Human development and tissue homeostasis depend on the regulated control of cellular proliferation and differentiation. DNA replication is essential to couple genome duplication and cell division with the establishment and maintenance of cellular differentiation programs. In eukaryotes, DNA replication is performed by a large machine known as the ‘replisome,’ which is strictly regulated in a cell cycle-dependent manner. Inherited mutations of replisome components have been identified in a range of genetic conditions characterised by developmental abnormalities and reduced organismal growth in addition to an involvement of the immune and endocrine systems and/or heightened tumour predisposition. Here, we review the current knowledge of the molecular genetics of replisome dysfunction disorders and discuss recent mechanistic insights into their pathogenesis, with a focus on the specific steps of DNA replication affected in these human diseases.

DNA Replication and Human Disease

Efficient and accurate DNA replication is essential for the maintenance of genome stability and the accomplishment of developmental and differentiation programs in mammals. In vivo and in vitro studies in budding yeast and other lower eukaryotes have provided a clear understanding of the key steps involved in the control and execution of DNA replication [1]. Although redundancy has emerged during the evolution of higher eukaryotes, the basic mechanisms and players that perform DNA replication appear to be remarkably conserved. Over the last two decades, many of the basic components of the mammalian DNA replication machinery have been identified and functionally characterised. Proteomic studies have also identified several mammalian-specific factors stably or transiently associated with the replisome (see Glossary), particularly in the presence of replication stress, with a prominent role in maintaining genome stability and human health [2,3].

Even though the vast majority of replisome factors are required for viability of mammalian cells, hypomorphic mutations of replisome components have been increasingly identified in human genetic disease. Indeed, the application of whole-genome sequencing has led to a significant expansion in the identification of mutations in components of the replication machinery and factors required to maintain replication fork stability. While leading to a deeper understanding of the molecular genetics of these conditions, this has promoted new opportunities to investigate the pathological consequences of dysfunctional DNA replication in human biology and disease.

The Mechanism of DNA Replication in Mammalian Cells

Duplication of genomic DNA is performed by a large multiprotein assembly, known as the ‘replisome,’ whose activation and activity are strictly regulated during the cell cycle by a multistep process (Figure 1 and Box 1). The first step of DNA replication takes place during the G1 phase of the cell cycle, when DNA replication origins are ‘licensed’ by the sequential assembly of origin recognition complex 1–6 (ORC1–6) together with cell division cycle 6 (CDC6) and Cdc10-
Glossary

**Adrenal insufficiency**: reduced function of the adrenal gland that leads to impaired secretion of cortisol and/or aldosterone. It can be caused by genetic, immunologic, or infiltrative/haemorrhagic insults.

**Consanguineous**: genetically descendent from the same ancestor as another person.

**DNA–protein crosslinks (DPCs)**: covalent linkage of proteins with a DNA strand. DPCs are one of the most deleterious forms of DNA damage because they constitute an allosteric block to transcription and replication. They can be induced endogenously (commonly through reactions with aldehydes or trapping of enzymatic intermediates onto the DNA) or through environmental carcinogens and chemotherapeutic agents.

**DNA replication origins**: specific genetic sequences where DNA replication is initiated.

**Fork reversal**: conversion of the classic three-way replication fork into a four-way junction (also known as ‘chicken foot structure’) due to reannealing of unwound parental duplex and annealing of the ones with strands.

**Hypomorphic allele**: Mutant allele that causes a partial loss of gene function. It is generally caused by reduced expression (at the mRNA and/or protein levels) or functional activity of the codified mutant protein.

**Hypoplasia**: congenital condition associated with underdevelopment of a specific tissue or organ. It is caused by a reduced and/or inadequate number of cells.

**Interstrand crosslinks (ICLs)**: DNA lesions generated by the covalent linkage between the Watson and Crick DNA strands. ICLs are highly toxic because they prevent strand separation, thus blocking DNA replication and transcription.

**Intra-S-phase checkpoint**: mechanism that controls genomic replication to be performed accurately and effectively during the S phase of the cell cycle.

**Male hypogonadism**: condition that results from the failure to produce physiological concentrations of testosterone, normal amounts of sperm, or both. It may arise from testicular disease (primary hypogonadism) or dysfunction of the hypothalamic–

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**Figure 1. Licensing and Activation of a Human DNA Replication Origin.** Cartoon depicting the three main steps required for initiation of DNA replication at a replication origin. Formation of the pre-replication complex (PRE-RC): The origin recognition complex 1–6 (ORC1–6) initially recruits cell division cycle 6 (CDC6) at replication origins to form an AAA+ ring-shaped complex that encircles DNA and promotes the subsequent recruitment of mini-chromosome maintenance 2–7 (MCM2–7)/Cdc10-dependent transcript 1 (CTT1) and loading of MCM2–7 double hexamers at a replication origin.

(Figure legend continued at the bottom of the next page.)
As a helpful assistant, I can provide you with the text content of the document in a plain format. The content seems to be discussing the regulation of DNA replication origins in eukaryotic cells, specifically focusing on the role of CDK-dependent phosphorylation and the GEMININ protein. The text also mentions the importance of maintaining genome integrity and the potential implications of defects in these processes.

For a more detailed understanding, I would recommend reviewing the original source material to gain a comprehensive understanding of the context and implications of the discussed mechanisms.
In addition to this, MDM two binding protein (MTBP; the ortholog of *S. cerevisiae* Sld7) interacts with TICRR/TRESLIN to promote CDC45 binding to MCMs and CMG assembly [11]. All together, these proteins form the so-called pre-initiation complex (PRE-IC) [1] (Figure 1).

PRE-IC engagement with MCM10 triggers double-stranded DNA (dsDNA) melting and unwinding by the CMG. Replication protein A (RPA) is recruited to the resulting single-stranded DNA (ssDNA), and two separate replisomes are established, which translocate along ssDNA in a 3′–5′ direction [12,13] (Figure 1). After POLΔ-dependent synthesis of short RNA-DNA primers, leading and lagging strands are extended by the conserved polymerases POLε and POLδ [14] (Figure 2). *In vitro* reconstitution of DNA replication, as well as genetic and proteomic evidence *in vivo* have established that, in unchallenged conditions, POLε synthesises the majority of leading strand whereas POLδ is responsible for lagging strand extension [14]. Importantly, while POLδ is indirectly tethered to the replication fork, POLα appears to be physically coupled to the CMG by the AND-1/CTF4 trimer, a ‘hub’ that links multiple CTF4-interacting peptide (CIP) box–containing proteins to the CMG helicase [15] (Figure 2).

