

Supplemental Data

**Defective DNA Polymerase α -Primase Leads to X-Linked
Intellectual Disability Associated with Severe
Growth Retardation, Microcephaly, and Hypogonadism**

Hilde Van Esch, Rita Colnaghi, Kathleen Freson, Petro Starokadomskyy, Andreas Zankl, Liesbeth Backx, Iga Abramowicz, Emily Outwin, Luis Rohena, Claire Faulkner, Gary M. Leong, Ruth A. Newbury-Ecob, Rachel C. Challis, Katrin Ōunap, Jacques Jaeken, Eve Seuntjens, Koen Devriendt, Ezra Burstein, Karen J. Low, and Mark O'Driscoll

Supplemental Note: Case Reports

Clinical descriptions

Family A

The clinical phenotype and linkage analysis in family A has been described before (Van Esch et al. 2005). In summary, the 6 affected males from this 5-generation family showed intellectual disability, growth retardation including microcephaly and hypogonadism. Heights, head circumferences and cognitive levels of carrier females and non-affected males are all within the normal ranges. Carrier females show skewing of X-inactivation (Fig1).

Family B

The index of family B is a 19 years old male born to healthy parents. He has a healthy sister and family history is negative. He has a moderate to severe developmental delay and started to have seizures at the age of 3 months that were difficult to control. His development was from then on severely impaired because of the frequent seizures. He showed pronounced hypotonia, severe feeding problems necessitating feeding via gastrostomy, progressive spasticity, scoliosis, cerebellar hypoplasia and hypogonadotropic hypogonadism. His healthy mother and sister are carriers of the mutation and show skewing of X-inactivation (Fig 1).

Family C

The index of family C is 5 years old and is the second child of healthy non-consanguineous parents (individual III.2). He has a healthy older brother and younger sister. He was born with an oesophageal atresia with tracheoesophageal fistula repaired day 3 of life. In addition a small interatrial communication with aneurysmal atrial septal tissue was identified in the first year of life. He has moderate developmental delay with speech and language delay being the most pronounced. He has severe microcephaly and short stature fitting in the primordial dwarfism

scale. He has some unusual phenotypic features including deep sacral dimples and a notable lack of subcutaneous fat tissue. His mother, maternal aunt and grandmother are all carriers. His maternal cousin (individual III.5) died at the age of 14 months following a stormy course from birth. He too had severe microcephaly and short stature. After birth he was diagnosed with pulmonary artery stenosis, a VSD and pulmonary atresia. Post operatively he developed a left thalamic bleed, and left subdural effusion. He had a bifid uvula and right vocal cord palsy of unknown aetiology. His medical files documented a hypogonadotrophic hypogonadism. All carriers females show extreme skewing of X-inactivation (Fig 1).

Family D

Individual D is the first child of healthy non-consanguineous parents of Caucasian ancestry. The pregnancy was completed by severe intrauterine growth retardation and maternal eclampsia. Individual D was born at 38 weeks of gestation with a weight of 1.685 kg, length 44.5 cm and HC of 28.5 cm. He continued to grow poorly with the head circumference being particularly affected. At 6 years 11 months, his weight was 14.6 kg (-4.5 SD), height 110.4 cm (-2.6 SD), HC 43 cm (-5.8 SD). Individual D has mild intellectual impairment with impulsive behaviour and a short attention span. He had normal brain MRI Imaging. He has a characteristic facial appearance with up-slanting palpebral fissures and a longish face. He has no other medical problems apart from spina bifida occulta at the L4/L5 level.

Family E

The proband is a 4 year-old male, former dichorionic diamniotic twin conceived via IVF procedure and born at 29 weeks 6 days of gestation. He was diagnosed with intrauterine growth restriction, and currently is below the 3rd centile for height, weight and head circumference. He is developmentally delayed but making progress with specialized speech, occupational, and physical therapy. He has map-like brown pigmentation in the shins but does not display any reticulate hyperpigmentation pattern or hypohidrosis; the skin is abnormally dry

throughout the body. Since birth, the proband has suffered from recurrent infections, including viral illnesses (parainfluenza, recurrent upper respiratory infections), bacterial infections (multiple cases of pneumonia, ear infections, and enterococcus infections), and fungal infections (invasive kidney fungal infection and candidal urinary tract infections). The complete blood count was normal and other than very mild decrease of IgG and IgM levels; there were no other evident changes in the initial evaluation. Lymphocyte proliferation in response to phytohaemagglutinin (PHA) and poke-weed mitogen (PWM) was also normal.

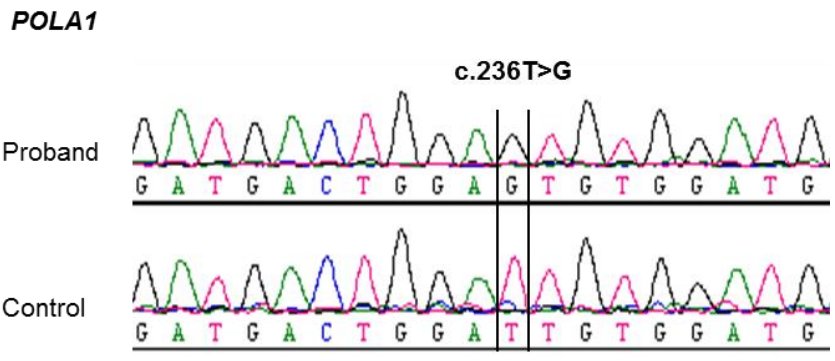
In addition to the *POLA1* variant, the proband and his mother were found to have an *STS* mutation (c.822-1G>A: IVS5-1G>A in intron 5), consistent with X-linked ichthyosis.

