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DNA Polymerase Epsilon Deficiency Causes IMAGe Syndrome with Variable Immunodeficiency

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During genome replication, polymerase epsilon (Pol ϵ) acts as the major leading-strand DNA polymerase. Here we report the identification of biallelic mutations in *POLE*, encoding the Pol ϵ catalytic subunit POLE1, in 15 individuals from 12 families. Phenotypically, these individuals had clinical features closely resembling IMAGe syndrome (intrauterine growth restriction [IUGR], metaphyseal dysplasia, adrenal hypoplasia congenita, and genitourinary anomalies in males), a disorder previously associated with gain-of-function mutations in *CDKN1C*. POLE1-deficient individuals also exhibited distinctive facial features and variable immune dysfunction with evidence of lymphocyte deficiency. All subjects shared the same intronic variant (c.1686+32C>G) as part of a common haplotype, in combination with different loss-of-function variants in *trans*. The intronic variant alters splicing, and together the biallelic mutations lead to cellular deficiency of Pol ϵ and delayed S-phase progression. In summary, we establish *POLE* as a second gene in which mutations cause IMAGe syndrome. These findings add to a growing list of disorders due to mutations in DNA replication genes that manifest growth restriction alongside adrenal dysfunction and/or immunodeficiency, consolidating these as replisome phenotypes and highlighting a need for future studies to understand the tissue-specific developmental roles of the encoded proteins.

DNA replication is a fundamental cellular process necessary to ensure the faithful transmission of genetic information. In eukaryotes, three highly conserved DNA polymerases, polymerase epsilon, delta, and alpha, act in concert at the replication fork. Polymerase epsilon (Pol ϵ) is the major enzyme responsible for the synthesis of the leading strand¹ and is consequently an essential gene.² *POLE* encodes the catalytic subunit of Pol ϵ (POLE1), and somatic and germline missense mutations affecting the proofreading domain of POLE1 have been associated with colon and endometrial cancer.^{3–6}

Microcephalic primordial dwarfism comprises a group of prenatal-onset extreme growth disorders characterized by intrauterine growth retardation, short stature, and microcephaly. Genes involved in cell cycle progression, including multiple components of the replication licensing machinery, have been identified as monogenic causes of this disorder.^{7–11} As the molecular basis for many affected individuals remains to be determined, we performed whole-genome sequencing studies to identify further genes and facilitate more comprehensive diagnosis.

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Table 1. Biallelic *POLE* Mutations (GenBank: NM_006231.3)

ID	Fam	Sex	Allele 1			Allele 2			Mat Allele	Pat Allele	Country of Origin
			Nucleotide Change	Amino Acid Consequence	MAF	Nucleotide Change	Amino Acid Consequence	MAF			
P1	1	M	c.2091dupC	p.Phe699Valfs*11	0	c.1686+32C>G	p.Asn563Valfs*16	0.000071	1	2	UK
P2	1	F	c.2091dupC	p.Phe699Valfs*11	0	c.1686+32C>G	p.Asn563Valfs*16	0.000071	1	2	UK
P3	2	M	c.62+1G>A	Essential Splice Site Intron 1	0	c.1686+32C>G	p.Asn563Valfs*16	0.000071	2	1	Ireland
P4	3	F	c.5940G>A	p.Trp1980*	0.000016	c.1686+32C>G	p.Asn563Valfs*16	0.000071	2	1	Australia
P5	4	M	c.4728+1G>T	Essential Splice Site Intron 36	0	c.1686+32C>G	p.Asn563Valfs*16	0.000071	2	1	USA
P6	5	F	c.3264_3275+13del	Essential Splice Site Intron 26	0.000016	c.1686+32C>G	p.Asn563Valfs*16	0.000071	1	2	Canada
P7	6	M	c.1A>T	p.?	0.000081	c.1686+32C>G	p.Asn563Valfs*16	0.000071	n/a	n/a	USA
P8	7	M	c.1A>T	p.?	0.000081	c.1686+32C>G	p.Asn563Valfs*16	0.000071	2	1	Ireland
P9	7	F	c.1A>T	p.?	0.000081	c.1686+32C>G	p.Asn563Valfs*16	0.000071	2	1	Ireland
P10	8	F	c.3019G>C	p.Ala1007Pro	0.000009	c.1686+32C>G	p.Asn563Valfs*16	0.000071	1	2	Ireland
P11	9	F	c.5265delG	Ile1756Serfs*5	0	c.1686+32C>G	p.Asn563Valfs*16	0.000071	2	1	Australia
P12	9	M	c.5265delG	Ile1756Serfs*5	0	c.1686+32C>G	p.Asn563Valfs*16	0.000071	2	1	Australia
P13	10	F	c.2049C>G	p.Tyr683*	0.000028	c.1686+32C>G	p.Asn563Valfs*16	0.000071	1	2	Australia
P14	11	M	c.6518_6519delCT	p.Ser2173Phefs*130	0.000089	c.1686+32C>G	p.Asn563Valfs*16	0.000071	2	1	USA
P15	12	M	c.801+2T>C	Essential Splice Site Intron 8	–	c.1686+32C>G	p.Asn563Valfs*16	0.000071	1	2	USA