Other essential components of the replication machinery are the replication factors C1–5 (RFC1–5) and chromosome transmission fidelity 18 (CTF18)–RFC2–5 complexes (or clamp loaders), which promote loading of the processive polymerase factor proliferating cell nuclear antigen (PCNA), a trimeric scaffold that encircles ssDNA–dsDNA junctions and promotes efficient synthesis on lagging and leading strands [14] (Figure 2). Upon extension of lagging-strand synthesis by POLδ, 5′ flap structures are generated that are processed by flap structure–specific endonuclease 1 (FEN1) or DNA replication helicase/nuclease 2 (DNA2; long flaps) before ligase 1–mediated ligation of end products [14] (Figure 2). In addition to this, CLASPIN (Mrc1 in *S. cerevisiae*) and the TIPIN/TIMELESS heterodimer (homologues of Csm3/Tof1) engage with the replisome to promote efficient DNA replication in unperturbed and perturbed conditions [1] (Figure 2). Finally, termination of DNA replication occurs stochastically in the genome upon replication fork convergence, which triggers ubiquitylation-dependent CMG disassembly and ligation of end products (Box 2).
Assembly of the CMG helicase and initiation of DNA replication have been studied extensively. However, less is known about the mechanisms that regulate convergence of replication forks and CMG disassembly at termination and the consequences of their deregulation [1]. The CMG helicase cannot be reloaded during S phase and appears to be remarkably stable even in conditions of replication stress [1]. Thus, its disassembly upon fork convergence must be regulated in a specific manner. Initial insights into this process came from the discovery of a dedicated ubiquitylation-dependent mechanism requiring the SCF\textsuperscript{Ddb1} ubiquitin ligase in budding yeast and Cdc48/p97 AAA+-ATPase in S. cerevisiae and Xenopus laevis, driving the disassembly of CMG helicase through ubiquitylation of its MCM7 subunit [116,117]. Subsequent studies identified CUL\textsuperscript{2LR} as the higher eukaryotic mediator of CMG ubiquitylation in conjunction with the CDC-48 cofactors UFD-1 and NPL-4, which are required for its disassembly [118–120]. Importantly, CMG unloading at termination sites occurs after the formation of fully ligated DNA products during in vitro replication in X. laevis egg extracts, which suggested that a specific conformational change must occur to promote MCM7 ubiquitylation and p97-dependent unfolding and disassembly of the CMG [121]. Consistent with this, in vitro reconstitution with purified budding yeast proteins recently showed that MCM7 ubiquitylation is normally repressed throughout fork elongation by the Y-shaped DNA structure of the replication fork itself, which is removed upon fork convergence and end-product ligation [122]. Efficient fork convergence and termination also necessitate activity of topoisomerase II and the Pit1 and Rrm3 DNA helicases [123]. In addition to CUL\textsuperscript{2LR}, a mitotic backup pathway has recently been discovered that involves the ubiquitin ligase TRAIP [124,125], which is also required for replisome disassembly at converged replication forks during interstrand crosslink repair in X. laevis egg extracts [95] and to allow mitotic DNA synthesis (MiDAS) and rescue of under-replicated DNA in mammalian cells [98]. All together, these studies point to an essential role for regulated CMG disassembly in genome stability and human health; yet, the consequences of its dysfunction are just starting to be unveiled.

### Human Genetic Diseases Linked to Defects in DNA Replication Dynamics

#### Meier-Gorlin Syndrome

Perturbation of the dynamics of DNA replication and the cell cycle is associated with a plethora of mendelian disorders, including microcephalic primordial dwarisms (MPDs), characterised by a core phenotype of pre- and postnatal growth restriction and microcephaly, with or without other developmental abnormalities [16]. A classic MPD is Meier-Gorlin syndrome (MGORS; MIM 224690), which is characterised by a triad of phenotypes: primordial dwarfism, microtia (small ears), and patellar aplasia/hypoplasia (absence or hypomorphic patellae) [17,18]. Intellect is usually preserved, and additional facial abnormalities can include microstomia (small mouth), micrognathia (underdevelopment of the jaw, lower), full lips, and a narrow nose with a high nasal bridge; mammary hypoplasia, abnormal genitalia (cryptorchidism and hypoplastic labia minora/majora), and pulmonary emphysema are also common [19] (Table 1).

In 2011, three seminal articles described the molecular genetics of MGORS and identified biallelic hypomorphic mutations in components of the PRE-RC, including ORC1–ORC4–ORC6–CDC6 and CDT1 [20–22]. Subsequently, a dominant gain-of-function mutation in GMMN (GEMININ) and hypomorphic mutations in MCM5 have been identified in patients affected by MGORS, further corroborating the unique genetic connection between the replication licensing system and this syndrome [23,24] (Table 1). Finally, an association of MGORS and craniosynostosis (prematurely closed cranial sutures) has been described in patients with biallelic loss of function mutations of the PRE-IC factor CDC45 [25] (Table 1).

#### Seckel Syndrome and Other MPDs

The most well-known MPD is Seckel syndrome (SS), which takes its name from the paediatrician, Helmut Seckel, who first described a group of patients affected by intra-/extraterine growth restriction with severe microcephaly and mental retardation in association with a "bird-headed" face due to a combination of receding forehead and chin with a large and beaked nose [26]. Several genes have been reported to be mutated in classical nonosteodyplastic SS (MIM 210600), which is characterised by severe microcephaly and mental retardation in the absence of specific osteodysplastic features. Seminal work by O’Driscoll et al. identified biallelic hypomorphic mutations in ATR in patients with SS, linking SS, for the first time, to DNA replication and the
Figure 3. The Intra-S-Phase Checkpoint. Schematic representation of the intra-S-phase checkpoint and its functions in the control of origin activation (A) and replication fork stability (B) [86]. Discontinuous DNA replication at leading or lagging strands generates extended regions of single-stranded DNA (ssDNA), rapidly covered by replication protein A (RPA). Accumulation of RPA at the replication fork promotes the independent recruitment of ATRIP, in concert with ATR, and ETAA1 [102–105]. The presence of a double-stranded DNA–ssDNA junction with a 5′–3′ free end allows RAD17-RFC2–5–dependent loading of the 9-1-1 complex, a

(Figure legend continued at the bottom of the next page.)
intra-S-phase checkpoint [27] (Table 1, Figure 3 and Box 3). Subsequent loss-of-function mutations in the ATR-binding protein ATRIP [28] (Table 1) further connected activation of the ATR pathway to the SS phenotype.