Van Esch, H., Zanni, G., Holvoet, M., Borghgraef, M., Chelly, J., Fryns, J.-P., and Devriendt, K. (2005). X-linked mental retardation, short stature, microcephaly and hypogonadism maps to Xp22.1-p21.3 in a Belgian family. *European Journal of Medical Genetics* 48, 145-152.

Figure S1

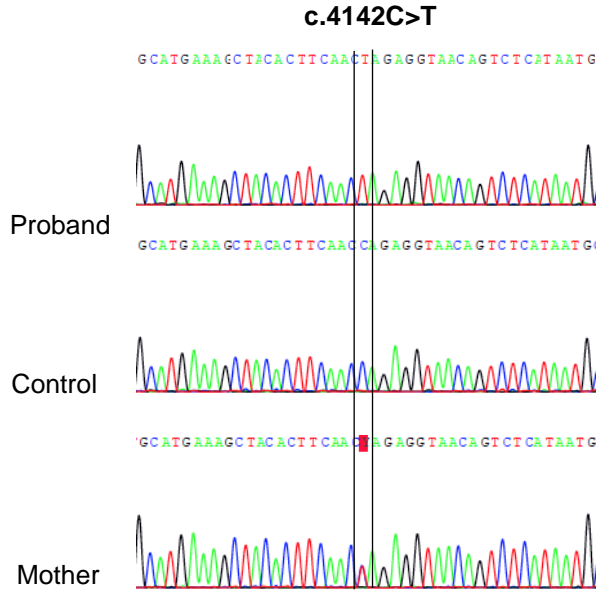
Molecular genetic investigations

Family A
(c.236T>G; p.Ile79Ser)



The c.236T>G mutation in the DNA sample of the index case (top) compared to a healthy, unrelated control.

Family B
(c.4142C>T; p.Pro1381Leu)

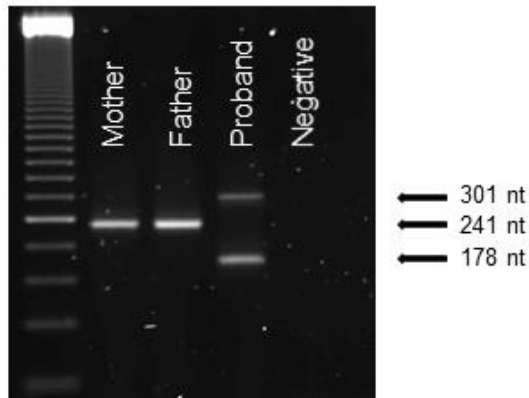


The c.4142C>T mutation in the DNA sample of the index case (top) compared to a healthy, unrelated control. The mother is a carrier of the mutation.

Family C

(c.507+1G>A; p.(Lys149_Glu169del,Thr170_Ser1462delins15*)

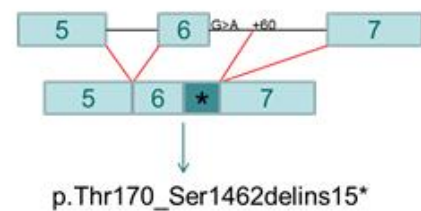
A (i)



B (i)



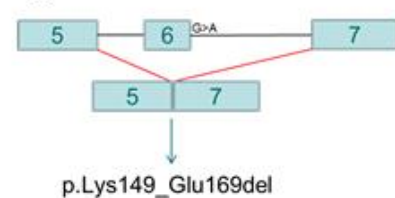
B (ii)



C (i)



C (ii)



The c.507+1G>A splice-site variant was predicted to completely abolish the donor splice site using 5 different splicing prediction programs (MaxEnt: -100.0%, NNSPLICE: -100.0%, Human Splicing Finder: -100.0%, SpliceSite Finder-like: -100.0%, GeneSplicer -100.0%, accessed through Alamut Visual v.2.7.1 software).

A. cDNA analysis of *POLA1* RNA transcripts from peripheral blood in mother, father and proband. Parents show a normally spliced RNA transcript containing exons 5, 6 and 7 (241nt).

The proband did not express the normal transcript, but expressed two abnormal transcripts of 301nt and 178nt.

B. (i) Sanger sequencing of the 301nt band revealed that this transcript contains an insertion of the first 60 nucleotides of intron 6, r.507_508ins507+1_507+60. (ii) Activation of a cryptic splice donor site within intron 6 is predicted to result in the insertion of 15 amino acids and the introduction of a premature termination codon p.(Thr170_Ser1462delins15*).

C. (i) Sanger sequencing of the 178nt band revealed that this transcript contains a deletion of exon 6, r.445_507del. (ii) This is an in-frame deletion, predicted to produce a protein product lacking the 21 amino acids encoded by exon 6, i.e. p.(Lys149_Glu169del).

