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Defective DNA polymerase α-primase leads to X-linked intellectual disability associated with severe growth retardation, microcephaly and hypogonadism.

Hilde Van Esch[1,2*], Rita Colnaghi[3], Kathleen Freson[4], Petro Starokadomskyy[5], Andreas Zankl[6,7,8], Liesbeth Backx[2], Iga Abramowicz[3], Emily Outwin[3], Luis Rohena[9], Claire Faulkner[10], Gary M. Leong[11], Ruth A Newbury-Ecob[12], Rachel C Challis[13], Katrin Õunap[14], Jacques Jaeken[15], Eve Seuntjens[16], Koen Devriendt[1], Ezra Burstein[5,16], Karen J Low[12], Mark O'Driscoll[3*]

Hilde Van Esch	**1.** Center for Human Genetics, University Hospitals Leuven, 3000 Leuven, Belgium
	2. Laboratory for the Genetics of Cognition, Department of Human Genetics, KU Leuven, 3000 Leuven, Belgium
Rita Colnaghi	**3.** Genome Damage & Stability Centre, University of Sussex, BN1 9RQ, United Kingdom
Kathleen Freson	**4.** Department of cardiovascular sciences, Center for Molecular and Vascular Biology, KU Leuven, 3000 Leuven, Belgium
Petro Starokadomskyy	**5.** Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75390 Texas, USA
Andreas Zankl	**6.** Department of Clinical Genetics, The Children's Hospital at Westmead, NSW 2145 Westmead, Australia
	7. Children's Hospital Westmead Clinical School, Sydney Medical School, The University of Sydney, NSW 2145 Westmead, Australia
	8. Bone Biology Division and Kinghorn Centre for Clinical Genomics, Garvan Institute of Medical Research, NSW 2010 Darlinghurst, Australia
Liesbeth Backx	**2.** Laboratory for the Genetics of Cognition, Department of Human Genetics, KU Leuven, 3000 Leuven, Belgium
Iga Abramowicz	**3.** Genome Damage & Stability Centre, University of Sussex, BN1 9RQ, United Kingdom
Emily Outwin	**3.** Genome Damage & Stability Centre, University of Sussex, BN1 9RQ, United Kingdom
Luis Rohena	**9.** Division of Genetics, Department of Pediatrics, San Antonio Military Medical Center, San Antonio, TX 78234 Texas, USA

Claire Faulkner	**10.** Bristol Genetics Laboratory, Southmead Hospital, BS10 5NB Bristol, United Kingdom
Gary M. Leong	**11.** Department of Paediatrics, Nepean Hospital, Nepean Clinical School, The University of Sydney, NSW 2747 Kingswood, NSW, Australia
Ruth A Newbury-Ecob	**12.** Clinical Genetics, St Michaels Hospital, University Hospitals NHS Trust, BS2 8HW Bristol, United Kingdom
Rachel C Challis	**13.** MRC Human Genetics Unit, Institute of Genetics and Molecular Medicine, University of Edinburgh, EH4 2XU Edinburgh, United Kingdom.
Katrin Õunap	**14.** Department of Clinical Genetics, United Laboratories, Tartu University Hospital and Institute of Clinical Medicine, University of Tartu, 50406, Estonia
Jacques Jaeken	**15.** Center for Metabolic Diseases, University Hospitals Leuven, 3000 Leuven, Belgium
Eve Seuntjens:	**16.** Developmental Neurobiology, Department of Biology, KU Leuven, 3000 Leuven, Belgium
Koen Devriendt	**1.** Center for Human genetics, University Hospitals Leuven, 3000 Leuven, Belgium
Ezra Burstein	**5.** Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75390 Texas, USA. **16.** Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390 Texas, USA.,
Karen J Low	**12.** Clinical Genetics, St Michaels Hospital, University Hospitals NHS Trust, BS2 8HW Bristol, United Kingdom
Mark O'Driscoll	**3.** Genome Damage & Stability Centre, University of Sussex, BN1 9RQ, United Kingdom

* Corresponding authors: Hilde Van Esch, MD, PhD

Center for Human Genetics
University Hospitals Leuven
Herestraat 49
3000 Leuven
Belgium
Tel: +32-16-345903
Fax: +32-16-346051

Email: Hilde.VanEsch@med.kuleuven.be

Mark O'Driscoll, PhD
Genome Damage & Stability Centre
University of Sussex
Falmer, Brighton BN1 9RQ
United Kingdom
Tel: +44 1273 877 515
Fax: +44 1273 678 121
Email: m.o-driscoll@sussex.ac.uk

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Abstract

Replicating the human genome efficiently and accurately is a daunting challenge, involving the duplication of upwards of three billion base pairs. At the core of the complex machinery that achieves this task are three members of the B family of DNA polymerases; DNA polymerase α, δ and ε. Collectively these multimeric polymerases ensure DNA replication proceeds at optimal rates approaching $2x10^3$ nucleotides/min, and with an error rate of less than one per million nucleotides polymerised. The majority of DNA replication of undamaged DNA is conducted by DNA polymerases δ and ε. DNA polymerase α-primase complex performs limited synthesis to initiate the replication process, along with Okazaki fragment synthesis on the discontinuous lagging strand. An increasing number of human disorders caused by defects in different components of the DNA replication apparatus have been described to date. These are clinically diverse, involving a wide range of features including variable combinations of growth delay, immunodeficiency, endocrine insufficiencies, lipodystrophy and cancer predisposition. Here, using various complementary approaches including classical linkage analysis, targeted next generating sequencing and whole exome sequencing, we describe distinct missense and splice-impacting mutations in *POLA1* in five unrelated families presenting with an X-linked syndrome involving intellectual disability, proportionate short stature, microcephaly and hypogonadism. *POLA1* encodes the p180 catalytic subunit of DNA polymerase α-primase. A range of replicative impairments could be demonstrated in lymphoblastoid cell lines, derived from affected individuals. Our findings describe the presentation of pathogenic mutations in a catalytic component of a B family DNA polymerase member; DNA polymerase α.

