Trends in Genetics

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Feature Review

Spotlight on the Replisome: Aetiology of DNA Replication-Associated Genetic Diseases

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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.

Highlights

DNA replication is performed by a multiprotein complex known as the 'replisome,' which is assembled and regulated in a cell cycle–dependent manner.

Hypomorphic mutations of components of the replisome lead to defective development, reduced growth, and altered tissue homeostasis.

Whole-genome sequencing studies significantly expanded the repertoire of mendelian diseases caused by mutation of the replication machinery.

Phenotypic analysis and mechanistic studies support defective replication origin assembly and activation and perturbation of replication fork stability as the pathogenetic mechanisms of replication-linked human genetic 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 G_1 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-

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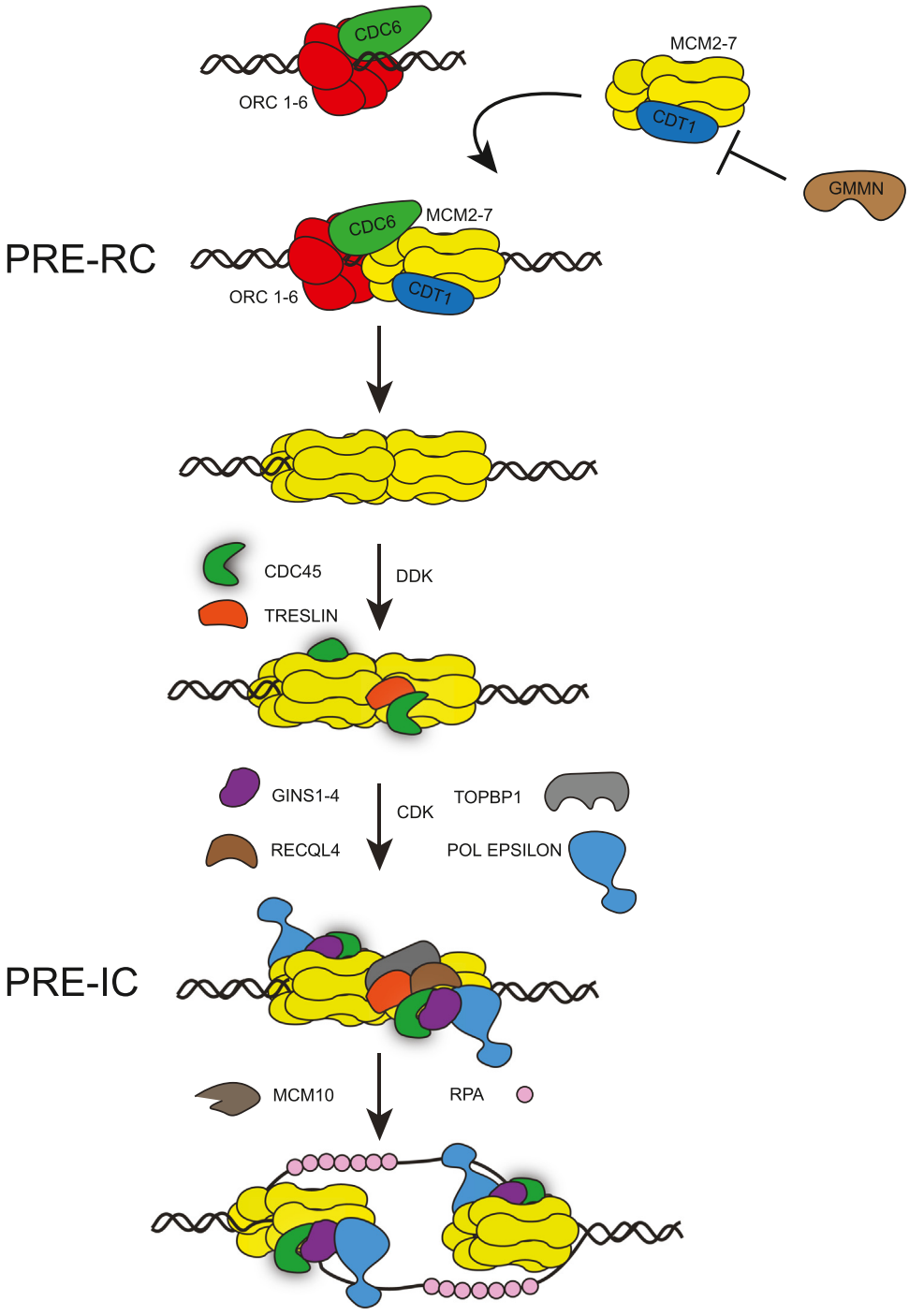

