

























Supplementary Information

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Genomics England Research Consortium

Ambrose J. C. ¹, Bleda M. ¹ , Boardman-Pretty F. ^{1,2} , Boissiere J. M. ¹, Boustred C. R. ¹, Caulfield M. J. ^{1,2}, Chan G. C. ¹, Craig C. E. H. ¹ , Daugherty L. C. ¹ , de Burca A. ¹, Devereau, A. ¹, Elgar G. ^{1,2} , Foulger R. E. ¹ , Fowler T. ¹ , Furió-Tarí P. ¹ , Hackett J. M. ¹, Halai D. ¹, Holman J. E. ¹, Hubbard T. J. P. ¹ , Kasperaviciute D. ^{1,2}, Kayikci M. ¹ , Lahnstein L. ¹, Lawson K. ¹ , Leigh S. E. A. ¹ , Leong I. U. S. ¹ , Lopez F. J. ¹, Maleady-Crowe F. ¹, Mason J. ¹ , McDonagh E. M. ^{1,2} , Moutsianas L. ^{1,2} , Mueller M. ^{1,2} , Need A. C. ^{1,2} , Odhams C. A. ¹ , Patch C. ^{1,2} , Perez-Gil D. ¹, Polychronopoulos D. ¹ , Pullinger J. ¹ , Rahim T. ¹ , Rendon A. ¹ , Rogers T. ¹, Ryten M. ¹, Savage K. ¹, Scott R. H. ¹, Siddiq A. ¹ , Sieghart A. ¹ , Smedley D. ^{1,2}, Smith K. R. ^{1,2} , Sosinsky A. ^{1,2} , Spooner W. ¹ , Stevens H. E. ¹ , Stuckey A. ¹ , Thomas E. R. A. ^{1,2} , Thompson S. R. ¹ , Tregidgo C. ¹, Tucci A. ^{1,2} , Walsh E. ¹ , Watters, S. A. ¹ , Welland M. J. ¹, Williams E. ¹ , Witkowska K. ^{1,2}, Wood S. M. ^{1,2}, Zarowiecki M. ¹ .

1. Genomics England, London, UK

2. William Harvey Research Institute, Queen Mary University of London, London, EC1M 6BQ, UK.

Supplementary Case Reports

Subject 1

This male child initially presented to community paediatrics at the age of 2 years with delayed development. Metopic ridging had been noted at birth, and following referral to the clinical genetics department at the age of 4 years he was seen in the craniofacial clinic aged 4.5 years for further assessment of his head shape. At this time he was noted to be scaphocephalic with frontal bossing and a trigonocephalic shape to his forehead. He had telecanthus, mild exorbitism with midface retrusion and bilateral ptosis; in addition he had a well-demarcated patch of numerous freckles restricted to his right shoulder, but there were no ocular signs of type 1 neurofibromatosis. There was no papilledema.

Computed tomography (CT) scan at this time showed a scaphocephalic head shape with mild trigonocephaly and shallow orbits, and confirmed fusion of the metopic, sagittal and lambdoid sutures; the coronal sutures were patent. Magnetic resonance imaging (MRI) showed a Chiari I malformation and lack of cerebrospinal fluid around the hindbrain region, but without any evidence of a syrinx. Fronto-orbital advancement and remodelling with lateral panel release was performed at the age of 5 years 5 months. On review aged 8 years he was doing well from a craniofacial point of view.

At the age of 4 years array comparative genomic hybridisation (aCGH) was performed using the ISCA 60k oligo array. This was reported as arr[hg19] 15q15.3(43,851,119-44,048,331)x1 pat,16p13.13p13.11(12,017,784-15,551,332)x3 mat. The paternal 15q15.3 deletion overlaps a Gold Standard deletion in Database of Genomic Variants (MacDonald et al., 2014) (reference gssvL39070) and is likely benign. However the large 16p13.13p13.11 duplication overlaps in its centromeric portion a genomic region frequently subject to duplications, which are associated with a very variable phenotype ranging from apparently normal to speech delay, learning disability and autism spectrum disorder (Allach El Khattabi et al., 2020).

These aCGH findings were not considered to explain the child's multiple suture synostosis, so targeted gene sequencing was performed including *ERF*, *TWIST1*, *ZIC1* and

mutation hotspots in *FGFR1*, -2 and -3. Following normal results, genome sequencing of the parent-child trio was undertaken as part of the 100,000 genomes project (100kGP). This revealed a third, previously undetected 314 kb chromosomal deletion involving chromosome 19, as described in the main text; the associated deletion of *ERF* likely explains the craniosynostosis present in this patient. However, a more detailed genotype-phenotype correlation analysis for the chromosome 19 deletion was not attempted, owing to confounding caused by the additional imbalances on chromosomes 15 and 16.