More recently, whole-exome sequencing (WES) studies have expanded the repertoire of single gene mutations causing MPD. These include components of the replication machinery and DNA repair factors, such as DNA2 and RBBP8/CTIP, and newly identified factors required for replication fork stability, such as TRAIP (TRAF-interacting protein) and DONSON (downstream neighbour of son) [29–33] (Table 1). Intriguingly, hypomorphic mutations of DONSON result in a spectrum of different phenotypic presentations. In addition to microcephaly, short stature, and limb abnormalities (MSSL) (MIM 617604), an RNA-sequencing approach led to the discovery of aberrant splicing in DONSON in patients with microcephaly-micromelia syndrome (MMS) (MIM 251230), a condition presenting with intrauterine growth restriction, severe microcephaly, craniofacial dysmorphisms, and limb malformations, usually lethal in the perinatal period [34,35] (Table 1). Last, mutations in DONSON have been identified in patients previously diagnosed with classic MGORS, further expanding the breadth of phenotypes related to partial loss of function of DONSON [36].

Mutation of Factors Required for Replication Origin Activation and/or DNA Synthesis at the Replisome

In addition to ‘classic’ MPD, defective growth and skeletal abnormalities are characteristics of several single-gene conditions involving components of the PRE-IC and/or replisome, in association or not with immune and endocrine system abnormalities. The PRE-IC component RECQL4 is mutated in three partially overlapping genetic conditions: Rothmund-Thomson syndrome (RTS), RAPADILINO syndrome, and Baller-Gerold syndrome. RTS (MIM 268400) features a characteristic and diagnostic facial rash known as poikiloderma, and a series of heterogeneous manifestations, which include reduced intra- and extraterine growth, sparse hair, eyelashes, and/or eyebrows, juvenile cataracts and skeletal abnormalities, including radial ray defects, absent or hypoplastic thumbs, hypoplasia/absence patella (similar to MGORS), syndactyly, and osteoporosis [37]. RTS also presents with heightened predisposition to cancer, particularly osteosarcoma (childhood) and spinocellular carcinoma (adult) [37] (Table 1).

In 1999, homozygous or compound heterozygous mutations in RECQL4 were described in a group of patients with RTS [38]. Hypomorphic mutations in RECQL4 have also been described in RAPADILINO (Radial hypoplasia, Patella hypoplasia and cleft or Arched palate, Diarrhoea and dislocated joints, Little size and limb malformation, slender Nose and nOrmal intelligence) (MIM 266280) [39] (Table 1) and Baller-Gerold syndrome (MIM 268400), characterised by coronal craniosynostosis, leading to abnormal skull shape, and radial aplasia [40] (Table 1).
Intriguingly, mutation of factors required for initiation of DNA replication frequently feature growth restriction and immunodeficiencies. In 2017, Cottineau et al. reported compound heterozygous hypomorphic mutations in GINS1 (PSF1), a component of the GINS1–4 complex, in a group of patients affected by intra- and extraterine growth restriction with neutropenia and natural killer (NK) cell deficiency (immunodeficiency 55 [IMD55]; MIM 617827) [41] (Table 1). Other features include mild facial dysmorphism, signs of autoimmunity, and tumour predisposition. Interestingly, this phenotype closely resembles patients with MCM4 truncating mutations associated with selective NK cell deficiency, reduced growth, and primal adrenal failure, also described as

| Gene mutation  | Step of DNA replication | MIM number link | Syndrome clinical features                                                                 | Refs          |
|---------------|-------------------------|----------------|-------------------------------------------------------------------------------------------|---------------|
| ORC1          | PRE-RC                  | 224690         | MGORS 1 (short stature, microtia, and patellae hypoplasia/aplasia)                         | [20,21]       |
| ORC4          | PRE-RC                  | 613800         | MGORS 2                                                                                  | [20,22]       |
| ORC6          | PRE-RC                  | 613803         | MGORS 3                                                                                  | [20]          |
| CDC6          | PRE-RC                  | 613805         | MGORS 5                                                                                  | [20]          |
| CDT1          | PRE-RC                  | 613804         | MGORS 4                                                                                  | [20]          |
| MCM5          | PRE-RC, PRE-IC, and replisome | 617564       | MGORS 8                                                                                  | [24]          |
| GMINN (GEMININ) | PRE-RC (inhibitor)     | 616835         | MGORS 6                                                                                  | [23]          |
| CDC45         | PRE-IC and replisome    | 617063         | MGORS 7 (Meier-Gorlin syndrome and/or craniosynostosis)                                   | [25]          |
| MCM4          | PRE-RC, PRE-IC, and replisome | 609981       | ID 54 (immunodeficiency 54) Growth restriction, adrenal insufficiency and NK cell deficiency | [42,43]       |
| RECQL4        | PRE-IC and DNA repair   | 268400, 218600, 266280 | • Rothmund-Thomson syndrome  
• Baller-Gerold syndrome  
• RAPADILINO syndrome | [37] [40] [39] |
| GINS1         | PRE-IC and replisome    | 617827         | ID 55 (immunodeficiency 55): growth restriction, neutropenia, NK cell deficiency          | [41]          |
| POLE1         | PRE-IC and replisome    | 615139, 618336 | FILS syndrome  
IMAGe syndrome                                           | [45] [46]     |
| POLE2         | PRE-IC and replisome    |               | Growth restriction and immunodeficiency                                                   | [49]          |
| MCM10         | Origin activation       |               | NK cell deficiency                                                                       | [44]          |
| POLA1         | Replisome               | 301220, 301030 | • XLPDR (X-linked pigmentary disorder, reticulate, with systemic manifestations)  
• Van Esch-O’Driscoll syndrome | [50] [52] |
| POLD1         | Replisome               | 615381         | • MPDL (mandibular hypoplasia, deafness, progeroid features, and lipodystrophy syndrome)  
• Growth restriction and immunodeficiency | [53] [55] |
| POLD2         | Replisome               |               | Growth restriction and immunodeficiency                                                   | [59]          |
| PCNA          | Replisome and DNA repair| 615919         | Growth restriction, hearing loss, neurodegeneration, premature ageing, telangiectasia, and photosensitivity | [56] |
| DNA2          | Replisome and DNA repair| 615807         | MPD                                                                                     | [29,30]       |
| ATR           | Intra-S-phase checkpoint | 210600         | Seckel syndrome                                                                         | [27]          |
| ATRIP         | Intra-S-phase checkpoint |               | Seckel syndrome                                                                         | [28]          |
| DONSON        | Replisome               | 617604, 251230 | • MSSSL (microcephaly, short stature, and limb abnormalities)  
• MMS (microcephaly-micromelia syndrome)  
• MGORS | [33,35] [34] [36] |
| TRAIP         | Replisome and DNA repair| 616777         | MPD                                                                                     | [32]          |
| RBBP8/CTIP    | Replisome and DNA repair| 606744, 251255 | • Seckel syndrome  
• Jawad syndrome                                          | [31]          |