MAIN TEXT

X-linked intellectual disability (XLID) is a heterogeneous disorder that can be classified as either non-syndromic, when cognitive impairment is the only feature, or as syndromic. In the latter, the cognitive impairment is associated with dysmorphic, metabolic and/or neurological features. Until now, over 140 XLID-associated genes have been identified [1], mainly through the implementation of comparative genome hybridization and next generation sequencing technologies [2; 3]. Many of these genes converge into a few common functional networks as ID proteins often participate in interconnected cellular and molecular processes, including neurogenesis, neuronal migration, synapse formation and function [4; 5]. Here we report that hypomorphic defects in the replicative DNA polymerase α cause a human XLID syndrome. In 5 families, we identified mutations in *POLA1* (Xp22.1-p21.3. MIM: 312040), which encodes the p180 catalytic subunit of the heterotetrameric DNA Polymerase α-primase (POLα). All affected individuals present with different degrees of intellectual disability and moderate to severe short stature, microcephaly, hypogonadism and variable congenital malformations (Fig 1). Written informed consent was obtained from all parents on behalf of the affected individuals, according to local ethical protocols and the principles of the Declaration of Helsinki. An overview of the clinical features of the affected individuals is presented in Table 1. More detailed clinical descriptions and pedigrees are provided in the Supplemental Note and Fig 1. The core clinical features consist of intellectual disability and developmental delay, ranging from mild to severe, pronounced proportionate short stature (ranging from -2 SD to -7.7 SD) and microcephaly (ranging from – 3.1 SD to -7.8 SD) pointing towards a clear growth deficiency syndrome of prenatal origin. Hypogonadism is also frequently evident. The index case of Family B also developed seizures and secondary neurological and orthopedic manifestations, which were not seen in the other individuals. In two affected individuals a congenital heart malformation was present at birth (family C). Although we cannot define a recognizable facial gestalt, mild upslant of the palpebral fissures are present in 4 cases (Fig 1A-G).

Of the three mammalian replicative DNA polymerases (POLα, POLδ and POLε), POLα-primase is the only polymerase that can initiate *de novo* DNA synthesis from licensed replication origins as well as mediating Okazaki fragment synthesis during lagging strand DNA replication [6-8]. POLα is also involved in other cellular processes such as DNA damage response signaling from stalled replication forks, telomere maintenance, and epigenetic regulation [9-14]. Interestingly, a recurrent deep intronic mutation in *POLA1* was recently found as causing X-linked reticulate pigmentary disorder (XLPDR. MIM: 301220), a primary immunodeficiency with autoinflammatory features, as well as skin hyperpigmentation and prototypical facial gestalt [15; 16]. This intronic mutation creates a novel exon 13a into the *POLA1* transcript, reducing the total amount of p180-POLα protein. XLPDR individuals do not exhibit intellectual disability, altered body growth or smaller head circumference, which might indicate tissue-specific differences in abnormal splicing. Conversely, our cases do not show any of the XLPDR related symptoms, except proband E who suffered from recurrent infections.

POLA1 mutations were identified either using classical linkage analysis followed by Sanger sequencing of all 17 genes present in the 6 cM interval (LOD score 2.6) (Family A) [17], by a custom-designed microcephaly/microcephalic dwarfism Sure Select capture panel consisting of 63 genes (Family C), by single WES (Family B) or by trio WES (Families D and E). In family A, a missense mutation, c.236T>G in exon 3 of *POLA1* leading to a p.Ile79Ser change was identified, which segregates with the disease in all 4 affected individuals and obligate carrier mothers (Fig1H). The sequence variant results in substitution of an isoleucine, a non-polar amino acid to a serine, a polar amino acid. This residue and its surrounding sequence is highly conserved and p.Ile79Ser was predicted to be deleterious by various *in silico* methods. (Fig S1, S2). In family B, exome analysis identified a missense mutation c.4142C>T leading to p.Pro1381Leu mutation. This mutation affects a conserved residue and

is also present in the unaffected mother, maternal grandmother and sister (Fig 1I, S1, S2). In family C, a splice-site variant, c.507+1G>A located in the donor splice site of intron 6 of *POLA1* was identified. The variant was identified in the affected proband and his affected maternal cousin (Fig 1J). The c.507+1G>A splice-site variant was predicted to completely abolish the donor splice site using 5 different splicing prediction programs. RNA studies were performed showing that c.507+1G>A prevents normal splicing and leads to the production of two abnormally-spliced transcripts (Fig S1). The larger c.507+1G>A transcript results from activation of a cryptic splice donor site within intron 6 leading to insertion of the first 60 nucleotides of intron 6. This is predicted to cause insertion of 15 amino acids and the introduction of a premature termination codon p.(Thr170_Ser1462delins15*) truncating the potential p180-POLα product upstream of the domains responsible for DNA binding and catalytic activity. The smaller c.507+1G>A transcript results from exon 6 skipping, leading to an in-frame deletion which is predicted to produce a protein product lacking 21 amino acids, i.e. p.(Lys149_Glu169del) (Fig S1). In Family D, a hemizygous deletion of exon 6 was identified via exome sequencing, leading to an in-frame deletion producing a protein lacking 21 amino acids as seen for the smaller transcript in Family C. This deletion arose *de novo* in the index case (Fig S1). Exome sequencing in Family E identified a *de novo* variant c.328G>A affecting the last nucleotide of exon 4 leading to p.Gly110Arg. Bioinformatic analysis predicts a high probability of intron 4 missplicing upon c.328G>A replacement and subsequent qRT-PCR analysis displayed a dramatic reduction of *POLA1* mRNA compared to cells derived from unaffected males, even more profound than that observed in XLPDR-derived fibroblasts (Starokadomskyy et al., 2016) (Fig S1). None of the above identified variants are present in the dbSNP, 1000 Genomes, ExAC or gnomAD databases, and were submitted to ClinVar, accession number XXXXX. All missense mutations affect conserved amino acids and are predicted to be deleterious by various *in silico* methods (Fig S2). In addition, in the 3 familial cases (Families A-C), all obligate female carriers show significant to complete skewing of X inactivation (Fig 1H-J).

