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Hisao Masai
Marco Foiani *Editors*

# DNA Replication

## From Old Principles to New Discoveries

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Editors

# DNA Replication

## From Old Principles to New Discoveries

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# Preface

The discovery of the double-helix structure in 1953 provided the basic concept of how genetic materials are duplicated. However, this also triggered the quest for understanding the whole picture of DNA replication. A semi-conservative replication was demonstrated in a historical experiment by Messelson and Stahl, and Arthur Kornberg discovered an enzyme responsible for synthesizing DNA. At that time, no one envisioned such complicated systems required to make copies of DNA.

The proposal of the replicon hypothesis by Jacob made a major impact in pointing the direction in which research of DNA replication in the following years would be led. In fact, the results of genetic studies in bacteria provided evidence for the presence of the factors (initiator and replicator) hypothesized in the model. This was striking and revealing, and the research of DNA replication in the following 50 years tried to recapitulate this finding in different organisms. The elegant single-molecule analyses of replicating DNA in mammalian cells done by Huberman and Riggs in 1966 were interpreted in the framework of the replicon model, becoming the basis for the "multiple replicon" hypothesis for the eukaryotic genomes.

Molecular genetic studies in *Escherichia coli*, in combination with development of recombinant DNA technology, clarified the structures of the replication origin (*oriC*), and "resolution and reconstitution" studies of the single-stranded DNA phage and eventually those of *oriC* replication elucidated the mechanisms of DNA chain elongation and initiation at the bacterial chromosome replicator.

A similar approach was taken for eukaryotes, and the studies using viruses as a model significantly contributed to the elucidation of eukaryotic DNA replication machinery. Genetic studies, in conjunction with newly developed methods for physical mapping of origins, in a unicellular eukaryote, yeast, led to identification of specific sequences that could serve as replicators. In contrast, the "initiator" remained elusive until Bell and Stillman discovered ORC (*O*rigin *R*ecognition *C*omplex), which appears to fulfill all the requirement to be qualified as the "initiator". All the data pointed to the adherence to the old "replicon" principle even in eukaryotes.

Fifty-four years after the replicon hypothesis, almost the entire process for eukaryotic DNA replication was reconstituted with purified proteins, and the

detailed mechanisms are bound to be discovered in the ensuing years. Given the large extent of conservation of most of the core replication factors between yeast and higher eukaryotes, the basic mechanisms of origin activation and assembly of a replisome would be conserved through evolution.

Compared to the replication machinery, the sequences that define the "replicator" appear to have great divergence between species. A genome-wide approach with NGS (Next Generation Sequencing) generated an enormous amount of new information on the profiles of replication origins in higher eukaryotes, but consensus sequences, similar to those found in the yeast replication origins, have not yet been discovered. An additional complication is the absence of sequence-specific DNA binding activity in mammalian ORC. These results suggest the existence of a determinant other than nucleotide sequences that dictate the assembly of a replication complex.

This book compiles various timely topics in DNA replication. The volume starts with a historical description on studies of eukaryotic DNA replication by Professor Thomas Kelly. Professor Kelly has made major contributions in this field for the past over 40 years through his studies on viral DNA replication, human genome replication, and yeast replication. The history of Professor Kelly's research itself represents how this field evolved and materialized into the current understanding of eukaryotic DNA replication. We are very honored and excited to have this chapter at the beginning of the book. This chapter is followed by two chapters describing replication origins in higher eukaryotes. Dr. Mirit Aladjem describes various features that define metazoan replication origins; those include sequence bias, open chromatin, and histone modifications. Drs. Nozomi Sugimoto and Masatoshi Fujita focus on chromatin remodeling factors that determine origin activity though promotion of replication licensing.

Studies in *E. coli*, starting from those on phage replicons to those of *oriC* plasmid, have led the field of DNA replication. In spite of evolutional distance from eukaryotes, the expertise from the bacterial systems have been proven to play leading roles in elucidating the mechanisms of eukaryotic DNA replication. Dr. Tsutomu Katayama describes the most recent discovery on the detailed mechanism of *E. coli oriC* replication and various modes of its regulation. Studies in Archaea have filled unique roles in shedding new light on regulation of eukaryotic DNA replication. Dr. Stephen Bell has contributed a chapter describing initiation and its regulation in Archaea replicons.

In the following two chapters, replication machinery for DNA chain elongation is discussed. Drs. Joseph Stodola and Peter Burgers deal with the mechanism of lagging strand DNA replication in eukaryotes, which is a critical step for DNA chain elongation and is also crucial for stable maintenance of genome, while Drs. Eiji Ohashi and Toshiki Tsurimoto discuss the multiple clamp and its loaders, which are now known to play major roles in coordinating the process of DNA replication with various other chromosome maintenance systems, including checkpoint/DNA damage repair systems, epigenetic transmission, and chromosome partition.

Replication termination is a recent hot topic. In bacteria, replication termination occurs when two replication forks collide 180° from the origin, and their progres-

sion is arrested at the *ter* signals bound with tus protein. On the eukaryotic chromosomes, combined actions of topoisomerase and a ubiquitin ligase are required to dislodge the replisome at the termination site.

The recent technical revolution in cryoelectron microscopy has enabled structural analyses of huge protein complexes at a resolution close to that achieved by X-ray crystallography. Application of this new technology has revealed the complex structures and operation mechanism of MCM (*m*inic*h*romosome *m*aintenance) and CMG helicase as well as more complex replisome assembly. Drs. Yuanliang Zhai and Bik-Kwoon Tye; Drs. Lin Bai, Zuanning Yuan, Jingchuan Sun, Roxana Georgescu, Michael O'Donnell, and Huilin Li describe their state-of-the-art analyses of yeast MCM2–7 double hexameter and the replisome complex, respectively.

Long-standing questions about DNA replication timing regulation are now being addressed in detail at a genome-wide level and detailed landscape of replication domains has been presented in various cell types. Drs. Peiyao Zhao, Juan Carlos Rivera-Mulia, and David Gilbert, a leading group in this area, discuss their current model on how replication domains are related to genome compartmentalization and chromatin architecture. The conserved Rif1 protein, originally identified as a telomere binding factor in yeast, was rediscovered as a critical regulator of replication timing in fission yeast. Rif1 also plays a major role in organizing the replication domains in mammalian cells. Dr. Sara Buonomo describes how chromatin architecture is regulated by Rif1 in mammalian cells to define the replication domains. Drs. Kenji Moriyama, Mong Sing Lai, and Hisao Masai describe functions of Rif1 in both fission yeast and mammalian cells, with particular emphasis on its ability to specifically recognize the G-quadruplex structure (G4), and also the potential roles of G4 in the regulation of chromosome functions. The presence of G4 is one of the most prevalent features associated with replication origins from higher eukaryotes. Dr. Marie-Noëlle Prioleau describes the genetic experiments that show the requirement for G4 in origin activity and discusses the potential roles of G4 in origin regulation.

Chromatin structures play central roles in almost all the metabolism of DNA. Drs. Constance Alabert, Zuzana Jasencakova, and Anja Groth discuss how chromatin is inherited during the course of DNA replication.

DNA replication is a part of cell cycle events. Therefore, how it is integrated in the global cell cycle regulation is an important issue, especially from the point of view that cell proliferation is regulated by the extracellular stimuli, and that it is this pathway that is often deregulated in cancer cells. Drs. Gavin Grant and Jeanette Cook discuss cell cycle regulation of the S phase, with particular emphasis on the events in G1 that are crucial for regulated execution of the S phase.

Modification of proteins with small polypeptides such as ubiquitin or SUMO permits rapid and reversible regulation of various biological reactions, and DNA replication is no exception. Drs. Tarek Abbas and Anindya Dutta describe how unperturbed DNA replication is regulated by protein ubiquitination and its relation to diseases. Drs. Sara Villa-Hernández, Avelino Bueno, and Rodrigo Bermejo describe protein ubiquitylation in cellular responses to perturbed DNA replication

or DNA damages. SUMOylation also recently has been shown to play important roles during DNA replication, which is covered by Drs. Lei Wei and Xiaolan Zhao.

DNA replication and transcription take place on the same template DNA, and how they are coordinated has been an intriguing issue. It is usually assumed that collision of replication and transcription can cause genomic instability and needs to be avoided if possible. However, especially on the eukaryotic genome where there are many origins, this is inevitable. Drs. Yathish Achar and Marco Foiani describe how cells coordinate replication with transcription.

The processes of DNA replication and its regulation are directly linked to maintenance of genomic stability. Overexpression of Cyclin E has been known to induce aberrant DNA replication that leads to genome instability. Drs. Leonardo Teixeira and Steven Reed describe how cyclin E overexpression induces genome instability. Repetitive DNA sequences and sequences capable of forming unusual DNA structures are ubiquitous on the human genome, and increasing evidence points to their pathogenic nature for various diseases. Chromosomal fragile sites, often composed of unusual repetitive sequences, have long been known and implicated in genome instability. Drs. Wenyi Feng and Arijita Chakraborty provide a comprehensive historical account of chromosomal fragile sites and provide a detailed discussion of their disease association and then how their expression is related to DNA replication. Advaitha Madireddy and Jeannine Gerhardt discuss the consequence of replication through repetitive DNA elements and how they could lead to specific diseases.

With the astounding amount of new information on replication origins and protein associations on the chromatin and protein–protein/inter-chromatin networks and with the long-awaited reconstitution system of eukaryotic DNA replication in hand, we are at one of the most exciting moments in the field of DNA replication. While we will gain detailed mechanistic insight into the molecular basis of eukaryotic DNA replication, the basic principle of initiation of eukaryotic chromosomal replication remains elusive. It is becoming clear that initiation of replication in higher eukaryotes is quite distinct from that in bacteria in that it can be initiated almost anywhere, albeit with varied efficiency, and the initiation event may be quite stochastic, pointing to the possibility that the bacterial replicator–initiator concept may not apply to regulation in higher eukaryotes.

We hope that this book will help readers to get a taste of the newest trends in this exciting field and will trigger a new wave of research in search of a new principle of DNA replication.

Tokyo, Japan — Hisao Masai
Milan, Italy — Marco Foiani

# Contents

# About the Editors

**Hisao Masai** Hisao Masai graduated from the University of Tokyo in 1981 and received his Ph.D. from the same university in 1986 for his work on DNA replication in *E. coli*, which he conducted at the DNAX Research Institute (Palo Alto, California) under the supervision of Dr. Ken-ichi Arai. He did postdoctoral studies at the same institute and became an assistant professor (1990) and then associate professor (1995) of the Institute of Medical Science, the University of Tokyo. In 2000, he moved to the Tokyo Metropolitan Institute of Medical Science to be the head of the Department of Cell Biology. He is currently a vice director of the institute, leading a laboratory of genome dynamics. He continues to work on the mechanisms of DNA replication using *E. coli*, fission yeast, mammalian cells, and animal models. One of his current interests is to elucidate the biological significance of G (guanine)-quadruplex in various chromosome transactions including DNA replication. Dr. Masai served on the editorial board of *The Journal of Biological Chemistry* (2009–2012). He is currently an editor for *Biochemical and Biophysical Research Communications* and an associate editor for *Genes to Cells*. The awards he has received include the Tokyo Metropolitan Governor's Award (2006), A-IMBN (Asian International Molecular Biology Network) Arthur Kornberg Memorial Award (2012), the Prize for Science and Technology (Research Category), and the Commendation for Science and Technology from the Minister of Education, Culture, Sports, Science and Technology (2017).

**Marco Foiani** Marco Foiani obtained his Ph.D. in molecular and cell biology from the University of Milan in 1985. After conducting postdoctoral research at the University of Milan and at NIH-NICHD, USA (laboratory of Dr. Alan Hinnebusch), he became a faculty member at the University of Milan in 1990, and in 2000, he became the head of the Genome Integrity Laboratory at IFOM, Milan. He also became a full professor of molecular biology at the University of Milan in 2002. Since 2009 he has been the scientific director of IFOM, an international cancer center that hosts 20 groups and 300 scientists. At IFOM, Dr. Foiani is responsible for strategic research planning, the development of programs aimed at results reduction into practice, and the establishment of national and international cooperation

programs and joint ventures. He is the founder of the European Nanomedicine Foundation (CEN) that aims to support multidisciplinary projects/teams in biomedicine. He is cofounder of the IFOM-IEO Campus, hosting IFOM, the European Institute of Oncology, the European School of Molecular Medicine, the Italian Institute of Technology, and Cogentech. Since 2012 he has directed a cancer genetics diagnostic laboratory within Cogentech. In 2004 he was elected an EMBO member and in 2010 a member of the Academia Europaea. He is currently a member of the editorial board of *Cell*.

# Chapter 1
# Historical Perspective of Eukaryotic DNA Replication

**Thomas Kelly**

**Abstract** The replication of the genome of a eukaryotic cell is a complex process requiring the ordered assembly of multiprotein replisomes at many chromosomal sites. The process is strictly controlled during the cell cycle to ensure the complete and faithful transmission of genetic information to progeny cells. Our current understanding of the mechanisms of eukaryotic DNA replication has evolved over a period of more than 30 years through the efforts of many investigators. The aim of this perspective is to provide a brief history of the major advances during this period.

**Keywords** DNA replication • Eukaryotes • Viral models • Origin of DNA replication • Prereplicative complex • Helicase • Initiator • Replisome

## 1.1 Introduction

The quest to understand how our genomes are duplicated began in earnest with the description of the double helix in 1953. The self-complementary structure of DNA immediately suggested how the information in the sequence of nucleotides could be copied during DNA replication and repaired after DNA damage. The first enzyme activities capable of synthesizing DNA were described within 5 years of the publication of the DNA structure (Bessman et al. 1956), but it would take many more years to achieve even a basic understanding of the complex machinery required to replicate genomes and to elucidate some of the mechanisms that control it. As one of the fundamental processes of life, DNA replication has been a central focus of molecular biology from the very beginning and remains so today.

Early studies of DNA replication focused on prokaryotic systems because of their relative simplicity. The work of many investigators established most of the basic principles that govern DNA replication in all organisms from prokaryotes to archaea and eukaryotes. In 1958 Meselson and Stahl demonstrated that the

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replication process in bacteria is semiconservative (Meselson and Stahl 1958). A few years later, Cairns showed that replication in *E. coli* begins at a single site in the circular chromosome and that DNA synthesis occurs on two arms of a forked structure (Cairns 1963). Taken together, these observations implied the existence of a replication machine that is loaded on the genomic DNA at an "origin" and subsequently moves along the DNA, unwinding the two parental strands and synthesizing new complementary strands. One of the key features of replication machines was discovered in 1968 when Okazaki showed by pulse-labeling experiments that newly synthesized DNA in *E. coli* cells consists of short fragments (Okazaki et al. 1968). Further analysis in vitro suggested that DNA replication is semi-discontinuous at each growing point (Olivera and Bonhoeffer 1972; Herrmann et al. 1972). One strand (the leading strand) is synthesized continuously 5′–3′ in the same direction as the fork moves, while the other strand (the lagging strand) is synthesized discontinuously 5′–3′ in the direction opposite to fork movement, producing short DNA fragments that are subsequently joined together. In the 30 years following the publication of the DNA structure, many of the components of prokaryotic replication machines were identified and characterized, including origin recognition proteins, DNA polymerases, processivity factors, primases, single-stranded DNA-binding proteins, topoisomerases, etc. This saga is the main subject of the superb monographs on DNA replication by Kornberg and more recent reviews (e.g., Kornberg 1981; Kornberg and Baker 1992; Johnson and O'Donnell 2005; Lewis et al. 2016). It was (correctly) surmised that many of the components of the eukaryotic replication machinery would have similar functions to their prokaryotic counterparts.

The identification and characterization of essential prokaryotic replication factors were largely accomplished by classical biochemical approaches – developing assays specific for putative replication functions and then purifying the active proteins(s) to near homogeneity from crude cell extracts. Initial work focused on viruses of *E. coli*, such as T4, T7, and φX174, because their genomes are relatively small and readily obtained. Some of these viruses (e.g., T4, T7) encode most of the factors required for the replication of their genomes, while others (e.g., ϕX174) rely largely on *E. coli* replication proteins. The isolation of many bacterial and phage mutants with conditional defects in DNA replication accelerated the identification and purification of replication proteins by in vitro complementation assays. Other proteins were purified by straightforward fractionation and reconstitution approaches. By 1980 most of the proteins required for propagation of a bacterial replication fork had been identified, and their functions characterized through studies of viruses and plasmids (Kornberg 1981). Understanding of the mechanisms involved in initiation of bacterial DNA replication during the subsequent decade required the development and analysis of cell-free systems capable of replicating plasmids containing *oriC*, the *E. coli* origin of DNA replication (Kornberg and Baker 1992). These studies established that initiation of bacterial DNA replication is largely controlled at the level of occupancy of the *oriC* origin by the DnaA initiator protein, consistent with the replicon model of Jacob et al. (1964).