Family D

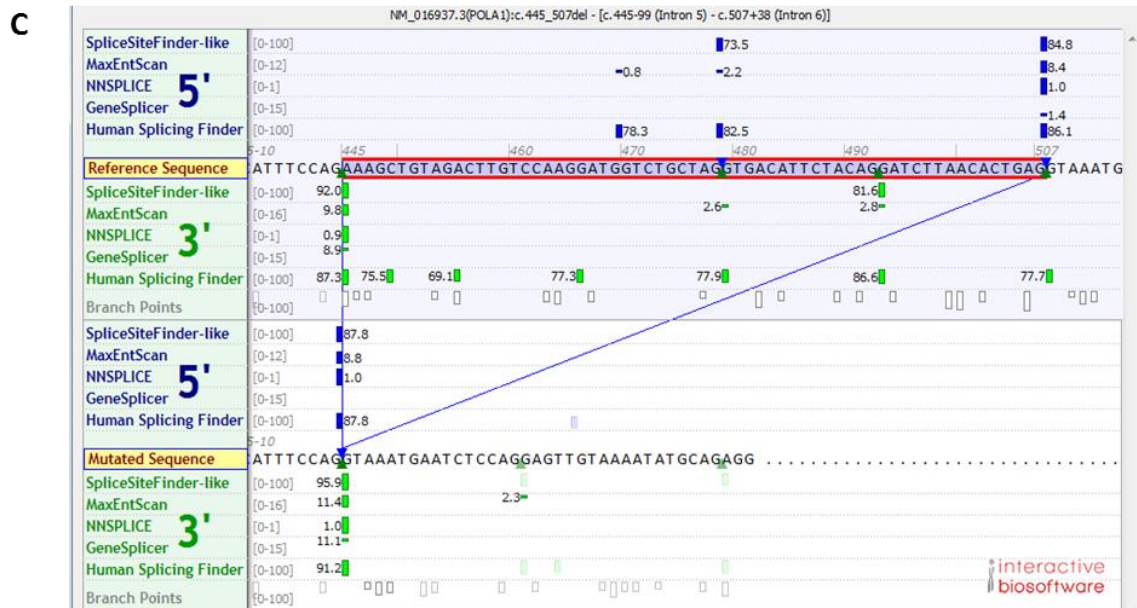
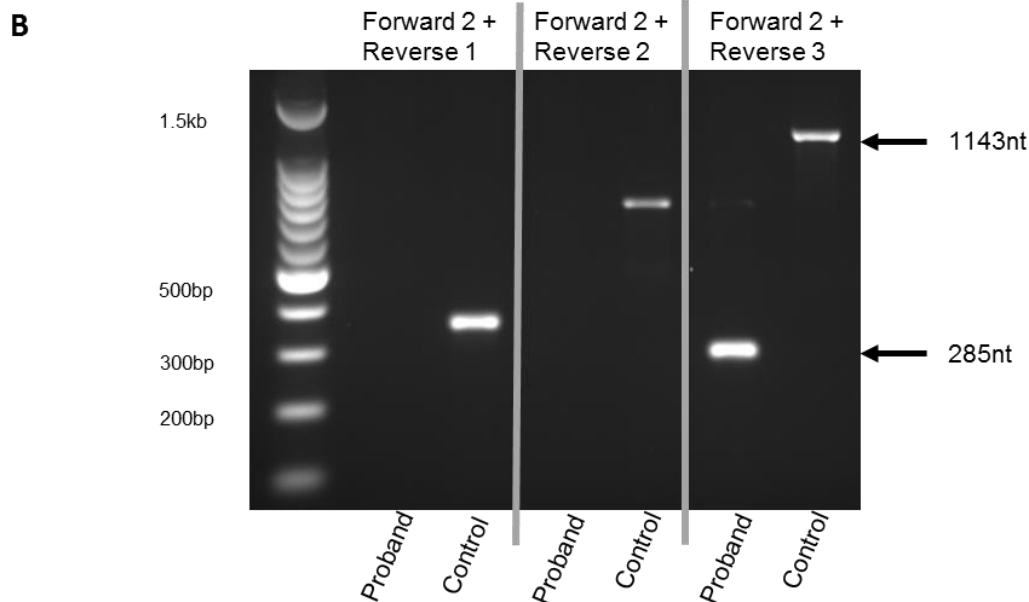
(c.445_507del; p.Lys149_Glu169del)

A

```

gtagcaggaagatcatggattaaggcgaaagtagaggatgggagctatagatgtaggaatgctatatacacagg
tggttgttaaaatctgtgaatgtagatgagcactcagagagtgggaatgaagaggcaaggactaactggctagg
tccttcctttcatatacatgtgtgtagtttggtagacattttaagtgcaaggctttctcatgttacctttctt
tatcatttccagAAAGCTGTAGACTTGTCCAAGGATGGTCTGCTAGGTGACATTCTACAGGATCTTAACACTGAG
gtaaatgaatctccaggagtgtgaaaatatgcagaggacatagactgaagaaacactctagtaattatacaaaac
agactattgccagaatttttcttttttttgaacagtagcttgcaactgttgcccaggctgggtgagcagtggtg
cgatcttggctcgttgcaacctctgattcccagggttccctcttccctgattcctgctcagcctcccagtagcta
ggattacaggcacctgccaccacgcccggtaaatatttggatatttactagagactaggtttcaactatgttggc
caggctggctcgaactccttacctcgtgatccgctgtcttggcctccaaagtgtgggattacaggcgtgag
ccactgtgccggcctgttgacagaattttttaaaatgtgaagaacgccaacaaaaaacccccacaaaaaaacaa
agaaaccccttcagattggtttatcccaccattttagggtgagtatttccagctgttagagctgtgtgattatg
tagatagggatgtgtgggtgtgtgttgaagtagtaagaatatacccaggagtcagttctgtgagtgctcactgc
ttgtgttcagttgctgtgggtgtatgttccctaaatagattgaagcctgtcaatctgcacaatcaggtataagagc
cttgtcttgaattctgagtggggaatgaaggaagagtgagtgaaactatccctgatagaactcaaacaggattagct
gcatgtgaaattgagatttggccatttccataaagactacgctctcatttagaagtgacttcttttaaATG
accatgattaaaaatgac
    
```