DNA polymerases are highly expressed during development, when rapid DNA replication and cell division is required [18; 19]. To further investigate POLα/*POLA1* in mammalian brain development, we assessed *Pola1* expression by *in situ* hybridisation in the embryonic and adult mouse brain. In the mouse forebrain, *Pola1* is expressed in those zones containing proliferating cells in the developing embryonic neocortex (Fig 2A) as well as in the lateral and medial ganglionic eminences (not shown). After birth, the gene is transcribed in cells that remain proliferating in the ventricular and subventricular zone of the striatum (Fig 2B). These data suggest that *Pola1* has a role in neurogenesis throughout life. Additionally, *pola1* expression by *in situ* hybridization in developing zebrafish embryos shows early and intense staining in the developing brain [20; 21], whilst Polα activity appears highest in isolated neurons from developing rat brain cerebral cortex when the mitotic activity is at its peak [22]. Conversely, an insertional mutation in *pola1* of zebrafish (*pola1* ^{hi1146Tg}) presented with central nervous system necrosis, small head and eyes, an inflated hindbrain ventricle, a thin and often curved body and a rounder yolk with no extension at day 2 [23]. At days 3-5, the necrosis spread throughout the body resulting in overt body wasting, small head and eyes [23].

We examined POLα protein levels in proband-derived cell lines from families A, B, C and E (Fig 2C-F). Cell lines from the Family D proband were unavailable. Using whole cell extracts (WCE) from lymphoblastoid cell lines (LCLs) derived from affected individuals from family A and B, we found POLα protein levels comparable to that of wild-type (WT) LCLs (Fig 2C, D). In contrast, WCE derived from family C probands' LCLs or dermal primary fibroblasts from family E proband, both showed marked reduction in POLα levels (Fig 2E, F). These findings are consistent with the RT-PCR analysis from each of these two families (Fig S1). We next assessed POLα enrichment on chromatin using LCLs derived from affected individuals. Whilst chromatin extracts from family A and B tended towards slightly reduced POLα levels compared to WT LCLs, the reduction did not reach statistical significance (Fig S3A, B). In contrast, chromatin extracts from family C LCLs showed approx. 60% reduction in POLα

levels compared to WT LCLs (Fig S3C). Furthermore, chromatin recruitment of the additional POLα-primase subunit proteins p68-POLA2 and p48-PRIM1 appeared unaffected in proband LCL extracts from families A, B and C, suggesting the stoichiometry of the POLα-primase component subunits is largely preserved (Fig S3D).

Reduced cellular proliferation represents a logical pathomechanism underlying growth retardation and microcephaly in human disorders such as the prototypical microcephalic primordial dwarfism of Seckel syndrome (MIM: 210600), or Meier-Gorlin syndrome (MIM: 224690), which is caused by mutations in multiple components of the DNA replication licensing machinery [24-26]. *C.elegans div-1* (division delayed) allele encoding the B subunit of DNA polymerase α-primase delays cell division and lethally disrupts cell polarity in embryos [27] whilst *POL1* mutants of *S. cerevisiae* and a *PolA1* mutant (p.Ser1180Phe) of the mouse mammary carcinoma line FM3A are each associated with temperature-sensitive growth delay [11; 28]. Nonetheless, we did not observe a marked delay in proliferation of proband LCLs from families A, B and C compared to WT (Fig S4). Therefore, we carefully assessed different aspects of DNA replication capacity in family C LCLs specifically, as these showed a pronounced reduction in POLα-primase expression and chromatin localisation (Fig 2E, Fig S3C). POLα is characterised by limited processivity and it also lacks 3' exonucleolytic proofreading capacity. Therefore, in contrast to POLδ and POLε, POLα is unsuited to efficiently and accurately duplicate long DNA templates [29; 30]. Using DNA fibre combing analysis of ongoing unperturbed DNA replication in LCLs obtained from the proband of family C and his unaffected father, we observed similar rates of replication fork progression (Fig 3A). This was perhaps not entirely unanticipated since POLα-primase doesn't replicate the bulk of the genomic DNA and *POLA1* encodes a core product of a fundamentally essential cellular process, hence any viable defects in this gene would have to be hypomorphic. POLα is essential for viability [23; 27; 31]; indeed *POLA1* has a negative residual variation intolerance/genic intolerance score of -0.795, indicating it is under substantial purifying

selection [15; 32]. This is further illustrated by the absence of microdeletions involving *POLA1* in males, both in control and diseases CNV databases, as well as the identification of a female X-autosome translocation disrupting *POLA1*. In this female, in contrast to what normally happens, the wild-type X chromosome remained active in all her cells, probably as a result of selection against cells that contained the non-functional *POLA1* [33]. DNA fibre combing analysis did reveal a reduction in new initiation events in family C proband LCLs of 5.9% (n= 170 fibres) compared to 9.6% (n= 178 fibres) observed in paternal LCLs, indicative of impaired 'productive' replication initiation [34; 35]. Consistent with this, we also observed increased inter-origin distance (IOD) in family C proband LCLs compared to those of the father (Fig 3B). This would also be consistent with possible impairments in dormant origin firing [34; 35].Furthermore, we found an increase in asymmetric forks and an accumulation of longer replication tracts in family C proband LCLs compared to those of the father (Fig 3C, D). Collectively, analyses of multiple replication fork parameters in these *POLA1* deficient LCLs demonstrated several phenotypes consistent with spontaneously diminished productive replication initiation under unperturbed exponential growth conditions. These replication phenotypes are reminiscent of those recently reported for Polε impairment [36].