It was clear early on that eukaryotic DNA replication was likely to be much more complex than prokaryotic DNA replication because eukaryotic genomes can be orders of magnitude larger than their prokaryotic counterparts. It follows that to

complete chromosome duplication in a timely fashion, DNA replication must start at many sites along the chromosomal DNA. Using fiber autoradiographic methods similar to those of Cairns, two graduate students at Caltech, Joel Huberman and Arthur Riggs, demonstrated in 1968 that a mammalian cell utilizes tens of thousands of origins and that replication is bidirectional from each origin (Huberman and Riggs 1968). It was also known that eukaryotic chromosomes are duplicated precisely once each cell cycle, so the requirement for multiple origins raised a number of regulatory issues that were not apparent in bacterial systems with single origins per chromosome. When *E. coli* cells are growing rapidly in rich media, they initiate DNA replication at *oriC* prior to the complete duplication of the chromosome. As a result, DNA synthesis is continuous throughout the cell cycle, and segments of the bacterial chromosome proximal to *oriC* are present in more than two copies (Skarstad and Katayama 2013). This scenario does not happen in eukaryotes where each segment of DNA is duplicated once and only once during a defined period of each cell cycle. These considerations strongly implied that the logic of replication control in eukaryotes would prove to be substantially different from that of prokaryotes and that the biochemical mechanism of initiation of eukaryotic DNA replication would be correspondingly complex. Because of this complexity, the pathway of initiation of eukaryotic DNA replication was not worked out in detail and recapitulated in cell-free systems until comparatively recently.

As described in the succeeding chapters of this volume, the complexity of eukaryotic DNA replication extends to the organization of the machinery at the replication fork. A number of the core components of the eukaryotic (and archaeal) replisomes are unrelated, or only distantly related, to their bacterial counterparts, suggesting that the prokaryotic and eukaryotic/archaeal replication machineries may have evolved largely independently (Edgell and Doolittle 1997; Leipe et al. 1999; Makarova and Koonin 2013). The eukaryotic replisome contains multiple DNA polymerases and a replicative helicase with a considerably more complex subunit composition than that of *E. coli.* In addition, several factors associated with the replisome have no clear prokaryotic counterparts. The reasons for this extra complexity are not yet completely clear, but may be related to the need for greater regulation of DNA chain elongation in large genomes. For example, the packaging of the nuclear DNA into nucleosomes and higher-order structures likely creates unique problems for the elongation machinery. In addition, the replication machinery must deal with multiple sources of endogenous and exogenous DNA damage and potentially other barriers to DNA synthesis, such as large transcriptional units, DNA-binding proteins, etc. While these obstacles are not unique to eukaryotes, the dimension of the problem may be magnified by the large number of replicons involved in the replication of eukaryotic genomes.

In this perspective, I describe some of the major milestones in the study of eukaryotic DNA replication over the past 35 years. There were numerous contributors to this story, and, generally speaking, each of the major advances described here was built on discoveries made in many different laboratories. Given the span of time covered and the great progress that has been made, this perspective can't be comprehensive and of necessity must be somewhat idiosyncratic. While many primary references are provided, they represent a tiny fraction of the important publi-

cations of the last three or four decades, so I have also cited review articles that contain more comprehensive reference lists in particular areas of investigation. In writing this historical review, I was struck by the number of different lines of investigation that have converged to give us our current picture of how genomes are duplicated in eukaryotic cells. The field has grown enormously over the years and is now relatively mature, but the excitement remains.

## 1.2 The Beginning: Viral Models for Eukaryotic DNA Replication

The study of eukaryotic DNA replication was hampered for many years by the primitive state of genetic approaches in most eukaryotes and the lack of simple systems for biochemical analysis. The impasse was overcome by the development and characterization of cell-free systems capable of replicating the genomes of animal viruses. This advance, inspired by the success of previous studies of bacterial virus DNA replication, opened a viable pathway for identifying and purifying cellular replication proteins and characterizing their mechanisms of action. The result was a rapid acceleration of the study of eukaryotic DNA replication.

The replication of the human adenoviruses (Ad) was the first to be established in a completely soluble cell-free system (Challberg and Kelly 1979). The genome of these viruses consists of a linear duplex DNA molecule of about 35 kb with two features that are significant for viral DNA replication: the ends of the genome have identical sequences, and the 5′ terminus of each DNA strand is covalently attached to a virus-encoded protein of 55 kDa (the terminal protein or TP). Extracts of adenovirus-infected human cell nuclei are capable of carrying out the complete replication of exogenously added viral DNA molecules by a mechanism that closely resembles viral DNA synthesis in vivo. Analysis of replication in vitro led to the identification of the minimal essential requirements for the reaction and defined the basic mechanisms involved in initiation and chain elongation (Challberg and Kelly 1982; Stillman 1983; Nagata et al. 1983; Sussenbach and van der Vliet 1984; de Jong et al. 2003). Three viral proteins are required for adenovirus DNA replication: an 80 kDa precursor to the adenovirus 55 kDa terminal protein (pTP), a DNA polymerase (Ad Pol), and a single-stranded DNA-binding protein (Ad DBP). DNA replication is initiated by a novel protein-priming mechanism in which the first nucleotide in the adenovirus genome is covalently linked to a serine residue in the pTP (Rekosh et al. 1977; Challberg et al. 1980, 1982; Desiderio and Kelly 1981; Enomoto et al. 1981; Tamanoi and Stillman 1982; King and van der Vliet 1994). The biochemical mechanism of the initiation reaction is quite interesting although not directly germane to cellular DNA replication. One novel feature of the reaction is the requirement for two cellular transcription factors, nuclear factor I (NF-1/CTF) and Oct-1 (NF-III/Otf-1/Oct-1), for efficient initiation (Nagata et al. 1982; Tamanoi and Stillman 1983; Pruijn et al. 1986; Rosenfeld and Kelly 1986; O'Neill et al. 1988). These two factors bind to sequences at the ends of the viral genome and act to stabilize the binding of a complex of the pTP and Ad Pol and to facilitate the

initial unwinding of the DNA. Together these cellular factors increase the efficiency of initiation by more than 100-fold. After initiation, daughter strand synthesis proceeds in the 5′–3′ direction by a strand displacement mechanism mediated by the adenovirus DNA polymerase and the Ad DBP (Challberg and Kelly 1982; Stillman 1983; Sussenbach and van der Vliet 1984). No separate helicase is required for duplex unwinding during chain elongation. The energy provided by hydrolysis of the nucleotide precursors and by the cooperative binding of the Ad DBP to single-stranded DNA is sufficient to drive strand displacement, which is further facilitated by a cellular topoisomerase. The products of this first round of DNA replication are a daughter duplex and a displaced parental single strand. Annealing of the self-complementary ends of the displaced single strand generates a duplex segment identical to the ends of the original viral genome. Following a second initiation event by the same protein-priming mechanism, complementary strand synthesis proceeds from one end of the genome to the other, generating the second daughter duplex.

Many features of adenovirus DNA replication differ from those of cellular DNA replication. The protein-priming mechanism represents an efficient solution to the end replication problem of linear DNA molecules but is not utilized for the ends of eukaryotic chromosomes. The adenovirus replisome is a remarkably efficient machine, requiring only a DNA polymerase and a single-stranded DNA binding protein, plus topoisomerase activity. At each growing point, only one of the two strands is synthesized, so there is no need for the complexities inherent in discontinuous DNA synthesis. On the other hand, the mechanism is unsuitable for cellular DNA replication because it exposes long regions of single-stranded DNA which is more sensitive to many DNA-damaging agents. Thus, the study of adenovirus DNA replication did not provide much insight into the normal mechanism of cellular DNA replication. However, the extremely rapid progress in defining the adenovirus replication mechanism after the initial development of the in vitro system clearly demonstrated the potential of the fractionation-reconstitution approach for studying DNA replication in eukaryotic cells and provided strong motivation to develop more informative models.

## 1.3 The SV40 DNA Replication System

A major turning point in the study of eukaryotic DNA replication was the discovery that extracts of primate cells infected with the polyomavirus SV40 could support the complete replication of added viral genomes (Li and Kelly 1984). Previous studies had indicated that SV40 offered many advantages as a model system. The viral genome is only about 5 kb and exists in infected cells as a minichromosome with a nucleoprotein structure like that of cellular chromatin. DNA replication initiates at a single origin and proceeds bidirectionally, similar to a single cellular replicon (Danna and Nathans 1972). Importantly, most of the proteins required for SV40 DNA replication are provided by the host cell, so biochemical dissection of the cell-free system provided a powerful approach for identifying cellular replication proteins and characterizing their mechanisms of action (Fig. 1.1a).

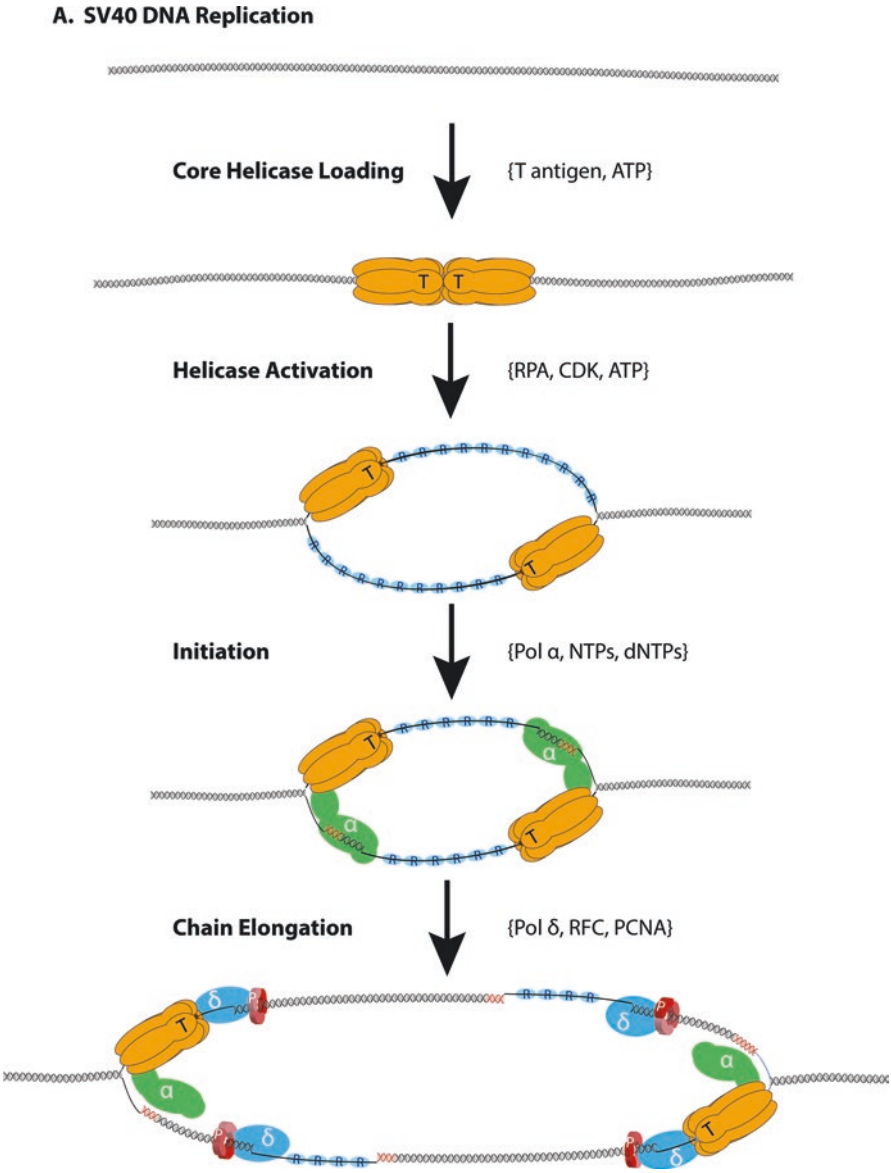


**Fig. 1.1** SV40 and cellular DNA replication. A diagrammatic representation of the major steps in the viral and cellular replication pathways and the protein requirements for each step. See the text for details. (The steps involved in maturation of Okazaki fragments, relaxation of supercoiling, replication termination, and decatenation are left out for clarity.) (**a**) SV40 DNA replication. T, T antigen; α, DNA polymerase alpha-primase; R, RPA; δ, DNA polymerase delta; P, PCNA. (**b**) Cellular DNA replication. M, MCM2-7; 45, Cdc45; G, GINS, ε, DNA polymerase epsilon; α, DNA polymerase alpha-primase; R, RPA; δ, DNA polymerase delta; P, PCNA

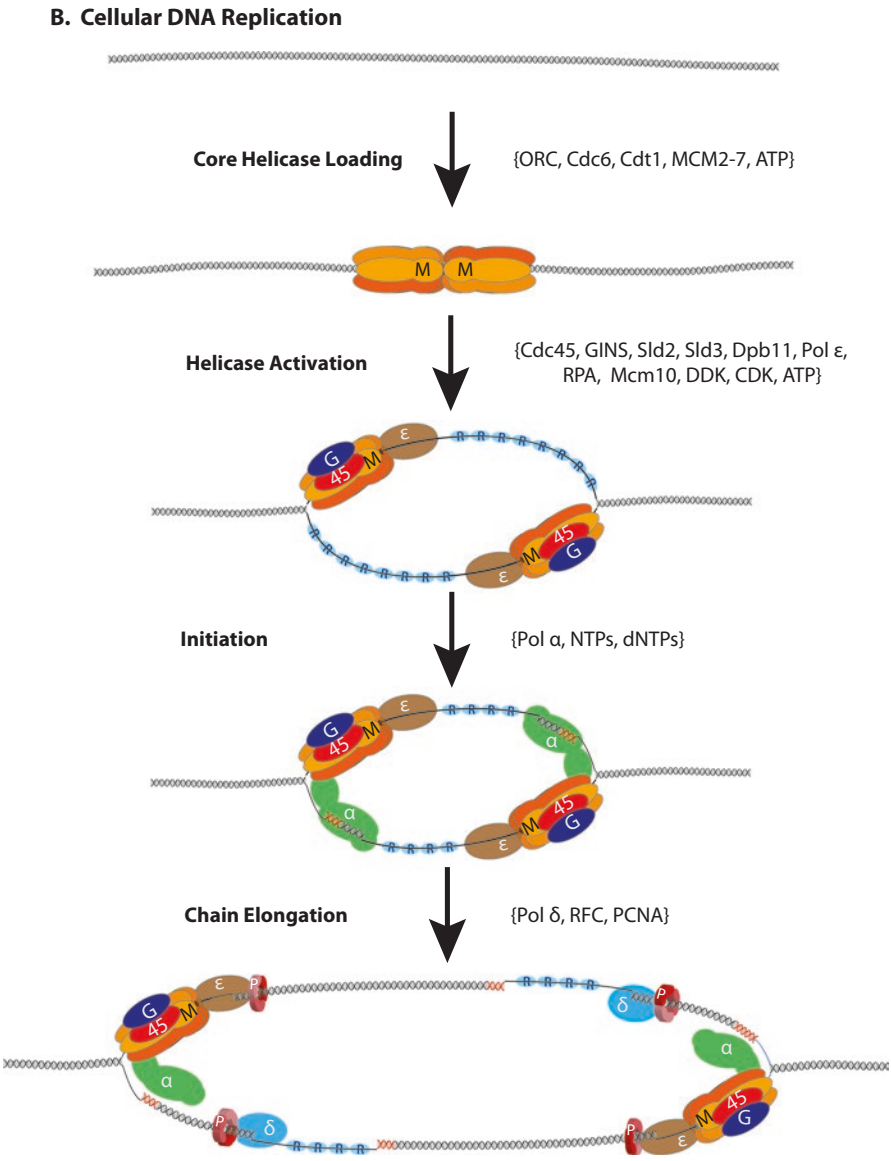


**Fig. 1.1** (continued)

The only SV40-encoded protein required for DNA replication in vivo is the viral T antigen, which binds to the origin of DNA replication and serves both as the initiator protein and as the replicative helicase (Tegtmeyer 1972; Tjian 1978; Delucia et al. 1983). In the initial description of the SV40 cell-free DNA replication system, it was demonstrated that extracts from uninfected primate cells, supplemented with purified T antigen, were sufficient for replication of DNA molecules containing the

SV40 origin of DNA replication (Li and Kelly 1984). Detailed studies demonstrated that T antigen monomers assemble into a double hexamer around the origin DNA in a reaction dependent upon ATP (Dean et al. 1992; Valle et al. 2000). The hexamers interact head-to-head via the N-terminal origin binding domains of T antigen and alter the structure of the origin DNA (Borowiec and Hurwitz 1988). In the presence of ATP and a single-stranded DNA-binding protein, each T antigen hexamer functions as a helicase to unwind the template DNA (Stahl et al. 1986; Dean et al. 1987; Wold et al. 1987). Helicase activity is dependent upon a C-terminal AAA+ module, which binds and hydrolyzes ATP to drive translocation of the hexamer in the 3′–5′ direction along the leading strand template at each replication fork (Fig. 1.1a). Structural studies of the T antigen helicase revealed a double-ring with one tier containing the AAA+ motor domains (Li et al. 2003a). The central channel contains basic residues that can interact with the DNA. The precise mechanism of T antigen helicase translocation on DNA is not yet understood, although structural studies have inspired some interesting models. In addition to serving as the replicative helicase, T antigen interacts with other replication proteins to organize the replisome and coordinate its activities (see more below).

Biochemical analysis of the cell-free system derived from human cells demonstrated that a number of cellular proteins were required for SV40 DNA replication in vitro. All of these proteins proved to be involved in cellular DNA replication and were subjects of extensive biochemical and structural studies in the ensuing years (Reviewed in Kelly 1988; Challberg and Kelly 1989; Stillman 1989; Hurwitz et al. 1990; Brush et al. 1995; Waga and Stillman 1998).

