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Mutations in the pre-replication complex cause Meier-Gorlin syndrome

Louise S Bicknell[1,20], Ernie M H F Bongers[2,20], Andrea Leitch[1], Stephen Brown[1], Jeroen Schoots[2], Margaret E Harley[1], Salim Aftimos[3], Jumana Y Al-Aama[4,5], Michael Bober[6], Paul A J Brown[7], Hans van Bokhoven[8], John Dean[9], Alaa Y Edrees[5], Murray Feingold[10], Alan Fryer[11], Lies H Hoefsloot[2], Nikolaus Kau[12], Nine V A M Knoers[13], James MacKenzie[7], John M Opitz[14], Pierre Sarda[15], Alison Ross[9], I Karen Temple[16], Annick Toutain[17], Carol A Wise[18], Michael Wright[19] & Andrew P Jackson[1]

Meier-Gorlin syndrome (ear, patella and short-stature syndrome) is an autosomal recessive primordial dwarfism syndrome characterized by absent or hypoplastic patellae and markedly small ears[1–3]. Both pre- and post-natal growth are impaired in this disorder, and although microcephaly is often evident, intellect is usually normal in this syndrome. We report here that individuals with this disorder show marked locus heterogeneity, and we identify mutations in five separate genes: *ORC1*, *ORC4*, *ORC6*, *CDT1* and *CDC6*. All of these genes encode components of the pre-replication complex, implicating defects in replication licensing as the cause of a genetic syndrome with distinct developmental abnormalities.

In the accompanying paper[4], we identified mutations in *ORC1* encoding a subunit of the origin recognition complex (ORC) in five individuals with microcephalic primordial dwarfism. The ORC multi-protein complex is loaded onto chromatin at DNA origins before the S phase in order to license replication. In conjunction with additional components CDT1, CDC6 and minichromosome maintenance (MCM) helicase proteins, it then forms the pre-replication complex (described in **Fig. 1**). Cellular studies have established that individuals with *ORC1* mutations (called here *ORC1* cases) had partial loss-of-function defects in replication licensing[4]. An orc1 zebrafish model recapitulated the dwarfism phenotype, as did morpholino depletion of another pre-replication complex component, mcm5. This led us to hypothesize that further pre-replication complex genes might cause primordial dwarfism; however, we did not find mutations in other ORC subunits.

In this study, the profound growth retardation and microcephaly in an individual with a complex lethal developmental syndrome led us to screen him for *ORC1* mutations. We found that this individual and his brother (called here P1 and P2; **Table 1** and **Supplementary Table 1**) were compound heterozygote for mutations in *ORC1* (p.Arg105Gln and p.Val667*fs*X24). The combination of truncating and missense mutations present in these individuals was in contrast to biallelic missense mutations seen in previous *ORC1* cases, suggestive of greater loss-of-function and consistent with a more severe phenotype. Unlike previous *ORC1* cases, the proband (P1) had an extensive number of developmental malformations including severe cortical dysplasia, congenital emphysema, absence of the pancreatic tail and retroflexion of the knees, as well as micropenis, blepharophimosis and cranial suture stenosis (**Fig. 1**). Notably, he also had marked microtia and absent patellae, features classically associated with Meier-Gorlin syndrome (MIM224690).

Consequently, we sequenced *ORC1* in 33 individuals with an established diagnosis of Meier-Gorlin syndrome and identified two further cases with mutations in *ORC1* (**Table 1** and **Fig. 2**). Both individuals, from families 2 and 3, respectively, were compound heterozygotes for the same *ORC1* mutations: a splice acceptor site mutation in conjunction with the previously identified c.314G>A