We next reasoned that additional impairments of DNA replication in *POLA1* LCLs could be context dependent; DNA replication under conditions of replication stress may represent that physiological context [37]. Disrupting the temporally coordinated balance between stem cell proliferation and differentiation programmes profoundly impacts upon brain and body growth [38; 39]. Rapidly proliferating murine embryonic stem cells exhibit constitutive replication stress and are highly dependent on replication-coupled pathways to preserve genome integrity and execute DNA replication efficiently and effectively [40]. Therefore, we reasoned that DNA replication in *POLA1*-mutated cells may be hypersensitive to replication stress conditions, particularly if dormant origin capacity was restricted due to a genetic defect of this nature [34; 35]. When we examined DNA fibres under conditions of replication stress by limiting deoxyribonucleotide availability via treatment with the ribonucleotide reductase inhibitor

hydroxyurea (HU), we observed an approximately 2-fold increase in stalled replication forks in combed fibres from family C probands' LCLs compared to paternal LCLs (Fig 4A). We next assessed DNA replication via pulse labelled EdU incorporation within LCL populations in a kinetic fashion following HU treatment (Fig 4B-D). Figure 4B shows representative EdU flow cytometry profiles from paternal and proband LCLs from family C, either untreated (Unt) or at different times following HU treatment. Family C probands' LCLs incorporated significantly less EdU upon HU-treatment compared to control LCLs (Fig 4B,C). This is a *POLA1*-dependent cellular phenotype as demonstrated by siRNA of *POLA1* in U2OS cells (Fig S5). Similarly, proband LCLs from family A and family B exhibited significantly reduced EdU incorporation indicative of impaired DNA replication following treatment with HU (Fig 4D). Collectively these results show that LCLs from affected individuals with distinct *POLA1* mutations exhibit reduced DNA replication under conditions of replication stress. A similar cellular response has been demonstrated for *ORC1*-mutated (MIM: 224690) and *MCM5*-mutated Meier-Gorlin syndrome (MIM: 617564) LCLs [41; 42]. Indeed pathogenic mutations in *MCM4* (MIM: 609981) and in the *GINS1* (MIM: 617827) component of the heterotetrametic Go-Ichi-Ni-San (GINS) complex, both encoding key components of the DNA replication apparatus, are each associated with cellular proliferation impairments, growth delay and natural killer (NK) cell deficiency [43-45].

In summary, we describe 9 affected individuals from 5 families presenting a syndrome involving a spectrum of developmental delay/intellectual disability, growth failure, microcephaly, hypogonadism and additional isolated abnormalities, associated with 5 different mutations in *POLA1* which encodes the catalytic subunit of the DNA Polymerase α-primase. The growth impairments were evident prenatally suggestive of their early origin *in utero*. LCLs from the proband of one such family spontaneously displayed altered replication fork parameters including reduced new initiation events, increased IOD and fork asymmetry and elongated replication tracts. All *POLA1*-mutant LCLs examined were additionally found to exhibit impaired DNA replication capacity under conditions of replication stress. These

data strongly suggest that cellular DNA replication deficits during development may underlie many of the clinical features observed in our families.

Interestingly, a recurrent intronic variant in *POLA1* has been shown to underlie XLPDR, a primary immunodeficiency associated with type I interferon-derived autoimflammatory features [15]. The elevated type I interferon signalling response underlying XLPDR has been shown to derive from a reduction in POLα-dependent synthesis of cytosolic RNA:DNA hybrid species [15]. Importantly, XLPDR cells with this specific intronic *POLA1* variant, do not exhibit a proliferative impairment and, except for the recurrent infections observed in proband E, no other phenotypic overlap with XLPDR was observed [15].

The remarkable fidelity of human DNA replication is a consequence of the combined and coordinate action of highly processive DNA polymerases, their intrinsic exonucleotytic proofreading activity and post-replicative DNA mismatch repair (MMR). Whilst POLα-primase initiates DNA replication and Okazaki fragment synthesis, it is not highly processive and does not possess an intrinsic proofreading activity. Processivity and proofreading are carried out by POLδ and POLε [29]. Germline mutations in components of the MMR pathway result in dramatically elevated spontaneous mutation frequencies and are associated with hereditary non-polyposis colorectal carcinoma (HNPCC)/Lynch syndrome (MIM: 120435) [46; 47]. Germline mutations within the exonucleolytic domain-encoding regions of *POLD1* (MIM: 174761) and *POLE* (MIM:174762), each encoding the catalytic subunit of the replicative polymerases POLδ and POLε respectively, have been identified as causing ultra-mutated colorectal ("polymerase proofreading-associated polyposis") and endometrial cancers (MIM: 612591 and 615083) [48-51]. Fascinatingly, differing mutations in *POLE* underlie a clinical spectrum including FILS syndrome (facial dysmorphism, immunodeficiency, livedo reticularis and short stature; MIM: 615083) [52] and IMAGe syndrome (intrauterine growth restriction, metaphyseal dysplasia, adrenal hypoplasia congenital and genitourinary anomalies in males; MIM:

614732), associated with variable immunodeficiency [36; 53]. Additionally, a *POLE2* (MIM: 602670) mutation has been identified in an individual with combined immunodeficiency, facial dysmorphism and autoimmunity associated with compromised lymphocyte proliferation [54]. Germline mutations in *POLD1* have been described as underlying a range of congenital disorders including MDP syndrome (mandibular hypoplasia, deafness and progeroid; MIM: 615381), lipodystrophy and atypical Werner's syndrome with short stature (MIM: 277700) [55-60]. Therefore, it appears that germline mutations in the core DNA replication polymerases can present as a wide range of phenotypes variably incorporating cancer predisposition, developmental/progeroid syndromes with or without growth failure, endocrine insufficiency and variable immunodeficiency. Our findings make an important additional contribution to this expanding knowledge base; namely, that hitherto undescribed hypomorphic *POLA1* mutations affecting the catalytic subunit of DNA POLα-primase are associated with multifaceted cellular DNA replicative deficits, and underlie an X-linked syndrome of intellectual disability, microcephaly, growth failure and hypogonadism.