No craniofacial features had been noted in the father, who was found to be a high-level mosaic (75% blood cells) for the chromosome 19 deletion.

Subject 2

This boy was born to non-consanguineous parents. Two previous pregnancies had resulted in a stillbirth at 39 weeks of unknown cause, and a healthy daughter. The father subsequently had three healthy children with a different partner.

Reduced fetal movements were noted in the 31st week of pregnancy and he was delivered by emergency Caesarean section at 31+5 weeks owing to suspected placental insufficiency. At birth he weighed 1.33 kg (-1.09 SD), he required ventilation for 24 hours, and remained on the special care unit for 2 months. During the first week of life he developed suspected necrotising enterocolitis, which settled with antibiotics. Cranial ultrasound showed a sub-ependymal haemorrhage of the left lateral ventricle, which resolved on repeat scan. He had presumed neonatal alloimmune thrombocytopenia (minimum count 58,000/ μ l) and received a 2-unit platelet transfusion; this also resolved by day 11. During subsequent weeks the OFC crossed the centile lines from 28.0 cm at birth (-0.76 SD) to 42.5 cm at 12 weeks (corrected; +1.12 SD), but subsequently stabilized.

Developmental milestones were delayed from early on, he sat aged 1 year; he started in mainstream education but moved to a school for special educational needs at the age of 7.5 years because of moderate learning difficulties.

Epilepsy started aged 6 years with occasional grand mal seizures, which appeared photosensitive. Initially he was treated with carbamazepine, this was later changed to sodium valproate which appeared effective at preventing the major episodes. Brain MRI at the age of 7 years was normal. At the age of 11 years he received a diagnosis of attention deficit hyperactivity disorder (ADHD), which showed marked improvement on treatment with methylphenidate, together with melatonin at bedtime.

From the age of 10 years he developed episodes of eyelid fluttering occurring up to several times per hour. These episodes deteriorated during a trial of valproate withdrawal. Electroencephalogram (EEG) at the age of 15 years confirmed photosensitivity; upon eyelid closure there were spikes post-centrally, which were increased during photic stimulation. A diagnosis of Jeavons syndrome was proposed.

At the age of 16 years, he could manage all activities of daily living and self-care. He engaged well when questions were directed to him and his reading age was 5-6 years. He was transitioning to college to learn independent living and employability skills.

On clinical genetic assessment at the age of 17 years, he was noted to have a narrow face, prominent eyes, mildly high palate, small chin, low frontal hairline with sparse hair, and pectus excavatum. His occipito-frontal circumference (OFC) was 55 cm (-0.07 SD), height 169.5 cm (-0.48 SD), and weight 45 kg (-2.15 SD).

Genetic testing using aCGH [Agilent ISCA 60K oligoarray] showed a deletion on chromosome 19, reported as arr[hg19] 19q13.2(42,532,353-42,723,970)x1 dn; testing of parental samples by fluorescence *in situ* hybridisation using the RP11-979P13 probe gave normal results, suggesting that it had arisen *de novo*. The deletion was evaluated as being of uncertain significance. A next-generation sequencing analysis of a 72-gene epilepsy/severe delay panel identified a deletion of exons 1-8 of *ATP1A3* (within the chromosome 19 deleted region), but was otherwise negative. Genome sequencing as part of the 100kGP confirmed the previously identified chromosome 19 deletion, but did not identify any other cause for his clinical presentation. As described in the main text, based on genome sequencing data, the chromosome 19 breakpoints were refined to

chr19:42,488,104-42,751,672. Analysis by breakpoint PCR confirmed the presence of the deletion, and showed that this was inherited from the father who demonstrated a low level of mosaicism in a DNA sample extracted from blood.

Subject 3

The male proband is the first and only child of healthy non-consanguineous white British parents. There was no family history of craniosynostosis or intellectual disability.

Polyhydramnios was noted during the pregnancy and he was born by emergency Caesarean section at 41 weeks' gestation with a birth weight of 4.08 kg (+1.06 SD). During the neonatal examination a systolic murmur was noted and he was referred for cardiology assessment.

Prominent epicanthic folds and ocular hypertelorism were noted; the echocardiogram demonstrated a mildly hypoplastic aortic arch, which has not required any active management.

He was seen for clinical genetics assessment at the age of 7 months. The OFC was 50 cm (+4.4 SD), indicating disproportionate macrocephaly compared to his length (72 cm, +1.2 SD) and weight (9.33 kg, +0.9 SD). Facially, telecanthus, prominent epicanthic folds and down-slanting palpebral fissures were observed. The remainder of the examination was normal. Both parents also exhibited macrocephaly (father's OFC was 60.3 cm; +3.63 SD and mother's OFC 59 cm; +4.22 SD), additionally the father was noted to have similar facial features to the proband including telecanthus, prominent epicanthic folds and down-slanting palpebral fissures. Array CGH showed a maternally inherited 11p11.2 duplication [arr(hg19) 11p11.2(47,892,568-48,664,526)x3 mat], confirmed using BlueGnome BlueFISH probes for BACs within the duplication interval (RP11-402H2 and RP11-358H20), which does not correspond to any rearrangement in the Database of Genomic Variants (DGV) or gnomAD SVs v2.1, but was not believed to be clinically significant based on inheritance from the clinically healthy mother. At this stage the family was recruited to 100kGP.