Abbreviations: ID, individual number; Fam, family number; Mat, maternal; Pat, paternal; n/a, not available. All subjects harbored a loss-of-function mutation in combination with an intronic variant on the alternate allele identified as part of a shared haplotype and found to alter splicing in RNA studies. MAF indicates minor allele frequency in European (non-Finnish) population observed in gnomAD. None of the variants were present in any Non-European population in gnomAD.

Whole-genome sequencing (WGS) of 48 individuals with microcephalic primordial dwarfism identified heterozygous *POLE* (GenBank: NM_006231.3) loss-of-function (LoF) variants in three subjects (P1, P3, P4; Table 1). These LoF variants were significantly enriched in our cohort compared to a control WGS dataset (GnomAD,¹² $p = 5.1 \times 10^{-5}$, Fisher's exact test, Table S1). As these variants were present in the unaffected parents, the WGS data were further evaluated and a second rare intronic variant in *POLE* identified, c.1686+32C>G (dbSNP: rs762985435). This was present in *trans* with the LoF mutation in all three probands (Table 1). Targeted sequencing of *POLE* and interrogation of existing whole-exome sequencing (WES) data in additional cases of primordial dwarfism identified five additional subjects compound heterozygous for LoF alleles and the c.1686+32C>G variant (P5–P9, Table 1). Notably, a clinical diagnosis of IMAGE syndrome (GeneReviews in Web Resources) (MIM: 614732) had been considered in individuals P1 and P3, with adrenal failure also reported in P5, P6, and P7. We therefore investigated cases of IMAGE syndrome drawn from other cohorts without an existing molecular diagnosis (i.e., *CDKN1C* mutation negative). These included three previously published IMAGE-affected case subjects.^{13,14} Analysis of their WGS data identified additional *POLE* LoF variants inherited in *trans* with the in-

tronic variant in individuals P11–P15 (Table 1). The c.1686+32C>G variant was part of a common haplotype in all individuals where WES/WGS performed, extending over 921 kbp (Figure S2, chr12:132341818–133263107, GRCh38). In P10 a missense variant (c.3019G>C) encoding a p.Ala1007Pro substitution was found, at a residue conserved to yeast (Figure S1) within the polymerase domain of the protein (Figure 1). All variants identified were sufficiently rare (MAF < 0.0001¹²) and, where DNA available, segregation in families was consistent with an autosomal recessively inherited disorder (Table 1).

Phenotypically, affected individuals had severe growth failure of prenatal onset (Figure 2, Table S2). IUGR was present in all case subjects (birth weight was -3.0 ± 0.8 SD) with significant short stature evident postnatally (height -8.1 ± 2.4 SD). While head circumference was also significantly reduced (OFC -5.4 ± 1.5 SD), this was less severe, resulting in a relative macrocephaly. Those affected had a common facial appearance with micrognathia, crowded dentition, long thin nose, short wide neck, and small, low-set, posteriorly rotated ears (Figure 2). 12 individuals had adrenal insufficiency and all affected males had genitourinary abnormalities including bilateral cryptorchidism and/or hypospadias, with the majority of case subjects fulfilling clinical criteria for IMAGE syndrome (GeneReviews in Web Resources; Table 2, Table S3, Supplemental Note).

