

# Defective DNA Polymerase $\alpha$ -Primase Leads to X-Linked Intellectual Disability Associated with Severe Growth Retardation, Microcephaly, and Hypogonadism

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Replicating the human genome efficiently and accurately is a daunting challenge involving the duplication of upward 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 polymerases  $\alpha$ ,  $\delta$ , and  $\epsilon$ . Collectively these multimeric polymerases ensure DNA replication proceeds at optimal rates approaching  $2 \times 10^3$  nucleotides/min with an error rate of less than one per million nucleotides polymerized. The majority of DNA replication of undamaged DNA is conducted by DNA polymerases  $\delta$  and  $\epsilon$ . The DNA polymerase  $\alpha$ -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 and involve a wide range of features, including variable combinations of growth delay, immunodeficiency, endocrine insufficiencies, lipodystrophy, and cancer predisposition. Here, by using various complementary approaches, including classical linkage analysis, targeted next-generation 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  $\alpha$ -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  $\alpha$ .

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,<sup>1</sup> mainly through the implementation of comparative genome hybridization and next-generation sequencing technologies.<sup>2,3</sup> Many of these genes converge into a few common functional networks because ID proteins often participate in interconnected cellular and molecular processes, including neurogenesis, neuronal migration, and synapse formation and function.<sup>4,5</sup> Here, we report that hypomorphic defects in the replicative DNA polymerase  $\alpha$  cause a human XLID syndrome. In five families, we identified mutations in *POLA1* (Xp22.1–p21.3, MIM: 312040), which encodes the p180 catalytic subunit of the heterotetrameric DNA polymerase  $\alpha$ -pri-

mase (POL $\alpha$ ). All affected individuals present with different degrees of intellectual disability and moderate to severe short stature, microcephaly, hypogonadism, and variable congenital malformations (Figure 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 Figure 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 toward a clear growth-deficiency syndrome of prenatal origin. Hypogonadism is also frequently evident. The index individual of family B also developed seizures and secondary

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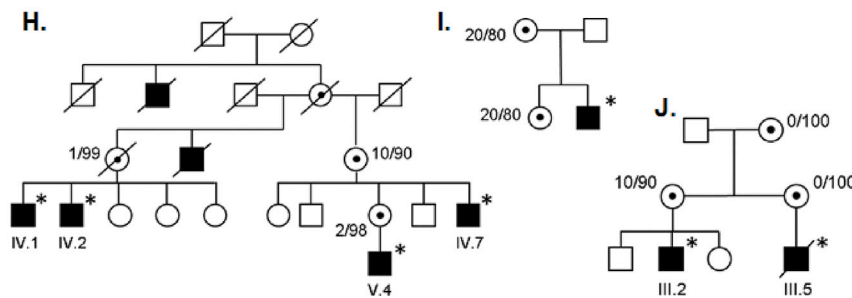


**Figure 1. Clinical Pictures and Pedigrees**

In clockwise order from top left: The index individual of family B at age 19 years (A); individual III-5 (family C) at newborn age (B); proband III-2 (family C) at age 5 years, demonstrating lack of subcutaneous fat, microcephaly, and proportionate short stature (C and D).

Lower panel: Pictures of the index individual of family D at age 6 months (E) and then at age 3 years (F); and a picture of the index individual 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 a mild upslant of the palpebral fissures.

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



neurological and orthopedic manifestations; these traits were not seen in the other individuals. In two affected individuals (in family C), a congenital heart malformation was present at birth. Although we cannot define a recognizable facial gestalt, mild upslant of the palpebral fissures is present in four affected individuals (Figures 1A–G).

Of the three mammalian replicative DNA polymerases (POL $\alpha$ , POL $\delta$ , and POL $\epsilon$ ), POL $\alpha$ -primase is the only polymerase that can initiate *de novo* DNA synthesis from licensed replication origins. It also mediates Okazaki-fragment synthesis during lagging-strand DNA replication.<sup>6–8</sup> POL $\alpha$  is also involved in other cellular processes such as DNA-damage-response signaling from stalled replication forks, telomere maintenance, and epigenetic regulation.<sup>9–14</sup> Interestingly, a recurrent, deep-intronic mutation in *POLA1* was recently found to cause X-linked reticulate pigmentary disorder (XLPDR; MIM: 301220), a primary immunodeficiency with autoinflammatory features, as well as skin hyperpigmentation and a prototypical facial gestalt.<sup>15,16</sup> This intronic mutation creates a novel exon 13a in the *POLA1* transcript, reducing the total amount of p180-POL $\alpha$  protein. XLPDR-affected individuals do not exhibit intellectual disability, altered body growth, or smaller head circumference, and this might indicate tissue-specific differences in abnormal splicing. Conversely, our affected individuals do not show any of

the XLPDR-related symptoms, except proband E, who suffered from recurrent infections.