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URLs

Online Mendelian Inheritance in Man, http://www.omim.org

Provean (protein variation effect analyser), http://provean.jcvi.org/index.php

PolyPhen, http://genetics.bwh.harvard.edu/pph/;

MutationTaster, http://mutationtaster.org/

Align GVGD, http://agvgd.hci.utah.edu/

ExAc, http://exac.broadinstitute.org/

Gnomad, http://gnomad.broadinstitute.org/

dbSNP, https://www.ncbi.nlm.nih.gov/snp

1000 Genomes, http://www.internationalgenome.org/

ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/

Genic intolerance, http://genic-intolerance.org/index.jsp

Legends to the figures

Table 1: Overview clinical features affected individuals

NA: not assessed

NP: not present

ASD: atrial septum defect

NIDDM: non-insulin dependent diabetes

ADHD: attention deficit hyperactivity disorder

VSD: ventral septal defect

TIQ: total intelligence quotient

Figure 1: Clinical pictures and pedigrees.

In clockwise order from top left: Index case of family B at age 19 years (**A**), individual III-5 (family C) at newborn age (**B**), proband III-2 (family C) aged 5 years demonstrating lack of subcutaneous fat, microcephaly and proportionate short stature (**C**,**D**). Lower panel: Picture

of index case of family D at age 6 months (**E**) and 3 years (**F**), picture of index case of family E at the age of 4 years (**G**). All individuals displayed proportionate short stature and microcephaly, as well as a pronounced nasal bridge and mild upslant of the palpebral fissures.

Below: Pedigrees of family A (H), family B (I) and family C (J). Carriers females show skewing of X-inactivation (next to symbol). Asterisks indicate the affected individuals that were tested and carry the respective mutation.

Figure 2: *Pola1* is expressed in proliferating progenitors during embryonic and postnatal neurogenesis in the mouse brain and *POLA1*-mutated cells exhibit variable POLα expression.

A. *Pola1* transcript (dark blue signal) is found in the proliferative zone of the embryonic neocortex. Scale bar: 200 µm. The dashed lines indicate an expanded area of the image.

B. Three weeks after birth, *Pola1* (dark blue signal) is expressed in the subventricular zone (SVZ) where postnatal neurogenesis occurs. Scale bar: 200 µm. The dashed lines indicate an expanded area of the image.

C. Increasing amounts of whole cell extract (WCE) from LCLs derived from a clinically unaffected, unrelated normal wild-type (WT) male individual and a *POLA1*-mutant individual from family A, were assessed for POLα expression levels. No difference in expression was observed.

D. Increasing amounts of WCE from LCLs derived from wild-type (WT) and a *POLA1*-mutant individual from family B were assessed for POLα expression levels. POLα expression was comparable.

E. Increasing amounts of WCE from LCLs derived from wild-type (WT) and a *POLA1*-mutant individual from family C were assessed for POLα expression levels. Here, POLα was markedly reduced compared to WT LCLs.

F. POLα levels were assessed using WCE derived from dermal fibroblast from WT, XLPDR-individual and the *POLA1*-mutant individual of family E. POLα was reduced in both instances of *POLA1* mutation.

Figure 3: *POLA1*-mutant LCLs spontaneously exhibit a range of DNA replication defects during unperturbed exponential growth.

A. Dual CldU (5-Chloro-2'-deoxyuridine) and IdU (5-Iodo-2'-deoxyuridine) labelled (20mins each as indicated) DNA fibre combing analysis of unperturbed exponentially growing LCLs from the unaffected father and family C proband (individual III.2) (CldU fibres: green. IdU fibres: red), demonstrated similar replication fork speeds between fathers' LCLs (mean: 0.95kb/min, median: 0.89 from n= 615 fibres) and family C proband (mean: 1.01kb/min, median: 1.01kb/min from n= 926 fibres).

B. Origins from family C proband's (individual III.2) LCLs exhibited significantly elevated inter-origin distance (IOD) compared to those of the father during unperturbed asynchronous growth conditions. Father's IOD median: 12.62μM (n=28) compared to family C proband IOD median: 20.75μM (n=20) (*p<0.05 Student's t test*). The schematic idealises the likely contrasting situation with regards to the bidirectional movement of fired origins (blue) in the father's LCLs compared to those of the proband. In the proband fewer new initiation events are seen (i.e. fewer new origins and reduced dormant origin firing capacity) and those that have fired are thus compelled to traverse greater distances.

C. The data shows and *XY* scatter plot of the length of fibres on the right and left hand-side of fired origins/ongoing forks visualised following DNA fibre combing of the fathers LCLs. Normally functional replicating forks exhibit left-right symmetry reflective of coordinate bidirectional movement. The dotted grey line indicates linear regression (R^2=0.4259 from n=18) showing a strong clustering of the forks consistent with symmetrical movement. The dotted blue lines are guide lines drawn to encapsulate the lengths of all of the forks assessed. Representative symmetrical fibres from the paternal LCLs are shown inset.

D. This *XY* scatter plot shows the length of fibres on the right and left hand-side of fired origins/ongoing forks derived from family C proband's LCLs. The wide dispersal of the data points with regards to the blue guide lines copied from paternal *XY* scatter plot shown in C indicates marked asymmetric movement of active replication forks. The dotted grey line denotes linear regression (R^2=0.0421 from n=33). Note also the preponderance of longer fork length (i.e. >15μM) observed in these fibres compared to those of the fathers shown in C. Representative fibres, demonstrating asymmetry, derived from family C proband's LCLs are shown inset.

Figure 4: *POLA1*-mutant proband LCLs exhibit DNA replication deficits following replication stress.