[1]Medical Research Council (MRC) Human Genetics Unit (HGU), Institute of Genetics and Molecular Medicine, Western General Hospital, Edinburgh, UK. [2]Department of Human Genetics, Institute for Genetic and Metabolic Disease, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. [3]Northern Regional Genetics Service, Auckland Hospital, Auckland, New Zealand. [4]Department of Genetic Medicine, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia. [5]Princess Al Jawhara Center of Excellence in the Research of Hereditary Disorders, King Abdulaziz University, Jeddah, Saudi Arabia. [6]Division of Genetics, Department of Pediatrics, A.I. DuPont Hospital for Children, Wilmington, Delaware, USA. [7]Department of Pathology, Aberdeen Royal Infirmary, Aberdeen, UK. [8]Department of Human Genetics, Nijmegen Centre for Molecular Life Sciences and Institute for Genetic and Metabolic Disease, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. [9]Department of Clinical Genetics, Ashgrove House, Foresterhill, Aberdeen, UK. [10]National Birth Defects Center, Waltham, Massachusetts, USA. [11]Department of Clinical Genetics, Royal Liverpool Children's Hospital, Alder Hey, Liverpool, UK. [12]Aberdeen Maternity Hospital, Foresterhill, Aberdeen, UK. [13]Department of Medical Genetics, University Medical Centre Utrecht, Utrecht, The Netherlands. [14]Human Genetics and Obstetrics and Gynecology, University of Utah, Medical Genetics, Salt Lake City, Utah, USA. [15]Service de Génétique Médicale, Centre de Référence Anomalies du Développement Centre Hospitalier Regional Universitaire de Montpellier, Hôpital Arnaud de Villeneuve, Montpellier, France. [16]Division of Human Genetics, University of Southampton, Faculty of Medicine, Southampton General Hospital, Southampton, UK. [17]Service de Génétique, Hôpital Bretonneau, Tours, France. [18]Sarah M. and Charles E. Seay Center for Musculoskeletal Research, Texas Scottish Rite Hospital for Children, Dallas, Texas, USA. [19]Northern Genetics Service, Newcastle Upon Tyne Hospitals National Health Service Trust, Central Parkway, Newcastle Upon Tyne, UK. [20]These authors contributed equally to this work. Correspondence should be addressed to A.P.J. (andrew.jackson@hgu.mrc.ac.uk) and E.M.H.F.B. (e.bongers@antrg.umcn.nl).

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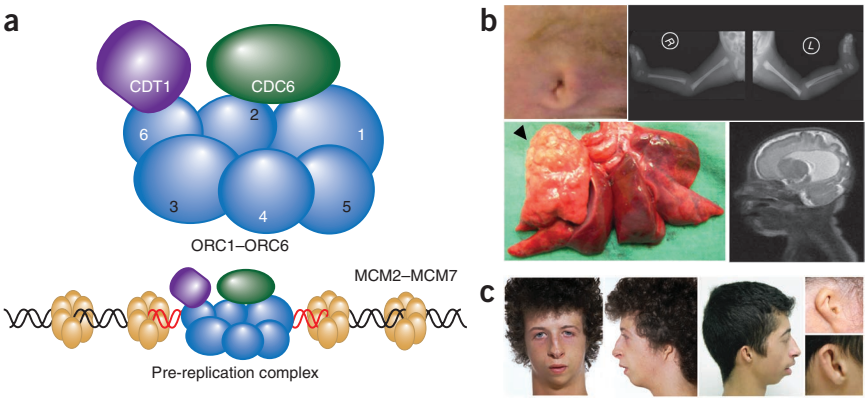

Figure 1 The pre-replication complex and Meier-Gorlin syndrome. (**a**) Genome replication is licensed by the binding of a number of specialized proteins to origins of replication that then form the pre-replication complex[9]. The first step in complex formation is the loading of the heterohexameric origin recognition complex (comprising ORC1–6 proteins) onto chromatin in an ATP-dependent manner during the M and G1 phases of the cell cycle. Further proteins, including CDC6 and CDT1, are then recruited to the pre-replicative complex that then permit reiterative loading of the multimeric MCM helicase. At the commencement of the S phase, replication is started by the MCM helicase unwinding the DNA and the recruitment of additional replication proteins. The five proteins implicated in Meier-Gorlin syndrome are highlighted in white text (ORC1, ORC4, ORC6, CDT1 and CDC6). (**b**) Individual P1 has a severe developmental malformation syndrome with marked microtia and extreme retroflexion and dislocation of the knees (top row, 'R', right; 'L' left). His malformations include lobar congenital emphysema (arrow head) and a severe cortical dysplasia of the brain. Parasagittal T2-weighted magnetic resonance imaging at age 1 month showing severe pachygyria, most severe frontally, along with ventricular enlargement. (**c**) Two individuals (P11 and P9) with classical Meier-Gorlin syndrome. (**b**,**c**) We obtained informed consent to publish the photographs from the subjects' parents.