We identified *POLA1* mutations either by using classical linkage analysis and then Sanger sequencing of all 17 genes present in the 6 cM interval (LOD score 2.6) (family A),<sup>17</sup> by a custom-designed microcephaly/microcephalic dwarfism Sure Select

capture panel consisting of 63 genes (family C), by single whole-exome sequencing (WES) (family B), or by trio WES (families D and E). In family A, a missense mutation in exon 3 of *POLA1* was identified, and this mutation, c.236T>G (p.Ile79Ser), segregates with the disease in all four affected individuals and obligate carrier mothers (Figure 1H). The sequence variant results in the replacement of an isoleucine, a non-polar amino acid, by a serine, a polar amino acid. This residue and its surrounding sequence are highly conserved, and p.Ile79Ser was predicted to be deleterious by various *in silico* methods (Figures S1 and S2). In family B, exome analysis identified a missense mutation, c.4142C>T, leading to a p.Pro1381Leu mutation. This mutation affects a conserved residue and is also present in the unaffected mother, maternal grandmother, and sister (Figures 1I, S1, and 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 (Figure 1J). The c.507+1G>A splice-site variant was predicted by five different splicing prediction programs to completely abolish the donor splice site. We performed RNA studies that showed that c.507+1G>A prevents normal splicing and leads to the production of two abnormally-spliced transcripts (Figure S1). The larger c.507+1G>A transcript results from the activation of a

**Table 1. Overview of the Clinical Features of Affected Individuals**

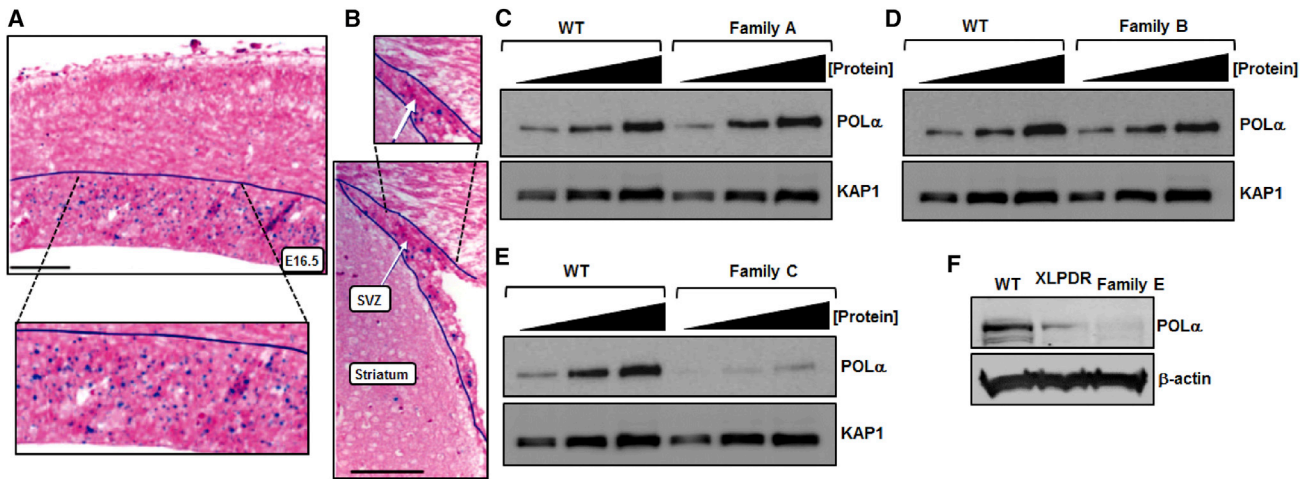
	Family A				Family B	Family C		Family D	Family E
	Individual V-4	Individual IV-7	Individual IV-1	Individual IV-2		Individual III-2	Individual III-5		
Gender	m	m	m	m	m	m	m	m	m
Country	Belgium				Belgium	UK		Australia	USA
Gene	POLA1				POLA1	POLA1		POLA1	POLA1
Chromosome change (Hg19), GenBank: NM_016937.3	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_Ser1462 delins15*		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 (g)	1,500 g	NA	NA	NA	2,700 g	1,786 g	1,729 g	1,688 g	840 g
Birth length (cm)	45 cm	NA	NA	NA	46.5 cm	NA	NA	44.5 cm	41 cm
Birth OFC (cm)	NA	NA	NA	NA	33 cm	30 cm	NA	28.5 cm	31.5 cm
Growth									
Age	6 years	28 years	46 years	44 years	16 years	5 years	14 months	6 years, 11 months	4 years, 5 months
Weight (kg)	10.5 kg (−7.9 SD)	NA	72 kg (+0.5 SD)	62 kg (−0.5 SD)	36.2 kg (−3.6 SD)	9.4 kg (−7 SD)	5.7 kg (−5 SD)	14.6 kg (−4.5 SD)	13.3kg (−1.9 SD)
Height (cm)	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 (cm)	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)	46 cm (−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)
Behavioral problems	ADHD	NP	NP	NP	hand stereotypies, autistic behavior	difficult behavior in association with frustration	NP	impulsive behavior, short attention span	shyness, weak eye contact, short attention
Hypotonia	childhood hypotonia	childhood hypotonia	NA	NA	childhood hypotonia	NP	yes	NP	yes