A. The level of replication fork stalling was investigated using a dual CldU and IdU labelling approach incorporating a hydroxyurea (HU) treatment. LCLs were first labelled with CldU (20mins), then treated with HU (2mM, 120mins), before a second label with IdU (60mins) to monitor fork recovery, as indicated. The middle panel shows a representative image of a labelled fibre demonstrating ongoing replication and a stalled replication fork. Under these conditions an approximately two-fold increase in the levels of stalled replication forks was observed in LCLs from family C proband, relative to paternal LCLs *(*p<0.05 Student's t test)*.

B. The impact of mildly stressing conditions (125μM HU) upon DNA replication was assessed via EdU-pulse incorporation (30mins) and flow cytometry using LCLs from family C. Representative flow cytometry panels are shown; the red boxed area denotes EdU positive cells. LCLs derived from the unaffected father and proband were either untreated (Unt) or treated with HU, and EdU incorporation was measured at the times indicated post-treatment. Consistent with the DNA fibre fork rate analysis shown in **Fig 3A.**, EdU incorporation in untreated LCLs was grossly comparable between those from the father and the proband. This was in contrast to the HU treated LCLs, where the proband showed markedly less EdU positive cells at each time point, when compared to LCLs from the father.

C. The bar chart shows EdU incorporation in untreated (Unt) and HU treated LCLs from the father, mother and proband of family C, from 4x independent experiments *(asterisks indicate p<0.05 Student's t test, compared to the equivalent parental time points).* The proband demonstrates significantly reduced EdU incorporation at each time point following HU, compared to the parental LCLs under these conditions.

D. The bar chart shows EdU incorporation in untreated (Unt) and HU treated LCLs from a clinically normal unrelated wild-type (WT) male individual and affected individuals from family A and family B, from 4x independent experiments *(asterisks indicate p<0.05 Student's t test, compared to the equivalent WT time point).* LCLs from all of the probands demonstrate significantly reduced EdU incorporation compared to WT following HU. This was most evident at 4hrs and 6hrs post-HU treatment.

Supplementary files.

Supplemental Note: Case Reports.

Figure S1: Molecular genetic investigations.

Figure S2: Conservation of missense variants and pathogenicity scores.

Figure S3: Chromatin localisation of POLα-primase subunits p180-POLα, p68-POLA2 and p4-PRIM1.

Figure S4: LCL proliferation analysis.

Figure S5: DNA replication following *POLA1* siRNA.

Material and Methods

Declaration of Interests

The authors declare no competing interests.

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	Family A				**Family B**	**Family C**		**Family D**	**Family E**
	Individual V-4	**IV-7**	**IV-1**	**IV-2**		**Individual III-2**	**III-5**		
Gender	M	M	M	M	M	M	M	M	M
Country	Belgium				Belgium	UK		Australia	USA
Gene	*POLA1*				*POLA1*	*POLA1*		*POLA1*	*POLA1*
Chr change (Hg19) NM_016937.3	c.236T>G				c.4142C>T	c.507+1G>A		c.445_507del	c.328G>A
Protein change	p.Ile79Ser				p.Pro1381Leu	p.(Lys149_Glu169del,Thr170_Ser1462delins15*)		p.Lys149_Glu169del	p.Gly110Arg
Mutation type	missense				missense	splice site		in frame deletion exon 6	splice site
Birth parameters									
Birth (weeks)	40 weeks	NA	NA	NA	39 weeks	38 weeks	38 weeks	38 weeks	29 weeks
Birth weight	1500 g	NA	NA	NA	2700 g	1786 g	1729 g	1688 g	840 g
Birth length	45 cm	NA	NA	NA	46,5 cm	NA	NA	44.5 cm	41 cm
Birth OFC	NA	NA	NA	NA	33 cm	30 cm	NA	28.5 cm	31,5 cm
Growth									
age	6 yrs	28 yrs	46 yrs	44 yrs	16 yrs	5 yrs	14 mo	6 yrs 11 mo	4 yrs 5 mo
Weight	10,5 kg (-7,9 SD)	NA	72 kg (+0,5 SD)	62 kg (-0,5 SD)	36,2 kg (-3,6 SD)	9,4kg (-7 SD)	5.7 kg (-5 SD)	14.6 kg (-4,5 SD)	13.3kg (-1.9 SD)
Height	98 cm (-4 SD)	150 cm (-4,1 SD)	158 cm (-2,9 SD)	160 cm (-2,6 SD)	137 cm (-5 SD)	95.2 cm (-3.5 SD)	59 cm (-7.7 SD)	110.4 cm (-2,6 SD)	95.8cm (-2 SD)
OFC	42,9 cm (-5,7 SD)	47,7 cm (-4,9 SD)	51,2 cm (-2,9 SD)	49,7 cm (-3,7 SD)	49,4 cm (-3,7 SD)	41 cm (-7.8 SD)	38 cm (-6.6 SD)	43 cm (-5,8 SD)	46cm (-3,1 SD)
Neurological									
Degree of DD/ID	mild (TIQ 71)	moderate (TIQ 57)	mild (TIQ 68)	moderate (TIQ 53)	severe	moderate	developmental delay	mild	mild (mainly speech delay)
Behavioural problems	ADHD	NP	NP	NP	hand stereotypies, autistic behaviour	difficult behaviour in association with frustration	NP	impulsive behaviour, short attention span	shyness, weak eye contact, short attention
Hypotonia	childhood hypotonia	childhood hypotonia	NA	NA	childhood hypotonia	NP	yes	NP	yes

