ORIGINAL ARTICLE

Specific combinations of biallelic *POLR3A* variants cause Wiedemann-Rautenstrauch syndrome

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ABSTRACT

Background Wiedemann-Rautenstrauch syndrome (WRS) is a form of segmental progeria presenting neonatally, characterised by growth retardation, sparse scalp hair, generalised lipodystrophy with characteristic local fatty tissue accumulations and unusual face. We aimed to understand its molecular cause.

Methods We performed exome sequencing in two families, targeted sequencing in 10 other families and performed in silico modelling studies and transcript processing analyses to explore the structural and functional consequences of the identified variants.

Results Biallelic *POLR3A* variants were identified in eight affected individuals and monoallelic variants of the same gene in four other individuals. In the latter, lack of genetic material precluded further analyses. Multiple variants were found to affect *POLR3A* transcript processing and were mostly located in deep intronic regions, making clinical suspicion fundamental to detection. While biallelic *POLR3A* variants have been previously reported in 4H syndrome and adolescent-onset progressive spastic ataxia, recurrent haplotypes specifically occurring in individuals with WRS were detected. All WRS-associated POLR3A amino acid changes were predicted to perturb substantially POLR3A structure/function.

Conclusion Biallelic mutations in *POLR3A*, which encodes for the largest subunit of the DNA-dependent RNA polymerase III, underlie WRS. No isolated functional sites in POLR3A explain the phenotype variability in POLR3A-related disorders. We suggest that specific combinations of compound heterozygous variants must be present to cause the WRS phenotype. Our findings expand the molecular mechanisms contributing to progeroid disorders.

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INTRODUCTION

Progeroid syndromes constitute a family of rare, clinically heterogeneous disorders having premature ageing as shared major feature[1] and having variable defective DNA repair or mitochondrial function, extracellular matrix defects, genome instability and/or altered chromosomal structure and organisation as the molecular causes underlying disease pathogenesis.[2–5] Among them, Wiedemann-Rautenstrauch syndrome (WRS; MIM 264090) is an infrequently described autosomal recessively inherited neonatal progeroid condition characterised by prenatal and early postnatal growth retardation, sparse scalp hair, generalised lipodystrophy with characteristic local fatty tissue accumulations and an unusual face characterised by a triangular shape, apparently low-set eyeballs partly covered by the lower eyelids, small mouth, pointed chin and natal teeth.[6]

POLR3A (MIM 614258) encodes for the largest subunit of the DNA-dependent RNA polymerase III, a polymerase that transcribes genes coding small RNAs, such as 5S rRNA and tRNAs.[7] POLR3A constitutes the active site together with POLR3B, and biallelic putative loss-of-function/hypomorphic mutations in both *POLR3A* and *POLR3B* (MIM 614366) are known to cause two forms of hypomyelinating leukodystrophy, with or without oligodontia and/or hypogonadotropic hypogonadism (MIM 607694 and 614381, respectively), which are thought to represent a single entity by others,[8] known as hypomyelination, hypodontia and hypogonadotropic hypogonadism syndrome (4H syndrome or 4H leukodystrophy (4H)).[9] Hypomyelination has been found to occur infrequently in WRS.[10–12] Despite the major differences between 4H and WRS, there is some overlap in phenotype, sustaining the suggestion that variants in *POLR3A* may be associated to both entities. More recently, hypomorphic biallelic variants in *POLR3A* were reported as cause of autosomal recessive adolescent-onset progressive spastic ataxia.[13–15] Of note, in some patients with WRS, progressive ataxia has been documented as well.[6] While the structural and functional validation of the putative disease-causing variants in *POLR3A* identified from our WES data were underway, biallelic variants in the same gene were reported in a single individual with WRS (c.190918G>A; p.Tyr637Cysfs*23, and c.2617C>T; p.Arg873*)[16] suggesting, but not proofing, that variants in this gene might underlie the disorder. Here, we report on the results of a multicentre collaborative project directed to identify and characterise the molecular basis of WRS. By

using WES in two affected individuals and target resequencing or Sanger sequencing in eight other subjects with WRS and two pairs of parents of deceased patients, we identified causative biallelic *POLR3A* variants in eight individuals and monoallelic *POLR3A* variants in the remaining four, strongly indicating that WRS is a genetically homogeneous disorder caused by defective POLR3A function.

SUBJECTS AND METHODS

Clinical samples

We collected a cohort of carefully phenotyped individuals with clinical manifestations fitting the disorder.[6] Most of the presently reported families and patients have been published (patients have the full number; parents are indicated by this number followed by b for father and c for mother): WRS001,[6] WRS005,[6] WRS0012,[6] WRS002 (patient number 2 in the report of Rautenstrauch and Snigula in 1977),[17] WRS003 (German origin, unpublished), WRS004,[18] WRS006b and WRS006c (father and mother, respectively, of patients number 1 and 2 in the Arboleda's report of 1997),[19] WRS007, WRS008, WRS009 (patients number 1, 2 and 3 in the report of Morales *et al*),[20] WRS010b and WRS10c (father and mother, respectively, of three affected sibs)[21] and WRS011 (Colombian origin, unpublished). In total, we studied 12 families and 15 affected patients. We refer to the original publications for detailed data per patient. A summary of the clinical features of all studied patients is presented in table 1. Informed consent was taken from the participants their legal representatives, and the study was approved by the local ethic committees in Amsterdam and Göttingen.