Forward 2
Exon 6
Reverse 1
Reverse 2
Reverse 3

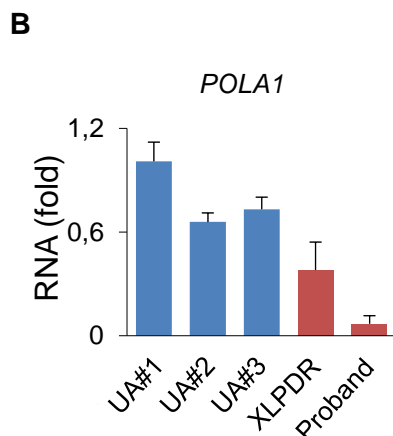
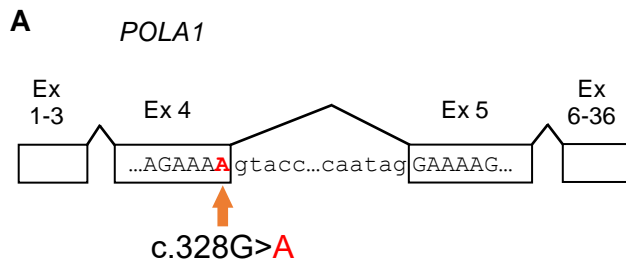


Analysis of exome data in the index of family D indicated a hemizygous deletion involving exon 6 of *POLA1*. This was confirmed by PCR, using different combinations of primers as shown in panels **A** and **B**. Only with primers Forward2 and Reverse3 a PCR product was obtained in the index, and subsequent sequencing of this product confirmed the deletion (the blue underlined sequence is present in the PCR product of 285 nt).

C. The deletion of exon 6 leads to an in-frame deletion of 21 amino acids.

Family E

(c.328G>A; p.Gly110Arg)



A. Schematic representation of *POLA1* and the location of the mutation (red arrow).

B. *POLA1* mRNA expression by qRT-PCR in dermal fibroblasts derived from 3 unaffected (UA) males, an XLPDR individual, and the proband described here. Data are representative of more than 5 replicates, bars – S.E.M. The experimental details are described in Materials and Methods section.

Figure S2**Conservation of missense variants and pathogenicity scores****Ile 79.****Family A: Ile79Ser**

Human	47	YK----YEV-EDFTGVYEEVDEEQYSKLVQARQDD--DWIVDDD---GIG	86
Chimpanzee	47	YK----YEV-EDFTGVYEEVDEEQYSKLVQARQDD--DWIVDDD---GIG	86
Rhesus macaque	47	YK----YEV-EDFTGVYEEVDEEQYSKLVQARQDD--DWIVDDD---GIG	86
Dog	53	YK----YEV-EDFTSVYEEVDEEQYSKLVQARQDD--DWIVDDD---GIG	92
Cow	47	YK----YEV-EDFTSVYEEVDEEQYSKLVQARQDD--DWIVDDD---GIG	86
Mouse	53	YK----YEV-EDLTSVYEEVDEEQYSKLVQARQDD--DWIVDDD---GIG	92
Rat	53	YK----YEV-EDLTSVYEEVDEEQYSKLVQARQDD--DWIVDDD---GIG	92
Chicken	94	LK----YEV-EEFTGVYDEIDEEQYSKIVRERQDD--DWIVDDD---GIG	133
Zebrafish	55	VK----YEV-EEFTSIYDEVDEEQYSKIVRERQDD--DWIIDD---GTG	94
Frog	38	VK----YEV-EEISSIYEEVDEEQYSKIVRDRQDD--DWIVDDD---GTG	77

Gly 110.**Family E: Gly110Arg**

Human	87	YVEDGREIF--DDD--LEDDALDADEK-----KDGKARNKDKRNV	123
Chimpanzee	87	YVEDGREIF--DDD--LEDDALDADEK-----KDGKARNKDKRNV	123
Rhesus macaque	87	YVEDGREIF--DDD--LEDDALDAGEK-----KDGKARNKDKRNV	123
Dog	93	YVEDGREIF--DDD--LEDDALDSHEK-----KDDKARTKDRNV	129
Cow	87	YVEDGREIF--DDD--LEDDALDSHEK-----KDNKACNKDKRTV	123
Mouse	93	YVEDGREIF--DDD--LEDDALDTCGK-----SDGKAHRKDRKDV	129
Rat	93	YVEDGREIF--DDD--LEDDALDTCGEG-----SDGKAHRKDRKDV	129
Chicken	134	YVEDGREIF--DED--LDDDALGSSKKG-----KTGKTSTIGKKNV	170
Zebrafish	95	YVEDGREIF--DEE--LDDDALG--PKTG-----KQAAKGGDSKKNV	130
Frog	78	YVEDGREIF--DDD--LEDNAL--ADSG-----KRAKGAPKDKSNV	112