Table 1. Molecular Genetics of Human Inherited Syndromes Caused by Mutations of Replisome Genes
immunodeficiency 54 (IMD54; MIM 60998) [42,43] (Table 1). More recently, Mace et al. described a patient with selective NK cell deficiency and increased susceptibility to cytomegalovirus infection caused by compound heterozygous mutations in MCM10 [44]. Thus, a group of genetic diseases affecting different steps of CMG formation and activation perturbs, in a specific manner, NK cell maturation.

Hypomorphic mutations of the catalytic subunit of POLε, POLE1, were initially described in a large consanguineous family affected by FILS syndrome (facial dysmorphism, immunodeficiency, livedo, and short stature) [45] (Table 1). Facial abnormalities include malar hypoplasia and high forehead with occasional signs of bone dysplasia. Patients also present with recurrent upper and lower respiratory tract infections due to B- and T-cell immunodeficiency. More recently, compound heterozygous mutations in POLE1 have been identified by WES in multiple patients affected by IMAGe syndrome (intrauterine growth restriction, metaphyseal dysplasia, adrenal hypoplasia, and genital anomalies in males) associated with variable immunodeficiency [46] (Table 1). IMAGe syndrome (MIM 614732) was initially described by Vilain et al., in cases of growth retardation associated with severe adrenal insufficiency [47]. Additional features include mild dysmorphism, bilateral cryptorchidism, a small penis, and hypogonadotropic hypogonadism. Heterozygous missense mutations in the PCNA binding domain of the CDK inhibitor p57 (CDKN1C) have been lately identified in individuals with IMAGe syndrome [48]. Distinct from classical IMAGe syndrome, mutations of POLE1 are associated with immunological dysfunction in addition to facial abnormalities comprising micrognathia, crowded dentition, long thin nose, short wide neck, and small, low-set, posteriorly rotated ears [46]. Osteopenia and developmental dysplasia of the hip (DDH) were also frequently observed together with café-au-lait patches. Two patients also developed lymphomas suggesting a specific lymphoma predisposition [46]. Intriguingly, severe immunodeficiency with facial dysmorphism and autoimmunity has also been associated with a homozygous splice-site mutation in POLE2, the second major subunit of POLε [49]. A severe B-cell differentiation defect in this condition led to an absence of circulating B cells and agammaglobulinemia with T-cell lymphopenia and neutropenia. Early-onset diabetes mellitus and hypothyroidism also suggest a significant autoimmune component [49].

A recurrent mutation in POLA1, codifying for the p180 catalytic subunit of POLα, was initially reported in the X-linked reticulate pigmentary disorder (XLPDR; MIM 301220), a primary

**Box 3. The Intra-S-Phase Checkpoint**

Replication origin activation and replisome progression are intimately connected during S phase. These events are fine-tuned both locally and over a distance to permit temporal activation of replication origins and to avoid exhaustion of dNTPs and replication factors. The intra-S-phase checkpoint has a particularly important role in this process by modulating origin usage and replication fork progression under both unchallenged and challenged conditions [86] (Figure 3). At the centre of this evolutionarily conserved pathway are the essential kinases ATR (ataxia-telangiectasia and Rad3-related; Mec1 in budding yeast) and its downstream effector CHK1 (checkpoint kinase 1; Rad53 in budding yeast). The trigger for ATR activation is the accumulation of RPA-coated ssDNA at the replication fork, a condition typically induced by uncoupling of dsDNA unwinding by the CMG helicase and DNA synthesis by processive DNA polymerases. RPA is recognised and bound by ATRIP (ATR-interacting protein), an ATR-binding protein, which promotes ATR loading at stalled replication forks [102,103]. ATR activation also requires TOPBP1, which is recruited at S- ended ssDNA–dsDNA junctions by the RAD9–RAD1–HUS1 (9–1–1) complex in a RAD17–RFC2/5-dependent manner [86,106] (Figure 3). More recently, an ATR-activating protein, Ewing tumour-associated antigen 1 (ETA1), has been identified and shown to directly bind RPA at stalled replication forks to promote ATR activation via a parallel pathway [104,105] (Figure 3). Once activated, ATR phosphorylates and activates, in a CLASPIN- and TIM/TIPIN-dependent manner, its downstream effector kinase CHK1 to promote cell cycle arrest, inhibition of origin firing, and stabilisation of replication forks to facilitate their repair and restart [86]. The first task is achieved via CHK1-dependent phosphorylation and inhibition of CDC25 phosphatases CDC25A, CDC25B, and CDC25C, which are required for CDK2 and CDK1 activity and progression throughout the cell cycle [86]. More recently, Saldikar et al. have also shown that ATR is activated during DNA replication via ETA1 to monitor S-phase progression and prevent a CDK1-dependent FOXM1 switch that transactivates the mitotic gene network [126].
immunodeficiency associated with interferon I-dependent systemic inflammation [50] (Table 1). In addition to unique facial features (frontally upswept hair and flared eyebrows), hypohidrosis, and hyperpigmentation, multiorgan inflammation involving the eyes, the intestine, and the urinary tract are the main features of this syndrome. Patients with XLDR also experience recurrent infections, predominantly in the respiratory tract, resulting in early-onset bronchiectasis and respiratory failure [50]. Interestingly, patients with XLDR also exhibit reduced NK cell numbers, particularly differentiated (CD3^-CD56^dim) cells, reminiscent of the previously described MCM4 mutation [51]. In addition to XLDR, Van Esch et al. reported five unrelated families with hypomorphic POLA1 mutations associated with syndromic X-linked growth restriction and microcephaly, intellectual disabilities, hypogonadism, and variable congenital abnormalities (MIM 301030) [52], more closely resembling syndromes caused by dysfunctional DNA replication initiation.