Exome sequencing and sequence data analysis and validation

DNA was extracted from whole blood. WES was performed in two families using SeqCap EZ MedExome kit (Roche) (WRS001 trio) and SeqCap EZ Human Exome Library v.3.0 (Roche)

Table 1 Main clinical manifestations in the presently reported group of individuals with Wiedemann-Rautenstrauch syndrome

Features	HPO term	Patients with biallelic variants in *POLR3A*	Patients with a single variant in *POLR3A*
Number		10	5
Sex		5F:5M	4F:1M
Consanguinity		4/10	0/4
Weight at birth <P3		8/10	3/4
Length at birth <P3		0/8	3/4
OFC at birth <P3		2/8	2/4
Weight at last exam <P3		4/7	?*
Height at last exam <P3		67	?*
OFC at last exam <P3		0/3	?*
Sparse scalp hair	0002209	9/9	4/4
Prominent scalp veins	0001043	9/9	4/4
Persistent open anterior fontanelle	0004474	10/10	4/4
Broad forehead	0000337	9/9	4/4
Triangular face	0000325	9/10	4/4
Eyebrows: broad (B), sparse (S)	00112290045075	3/6 (B); 5/6 (S)	1/4 (B); 4/4 (S)
Low-set eyeballs	–	9/9	3/4
Lower eyelid covering part of cornea	–	8/9	4/4
Convex nasal ridge	0000444	8/10	3/4
Narrow mouth	0000160	4/9	3/4
Thin upper lip vermilion	0000219	10/10	4/4
Downturned corners of mouth	0002714	9/9	4/4
Natal tooth	0000695	10/10	3/4
Teeth (hypodontia); delayed eruption	00006680000684	8/8	?*
Pointed chin	0000307	9/9	4/4
Abnormality of the ear	0000598	9/9	3/4
Atrophic skin with prominent veins	0001015	10/10	4/4
Decreased adipose tissue	0040063	10/10	4/4
Localised fat accumulation	–	5/9	1/2
Abnormality of the skeletal system	0000924	5/6	2/2
Tremor	0001337	2/8	?*
Hypertonia	0001276	6/9	2/4
Ataxia or hypotonia	00012510001290	2/9	1/3
Hypomyelination	0003429	0/4	?*
Intellectual disability	0001249	6/8	?*
Abnormality of the eye	0000478	5/8	1/1
Hearing impairment	0000365	1/5	?*
Endocrine anomalies	0000818	3/8	?*
Hypertriglyceridaemia	0002155	2/4	?*

*Affected individuals died in the first weeks of life and only limited studies could be performed. HPO, Human Phenotype Ontology; OFC occipitofrontal circumference.

(WRS002 and her father) for target enrichment. Sequencing was carried out on the HiSeq2500 (WRS001 trio) and HiSeq2000 (WRS002a and her father) platforms (Illumina), according to the manufacturer's protocols. The obtained reads were aligned to the human reference genome (UCSC GRCh37/hg19) using Burrows-Wheeler aligner v.0.7.10 (WRS001) and v.0.7.12 (WRS002).[22] PCR duplicates were removed using Picard MarkDuplicates v.1.111 (WRS001) and v.1.119 (WRS002). Realignment of sequences containing indels and base quality recalibration were performed by Genome Analysis Toolkit (GATK) v.3.3 (WRS001) and v.3.5 (WRS002).[23] SNPs and small indels were identified by means of GATK HaplotypeCaller algorithm. SnpEff toolbox v.4.0 (WRS001) and v.4.2 (WRS002) were used to predict the functional impact of variants.[24] Annotation of the variants was performed by GATK v.3.3 VariantAnnotator (WRS001), and dbNSFP v.3.2 and SnpEff toolbox v.4.2 (WRS002). Variants with depth <5 reads, an allele frequency >1% in 1000Genomes, ESP6500, dbSNP142, ExAC/GnomAD and in-house databases were filtered out. Pathogenicity of variants were evaluated using the decision-support software AlamutVisual v.2.8.

Sanger sequencing (BigDye Terminator v.3.1 Cycle Sequencing Kit, Applied Biosystems) was performed using an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems) (WRS003 and WRS004) and targeted parallel sequencing (SeqCap EZ choice kit, Roche NimbleGen) on a NextSeq550 instrument (Illumina) (WRS005, WRS006b, WRS006c, WRS007, WRS008, WRS009, WRS010b, WRS010c, WRS011 and WRS012). For the latter, the generated Fastq files were aligned to the human reference genome GRCh37/hg19, and the BWA Enrichment application of BaseSpace (Illumina) was used to analyse the reads and generate BAM and vcf files. Variants were annotated by GATK and Variant Studio software (Illumina) and TGexTM software (LifeMap Sciences) and manually analysed using IGV (Integrated Genomics Viewer). By using the same WES workflow reported above (SeqCap EZ MedExome kit), target NGS of the exons of *POLR3A* on parental DNA specimens of individuals WRS007 and WRS011, whose DNA was not available for additional analyses, was performed to assess further the extent of variation within the entire *POLR3A* coding sequence and exclude occurrence of putative relevant variants in other genes potentially compatible with transmission of the disorder as a recessive trait.

RNA was extracted from fresh blood of subject WRS002 (Paxgene blood RNA system, Qiagen), and primers were designed to amplify the portions of the *POLR3A* mRNA encompassing exons 5–9, exons 12–15 and exons 24–28 by using random primed cDNA as template (RevertAid First Strand cDNA synthesis Kit, Thermo Fisher Scientific) and validate the predicted disruptive impact of the identified splice site variants.