Pro 1381.**Family B: Pro1381Leu**

Human	1362	LQF---SRTGPLCP-----ACMK--ATLQPEYSDKSlyTQLCFYRYI	1398
Chimpanzee	1281	LQF---SRTGPLCP-----ACMK--ATLQPEYSDKSlyTQLCFYRYI	1317
Rhesus macaque	1362	LQF---SRTGPLCP-----ACMK--ATLRPEYSDKSlyTQLCFYRYI	1398
Dog	1368	LQF---SRNGPLCQ-----VCMK--ATLRPEYSDKSlyTQLCFYRYI	1404
Cow	1362	LQF---SRNGPLCQ-----VCMK--ATLRLEYSDKSlyTQLCFYRYI	1398
Mouse	1366	LHF---SRNGPLCP-----VCMK--AVLRPEYSDKSlyTQLCFYRYI	1402
Rat	1369	LHF---SRNGPLCP-----ACMK--AVLRPEYSDKSlyTQLCFYRYI	1405
Chicken	1438	LSF---SRSGPICQ-----ACRK--AILRPEYSDKALYtQLCFYRYI	1474
Zebrafish	1370	IAF---SRSGPICP-----ACLR--STLKPeySEKALYNQLSFYRYI	1406
Frog	1366	LSF---SRNGPICQ-----ACTK--ATLRSEYPEKALYtQLCFYRFI	1402

Conservation of the *POLA1* missense variants. Alignments were generated using MUSCLE

(version 3.6 using maxiters 2), comparing the amino acid sequences within and around the sites of the three human missense mutants identified in this study (highlighted in yellow), to that of other vertebrates. The high degree of conservation of Ile79 and Gly110 is evident. Pro 1381 is also highly conserved. It's worth noting that as aa1381 is Leu in the cow POL α , sequence identity across the entire cow POL α compared to human is 90.6%. This is lower than that of chimpanzee (98.2%) and rhesus macaque (97.7%). Specific accession numbers are as follows; Human: [NP_058633.2](#), Chimpanzee: [XP_003317445.2](#), Rhesus macaque: [XP_001091195.1](#) Dog: [XP_005641271.1](#), Cow: [NP_001192994.1](#), Mouse: [NP_032918.1](#),

Rat: NP_445931.1, Chicken: XP_416792.3, Zebrafish: XP_005162809.1, Frog:
XP_004911812.1.

Software	Variant & Score	Classification comment
PolyPhen2	I79S: 1 G110R: 0.247 P1381L: 0.001	Probably damaging Benign Benign
PROVEAN	I79S: -4.190 G110R: -1.365 P1381L: -2.919	Deleterious Neutral Deleterious
MutationTaster	I79S: 142 G110R: 125 P1381L: 98	Disease causing Disease causing Disease causing
Align GVGD	I79S: Class C65 G110R: Class C65 P1381L: Class C65	Most likely to interfere with function Most likely to interfere with function Most likely to interfere with function

Pathogenicity predictions. A summary of the pathogenicity predictions for each missense mutation generated using the following pathogenicity prediction software tools:

PolyPhen2 (<http://genetics.bwh.harvard.edu/pph2/>),

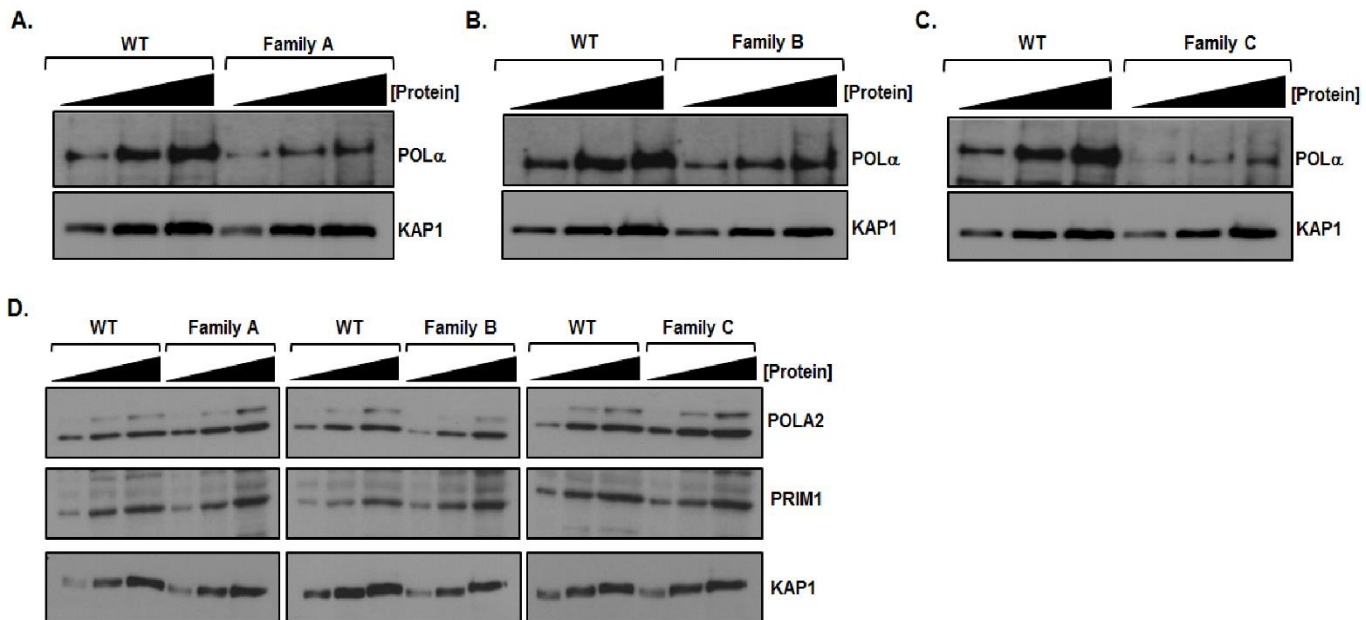
PROVEAN (<http://provean.icvi.org/index.php>),