Figure 1. Licensing and Activation of a Human DNA Replication Origin. Cartoon depicting the three main steps required for initiation of DNA replication at a replication origin. Formation of the pre-replication complex (PRE-RC): The origin recognition complex 1–6 (ORC1–6) initially recruits cell division 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 (CDT1) and loading of MCM2–7 double hexamers at a replication origin.

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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–

Box 1. Mechanisms of Control of Origin Licensing and Activation

Sophisticated mechanisms have evolved to control the major steps of DNA replication, from PRE-RC complex formation and replication origin activation to replisome activity and replication fork stability. Defects in any one of these mechanisms can significantly impact genome stability and human development and are associated with congenital human disease. Regulation of licensing of DNA replication origins is particularly crucial for the maintenance of genome integrity. Accordingly, loading of MCM2–7 on chromatin is limited to the G_1 phase of the cell cycle by several mechanisms that control overall levels and/or localisation of the licensing factors ORC1–6, CDC6, and CDT1 [110].

Early studies in budding yeast defined two separate phases of the cell cycle, characterised by low or high CDK activity associated with licensing or activation of DNA replication origins in a mutually exclusive manner. CDK activity, while promoting activation of DNA replication origins, restrains PRE-RC complex formation and origin re-licensing through inhibitory phosphorylation [111]. In mammalian cells, several CDK-independent mechanisms have also emerged, targeting the licensing factor CDT1 [110]. Thus, in addition to CDK-dependent phosphorylation and SKP2-mediated ubiquitylation/degradation, the CRL4-CDT2 ubiquitin ligase targets CDT1 for replication-coupled destruction in a PCNA-dependent manner [112]. Furthermore, CDT1 association with the S phase–specific inhibitor, GEMININ, inhibits PRE-RC assembly by sequestering CDT1 in an inactive complex that is unable to interact with or recruit MCM2–7 [69,70] (Figure 1).

Importantly, during the G_1 phase an excess of MCM2–7 double hexamers are loaded onto genomic DNA. Known as the MCM paradox, this phenomenon has been associated with the presence of accessory or 'dormant' replication origins. Dormant origins can be activated under conditions of replicative stress [113,114] in order to rescue replication fork stalling and facilitate completion of genome-wide replication.

Initiation of DNA replication, upon DDK and CDK activation, takes place at thousands of genomic sites in a strictly regulated manner [115]. Although specific sequence and chromatin features recently emerged as regulators of replication origin activity, in metazoans, flexibility in origin usage couples DNA replication and genome structure and function (e.g., transcriptional programs) [115]. In addition to this, a series of chromatin marks, including histone acetylation and methylation, have been associated with PRE-RC activity and/or origin activation [115]. Finally, replication origins are activated in a temporally regulated manner to prevent exhaustion of replication factors and dNTPs and allow accurate and complete DNA replication before transition into G_2/M phase [115].

dependent transcript 1 (CDT1), which promote loading of inactive mini-chromosome maintenance 2–7 (MCM2–7) double hexamers at DNA replication origins [4] (Figure 1). Historically, the factors required for licensing of replication origins are also known as components of the pre-replication complex (PRE-RC) [1].

At the G_1–S transition, a series of Dbf4-dependent kinase (DDK) and cyclin-dependent kinase (CDK) phosphorylation events drive assembly of the eukaryotic processive helicase CMG, which comprises CDC45, MCM2–7, and the Go-Ichi-Ni-San (GINS) complex (composed of PSF1, PSF2, PSF3, and SLD5; also known as 'GINS1–4' in mammals [5]). While MCMs represent the key targets of DDK activity, CDKs phosphorylate the essential pre-initiation factors TopBP1-interacting checkpoint and replication regulator (TICRR)/TRESLIN and RECQ-like helicase 4 (RECQL4; Sld3 and Sld2 in budding yeast), allowing BRCT-dependent binding to DNA topoisomerase II–binding protein 1 (TOPBP1; Dpb11 in *Saccharomyces cerevisiae*) and recruitment of CDC45 and GINS1–4 in concert with DNA polymerase epsilon (Pol ε) [6–