The proband's subsequent speech and language attainment was normal. He was vocalising at 5-8 months, babbling at 11-12 months, first words were at 15-16 months and he

had 2-3 word sentences at 2-2.5 years. There were no concerns regarding social development, behaviour, vision or hearing. When reviewed at the age of 7 years, he was attending mainstream school with no concerns. He continued to be macrocephalic (OFC 58 cm, +4.34 SD), with height 116 cm (-1.07 SD) and weight 23.8 kg (+0.15 SD). There was no sutural ridging or signs of craniosynostosis.

Following the detection of the heterozygous *ERF* deletion in the 100kGP genome sequencing data, independent clinical confirmation was obtained in both the proband and his father by multiplex ligation-dependent probe amplification (MLPA), using kit P479-A1 (MRC Holland). The proband has been referred for formal craniofacial assessment, but no cranial imaging has been performed to date.

Subject 4

This boy, who has a healthy twin sister, was conceived by *in vitro* fertilisation to unrelated parents when the father was aged 43 years and mother 37 years. A twin pregnancy resulted which proceeded without problems, although the proband was noted to be in breech position. He was delivered second by planned Caesarean section at 37 weeks' gestation, weighed 3.005 kg (-0.89 SD) and did not require resuscitation at birth. A left torticollis, noted at his six week check, was managed with physiotherapy.

Developmental delay was apparent from early on. He sat at 8 months and walked at 18 months; at two years his motor skills were noted to be poor, with low muscle tone. His first words were at 3 years. Investigation of the cause of his developmental delay by aCGH using the CytoSure Constitutional v3 oligo array showed a *de novo* deletion classified as arr[hg19] 19q13.2(42632509-42756260)x1.

As the deletion included the *ERF* gene, he was referred for a craniofacial assessment at the age of 3 years 7 months. His OFC was 54.5 cm (+3.09 SD) and cephalic index 0.73 (normal range 0.76-0.83); he was noted to have right occipital flattening and ipsilateral frontal bossing suggestive of positional plagiocephaly. CT scan of the skull revealed sagittal synostosis, with normal appearance of the brain, normal intracerebral fluid spaces, and no

Chiari malformation. Fundoscopy under anaesthesia excluded papilloedema. In view of the increased risk of raised intracranial pressure (ICP) associated with *ERF* haploinsufficiency he was further investigated by ICP monitoring, which showed a mean pressure of 14 mm Hg with 14 overnight episodes of increased pressure (B waves). He was noted to have large adenoid glands bilaterally and to mouth-breathe and snore during sleep. This was associated with bilateral glue ear. A sleep study confirmed mild-moderate respiratory obstruction. In view of these findings he was prioritized for adenoidectomy and insertion of grommets at the age of 4 years 5 months. This resulted in marked symptomatic improvement, with some evidence of developmental catch-up.

Formal developmental assessment at the age of 63 months using the Schedule of Growing Skills revealed equivalent ages in months as follows: locomotor, 30; manipulative, 24; visual, 24; speech and language, 15; interactive social, 24; self-care social, 24; cognitive, 18-24, indicating moderate-severe global developmental delay. He could speak about 100 word approximations with good comprehension, but was not putting two words together. He continued in nappies and his tone was low, with dribbling, unsteady walking and difficulty maintaining a sitting position. His social communication difficulties were assessed to be out of keeping with his developmental delay and, combined with a positive Autism Diagnostic Observation Schedule (ADOS) assessment, a formal diagnosis of autism was confirmed. Initially he attended a normal school with 1:1 support but had to repeat his reception year and he is scheduled to move to a special school.

Whole genome sequencing was performed to refine the breakpoint positions, which, as presented in the text, were defined as chr19:42537012-42801688 (hg19).

Subject 5

This boy, the fifth child of consanguineous (first cousin) parents from Yemen, was born at term by normal vaginal delivery. He showed moderate global developmental delay, first walking at 18 months and with his first words at the age of 3 years. An atrial septal defect

was diagnosed at the age 13 years, shortly after he moved to the UK, but has not required surgical intervention.

On review at the age of 14.2 years, he was attending a school for special educational needs and was noted to be hyperactive with poor concentration, and noted to be very sociable. He was able to speak in short sentences, could read numbers 1-10 and knew letters but could not write any words. He had an unsteady gait with frequent falls. His general health was good, with occasional severe headaches, but no visual or hearing problems and normal sleeping pattern. He had no seizures.