MutationTaster (<http://mutationtaster.org/>)

Align GVGD (<http://agvgd.hci.utah.edu/>).

Figure S3

Chromatin localisation of POL α -primase subunits p180-POL α , p68-POLA2 and p48-PRIM1.



A. Increasing amounts of chromatin extracts from wild-type (WT) and family A patient LCLs were assessed for POL α levels. A very modest reduction in POL α chromatin retention was seen in extracts from Family A, although this not a significant reduction.

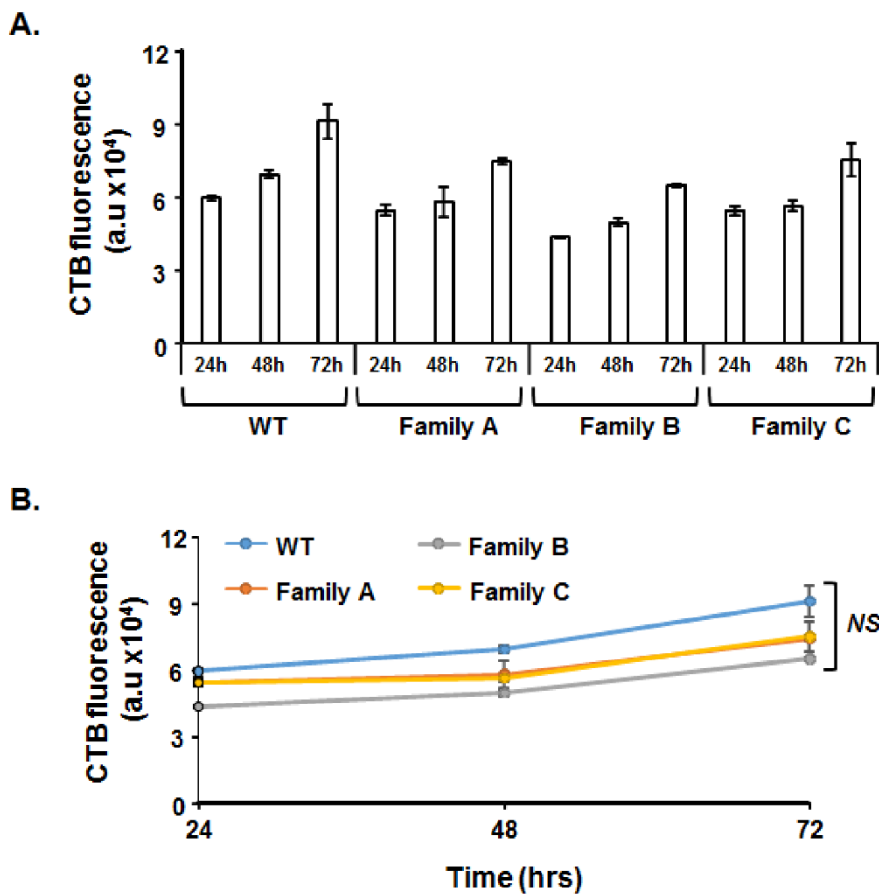
B. Chromatin extracts from family B LCLs exhibited a similar finding to that of family A.

C. Consistent with WCE analysis, *POLA1*-mutant LCLs from family C also exhibited a marked reduction of POL α on chromatin.

D. Western analysis of chromatin extracts for the POL α -primase subunits p68-POLA2 and p48-PRIM1 from each of the proband LCLs from families A, B and C did not reveal any marked differences in subunit chromatin retention. This suggests that the stoichiometry of POL α -primase components enriched on chromatin is preserved.

Figure S4

LCL proliferation analysis.