(Continued on next page)

**Table 1. Continued**

	<b>Family A</b>				<b>Family B</b>	<b>Family C</b>		<b>Family D</b>	<b>Family E</b>
	<b>Individual V-4</b>	<b>Individual IV-7</b>	<b>Individual IV-1</b>	<b>Individual IV-2</b>		<b>Individual III-2</b>	<b>Individual III-5</b>		
Epilepsy	NP	NP	NP	NP	epilepsy from age 3 months, therapy resistant	NP	NP	NP	NP
Brain abnormalities and/or 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	–	–
<b>Facial Features</b>									
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, fifth finger clinodactyly
<b>Gastro-Intestinal</b>									
	NP	NP	NP	NP	gastrostomy	esophageal atresia with tracheoesophageal fistula (repaired postnatal day 3)	NP	NP	tube feeding
<b>Heart</b>									
	normal	NA	NA	NA	normal	small ASD	pulmonary artery stenosis, VSD, pulmonary atresia	normal	normal
<b>Urogenital and Endocrine</b>									
Hypogonadotropic hypogonadism	NA	yes	yes	yes	yes, small testes (10 ml)	NA	yes	NA	left testicular atrophy, small right testicle
<b>Senses</b>									
Vision	normal	normal	normal	normal	normal	normal	normal	normal	intermittent esotropia, astigmatism
Other	–	–	–	NIDDM	secondary scoliosis	sacral dimples	sacral dimples, bifid uvula	–	recurrent infections

Abbreviations are as follows: NA = not assessed; NP = not present; OFC = occipital frontal circumference; ASD = atrial septum defect; NIDDM = non-insulin-dependent diabetes; ADHD = attention deficit hyperactivity disorder; VSD = ventral septal defect; and TIQ = total intelligence quotient.



**Figure 2. *Polα* Is Expressed in Proliferating Progenitors During Embryonic and Postnatal Neurogenesis in the Mouse Brain, and *POLA1*-Mutated Cells Exhibit Variable *POLα* Expression**

(A) The *Polα* transcript (dark blue signal) is found in the proliferative zone of the embryonic neocortex. The scale bar represents 200  $\mu$ m. The dashed lines indicate an expanded area of the image.  
 (B) Three weeks after birth, *Polα* (dark blue signal) is expressed in the subventricular zone (SVZ), where postnatal neurogenesis occurs. The scale bar represents 200  $\mu$ m. 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-affected 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 the WT and a *POLA1*-mutant-affected individual from family B were assessed for *POLα* expression levels. *POLα* expression was comparable.  
 (E) Increasing amounts of WCE from LCLs derived from the WT and a *POLA1*-mutant-affected individual from family C were assessed for *POLα* expression levels. Here, *POLα* was markedly reduced in affected cells compared to in WT LCLs.  
 (F) *POLα* levels were assessed via WCE derived from dermal fibroblasts from the WT, an XLPDR-affected individual, and the *POLA1*-mutant-affected individual from family E. *POLα* was reduced in both instances of *POLA1* mutation.

