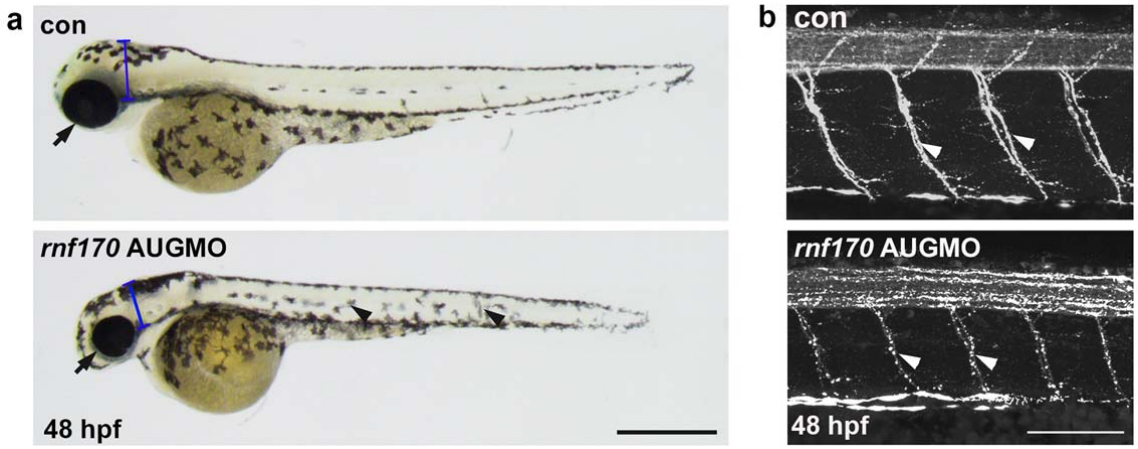


Supplementary Figure 5: Validation of targeted *rnf170* knockdown by antisense morpholino injections into zebrafish embryos followed by RT-PCR. **(a)** Schematic representation of the two-morpholino target sites (E3MO and E4MO, red bars). Primer sets were designed to validate either E3MO (primers: E2F, I2R, E3R. Blue arrows) or E4MO (primers: E3F, I3R, E4R. Black arrows) by RT-PCR. **(b)** RT-PCR performed on 48 hpf *rnf170* morphant or

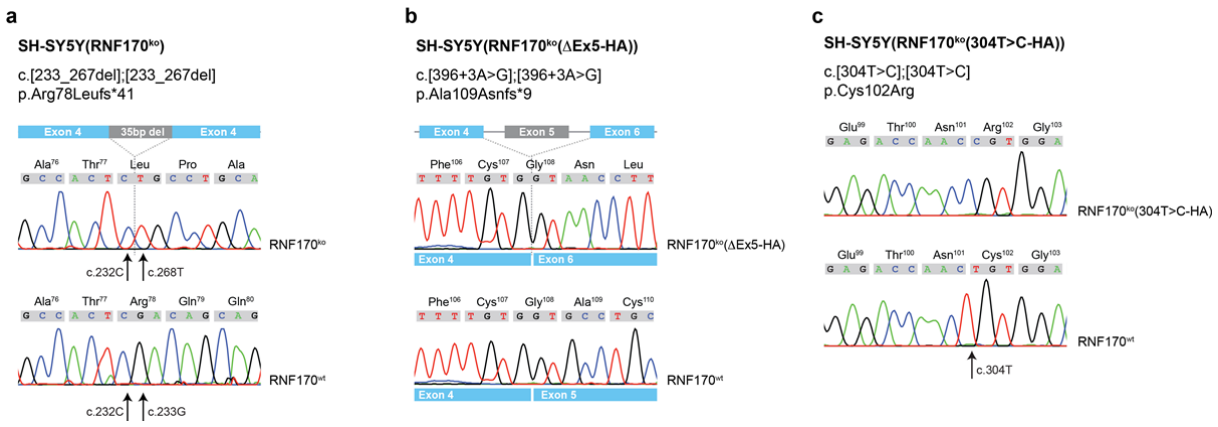
control morphant embryos (con). Injections of E3MO causes inappropriate splicing of intron 2 and predicted premature stop, as indicated by the presents of an expected 226 bp amplicon in the E3MO lane, compared to its absence in the con lane (primer pair E2F;I2R). Concurrently, amplification using exonic primers (E2F:E3R) results in the expected amplicon of 196 bp in only the con embryos, compared to multiple additional larger products found in the E3MO lane. Similarly, injections of E4MO results in the inclusion of intron 3 and predicted premature stop, as indicated by the presents of an expected 169 bp product (primer pair E3F;I3R) in the rnf170 morphant samples compared to its absence in con embryos. Amplification using exonic primers (E3F:E4R) shows an expected amplicon at approximately 158 bp in control embryos but an additional larger product in the E4MO injected embryos, indicative of the intronic inclusion. Amplification of a 300bp GAPDH amplicon was used as a positive control. **(c)** Quantification of morpholino efficacy by measuring the relative amplicon intensity between expected exonic amplicons (E2F:E3R, E3F:E4R) from control morpholino injected embryos verses morphants. Each band was normalized against GAPDH before relative intensity against control was calculated.