SYBR green-based real-time PCR assay was performed using a QuantStudio 12K Flex Real-Time PCR System (Life Technologies) on genomic DNA samples. The threshold cycle (C_t) values were determined by the QuantStudio 12k Flex Software (Life Technologies), data were normalised using *hTERT* as reference and relative quantification to determine copy number was made using the $2^{-\Delta\Delta Ct}$ calculation method.[25]

In silico structural analyses

Homology modelling of human POLR3A was performed based on the Protein Data Bank (PDB) structure 5fj8 representing the yeast DNA-directed RNA polymerase III (Pol III) elongation complex.[26] In that structure, Pol III subunit RPC1, which shares 49% amino acid identity with the human homologue POLR3A (online supplementary figure 1), was used as template. After removing all side chain atoms from the complex, the backbone atom-only residues in RPC1 were formally renamed and renumbered (in PDB format) to as the corresponding ones of human POLR3A according to the pairwise sequence alignment using dedicated tools developed in-house and parsed for side chain construction to SIDEpro.[27] In the complex, residues from other subunits having direct interactions with POLR3A residues affected by the WRS-causing variants resulted to be conserved. Those residues were renumbered as the homologous residues and the parent subunits were renamed to the corresponding human proteins. The nucleic acid stretches bound to the elongation complex were left in same binding pose to the model, and molecular graphics were produced with PyMOL.

RESULTS

WES analysis

WES data filtering yielded compound heterozygosity for a maternally inherited intronic variant, c.1909+18G>A, and a paternally inherited missense change, c.3206G>A (p.Arg1069Gln), in *POLR3A* as the most excellent candidate event underlying the trait in WRS001 (table 2). c.1909+18G>A was predicted to affect splicing by creating an intronic cryptic donor site and introducing a premature stop codon and had been reported as pathogenic mutation in 4H.[9 28] Similarly, c.3206G>A has a very low frequency in GnomAD (0.002%), affected a residue conserved through evolution (figure 1A) and was predicted to be deleterious by Sorting Intolerant From Tolerant (SIFT), probably damaging by polyphen-2, and disease causing by MutationTaster. A different change in the same codon (c.3205C>T and p.Arg1069Trp) had been reported in 4H.[29] We were unable to initially find any good candidate after filtering the WES data set for subject WRS002.

Scan of the private/rare nonsynonymous variants in *POLR3A*, however, allowed to identify three intronic heterozygous (despite the consanguinity among the parents of WRS002) variants, c.3337–11T>C, c.1909+22G>A and c.1048+5G>T, two of which (c.3337–11T>C and c.1909+22G>A) paternally inherited (mother's DNA was not available for testing) (table 2). The concomitant occurrence in cis of the c.1909+22G>A and c.3337–11T>C intronic changes is highly unusual. c.1909+22G>A represents a relatively common variant occurring in the population, was predicted to introduce a cryptic donor splice site and had previously been reported without the presence of the additional c.3337–11T>C on the same allele in patients with 4H.[29] Based on the relatively higher occurrence of this intronic variant, we expected a mild effect on transcript processing, representing a hypomorphic variant, and therefore was not expected to be able to sufficiently reduce the functionality of the transcripts for a recessive allele. However, the occurrence of the variant, c.3337–11T>C, on the same allele was predicted to increase the number of incorrectly spliced *POLR3A* transcripts to a level, which would have been sufficient to cause the disease in combination with a loss-of-function mutation as a second allele. Consistent with this model, the c.3337–11T>C had not been reported in public databases previously and was predicted to alter a different splice site. To verify the hypothesis of a biallelic hit affecting *POLR3A* transcript processing, RNA was extracted from subject WRS002 to validate the predicted disruptive impact of the splice site variants c.1048+5G>T, c.1909+22G>A and c.3337–11T>C (figure 2). The variant c.1048+5G>T caused exon 7 extension and resulted in an insertion of 177 bp intronic sequence, which was predicted to result in a frameshift and premature stop of translation (p.Arg353Profs*24). Similarly, the

Table 2 Presently reported group of individuals with Wiedemann-Rautenstrauch syndrome and their variants in *POLR3A*

Family code	Mutation analysis	Genomic region	Variants	Inheritance	rs	MAF (GnomAD)
WRS001	WES	chr10:79744964	c.3206G>A (p.Arg1069Gln)	Father	rs778985686	0.002%
		chr10:79769277	c.1909+18G>A (p.?)	Mother	rs267608677	
WRS002	WES	chr10:79743781	c.3337–11T>C (p.?)	Father		
		chr10:79769273	c.1909+22G>A (p.?)	Father	rs191875469	0.1%
		chr10:79781613	c.1048+5G>T (p.?)	Mother	rs890755853	0.0004%
WRS003	Sanger sequencing	chr10:79743781	c.3337–11T>C (p.?)	Father		
		chr10:79769273	c.1909+22G>A (p.?)	Father	rs191875469	0.1%
		chr10:79760738	c.2474C>G (p.Ser825*)	Mother		
WRS004	Sanger sequencing	chr10:79743781	c.3337–11T>C (p.?)	Father		
		chr10:79769273	c.1909+22G>A (p.?)	Father	rs191875469	0.1%
		chr10:79769404	c.1800C>T (p.Ile600Ile)	Mother		
WRS005	Targeted parallel sequencing	chr10:79753126	c.2617–1G>A (p.?)	Father	rs181087667	0.002%
		chr10:79743781	c.3337–11T>C (p.?)	Mother		
		chr10:79769273	c.1909+22G>A (p.?)	Mother	rs191875469	0.1%
WRS006	Targeted parallel sequencing*	chr10:79741306	c.3772_3773delCT (p.Leu1258Glyfs*12)	Father		
		/	c.=	Mother	/	/
WRS007	Targeted parallel sequencing	/	c.=	Father	/	/
		chr10:79741306	c.3772_3773delCT (p.Leu1258Glyfs*12)	Mother		
WRS008	Targeted parallel sequencing	chr10:79739920	c.4003G>A (p.Gly1335Arg)	Father	rs768222183	0.0004%
		chr10:79737218	c.*18C>T (p.?)	Mother		0.0004%
WRS009	Targeted parallel sequencing	chr10:79789163	c.3G>T (p.Met1?)	Father		0.0004%
		chr10:79737218	c.*18C>T (p.?)	Mother		0.0004%
WRS010	Targeted parallel sequencing[1]	chr10:79741203	c.3874G>A (p.Asp1292Asn)	Father	rs757209071	0.001%
		/	c.=	Mother	/	/
WRS011	Targeted parallel sequencing	/	c.=	Father	/	/
		chr10:79753034	c.2707G>A (p.Gly903Arg)	Mother		
WRS012	Targeted parallel sequencing	chr10:79743715	c.3392A>G (p.Lys1131Arg)	Father	rs138305578	0.003%
		nd	deletion comprising exons 12–15	Mother		