cryptic splice donor site within intron 6, leading to an insertion of the first 60 nucleotides of intron 6. This is predicted to cause an 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 and leads to an in-frame deletion, i.e., p.Lys149\_Glu169del, that is predicted to produce a protein product lacking 21 amino acids (Figure S1). In family D, a hemizygous deletion of exon 6 was identified via exome sequencing, and this deletion led 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 individual (Figure S1). Exome sequencing in family E identified a *de novo* variant, c.328G>A, affecting the last nucleotide of exon 4 and leading to a p.Gly110Arg mutation. 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 in affected cells compared to cells derived from unaffected males; the reduction was even more profound than that observed in XLPDR-derived fibroblasts<sup>15</sup> (Figure S1). None of the above identified variants are present in the dbSNP, 1000 Genomes, ExAC, or gnomAD databases, and we have submitted them to ClinVar (see Accession Numbers). All missense mutations affect conserved amino

acids and are predicted to be deleterious by various *in silico* methods (Figure S2). In addition, in the three familial cases (families A–C), all obligate female carriers show significant to complete skewing of X inactivation (Figures 1H–J).

DNA polymerases are highly expressed during development, when rapid DNA replication and cell division is required.<sup>18,19</sup> To further investigate *POLα*/*POLA1* in mammalian brain development, we assessed *Polα* expression by *in situ* hybridization in the embryonic and adult mouse brain. In the mouse forebrain, *Polα* is expressed in those zones containing proliferating cells in the developing embryonic neocortex (Figure 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 (Figure 2B). These data suggest that *Polα* has a role in neurogenesis throughout life. Additionally, *polα* expression by *in situ* hybridization in developing zebrafish embryos shows early and intense staining in the developing brain,<sup>20,21</sup> whereas *Polα* activity appears highest in isolated neurons from developing rat-brain cerebral cortex when the mitotic activity is at its peak.<sup>22</sup> Conversely, an insertional mutation in zebrafish *polα1* (*polα1*<sup>hi1146Tg</sup>) led to central nervous system (CNS) necrosis, a small head and eyes, an inflated hindbrain ventricle, a thin and often curved body, and a rounder yolk with no extension at day 2.<sup>23</sup> At days 3–5, the necrosis spread throughout the body, resulting in overt body wasting and a small head and eyes.<sup>23</sup>