Supplementary Figure 6: rnf170 is expressed in the developing brain and intersomitic regions and rnf170 knockdown affects motorneuron development. (a) Top two panels show lateral and dorsal views, respectively, of a representative 48 hpf embryo stained using an antisense probe against rnf170 transcript. The two bottom panels show lateral and dorsal views, respectively, of a representative 48 hpf embryo stained using a sense control probe against *rnf170*. Scale bar: 200 µm. **(b)** Staining for acetylated tubulin in larvae 48 hpf shows disorganized caudal primary motorneurons in *rnf170* knockdown embryos. Localisation of antigen signal appeared punctate and intermittent in *rnf170* morphants compared to controls (arrows). Scale bar represents 100 µm. **(c)** At 4dpf *rnf170* morphant verses control MO injected embryos continue to show reduced motorneuron staining in the myotome, whilst acetylcholine receptors (AchR) persists. Motorneurons (arrows) are stained using acetylated Tubulin (red), AchR are marked using bungarotoxin (green). Scale bar represents 100 µm.



Supplementary Figure 7: rnf170 knockdown at the translational recognition start site results in a similar phenotype to *rnf170* splice morphants. (a) *rnf170* AUG morphants display shortened body axis, micropthalmia (arrows), microcephaly (brackets) and alterations in pigmentation (arrow heads). Scale bar represents 500 µm. **(b)** Staining for the axonal marker acetylated tubulin at 48 hpf (arrow heads), *rnf170* AUG morphants display punctate and intermittent antigen localisation when compared to control MO injected embryos. Scale bar represents 100 µm.



Supplementary Figure 8: (a) Sanger sequencing confirms presence of a 35bp deletion in the RNF170 gene that was introduced by a CRISPR/Cas9 approach. **(b)-(c)** SH-SY5Y(RNF170^{ko}) cells were stably transfected with RNF170 constructs carrying RNF170 mutations.

Supplementary Tables

Supplementary Table 1: Variants in RNF170 identified in the present study

family	genomic variant (hg19)	zygosity	cDNA	protein effect	mutation type	gnomAD alleles
A	chr8:42720556T>C	hom	NM_030954.3: c.396+3A>G	p.Ala109Asnfs*9	splice	1/245854
B	chr8:42725165A>G	hom	NM_030954.3: c.304T>C	p.Cys102Arg	missense	1/246108
C	chr8:42704626_42729012delinsTTTTGGT	hom	c.? [delEx4_7]	p.?	CNV	absent
D	chr8:42711560_42711561delTC	hom	NM_030954.3: c.518_519delAG	p.Arg173Asnfs*49	deletion	absent

115 **Supplementary Table 2: Information on exome sequencing and bioinformatics pipelines**