On examination he was noted to have a long face, wide mouth with full lips, long philtrum, normal teeth and palate. His OFC was 50 cm (-3.6 SD), height 136.3 cm (-3.3 SD) and weight 30.1 kg (-3.1 SD). He had mild brachydactyly of his hands and feet with broad halluces. Multiple area of hyperpigmented skin appeared to be related to old scars, but one true café-au-lait macule was present on his left shoulder.

A skull radiograph was normal. A standard karyotype was normal (46,XY) and fluorescence *in situ* hybridization (FISH) analysis of 7q11.23 and fragile X testing was negative. Array CGH, performed as part of the DDD study using a customized Agilent array (genomic plus 5 probes per exon), identified the deletion arr[hg19] 19q13.2(42,492,136-42,756,726)x1. Exome sequencing using Agilent Sure Select Exome Plus capture probes, performed on the proband only, did not identify any pathogenic variants. The chromosome 19 deletion was confirmed by aCGH performed by a clinical diagnostic lab; parental samples were not available for analysis.

The patient is registered in the DECIPHER project with reference ID 272468.

Subject 6

This boy, currently aged 7 years, is the second of three children born to non-consanguineous parents. At birth he was noted to have mild macrocephaly (OFC +1SD) and macrosomia, although there had been no evidence of maternal diabetes mellitus during the pregnancy. Dysmorphic facial features were flat supra-orbital ridges, malar hypoplasia, short

philtrum, and large mouth with full lips; in the extremities, he had broad thumbs and halluces and mild brachydactyly. He later presented with speech delay and was subsequently assessed to have mild intellectual deficiency. There was no evidence of craniosynostosis, and cerebral MRI was normal.

Investigation by aCGH revealed two apparently *de novo* deletions in the patient, categorized as arr[hg19] 1q21.1q21.2(146,641,601-147,356,634)x1 dn; 19q13.2(42,702,762-42,754,032)x1 dn. The breakpoints of the chromosome 1 deletion correspond to BP3-BP4, which is associated with a variable presentation of learning disability (Bernier et al., 2016). Further scrutiny of the chromosome 19q deletion revealed that the first flanking non-deleted probes were at 19:42,673,249-42,673,308 and 19:42,790,697-42,790,756. Quantitative PCR and FISH for both deletions was negative in the parents, confirming the array findings. A quantitative PCR was also performed for the flanking *C/C* gene (42,788,912-42,788,977) and no deletion was detected in the patient. A more detailed genotype-phenotype correlation analysis for the chromosome 19 deletion was not attempted, owing to confounding caused by the additional imbalance on chromosome 1 present in this patient.

The patient is registered in the DECIPHER project with reference ID 381692.

Supplementary Materials and Methods

Bioinformatics analysis and copy number variant (CNV) validation

From Illumina short-read whole genome sequencing (WGS) data in the 100kGP, we initially interrogated the Canvas and Manta callsets for CNV/SV (structural variant) annotations at the *ERF* locus (region of interest: chr19:42459898-42803164, hg19; chr19:41955746-42299012, hg38) in 128 affected individuals (from 114 families) with craniosynostosis (as the primary phenotype) available in the Research Environment (main programme release v10). To identify additional cases with CNV/SV at the *ERF* locus, independently of the phenotype, bioinformatic screening was performed to scrutinize the same region of interest in all 74,008 genomes of participants from families affected with rare disorders available in the 100kGP

(v10). For each of these genomes, CNV/SV annotations at the region of interest were extracted (using bcftools query) from the SV.vcf files (containing the Canvas/Manta calls) available in the Research Environment. WGS of gDNA from Subject 4 was performed by Novogene (150 paired-end reads on an Illumina NovaSeq 6000 sequencer) and bioinformatic analysis was performed as previously described (Miller et al., 2017). Candidate variants (absent in gnomAD SVs and DGV gold standard datasets in UCSC, affecting genes and/or conserved elements/regions) were visualized in IGV and by using Samplot (Belyeu et al., 2020), to confirm that these were likely real variants based on read-pair information, the presence of split reads, and/or changes in read depth.