Mutations of components of the POLδ complex are associated with multiple syndromic conditions. A recurring heterozygous single-codon deletion in POLD1 affecting the polymerase site causes the autosomal dominant and multisystem disorder mandibular hypoplasia, with deafness, progeroid features, and Lipodystrophy (MDPL; MIM 615381) [53]. Facial features include a beaked nose, prominent eyes, crowded teeth, small mouth and uvula, and long eyelashes, and they are often associated with hypogonadism and cryptorchidism in males and metabolic anomalies such as insulin resistance. Radiologic skeletal abnormalities are also present in some individuals. A mutation in POLD1 has also been described in patients with atypical Werner syndrome in the absence of MDPL signs but with reduced growth [54]. More recently, mutations in both POLD1 and POLD2 have been implicated in a syndromic growth restriction and immunodeficiency characterised by T- and B-cell reductions with NK deficiency, closely resembling POLδ hypomorphic patients [55]. Finally, a homozygous missense mutation in PCNA causing a S228I substitution (MIM 615919) was identified in four patients with a syndromic growth restriction associated with neurodegeneration, hearing loss, premature ageing, telangiectasia, and photosensitivity. Both clinical presentation and dissection of the molecular pathogenesis suggest that this condition is more likely to reflect a DNA repair defect [56].

Mutation of Replisome-Associated Factors Required for DNA Repair and Sister Chromatin Cohesion

DNA replication is tightly linked to sister chromatin cohesion and DNA repair [1]. As such, a series of genetic syndromes described as DNA repair disorders are associated with defective S-phase progression and abnormal processing of replication intermediates at stalled replication forks. The most prominent examples are the RECQL helicase syndromes Bloom syndrome (BLM) and Werner syndrome (WRN), Fanconi anaemia, and Schinke immune-osseous dysplasia (SIOD) disorder [57–59]. Importantly, core features of these conditions comprise reduced growth and developmental abnormalities in association with haematological/immunological dysfunctions.

In addition to DNA repair disorders, compound heterozygous loss-of-function mutations of the E3 SUMO ligase NSMCE2 (also known as MMS21), a member of the structural maintenance of chromosomes 5–6 (SMC5–6) complex, have been reported in a primordial dwarfism combined with severe insulin resistance and gonadal failure in association with signs of replication stress (MIM 617253) [60]. Interestingly, homozygous or compound heterozygous mutations of another component of the SMC5–6 complex, NSMCE3, were subsequently discovered in a different chromosomal instability syndrome associated with severe T- and B-cell immunodeficiency (MIM 608243) [61]. Finally, a group of autosomal recessive microcephalic disorders have been
associated with hypomorphic mutations of components of condensin I and II complexes, decatenation failure at mitosis and chromosome mis-segregation [62].

Pathogenesis of Genetic Diseases Caused by Mutation of Components of the Replisome
Human genetic disorders caused by mutation in components of the replication machinery share a group of recurrent clinical manifestations, with reduced intra- and extraterine growth being the central theme. Hence, defective development during the embryonal stage, driven by replication stress, represents the unifying feature.

Impaired licensing of Replication Origins (MGORS)
A paradigmatic example is MGORS, whose molecular genetics is tightly linked to the licensing machinery and MCM2–7 loading at replication origins. Initial studies in patient-derived cell lines and zebrafish connected hypomorphic mutations of PRE-RC components to reduced chromatin loading of MCMs, slower progression through S phase, and reduced growth in zebrafish [21]. Several MCM hypomorphic mice have been generated, which exhibit defective development through embryonic stages and strain-dependent tumour predisposition, a feature not classically associated with MGORS [63,64]. Mcm4Chaos 3, a hypomorphic allele of MCM4, has been associated with mammary adenocarcinomas in the C3H genetic background and a wider spectrum of cancers in outbred strains [63,65]. Similarly, an Mcm2 hypomorphic mouse model showed a strain-specific predisposition to thymic lymphoblastic lymphoma [66]. Studies using mouse cells hypomorphic for MCMs have led to insights into the regulation and developmental role of dormant origins. Mouse embryonic fibroblasts (MEFs) homozygous for Mcm4Chaos 3 or an Mcm2 hypomorphic allele fail to activate dormant origins and show signs of replication fork asymmetry and chromosomal instability [65,66]. Genetic analysis of lymphomas from Mcm2 hypomorphic mice showed the accumulation of atypical small (less than 0.5 kb) deletions in these tumours, suggesting recombination between nearby stalled replication forks as the pathogenetic mechanism [67]. Organismal, developmental, and tissue-specific dynamics of loading and activation of replication origins are likely to play a fundamental role in the phenotypic expression of this condition, as also inferred from studies in embryonic stem cells and neuroprogenitors [64,68].