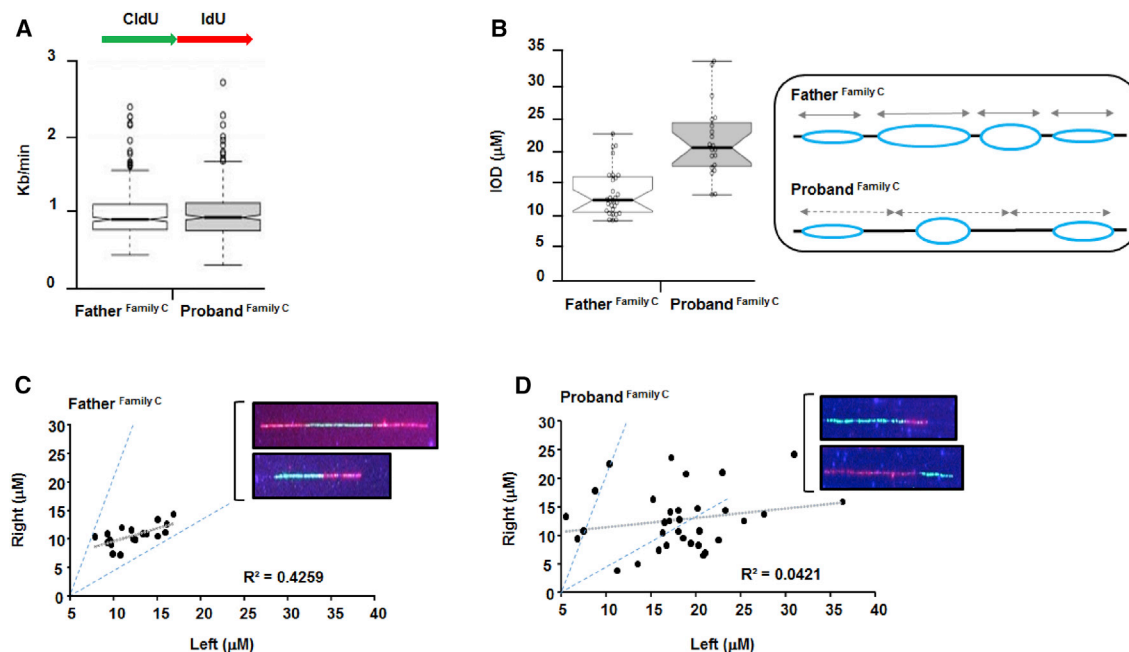


We examined POL $\alpha$  protein levels in proband-derived cell lines from families A, B, C, and E (Figures 2C–F). Cell lines from the family D proband were unavailable. By using whole-cell extracts (WCEs) from lymphoblastoid cell lines (LCLs) derived from affected individuals from families A and B, we found POL $\alpha$  protein levels comparable to those of wild-type (WT) LCLs (Figures 2C and 2D). In contrast, WCEs derived from the family C proband's LCLs or from dermal, primary fibroblasts from the family E proband both showed marked reduction in POL $\alpha$  levels (Figures 2E and 2F). These findings are consistent with the RT-PCR analysis from each of these two families (Figure S1). We next assessed POL $\alpha$  enrichment on chromatin by using LCLs derived from affected individuals. Although chromatin extracts from families A and B tended toward slightly reduced POL $\alpha$  levels compared to those of WT LCLs, the reduction did not reach statistical significance (Figures S3A and S3B). In contrast, chromatin extracts from family C's LCLs showed an approximately 60% reduction in POL $\alpha$  levels compared to those of WT LCLs (Figure S3C). Furthermore, chromatin recruitment of the additional POL $\alpha$ -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 POL $\alpha$ -primase component subunits is largely preserved (Figure S3D).

Reduced cellular proliferation represents a logical pathomechanism underlying growth retardation and microcephaly in human disorders such as Seckel syndrome (MIM: 210600), which presents with prototypical microcephalic primordial dwarfism, or Meier-Gorlin syndrome (MIM: 224690), in which the dwarfism is caused by mutations in multiple components of the DNA replication-licensing machinery.<sup>24–26</sup> The *C.elegans* *div-1* (division delayed) allele encoding the B subunit of DNA polymerase  $\alpha$ -primase delays cell division and lethally disrupts cell polarity in embryos,<sup>27</sup> whereas *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.<sup>11,28</sup> Nonetheless, we did not observe a marked delay in proliferation of proband LCLs from families A, B, and C compared to in the WT (Figure S4). Therefore, we carefully assessed different aspects of DNA-replication capacity in family C's LCLs specifically, as these showed a pronounced reduction in POL $\alpha$ -primase expression and chromatin localization (Figures 2E and S3C). POL $\alpha$  is characterized by limited processivity, and it also lacks 3' exonucleolytic proofreading capacity. Therefore, in contrast to POL $\delta$  and POL $\epsilon$ , POL $\alpha$  is unsuited to efficiently and accurately duplicate long DNA templates.<sup>29,30</sup> By using DNA-fiber-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 (Figure 3A). This was perhaps not entirely unanticipated because POL $\alpha$ -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. POL $\alpha$  is essential for viability,<sup>23,27,31</sup> indeed, *POLA1* has a negative residual-variation intolerance score of  $-0.795$ , indicating it is under substantial purifying selection.<sup>15,32</sup> This is further illustrated by the absence of microdeletions involving *POLA1* in males, both in the control and diseases copy number variation (CNV) databases, as well as by the identification of a female with 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*.<sup>33</sup> DNA-fiber-combing analysis did reveal a reduction in new initiation events in the family C proband's LCLs of 5.9% ( $n = 170$  fibers), compared to 9.6% ( $n = 178$  fibers) observed in the paternal LCLs, a difference indicative of impaired “productive” replication initiation.<sup>34,35</sup> Consistent with this, we also observed increased inter-origin distance (IOD) in the family C proband's LCLs compared to those of the father (Figure 3B). This would also be consistent with possible impairments in dormant origin firing.<sup>34,35</sup> Furthermore, we found an increase in asymmetric forks and an accumulation of longer replication tracts in the family C proband's LCLs compared to those of the father (Figures 3C and 3D). 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 Pole impairment.<sup>36</sup>