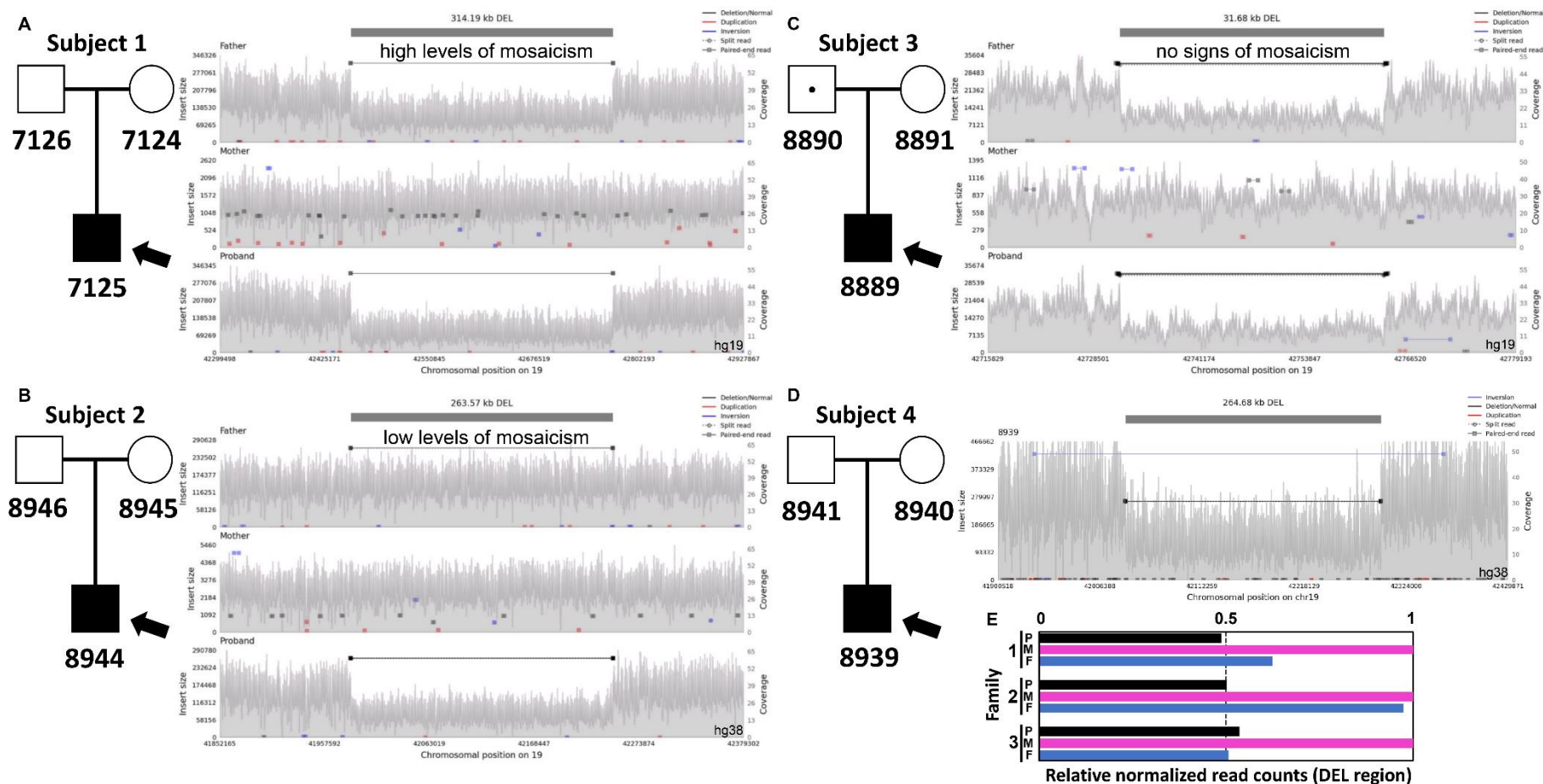
In cases where mosaicism was suspected based on (i) the presence/levels of SNPs from the non-deleted allele within the deleted region, (ii) the read depth and/or (iii) the presence/levels of abnormal reads supporting the CNV, we used the following approach to quantify the levels of mosaicism. From the WGS data, the count number of reads (obtained from the BAM files available in the Research Environment of the 100kGP using samtools view) within the deleted region were normalized to the total number of reads outside the deletion on chromosome 19, and the ratio obtained was divided by the equivalent figure for the parent without the deletion. The value x of the normalized read counts (Supp. Figure S1E) was used to estimate the proportion p of cells bearing the deletion using the formula $p = 2(1-x)$.

Validation and segregation analysis (using parental samples, if available) of the variants identified by the WGS data were undertaken by breakpoint PCR and dideoxy-sequencing of PCR products from genomic DNA using BigDye Terminator v3.1 (Applied Biosystems). In Subject 4, segregation analysis (NlaIV restriction digest of PCR product) of the rare chr19:g.42783791G>C (hg19) hemizygous variant identified in this proband's WGS data was used to determine the parental origin of the identified deletion. Primer sequences are provided in Table S2.