Epilepsy	NP	NP	NP	NP	epilepsy from age 3 mo, therapy resistent	NP	NP	NP	NP
Brain abnormalities/MRI	normal MRI imaging	normal MRI imaging	NA	NA	cerebellar atrophy	slight thickening of pituitary stalk	cerebral atrophy; postoperative left thalamic bleed	normal MRI imaging	normal MRI imaging
Other					spasticity		vocal cord palsy		
Facial features									
Dysmorphic features	retrognathia	NP	NP	NP	large mouth, downturned corners of the mouth	short palpebral fissures, upslanting, prominent ears, brachymesophalangy	retrognathia, flat nasal bridge, small nose	upslanting palpebral fissures, long face	small ears, shallow orbits, upslanting palpebral fissures, 5th finger clinodactyly
Gastro-intestinal									
	NP	NP	NP	NP	gastrostomy	oesophageal atresia with tracheosophageal fistula (repaired day3)	NP	NP	tube feeding
Heart									
	normal	NA	NA	NA	normal	small ASD	pulmonary artery stenosis, VSD, pulmonary atresia	normal	normal
Urogenital/Endocrine									
Hypogonadotrophic hypogonadism	NA	yes	yes	yes	yes, small testes (10 ml)	NA	yes	NA	left testicular atrophy; small right testicle
Senses									
Vision	normal	normal	normal	normal	normal	normal	normal	normal	intermittent esotropia; astigmatism
Other				NIDDM	secondary scoliosis	sacral dimples	sacral dimples, bifid uvula		recurrent infections

Figure 1

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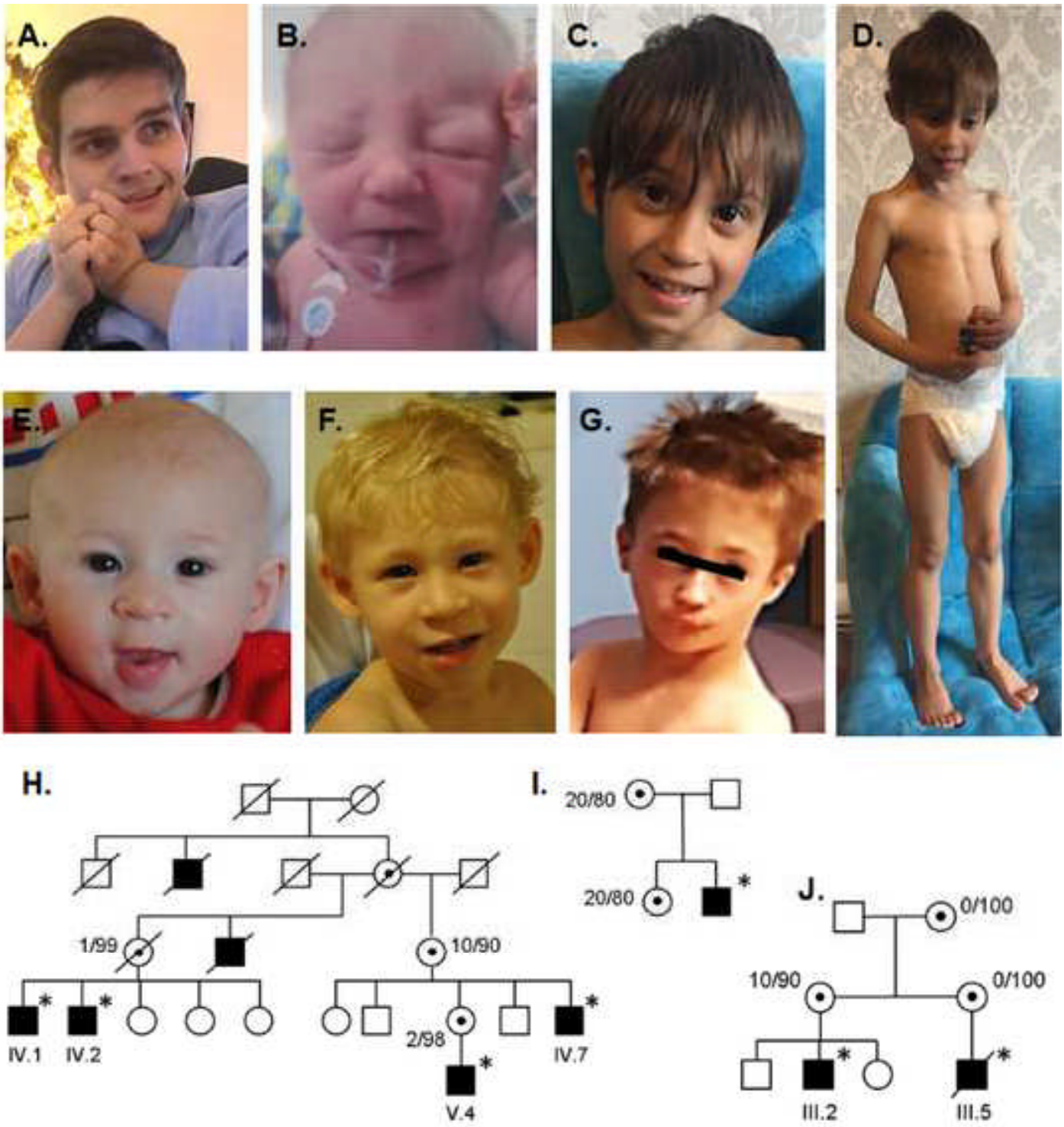