The first cellular protein identified by fractionation of the SV40 system was RPA, the eukaryotic single-stranded DNA-binding protein (Wobbe et al. 1987; Wold and Kelly 1988; Fairman and Stillman 1988; Wold 1997; Chen and Wold 2014). The three nonidentical subunits of RPA contain multiple OB folds that bind single-stranded DNA. RPA was subsequently found to be subject to multiple post-translational modifications and to interact with many cellular proteins involved in a wide range of transactions involving DNA (Chen and Wold 2014). The contrast of the multi-subunit structure of RPA with the simpler bacterial single-stranded DNA-binding protein (SSB) was an early hint that the eukaryotic replisome would prove to be much more complex than that of prokaryotes.

An unexpected discovery that emerged from analysis of the SV40 system was that viral DNA synthesis in vitro is dependent on more than one cellular DNA polymerase. The eukaryotic DNA polymerase α had been discovered many years prior to the development of the SV40 system and was thought to be the major, perhaps the only, replicative DNA polymerase in eukaryotic cells on the basis of many indirect lines of evidence (Campbell 1986; Lehman and Kaguni 1989). After initial difficulties with biochemical characterization, the enzyme was eventually shown to contain four subunits, one of which harbors the polymerase activity. The two smallest subunits comprise a primase enzyme (Tseng and Ahlem 1982; Kaguni et al. 1983; Plevani et al. 1985). The primase catalyzes de novo synthesis of RNA primers on single-stranded DNA templates that can be further extended into nascent DNA chains by the DNA polymerase activity of the enzyme. By antibody depletion and

fractionation/reconstitution experiments, it was demonstrated that DNA polymerase α is absolutely required for SV40 DNA replication in vitro. In the presence of T antigen and RPA, which are sufficient to drive extensive DNA unwinding, DNA polymerase α can initiate DNA synthesis on DNA molecules containing the SV40 origin and catalyze DNA synthesis on both the leading and lagging strand templates (Li and Kelly 1984; Murakami et al. 1986, 1992; Wold et al. 1988; Fig. 1.1a).

DNA polymerase δ, a second eukaryotic DNA polymerase, was identified in the 1970s but had been largely ignored (Byrnes et al. 1976). The activity of DNA polymerase δ was initially distinguished from that of DNA polymerase α because it contained an associated 3′–5′ proofreading exonuclease activity, which was lacking in DNA polymerase α. The first clue that this polymerase might play a role in SV40 DNA replication was the discovery that PCNA, a 37 kDa protein essential for SV40 DNA replication in vitro, was identical to a previously identified factor that increased the processivity of DNA polymerase δ (Tan et al. 1986; Prelich et al. 1987). The requirement for DNA polymerase δ was subsequently confirmed by direct reconstitution of the SV40 replication reaction with purified proteins (Lee et al. 1989; Weinberg and Kelly 1989; Tsurimoto et al. 1990). As described in more detail below, DNA polymerase δ catalyzes the bulk of DNA synthesis on both the leading and lagging strands of SV40 (Fig. 1.1a).

The PCNA processivity factor required for efficient DNA synthesis by DNA polymerase δ was reminiscent of the sliding clamps previously described in the prokaryotic T4 and *E. coli* systems (Tan et al. 1986; Prelich et al. 1987; Tsurimoto and Stillman 1990). It was ultimately shown by elegant biochemical and structural studies that PCNA, the *E. coli* β-clamp, the T4 gp45 sliding clamp, and archaeal sliding clamps are ring-shaped proteins with pseudo sixfold symmetry that accommodate duplex DNA in a topological linkage (Jeruzalmi et al. 2002). The general structure of the rings and the process by which they are loaded onto DNA have been highly conserved in evolution. In addition to its role in mediating processive DNA synthesis, PCNA plays major roles in other processes, such as Okazaki fragment maturation, DNA repair, recombination, chromatin assembly, cell cycle control, etc. (Moldovan et al. 2007).

The eukaryotic clamp loader RF-C, identified initially as a fraction required for SV40 DNA replication, consists of five subunits, each of which contains an AAA+ domain (Tsurimoto and Stillman 1989; Lee et al. 1991; Cai et al. 1996; Bowman et al. 2005). RF-C, like prokaryotic and archaeal clamp loaders, functions as a machine that couples the energy of ATP hydrolysis to open PCNA and load it at a primer terminus (Bowman et al. 2005; Yao et al. 2006). After dissociation of the RF-C loader, DNA polymerase δ associates with the loaded PCNA to form a highly processive complex. Much has been learned about the structural basis of the specificity of RF-C for primer termini and about the biochemical mechanism of clamp loading (Yao and O'Donnell 2012). On the lagging strand, RF-C and other clamp loaders can also function to unload PCNA rings from double-stranded DNA after completion of Okazaki fragment synthesis and ligation so that they can be recycled.

Studies of SV40 DNA replication in vitro also allowed the detailed analysis of the roles of DNA topoisomerases (Yang et al. 1987). It was shown that topoisomer-

ase activity is required during DNA chain elongation to remove supercoils generated by DNA unwinding and that this function can be mediated by either type I or type II topoisomerase. Human topoisomerase I binds to T antigen and may play the predominant role in relieving topological stress at replication forks (Simmons et al. 1996). Interestingly, yeast topoisomerase I co-purifies with the cellular helicase, suggesting that it may also travel with the replication fork (Gambus et al. 2006). Topoisomerase activity is additionally required for the decatenation and segregation of completed daughter DNA molecules synthesized in vitro, but this activity can only be provided by type II topoisomerase. These observations are entirely consistent with studies of SV40 DNA replication in vivo (Sundin and Varshavsky 1980, 1981).

Analysis of the interactions among the proteins required for SV40 DNA replication provided insights into the functional organization of the replication fork, many of which are relevant to understanding the cellular replication fork (Reviewed in Kelly 1988; Challberg and Kelly 1989; Stillman 1989; Hurwitz et al. 1990; Waga and Stillman 1998; Fig. 1.1a). Movement of the replication fork, driven by the T antigen helicase motor, generates single-stranded DNA bound by RPA. During this process, the complex of DNA polymerase δ with PCNA advances, synthesizing the leading strand, while a region of the lagging strand template accumulates in single-stranded form prior to synthesis of a primer by DNA polymerase α. Interestingly, it was observed that DNA polymerase α, in the absence of other factors, is completely unable to initiate primer synthesis on DNA templates that are coated with bound RPA (Collins and Kelly 1991; Melendy and Stillman 1993). This observation led to the discovery that T antigen promotes primer synthesis via specific interactions with DNA polymerase α and RPA that presumably destabilize bound RPA and allow access of primase to the template (Collins and Kelly 1991; Dornreiter et al. 1992; Collins et al. 1993; Melendy and Stillman 1993; Zhou et al. 2012). Thus, the T antigen helicase-DNA polymerase α complex constitutes a mobile primosome that is active in primer synthesis and likely limits priming to the vicinity of replication forks. Presumably, functionally similar interactions involving the cellular helicase or associated factors are required for priming by DNA polymerase α at cellular replication forks.

Another important phenomenon discovered in the SV40 system is switching among DNA polymerases (Lee et al. 1989; Weinberg and Kelly 1989; Tsurimoto et al. 1990; Waga et al. 1994; Waga and Stillman 1994, 1998; Fig. 1.1a). Polymerase switching is unique to eukaryotic DNA replication and occurs in both viral and cellular systems. DNA synthesis is initiated on both the leading and lagging strand templates by the primase activity of DNA polymerase α. After synthesis of a short RNA primer, DNA polymerase α extends it into an "initiator DNA" chain. In the case of SV40, DNA polymerase α is subsequently replaced by the complex of PCNA and DNA polymerase δ, which carries out the bulk of DNA synthesis. The mechanism of this polymerase switch involves a competition between DNA polymerase α and RF-C for the primer terminus (Tsurimoto et al. 1990; Tsurimoto and Stillman 1991a, b; Waga et al. 1994). Since DNA polymerase α is not highly processive, RF-C will gain access to the primer terminus at some point during synthesis of

the initiator DNA. When this happens, RF-C loads PCNA and then dissociates, allowing the binding of DNA polymerase δ to PCNA. The PCNA-DNA polymerase δ complex is stable, creating a highly processive polymerase engine capable of synthesizing long nascent DNA strands. On the leading strand, the polymerase switch is only required once, while on the lagging strand, one switching event occurs for each Okazaki fragment. DNA polymerase δ efficiently completes the synthesis of Okazaki fragments, after which they are joined together by DNA ligase in a maturation process that involves removal of the RNA primer and much of the initiator DNA (Waga and Stillman 1994; Balakrishnan and Bambara 2013). DNA polymerase switching plays an important role in maintaining the integrity of the genome. DNA polymerase δ has a proofreading exonuclease, but DNA polymerase α does not, so the polymerase switch, together with the removal of the primer and much of the initiator DNA during completion of Okazaki fragment synthesis, helps ensure the fidelity of DNA replication. Subsequent genetic and biochemical studies in yeast revealed the existence of a third eukaryotic DNA polymerase, DNA polymerase ε (Pol ε), that functions in cellular DNA replication but apparently not in SV40 DNA replication (Budd et al. 1989; Morrison et al. 1990; Araki et al. 1992; Zlotkin et al. 1996; Pospiech et al. 1999). Multiple recent studies indicate that DNA polymerase ε plays the major role in the synthesis of the leading strand during cellular DNA replication, while DNA polymerase δ synthesizes most of the DNA on the lagging strand (Burgers et al. 2016). It follows that a switch from DNA polymerase α to DNA polymerase ε occurs at the terminus of the leading strand shortly after synthesis of the first primer. The mechanism of this switch is not yet known. However, yeast DNA polymerase ε is quite processive, even in the absence of RF-C and PCNA, so it may simply replace the less processive DNA polymerase α after synthesis of a short segment of initiator DNA. In the yeasts *S. cerevisiae* and *S. pombe*, the catalytic activity of DNA polymerase ε is not essential, although DNA replication is somewhat abnormal in its absence (Dua et al. 1999; Kesti et al. 1999; Feng and D'Urso 2001). In this circumstance DNA polymerase δ is presumably responsible for most DNA synthesis on both the leading and lagging strand templates, as is the case with SV40.

The SV40 system also focused attention on the potential for regulation of DNA replication by protein phosphorylation. Viral DNA replication in vivo and in vitro was found to be completely dependent upon phosphorylation of T antigen on Thr-124 by cyclin-dependent kinase (McVey et al. 1989, 1993; Moarefi et al. 1993). The unphosphorylated protein is competent to bind to the origin and to induce structural distortions in origin DNA and is also competent to catalyze unidirectional unwinding in helicase assays with artificial DNA substrates containing single- and double-stranded DNA. However, the unphosphorylated protein is completely inactive in bidirectional helicase activity from the SV40 origin, indicating that phosphorylation controls a step subsequent to assembly of the T antigen double hexamer on the DNA, possibly an interaction between T antigen hexamers that is required for the initial unwinding of the origin. Interestingly, it was observed that phosphorylation of other residues in T antigen, notably Ser-120 and Ser-123, is inhibitory to initiation of DNA replication and, again, the modifications appear to regulate a step sub-

sequent to assembly of the double hexamer (Virshup et al. 1989, 1992; Scheidtmann et al. 1991; Cegielska and Virshup 1993; Cegielska et al. 1994). Thus, T antigen helicase function is regulated both positively and negatively by phosphorylation. The biological role of this regulation is still unclear, but it may function in part to coordinate SV40 replication with the cell cycle, to enhance the efficiency of production of viral genomes. Consistent with this idea, human CDK can promote SV40 DNA replication in G1 extracts that are normally deficient in replication activity (D'Urso et al. 1990). These discoveries presaged the description of the (much more complex) control of the activation of the cellular helicase by protein phosphorylation (see below).

As the foregoing indicates, analysis of SV40 DNA replication in vitro by many investigators resulted in the identification of key cellular replication proteins and generated many insights into their mechanisms of action. Naturally, these advances kindled great interest in identifying the cellular counterparts of T antigen – the molecules that are required for origin recognition in cellular chromosomes and the molecules that comprise the cellular helicase. A related issue of great interest was how initiation of replication in cells is controlled to ensure that each segment of the genome is faithfully duplicated once each cell cycle. These are the main issues discussed in the remainder of this perspective. To attack these problems, attention shifted from SV40 to other model systems. The analysis of DNA replication in budding yeast proved particularly fruitful because of the availability of highly developed genetic approaches and the existence of defined origins of replication. Fission yeast provided many novel insights as well and, in many cases, complemented the work in budding yeast. Many advances also came from studying replication in extracts of eggs of *Xenopus laevis*. The *Xenopus* system lacks genetics and is not particularly amenable to large-scale fractionation/reconstitution approaches. However, it can be used effectively to test specific requirements for cellular replication by antibody depletion-reconstitution experiments. It is also especially useful for analyzing the regulation of cellular DNA replication.

Because of methodological advances, there was also a gradual shift away from classical approaches for identifying cellular replication proteins by fractionation of crude extracts toward more efficient methods. The products of putative replication genes identified in genetic screens or homology searches were expressed as tagged variants that could be quickly purified and biochemically characterized. But reconstitution with purified proteins remained the gold standard for defining the minimal constellation of proteins required for eukaryotic DNA replication.

## 1.4 Cellular Origins and Initiators

The first cellular origins of DNA replication to be characterized were those of the budding yeast *S. cerevisiae* (Hsiao and Carbon 1979; Stinchcomb et al. 1979; Newlon 1988; Fangman and Brewer 1991; Campbell and Newlon 1991). In early genetic studies, it was discovered that certain chromosomal DNA segments, called

autonomously replicating sequences or ARSs, could increase the transformation efficiency and replication stability of yeast plasmids. It was subsequently shown by direct physical assays that ARS elements represent the start sites for initiation of bidirectional replication in budding yeast (Brewer and Fangman 1987; Huberman et al. 1987). Detailed analysis revealed that ARS elements share a common sequence of 11 bp called the ARS consensus sequence, as well as adjacent, less highly conserved sequence elements (Broach et al. 1983; Celniker et al. 1984; Van Houten and Newlon 1990; Marahrens and Stillman 1992; Breier et al. 2004). The strong sequence specificity and other properties of *S. cerevisiae* origins of DNA replication were similar to the familiar prokaryotic origins of DNA replication and the origins of eukaryotic viruses like SV40. Importantly, this sequence specificity would prove crucial for identifying the cellular proteins that assemble at origins of replication prior to the onset of DNA synthesis. Central among these is the origin recognition complex (ORC), which was detected as a protein that binds specifically to ARS elements and is essential for initiation of DNA replication (Bell and Stillman 1992; Bell et al. 1993). The discovery of ORC was a major advance that opened the way to study the early events in cellular DNA replication.

ORC was found to have six subunits, five of which are related to the AAA+ protein family of ATPases (Bell et al. 1995; Bell and Stillman 1992; Bell 1995; Bell and Dutta 2002; Speck et al. 2005; Clarey et al. 2006). High-affinity binding of ORC to ARS elements is dependent upon ATP binding to the largest subunit, Orc1. In the years following the identification of ORC, a general picture of the first steps in cellular replication emerged from studies in yeast cells and the *Xenopus* egg extract system. These steps result in the loading of the core replicative helicase onto the origin DNA. An early finding that opened a path toward defining the helicase loading reaction was the identification of a protein assembly at yeast origins of DNA replication that is referred to as the prereplicative complex or pre-RC (Diffley and Cocker 1992; Diffley et al. 1994). The pre-RC was originally characterized as a distinct pattern of nuclease protection in genomic footprinting experiments that was observed at yeast origins during the G1 phase of the cell cycle. The requirements for pre-RC formation and the protein composition of the complex were not immediately apparent but were rapidly determined by a variety of experimental approaches (Cocker et al. 1996; Rowles et al. 1996; Coleman et al. 1996; Romanowski et al. 1996b; Donovan et al. 1997; Tanaka et al. 1997; Aparicio et al. 1997; Nishitani et al. 2000; Maiorano et al. 2000; Labib et al. 2001; Devault et al. 2002; Tanaka and Diffley 2002). These experiments demonstrated that in the initial step of pre-RC formation, ORC recruits the Cdc6 protein to the origin. Cdc6 is a AAA+ protein in the same clade as the Orc1-5 subunits. The complex of ORC and Cdc6 then recruits the Cdt1 protein and the MCM2-7 complex. Cdt1, a factor first identified in fission yeast, binds to MCM2-7 and facilitates its interaction with ORC-Cdc6 bound at the origin. The MCM2-7 complex is the core of the eukaryotic replicative helicase. It is loaded onto the origin DNA by ORC-Cdc6-Cdt1 in a reaction requiring ATP hydrolysis (Fig. 1.1b). The nature of the association of MCM2-7 with DNA was not clear from these early experiments but was eventually determined by elegant biochemical studies with purified components (Evrin et al. 2009; Remus et al. 2009; Kawasaki

et al. 2006). Two hexameric rings of MCM2-7 are loaded in a head-to-head configuration with duplex DNA passing through a central channel. In the loading reaction, ORC and Cdc6 function as a molecular machine to close a gate between the Mcm2 and Mcm5 subunits, leaving the MCM2-7 rings topologically linked to the DNA. The current view is that the two hexamers are loaded in separate steps which are dependent on distinct Cdc6 and Cdt1 molecules (Bell and Kaguni 2013; Sun et al. 2014; Ticau et al. 2015). Once the MCM double hexamer is loaded on the DNA, subsequent events in initiation of DNA replication do not require Cdc6, Cdt1, or ORC, and in vitro studies suggest that these factors may dissociate from the DNA (Rowles et al. 1999; Hua and Newport 1998; Gros et al. 2014; On et al. 2014; Ticau et al. 2015). Thus, loaded double hexamers of MCM2-7 mark the potential sites of initiation of DNA replication in chromosomal DNA at the end of the G1 phase of the cell cycle. The transformation of MCM2-7 into an active helicase and the assembly of the other replisome components begin at the onset of S phase and require the CDK and DDK protein kinases (more about this below).