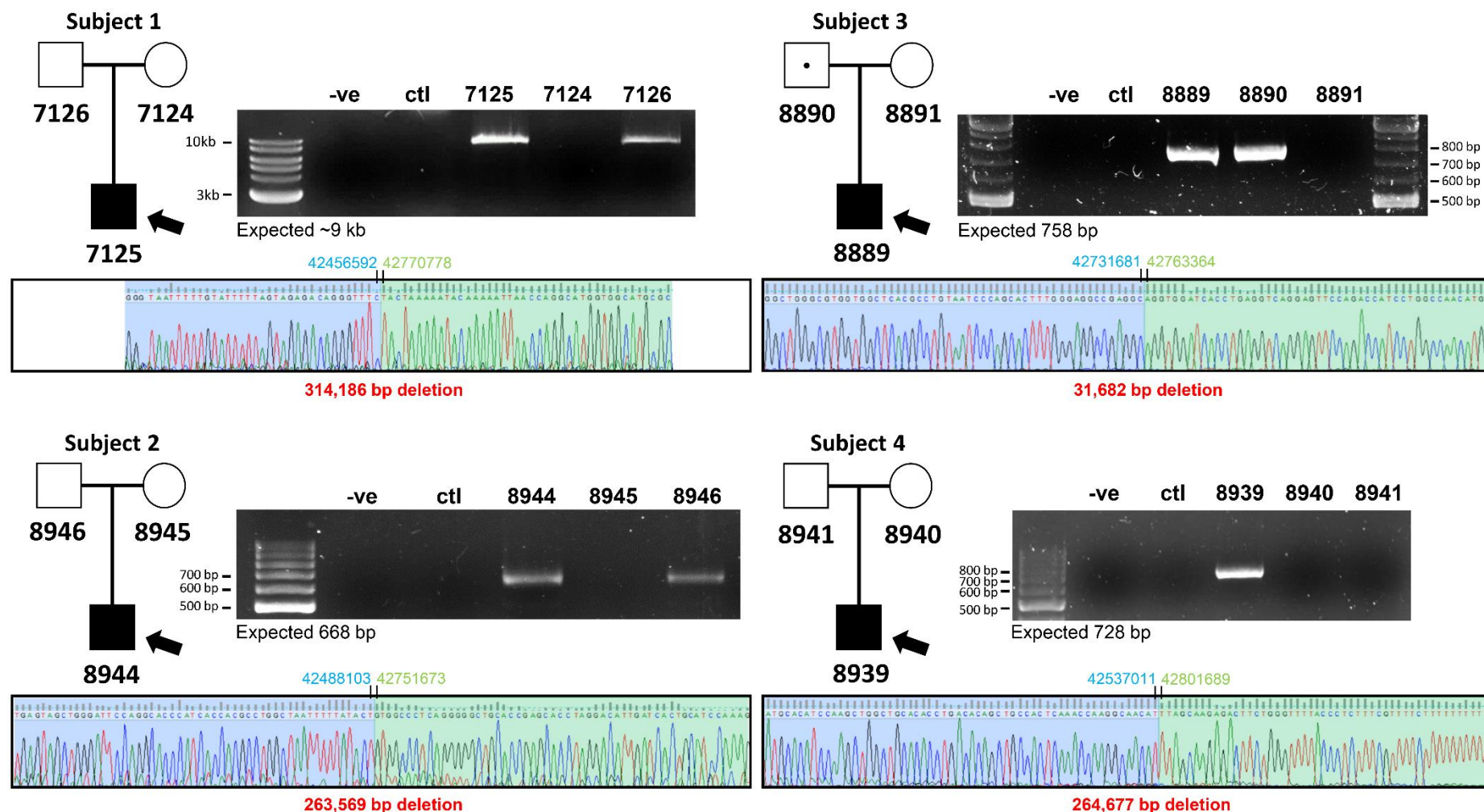
Additional cases harboring heterozygous deletions around *ERF* identified by aCGH where retrieved from the DECIPHER database (<https://decipher.sanger.ac.uk/>) (Firth et al., 2009).

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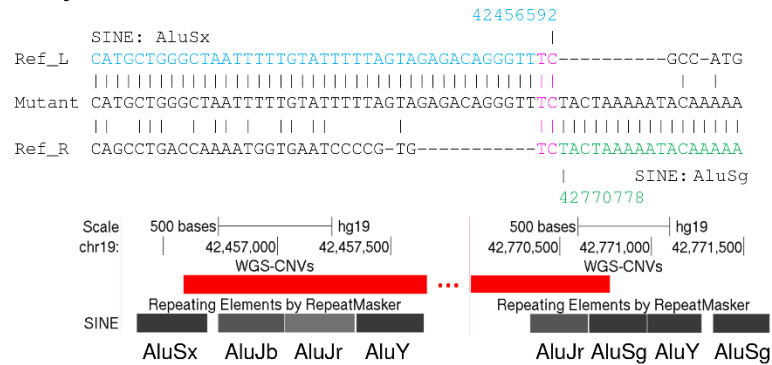


Supp. Figure S1. Overview of deletions in the neighbourhood of *ERF* identified by WGS. Simplified pedigrees and Samplot figures (A-D) showing coverage in gray (right y axis) and insert size between paired-end reads (left y axis), with split and discordant reads shown as lines. In E the relative normalized read counts (calculated from the WGS data) in the three families sequenced as trios are shown; for each subject, the count number of reads within the deleted region was calculated relative to the total number of reads outside the deletion on chromosome 19, and the ratio normalized to the value obtained for the parent from whom the deletion had not arisen (the mother in each of Subjects 1-3), defined as having value = 1. P, proband; M, mother; F, father.