mutation that encodes the p.Arg105Gln amino acid substitution[4], again in contrast to the biallelic missense mutations observed in the previous study. In family 2, the c.314G>A mutation was inherited on a haplotype shared with family 1 (**Supplementary Fig. 1**), consistent with common ancestry. However, the c.314G>A mutation in family 3 and the two splice acceptor site mutations in families 2 and 3

Table 1 Mutations in five genes encoding pre-replication complex proteins in individuals with Meier-Gorlin syndrome

Family	Case	Ancestry	Gene	Nucleotide alterations	Amino acid alterations	Exon(s)	Segregation	Parental consanguinity	Gender	Current height (s.d.)	Current OFC (s.d.)	Microtia	Absent or small patellae
F1	P1	UK	*ORC1*	[c.314G>A] + [c.1999_2000delGTinsA]	p.Arg105Gln + p.Val667*fs*X24	4; 13	Het, M, P	No	M	−9.6	−9.8	+	+
F1	P2	UK	*ORC1*	[c.314G>A] + [c.1999_2000delGTinsA]	p.Arg105Gln + p.Val667*fs*X24	4; 13	Het, M, P	No	M	NA	NA	+	U
F2	P3	USA	*ORC1*	[c.314G>A] + [c.1482-2A>G]	p.Arg105Gln + intron 9 splice acceptor site	4; intron 9	Het, M, nps	No	M	−6.6	U	+	+
F3	P4	UK	*ORC1*	[c.314G>A] + [c.1482-2A>G]	p.Arg105Gln + intron 9 splice acceptor site	4; intron 9	Het, M, P	No	F	−6.9	−4.0	+	+
F4	P5	USA	*ORC4*	[c.521A>G] + [c.874_875insAACA]	p.Tyr174Cys + p.Ala292*fs*X19	8; 11	Het, M, P	No	F	−6.4	U	+	+
F5	P6	USA	*ORC4*	c.521A>G	p.Tyr174Cys	8	Hom, M, P	Yes	F	−4.2	−2.1	+	−
F5	P7	USA	*ORC4*	c.521A>G	p.Tyr174Cys	8	Hom, M, P	Yes	F	−4.1	−3.0	+	−
F6	P8	TR	*ORC6*	[c.257_258delTT] + [c.695A>C]	p.Phe86X + p.Tyr232Ser	3; 7	Het, M, P	Yes	F	−3.3	−1.6	+	+
F6	P9	TR	*ORC6*	[c.257_258delTT] + [c.695A>C]	p.Phe86X + p.Tyr232Ser	3; 7	Het, M, P	Yes	M	−2.4	−2.1	+	+
F6	P10	TR	*ORC6*	[c.257_258delTT] + [c.695A>C]	p.Phe86X + p.Tyr232Ser	3; 7	Het, M, P	Yes	M	−3.2	−2.3	+	+
F7	P11	NZ	*CDT1*	[c.1385G>A] + [c.1560C>A]	p.Arg462Gln + p.Tyr520X	9; 10	Het, M, P	No	M	−4.7	+0.1	+	+
F8	P12	UK	*CDT1*	[c.196G>A] + [c.351G>C]	p.Ala66Thr + p.Gln117His (exon 2 splice donor site)	1; 2	Het, M, P	No	F	−5.1	−5.0	+	+
F9	P13	USA	*CDT1*	[c.1385G>A] + [c.1560C>A]	p.Arg462Gln + p.Tyr520X	9; 10	Het, M, P	Yes	F	−4.7	−1.3	+	+
F9	P14	USA	*CDT1*	[c.1385G>A] + [c.1560C>A]	p.Arg462Gln + p.Tyr520X	9; 10	Het, M, P	Yes	F	−3.9	−1.0	+	+
F9	P15	USA	*CDT1*	[c.1385G>A] + [c.1560C>A]	p.Arg462Gln + p.Tyr520X	9; 10	Het, M, P	Yes	M	−1.6	+1.7	+	+
F10	P16	UK	*CDT1*	[c.351G>C] + [c.1385G>A]	p.Gln117His (exon 2 splice donor site) + p.Arg462Gln	2; 9	Het, nps	Yes	F	−3.3	−0.5	+	+
F11	P17	SA	*CDT1*	[c.1081C>T] + [c.1357C>T]	p.Gln361X + p.Arg453Trp	7; 9	Het, M, P	No	F	−0.4	−2.1	+	+
F12	P18	FR	*CDC6*	c.968C>G	p.Thr323Arg	7	Hom, M, P	Yes	M	−4.1	−3.3	+	+