*Mutation analysis was performed on the two unaffected parents. '/' indicates no variant detected.
MAF, minor allele frequency; nd, not determined.

other two intronic variants occurring as a haplotype were found to affect transcript processing: c.1909+22G>A having a mild effect on exon 14 splicing, and c.3337–11T>C causing skipping of exon 26. For the latter mutant haplotype, the same disruptive impact of both variants was demonstrated on RNA of a second unrelated affected individual, WRS004, who carried the same pathogenic allele (see below and table 2). Skipping of exon 14 caused by the c.1909+22G>A change predicts a frameshift and early stop of translation (p.Pro591Metfs*9), while the observed splicing defect caused by the c.3337–11T>C variant leads to an in-frame deletion, namely p.I1113_E1143del.

POLR3A mutation analysis

Based on our findings and reinforced by the report by Jay and colleagues,[16] scanning of the entire *POLR3A* coding sequence and flanking intronic stretches was performed by Sanger/parallel sequencing. Biallelic variants in *POLR3A* were identified in five affected subjects, while a single variant was identified in the remaining five cases (table 2). The cis-acting c.3337–11T>C and c.1909+22G>A mutant allele were identified in three additional patients originating from different countries, who were compound heterozygotes for a nonsense variant (c.2474C>G, p.Ser825*) (WRS003), a synonymous change predicted to affect splicing (c.1800C>T, p.Ile600Ile) (WRS004 and two affected siblings) and a splice site variant (c.2617–1G>A) (WRS005), consistently indicating defective POLR3A function as the mechanism implicated in disease pathogenesis. RNA was available for subject WRS004, allowing to verify the impact of the c.1800C>T change, which located in exon 14 and similarly to c.1909+22G>A, was predicted to alter proper splicing of exon 14. The variant c.1800C>T on one allele and the variants c.1909+22G>A and c.3337–11T>C on the other allele both together resulted in an increased skipping of exon 14 (figure 2) compared with the effect of c.1909+22G>A alone, resulting in a frameshift and premature protein truncation (p.Pro591Metfs*9). Similarly, the c.2617–1G>A variant affects an acceptor splice site already associated with 4H.[9] Unexpectedly, the father of WRS004, who was specifically investigated and found to be apparently unaffected (although we cannot exclude completely he will proof to become affected at a later age), was found homozygous for the mutant allele carrying the c.3337–11T>C and c.1909+22G>A variants, indicating that this mutant allele does not cause the phenotype when present in homozygous state and that a specific mutation signature indicated by the combination of compound heterozygous mutations in *POLR3A* is necessary to cause WRS. WRS008 carried an extremely rare paternally inherited variant, c.4003G>A (p.Gly1335Arg), affecting a residue invariantly conserved among vertebrates and a previously unreported maternal variant c.*18C>T. The latter was also found in WRS009, who was compound heterozygote for a second missense change affecting the starting initiation translation site, c.3G>T (p.Met1?), which was predicted to lead to absent translation since no other ATG codons are predicted suitable for more downstream initiation