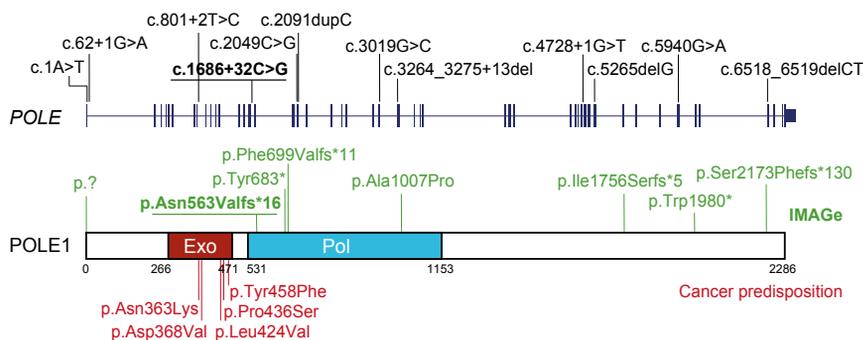


Figure 1. Mutations Causing *POLE*-Associated IMAGe Syndrome Are Distinct from Mutations Conferring a Non-syndromic Susceptibility to Cancer

Schematic of the *POLE* gene, which encodes POLE1, the catalytic subunit of DNA polymerase epsilon. Domains: Pol, polymerase; Exo, exonuclease. Mutations identified in *POLE* subjects indicated above gene and protein (green). Recurrent intronic mutation underlined. For comparison, heterozygous germline missense mutations located in the exonuclease domain predisposing to colorectal cancer and other malignancies highlighted below (red).

Osteopenia and developmental dysplasia of the hip (DDH) were frequently observed and café-au-lait patches were notably present in a third of individuals.

A single homozygous intronic variant (c.4444+3A>G) in *POLE* has previously been reported to be associated with immunodeficiency, lymphopenia, and short stature (facial dysmorphism, immunodeficiency, livedo, and short stature, aka FILS syndrome [MIM: 615139]).^{15,16} Five affected individuals identified in this study also had increased susceptibility to respiratory tract infections, with lymphocyte subset deficiencies and/or IgM hypogammaglobulinemia identified in P1, P3, P4, P8, P9, P14, and P15 (Table 2, Table S4). Deficiency of natural killer cells was present in P1, P3, and P8. P1 had the most profound immunodeficiency, developing CMV pneumonitis and then subsequently developed EBV haemophagocytic lymphohistiocytosis, requiring an allogeneic bone marrow transplant. Notably, this subject's sister (P2), who had the same compound heterozygous *POLE* mutations, died at 22 months from HSV infection. Therefore, our findings

establish that the phenotype spectrum of biallelic *POLE* mutations extends from IMAGe syndrome to include immunodeficiency, in line with the phenotype and pathogenicity of the previously reported c.4444+3A>G mutation.^{15,16}

To establish whether the c.1686+32C>T variant affected the *POLE* transcript, RNA studies were performed on primary fibroblast lines derived from two subjects (P1, P3). RT-PCR using primers spanning *POLE* intron 15 demonstrated the presence of a larger PCR product (Figure 3), which capillary sequencing established to be due to retention of part of intron 15 within *POLE* transcripts (Figure S3). A minigene assay was then performed to assess splicing of this genomic segment and to directly confirm the contribution of the c.1686+32C>G variant. This demonstrated that the c.1686+32C>G variant markedly impaired splicing of the usual exon 15 splice donor site, leading to preferential use of a downstream alternate splice donor site in intron 15, although some canonical splicing also occurred (Figure 3). The inclusion of 47 bp of intronic