Origin licensing is subjected to sophisticated control mechanisms. In vertebrates, a cell cycle-regulated factor, GEMININ, keeps CDT1 under control during the S and G2 phases of the cell cycle [69,70]. GEMININ is normally degraded during mitosis and G1 by anaphase-promoting complex (APC)-mediated ubiquitination and proteolysis, which is dependent on a classical destruction box (D-box) located in its N-terminal domain [69]. Although MGORS generally presents with an autosomal recessive pattern of inheritance due to biallelic hypomorphic mutations of ORC1-ORC4-ORC6-CDC6-CDT1 and MCM5, heterozygous de novo mutations in the 5’ coding region of GEMININ were also recently reported [23]. Strikingly, these genetic alterations result in the expression of GEMININ protein products specifically lacking the N-terminal D-box, which confers increased stability throughout the cell cycle and insufficient origin licensing [23]. Partial loss of function mutations of CDC45 have also been associated with cases of MGORS and/or craniosynostosis [25]. In budding yeast, Cdc45 and Sld3/Treslin are recruited to DNA replication origins in a DDK-dependent but CDK-independent manner during the G1 phase of cell cycle [1,71], acting as a ‘second’ licensing step of DNA replication origins. The absence of CDK-dependent commitment to origin activation, a ‘point of no return,’ might thus help explain the association of CDC45 mutations with MGORS. The targeting of specific replication origins
Key Figure
Pathogenetic Mechanisms of Human Genetic Diseases Caused by Mutations of the Replication Machinery

(A) Hypomorphic mutation of CDC6  Hypomorphic mutation of CDT1  Hypomorphic mutation of MCM5
Hypomorphic mutation of ORC1-4-6
MCM2-7

Gain of function mutation of GEMININ

Reduced licensing of replication origins

(B) MCM10 mutation
DDK  CDK
Hypomorphic mutation of POLE1-POLE2
MCM4 mutation
Hypomorphic mutation of POLA1

Reduced DNA replication origin activation or initiation of DNA synthesis at the replisome

(C) Hypomorphic mutation of ATR-ATRIP
Hypomorphic mutation of CTIP-DNA2
Hypomorphic mutation of DONSON
Hypomorphic mutation of TRAIP

Replication fork destabilisation and collapse
Unscheduled origin activation

Disruption of replication fork stability

(See figure legend at the bottom of the next page.)
(e.g., early replicating) and/or a specific pattern of developmental expression and/or regulation might also play a role.

**Reduced Replication Origin Activation and/or DNA Synthesis at the Replisome**

Replication genetic diseases affecting the CDK-dependent step of origin activation share similar phenotypes (Figure 4). Examples are the recently identified mutations of **GINS1, MCM4, and POLE1** in immunodeficiency 55 and 54 and IMAGe syndrome [41–43,46]. Classic IMAGe syndrome is caused by mutations of **CDKN1C**, which codifies for the p57 CDK inhibitor [48]. Mutations of p57 in patients with IMAGe syndrome occur within its PCNA-interacting protein box (PIP-box) domain and prevent PCNA binding and proteasomal degradation [72,73]. Hence, increased p57 stability during S phase and deregulated CDK activity might perturb origin activation. An intriguing pathogenetic case is constituted by mutations of **MCM4**, reported in patients with NK cell deficiency, growth restriction, and adrenal insufficiency [42,43]. Identified cases presented with an **MCM4** splice-site mutation and generation of two N-terminal truncated versions of MCM4 lacking 50 or 74 amino acids (aa), respectively [42,43]. Importantly, this truncation does not affect formation of the MCM2–7 complex or MCMs levels on chromatin, differing from a PRE-RC assembly defect and **Mcm4chaos** mutant mouse cells [42]. In budding yeast, the N-terminal domain of MCM4 is targeted by several kinases, such as CDK, DDK, and Mec1/ATR, to control initiation of DNA replication and coordinate the checkpoint response. Hence, aa 74–178 of MCM4 contain an essential inhibitory domain released by DDK phosphorylation, a key priming event for the formation and activation of the CMG. Conversely, the proximal N-terminal residues contain several CDK target sites, which are important for activation of DNA replication origins [74]. Accordingly, Sheu et al. have shown that a MCM4 strain lacking the essential CDK targets exhibits reduced origin activation and a compensatory increase in fork speed [75].

Although humanised mouse models of **MCM4, GINS1, and POLE1** mutations have not been reported, Bellelli et al. recently characterised a mouse model lacking the POLE4 subunit of POLε, which presented with reduced growth, craniofacial abnormalities, skeletal dysplasia, and diminished T and B cells, closely resembling features of IMAGe syndrome [76]. Strikingly, they also observed a slight but significant predisposition to B- and T-cell lymphomas, similar to that reported by Logan et al. in patients with **POLE1** mutations [46]. **Pole4**-knockout (Pole4-KO) mouse cells exhibit instability of the POLε complex and reduced origin activation. The striking similarities with patients with **POLE1** mutations suggest that the Pole4-KO mice

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**Figure 4.** (A) Reduced licensing of DNA replication origins in Meier-Gorlin syndrome (MGORS). Hypomorphic mutations of origin recognition complex 1–4–6 (ORC1–4–6), cell division cycle 6 (CDC6), Cdc10-dependent transcript 1 (CDT1), or mini-chromosome maintenance 5 (MCM5) impair chromatin loading of inactive MCM2–7 double hexamers and cause autosomal recessive MGORS [20–22,24]. De novo mutations of GEMININ prevent interaction of CDT1 with MCM2–7 and reduce MCMs chromatin loading, causing autosomal dominant MGORS [23]. (B) Reduced activation of DNA replication origins or dysfunctional initiation of DNA replication cause a spectrum of diseases with reduced growth and/or immunodeficiency. Mutations of **MCM10** affect CMG activation, whereas mutations of Go-Ichi-Ni-San 1 (GINS1) lead to GINS1–4 complex instability and reduced CMG assembly [41,44]. Similarly, hypomorphic mutations of **POLE1/POLE2** lead to reduced levels of POLε, preventing stable CMG formation [45,46,49]. MCM4 N-terminal truncations lead to loss of CDK-mediated phosphorylation of MCM4, affecting origin activation [42,43]. Finally, hypomorphic mutations in **POLA1 and POLE1/POLE2** cause reduced initiation of DNA synthesis [52,55]. (C) Mutations of components of the intra-S-phase checkpoint or factors required for replication fork stability cause microcephalic primordial dwarfism (MPD). Hypomorphic mutations of **ATR and ATRIP** cause reduced levels of the ATR/ATRIP complex and cause Seckel syndrome (SS) [27,28]. Hypomorphic mutations of RBBP8/CTIP and DNA2 impair protection and/or processing of replication intermediates and impact the ATR signalling causing SS/MPD [29–31]. Similarly, hypomorphic mutations of TRAIP affect the processing of replication intermediates at stalled replication forks and cause MPD [32]. Finally, hypomorphic mutations of DONSON cause a spectrum of MPDs by impairing replication fork stability and/or the ATR-dependent checkpoint [33–36].
likely represent a hypomorphic Pol epsilon model [76]. Indeed, a recently reported Pole3-KO mouse model presented with reduced intrauterine growth and lethality in a C57BL/6 background, closely mimicking Pole4-KO mice [77]. Intriguingly, Siamishi et al. also reported the generation of a Pole3 mouse model lacking the acidic C-terminal domain of POLE3 that was previously shown to support POLE3–POLE4 binding to histones H3–H4 [77,78]. Although this mouse model exhibited no major phenotypic abnormalities, suggesting compensatory mechanisms, the gradual increased substitution of the negatively charged residues in the C-terminus of POLE3 with positive ones led to the development of an increasingly severe B- and T-cell deficiency [77]. This observation suggests that loss of interactions with histones and/or other factors during DNA replication might impair differentiation of lymphocyte precursors, likely through an epigenetic mechanism [79].