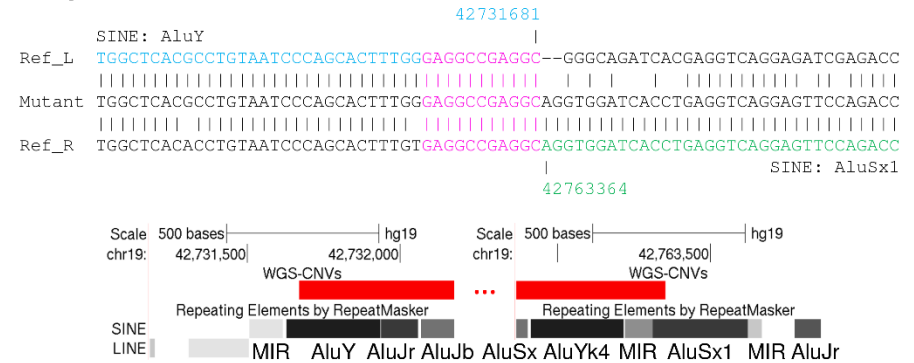


Supp. Figure S2. Validation of the CNVs by breakpoint PCR and dideoxy-sequencing. For each analysed subject, the sample ID for probands and parents are indicated in each family tree. Expected size of the PCR product is indicated below each agarose gel. Sequencing traces from each proband's PCR product are shown at the breakpoints, with highlighting showing the centromeric (blue) and telomeric (green) sides. Genomic coordinates (chromosome 19, hg19) of the bases flanking each deletion are indicated at the top of the sequencing traces, and the size of the deletion is shown at the bottom.

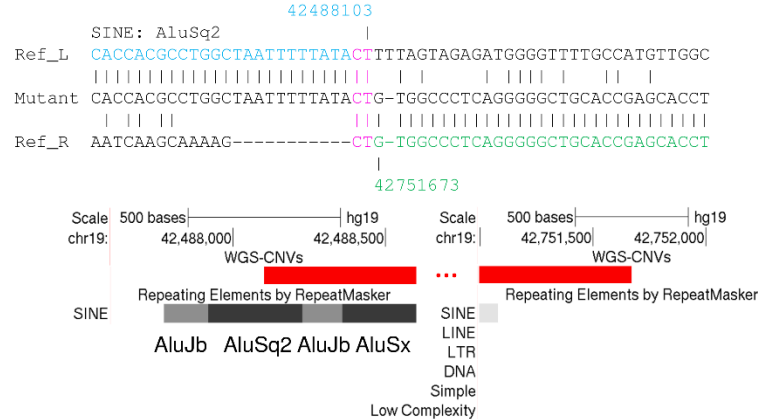
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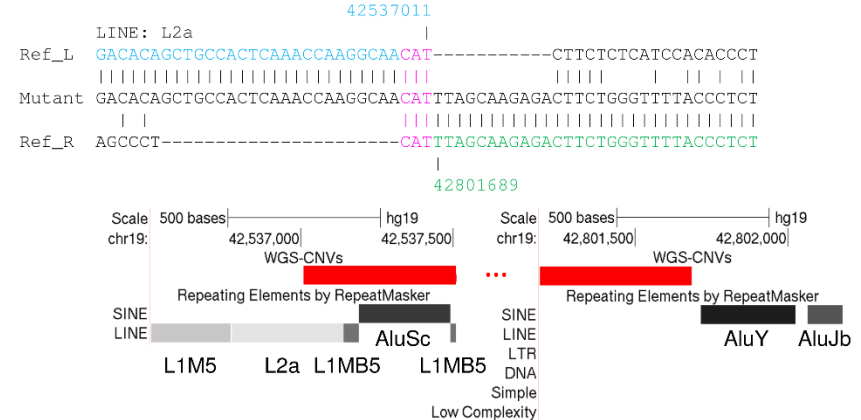
Subject 3



Subject 2



Subject 4



Supp. Figure S3. Analysis of sequences at the breakpoints. For each subject, the upper panel shows the DNA sequences across the deletions identified by WGS compared with the respective normal sequences at the centromeric (left, in blue) and telomeric (right, in green) ends of the breakpoints. Regions of homology at the breakpoints are highlighted in pink. The lower panels show the repetitive elements present in each breakpoint region (UCSC Genome Browser; hg19), compared to the ends of each deletion (paired red bars connected by three dots).