It was quickly established that the early events in DNA replication just described are largely conserved in all eukaryotes. ORC molecules were identified in eukaryotic species other than *S. cerevisiae*, including humans and other metazoa, and the general requirements for pre-RC assembly proved to be universal (Bell and Dutta 2002). However, studies in other systems did not reveal the strong sequence specificity of budding yeast ARS elements. It gradually became apparent that DNA replication origins in most eukaryotes conform to a paradigm that is somewhat different from that of *S. cerevisiae*. A well-characterized example is the fission yeast *S. pombe*. It was possible to isolate segments from *S. pombe* chromosomal DNA that, like ARS elements in budding yeast, confer stable plasmid replication in fission yeast cells (Dubey et al. 1994, 1996; Clyne and Kelly 1995; Dai et al. 2005). However, the properties of the active segments are different from those of budding yeast: they are much larger than budding yeast ARS elements, and they exhibit little conservation of nucleotide sequence. The average AT content of the sequences active as origins is greater than that of the fission yeast genome (Segurado et al. 2003; Dai et al. 2005), an observation that was explained, at least in part, by the discovery that *S. pombe* ORC binds to chromosomal DNA via multiple copies of a DNA-binding motif called the AT hook (Chuang and Kelly 1999; Kong and DePamphilis 2001). But fission yeast origins do not share a common consensus sequence. In fact, the best predictors of the ability of a segment of the fission yeast genome to function as a plasmid origin are AT content and length (Segurado et al. 2003; Dai et al. 2005). At least half the intergenic regions in the fission yeast genome can exhibit detectable origin activity in plasmid assays. It was suggested that the properties of fission yeast origins are best explained by a stochastic model in which ORC can bind and drive initiation of DNA replication at many potential sites in the genome with little intrinsic sequence specificity, and during each cell cycle these potential sites are chosen largely at random with perhaps some preference for AT-rich intergenic DNA (Dai et al. 2005). Subsequent DNA combing experiments demonstrated that the distribution of distances between start sites in a fission yeast genome is exponential, which is completely consistent with a stochastic model for

the spatial distribution of potential origins (Patel et al. 2006; Kaykov and Nurse 2015). The firing of potential origins appears to be stochastic in time as well as space, although the rates of firing per unit time may vary from origin to origin (Heichinger et al. 2006; Kaykov and Nurse 2015).

The nature of origins of DNA replication in metazoan organisms is still not well understood, but the available evidence suggests that there are many potential origin sites and that such sites lack strong sequence determinants (Leonard and Mechali 2013). Consistent with this impression is the observation that purified metazoan ORC molecules, such as those of *Drosophila* or human cells, do not exhibit a strong preference for any particular DNA sequence (Vashee et al. 2003; Remus et al. 2004). Human ORC can drive replication of essentially any DNA molecule in a replication system derived from *Xenopus* eggs (Vashee et al. 2003).

It is important to recognize that even in the case of *S. cerevisiae,* the highly specific interactions between ORC and the ARS consensus sequences are not essential for the fundamental mechanism by which ORC promotes initiation of DNA replication. Specific binding by ORC as observed in budding yeast simply affects the distribution of ORC molecules over the genome. In cell-free systems with *S. cerevisiae* proteins, ORC can efficiently initiate DNA replication on DNA molecules lacking ARS elements (Gros et al. 2014; On et al. 2014). Moreover, deleting chromosomal ARS elements from an *S. cerevisiae* chromosome has surprisingly little effect on the efficiency of DNA replication (Dershowitz et al. 2007). Thus, it seems likely that initiation of DNA replication in most, if not all, eukaryotes may occur on essentially any DNA molecule with some efficiency. *S. cerevisiae* may have acquired the capacity to initiate DNA replication at more specific sites because its chromosomes are relatively small. If replication initiation were purely random, there would be some probability that a small chromosome would not be duplicated in a significant fraction of cell cycles.

Why have eukaryotes largely departed from the prokaryotic paradigm of highly specific origin sequences enshrined in the replicon model? One possibility is that the distribution of chromosomal sites that are available for ORC binding and initiation may vary considerably in eukaryotic cells in different physiological states (e.g., different states of development, different states of differentiation, different tissue environments, etc.) because the composition and organization of chromatin and the pattern of gene expression are different in such states. Thus, the ability to initiate DNA replication in almost any accessible region may be advantageous in the face of the increased complexity of eukaryotic biology.

Because of the many potential origins of replication, the dynamics of DNA replication in an individual eukaryotic cell is extremely complex and is different from one cell cycle to the next. The factors that determine the sites where replication is initiated and the timing of the replication of each genomic sequence are not yet understood in detail in any organism. The overall pattern of DNA replication is established by events that occur at two stages of initiation: the loading of the core helicase in G1 and the activation of the helicase at the onset of S phase. The distribution of loaded MCMs obviously depends upon the relative affinity of ORC for particular DNA sequences, which may be affected to some degree by other components

of the pre-RC (e.g., Cdc6) or by interactions with other cellular proteins (e.g., Beall et al. 2002; Speck and Stillman 2007). The distribution of loaded MCMs is also greatly influenced by competition with the multitude of other chromatin factors that bind to chromosomal DNA. This competition affects the accessibility of chromosomal loci to the helicase loading factors and probably explains why many studies have correlated initiation of DNA replication with so-called open chromatin domains (Berbenetz et al. 2010; Eaton et al. 2011; Leonard and Mechali 2013). Once S phase begins, the loading of MCMs is precluded (see below), so their number and their approximate locations are fixed (although there is some recent evidence that they may have limited mobility) (Gros et al. 2015). Therefore, subsequent events are largely determined by the pattern of activation of loaded MCMs by the CDK and DDK protein kinases to generate functional replisomes. This pattern is also extremely complex. Not all loaded MCMs are activated in a given cell cycle – many are functionally inactivated when they are replicated passively by a replisome originating at another site (Friedman et al. 1997; Santocanale et al. 1999; Vujcic et al. 1999). Thus, the determination of which loaded MCMs are activated and which are not is highly dependent upon their order of activation, and this is likely to be largely stochastic in a given cell cycle. There is no convincing evidence that the ordering of helicase activation in the cell cycle is deterministic, but, as discussed later in this perspective, the relative rates of activation of loaded MCMs at different sites can differ, so that on average activation at some sites occurs earlier than at others. In the end, the timing of replication of a particular sequence in the genome during a given cell cycle is a function of (1) its distance from the flanking initiation sites, (2) the times of initiation at those sites, and (3) the rates at which the forks move after initiation. These factors, particularly the first and second, are subject to variation from one cell cycle to the next. This is an active area of research, and it is expected that further insights will come from increasing the spatial and temporal resolution of single molecule and ensemble approaches to studying replication timing and from improving the methods of analysis in silico.

## 1.5 CMG the Cellular Helicase

Following the discovery of ORC, the cellular initiator protein, another 15 years were required to definitively identify the active form of the cellular helicase. This advance was driven by two separate lines of investigation that eventually came together. Genetic studies in budding yeast defined a group of genes required for the stable maintenance of plasmids as autonomously replicating units in cells (Moir et al. 1982; Maine et al. 1984). Although isolated by several different assays, it was eventually appreciated that the corresponding proteins form a complex, which was named the MCM2-7 complex (for minichromosome maintenance 2-7). The six subunits of the MCM2-7 complex were shown to be related members of the AAA+ superfamily of ATPases (Chong et al. 1996), and a number of studies suggested that they are required for initiation of DNA replication (Yan et al. 1991; Hennessy et al.

1990; Chong et al. 1995; Madine et al. 1995; Romanowski et al. 1996a). The first clue regarding their potential function was the discovery that a sub-complex of MCM4-6-7 purified from human cells possessed ATP-dependent helicase activity in vitro (Ishimi 1997). This insight was followed by the demonstration that the single archaeal MCM protein forms multimeric complexes with robust helicase activity (Kelman et al. 1999; Chong et al. 2000). Analysis of the distribution of MCM proteins in yeast chromosomes during S phase strongly suggested that the MCM complex travels with replication forks, and it was subsequently shown that depletion of MCM subunits interrupts replication fork progression (Aparicio et al. 1997; Labib et al. 2000). Experiments in *Xenopus* egg extracts confirmed that inhibition of MCM activity prevents efficient DNA unwinding (Pacek and Walter 2004). In the course of these studies, it was observed that Cdc45, another protein thought to be involved in initiation of DNA replication, interacts with the MCM complex and is also required for both DNA unwinding and fork progression (Hopwood and Dalton 1996; Aparicio et al. 1997; Pacek and Walter 2004). While these studies, taken together, established the likelihood that the MCM complex was a required component of the cellular helicase, it did not seem to be sufficient for helicase activity, since purified yeast MCM2-7, containing all six subunits, did not have detectable helicase activity under the usual assay conditions.

A second line of investigation that proved critical for the identification of the cellular helicase was a product of the discovery that DNA polymerase ε is required for DNA replication in yeast (Morrison et al. 1990; Araki et al. 1992). A network of proteins that exhibited genetic and physical interactions with each other and with DNA polymerase ε was discovered and characterized. The key players in this network were Dpb11, Sld2, Sld3, and a complex of four proteins called the GINS complex (Araki et al. 1995; Kamimura et al. 1998, 2001; Takayama et al. 2003). It was observed that GINS colocalizes with Cdc45 and MCM2-7 at sites of DNA unwinding during DNA replication and is required to maintain the association of Cdc45 with MCM2-7 during DNA chain elongation (Aparicio et al. 1997; Gambus et al. 2006; Pacek et al. 2006). The definitive biochemical definition of the cellular helicase was a result of experiments aimed at purifying the Cdc45 protein from extracts of *Drosophila* embryos (Moyer et al. 2006; Ilves et al. 2010). The purification yielded a complex of 11 polypeptides that included MCM2-7 and GINS, as well as Cdc45, and is now referred to as CMG. Strikingly, the purified CMG complex possessed robust 3′–5′ helicase activity on partially duplex DNA molecules. Reconstitution of the complex with recombinant proteins revealed that the presence of GINS and Cdc45 dramatically changed the properties of the MCM2-7 complex, increasing its affinity for DNA and stimulating its ATPase activity by two orders of magnitude (Ilves et al. 2010; Costa et al. 2011, 2014). Since GINS and Cdc45 do not have ATPase active sites, the observed stimulation is due to allosteric remodeling of the core MCM2-7 helicase engine. More recent studies have examined the structure of the CMG in the presence of single-stranded DNA and non-hydrolysable ATP (Costa et al. 2014). In this complex the AAA+ motor domains of the MCM2-7 hexamer form a cracked-ring, right-handed spiral with the crack at the MCM2-5 interface as predicted from biochemical studies (Bochman and Schwacha 2008). GINS

and Cdc45 bridge the gap creating a topologically closed toroid. The CMG prefers to bind to single- stranded DNA as expected from previous work demonstrating that a single strand of DNA (the leading strand template) is enclosed by the central channel of the replicative helicase at the replication fork (Fu et al. 2011). The structure and biochemical properties of the CMG are consistent with a steric exclusion model in which unwinding occurs as a result of exclusion of the lagging strand template from the central channel as the helicase engine translocates in the 3′–5′ direction on the leading strand template (Kaplan et al. 2003; Fu et al. 2011; Fig. 1.1b). The molecular details of DNA unwinding by the CMG helicase are not yet clear. Much of the thinking about this issue has come from detailed structural studies of the homohexameric helicases of archaea, SV40 and papillomavirus (Gai et al. 2004; Singleton et al. 2007; Enemark and Joshua-Tor 2008; Hauk and Berger 2016). In these cases, it has been suggested that cooperative structural changes in the AAA+ motors that are mediated by cycles of sequential ATP binding and hydrolysis around the ring drive the motion of DNA-binding loops in the central channel to propel the helicase along the DNA. The same general mechanism may hold for the heterohexameric CMG as well, but there are some data that are not completely consistent with it. For example, only two of the six ATP-binding sites in CMG are absolutely essential for helicase activity (Ilves et al. 2010). In recent structural studies, two alternative states of the CMG have been observed, one compact and the other extended (Yuan et al. 2016; Abid Ali et al. 2016). This observation has led to the proposal that the helicase moves by a ratcheting or inchworm-like motion. The precise mechanism of this critical feature of replisome function awaits further analysis.

## 1.6 Activation of the Cellular Helicase and Building the Replisome

The realization that the core replicative helicase, MCM2-7, was loaded onto DNA in a form completely inactive in DNA unwinding raised the pivotal question of how this structure is converted into an active CMG helicase within a functional replisome during S phase. The transformation of the double-hexamer encircling duplex DNA into an active CMG helicase capable of translocating on single-stranded DNA and the recruitment of additional components to form the complete replisome are still not understood completely, but great progress has been made (Fig. 1.1b). The process is quite complex, involving multiple protein-protein interactions regulated by protein phosphorylation. All of the essential factors are now known, but many aspects of the mechanisms involved in the process remain mysterious (Tanaka and Araki 2013; Yeeles et al. 2015, 2017; Devbhandari et al. 2017; Kurat et al. 2017).

CMG assembly and activation are promoted by two protein kinases, cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK). Each kinase has a regulatory subunit – a cyclin in the case of CDK and Dbf4 in the case of DDK – whose abundance increases at the onset of S phase. The kinases and their regulatory subunits were discovered by multiple lines of investigation, and their positive role

in DNA replication has long been known (Siddiqui et al. 2013). The identification of the key substrates of these enzymes and the elucidation of their roles in CMG assembly and activation were products of a number of independent studies that have only recently converged.

In addition to the protein kinases, the efficient assembly and activation of the replicative helicase are dependent upon several proteins that are not present in the final active helicase. These proteins, Sld2, Sld3, Sld7, and Dpb11 were identified, along with GINS, as factors that interact directly or indirectly with DNA polymerase ε, which becomes associated with the CMG during its assembly (Araki et al. 1995; Kamimura et al. 1998, 2001; Takayama et al. 2003; Tanaka and Araki 2013). Many interactions have been identified among the proteins involved in CMG assembly and probably more will be found (Tanaka and Araki 2013; Deegan et al. 2016). Detailed understanding of the overall pathway will require extensive biochemical and structural studies. At this point in time, the evidence suggests that assembly of the CMG proceeds in (at least) two steps driven by DDK and CDK. DDK phosphorylation of the MCM2-7 double hexamer drives its association with Sld3, Sld7, and Cdc45 (Hardy et al. 1997; Kamimura et al. 2001; Randell et al. 2010; Sheu and Stillman 2010; Heller et al. 2011). In this reaction Sld3 binds to phosphorylated Mcm4 or Mcm6 and then recruits Cdc45 (Deegan et al. 2016). Importantly, recruitment of Sld3 and Cdc45 to MCM2-7 is not dependent upon CDK activity, but the stability of the complex is increased following CDK activation. Sld7 is not essential for this reaction but may increase its efficiency. In budding yeast, it was observed that association of Sld3, Cdc45, and MCM2-7 can occur to a limited extent in cells blocked in G1 phase with alpha factor presumably because of the presence of a low level of active DDK (Aparicio et al. 1999; Kanemaki and Labib 2006; Tanaka et al. 2011; Heller et al. 2011). Subsequent analysis of a cell-free yeast replication system demonstrated clearly that purified DDK can induce the association of the three proteins in extracts derived from alpha factor blocked cells (Heller et al. 2011).

The final assembly of the CMG helicase is driven by CDK phosphorylation of Sld2 and Sld3, which allows the formation of a complex of the two proteins with Dpb11 (Masumoto et al. 2002; Tak et al. 2006; Zegerman and Diffley 2007; Tanaka et al. 2007). Dpb11 contains four BRCT domains. The amino-terminal pair binds phospho-Sld3 and the carboxyl-terminal binds phospho-Sld2. Phosphorylated Sld2 is present in a complex containing Dpb11, GINS, and DNA polymerase ε, which is referred to as the pre-loading complex (Muramatsu et al. 2010). The protein-protein interactions required to form the pre-loading complex are not known in detail, but there is good evidence that the Dpb2 subunit of DNA polymerase ε plays an important structural role in the assembly of the CMG helicase and in maintaining association of the helicase with the leading strand DNA polymerase at the fork (Muramatsu et al. 2010; Sengupta et al. 2013). The binding of Sld2 and Sld3 to Dpb11 thus brings together all of the components required to form the CMG. The initiation of DNA unwinding at an origin requires one additional protein, MCM10, whose role at the molecular level is not yet clear (Kanke et al. 2012; van Deursen et al. 2012; Watase et al. 2012; Yeeles et al. 2015). In vivo and in vitro studies indicate that the stable association of MCM10 with origins occurs after MCM2-7 loading, and its

accumulation is dependent upon DDK and CDK. DNA unwinding also requires the single-stranded DNA-binding protein RPA and, for circular templates, a DNA topoisomerase. How the interactions among this multitude of proteins promote the assembly of the active CMG helicase, remodel the association of the helicase with the two DNA strands, and initiate DNA unwinding are fascinating issues that will be a major focus of future work.