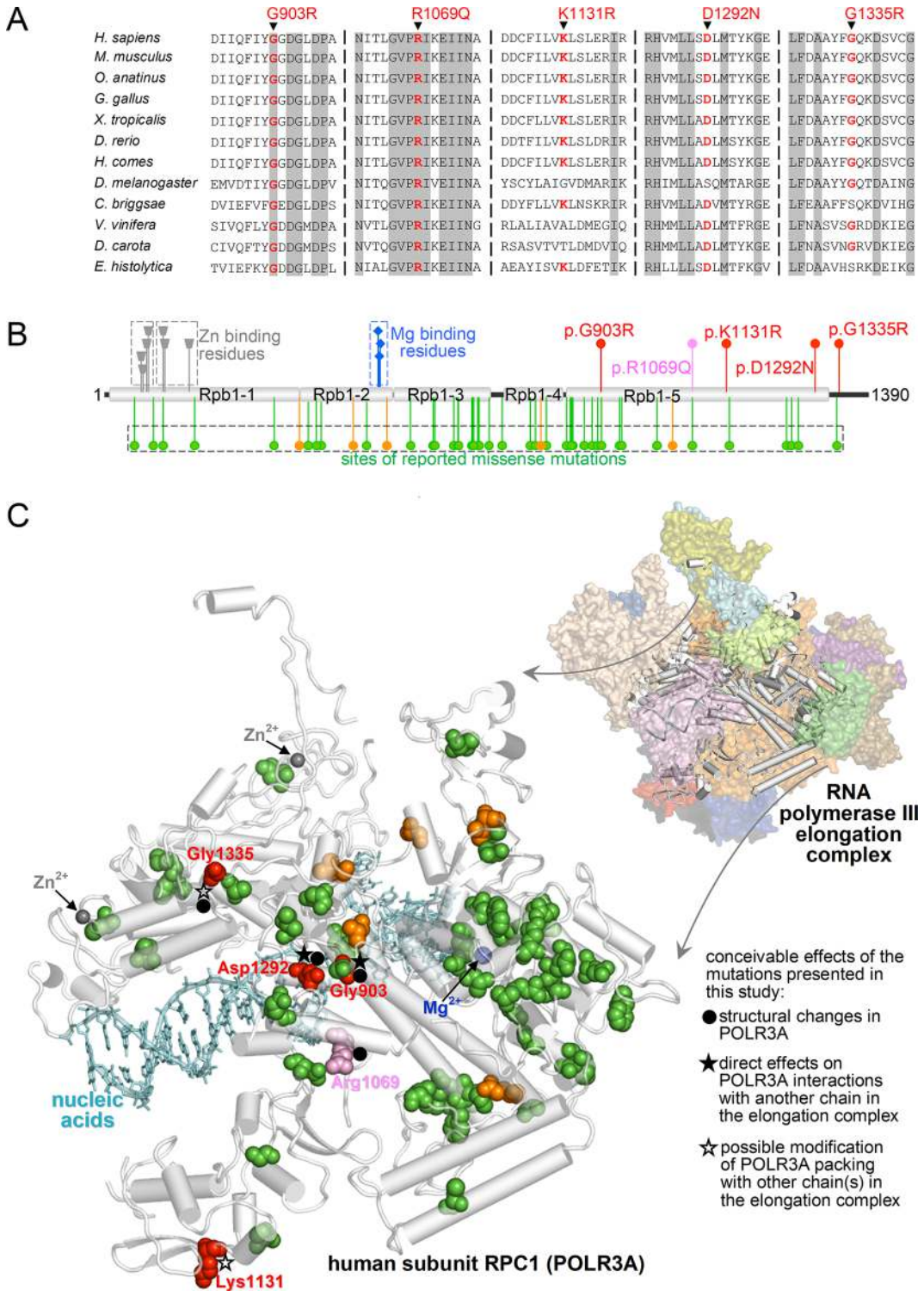

Figure 1 Structure of POLR3A and the RNA Pol III elongation complex and location of affected POLR3A residues in human disease. (A) DNA-directed RNA polymerase III subunit RPC1 (POLR3A) sequence alignment among eukaryotes around the amino acid residues affected by the disease-associated missense variants (invariant residues are greyed). (B) POLR3A protein scheme with domains and residues affected by the disease-associated missense variants (identified in this study, red; previously reported in 4H, green; previously reported in spastic ataxia, orange). One of the identified missense changes, p.Arg1069Gln, had previously reported in 4H. (C) Homology model of the RNA polymerase III elongation complex and enlarged view of the POLR3A subunit without the other subunits of the complex. Residues affected by disease-associated missense substitutions are shown and coloured as reported above.

of translation (http://atgpr.dbcls.jp/). The functional validation of the c.*18C>T change was unfortunately not possible due to lack of biological material from the patients. Variants in the 3′ untranslated region (UTR) of genes have rarely been reported as causes of Mendelian traits by altering microRNA recognition sites or proper binding to regulatory proteins.[30 31] While dedicated validation studies are required to assess the impact of this specific nucleotide substitution on processes controlling *POLR3A* mRNA stability/degradation or translation, the possibility that an unidentified pathogenic variant occurs in cis with