We next reasoned that additional impairments of DNA replication in *POLA1* LCLs could be context dependent, and DNA replication under conditions of replication stress may represent that physiological context.<sup>37</sup> Disrupting the temporally coordinated balance between stem-cell proliferation and differentiation programs profoundly impacts upon brain and body growth.<sup>38,39</sup> 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.<sup>40</sup> 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.<sup>34,35</sup> We examined DNA fibers under conditions of replication stress by limiting deoxyribonucleotide availability via treatment with the ribonucleotide reductase inhibitor hydroxyurea (HU), and we observed an approximately 2-fold increase in stalled replication forks in combed fibers from the family C proband's LCLs compared to paternal LCLs (Figure 4A). We next assessed DNA replication via pulse-labeled EdU incorporation within LCL populations in a kinetic fashion after HU treatment (Figures 4B–D). Figure 4B shows representative EdU flow-cytometry profiles from family C paternal

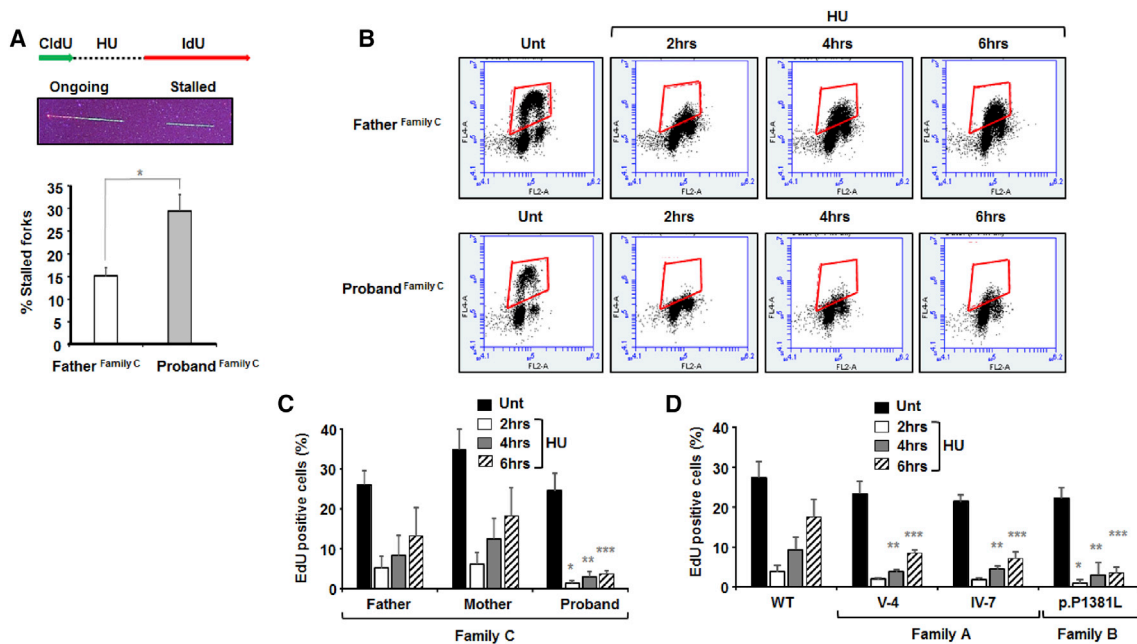


**Figure 3. *POLA1*-Mutant LCLs Spontaneously Exhibit a Range of DNA-Replication Defects During Unperturbed, Exponential Growth** (A) Dual CldU (5-Chloro-2'-deoxyuridine)- and IdU (5-Iodo-2'-deoxyuridine)-labeled (for 20 min each, as indicated) DNA-fiber-combing analyses of unperturbed, exponentially growing LCLs from the unaffected father and family C proband (individual III-2) demonstrated similar replication-fork speeds between the fathers' LCLs (mean: 0.95kb/min, median: 0.89, from  $n = 615$  fibers) and the family C proband's (mean: 1.01kb/min, median: 1.01kb/min, from  $n = 926$  fibers). CldU fibers are shown in green and IdU fibers are shown in red. (B) Origins from the family C proband's (individual III-2) LCLs exhibited significantly elevated inter-origin distance (IOD) compared to that in those of the father during unperturbed, asynchronous growth conditions. The father's IOD median was 12.62  $\mu\text{M}$  ( $n = 28$ ), compared to the family C proband's IOD median of 20.75  $\mu\text{M}$  ( $n = 20$ ) ( $p < 0.05$ ; Student's  $t$  test). The schematic idealizes the most likely contrasting situation with regard to the bidirectional movement of fired origins (blue) in the father's LCLs compared to that in 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) In the XY scatterplot, the data show the length of fibers on the right- and left-hand-sides of fired origins and/or ongoing forks, visualized after the father's LCLs were subjected to DNA fiber combing. Normally, functional replicating forks exhibit left-right symmetry reflective of coordinated bidirectional movement. The dotted gray line indicates linear regression ( $R^2 = 0.4259$  from  $n = 18$ ) showing a strong clustering, which is consistent with symmetrical movement, of the forks. The dotted blue lines are guide lines drawn to encapsulate the lengths of all of the forks