This involves the formation of a transient intermediate known as 'OCCM' (ORC-CDC6-CDT1-MCM) [4]. Licensing of replication origins is restricted to the G_1 phase by several mechanisms, which include the vertebrate-specific factor GEMININ, which binds to CDT1 during the S and G_2 phases of the cell cycle to prevent its interaction with MCMs [69,70]. Formation of the pre-initiation complex (PRE-IC): Dbf4-dependent kinase (DDK) initially phosphorylates MCMs and promotes binding of TopBP1-interacting checkpoint and replication regulator (TICRR)/TRESLIN and CDC45 to replication origins [1]. Subsequently, cyclin-dependent kinases (CDKs) phosphorylate TICRR/TRESLIN and RECQ-like helicase 4 (RECQL4) and induce binding to TOPBP1 and recruitment of Go-Ichi-Ni-San (GINS) complex (GINS1–4) in concert with POLε [6–10]. Origin activation (or firing): Recruitment of MCM10 leads to activation of the CMG (CDC45/MCM2-7/GINS1-4) helicase, thus promoting double-stranded DNA (dsDNA) melting and single-stranded DNA (ssDNA) extrusion from the MCM2–5 gate [12]. dsDNA unwinding by the CMG causes the formation of ssDNA, which is rapidly coated by replication protein A (RPA), leading to the formation of two symmetric replication forks that travel in opposite directions [13].

pituitary unit (secondary hypogonadism or hypogonadotropic hypogonadism).
Mendelian disorders: disorders that result from a mutation at a single genetic locus in an autosome or sex chromosome. They might present in a dominant or recessive mode of inheritance.
Microcephaly: genetic or acquired condition in which the head of the newborn is smaller than normal, as diagnosed by measurement of the occipitofrontal circumference. It is caused by congenital insufficiency during foetal brain development, and its extent generally correlates with the severity of mental retardation.
Origin licensing: first step required for DNA replication. It involves loading of the replicative helicase at replication origins by components of the pre-replication complex.
Primary immunodeficiency: genetic disorder of the immune system, generally resulting in development of recurrent and/or more severe infections.
Primordial dwarfism: group of genetic disorders characterised by prenatal and postnatal growth retardation. This results in individuals extremely small (dwarfs) for their age even as a foetus.
Pulmonary emphysema: abnormal and permanent enlargement of airspaces distal to terminal bronchioles associated with alveolar wall destruction.
Replication fork: DNA structure generated when dsDNA unwinding by the replicative helicase is coupled to synthesis of the complementary strands.
Replication fork collapse: failure to stabilise a stalled replication fork that results in its inability to resume DNA synthesis and processing into a double-strand break.
Replication stress: alteration of the dynamics of DNA replication associated with fork stalling and slowing, accumulation of ssDNA at the replication fork, and activation of the intra-S-phase checkpoint.
Replisome: large machinery that carries out DNA replication during the S phase of the cell cycle. It consists of a helicase complex that unwinds dsDNA in conjunction with DNA polymerases that copy it and a series of nucleases and ligases that process lagging strands.
Stalled replication forks: replication forks that have prematurely stopped synthesizing DNA.
Syndactyly: congenital defect in which two or more digits are fused together. It

might be genetically inherited, isolated in the context of a specific syndrome, or caused by exposure to environmental factors during pregnancy.
Whole-exome sequencing (WES): high-throughput technique for sequencing of all the protein-coding regions (or the exome) of a specific genome.

10]. 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 POLα-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).