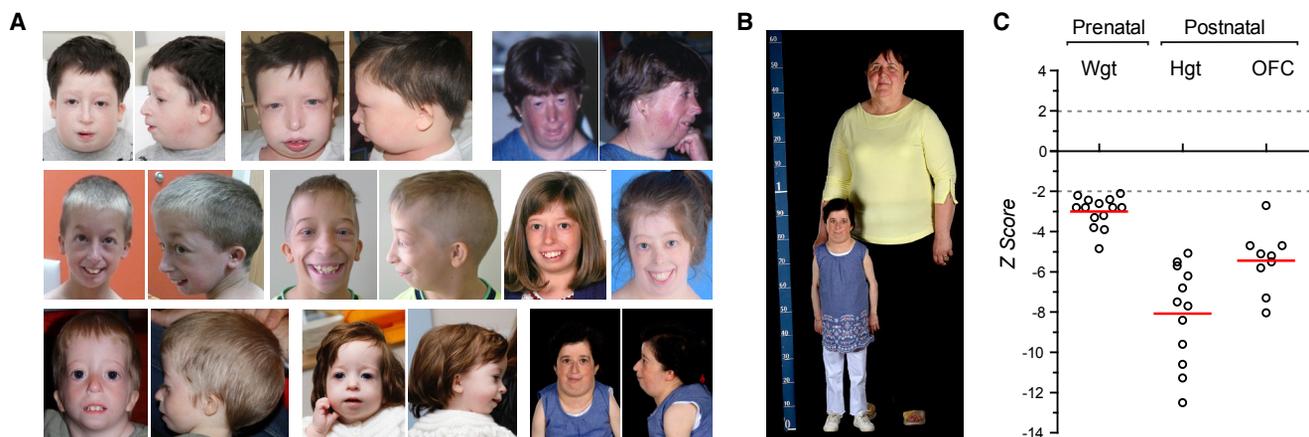


Figure 2. Individuals with Biallelic *POLE* Mutations Have Severely Impaired Pre- and Post-natal Growth and a Recognizable Facial Gestalt

(A) Photographs of *POLE*-deficient subjects demonstrating facial similarities. Written consent obtained from all families for photography.

(B and C) Severe pre-natal onset growth restriction occurs in *POLE*-deficient individuals.

(B) Adult *POLE*-deficient subject next to a control individual of average stature.

(C) Growth is severely impaired pre- and postnatally. Z-scores (standard deviations from population mean for age and sex) for birth weight and postnatal height and head circumference (OFC). Dashed lines 95% confidence interval for general population. Circles, individual subject data points; red bars, mean values.

Table 2. Individuals with Biallelic Mutations in *POLE* Were Clinically Diagnosed with Primordial Dwarfism and Features of IMAGE Syndrome

ID	Fam	Sex	Age	I	M _{+SI}	A	Ge	-I	Other Features
P1	1	M	18	Y	Y	Y	Y	Y	scoliosis, osteopenia, small patella, seizures, gastrostomy, eczema
P2	1	F	1	Y	Y	Y	-	Y	-
P3	2	M	7	Y	Y	Y	Y	Y	midline accessory incisor, osteopenia, infant eczema
P4	3	F	50	Y	Y	N	-	Y	IgM paraproteinaemia
P5	4	M	12	Y	NA	Y	Y	Y	hypopituitarism, T cell lymphoma, gastrostomy, absent patella
P6	5	F	10	Y	Y	Y	-	Y	bilat coxa valga, 11 ribs, 6 lumbar vertebrae, scoliosis, gastrostomy, infant eczema
P7	6	M	13	Y	Y	Y	Y	N	hypopituitarism, atrial septal defect, brachydactyly, gastrostomy
P8	7	M	3	Y	Y	N	Y	Y	DDH, gastrostomy
P9	7	F	2	Y	Y	N	-	Y	DDH, gastrostomy
P10	8	F	39	Y	Y	Y	-	N	DDH, 11 ribs, clinodactyly, osteopenia, café au lait patches
P11	9	F	0.2	Y	NA	Y	-	Y	café au lait patch
P12	9	F	12	Y	Y	Y	-	N	-
P13	10	M	22	Y	Y	Y	Y	N	DDH, café au lait patch
P14	11	F	18	Y	Y	Y	-	Y	gastrostomy, hypercalcaemia in infancy, café au lait patches, DDH, kyphoscoliosis
P15	12	M	31	Y	NA	Y	Y	Y	café au lait patches, seizures, osteopenia, osteoporosis, nodular sclerosis, Hodgkin's lymphoma

Abbreviations: ID, individual number; Fam, family number; I, intrauterine growth restriction; M_{+SI}, skeletal involvement: metaphyseal dysplasia or other skeletal abnormalities reported in CDKN1C IMAGE-affected individuals (NA, not assessed); A, adrenal insufficiency; Ge, genitourinary abnormalities in males (- female, genitourinary anomalies not applicable); -I, immunodeficiency, either increased susceptibility to infections or documented lymphopenia/hypogammaglobinemia; DDH, developmental dysplasia of the hip; Y, yes; N, no. See Tables S1–S4 for extended clinical data and morphometrics.