Mutations of the POLα subunit POLA1 have been described in patients with reduced growth, immunodeficiency, and hypogonadism [52]. Characterisation of patient-derived cells showed increased fork asymmetry and enhanced interorigin distance, suggestive of dysfunctional initiation of DNA replication, resembling an insufficiency of initiation factors [52] (Figure 4). Mechanistically, POLα is strictly required for the synthesis of RNA-DNA primers extended by the processive DNA polymerases, thus explaining the functional defect in initiation of DNA replication (Figure 4).

Biallelic mutations of POLD1 and POLD2 have also been identified in patients with reduced stature, T- and B-cell lymphopenia, and NK cell deficiency, resembling POLE1 mutations in IMAGe syndrome [55]. Mutations in both POLD1 and POLD2 significantly reduced expression of POLδ complex components in patient-derived cells, suggesting a POLδ hypomorphic condition [55]. Analysis of replication dynamics in patient-derived cells showed a combination of reduced origin activation and increased fork speed, suggestive of dysfunctional replication initiation, but not a replication fork progression defect [55]. In budding yeast, POLδ is required for initiation of leading strand DNA replication and the establishment of two functional replisomes at replication origins [80,81]. Despite not being physically tethered to the CMG helicase, single-molecule analysis recently suggested that POLδ is stable at replication forks, pointing to a mechanism of continuous recycling [82]. Thus, although reduced levels of POLδ might limit ‘functional’ activation of replication origins, once POLδ is engaged at a replication fork, it might remain associated and promote extensive DNA synthesis.

**Defective Intra-S-Phase Checkpoint and Replication Fork Stability (SS and Other Cases of MPD)**

The other paradigmatic genetic condition affecting replication fork dynamics is SS. Important insights into the pathogenesis of this condition initially came from the development of a humanised Atr hypomorphic mouse. While presenting with phenotypic alterations remarkably similar to those of human patients, this model provided evidence for in utero replicative stress as a driving force of adult phenotypic alterations [83]. Atr Seckel mouse cells and patient-derived fibroblasts exhibit reduced fork extension rates and interorigin distance suggestive of disrupted control of replication origin activation and fork extension/stability by the intra-S-phase checkpoint [84–86] (Box 4). A combination of altered replication origin activation and replication fork instability likely drive permanent fork stalling, abnormal replication fork processing, and irreversible DNA damage.

ATR is indeed required for the control of replication origin activation under unperturbed conditions and is normally activated during S phase [86]. While lagging strand DNA synthesis has been suggested to promote ATR activation during normal DNA replication [87], recent
work by Forey et al. showed that Mec1/ATR is activated at the onset of S phase, at a subset of early replication origins, to promote increased deoxynucleoside triphosphate (dNTP) synthesis and sustain genome-wide DNA replication [88]. Multiple lines of evidence suggest that defective dNTP metabolism might play a role in the pathogenesis of replication stress induced by ATR hypomorphic mutations. First, nucleoside supplementation can partially rescue proliferation rates and replication stress in Atr hypomorphic MEFs [85]. Second, in a mouse model, increased dosage of Rrm2, the gene codifying for the limiting subunit of ribonucleotide reductase, can prolong survival of Atr Seckel mice and reduce the burden of symptoms [85]. While hypomorphic mutations of ATRIP directly affect the levels of ATR and result in an ATR hypomorphic condition [28], the molecular mechanisms responsible for the development of severe cases of SS/MPD in patients with other genetic alterations is more complex. SS/MPD causal mutations in RBBP8/CTIP lead to the production of an abnormal C-terminal truncated product compromising MRN (MRE11-RAD50-NBS1) interaction and a CDK phosphorylation site. Importantly, this mutant protein retains its dimerisation domain and can act as a dominant negative reducing double-strand break (DSB) resection, RPA accumulation on ssDNA, and consequently ATR signalling in response to DSB-inducing agents [31]. Despite being mainly known for its role in DSB resection, CTIP is essential for early embryonic development, suggesting an essential role during DNA replication [89]. Although CTIP-mediated end resection might be necessary to process replication intermediates and generate ssDNA and ATR signalling during DNA replication, recent work suggested a paradoxical role for CTIP in the protection of newly replicated strands from DNA2-dependent nucleolytic degradation [80]. In agreement with this hypothesis, mutations of CTIP have also been identified in patients with familial breast cancer [91]. Intriguingly, Shaheen et al. also reported a hypomorphic mutation of BRCA2, a prominent homologous recombination factor involved in DSB repair and fork protection, in patients with MPD in the absence of pathologic involvement of the bone marrow, which precluded a diagnosis of Fanconi anaemia [30]. Instability of
replication fork intermediates undergoing fork reversal might explain these phenotypic associations.