Following the assembly of an active helicase capable of unwinding DNA, other factors are recruited to create a complete replisome at each replication fork (Fig. 1.1b). Exploration of replisome organization and function are at an early stage, but some general features are becoming evident from increasingly sophisticated reconstitution experiments and improved structural studies by cryo-EM. A major milestone was the reconstitution of initiation of *S. cerevisiae* DNA replication with a minimal set of purified proteins (Yeeles et al. 2015). More recently the replication of both naked DNA and chromatin templates was reconstituted in vitro (Kurat et al. 2017; Yeeles et al. 2017; Devbhandari et al. 2017). These advances represent the culmination of years of efforts by many laboratories to define the requirements for eukaryotic DNA replication. Importantly, the work has confirmed the minimal protein requirements for the several stages of DNA replication predicted by previous work (Fig. 1.1b): (1) the loading of the MCM2-7 complex requires ORC, Cdc6, and Cdt1; (2) the formation and activation of the CMG helicase additionally requires Cdc45, GINS, Sld3/7, Sld2, Dpb11, DNA polymerase ε, DDK, CDK, and MCM10; (3) the initiation of DNA synthesis additionally requires RPA and DNA polymerase α; (4) the efficient elongation of leading and lagging strands additionally requires RFC, PCNA, topoisomerase I or II, and DNA polymerase δ; and (5) the maturation of Okazaki fragments additionally requires Fen1 and DNA ligase. All together eukaryotic DNA replication on naked DNA templates requires a minimum more than 40 distinct polypeptide chains.

The reconstitution studies have opened the way to a deeper understanding of the fundamental mechanisms of replication and have already provided several interesting mechanistic details (Devbhandari et al. 2017; Yeeles et al. 2017). For example, it was demonstrated directly that rapid leading strand DNA synthesis at rates comparable to those observed in vivo requires DNA polymerase ε together with the processivity factor PCNA. DNA polymerase δ can support leading strand synthesis when DNA polymerase ε is defective in catalysis, but it functions at a significantly slower rate. On the other hand, DNA polymerase δ in complex with PCNA is essential for the complete synthesis and efficient joining of nascent Okazaki fragments on the lagging strand. Only DNA polymerase δ can carry out the strand displacement synthesis required for primer removal by the Fen1 endonuclease during Okazaki fragment maturation (Devbhandari et al. 2017). Reconstitution studies have also demonstrated that maximal rates of DNA replication require the nonessential protein factors Mrc1 and Csm3/Tof1, which had previously been shown to be associated with active replisomes. The functional role of these factors is not yet clear, but it has been suggested that they accelerate the rate of DNA unwinding by the CMG helicase (Yeeles et al. 2017).

The replication of chromatin templates has also been explored in the reconstituted yeast replication systems (Kurat et al. 2017; Devbhandari et al. 2017). Interestingly, chromatin appears to enforce the origin specificity of *S. cerevisiae* DNA replication. As mentioned earlier, the replication of naked DNA templates in vitro does not exhibit the strong origin dependence characteristic of replication in vivo. However, assembly of the template into chromatin significantly increases the dependence of replication on a canonical yeast origin (Kurat et al. 2017; Devbhandari et al. 2017). The evidence suggests that this effect is due to suppression of ORC binding to non-specific sites (Kurat et al. 2017). Binding of ORC to the origin is less affected by nucleosomes because of its high affinity for specific origin sequences and because origin sequences tend to exclude nucleosomes even in the absence of ORC. The latter phenomenon is reminiscent of the observation that initiation of DNA replication in mammalian cells is correlated with open chromatin domains. With the minimal set of replication proteins listed above, assembly of the CMG helicase readily occurs on chromatin templates, but replisome progression is blocked. A recent study demonstrated that FACT (facilitates chromatin transcription), a dimeric histone chaperone, is sufficient to overcome the block and allow replication to proceed (Kurat et al. 2017). The precise role of FACT is unclear, but it has been suggested that it may function by displacing nucleosomes ahead of the fork or by facilitating the transfer of nucleosomes to the nascent DNA behind the fork. The addition of FACT alone to the minimal reconstituted system, though sufficient for replication, does not restore the full rate of DNA replication observed with naked DNA templates. Several additional factors, including the chaperone Nhp6; the chromatin remodelers, INO80 and ISW1A; and the histone acetyltransferases, pNuA4 and pSAGA, further stimulate the rate of fork movement (Kurat et al. 2017). These seminal studies indicate that detailed analysis of the factors and mechanisms that allow the replication machinery to progress through chromatin (and other protein-DNA complexes) is now feasible, and these issues will clearly be the focus of much future work.

Like the replisomes of prokaryotes and SV40, protein-protein interactions between the replicative helicase and other replisome components are a major organizational principle of the eukaryotic replisome. As described above, the leading strand DNA polymerase, Pol ε, associates with the components of the CMG during helicase assembly. Studies of replisomes isolated from yeast or reconstituted in vitro indicate that DNA polymerase epsilon is bound directly to the CMG (Langston et al. 2014; Georgescu et al. 2015a, b; O'Donnell and Li 2016). An interaction between Psf1 in GINS with the Dpb2 subunit of Pol ε is required to maintain association of the polymerase with the replisome, and crosslinking studies suggest additional contacts with MCM proteins and Cdc45 (Sengupta et al. 2013; Sun et al. 2015). DNA polymerase α-primase may also be tethered to the core CMG. In this case the tether is provided, at least in part, by the protein Ctf4, which was originally identified in a screen for factors affecting the fidelity of chromosome segregation (Zhu et al. 2007; Gambus et al. 2009; Tanaka et al. 2009; Simon et al. 2014; Samora et al. 2016; Sutani and Shirahige 2016; Villa et al. 2016). Recent structural studies have shown that Ctf4 forms a trimer capable of binding to similar motifs present in

the Sld5 subunit of GINS, the catalytic subunit of DNA Pol α, and other proteins (Simon et al. 2014). Linking of Pol α to the CMG likely increases the efficiency of priming of Okazaki fragments on the lagging strand of replication forks. Interactions of the lagging strand DNA polymerase δ and the RF-C clamp loader with other components of the replisome have not yet been observed but may well be important for replisome function.

## 1.7 Replication Control

As noted at the outset of this review, a distinguishing feature of eukaryotic DNA replication is an elaborate and finely tuned set of control mechanisms that ensure the accurate and timely completion of DNA replication and coordinate replication with other events that may occur during a cell cycle. The problem of control is complicated by the existence of thousands of potential sites of initiation in a genome, none of which can fire more than once. Moreover, the replication apparatus must deal with various kinds of damage and other threats to the fidelity of the replication process. While there has been considerable progress, there remains much to be learned about the control of DNA replication, particularly in metazoan organisms.

### *1.7.1 The Two-State Model for Initiation*

Cell fusion experiments performed by Rao and Johnson published in 1970 revealed some of the features of replication control (Rao and Johnson 1970). Their work established that factors present in S phase cells can trigger the initiation of DNA replication in G1 phase nuclei but not G2 phase nuclei. Thus, there appeared to be (at least) two chromosomal states: one that is competent for initiation of DNA replication upon exposure to the S phase activator(s) and one that is not. On the basis of these experiments and subsequent studies in the *Xenopus* egg extract system, it was recognized in a general way that if some factor(s) required for establishment of the competent chromosomal state in G1 phase (a "licensing" factor(s)) were to be inactivated as a consequence of initiation of DNA synthesis and only restored in the following G1 phase, replication of the genome would be limited to a single round in each cell cycle (e.g., Blow and Laskey 1988; Blow 1993). However, the molecular processes responsible for controlling DNA replication remained obscure until the 1990s when the factors that establish the permissive chromosomal state in G1 and block it afterward were largely identified by the convergence of several lines of investigation in budding and fission yeast and the *Xenopus* egg extract system.

An important observation that shaped thinking about replication control was that yeast origins exist in distinct chromatin states before and after the onset of DNA replication (Diffley et al. 1994). In the post-replicative state, the genomic footprint

produced by DNase digestion closely resembled that produced by ORC in vitro, while in the prereplicative state the genomic footprint was larger, suggesting the presence of an additional protein(s). The significance of this observation was twofold: first it suggested that the prereplicative state might be the physical correlate of the state of initiation competence observed by Rao and Johnson, and second, it focused attention on the question of how the formation of the prereplicative state is controlled. It gradually became clear that the establishment of this state corresponds to loading the core MCM2-7 helicase on DNA and that this event is restricted to the G1 phase of the cell cycle by a number of regulatory mechanisms. Since activation of the loaded core helicase is restricted to S phase when further helicase loading is blocked, the outcome is a single round of genome duplication.

Experiments over the last two decades have shown that inhibition of the helicase loading reaction can be coupled to the onset of DNA replication in a number of different ways. Early studies led to the appreciation that CDK, in addition to its positive role in triggering initiation of DNA replication, also functions to inhibit loading of new MCM2-7 complexes on the DNA. Work in fission yeast demonstrated that several kinds of genetic manipulations that reduced CDK activity could induce rereplication in a single cell cycle (Broek et al. 1991; Hayles et al. 1994; Moreno and Nurse 1994; Jallepalli and Kelly 1996). For example, when mutants with a temperature-sensitive allele of *cdc2*$^{+}$, synchronized in G2 phase, were shifted to high temperature to inactivate CDK activity and then returned to a permissive temperature, they underwent a second round of DNA replication prior to mitosis. Similar results were obtained by manipulating the level of an inhibitor of CDK activity. In perhaps the most striking example of this phenomenon, deletion of Cdc13, the major mitotic B cyclin in fission yeast, led to multiple rounds of DNA replication in the absence of mitosis (Hayles et al. 1994). This work, taken together, demonstrated quite clearly that CDK plays a negative role in controlling initiation of DNA replication. Studies in *S. cerevisiae* confirmed this conclusion and, importantly, showed that CDK acts by suppressing the assembly of prereplicative complexes (Dahmann et al. 1995). Thus, by 1996 the basic logic of control of DNA replication was understood (Reviewed in Diffley 1996). ORC, Cdc6, and Cdt1 generate prereplicative complexes from the end of mitosis through G1 when CDK activity is low. An increase in CDK activity activates prereplicative complexes at the onset of S phase (Li et al. 2003b; Takeda et al. 2005; Sugimoto et al. 2004), and the very same kinase activity acts to prevent further assembly of prereplicative complexes. Very soon after these seminal studies in yeast, it was demonstrated in the *Xenopus* egg extract system that similar control mechanisms are operative in vertebrates (Hua et al. 1997). The identification of the targets of negative regulation by CDK became the next key issue, and it was found that all of the players required for loading the core helicase are potential CDK targets in budding yeast (Tanaka et al. 1997; Drury et al. 1997; Nguyen et al. 2000, 2001; Li et al. 2003b; Arias and Walter 2007; Yeeles et al. 2015; Labib et al. 1999). In S phase CDK phosphorylation of Cdc6 marks it for ubiquitin-mediated proteolysis, and the MCM2-7 complex is exported from the nuclease by a CDK-dependent mechanism. In addition, CDK phosphorylation of yeast ORC subunits inhibits loading of the core helicase by a

mechanism that is not yet clear. In fission yeast CDK phosphorylation of Cdc18 (homologue of Cdc6) drives its destruction by ubiquitin-mediated proteolysis, but this mechanism is redundant with a CDK-independent mechanism that targets Cdt1 (Nishitani and Nurse 1995; Jallepalli et al. 1997; Nishitani et al. 2000; Gopalakrishnan et al. 2001; Guarino et al. 2011). In human cells Cdt1 is targeted for proteolysis by a CDK-dependent pathway (Liu et al. 2004).

Studies of the control of DNA replication in the *Xenopus* system uncovered additional mechanisms that are independent of CDK (See Arias and Walter 2007 for review). In metazoans, loading of MCM2-7 is inhibited in S phase by a protein factor called geminin that binds to Cdt1 and sequesters it in an inactive form (McGarry and Kirschner 1998; Wohlschlegel et al. 2000; Tada et al. 2001; Lee et al. 2004; Cook et al. 2004; Lutzmann et al. 2006). Cdt1 is also targeted for replication-dependent proteolysis by an interesting mechanism that is dependent upon specific interaction of Cdt1 with the replication processivity factor, PCNA (Arias and Walter 2005, 2006; Senga et al. 2006). PCNA-dependent proteolysis of Cdt1 is also observed in fission yeast (Ralph et al. 2006; Guarino et al. 2011). Thus, it has become apparent that negative control of replication can involve several partially redundant mechanisms and these may differ to some extent in different eukaryotes. So far, it appears that metazoans inhibit core helicase loading in S phase largely by degrading or blocking the activity of Cdt1.

### *1.7.2 Replication Timing*

The development of methods to physically map sites of initiation in the budding yeast genome led to the discovery that initiation events occur throughout S phase and that the average time of activation of a particular yeast origin depends in part on its chromosomal context (Fangman and Brewer 1991). Why is the activation of loaded MCM2-7s distributed across S phase rather than being concentrated at the beginning of S phase? One possibility is to ensure the completion of DNA replication without sacrificing efficiency. Since loading of additional core helicases is prevented during S phase, DNA replication will be incomplete if forks converging from adjacent initiation sites should stall or collapse. The probability of such an event can be reduced by increasing the number of loaded MCM2-7 hexamers across the genome. However, activating all such hexamers at the same time would be wasteful of resources, since many of them, perhaps the majority, will not be needed. By spreading the activation of loaded hexamers over time, many will be inactivated by passive replication, but those that lie in regions that are, for whatever reason, late to replicate can still be activated to complete replication. Since the loci of stalled or collapsed forks cannot be known a priori, there is no obvious penalty for an activation mechanism that is completely stochastic in time. Thus, it is possible that the average number of potential origins (loaded MCM2-7 hexamers) in cells is determined by a trade-off between reducing the chance of leaving a segment of DNA

unreplicated and using more resources than necessary to load and activate helicases.

Recent evidence suggests that the activation of loaded MCM2-7 hexamers in S phase is largely stochastic in time (see, e.g., Bechhoefer and Rhind 2012). It follows that the average time of initiation at a particular site reflects the relative rate constant for activation of loaded MCM complexes at that site. Other things being equal, an increase or decrease in the probability of activation per unit time would be expected to shift the average time of replication of the neighboring DNA to an earlier or later time, respectively. Variation in initiation timing appears to hold for eukaryotes other than budding yeast, but analysis is more difficult in organisms with less defined origins of DNA replication. The mechanisms responsible for modulating the probability of activation of loaded MCMs per unit time are under active investigation. Emerging evidence suggests that regulation of the activity of DDK in the vicinity of potential origins may be one major mechanism for altering initiation timing. For example, it has been reported that the pericentromeric regions of the *S. pombe* genome initiate replication early in S phase on average, and this phenomenon has been explained by recruitment of DDK by the pericentromeric protein HP1 (Kim and Huberman 2001; Hayashi et al. 2009). On the other hand, subtelomeric sequences in fission yeast (and other eukaryotes) initiate DNA synthesis later than the bulk of the genome, and recent work indicates that the telomere-binding protein Rif1 is required for late replication (Hayano et al. 2012). It has been suggested that Rif1 recruits a cellular phosphatase that inhibits activation of loaded MCMs to replisomes by the DDK protein kinase (Dave et al. 2014; Hiraga et al. 2014; Mattarocci et al. 2014). Other mechanisms that affect replication timing of particular regions of the genome have been described, and it is likely that their numbers will grow.

A very important example of the control of the efficiency of activation of loaded MCMs is the so-called intra-S phase checkpoint. This signal transduction mechanism recognizes when replication forks are slowed, which can occur, for example, if the levels of nucleotide precursors are suboptimal. In this situation a checkpoint-dependent protein kinase is activated and phosphorylates Sld3 and Dbf4, two proteins required for the transition of loaded MCMs to active replisomes (Santocanale and Diffley 1998; Shirahige et al. 1998; Lopez-Mosqueda et al. 2010; Zegerman and Diffley 2010). These phosphorylation events inhibit further activation of loaded MCMs and thus, preferentially, affect origins that normally fire later in S phase. The resulting reduction in the number of active replicons dynamically lessens the cellular requirement for precursors and other replication factors. These examples all suggest that DDK and the protein-protein interactions that it facilitates during helicase activation may be central targets of the mechanisms controlling the timing of initiation within the genome. The biological meaning of timing control seems fairly obvious in some cases – e.g., checkpoint control of initiation in response to perturbations of DNA replication – but remains obscure in other cases, e.g., late replication of telomere proximal DNA and early replication of pericentromeric DNA.

## 1.8 Termination of DNA Synthesis and Disassembly of the Replisome

Termination of DNA synthesis occurs when two convergent forks meet. Early studies of the termination of SV40 DNA replication focused on the roles of DNA topoisomerases as forks converge (Sundin and Varshavsky 1980, 1981). Topoisomerases are required to prevent the buildup of positive writhe in the DNA as the parental strands are unwound. Positive writhe increases the energetic cost of unwinding and, if not removed, can eventually slow or stop fork progression. If the replication fork is free to rotate in space, positive writhe can be manifested as supercoils in the unreplicated DNA in front of the fork or as interlinks (pre-catenanes) of the two daughter duplexes behind the fork. The former can be removed by the action of either a type I or type II topoisomerase, while the latter can only be removed by a type II topoisomerase. As two forks converge, however, the target size for topoisomerase action in front of the fork is progressively reduced. Studies of SV40 DNA replication revealed that replication forks slow as they approach one another and suggested that completion of DNA synthesis at converging forks is highly dependent upon type II topoisomerase, presumably functioning behind the replication fork (Levine et al. 1970; Tapper and DePamphilis 1978; Sundin and Varshavsky 1981; Ishimi et al. 1992). Subsequent work in yeast and the *Xenopus* egg extract system has not revealed a general requirement for type II topoisomerase for termination of cellular DNA replication, although the enzyme may play a special role at barriers to fork progression (DiNardo et al. 1984; Lucas et al. 2001; Baxter and Diffley 2008; Fachinetti et al. 2010). Like SV40, both type I and type II topoisomerases function to remove positive writhe during DNA chain elongation, but DNA replication can be completed in a timely fashion in the absence of type II topoisomerase. Type I topoisomerase appears to be sufficient for complete duplication of the genome. Following completion of replication, type II topoisomerase is uniquely required for decatenation of newly synthesized daughter duplexes to allow chromosome segregation during mitosis (DiNardo et al. 1984).