DNA in the variant transcript results in a frameshift, which would lead to premature termination (p.Asn563Valfs*16). While this transcript might be targeted for nonsense-mediated decay, any translated protein would also be non-functional given that this frameshift occurs at the start of the polymerase catalytic domain. Combined with a LoF mutation on the second allele, substantial reduction in POLE1 was therefore anticipated. Subsequent immunoblotting of total protein extracts from primary fibroblasts from affected subjects confirmed that POLE1 levels were indeed markedly depleted (Figure 3; 5% ± 3% for P1 and 11% ± 4% P3, relative to the mean of both control subjects and normalized to vinculin loading control; mean ± SD for n = 2 independent experiments), with chromatin fractionation experiments demonstrating reduction of POLE1 in both soluble and chromatin-bound fractions (Figure S4). Taken together with the consistent clinical phenotype across case subjects, we concluded that the identified *POLE* variants were pathogenic, resulting in a phenotype spectrum substantially overlapping IMAGE syndrome.

In keeping with an essential requirement for *POLE* in eukaryotes,² the “leaky” c.1686+32C>G splice mutation

permitted residual expression of functional POLE1 in all case subjects. This mutation in *trans* with truncating mutations would then be expected to lead to marked but partial loss of function. As *POLE* encodes POLE1, the catalytic subunit of the major leading-strand DNA polymerase Pol ε, reduced chromatin levels of POLE1 would therefore be expected to impact on the availability of Pol ε DNA polymerase activity during its canonical function in DNA replication. Consistent with this, time-course FACS analysis demonstrated delayed cell-cycle progression of BrdU-labeled primary fibroblasts from P1 and P3, indicative of impaired S-phase progression (Figure 3). While no viable model of POLE1 deficiency exists, a *Pole4*^{-/-} mouse has been generated, which is similarly deficient for the Pol ε holoenzyme.¹⁷ This mouse also has significant prenatal onset growth failure, reduced brain size, and markedly reduced lymphocyte levels. Analysis of embryonic fibroblasts derived from this mouse alongside *POLE* primary human fibroblasts (derived from P1 and P3 in this study) established that in both cases Pol ε deficiency leads to reduced levels of chromatin-loaded Pol ε complexes, resulting in replication stress arising from reduced numbers of active replication origins.¹⁷