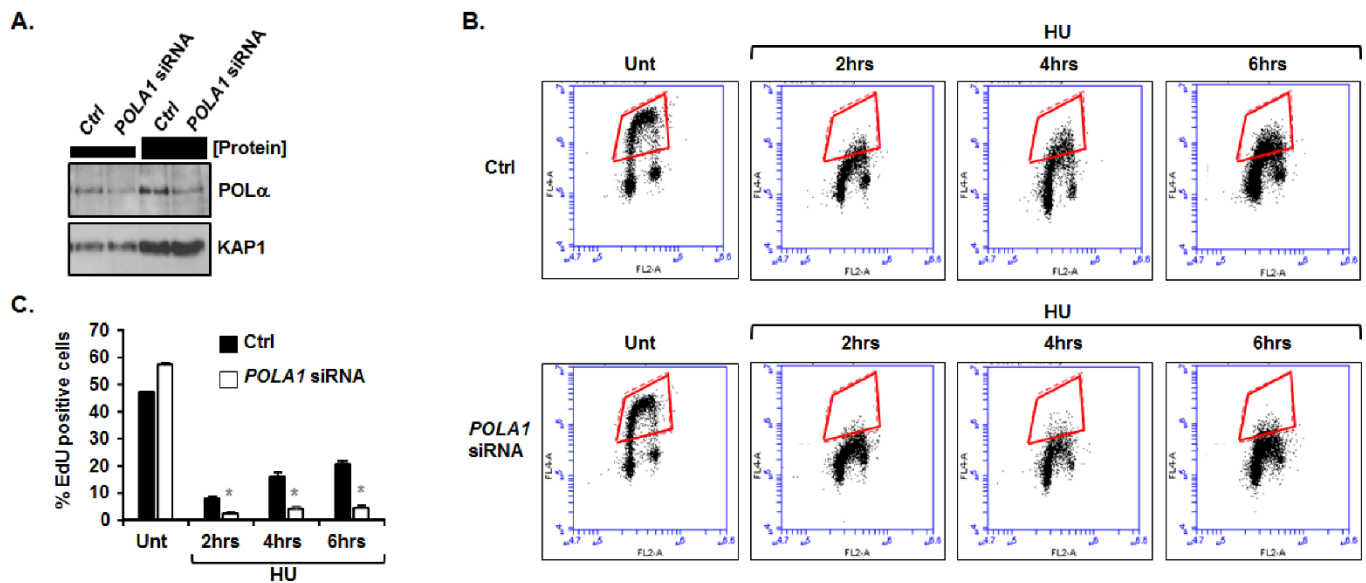


A. 1×10^4 cells were seeded in $100 \mu\text{l}$ in triplicate into a 96 well plate and proliferation was assessed over 24hrs-72hrs using CellTitre-Blue fluorescence proliferation assay. The fluorescence output from each LCL at 24h, 48h and 72h is shown as a bar chart. From Fig 4C; % EdU positive cells (30 min pulse) from untreated (Unt) LCLs from the father (25.86 ± 3.57) and mother (34.78 ± 5.17) compare highly to that of the family C proband ($24.44 \pm 4.28\%$). From Fig 4D a similar outcome is evident; % EdU positive cells from untreated (Unt) LCLs from WT ($26.84 \pm 4.92\%$) are highly similar to those of both affected individuals from Family A (V-4: $23.31 \pm 3.03\%$ and IV-7: $21.31 \pm 1.59\%$) and the proband of family B ($22.06 \pm 2.72\%$).

B. This is a line graph depiction of the same fluorescence shown in A, indicating the similar slope between the wild-type (WT) LCLs and those of the probands from families A, B and C. (WT: $m > 652.4$, Family A: $m > 413.2$, Family B: $m > 444.3$, Family C: $m > 432$). NS; not statistically significant at 72hrs $p = 0.376$ (Student's *t* test).

Figure S5

DNA replication following *POLA1* siRNA



A. U2OS cells were subjected to *POLA1* siRNA (24hrs) with the intention of obtaining a partial and not complete reduction in POL α expression levels. Under these conditions, a reduction of ~60% in POL α levels from whole cell extracts was observed following *POLA1* siRNA compared to control (Ctrl) scrambled siRNA.

B. The impact of mildly stressing conditions (125 μ M HU) upon DNA replication was assessed via EdU-pulse incorporation (30mins) in untreated (Unt) cells, and at the times indicated post treatment with HU, using flow cytometry of U2OS following control (Ctrl) scramble siRNA and *POLA1* siRNA. Representative flow cytometry panels are shown; the red hatched area denotes EdU positive cells. The panels show a marked reduction in EdU incorporation in the *POLA1* siRNA cells at all-time points following HU, compared to the control (Ctrl) conditions.

C. The bar chart shows EdU incorporation in untreated (Unt) and HU treated U2OS following control (Ctrl) and *POLA1* siRNA, indicating the significant reduction in DNA replication under these conditions in U2OS cells under-expressing POL α (*POLA1*) (*asterisks indicate p*<0.05 *Student's t test*).

Materials and methods

Cell culture

EBV-transformed lymphoblastoid cell lines (LCLs) derived from affected individuals, indicated parents or an unrelated wild-type (WT) phenotypically normal individual were cultured in RPMI1640 with 15% foetal calf serum (FCS), antibiotics (Pen-Strep) and L-Gln. Cells were maintained at 37°C in humidified incubators under 5% CO₂. LCL proliferation was assessed using CellTitre-Blue reagent kit (G8080) according to manufacturers' instructions (Promega). U2OS cells were similarly maintained but cultured in DMEM supplemented with 10% FCS, Pen-Strep and L-Gln.

Antibodies

DNA Pol α (G16) goat polyclonal (sc-5921), POLA2 (D2) mouse monoclonal (sc-398255) and PRIM1 (H9) mouse monoclonal (sc-390265) were obtained from Santa Cruz Technologies Ltd and used for blotting extracts from the LCLs. For the dermal fibroblasts we used anti- POLA1 from ThermoFisher (PA5-36147) and anti- β -Actin from Sigma-Aldrich (A5441). The antibodies used for DNA fibre combing included mouse anti-BrdU (Becton Dickinson 347580) for IdU detection, rat anti-BrdU (Abcam Ab 6326) for CldU detection, rat anti-single strand DNA (Genomic Vision). Secondary antibodies were Alexa Fluor (Invitrogen) anti-rat 488 (A21208), anti-mouse 594 (A31624) and anti-rabbit 647 (A31573).