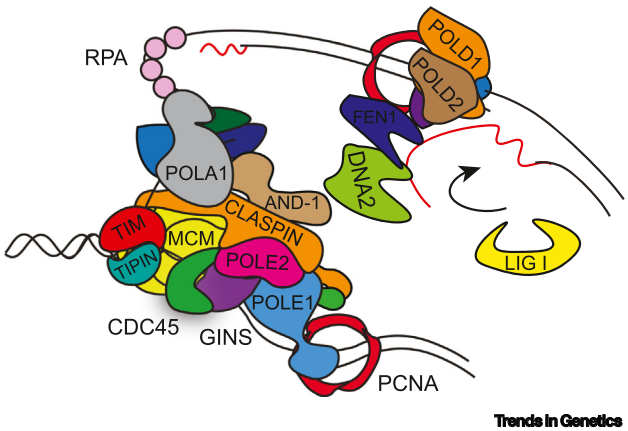

Figure 2. Structure of a Replication Fork and Lagging-Strand DNA Synthesis. Representative cartoon of the main components of the mammalian replisome. The CMG helicase, in concert with POLε, forms the core of the unwinding machine [14]. The TIM–TIPIN complex sits ahead of the replication fork and grips double-stranded DNA [101]. CLASPIN/MRC1 interact with several components of the replisomes, including TIPIN, MCMs, AND-1/CTF4, and POLε [101]. POLα is indirectly tethered to the CMG by the interaction with AND-1/CTF4, a trimeric complex that links the CMG to other components of the replication machinery endowed with a CTF4-interacting peptide (CIP) domain [15]. POLε stably interacts with the CMG via its *POLE1* and *POLE2* subunits and synthesises the leading strand in a continuous manner. POLα synthesises RNA-DNA primers at the lagging strand subsequently extended by POLδ. FEN1 and/or DNA2 cut the 5′ flaps generated by discontinuous lagging-strand replication, which are finally ligated by ligase I [14].

Box 2. Termination of DNA Replication

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 consequence 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^{Dia2} 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 $CUL\text{-}2^{LRR\text{-}1}$ 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 Pif1 and Rrm3 DNA helicases [123]. In addition to $CUL2^{LLR1}$, 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 dwarfisms (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 lower jaw), 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-/extrauterine 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 nonosteodysplastic 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