Mutations are described numbered from the first nucleotide of the initiation codon in the nucleotide sequence. For each mutation, 380 control chromosomes were screened and found to be negative for the sequence change. The protein truncation p.Tyr520X in *CDT1* is within the last exon and therefore the transcript is not expected to undergo nonsense-mediated mRNA decay. Additionally, in *CDT1*, the mutation c.351G>C confers a non-conservative amino acid substitution (p.Gln117His), however, the nucleotide lies within the exon 2 splice donor site, and therefore this mutation is expected to result in an inframe deletion of exon 3 from the transcript and resulting protein. Hom, homozygous in affected individual; Het, compound heterozygous in affected individual; M, mutation identified in mother; P, mutation identified in father; nps, parental sample(s) not available; TR, Turkey; NZ, New Zealand; FR, France; SA, Saudi Arabia; s.d., standard deviation; OFC, occipito-frontal circumference; NA, not available; U, unknown.

appear to have arisen independently. Notably, P3 in family 2 is the original individual described by Gorlin[2], unequivocally establishing that mutations in *ORC1* cause Meier-Gorlin syndrome.

Sequencing the genes encoding other ORC subunits then identified pathogenic mutations in two further subunits of the ORC (ORC4 and ORC6) in three individuals with Meier-Gorlin syndrome (**Table 1** and **Supplementary Table 1**). We found monozygotic twins P6 and P7 to be homozygous for a missense mutation (c.521A>G, p.Tyr174Cys) in *ORC4* (this family is also reported in reference 5, having been independently ascertained). The substituted tyrosine was conserved throughout evolution in organisms as distant as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* and was located within box VI of the ATPase associated with a wide range of cellular activities (AAA) domain[6] (**Fig. 2**), a domain that is essential for ATP-dependent complex formation of ORC4 with other ORC subunits[7,8]. A second individual, P5, is a compound heterozygote for this same p.Tyr174Cys substitution (present on the ancestral haplotype shared with P6 and P7; **Supplementary Fig. 1**) in conjunction with a 4-bp insertion mutation causing a translational frameshift and premature protein termination.

In family 6, three affected children were compound heterozygotes for mutations in *ORC6*. Similar to P5, these children inherited one loss-of-function mutation caused by a 2-bp deletion and a missense mutation resulting in the substitution (p.Tyr232Ser) of an amino acid completely conserved from fungi to humans located at the C terminus of the ORC6 protein (**Fig. 2**).

Given that mutations in genes encoding multiple components of the ORC complex cause Meier-Gorlin syndrome, we next examined whether additional components of the pre-replication complex could be similarly implicated. CDT1 and CDC6 are recruited to the pre-replication complex following assembly of the full ORC complex on chromatin[9]. As the region mutated in ORC6 is involved in CDT1 protein binding in *S. cerevisiae*[10], we next screened our Meier-Gorlin–syndrome cohort for mutations in *CDT1*. We identified five families with compound heterozygous mutations in *CDT1* (**Table 1**). In three of these families, one of the mutations was c.1385G>A, causing the substitution p.Arg462Gln in the C-terminal winged helix domain of CDT1 (**Fig. 2**). This residue has been implicated in MCM helicase complex binding through mutagenesis of the orthologous mouse residue (p.Arg474Gln)[11], the same amino-acid substitution resulting from the human mutation we have identified here.

Finally, screening of *CDC6* identified one individual with Meier-Gorlin syndrome (P18) homozygous for a missense mutation (c.968C>G) resulting in the substitution p.Thr323Arg. This residue is absolutely conserved from fungi to humans (**Fig. 2**) and lies within the box VII motif of the AAA domain (**Fig. 2**)[6], an ATP binding domain that is essential for CDC6 function in DNA replication[12].

We report here that mutations in multiple components of the pre-replication complex cause Meier-Gorlin syndrome and, despite finding marked genetic heterogeneity, identify an underlying functional homogeneity in this disorder. The functional studies of ORC1 in the accompanying manuscript[4] establish that impairment of ORC1 function is associated with extreme growth failure in humans. Here our molecular genetic findings implicate the entire pre-replication complex in primordial dwarfism syndromes, providing compelling genetic evidence supporting the notion that impaired replication licensing causes growth failure.