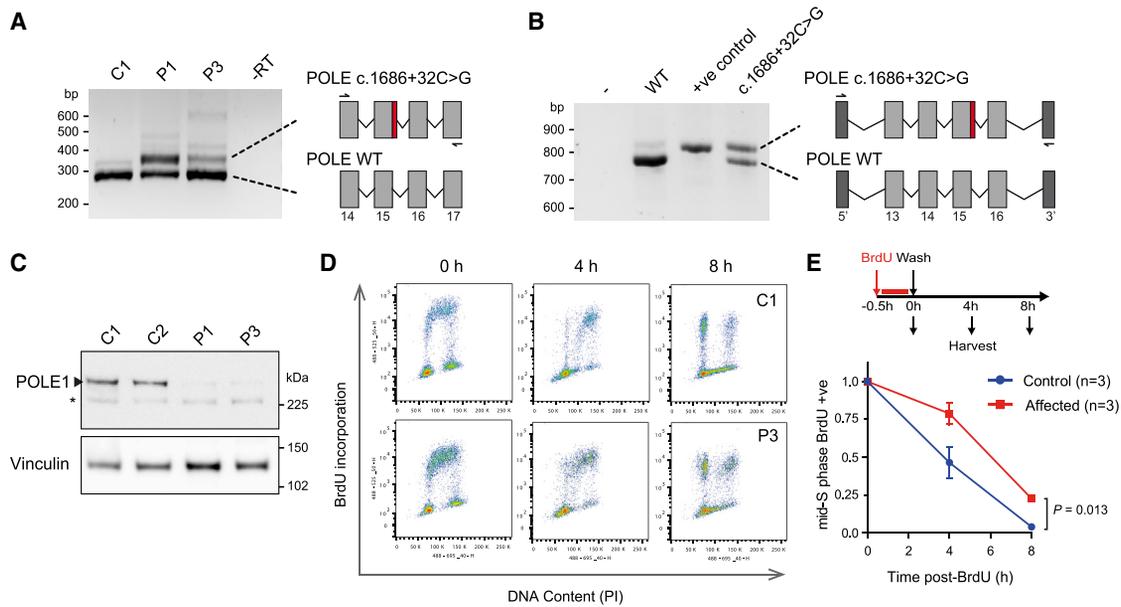


Figure 3. Common Intronic Variant Identified Causes Aberrant Splicing and *POLE*-Deficient Cells Show Deficiency of Polymerase Epsilon and Slowed S-phase Progression

(A) The c.1686+32C>G mutation causes aberrant splicing of intron 15 in subject cells. RT-PCR of *POLE* transcripts from primary fibroblasts. Primers indicated by arrows in schematic. P1, P3, *POLE*-deficient subjects; C1, C2, control subjects.
 (B) Minigene assay demonstrating that aberrant splicing is a direct consequence of the c.1686+32C>G mutation. +ve control, point mutation in splice donor site, c.1686+1G>A. 5' & 3' indicate artificial vector-associated exons.
 (C) *POLE1* levels are markedly reduced in subject fibroblasts. Immunoblot of total cell extracts. *POLE1* antibody raised against AA1-176. Vinculin, loading control. * non-specific band.
 (D and E) Fibroblast cells from affected individuals exhibit delayed S phase progression. Schematic, experimental set-up.
 (D) Representative FACS plots.
 (E) Quantification of n = 3 affected and n = 3 control cell lines from representative experiment (of n = 3 expts with n ≥ 2 biological replicates per group). Mid-S-phase mean (±SEM) BrdU-labeled cells, normalized to t = 0 time point are plotted for each group. p value, two-way ANOVA.

IMAge syndrome has previously been found to be caused by dominant gain-of-function mutations in the imprinted gene, *CDKN1C*.^{18,19} Here, we establish mutations of *POLE* as an autosomal-recessive cause of the IMAge phenotype. These mutations contrast with heterozygous germline and somatic cancer-predisposing mutations that affect the exonuclease domain of *POLE1*^{3–6} (Figure 1). IMAge and cancer mutations are likely to have differing functional outcomes, respectively leading to deficient DNA replication or to impaired proof-reading.²⁰ Hence, a similar cancer predisposition in *POLE1*-deficient individuals or *POLE* heterozygous carriers cannot be assumed. However, P5 developed a T cell lymphoma at age 11 and P15 developed Hodgkin's lymphoma at age 28. Given also the increased lymphoma rates in *Pole4*^{-/-} mice,¹⁷ *POLE1* deficiency may therefore confer an increased risk of lymphoma.

All *CDKN1C* IMAge mutations cluster within its proliferating cell nuclear antigen (PCNA) binding domain,^{18,19} targeting the PCNA binding PIP-box motif.²¹ As PCNA loads with Pol ε at replication initiation (Figures 4 and S5), the phenotypic overlap with *POLE*-associated IMAge syndrome suggests a mechanistic link. Supporting this notion, biochemical studies of a *Xenopus* homolog suggests that CDKN ubiquitination and subsequent degradation

is mediated by PCNA/polymerase loading^{23,24} (Figure S5). Furthermore, single homozygous mutations in *MCM4*^{11,25} (MIM: 609981) and *POLE2*²⁶ have been associated with IUGR and short stature, alongside immunodeficiency, respectively with and without adrenal failure. Likewise, several families with *GINS1* biallelic mutations have been reported to be associated with pre/postnatal growth restriction, chronic neutropenia, and NK cell deficiency (MIM: 610608).¹⁰ Hence, the identification of a cohort of individuals with *POLE* mutations that encompasses all these features consolidates this as a group of replisome-associated disorders (Figure 4, Table S5). Replication stress and p53-mediated cell death¹⁷ likely explain the immunodeficiency as well as global growth failure in *POLE1*-deficient individuals. However, why impaired replisome function should have a particularly strong impact on specific lymphoid lineages (T/B cells in *POLE1/2*-deficient subjects and NK cells in *MCM4/GINS1*-deficient individuals) or on adrenal cortical cells is unclear. Notably, another distinct form of primordial dwarfism, Meier-Gorlin syndrome (defined by the triad of short stature, patella hypoplasia, and microtia [MIM: 224690]) is also caused by biallelic (or *de novo*) mutations in genes involved in replication licensing and initiation^{7–9,27,28} (Figure 4). Further studies to understand the specific role(s) of the encoded