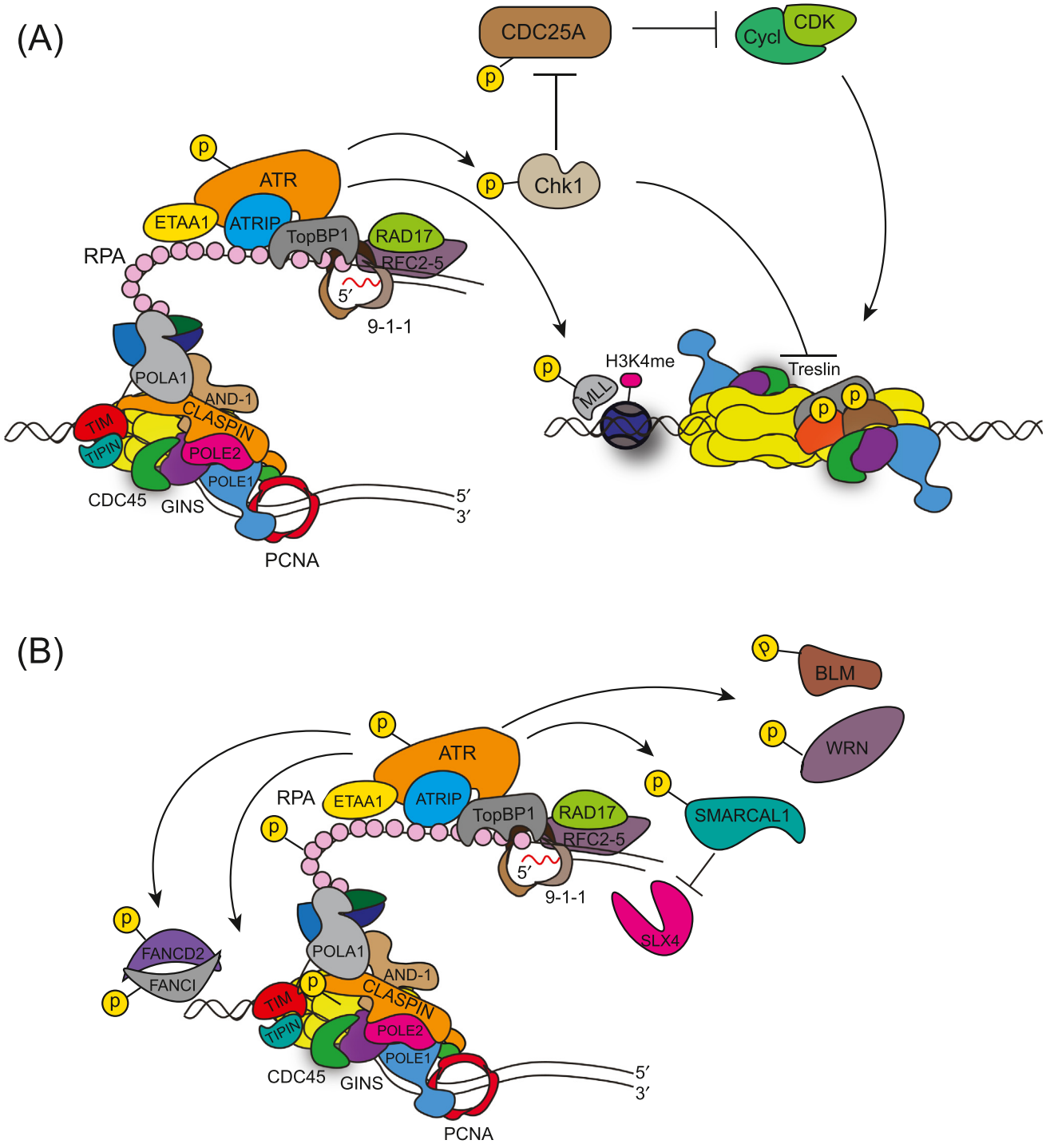

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Figure 3. The Intra-S-Phase Checkpoint. Schematic representation of the intra-S-phase checkpoint and its functions in the control of origin activation (A) and replication fork stability (B) [86]. Discontinuous DNA replication at leading or lagging 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

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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 extrauterine 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).

heterotrimeric, proliferating cell nuclear antigen (PCNA)-like complex composed of RAD9-HUS1-RAD1 [86]. The 9-1-1 complex promotes the stable recruitment of TOPBP1, which is required for ATR activation [106]. Once activated, ATR phosphorylates and activates its downstream effector kinase CHK1, leading to the full activation of the intra-S-phase response [86]. (A) Control of origin activation by the intra-S-phase checkpoint. CHK1 phosphorylates cell division cycle 25 (CDC25) phosphatases such as CDC25A, promoting its proteasomal degradation, thus indirectly affecting CDK-CYCLIN activation, which is required for formation of the pre-initiation complex (PRE-IC) [86]. In addition to this, CHK1 directly interacts with and phosphorylates TopBP1-interacting checkpoint and replication regulator (TICRR)/TRESLIN, whereas ATR can directly block origin activation by phosphorylating the histone methyltransferase mixed-lineage leukaemia (MLL) and promoting H3K4 methylation and blocking of CDC45 recruitment [107,108]. (B) Control of replication fork stability and repair by the intra-S-phase checkpoint. ATR phosphorylates several components of the replisome and can directly or indirectly affect their function. Among them, ATR phosphorylates the replication fork reversal factor SMARCAL1 to prevent abnormal replication fork intermediate transactions and processing by the SLX4 nuclease [109]. In addition to this, ATR directly phosphorylates components of the Fanconi anaemia pathway, RPA, MCMs (mini-chromosome maintenance), and several helicases and nucleases involved in replication fork remodelling [86].

Table 1. Molecular Genetics of Human Inherited Syndromes Caused by Mutations of Replisome Genes

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]
GMNN (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	[55]
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	• MSSL (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] [31]

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 extrauterine 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

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 dysmorphia, 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 dysmorphia 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 5′-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 (ETAA1), 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, Saldivar *et al*. have also shown that ATR is activated during DNA replication via ETAA1 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 XLPDR also experience recurrent infections, predominantly in the respiratory tract, resulting in early-onset bronchiectasis and respiratory failure [50]. Interestingly, patients with XLPDR also exhibit reduced NK cell numbers, particularly differentiated ($CD3^{-}CD56^{dim}$) cells, reminiscent of the previously described *MCM4* mutation [51]. In addition to XLPDR, 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 POLε 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 Schimke 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 extrauterine 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)