DNA2 is a nuclease involved in the removal of 5′-flap structures during lagging strand DNA maturation and long-path BER [14]. Biallelic hypomorphic variants in DNA2 have been reported in patients with severe MPD [29,30]. DNA2 is also required for Mec1 activation in budding yeast at lagging strands and during unchallenged DNA replication [87,92]. Whether mammalian DNA2 is necessary, along with ATRIP and ETAA1, for ATR activation remains unclear. Nevertheless, DNA2 has a prominent role in mammals in remodelling stressed replication forks, where, in cooperation with WRN, it promotes resection of reversed forks to generate a 5′-3′ end necessary for TOPBP1 recruitment and ATR activation [86,93]. Therefore, DNA2, in addition to its role on the lagging strand, might be required to process replication intermediates and promote sustained activation of ATR under both challenged and normal DNA replication.

A severe MPD syndrome is also caused by hypomorphic mutations in the essential RING E3 ubiquitin ligase, TRAIP (TRAF-interacting protein) [32]. Identified as a PCNA-interacting protein enriched at replication forks, TRAIP travels with the replisome and promotes RPA accumulation and ATR activation in response to replication stress-inducing agents, including mitomycin-C (MMC) [94]. Subsequent work conducted at the Walter laboratory identified TRAIP as the ubiquitin ligase required for CMG unloading and activation of the Fanconi anemia pathway at interstrand crosslinks (ICLs) [95]. Importantly, CMG unloading is also required for fork reversal and incision of the crosslink at ICLs [96]. This might help explain defective RPA accumulation and ATR signalling upon TRAIP deficiency and its pathogenetic alteration in MPD [32,94]. In addition to this, TRAIP also ubiquitylates DNA–protein crosslinks (DPCs) to promote their proteasomal degradation and also ubiquitylates stalled CMGs during mitosis to promote mitotic DNA synthesis (MiDAS) [97,98]. Which of these functions, when compromised, leads to defective growth and impaired neuronal development in MPD remains to be established.

Finally, mutation of DONSON in MPD results in reduced protein levels and/or subcellular localisation, pointing to an hypomorphic pathogenetic mechanism [33]. Loss of Donson is lethal in mice, which suggests an essential role during DNA replication [34]. In accordance with this, mechanistic studies in human cells and patient-derived cell lines established DONSON as a novel component of the replisome, required for replication fork stability and activation of the intra-S-phase checkpoint [33]. More recently, work by Zhang et al. has provided evidence that DONSON is particularly enriched in replisomes in early replicating domains, suggesting a specific function in challenged and unchallenged conditions in euchromatin replication [99]. In summary, the aforementioned group of genetic syndromes caused by mutations in ATR-ATRIP, DNA2, CTIP, and the newly identified TRAIP and DONSON replisome components share a common mechanistic basis linked to defective maintenance of replication fork stability and/or ATR signalling (Figure 4).

Concluding Remarks

The identification, in the last decade, of several genetic conditions caused by mutation of the replication machinery, significantly strengthened our perception of the role of DNA replication in genome stability, human development, and health. The progress in genetic sequencing made available more accurate molecular diagnosis for both genetic counselling and the clinical management of patient comorbidities. Furthermore, understanding of the genetic and dynamics of these conditions provided avenues for targeted therapies, particularly in the context of neoplastic manifestations and haematological dysfunctions. The recent extraordinary progress in genetic sequencing has enabled more accurate molecular diagnosis for both genetic counselling and the clinical management of patient comorbidities.
therapies and genetic manipulation (e.g., CRISPR/Cas9 editing) also hold promise to further reduce the gap between molecular diagnosis and effective treatment.

Here, we review the molecular genetics of replication-linked human genetic diseases and discuss their pathogenetic mechanisms based on the steps of DNA replication affected and the most recent understanding of its dynamics (Figure 4). However, despite the recent advancements, many questions remain to be addressed (see Outstanding Questions).

Mechanistically, which regions of the genome are affected by reduced loading of MCM2–7 in MGORS, and why? Where in the genome do replication origins fail to be activated in CMG-POLɛ hypomorphic conditions? Which genomic regions undergo replication fork collapse, and how is this triggered?

There is also a need to extend the molecular studies to systems that recapitulate these genetic conditions and the stages of embryonic development and tissue homeostasis that are affected. Evaluating the dynamics of origin loading and activation have proved to be significantly different in embryonic and adult stem cells, as well as in differentiated cells from different tissues. Addressing these questions will be fundamental to explain the involvement of particular cell populations in these diseases and the roles exerted by specific replisome components. This is particularly relevant for cell populations of the immune system, such as NK cells in MCM4, MCM10, and GINS1 mutant patients and lymphocytes in POLE1/2 and POLD1/2 hypomorphic individuals. Similarly, the specific development of adrenal insufficiency in patients with IMAGe syndrome and MCM4 mutations remains to be explained. The exploitation of mouse models that resemble these syndromes, such as the Atr Seckel and Pole4-KO mice, could help extend the study of replication origin activity to an in vivo context with its tissue-specific dynamics. The use of organoids could also provide important insights into the mechanistic basis of these diseases.

In addition to this, the function of recently identified replisome-associated factors remain enigmatic or poorly defined. A paradigmatic example is DONSON, whose hypomorphic mutations confer a spectrum of developmental disorders ranging from MGORS to MMS [33–36]. Similarly, which of the functions recently described for the ubiquitin ligase TRAIP is responsible for the severe MPD caused by TRAIP hypomorphic mutations? Furthermore, although termination of DNA replication represents a relatively recent area of investigation, the consequences of its dysfunction for human health remain unclear. Moreover, the mechanisms responsible for replication fork collapse in SS and similar disorders remains to be established. Dysfunctional protection of newly replicated DNA has recently emerged as an important pathogenetic mechanism in both genetic and acquired human diseases, and we speculate that it might play an important role also in SS and MPD pathogenesis.

Finally, an area to be further explored is the mechanisms that couple DNA replication with the maintenance of the epigenetic information [79]. Understanding if and how mutations of specific components of the replisome, such as MCM2, POLɛ, and POLɛ impact this process remains to be defined and might reveal new insights into the pathogenesis of replication-associated human genetic disease.

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