Another event that occurs when replication terminates at converging forks is the disassembly of the CMG helicase, the core of the replisome. As discussed earlier in this review, the helicase is loaded onto DNA and activated in a complex and highly regulated series of reactions. The CMG is quite stable and presumably remains associated with the replication fork until the termination of DNA synthesis. Early studies revealed that MCM proteins are progressively lost from chromatin during S phase, and more recent work indicates that disassembly of the CMG following the convergence of replication forks is an active process that is also highly regulated (Maric et al. 2014). The CMG helicase is ubiquitylated on the MCM7 subunit after the completion of DNA synthesis. The modification requires a ubiquitin ligase containing the F-box protein, Dia2, which is essential for CMG disassembly. Disassembly is also dependent upon the Cdc48 segregase, a AAA+ family member that associates with ubiquitylated CMG. The action of Cdc48 results in the dissociation of GINS and Cdc45 from the CMG helicase, after which the MCM2-7 hexamer

dissociates from the DNA. It is clearly important that the disassembly reaction only occurs after completion of DNA synthesis and not during active DNA chain elongation. The signal responsible for activating the disassembly machinery has not yet been identified. One interesting speculation is that ubiquitylation of CMG is triggered when completion of DNA synthesis leaves the CMG encircling double-stranded DNA instead of single-stranded DNA (Dewar et al. 2015).

## 1.9 The Future

After more than 35 years of research, the basic features of DNA replication in eukaryotic cells are reasonably well understood. The minimal set of proteins is known, many of their interactions and functions have been defined, and some aspects of regulation of DNA replication are understood at least in outline. Many important and interesting issues remain, some of which have implications for human disease. It is expected that the field will continue to build on the legacy of success that it has enjoyed over the last three to four decades. The following are some areas that are likely to see important advances in the next decade.

The temporal program of replication in individual eukaryotic cells remains of considerable interest, and the central issues are how potential origins are distributed and how they are activated in space and time. There are now excellent tools that can be used to measure replication of individual segments of the genome at different points in the cell cycle under different physiological conditions. These methods will improve in resolution and will likely be coupled to increasingly sophisticated in silico modeling approaches to gain a deeper appreciation of the processes that affect the pattern of replication across the genome. This issue is of particular importance for understanding the mechanisms that underlie the correlation between replication timing and the frequency of mutations in the human genome (Stamatoyannopoulos et al. 2009; Lang and Murray 2011; Woo and Li 2012).

Much remains to be learned about the details of helicase activation and replisome assembly and function. Structural studies by X-ray diffraction and cryo-EM coupled with mechanistic biochemical approaches, including single-molecule experiments, will play an increasingly important role. The path to deeper understanding has been opened by the recent reconstitution of core replication reactions with purified yeast proteins. It will be important in the coming years to extend these approaches to higher eukaryotes. Experiments in the *Xenopus* egg extract system have revealed a number of differences in the regulation of DNA replication between vertebrates and yeast, and it is likely that more differences will be uncovered.

Another question of vital interest is how the replication machinery coexists with other nuclear processes, including transcription, chromatin assembly, and various repair processes. A closely related issue is how replication deals with proteins and RNAs that compete with replication proteins for access to the DNA substrate. These factors can clearly affect both the initiation of DNA replication and the elongation of replication forks and can now be studied in vitro with purified proteins. As illus-

trated by recent studies of Csm3/Tof1 and Mrc1, the recent reconstitution of yeast DNA replication will make it possible to define the functional roles of proteins that normally travel with the replisome but are not absolutely essential for DNA replication (Yeeles et al. 2017). Such proteins may function to allow forks to deal with barriers to chain elongation or other factors that may perturb ongoing DNA replication. It is expected that progress in understanding the role of these additional factors will now be rapid.

Finally, a question that is of great significance for disease and aging is how the replication machinery deals with DNA damage and how replication is integrated with the cellular DNA damage response. It has become apparent that most somatic mutations are the result of encounters of the replication apparatus with damage of various kinds in the template. With the tools in hand, it should be possible to gain a much deeper understanding of the results of such encounters. There is no doubt that such understanding would contribute to building better approaches to prevention and therapy of cancer and perhaps provide insights into the origins of somatic mutations that contribute to aging.

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# Chapter 2
# Regulation of Replication Origins

**Anna B. Marks, Haiqing Fu, and Mirit I. Aladjem**

**Abstract** In eukaryotes, genome duplication starts concomitantly at many replication initiation sites termed replication origins. The replication initiation program is spatially and temporally coordinated to ensure accurate, efficient DNA synthesis that duplicates the entire genome while maintaining other chromatin-dependent functions. Unlike in prokaryotes, not all potential replication origins in eukaryotes are needed for complete genome duplication during each cell cycle. Instead, eukaryotic cells vary the use of initiation sites so that only a fraction of potential replication origins initiate replication each cell cycle. Flexibility in origin choice allows each eukaryotic cell type to utilize different initiation sites, corresponding to unique nuclear DNA packaging patterns. These patterns coordinate replication with gene expression and chromatin condensation. Budding yeast replication origins share a consensus sequence that marks potential initiation sites. Metazoan origins, on the other hand, lack a consensus sequence. Rather, they are associated with a collection of structural features, chromatin packaging features, histone modifications, transcription, and DNA-DNA/DNA-protein interactions. These features confer cell type-specific replication and expression and play an essential role in maintaining genomic stability.

**Keywords** DNA replication • Cell cycle regulation • Replication origin licensing • Chromatin organization • Histone modification • Replication timing

## 2.1 Initiation of DNA Replication

Origins of replication are defined as chromosomal sites where double-stranded DNA unwinds to form single-stranded DNA templates for genome duplication. Genetically, the *cis*-acting sequences that determine the locations of replication initiation events are termed replicators (Jacob et al. 1963). Replicators can confer the ability to start replication when transferred from their original locations to ectopic sites. Replicators

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interact with trans-acting factors, termed initiators, to facilitate DNA replication. In eukaryotes, initiators are highly conserved, as all eukaryotes share a group of essential DNA-binding protein complexes that form pre-replication complexes. Pre-replication complexes assemble on chromatin in a process termed "replication licensing," and the components of pre-replication complexes are orthologous in all eukaryotes (Aladjem 2007; DePamphilis 1999; Fragkos et al. 2015; Masai et al. 2010; Remus and Diffley 2009). Conversely, the chromatin features associated with eukaryotic replicators vary and are often cell type and/or developmental specific (Aladjem 2007; Besnard et al. 2012; Cayrou et al. 2015; Smith and Aladjem 2014; Smith et al. 2016).

The conserved proteins that form pre-replication complexes (Fig. 2.1) include a DNA-binding origin recognition complex (ORC) that serves as a platform to recruit

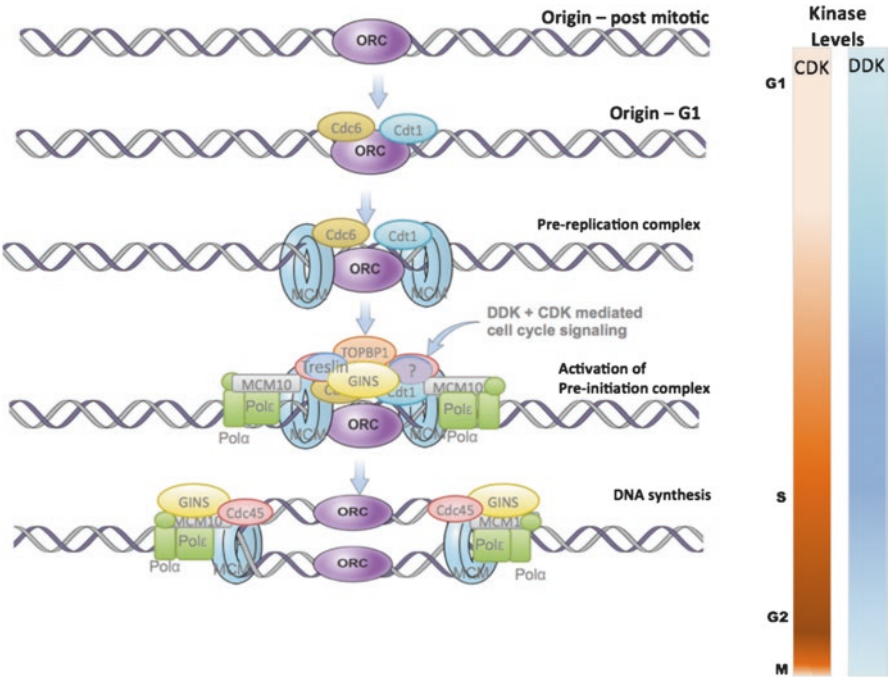


**Fig. 2.1** Pre-replication complex proteins bind in a stepwise manner throughout G1. Recruited during the M to G1 transition, the origin recognition complex (ORC) is a platform to recruit a conserved group of helicases, polymerases, and accessory proteins that catalyze the initiation of DNA replication. ORC binds to chromatin as cells emerge from mitosis. Licensing factors Cdc6 and Cdt1 bind to ORC, followed by the inactive form of the replicative helicase MCM2-7. Additional proteins are required to activate the MCM helicase and initiate DNA replication. Specifically, the inactive MCM2-7 helicase then interacts with CDC45, MCM10, and GINS (Sld5, Psf1, Psf2, Psf3) to form the complete helicase (CMG) complex. Cyclin-dependent kinase (CDK)- and Dbf4-dependent kinase (DDK)-mediated phosphorylation activates proteins and allows Cdc45 interacts with Treslin (Sld3 in yeast). Sld2/RecQL4 and DPB11/TopBP1 are then recruited to the complex. Chromatin-associated DNA polymerases (Pol-α and pol-δ), replication protein A (RPA), CMG, and Dpb11/TopBP1 then initiate DNA replication

a conserved group of helicases, polymerases, and accessory proteins that catalyze the initiation of DNA replication. Assembly of the conserved pre-replication and pre-initiation complexes at potential replication origins occurs stepwise during the G1 phase of each the cell cycle (Aladjem 2007; DePamphilis 1999; Fragkos et al. 2015; Remus and Diffley 2009). First, ORC binds to chromatin as cells emerge from mitosis. Then, licensing factors CDC6 and CDT1 bind to ORC, which allows for the binding of the inactive form of the replicative helicase MCM2-7. The resulting pre-replication complex recruits additional proteins required to activate the MCM helicase and initiate DNA replication (Boos et al. 2013; Sansam et al. 2015; Sheu et al. 2016; Tanaka and Araki 2013). The chromatin-bound but inactive MCM2-7 helicase then interacts with additional components, CDC45, MCM10, and GINS (Sld5, Psf1, Psf2, Psf3), to form the complete helicase (CMG) complex. Prompted by cyclin-dependent kinase (CDK)- and Dbf4-dependent kinase (DDK)-mediated phosphorylation, CDC45 interacts with Treslin (Sld3 in yeast), which recruits Sld2/RecQL4 and DPB11/TopBP1 (Bruck et al. 2015; Depamphilis et al. 2012; Masai et al. 2010; Remus and Diffley 2009; Zegerman and Diffley 2007). Chromatin-associated DNA polymerases (Pol-α and pol-δ), replication protein A (RPA), CMG, and Dpb11/TopBP1 then initiate DNA replication (Abid and Costa 2016; Kanemaki and Labib 2006; Takayama et al. 2003).

The assembly of pre-replication complexes and the subsequent initiation are tightly coupled with cell cycle progression by the phosphorylation activities of two kinases, CDK and DDK. Helicase recruitment, in an inactive form, can only occur at low kinase levels (Bell 2002; Remus and Diffley 2009), whereas the activities of DDK and CDK are required at subsequent steps to activate the helicase and initiate replication (Boos et al. 2013; Remus and Diffley 2009; Sansam et al. 2015; Sheu et al. 2016; Tanaka and Araki 2013). The need for low kinase levels in the early stages of pre-replication complex assembly implies that such complexes cannot be assembled once DNA replication has started, insuring orderly cell cycle progression as well as preventing re-replication of cellular DNA, a hallmark of genomic instability (Abbas et al. 2013; Hanlon and Li 2015; Remus and Diffley 2009; Richardson and Li 2014).

Although the events that lead to initiation of DNA replication occur at all potential replication origins, replication initiation occurs with a remarkably consistent order in most cells (Besnard et al. 2012; Cayrou et al. 2011; Martin et al. 2011) to create a coordinated replication timing program (Koren et al. 2014; Mukhopadhyay et al. 2014; Rhind and Gilbert 2013b). The binding patterns of pre-replication complexes do not provide clues to the principles of origin choice as ORC does not exhibit sequence-specific DNA binding (Miotto et al. 2016) and replication origins in most eukaryotes do not share a clear common consensus (Aladjem 2007; Bartholdy et al. 2015; Leonard and Mechali 2013; Masai et al. 2010). Interactions between replication origins and components of pre-replication complexes, therefore, are essential for initiation but cannot intuitively explain the consistent replication patterns observed in most mitotic cell cycles.

## 2.2 Genetic Features and Local Determinants of Replication Origins

Replication origins in viruses and in some prokaryotes and eukaryotes exhibit distinct sequence features that facilitate interactions with unique initiator proteins. In DNA tumor viruses, replication origins colocalize with replicator sequences that bind specialized initiators (e.g., SV40 T-antigen and BPV E1 protein) to catalyze DNA unwinding and recruit the host replication machinery (Fanning and Zhao 2009). In budding yeast, replication origins contain an AT-rich, 11 bp consensus ORC binding sequence. High frequency of initiation from yeast replication origins also requires accessory sequences that affect chromatin structure by dictating the efficiency of ORC binding as well as directing initiation in unique chromosomal environments (Hoggard et al. 2013; Marahrens and Stillman 1992; Palacios DeBeer et al. 2003).

In metazoa, replication origin sequences exhibit high heterogeneity (Aladjem 2007; Bartholdy et al. 2015; DePamphilis 1999; Fragkos et al. 2015) and do not share a clear consensus, consistent with the observation that not all potential replication origins initiate replication in all cells each cell cycle. There are two sources of replication origin heterogeneity. First, a large fraction of replication origins exhibit consistent initiation in particular cell types and not in other cells (Besnard et al. 2012; Cayrou et al. 2015; Martin et al. 2011; Smith et al. 2016). For example, less than 50% of all origins identified in a survey of eight cell lines initiated replication in all cells within that cohort, and a large fraction (about 15%) only initiated replication in a single cell line (Smith et al. 2016). Second, within populations of cells of the same type, most replication origins initiate replication stochastically in a fraction of cell cycles. In most somatic metazoan cells, only 10–20% of all potential origins actually initiate replication each cell cycle, suggesting that most origins exhibit flexible initiation patterns (Cayrou et al. 2015). When such flexible origins remain "dormant" and do not initiate replication, they replicate passively from adjacent replication forks (Fig. 2.2). Notably, however, the presence of excess replication origins plays a role in genome preservation: a marked reduction in the frequency of licensed but dormant origins, achieved either by targeted genetic deletions of origins or by mutating replication licensing factors, increases genomic instability (Abbas et al. 2013; Besnard et al. 2012; Blow et al. 2011; Fragkos et al. 2015; Kawabata et al. 2011; Marks et al. 2016). These observations suggest that flexible or consistent dormant origins, which rarely or never initiate replication during normal cell cycle progression, might serve as backup origins when replication forks stall.

The exact sequence features that mark replication origins and determine the frequency of initiation at each origin remain to be elucidated. Budding yeast potential replication origins share a consensus sequence that is recognized by the ORC complex. Yeast origins can all initiate replication on plasmids (Marahrens and Stillman 1992; Masai et al. 2010), but chromatin context plays a role in determining the activation rates of particular chromosomal origins (Hoggard et al. 2013; Knott et al. 2012). Metazoan replication origins do not exhibit a prominent single consensus sequence (Aladjem 2007; Fragkos et al. 2015; Mechali et al. 2013; Urban et al.