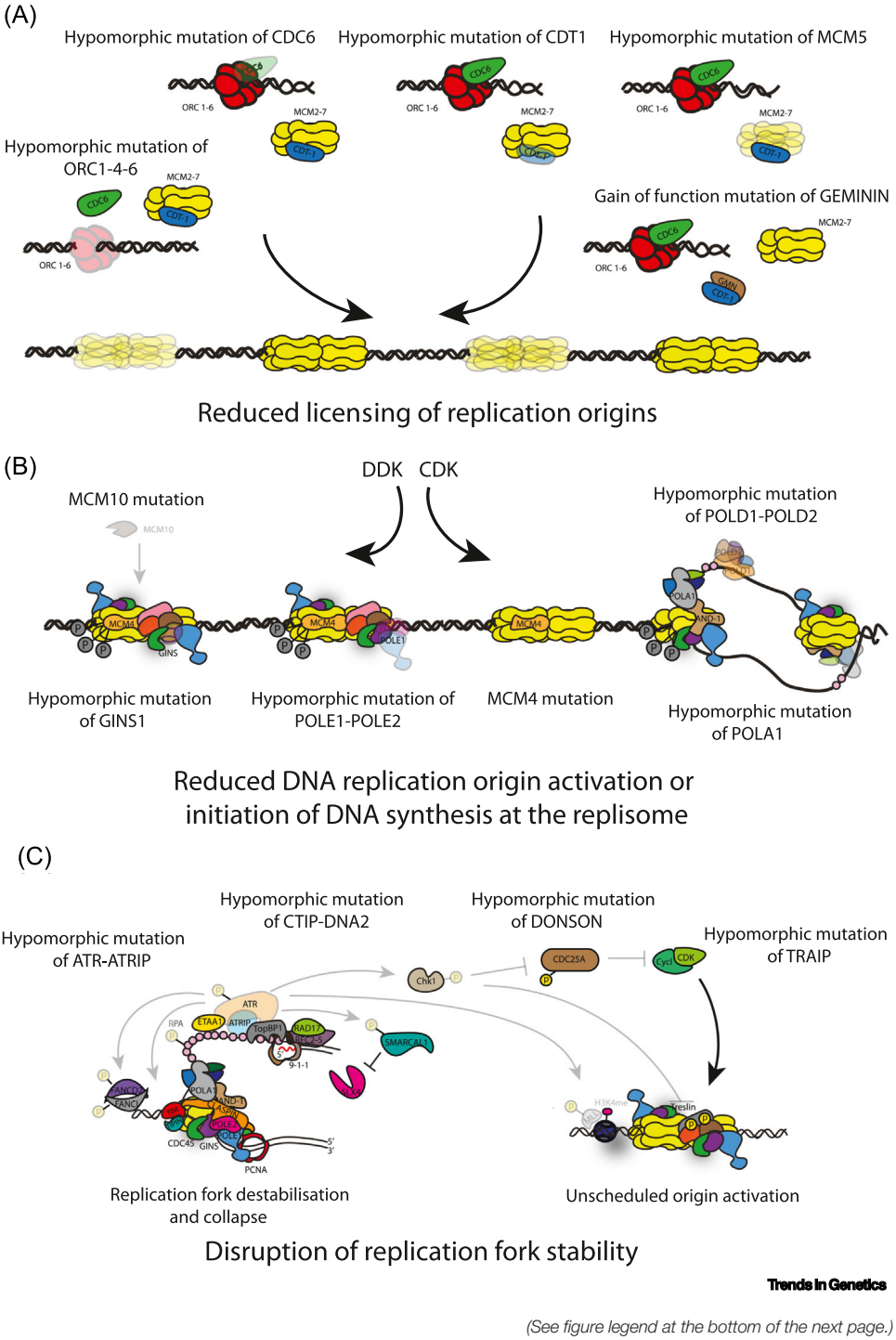
A paradigmatic example is MGORS, whose molecular genetics is tightly linked to the licensing machinery and MCM2–7 loading at replication origins. Initial studies in patient-derived cell lines and zebrafish connected hypomorphic mutations of PRE-RC components to reduced chromatin loading of MCMs, slower progression through S phase, and reduced growth in zebrafish [21] (Figure 4). 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]. *Mcm4*^{Chaos3}, 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 *Mcm4*^{Chaos3} 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 G_2 phases of the cell cycle [69,70] (Figure 1). GEMININ is normally degraded during mitosis and G_1 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] (Figure 4).