Cell extracts

LCLs were washed in PBS then lysed in Urea-based lysis buffer (9M urea, 50mM Tris-HCl at pH 7.5, 10mM β -mercaptoethanol), followed by a sonication at 30% amplitude (12 sec). Protein concentration was determined by Bradford Assay. For chromatin extract preparation cells were washed in ice-cold PBS, resuspended in hypotonic buffer (10 mM HEPES pH 7.5, 5mM KCl, 1.5 mM MgCl₂, 1mM DDT, 10 mM NaF, 1 mM Na₂VO₃, 10 mM β -glycerophosphate, 0.5% IGEPAL and Roche protease inhibitor cocktail) and incubated on ice for 15 min. The sample was pelleted and washed twice in hypotonic buffer and resuspended in hypotonic buffer containing 0.5 M NaCl and incubated for 15 min on ice. Resultant chromatin was then

pelleted (17,000xg for 10 min) and solubilised in urea-based lysis buffer. For the dermal fibroblasts, whole cell lysates were prepared using Triton lysis buffer (25mM HEPES, 100mM NaCl, 10mM DTT, 1mM EDTA, 10% glycerol, 1% Triton X-100) supplemented with 1mM sodium orthovanadate and protease inhibitors (Roche).

Fibre combing and microscopy

DNA fibres were combed from genomic DNA agarose plugs using the FiberComb Molecular Combing System (Genomic Vision) and processed for immunofluorescence according to the manufacturer's instructions. Images were captured and processed using the Olympus IX70 Fluorescence microscopy platform. A conversion factor of 2kb/ μm was used as is standard for combed fibres and fibre track lengths processed using BoxPlotR.

Flow cytometry

LCLs were treated with 125 μM HU for the indicated times and pulse labelled with EdU (10 μM) 30mins prior to the end of each time point, then processed using Click-iT EdU Alexa Fluor-647 imaging kit according to the manufacturer's instructions (ThermoFisher Scientific). Samples were counterstained with propidium iodide (5 $\mu\text{g/ml}$), analysed using BD Accuri C6 Plus flow cytometer and profiles processed using BD CSampler software.

siRNA

U2OS were transfected with *POLA1* siRNA oligonucleotides using Lipofectamine RNAmix (Invitrogen) and assessed 24hrs post-transfection. A combination of two 3'-UTR directed *POLA1* oligonucleotides was used; sense, 5'-UCGUAAGCAUCAUAGAAAUUU-3' and sense, 5'-CAAUUAACCCGGUCUAAAUU-3'.

In situ hybridisation

Digoxigenin (DIG) labelled sense and antisense PCR probes (DIG RNA labelling kit, Roche) were designed using the F primer with a SP6 promotor site: 5'-CATTAGGTGACACTATAGAAAGGGAGTTTTGCAGCTTCC-3' and a R primer with a T7 promotor site: 5'-TAATACGACTCACTATAGGGAGGTGGTGGAGTTATTTGAGC-3'. By

means of sequencing we verified the PCR product obtained for *Pola1*. The labelled RNA sample was purified by ethanol precipitation and the sample was size separated on a 2% agarose gel. Timed pregnant (E0.5 is the morning of the day of vaginal plug) Swiss females were dissected and brains of E16.5 mouse embryos were collected and washed in ice-cold PBS. Postnatal brains were isolated after perfusion with PBS and 4% paraformaldehyde. All material was fixed overnight with 4% paraformaldehyde followed by progressive alcohol-assisted dehydration and paraffin embedding. Frontal 6 μ m thick sections were processed for *in situ* hybridization using an automated platform (Ventana Discovery, Ventana Medical Systems; details of procedures can be obtained at request), dehydrated and mounted with Eukitt (Sigma). Photographs were taken on a Leica DM5500B microscope.

qRT-PCR Family E

RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Total RNA (5 μ g) was used for cDNA synthesis utilizing the Superscript III strand synthesis system (Invitrogen). Quantitative real-time RT-PCR was performed using a Mastercycler (Eppendorf, Germany) following manufacturer's recommendations. SYBR Green based detection (Invitrogen) was employed using the following gene specific primers:

Target	Forward	Reverse
ACTB	GCGGGAAATCGTGCGTGACATT	GATGGAGTTGAAGGTAGTTTCGTG
POLA1 full	GCTATGTGGAAGATGGCCGA	TGTTCCGGTTTTGTCACTGCG

Experiments were performed in duplicate, data were normalized to housekeeping genes, and the relative abundance of transcripts was calculated by the comparative $\Delta\Delta C_t$ method.

X-chromosome inactivation

Lymphocyte-derived genomic DNA was subjected to the androgen-receptor gene methylation assay for assessment of the methylation status [Allen et al., 1992]. A ROX-labeled genotyping marker 100-500 (Applied Biosystems) was added and the samples were

separated on an ABI3130xl automated DNA sequencer (Applied Biosystems) and analyzed with the GeneMapper analysis software (Applied Biosystems) for peak position and area intensity calculations.

Allen, RC., Zoghbi, HY., Moseley, AB., Rosenblatt, HM., Belmont, JW. (1992). Methylation of HpaII and HhaI sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation. *Am. J. Hum. Genet.* 51(6), 1229-39.