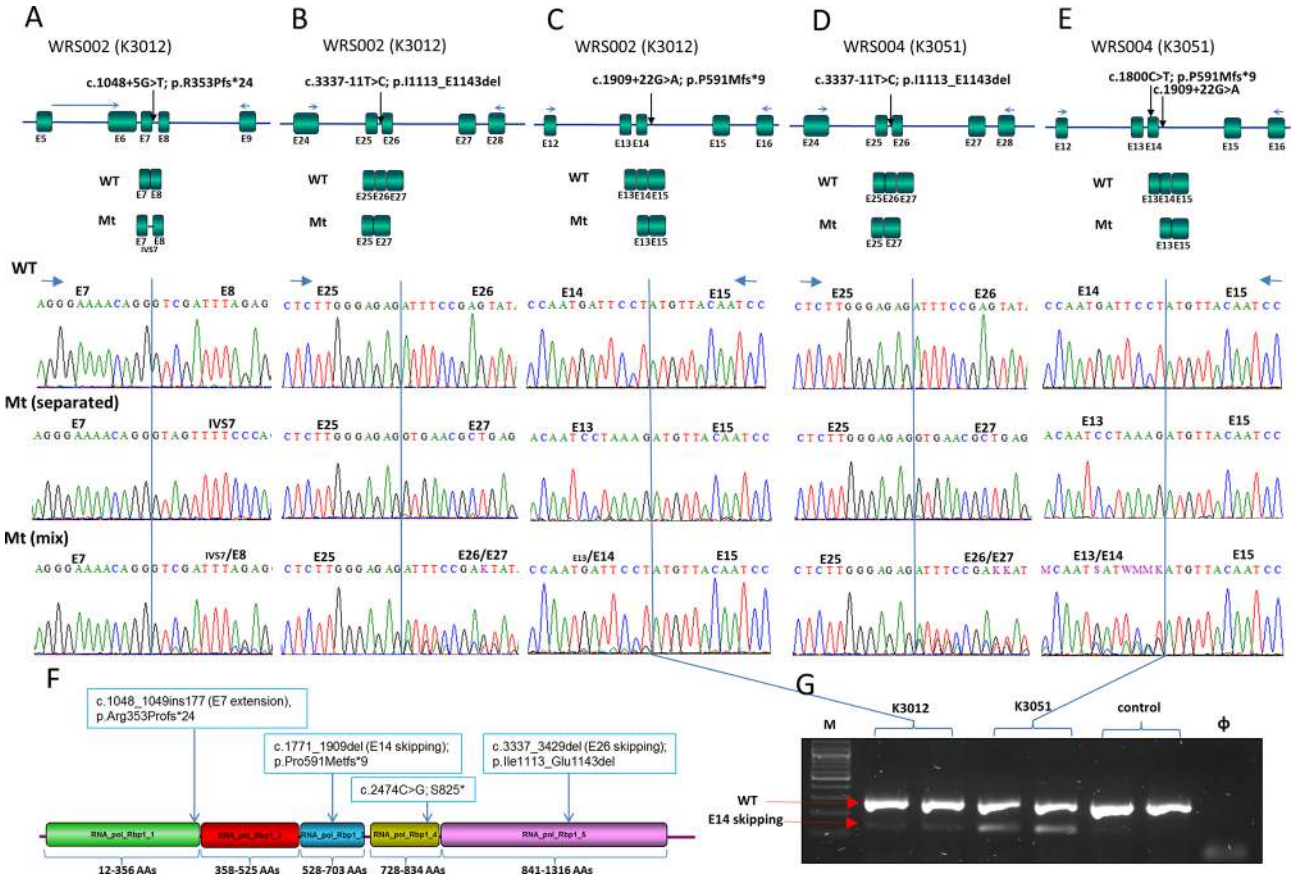

Figure 2 cDNA analyses of splice-site variants identified in WRS. (A) Amplification of *POLR3A* cDNAs of WRS002 carrying the c.1048+5 splice site variant, and a healthy control individual. The variant resulted in an exon 7 extension towards exon 8. (B) Individual WRS002 carrying the c.3337–11T>C splice site variant. The cDNA analysis indicated exon 26 skipping. (C) The c.1909+22G>A splice site variant in WRS002 had a mild effect on transcript processing resulting in exon 14 (E14) skipping. (D) Individual WRS004 carries the c.1909+22G>A and c.3337–11T>C mutant allele. The c.3337–11T>C splice site variant of this allele causes skipping of exon 26. (E) WRS004 was found to carry in addition to the mutant c.1909+22G>A and c.3337–11T>C allele also the synonymous variant c.1800C>T in E14. The cDNA analysis showed that both c.1800C>T and c.1909+22G>A had a strong additive effect on splicing resulting in exon 14 (E14) skipping. The primer locations for cDNA amplification are indicated with an arrow on the schematic view of POLR3A exons. Predicted effects on the proteins are indicated. (F) POLR3A protein domains and mutation locations. (G) Image of a gel electrophoresis indicating the E14 skipping in cases K3012 and K3051 and one control sample. Each sample was done in replicate. M: marker, ϕ: negative control. The upper band represents WT and lower band results from the E14 skipping. K3012 who carries only the c.1909+22G>A showed a very weak lower band, whereas K3051 who carries in addition the E14 variant c.1800C>T showed an increased E14 skipping. Mt (separated), mutant band was cut out of the agarose gel and sequenced; Mt (mix), PCR product was directly sequenced; WT, wild type.

the c.*18C>T change as a shared haplotype in the two subjects cannot be ruled out.

Since a single heterozygous variant was identified in five individuals (WRS006, WRS007, WRS010, WRS011 and WRS012; WRS006 and WRS010 by studying parents) (table 2), we expected that a second hit affecting the gene in these patients might involve intronic or regulatory regions, or structural rearrangements, including large intragenic deletions/duplications. Based on this hypothesis, parallel sequencing output was analysed for the detection of exonic deletions using the normalised mean coverage of individual exons. An intragenic deletion comprising exons 12–15 was predicted in WRS012. To validate this finding, a PCR assay was performed on genomic DNA samples from the propositus and her mother. Primers were designed to amplify each of exons 12, 13 and 15. The assay confirmed the presence of the deletion in both subjects, establishing the compound heterozygosity for the missense change, p.Lys113Arg, and the large intragenic deletion in WRS012 (table 2). No biological material was available for analysing the disruptive effect of the deletion in terms of transcript processing and/or stability; based on the *POLR3A* coding sequence, however, deletion of the four exons is predicted to cause a frameshift and premature termination (542 vs 1390 residues), strongly supporting loss of function. Unfortunately, lack of additional DNA specimens of the four deceased patients with a single heterozygous variant prevented further studies for small structural rearrangements or mutations involving regulatory regions of the gene. WES analysis performed using available parental DNAs of subjects WRS007 and WRS011 allowed to confirm the c.3772_3773delCT (p.Leu1258fs, mother of WRS007) and c.2707G>A (p.Gly903Arg, mother of WRS011) changes, but failed in identifying any additional functionally relevant hit within the entire *POLR3A* coding exons and adjacent intronic stretches in the paternal samples, suggesting that the 'missing' variants likely reside in deep intronic regions or involve untranscribed regulatory sequences of the gene. WES analyses excluded the occurrence of functionally relevant variants in parental exomes compatible with a recessive inheritance model for the trait. This finding and the consideration that *POLR3A* is among the most mutation-intolerant genes

(Residual Variation Intolerance Score (RVIS)=−1.92; %ExAC RVIS=1.4%) further supports the clinical relevance of the identified single hits in the gene.