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 G_1 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



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(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 $Mcm4^{chaos}$ 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 yeast 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

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 *POLD1*/*POLD2* 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

Box 4. Control of Origin Activation and Replication Fork Stability by the Intra-S-Phase Checkpoint

Multiple mechanisms are responsible for the inhibition of origin activation mediated by the intra-S-phase checkpoint. Importantly, the ATR–CHK1 axis regulates origin firing in unperturbed conditions, because inhibition of ATR and CHK1 induces increased origin firing and reduced interorigin distance in the absence of replication stress, suggesting a global deregulation of origin activation both locally and at distance [109,127]. An important role in this context is exerted by the inhibitory phosphorylation and degradation of CDC25A mediated by CHK1, which reduces cyclin A-CDK2 activity, thus preventing origin activation [86] (Figure 3). In addition to this, several other mechanisms have been proposed to prevent formation of the PRE-IC upon ATR activation. In budding yeast, the essential targets of Rad53 inhibitory activity reside in Sld3 (TICRR/TRESLIN) and Dbf4 (subunit of the CDC7-DBF4 kinase complex) [128,129]. In mammalian cells, the situation is more complex; CHK1 directly interacts with and phosphorylates TICRR/TRESLIN, whereas ATR can also phosphorylate the histone methyltransferase mixed-lineage leukaemia (MLL) to promote H3K4 methylation and inhibit CDC45 chromatin loading at replication origins [107,108].

Another essential activity of the intra-S-phase checkpoint is the maintenance of replication fork stability. Although recent studies in budding yeast and mammalian cells have shown that replisome stability is independent of Mec1/ATR activity, loss of ATR kinase activity is associated with an inability to rescue fork stalling and resume DNA replication [2,130]. The mechanism behind this phenomenon is still incompletely characterised, but multiple ATR-dependent activities likely cooperate to remodel and stabilise stalled replication fork intermediates to prevent their abnormal processing by nucleases, leading to **replication fork collapse** [86]. For instance, ATR phosphorylates the fork remodelling enzyme SMARCAL1 to prevent SLX4-mediated cleavage of replication forks leading to DSB formation [109]. ATR also directly phosphorylates MCMs and Fanconi anaemia proteins to regulate replisome progression and repair as well as helicases of the RECQ family, such as BLM and WRN, which are involved in the metabolism of replication intermediates [86]. Finally, ATR has been shown to directly promote fork slowing and **reversal** to prevent excessive processing of replication fork stalling events [131].

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 [90]. 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 anaemia 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 at 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 gene

Outstanding Questions

Studies in the last two decades have unravelled the mechanistic basis and steps of DNA replication in eukaryotes. However, how dysfunctional DNA replication induces human genetic disease remains to be deciphered. For instance, how are PRE-RC factors assembled at replication origins in Meier-Gorlin syndrome? Where and how in the genome is origin activation perturbed upon hypomorphic mutation of initiation factors such as *GINS1* and *POLE1*?

Although sharing a common set of clinical features, such as reduced intra- and extrauterine growth, many human genetic diseases caused by mutations of replisome components are characterised by the involvement of specific tissues or cell populations. The reason for this phenomenon is unclear. For instance, why do mutations of *MCM4* and *POLE1* specifically affect adrenal gland development? Why do mutations of *POLE1-POLE2* and *POLD1-POLD2* lead to a reduction of T and B cells, whereas mutations of *MCM4*, *MCM10* and *GINS1* induce a selective NK cell deficiency? Studying the dynamics of DNA replication and cellular differentiation in these subpopulations might help to unravel these clinical associations.

The ATR-dependent intra-S-phase checkpoint has a fundamental role in coordinating origin activation and replication fork progression and stability. Although mutation of several components of the replication machinery and/or intra-S-phase checkpoint leads to MPD, the underlying molecular defect remains to be defined. More specifically, what are the genetic lesions that drive defective embryonic development and tissue homeostasis? Where are they occurring in the genome?

Recently, mutations of new factors associated with the replisome, such as *TRAIP* and *DONSON*, have been discovered in MPD. However, the specific function of these proteins and how their hypomorphic mutations induce dysfunctional DNA replication remain to be characterised. For example, mutations of *DONSON* cause a spectrum of clinical manifestations ranging from MGORS to MMS,

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-POLε 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 [100].

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, POLα, and POLε impact this process remains to be defined and might reveal new insights into the pathogenesis of replication-associated human genetic disease.

suggestive of multiple roles in DNA replication and the intra-S-phase checkpoint.

POLα and POLε are also involved in the maintenance of epigenetic integrity during DNA replication. Is hypomorphic mutation of these two components of the replisome impacting epigenetic stability during cellular proliferation and differentiation?

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