Fam-ID	Sequenced individuals	Sequencing center	Instrument	Experiment type	Library/ Exome capture	Reads	Mapped reads	% Mapped	Mean coverage	Coverage 20X	alignment tool	variant caller
A	A.4, A.5	Hudson Alpha (Huntsville, Alabama)	Illumina HiSeq X HD v2.5	WGS	TruSeq DNA PCR-Free Library Prep	870,404,738 (A.4) / 814,593,329 (A.5)	868,079,887 (A.4) / 812,703,476 (A.5)	99.73 (A.4) / 99.77 (A.5)	34.90 (A.4) / 34.60 (A.5)	95.5% (A.4) / 93.0% (A.5)	BWA v.0.7.1.2	Freebayes
B	B.4	Novogene (Beijing, China)	Illumina HiSeq 2500	WES	Agilent SureSelect Human All ExonV5/V6	44,055,430	43,991,117	99.85	99.9%	94.1%	BWA v.0.7.8-r455	GATK v3.1
C	C.1, C.2, C.4	Helmholtz Center Munich, Germany	Illumina HiSeq 4000	WES (Trio)	Agilent SureSelect Human All Exon V6	118,305,358	118,076,733	99.81	126.51	98.16%	BWA v.0.5.8	SAMtools v.0.1.7, ExomeDepth, Pindel
D	D.3, D.4	Yale Center for Genome	Illumina HiSeq 4000	WES	IDT xGen® Exome Research	38,541,168(D.1)/41, 791,764 (D.2)	38,514,682(D.1)/41, 773,100 (D.2)	99.93% (D.1)/99.96% (D.2)	26.789(D.1)/ 29.024(D	95.8%/97%	BWA 0.7.12-r1039	GATK v3.6-0-g89b7209

	Analysis	Panel v1.0	.2)	HaplotypeCaller

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119 **Supplementary Table 3: List of primers used**

Primer name	Sequence (5'->3')
Primers for confirmation of gDNA variants (Fig. 1)	
Fam A_F1	AGGAAGCTACGATCATGCCA
Fam A_R1	AAGGGTTGGCTGGATGAAGT
Fam B_F1	CGTTTACAGTTTGATGAGGGTTACA
Fam B_R1	TTGGTTGACAAGTAGAGCAGGAT
Fam C_F1	GCCAGTCAGTGGTGAGTGAG
Fam C_R1	GTCCATTGGCACCATTTTTC
Fam C_F2	GAAAGAAGCCCATGTTTCCA
Fam C_R2	TTCACCCAGAAAACCAGGAG
Fam D_F1	GCCATGGGTCCTTCTGTTTG
Fam D_R1	CGCGCTAGGTTCTTTGGTTT
Confirmation of splicing defect in Fam A (cDNA) (Fig. 1c)	
Fam A_F_cDNA	CTTCAAACAGAACAGGATGCAC
Fam A_R_cDNA	GGGGGCCTTTTCTGGATGTT
qRT-PCR primers (Fig. 1e)	
RNF170_F	GGCAGTTGTGGTCAGTTTCG
RNF170_R	CAGGTGCATCCTGTTCTGTTTG
RNF10_F	CAC CCA CTG CCA GTC AGG GC
RNF10_R	TCC CCG TCG CTG TCC ACA GG
RNF111_F	GCAGAATGCAGCAGAAGTTG
RNF111_R	CCATTCTTGCAGAAGTGGTTG
RPLPO_F	CCCGAGAAGACCTCCTTTTT
RPLPO_R	GGGTTGTAGATGCTGCCATT
Confirmation of knockout in SH-SY5Y cells (Fig. 8a)	
SH-SY5Y_F	GTGTTCCAATGTGTGCACCTG

SH-SY5Y_R	CCCAAGTATAGCGTTGTTTGCTT
Confirmation of plasmid mutations (Fig. 8b+c)	
plsmd_RNF170_F	GCCACTCGACAGCAGTTCTA
plsmd_RNF170_R	GGTCATCTAGTTAGCCTTTGGGTT

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References

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2. Li H, Durbin R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* **26**, 589-595 (2010).
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