assessed. Representative symmetrical fibers from the paternal LCLs are shown inset. (D) This XY scatterplot shows the length of fibers on the right- and left-hand-sides of fired origins and/or ongoing forks derived from the family C proband's LCLs. The wide dispersal of the data points with regard to the blue guide lines (copied from the paternal XY scatterplot shown in [C]) indicates marked asymmetric movement of active replication forks. The dotted gray line denotes linear regression ( $R^2 = 0.0421$  from  $n = 33$ ). Note also the preponderance of longer fork lengths (i.e.,  $>15 \mu\text{M}$ ) observed in these fibers compared to the lengths of those of the father's fibers (shown in [C]). Representative fibers that are derived from the family C proband's LCLs and that demonstrate asymmetry are shown inset.

and proband LCLs, either untreated (Unt) or at different times after HU treatment. The family C proband's LCLs incorporated significantly less EdU upon HU-treatment compared to control LCLs (Figures 4B and 4C). This is a *POLA1*-dependent cellular phenotype, as demonstrated by siRNA of *POLA1* in U2OS cells (Figure S5). Similarly, after treatment with HU, proband LCLs from family A and family B exhibited significantly reduced EdU incorporation, which is indicative of impaired DNA replication (Figure 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.<sup>41,42</sup> 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.<sup>43–45</sup>

In summary, we describe nine affected individuals, from five families, who present with a syndrome involving a spectrum of developmental delay/intellectual disability, growth failure, microcephaly, hypogonadism, and additional, isolated abnormalities; this syndrome is associated with five different mutations in *POLA1*, which encodes the catalytic subunit of the DNA polymerase  $\alpha$ -primase. The growth impairments were evident prenatally, suggesting an early origin *in utero*. LCLs from the proband of one affected family spontaneously displayed altered replication-fork parameters, including reduced new-initiation events, increased IOD and fork asymmetry, and elongated replication tracts. All the *POLA1*-mutant LCLs we



**Figure 4. POLA1-Mutant Proband LCLs Exhibit DNA-Replication Deficits After Experiencing Replication Stress**

(A) The level of replication-fork stalling was investigated via a dual CldU- and IdU-labeling approach that incorporated a hydroxyurea (HU) treatment. LCLs were first labeled with CldU (for 20 min) and then treated with HU (2 mM, for 120 mins) before a second labeling with IdU (for 60 mins) to monitor fork recovery, as indicated. The middle panel shows a representative image of a labeled fiber demonstrating ongoing replication and a stalled replication fork. Under these conditions, an approximately 2-fold increase in the levels of stalled replication forks was observed in LCLs from the family C proband, relative to the levels in the paternal LCLs (\* $p < 0.05$ , Student's  $t$  test; error bars = standard deviation).