Figure 2

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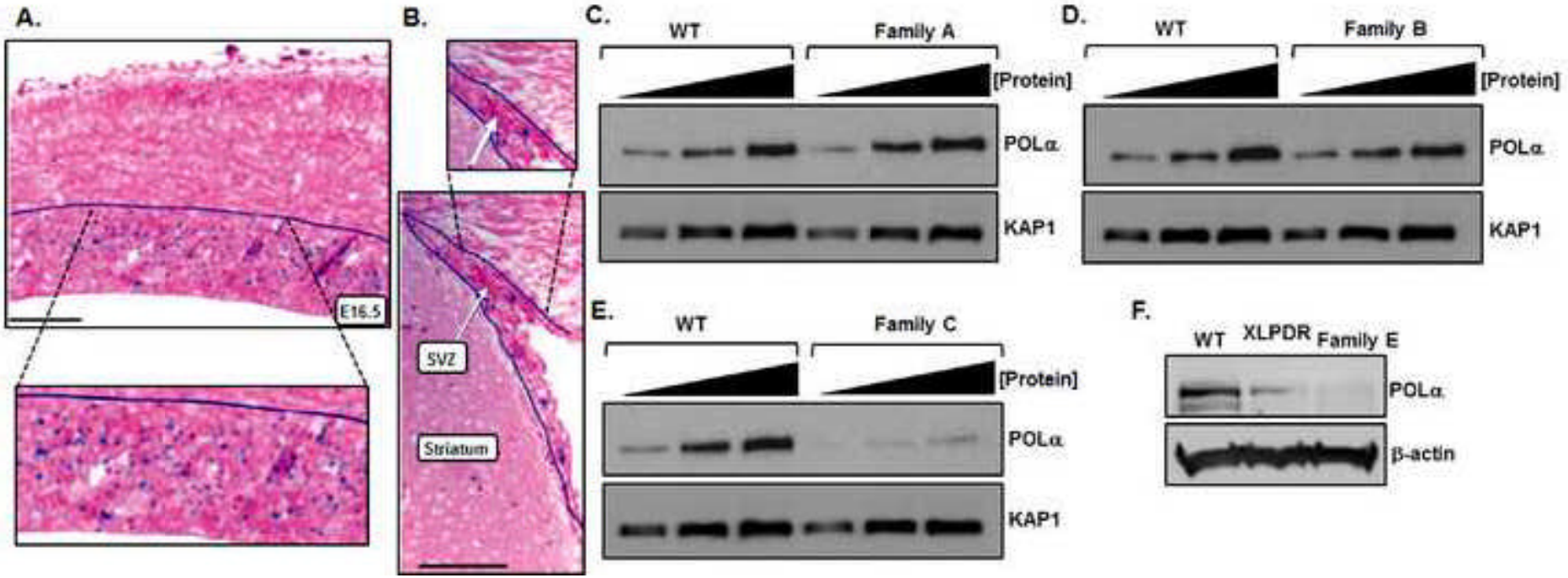

Figure 3

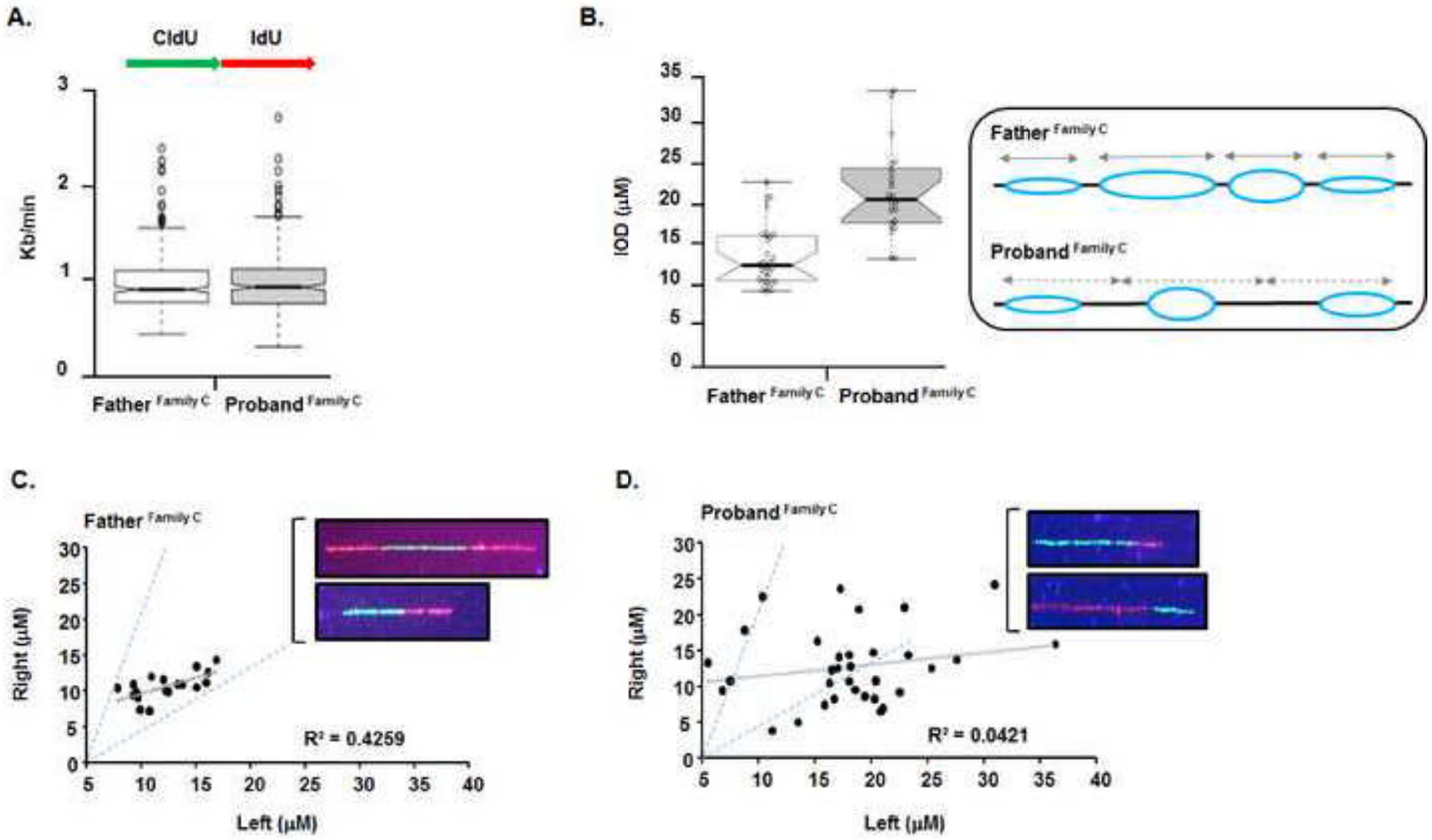

Figure 4

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