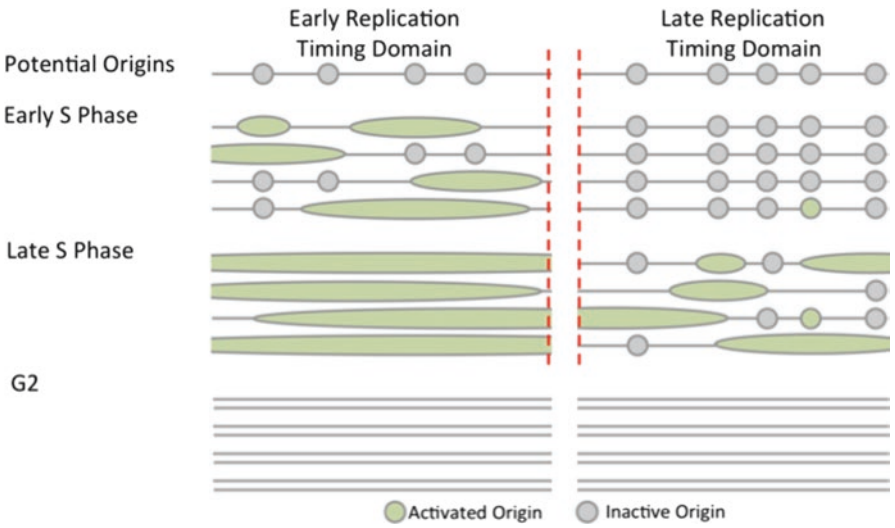


**Fig. 2.2** While the profiles of activated replication origins are similar within cell line, specific replication origin chosen by cells within that population varies in location and replication time. Most origins exhibit flexible initiation patterns, where the specific origins activated differ between cells. When such flexible origins remain "dormant" and do not initiate replication, they replicate passively from adjacent replication forks

2015) but share several sequence features (Fig. 2.3a) including regions that exhibit strand asymmetry, CpG islands, G-quadruplexes, transcription start sites, origin G-rich repeated elements (OGREs), and regions of DNase hypersensitivity (Besnard et al. 2012; Cayrou et al. 2015; Foulk et al. 2015; Martin et al. 2011; Mukhopadhyay et al. 2014; Rao et al. 2014). Of those, genetic association studies on phased genome revealed strong high association with strand asymmetry (Bartholdy et al. 2015).

The primary DNA sequence at replication origins can determine the ability to initiate replication at ectopic sites (Aladjem et al. 1998; Liu et al. 2003), possibly via affecting chromatin modifications (Chen et al. 2013; Conner and Aladjem 2012; Fu et al. 2006; Liu et al. 2003). For example, the replication origin at the human *HBB* locus can create an open chromatin conformation at ectopic sites (Fu et al. 2006). A group of replication origins colocalizes with ubiquitous chromatin opening elements (UCOES), which maintain open chromatin structure by recruiting transcription factors (Conner and Aladjem 2012; Majocchi et al. 2014) and protecting transcriptional activity despite local repressive epigenetic features (Flickinger 2015). Hence, recruiting chromatin modifiers could allow replication origins to alter the local environment and create a context permissive for both transcriptional activity and replication initiation (Aladjem 2007; Hassan-Zadeh et al. 2012; Huang et al. 2011).

In agreement, local histone modifications correlate with, and can determine, replication origin locations (Feng et al. 2006; Leonard and Mechali 2013; Mechali et al. 2013; Rhind and Gilbert 2013b; Smith and Aladjem 2014; Smith et al. 2016; Vogelauer et al. 2002). Comparisons of initiation sites with histone features and

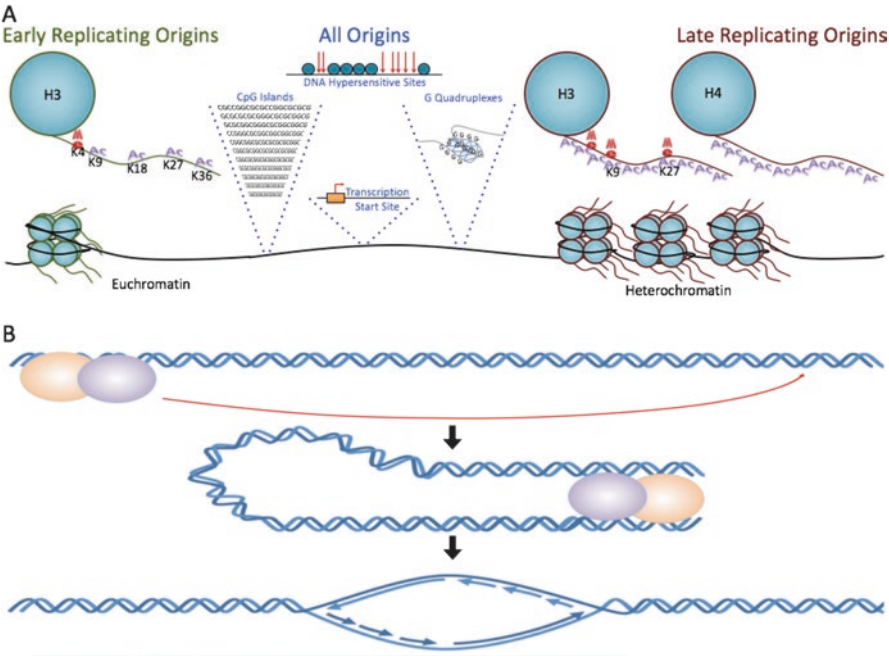


**Fig. 2.3** (**a**) Metazoan replication origins share several sequence features. Origins generally associate with regions that exhibit strand asymmetry, CpG islands, G-quadruplexes, transcription start sites, origin G-rich-repeated elements (OGREs), and regions of DNase hypersensitivity. In agreement, local histone modifications correlate with and can determine replication origin locations and timing. Early replicating regions associate with H3K4me1/2/3, H3K9ac, H3K18ac, H3K36me3, and H3K27ac. These histone modifications also associate with open chromatin and are enriched in moderately active transcription start sites. Late replicating regions tend to associate with H3 and H4 hypoacetylation, H3K9 and H3K27 methylation, and are found in heterochromatic regions. (**b**) Distal DNA sequences affect origin activity and transcriptional through long-distance interactions. Such interactions can be mediated via protein interaction with enhancers and locus control regions, by chromatin remodeling factors and transcriptional activators that bind enhancers and locus control regions, and by long noncoding RNAs

replication timing domains identify certain histone modifications as strong indicators of origin utilization (Fig. 2.3a). For example, early replicating regions associate with H3K4me1/2/3, H3K9ac, H3K18ac, H3K36me3, and H3K27ac (Smith et al. 2016). Origins that associate with those chromatin features also localize in open chromatin and are enriched in moderately active transcription start sites (Besnard et al. 2012; Martin et al. 2011). Replication origins that initiate replication early during S-phase tend to associate with open chromatin features and are often activated in many cell types. In contrast, late replicating regions tend to associate with H3 and H4 hypoacetylation and H3K9 and H3K27 methylation and often initiate replication in a cell type-specific manner (Cayrou et al. 2015; Mechali et al. 2013; Smith et al. 2016). The tendency to initiate replication in most cells or in a particular cell type does not depend on cancer status, as common and cell type-specific initia-

tion activity correlates with cellular lineages rather than with cancer or noncancer (Smith et al. 2016).

Transcriptional activity of local genes and cellular differentiation state can alter the replication timing program. For example, *Xenopus* early embryos do not exhibit a strong preference for initiation sites, correlated with the absence of transcription (Mechali and Kearsey 1984). In those embryos, induced transcription either through development or by tethering specific transcription factors resulted in increased localized initiation (Fragkos et al. 2015; Mechali et al. 2013). In somatic dividing cells, replication origins often associate with transcription start sites at active genes (Valenzuela et al. 2011) and, in particular, with transcription start sites at moderately active transcribed regions (Martin et al. 2011; Sequeira-Mendes et al. 2009). However, this association is diminished in highly transcribed regions, suggesting that transcription and replication regulate each other to avoid disruptions and polymerase collision events (Martin et al. 2011; Sequeira-Mendes et al. 2009). In agreement, transcriptional activity coordinates with the replication timing program (Rivera-Mulia et al. 2015), and replication delays often accompany gene silencing.

## 2.3 Influences of Global Chromatin Organization on Replication Initiation

On a larger scale, replication timing domains, each containing multiple replication origins that replicate concomitantly (Bartholdy et al. 2015; Mukhopadhyay et al. 2014), exhibit high concordance with large-scale chromatin organization units termed topologically associated domains that encompass several hundred kilobases to megabases (Hiratani et al. 2010; Lieberman-Aiden et al. 2009; Mattarocci et al. 2014; Moindrot et al. 2012; Rao et al. 2014; Rhind and Gilbert 2013b; Yaffe et al. 2010). This association suggests that the time of activation of replication origins reflects a fundamental structural property of the nucleus (Hiratani et al. 2010; Moindrot et al. 2012; Pope et al. 2014; Rhind and Gilbert 2013b; Yaffe et al. 2010). In agreement, replication origins are known to associate nuclear structural features such as matrix attachment sites (MARS), scaffold attachment sites (SARs), and stabilizing anti-repressor elements (STARs) (Mechali et al. 2013; Smith and Aladjem 2014) as well as with lamins and cohesins (Cayrou et al. 2015; Smith and Aladjem 2014).

High-resolution whole-genome analyses reveal that replication timing domains often reflect chromatin modifications (Dileep et al. 2015; Pope et al. 2014). Early replicating regions often associate with transcriptionally active topological domains, whereas late replicating origins often associate with heterochromatin. The effects of the primary sequence on replication origin activity and replication timing were assessed using analyses on phased genomes, which permit identification of paternal vs. maternal origins to characterize the effects of specific sequence variations on origin activity (Bartholdy et al. 2015; Mukhopadhyay et al. 2014), and by identify-

ing inherited alleles that affect replication timing following the sequencing of 161 individual proliferating cell samples (Koren et al. 2014). These analyses have demonstrated that *cis*-acting genetic elements determine, at least in part, the locations of megabase-scale replication timing domains. In cancer cells, the replication of entire chromosomes could be delayed in a sequence-specific manner, and interactions with long noncoding RNAs could alter the timing of replication for entire chromosomes (Donley et al. 2015).

Proteins that catalyze distinct histone modifications can facilitate or modulate initiation in groups of replication origins. HBO1, a histone acetyltransferase that modifies H4K5 and H4K12, binds near origins of replication by associating with ORC1 and Cdt1 (Iizuka et al. 2006; Miotto and Struhl 2010). The chromatin decondensation promoted by HBO1 is enhanced near H3K4me3 and reduced near H3K20me1/2/3 (Huang et al. 2006; Kim et al. 2006; Saksouk et al. 2009). While histone acetyltransferase HBO1 is associated with early replicating origins, histone methyltransferase PR-Set7 and heterochromatin-associated proteins ORC-associated protein (ORCA) and HP1 are associated with late replicating origins. ORCA/LRWD1 stabilizes ORC on origins and promotes late replication by perpetuating histone compaction near the repressive methylation of H3K9, H4K20, and H4K27 (Chakraborty et al. 2011; Giri et al. 2015). HP1 is also known to stabilize ORC on origins via interaction with ORC2 and ORC3 and to bind to methylated H3K9 to establish late replicating domains (Chakraborty et al. 2011; Schwaiger et al. 2010). H4K20me1 serves as a binding domain for other histone modifiers, like Suv4, promoting further chromatin compaction (Tardat et al. 2010). Cells depleted in further methylation of H4K20 are also shown have reduced ORCA and ORC1 binding (Sherstyuk et al. 2014).

In addition to modifying histones near origins of replication, some *trans*-acting factors, like Rif1, Taz1, and FKH1/2, facilitate the recruitment of replication factors to origins. Taz1 and Rif1 help promote replication initiation in heterochromatic, telomeric regions (Cornacchia et al. 2012; Hayano et al. 2012; Tazumi et al. 2012; Yamazaki et al. 2012). Both Taz1, which prevents early-S replication activation, and Rif1, which recruits protein phosphatase 1 (PP1) and modulates the chromatin binding of pre-initiation complex components (Dave et al. 2014; Foti et al. 2016; Hiraga et al. 2014; Kanoh et al. 2015), are associated with late replication. Taz1 and Rif1 might delay replication by interfering with DDK phosphorylation of Mcm2-7 and associating with nuclear architectures that anchor heterochromatin (Foti et al. 2016; Tazumi et al. 2012). Inhibition of MCM phosphorylation subsequently interferes with CDC45 and Sld3 loading (Francis et al. 2009; Tazumi et al. 2012). The replication and DNA repair features regulated by Rif1 are conserved across eukaryotes (Mattarocci et al. 2014). Rif1 organizes replication timing domains by associating with G-quadruplexes to suppress replication (Foti et al. 2016; Kanoh et al. 2015; Mattarocci et al. 2014).

Unlike Taz1 and Rif1, FKH1/2 can promote early replication by recruiting replication factors to early replicating DNA (Knott et al. 2012). In addition, FKH1/2 overexpression advances the replication time of late replicating origins (Knott et al. 2012). These proteins help either activate or repress replication by facilitating inter-

chromosomal interactions (Musialek and Rybaczek 2015). FKH1/2 advance the time of initiation by acting during the late G1 phase of the cell cycle (Peace et al. 2016), indicating that replication timing can be reset subsequently to origin licensing.

Distal DNA sequences affect transcriptional activity and origin activity through long-distance interactions (Aladjem et al. 1998; Gerhardt et al. 2014; Norio et al. 2005). Such interactions (Fig. 2.3b) can be mediated via protein interaction with enhancers and locus control regions (Huang et al. 2011) or by chromatin remodeling factors and transcriptional activators that bind enhancers and locus control regions (Aladjem 2007; Fragkos et al. 2015). RepID, a protein that interacts with a group of replication origins, is associated with an origin-activating chromatin loop between the origin and the locus control region at the human *HBB* locus (Zhang et al. 2016). Long-distance interactions that modulate replication timing can also be mediated by long noncoding RNAs (lncRNAs) such as *Xist* and *HOTAIR*, which guide histone and chromatin remodeling proteins to specific DNA sequences and facilitate chromatin interactions (Fragkos et al. 2015; Nagano and Fraser 2011). lncRNAs can stabilize ORC to origins in viruses (Fragkos et al. 2015; Nagano and Fraser 2011) and can affect the timing of replication for entire chromosomes in cancer cells (Donley et al. 2015).

## 2.4 Role of Replication Origins

The apparent excess of potential replication origins and the absence of sequence-specific initiation during early embryogenesis both suggest that a consistent replication initiation program is not a mechanistic requirement for genome duplication. Replication also proceeds stochastically with no apparent replication timing domains within the human inactive X chromosome (Koren et al. 2014), again suggesting that a consistent replication timing program is not required merely to insure genome duplication. The replication program could be established to coordinate replication with other chromatin transactions, primarily transcription. Consistent replication initiation sites could facilitate genome integrity by coordinating replication with transcription and chromatin assembly on the shared chromatin template. The consistent replication timing programs establish regions that replicate late during S-phase and might serve to establish and maintain specific nuclear compartments, such as heterochromatin. Late replication of heterochromatin can be required to preserve the structural integrity of the nucleus by preventing rapid, massive chromatin decondensation and re-condensation that could and lead to DNA damage (Bustin and Misteli 2016).

Although the severe effects of changes in replication timing support a critical role for replication timing regulation in maintaining genomic stability, recent mathematical models suggest that the relative efficiencies of initiation at replication origins are sufficient to determine the organization of replication timing domains. A mathematical model can predict replication timing with high accuracy in human cells without assuming any “replication timing factor,” using two variables: the

known distribution of replication origins as correlates of DNase hypersensitive sites and the assumption that replication initiation is restricted by the availability of a single rate-limiting activator (Gindin et al. 2014). A second model (Lob et al. 2016) was also able to predict the general progression of DNA replication and in addition predicted the three-dimensional spatial organization of replication events based on higher chromatin organization, assuming spontaneous stochastic initiation within euchromatin and facultative heterochromatin. Again, this model did not assume a replication timing factor, and replication timing could be deduced without such a factor assuming concomitant inhibition of replication initiation at distances below the size of chromatin loops and a domino-like effect by which replication at a particular origin would induce initiation from adjacent origins. A third model was able to predict replication timing in yeast with high accuracy relying on the density of the MCM replicative helicase, assuming a high level of MCMs at early origins (Das et al. 2015). Together, all models suggest that the spatial distribution of replication origins determines the temporal organization of replication.

The ability to modify the spatial and temporal initiation profile also allows a cell to accommodate its specific transcription program. A large fraction of the human genome exhibits changes in the order of replication during nuclear reorganization associated with differentiation and development (Pope et al. 2014; Rhind and Gilbert 2013a; Rivera-Mulia and Gilbert 2016). Consistent with the need to activate initiation at distinct times to accommodate changes in transcription, cell type-specific replication origins are often located in regions that exhibit differentiation-specific and tissue-specific gene expression (Gerhardt et al. 2014; Norio et al. 2005). Origins that are activated in a narrow set of distinct cell types tend to initiate replication late in S-phase in regions that contain few and sparse origins, whereas origins that are commonly activated in many cell type initiate replication throughout S-phase (Smith et al. 2016). Since chromatin and histone modifications influence transcription and replication patterns, varying recruitment of modifiers and other proteins by transcription factors can markedly influence the replication program and vice versa (Bar-Ziv et al. 2016). Massive alterations in replication initiation patterns can be programmed, associated with activation of a differentiation cascade leading to changes in gene expression patterns (Gerhardt et al. 2014; Norio et al. 2005). Conversely, since transcription can hinder initiation of DNA replication on the common chromatin template (Martin et al. 2011), regions that exhibit massive differentiation-induced transcription might contain fewer and sparser replication origins due to the paucity of genetic elements that can support initiation. The precise timing of origin activation within replication timing domains is determined anew after each mitotic cell division (Wu and Gilbert 1996), facilitating dynamic and flexible changes in replication order (Rhind and Gilbert 2013a).

The excess of replication origins might also play a regulatory role to facilitate genomic stability by allowing for timely, accurate replication under stress. Activation of stress responses in actively proliferating cells, including changes in the rate of replication fork progression, can signal for changes in the utilization of certain origins. Conversely, alterations in the frequency of initiation can affect the rate of DNA synthesis. In yeast, replication can proceed upon depletion of the most or all replica-

tion origins in specific chromosomes, but those cells exhibit elevated chromosome loss rates (Dershowitz et al. 2007). In addition, a lower number of potential origins can increase the abundance of DNase hypersensitive regions, chromosome fragility, and chromosomal rearrangements (Huang and Koshland 2003; Lengronne and Schwob 2002).