Sup. Table S1. Names, positions, and constraint scores of genes around *ERF*

Name	Location (GRCh38)	Description	Morbid	Phenotype MIM number	DDG2P	%HI	gnomAD pLI	gnomAD o/e	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6
<i>RPS19</i>	19:41860255-41872925	ribosomal protein S19	Y	Diamond-Blackfan anemia 1, AD (105650)	Confirmed: Monoallelic	23.95	0.92	0 (0 - 0.37)	chr19:41952441-42266625 (hg38)					
<i>CD79A</i>	19:41877279-41881372	CD79a molecule	Y	Agammaglobulinemia 3, AR, (613501)		45.28	0.69	0.16 (0.06 - 0.5)		chr19:41983952-42247520 (hg38)				
<i>ARHGEF1</i>	19:41883173-41930150	Rho guanine nucleotide e	Y	?Immunodeficiency 62, AR, (618459)		47.07	0.77	0.21 (0.13 - 0.34)			chr19:42227530-42259211 (hg38)			
<i>ERFL</i>	19:41907705-41928516	ETS repressor factor like				NA	NA	NA				chr19:42032860-42297536 (hg38)		
<i>RABAC1</i>	19:41956681-41959321	Rab acceptor 1				48.63	0.01	0.51 (0.26 - 1.07)					chr19:41987984-42252574 (hg38)	
<i>ATP1A3</i>	19:41966582-41997497	ATPase Na+/K+ transport	Y	Alternating hemiplegia of childhood 2, AD, (614820);	Confirmed: Monoallelic	43.12	1	0 (0 - 0.06)						chr19:42198610-42249880 (hg38)
<i>GRIK5</i>	19:41998321-42069498	glutamate ionotropic receptor kainate type subunit 5				38.12	0.99	0.15 (0.09 - 0.28)						
<i>ZNF574</i>	19:42068477-42081552	zinc finger protein 574				56.57	0.08	0.27 (0.15 - 0.51)						
<i>POU2F2</i>	19:42086110-42196585	POU class 2 homeobox 2				29.22	1	0.04 (0.01 - 0.2)						
<i>DEDD2</i>	19:42198598-42220140	death effector domain containing 2				54.29	0.06	0.34 (0.17 - 0.77)						
<i>ZNF526</i>	19:42220312-42228201	zinc finger protein 526				72.83	0.52	0.21 (0.1 - 0.47)						
<i>GSK3A</i>	19:42226225-42242625	glycogen synthase kinase 3 alpha				20.18	1	0 (0 - 0.13)						
<i>ERF</i>	19:42247569-42255128	ETS2 repressor factor	Y	Craniosynostosis 4, AD, (600775); Chitayat syndrome, Confirmed: Monoallelic		36.31	0.99	0.06 (0.02 - 0.26)						
<i>CIC</i>	19:42268537-42295797	capicua transcriptional re	Y	Mental retardation, autosomal dominant 45, AD, (61 Probable: Monoallelic		37.92	1	0.08 (0.04 - 0.18)						
<i>PAFAH1B3</i>	19:42297033-42303546	platelet activating factor acetylhydrolase 1b catalytic subunit 3				29.13	0	0.49 (0.27 - 0.97)						
<i>PRR19</i>	19:42302098-42310821	proline rich 19				71.72	0.81	0.1 (0.04 - 0.48)						
<i>TMEM145</i>	19:42313309-42325064	transmembrane protein 145				30.64	0	0.53 (0.36 - 0.81)						

Protein coding gene names, coordinates, description and haploinsufficiency score information were downloaded from Decipher v11.0. Gene constraints scores, the probability of being loss-of-function intolerant (pLI) and the ratio of the observed / expected (oe) number of loss-of-function variants were obtained from gnomAD v2.1.1. NA = not available

Sup. Table S2. Primer sequences

PCR: BP analysis	Forward (5'-3')	Reverse (5'-3')
ERF-subject1	GTCATTACAAACTAGGCAGCTAACC	CAGCAGTGCTAGGGTCTGAACC
ERF-subject2	GAATGGTATTTCTAAGATAGTCCC	TGGTCAAGCCTTATTCTGTATTGG
ERF-subject3	GCAGAGATGAGTCAGGCCTAAGG	TAGGGTAGAGTGCTCTTGTTATCC
ERF-subject4	AACACTCCCACAAATCCACAGG	CTAAGTGATACTTGGAAGAGGC
PCR: segregation analysis (SNV)	Forward (5'-3')	Reverse (5'-3')
ERF-subject4-SNV	GACATTTGAGCTGAAGTATGAGG	CTACAGTTACCAAGACTCACTCC