Though most of the affected individuals described in this paper have typical features of Meier-Gorlin syndrome[1–3], we saw considerable phenotypic variation from mutations in these genes, particularly with the *ORC1* cases reported here and in the accompanying paper[4]. Growth parameters vary markedly (height ranges from −0.4 to −9.6 standard deviations (s.d.) and head circumference from +1.7 to −9.8 s.d. from the population mean) with cognition varying from normal to severe neurological impairment associated with a severe cortical dysplasia. Thus, although microtia and small patellae are the best predictors of mutations in the pre-replication complex, the disease spectrum associated with these genes could extend from cases described as microcephalic osteodysplastic primordial dwarfism I or III to those with non-syndromic primordial dwarfism (for further discussion of clinical phenotypes, see the **Supplementary Note**).

Impaired replication might be expected to affect growth of all tissues equally; therefore, it is noteworthy that specific tissues are disproportionately reduced in size, most readily apparent in the ears and patellae. Most strikingly, major congenital malformations

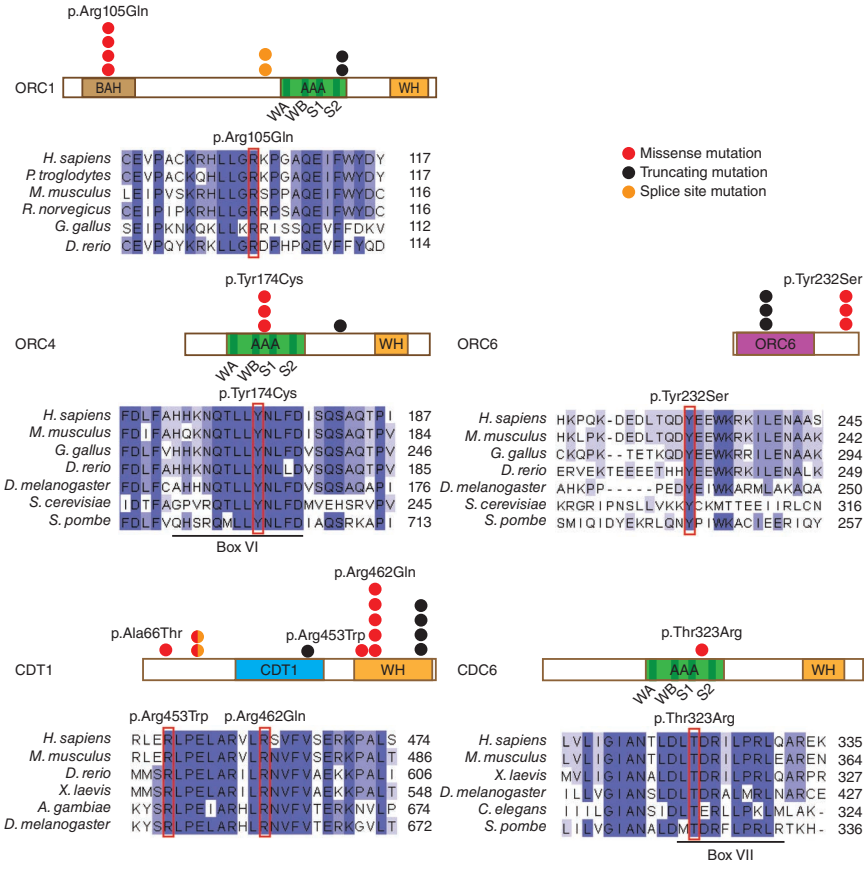

Figure 2 Pre-replication complex proteins mutated in Meier-Gorlin syndrome. Schematics for each protein depicting known protein domains with positions of mutations shown by filled circles. Each filled circle represents one affected individual. ClustalW2 alignment of protein residues surrounding substituted amino acids (with the positions of substituted resides indicated by red boxes). WA, Walker A; WB Walker B, S1, Sensor 1; S2, Sensor 2 motifs. BAH, bromo-associated homology domain; AAA, ATPase associated with a wide range of cellular activites; WH, winged helix domain.

were also evident in several cases affecting limb, genitourinary, lung and brain development. This finding suprisingly links mutations in a chromatin-bound protein complex regulating replication with multiple congenital malformations. Such an association between chromatin-bound complexes involved in essential cellular functions and development is not unprecedented. Notably, in Cornelia de Lange syndrome (MIM122470), mutations in the cohesin complex also cause severe growth retardation, though with very different developmental consequences[13].