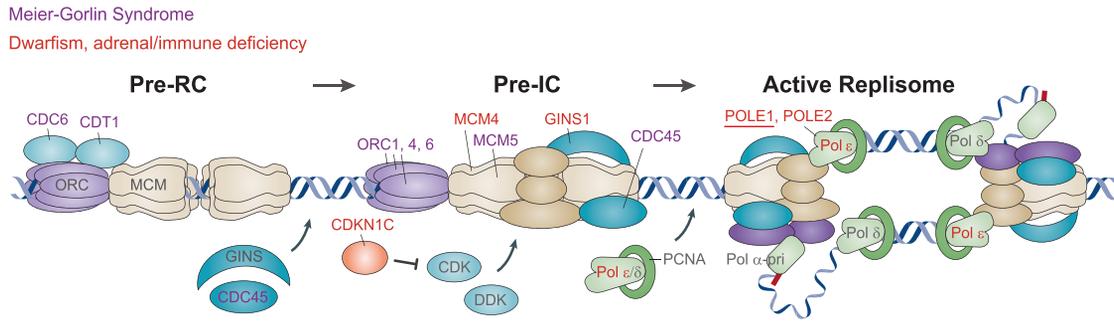


Figure 4. POLE1 Deficiency Links CDKN1C-IMAGE Syndrome¹⁸ with Other Replisome-Associated Disorders

Schematic of replication initiation (adapted by permission from Gaillard et al.²² copyright 2015 Macmillan Publishers), highlighting the sequential action of replisome-associated proteins, mutation of which causes MGS (blue text) and those that are associated with dwarfism with adrenal insufficiency and/or immune deficiency, including IMAGE syndrome (red text). During replication licensing, MCM helicases (MCM2-7) are loaded at replication origins by the ORC complex (ORC1-6) with CDC6 and CDT1 to form the pre-replicative complex (pre-RC). Subsequently, loading of additional replisome protein occurs, regulated by DDK and CDK kinases, to form the pre-initiation complex (pre-IC), that contains the CMG (CDC45, MCMs, GINS) complex. CDKN1C inhibits CDK activity. In the active replisome, Primase-Pol α initiates DNA synthesis with strands extended by the PCNA-associated DNA polymerases δ and ϵ . POLE1 and POLE2 are part of the Pol ϵ holoenzyme.

replication proteins during development, along with the cellular and biochemical basis for the relationship between CDKN1C and Pol ϵ , will therefore be of interest.

Supplemental Data

Supplemental Data include Supplemental Note, seven figures, five tables, and Supplemental Material and Methods and can be found with this article online at <https://doi.org/10.1016/j.ajhg.2018.10.024>.

Consortia

Members of the Scottish Genome Partnership include Timothy J. Aitman, Andrew V. Biankin, Susanna L. Cooke, Wendy Inglis Humphrey, Sancha Martin, Lynne Mennie, Alison Meynert, Zosia Miedzybrodzka, Fiona Murphy, Craig Nourse, Javier Santoyo-Lopez, Colin A. Semple, and Nicola Williams.

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Declaration of Interests

The Department of Molecular and Human Genetics at Baylor College of Medicine receives revenue from the genetic testing services offered by Baylor Genetics.

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Web Resources

dbSNP, <https://www.ncbi.nlm.nih.gov/projects/SNP/>
 GenBank, <https://www.ncbi.nlm.nih.gov/genbank/>
 GeneReviews, Bennett, J., Schrier Vergano, S.A., and Dearthoff, M.A. (1993). IMAGE syndrome, <https://www.ncbi.nlm.nih.gov/books/NBK190103/>
 gnomAD Browser, <http://gnomad.broadinstitute.org/>
 OMIM, <http://www.omim.org/>

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