Structural analyses

POLR3A variants reported in individuals with 4H are spread over the gene (figure 1B) and so are the presently reported variants in WRS. Among them, five missense changes affected conserved residues located at the C-terminal portion of the protein (figure 1A). To explore the structural and functional impact of missense *POLR3A* variants identified in WRS, POLR3A modelling was performed using the available structure of yeast RNA Pol III elongation complex.[26] The five amino acid substitutions mapped in locations of the RPC1/POLR3 subunit predicted to undergo substantial structural rearrangements or impact on the stability of the complex (figure 1C), with four of them expected to affect POLR3A interaction with other subunits (see online supplementary figure 2 for full alignment with orthologs highlighting residues directly involved in the intermolecular binding network within the complex). Yeast RPC1 directly interacts with the RPABC1 subunit, which corresponds to POLR2E in the human Pol III elongation complex. A number of mutated residues were observed to map in regions facing or very close to the RPC1/POLR3A-RPABC1/POLR2E interface. Specifically, Gly^{903} directly contributes to this interface by forming an H-bond with the side chain of Thr^{199} of the POLR2E subunit, and the Gly-to-Arg substitution caused by the c.2707G>A change introduces a much larger and cationic residue, which is expected to disrupt normal functioning of the interface (online supplementary figure 3A). Asp^{1292} is closely located to Gly^{903} and plays an important role in stabilising the POLR3A–POLR2E interaction as its side chain forms an intramolecular salt-bridge with Arg^{1264} and two intermolecular salt-bridges with Arg^{195} and Arg^{207} (online supplementary figure 3B). The Asp-to-Asn substitution resulting from the c.3874G>A change is predicted to cause a loss of these ionic interactions and consequently a substantial disruption of the RPC1-RPABC1 interface. Similarly, Arg^{1069} is located close to the POLR3A-POLR2E interface and interacts with Thr^{1064} (hydrogen bonding) and Glu^{1270} (salt-bridge) intramolecularly (online supplementary figure 3C). The p.Arg1069Gln amino acid change is predicted to affect these interactions and the POLR3A-POLR2E interface, although perturbation of POLR3A interactions with the nucleic acid molecule cannot be ruled out. Lys^{1131} is found within a calix-like shaped group of side chains from neighbouring residues with whom it interacts by salt-bridges, polar interactions and hydrophobic interactions via the aliphatic moieties (online supplementary figure 3D). The residue is close to the region of interaction between POLR3A and POLR3K, the human orthologue of yeast RPC10, mediated by inter-backbone H-bonding of the two proteins. Only a few residues are involved in this interaction, and the binding of the two subunits is labile. The p.Lys1131Arg substitution is expected to perturb this interaction. Finally, Gly^{1335} is located proximal to the interacting surfaces of POLR3A and POLR3B, the vertebrate homologue of yeast RPC2 (online supplementary figure 3E). The p.Gly1335Arg substitution introduces a cationic and much larger residue and is predicted to cause substantial structural perturbation of the region, impairing interactions between the two subunits. Overall, all presently reported WRS-associated POLR3A amino acid changes are predicted to perturb substantially the structure of the POLR3A subunit and its binding to other subunits of the complex, predicting major functional consequences.

DISCUSSION

Progeroid disorders have previously been established to be caused by mutations affecting genome stability, DNA repair, nuclear organisation, chromatin structure and epigenetic regulation, as well as mitochondrial function and extracellular matrix composition. Here we provide evidence that defective *POLR3A* function causes WRS. *POLR3A* encodes for the largest subunit of DNA-dependent RNA polymerase III and constitutes the catalytic core together with POLR3B. This polymerase synthesises small RNAs, such as 5S rRNA and tRNAs. Reduction of POLR3A leads to reduction of the total pool of tRNAs and a deregulated transcription of certain types of ncRNAs.[32] Some of these ncRNAs such as 7SL RNA and 7SK RNA, regulate the activity of the DNA-dependent RNA polymerase II, so *POLR3A* variants can also affect levels of polymerase II-transcribed genes. The exact pathogenesis explaining the mechanism behind the various signs and symptoms that go along with decreased POLR3A levels, such as myelination, dental, bone and fat tissue abnormalities, remains unclear.

We documented biallelic hits in 8 of the 12 families included in the study. All identified variants were private or rare, and many of them are intronic and have been proven to affect transcript processing. In one proband (WRS012) compound heterozygosity for a missense variant (p.Lys1131Arg) and an intragenic deletion encompassing three exons was observed. Two patients shared a heterozygous previously unreported variant in the 3′UTR of the gene, but unfortunately lack of additional DNA/RNA specimens prevented further studies. Structural analyses make disruption of normal POLR3A function likely in all missense variants. The remaining four affected subjects were found to carry a single pathogenic variant. While we failed in detecting the second hit, we hypothesise that large intragenic deletions/insertions or variants affecting intronic regions or regulatory motifs controlling *POLR3A* expression might be involved in these individuals. Additional DNA specimens to validate this hypothesis was not available. The involvement of *POLR3A* variants in introns in most patients may explain why the molecular bases of WRS has remained unknown for long time. Also in patients with spastic ataxia, the recurrent deep intronic variant in *POLR3A*, c.1909+22G>A, has only recently been reported.[13] The c.1909+22G>A variant is not found in individuals with WRS without the presence of the variant c.3337–11T>C on the same allele, as demonstrated here in seven individuals from four families. Our cDNA studies provide evidence that both variants alter splicing suggesting a combined functional effect on the *POLR3A* transcripts. The presence of this cis-acting c.3337–11T>C and c.1909+22G>A mutant allele only in individuals with WRS points towards an important difference in mutation signatures associated with WRS and patients with 4H or spastic ataxia. The concomitant occurrence in cis of the c.1909+22G>A and c.3337–11T>C intronic changes is highly unusual. c.1909+22G>A represents a relatively common variant occurring in the population, was predicted to introduce a cryptic donor splice site and had previously been reported without the presence of the additional c.3337–11T>C on the same allele in patients with 4H. Based on the relatively higher occurrence of this intronic variant, we expected a mild effect on transcript processing, representing a hypomorphic variant, and therefore was not expected to be able to sufficiently reduce the functionality of the transcripts for a recessive allele. However, the occurrence of the variant, c.3337–11T>C, on the same allele was predicted to increase the number of incorrectly spliced POLR3A transcripts to a level, which would have been sufficient