The cellular mechanisms by which prereplication complex (pre-RC) components cause the specific developmental phenotypes reported here are not immediately evident. It may be that proliferation of certain cell types, such as chondrocytes in the ears and patellae, are particularly sensitive to reduced pre-RC function. Alternatively, these and the other developmental anomalies could result from other cellular functions of the pre-RC components, as has been found for individual ORC proteins[14].

Previous primordial dwarfism genes have DNA damage response and centrosome functions[15–19], whereas here we find mutations in genes involved in licensing of DNA replication origins. All are critical components required for cell cycle progression. Pathogenesis is therefore likely to be the result of impaired cellular proliferation, resulting in reduced cell number and consequently global growth failure. Although in this framework genes regulating replication are rational candidates for primordial dwarfism, it is most surprising to find specific congenital malformations occurring as the consequence of mutations in the pre-replication complex. This suggests an unanticipated link between the regulation of replication and development that warrants future investigation.

URLs. UniSTS, http://www.ncbi.nlm.nih.gov/unists; ExonPrimer, http://ihg2.helmholtz-muenchen.de/ihg/ExonPrimer.html; ClustalW2, http://www.ebi.ac.uk/Tools/clustalw2/index.html; Conserved Domains Database, http://www.ncbi.nlm.nih.gov/cdd.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Accession numbers. The reference gene sequences referred to in this study are available in the RefSeq database under the following accession codes: *ORC1,* NM_004153.2; *ORC4,* NM_181742.3; *ORC6,* NM_014321.3; *CDT1,* NM_030928.3; and *CDC6,* NM_001254.3.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

L.S.B. performed microsatellite genotyping. L.S.B., S.B. and J.S. performed mutation screening of cases and controls with the help of A.L., E.M.H.F.B., L.H.H., H.v.B. and N.V.A.M.K. M.E.H. performed chromosome breakage analysis. E.M.H.F.B. and A.P.J. clinically characterized the Meier-Gorlin syndrome cases and performed review of phenotypes and sample collection. S.A., J.Y.A.-A., M.B., P.A.J.B., H.v.B., J.D., A.Y.E., M.F., A.F., N.K., N.V.A.M.K., J.M., J.M.O., P.S., A.R., I.K.T., A.T., C.A.W. and M.W. contributed clinical cases and clinical data for the study. A.P.J. and L.S.B. wrote the paper with E.M.H.F.B.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Research subjects. All affected individuals in this study had previously been published as cases with Meier-Gorlin syndrome or fulfilled diagnostic criteria for Meier-Gorlin syndrome (microtia, absent or small patellae and short stature). Genomic DNA was isolated from peripheral blood from the affected children and family members. No cytogenetic abnormalities were observed on routine karyotyping of these cases with Meier-Gorlin syndrome (n = 10 cases). Furthermore, no significant increase of either spontaneous or radiation-induced chromosome breaks was observed (two cases versus two controls, ≥100 metaphases assessed per individual; $P \geq 0.7$, χ^2 test). Informed consent was obtained from all participating families and the studies were approved by the Scottish Multicentre Research Ethics Committee (04:MRE00/19), the Regional Committee on Research Involving Human Subjects Nijmegen-Arnhem (0006-0119) or the University of Texas Southwestern Medical Center at Dallas (IRB #032008-066). Informed consent was obtained for the publication of photographs.

Sequencing of candidate genes. Primers were designed using ExonPrimer (**Supplementary Table 2**). Coding exons and exon-intron boundaries of each gene were screened by bi-directional capillary sequencing on an ABI 3730 gene sequencer. PCR conditions are available upon request. Sequence analysis and mutation detection was performed using Mutation Surveyor v. 2.61 (Softgenetics LLC).

Microsatellite genotyping. Microsatellites were selected on the basis of heterozygosity and proximity to each gene. Primer sequences for microsatellites were obtained from UniSTS. Amplicons were separated by capillary electrophoresis on an ABI 3730 sequencer, and allele sizes were obtained using GeneMapper (Applied Biosystems).

Protein alignments. Reference sequences for all proteins were obtained from RefSeq, and alignments were performed using ClustalW2. Protein domains were mapped using data obtained from the Conserved Domain Database (CDD).

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