(B) The impact of mildly stressing conditions (125  $\mu$ M HU) upon DNA replication was assessed in LCLs from family C via EdU-pulse incorporation (for 30 min) and flow cytometry. Representative flow cytometry panels are shown; the area boxed in red denotes EdU-positive cells. LCLs derived from the unaffected father and the proband were either untreated (Unt) or treated with HU, and EdU incorporation was measured at the times indicated post-treatment. Consistent with the DNA-fiber fork-rate analysis shown in Figure 3A, EdU incorporation in untreated LCLs was grossly comparable between the father and the proband. This was in contrast to the HU-treated LCLs, where the proband showed markedly fewer EdU-positive cells at each time point compared to 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 4 $\times$  independent experiments (asterisks indicate  $p < 0.05$  [Student's  $t$  test], compared to the equivalent parental time points; error bars = standard deviation). The proband LCLs demonstrate significantly reduced EdU incorporation at each time point after HU treatment, 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 4 $\times$  independent experiments (asterisks indicate  $p < 0.05$  [Student's  $t$  test], compared to the equivalent WT time point; error bars = standard deviation). LCLs from all of the probands demonstrate significantly reduced EdU incorporation compared to WT LCLs after HU treatment. This was most evident at 4 and 6 hr post-HU treatment.

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 these families.

Interestingly, a recurrent intronic variant in *POLA1* has been shown to underlie XLPDR, a primary immunodeficiency associated with type I-interferon-derived autoinflammatory features.<sup>15</sup> The elevated type I-interferon-signaling response underlying XLPDR has been shown to derive from a reduction in  $\text{POL}\alpha$ -dependent synthesis of cytosolic RNA:DNA hybrid species.<sup>15</sup> 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, we observed no other phenotypic overlap with XLPDR.<sup>15</sup>

The remarkable fidelity of human DNA replication is a consequence of the combined and coordinated action of

highly processive DNA polymerases, their intrinsic exonucleolytic proofreading activity, and post-replicative DNA mismatch repair (MMR). Although  $\text{POL}\alpha$ -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  $\text{POL}\delta$  and  $\text{POLE}$ .<sup>29</sup> 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) or Lynch syndrome (MIM: 120435).<sup>46,47</sup> 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  $\text{POL}\delta$  and  $\text{POLE}$ , respectively, have been identified as causing ultra-mutated colorectal ("polymerase proofreading-associated polyposis") and endometrial cancers (MIM: 612591 and 615083).<sup>48–51</sup>



Fascinatingly, differing mutations in *POLE* underlie a clinical spectrum that includes FILS syndrome (facial dysmorphism, immunodeficiency, livedo reticularis, and short stature; MIM: 615083)<sup>52</sup> and IMAGe syndrome (intrauterine growth restriction, metaphyseal dysplasia, adrenal hypoplasia, and congenital and genitourinary anomalies in males; MIM: 614732), and both diseases are associated with variable immunodeficiency.<sup>36,53</sup> Additionally, a *POLE2* (MIM: 602670) mutation has been identified in an individual with combined immunodeficiency, facial dysmorphism, and autoimmunity associated with compromised lymphocyte proliferation.<sup>54</sup> Germline mutations in *POLD1* have been described to underlie 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).<sup>55–60</sup> Therefore, it appears that germline mutations in the core DNA-replication polymerases can present as a wide range of phenotypes and variably incorporate cancer predisposition, developmental and/or 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 POL $\alpha$ -primase are associated with multifaceted cellular DNA-replicative deficits, and they underlie an X-linked syndrome of intellectual disability, microcephaly, growth failure, and hypogonadism.

### Accession Numbers

ClinVar accession numbers for the variants reported in this paper were not available from ClinVar as of the date this article was finalized for press; please contact the corresponding authors for the numbers.

### Supplemental Data

Supplemental Data can be found online at <https://doi.org/10.1016/j.ajhg.2019.03.006>.

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### Declaration of Interests

The authors declare no competing interests.

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### Web Resources

1000 Genomes, <http://www.internationalgenome.org/>  
Align-GVGD, <http://agvgd.hci.utah.edu/>  
ClinVar, <https://www.ncbi.nlm.nih.gov/clinvar/>  
dbSNP, <https://www.ncbi.nlm.nih.gov/snp>  
ExAC, <http://exac.broadinstitute.org/>  
Genic Intolerance, <http://genic-intolerance.org/index.jsp>  
gnomAD, <https://gnomad.broadinstitute.org/>  
MutationTaster, <http://mutationtaster.org/>  
Online Mendelian Inheritance in Man, <http://www.omim.org>  
Provean (Protein Variation Effect Analyzer), <http://provean.jcvi.org/index.php>  
PolyPhen, <http://genetics.bwh.harvard.edu/pph/>

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