to cause the disease in combination with a loss-of-function mutation as a second allele. A more profound functional impairment of POLR3A seems to be necessary to cause the WRS phenotype rather than a different mechanistic effect of mutations. The results of the cDNA analyses demonstrate that both splicing variants do have an effect—although mild—on proper transcript generation, leading to a frameshift and early protein truncation (c.1909+22G>A; p.Pro591Metfs*9) and a larger in-frame deletion (c.3337–11T>C; p.Ile1113_Glu1143del), both of which are predicted to substantially alter protein function by disrupting important protein domains. Therefore, an additive functional effect of both variants of this mutant allele seems likely. Future functional studies will focus on the exact functional effects of WRS-associated deep intronic splice-site mutations. Currently, partial loss of function is likely, but we cannot exclude incorporation of mutant POLR3A into the DNA-dependent RNA polymerase III complex causing specific mechanistic alterations of complex function. Moreover, the homozygosity for this allele in a healthy parent suggests that the presence of only this allele is insufficient to alter the POLR3A function that a phenotype will arise. Therefore, specific combinations of compound heterozygous mutations are assumed to be required to cause the WRS phenotype. Based on the mutation profiles detected in our cohort of patients with WRS, we suggest that the biallelic mutation signature includes one variant with a strong functional effect on the protein (strong spice-site or truncating mutations) and one milder hypomorphic variant (often a deep intronic variant partially affecting splicing).

In general, involvement of variants in regulatory regions causing splicing has been underestimated in the past due to the focusing of searches on mainly coding variants or intronic variants near the exon–intron boundary directly involved in the splicing process. Several authors have suggested that splicing mutations are in fact the most frequent cause of hereditary disorders.[33 34] Furthermore, the model of Xiong *et al*[35] predicts that substantial and unexpected splicing can arise from variants within introns and exons, including those at a far distance from the splice site. For example, among intronic variants that are more than 30 nucleotides away from a splice site, known disease causing variants alter splicing nine times more frequent than common variants; among missense exonic disease variants, those that least impact protein function are over five times more likely to alter splicing than other variants.[35] WRS is not the only syndrome caused by recurrent intronic variants,[36] but the frequency of reported variants affecting splicing seems to be higher than in other known syndromes. It remains uncertain whether indeed the frequency is higher in WRS or whether these variants remain undetected in other entities at present. Likely the increasing use of whole genome sequencing technologies will allow detection of intronic variants more easily and indicate the true frequency. Until then the difficulty in detecting deep intronic variants in *POLR3A* by exome sequencing indicates a clinical suspicion is fundamental to indicate the need for more detailed molecular analyses.[37]

Our findings provide genetic proof that biallelic mutations in *POLR3A* are the causative event underlying WRS and confirm the suggestive observation based on a single affected individual.[16] Biallelic loss-of-function variants in *POLR3A* and its interactors *POLR3B* and *POLR1C* are known to cause a series of related but still different entities. *POLR3A* variants can cause 4H (MIM 607694), hypomyelinating leukodystrophy type 8 (MIM 614381) and progressive spastic paraplegia and ataxia. Some of the presently reported patients developed progressive neurological anomalies such as tremor, ataxia, hypertonia and nystagmus (table 1), but these are absent in several reliably diagnosed individuals as well.[6] Variants in *POLR1C* can cause hypomyelinating leukodystrophy type 11 (MIM 614494) and variants in *POLR3B* have been found in individuals with cerebellar hypoplasia-endosteal sclerosis (CHES; MIM 213002).[38] This entity shows overlap with both 4H and WRS as well, but individuals with CHES lack the facial phenotype and lipodystrophy as present in WRS and show osteosclerosis, which is absent in both WRS and 4H.[6] The three entities, WRS, 4H and CHES, share the unusual dentition and disturbed growth,[6 8 38] but growth has not been stated to be abnormal in the individuals with progressive ataxia. Several mutations occur in two or more of the four disorders that can result because of *POLR3A* variants, although this has only been found on a single allele until now. One may speculate that the cause for the difference in phenotype between individuals with *POLR3A* variants is based on the reduction of the levels of POLR3A and is influenced by other genetic or epigenetic factors as well.

The present study should facilitate future recognition of WRS as we show that in all individuals in whom the diagnosis WRS was clinically reliably determined variants in *POLR3A* are present. WRS is allelic with 4H, spastic ataxia and related to CHES and shows (admittedly limited) overlap with these entities. Careful clinical analysis of all four entities for signs and symptoms occurring in one of the entities is indicated to evaluate the clinical overlap more carefully. An explanation for the major clinical differences in individuals sharing the same *POLR3A* variant on one allele and the mechanistic link between defective POLR3A function and the developmental processes affected in the four disorders remain lacking. By pointing to defective synthesis of tRNA, rRNA and other ncRNA pools as the event implicated in WRS, however, our findings expand the molecular mechanisms contributing to progeroid disorders.

Electronic database information

- 1000 Genomes: http://www.1000genomes.org.
- AlamutVisual software: http://www.interactive-biosoftware.com/alamut-visual/.
- ATGpr: http://atgpr.dbcls.jp/.
- ClinVar: http://www.ncbi.nlm.nih.gov/clinvar/.
- ExAC Browser: http://exac.broadinstitute.org/.
- Genic intolerance: http://genic-intolerance.org/.
- HGMD: http://www.hgmd.cf.ac.uk/ac/index.php.
- NHLBI Exome Sequencing Project (ESP) Exome Variant Server: http://evs.gs.washington.edu/EVS/.
- OMIM: http://www.omim.org/.
- Pfam: http://pfam.xfam.org/.
- PyMOL: http://www.pymol.org.

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Contributors SP, YL, EA, CEA-B, DC, JA, CM, AN and GY performed biological sample processing, WES data interpretation, sequence data validation and segregation analyses, mutation screening, SNP. array analyses and data curation; EB carried out the structural analyses; DB, LA-G, MA, GA,. GG-K, TK, SM, JO, AS, GV, HA, CL-O and RCH recruited patients, collected biological samples and performed clinical evaluations; FB, AB and AC performed the WES data processing and analyses; BW, MT and RCH conceived the study, analysed and interpreted the data and wrote the manuscript.

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