Mammalian cells often exhibit increased frequency of replication initiation events (activation of "dormant origins") in response to events that slow the progression of replication forks, including changes in nucleotide pool levels (Anglana et al. 2003), exposure to histone deacetylase inhibitors (Conti et al. 2010) diminished homologous recombination (Daboussi et al. 2008) and dysfunctional DNA modifying enzymes such as topoisomerase I (Tuduri et al. 2009) and Mus81 endonuclease (Fu et al. 2015). Since the enhanced frequency of initiation in those cases associates with a mild decrease in replication fork progression rates, it is unclear whether the overall increase in initiation frequency indicates a global compensatory mechanism linking replication fork rates and origin activity or reflects local changes in a group of loci (e.g., fragile sites) that are particularly prone to potentially genotoxic lesions under conditions of slow replication fork progression.

## 2.5 Conclusions and Future Questions

Cells duplicate their genomes starting from many origins and proceeding along a well-established program that sequentially replicate the entire genome. Consistent replication origins are evident in most cells, although they are not essential for complete genome duplication. Origin activation dynamics might therefore primarily play a role in establishing local and global chromatin structure and facilitate the cellular response to adverse events that perturb the replication process.

Although the ability to initiate DNA replication can partially be conferred by the primary DNA sequence, most replication origins exhibit flexible initiation, as their activation in a fraction of cells is affected by the chromatin environment and by interactions with distal DNA elements. This flexible initiation program facilitates coordination between replication and transcription and preserves genome stability by maintaining a group of "reserve" potential replication origins that can be activated if replication at adjacent replicons stalls.

Understanding the molecular interactions at replication origins is critical for establishing a complete picture of how cells coordinate chromatin transactions, including transcription, chromatin decondensation and compaction, and DNA synthesis. Despite rapid progress in mapping the locations and timing of replication initiation events, we have yet to identify the corresponding molecular interactions that dictate initiation of DNA replication at particular sites. Future progress in addressing these issues will be achieved by identifying the exact combination of DNA-binding proteins or chromatin modifiers that activate DNA replication. The evident flexibility of the replication program also necessitates studies that characterize cell cycle signaling pathways that repress replication from origins that remain

"dormant" during particular cell cycles and modulate replication initiation to coordinate with changes in the transcription program. Finally, future studies characterizing how cell cycle checkpoint pathways affect molecular interactions at replication origins could lead to a better understanding of cellular responses to potentially genotoxic stress.

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# Chapter 3
# Molecular Mechanism for Chromatin Regulation During MCM Loading in Mammalian Cells

**Nozomi Sugimoto and Masatoshi Fujita**

**Abstract** DNA replication is a fundamental process required for the accurate and timely duplication of chromosomes. During late mitosis to G1 phase, the MCM2-7 complex is loaded onto chromatin in a manner dependent on ORC, CDC6, and Cdt1, and chromatin becomes licensed for replication. Although every eukaryotic organism shares common features in replication control, there are also some differences among species. For example, in higher eukaryotic cells including human cells, no strict sequence specificity has been observed for replication origins, unlike budding yeast or bacterial replication origins. Therefore, elements other than beyond DNA sequences are important for regulating replication. For example, the stability and precise positioning of nucleosomes affects replication control. However, little is known about how nucleosome structure is regulated when replication licensing occurs. During the last decade, histone acetylation enzyme HBO1, chromatin remodeler SNF2H, and histone chaperone GRWD1 have been identified as chromatin-handling factors involved in the promotion of replication licensing. In this review, we discuss how the rearrangement of nucleosome formation by these factors affects replication licensing.

**Keywords** Replication • Pre-RC • Cdt1 • MCM • HBO1 • SNF2H • GRWD1 • Histone • Nucleosome

## 3.1 Introduction

One of the fundamental events in the cell cycle is complete and precise duplication of the genome. Although major features of replication are well conserved among all organisms, there are also some differences. In all eukaryotes, DNA replication is thought to begin at origins of replication. In *S. cerevisiae*, autonomously replicating

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sequences (ARSs) have been identified as origins (Stinchcomb et al. 1979; Rao et al. 1994; Wyrick et al. 2001). In *S. pombe*, although no consensus sequence has been identified, AT-rich regions serve as potential origins (Segurado et al. 2003; Yompakdee and Huberman 2004; Dai et al. 2005; Hayashi et al. 2007). In mammalian cells, DNA replication origins do not have such consensus sequences (Vashee et al. 2003; Schaarschmidt et al. 2004; Martin et al. 2011; Valenzuela et al. 2011; Besnard et al. 2012; Petryk et al. 2016). Although metazoan replication origins have been shown to possess some characteristic features, genome-wide studies have shown that most efficient origins in mammalian cells are strongly associated with human CpG island (CGI) promoters (Cadoret et al. 2008). The association with CGI has also been confirmed in mouse cells (Sequeira-Mendes et al. 2009; Cayrou et al. 2011). A conserved G-rich motif named OGRE (origin G-rich repeated elements) is present in most of mouse and *Drosophila* origins, and OGRE motifs can form a G-quadruplex (G4) (Cayrou et al. 2011, 2012). A genome-wide association between origins and G4 motifs has also been found in human cells (Besnard et al. 2012). In fact, some G4 motifs could promote replication initiation (Valton et al. 2014), and human ORC binds preferentially to G4 motifs on single-stranded DNA (Hoshina et al. 2013). Therefore, G4 structures could mediate ORC recruitment to initiation sites.

To initiate DNA replication, it is essential to form prereplicative complexes (pre-RCs). In late M to early G1 phase, pre-RCs are formed on chromatin (Fig. 3.1a). The pre-RC assembly, known as "licensing," involves the binding of ORC to DNA. In budding yeast, ORC interacts with ARSs in a sequence-specific manner (Bell and Stillman 1992). In fission yeast, Orc4, a subunit of the ORC complex, has unique sequence motifs called "AT hooks" and binds to replication origin DNA via these motifs (Chuang and Kelly 1999). In metazoan, including human cells, ORC binds to DNA without sequence specificity and tends to interact with open chromatin regions containing active chromatin marks such as histone H3 acetylation and H3K4 methylation (Vashee et al. 2003; Schaarschmidt et al. 2004; Dellino et al. 2013; Miotto et al. 2016).

ORC bound to origins, in conjunction with CDC6 and Cdt1, loads MCM double hexamers onto DNA. Once MCM complexes are loaded, the origin becomes "licensed" and is ready to be activated. As cells enter S phase, the licensing functions of ORC, CDC6, and Cdt1 are suppressed to inhibit MCM reloading (Fig. 3.1b). In addition, to activate MCM replicative helicase, two kinases, CDK and CDC7, phosphorylate fork components and facilitate CDC45 and GINS recruitment (Araki 2011; Heller et al. 2011; Yeeles et al. 2015; Bleichert et al. 2017).

Generally, eukaryotic DNA is packaged into nucleosomes. The nucleosome contains 146 bp of DNA wrapped around an octameric complex of H2A, H2B, H3, and H4 (Horn and Peterson 2002). The N-terminal tails of the histones protrude from the nucleosome. These histone tails are important for higher-order chromatin folding. Because nucleosomes prevent DNA-related processes, they must first be disassembled or moved transiently to allow DNA replication, DNA repair, and transcription machineries to access the DNA. At the replication fork during S phase, two histone chaperones, CAF1 and ASF1, aid histone eviction and redeposition (MacAlpine and Almouzni 2013). The FACT histone chaperone binds to several replisome components

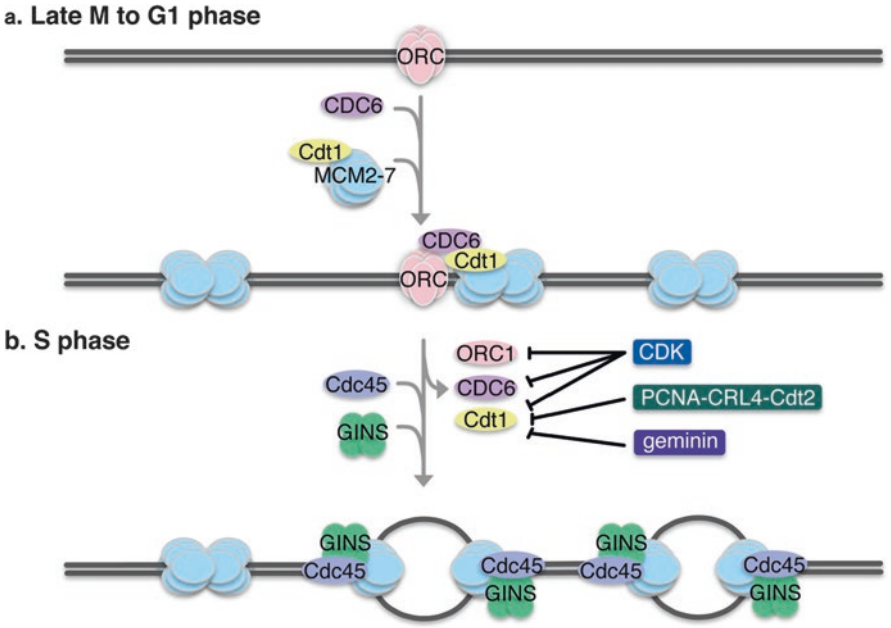


**Fig. 3.1** A model for regulation of pre-RC formation and its firing in human cells. (**a**) During late M to G1 phase, the ORC, CDC6, Cdt1, and MCM2-7 proteins are sequentially assembled on replication origins (including dormant origins). The MCM complexes are loaded in excess onto chromatin and may be distributed to locations distant from the ORC binding sites. These ORC-distal MCMs may also function as origins (including dormant ones). (**b**) When cells enter S phase, CDK and CDC7 kinases are activated and facilitate loading of CDC45, GINS, and other proteins to activate the helicase activity of MCM. This triggers the unwinding of DNA and the subsequent loading of DNA polymerases and other factors onto chromatin to start DNA replication. To prevent relicensing (i.e., reloading of MCM) during S phase, it is important to downregulate the function of MCM loaders. A major pathway for this purpose is through regulation of Cdt1. Cdt1 activity is regulated by ubiquitin-mediated degradation and its specific inhibitor, geminin. Part of the mechanisms for inhibition of relicensing is regulation of CDC6 and ORC1. CDC6 is exported from the nucleus in a CDK phosphorylation-dependent manner. ORC1 is phosphorylated by CDK and is subsequently degraded by ubiquitin-mediated proteolysis

and travels with the replication fork (Wittmeyer and Formosa 1997; Gambus et al. 2006; Tan et al. 2006; VanDemark et al. 2006). Conditional knockout of SSRP1, a small subunit of FACT, reduces fork speed in chicken DT40 cells (Abe et al. 2011). In in vitro replication with purified yeast replication proteins and fully chromatinized templates, replisome progression requires the FACT (Kurat et al. 2017). In addition, several studies have shown that MCM2 interacts with histones H3-H4 and histone chaperone ASF1 and acts as a histone chaperone to assist disassembly and assembly of nucleosomes during fork progression (Ishimi et al. 2001; Groth et al. 2007; Jasencakova et al. 2010; Huang et al. 2015; Richet et al. 2015). However, little is known about nucleosomal regulation during pre-RC formation, especially in human cells. This review will focus on recent findings addressing this issue.

## 3.2 Factors That Promote Pre-RC Formation

Broadly, three families of functionally distinct protein complexes have been implicated in transient nucleosome eviction, disassembly, and/or movement: (1) ATP-dependent chromatin remodeling proteins, (2) histone acetyltransferases (HAT), and (3) histone chaperones (Aalfs and Kingston 2000; Hammond et al. 2017). "Chromatin openness" is important not only for transcription but also for replication licensing, origin firing, and other nuclear transactions. "Open chromatin regions" may represent transiently generated nucleosome-free or nucleosome-low DNA regions (Fig. 3.2). In general, when activating transcription, transcription factors promote chromatin openness by recruiting these chromatin-handling factors.

In yeast, ARS-binding factor 1 (ABF1), a transcription factor, is implicated in formation of nucleosome-free regions at its binding sites and activates transcription of many genes (Springer et al. 1997; Badis et al. 2008; Hartley and Madhani 2009). ABF1 binds to several ARSs and alters local chromatin structure (Venditti et al. 1994; Hu et al. 1999). In an in vitro system, ABF1-dependent nucleosomal arrangement of *ARS1* is required for efficient ORC loading (Lipford and Bell 2001). Furthermore, ABF1 binds to the promoters of many ribosomal genes and recruits Esa1, an essential histone acetyltransferase (Reid et al. 2000).

In higher eukaryotes, numerous studies have shown that transcription factors contribute to replication licensing. In *Drosophila*, Myb, a sequence-specific transcription factor, is required for site-specific replication at ACE3 and Ori-β (Beall et al. 2002). In human, mouse, and *Xenopus*, c-myc interacts with pre-RC components and regulates origin activity (Dominguez-Sola et al. 2007). Binding of *Drosophila* ORC to origin DNA is facilitated by E2F1-Rb (Bosco et al. 2001). These studies indicate that transcription factors bind to specific DNA sequences, thereby efficiently recruiting ORC via physical interactions. However, they could also promote MCM loading by recruitment of chromatin-handling factors. As another example, ORCA recruits ORC to chromatin at human heterochromatic regions (Shen et al. 2010).

## 3.3 Cdt1 Strongly Stimulates Pre-RC Formation and Its Activity Is Tightly Regulated by Multiple Mechanisms in Human Cells

Cdt1 is essential for loading of the MCM2-7 complex onto chromatin and physically interacts with several subunits of the MCM complex (Tanaka and Diffley 2002; Randell et al. 2006; You and Masai 2008). In human cells, Cdt1 strongly promotes MCM loading (Vaziri et al. 2003; Sugimoto et al. 2009), and accordingly, its activity is tightly regulated by multiple mechanisms (Fig. 3.1b). It was originally shown that geminin directly binds to Cdt1 and inhibits its activity (McGarry and Kirschner 1998; Wohlschlegel et al. 2000; Tada et al. 2001). Geminin is a substrate

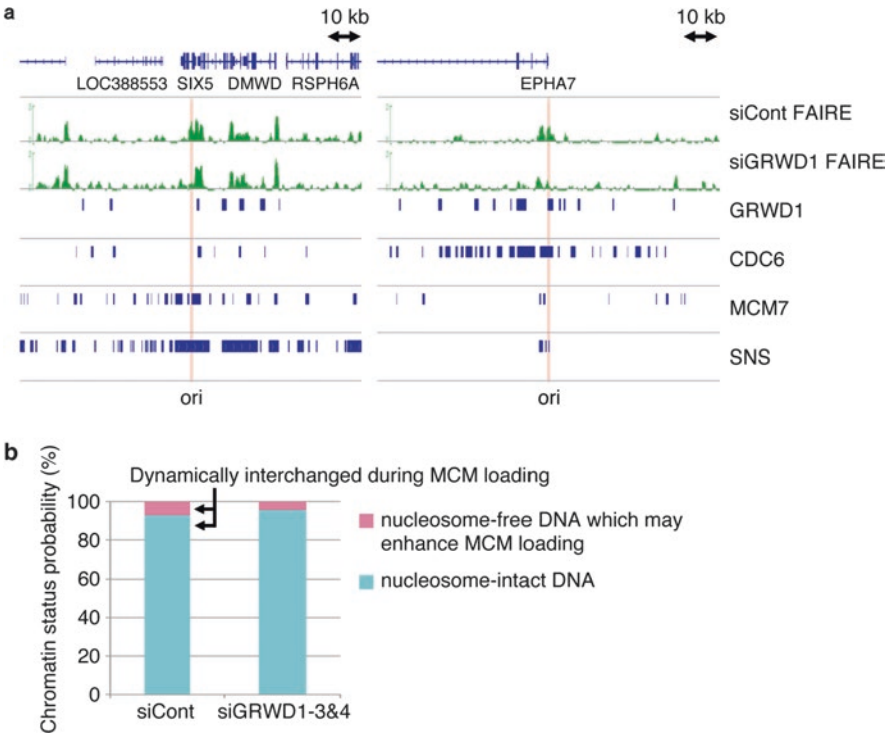


**Fig. 3.2** "Open chromatin region" may have transient nucleosome-free or nucleosome-low DNA at a low frequency. (**a**) Open chromatin regions are associated with DNA replication origins. Here, chromatin openness is estimated by FAIRE (formaldehyde-assisted isolation of regulatory elements), a method of isolating genomic regions with no or depleted nucleosomes (Sugimoto et al. 2015). ChIP-seq data for CDC6 and MCM7 are also from Sugimoto et al. (2015). Short nascent strand (SNS) data are from Besnard et al. (2012). *SIX5* and *EPHA7* origins (Liu et al. 2012) are shown as examples of origins having GRWD1-dependent open chromatin structure. (**b**) In quantitative FAIRE-qPCR assays at several replication origins, the percentages of FAIRE signals are ~10% or less (Sugimoto et al. 2015). Therefore, theoretically, only a small fraction of DNA may be in a "nucleosome-free" form even in such "open chromatin" regions. Such nucleosome-free DNA may appear transiently and dynamically, which may generate spaces for efficient MCM loading. At a subset of replication origins, maintenance of "chromatin openness" is dependent on GRWD1 (Sugimoto et al. 2015). Given that most DNA is organized into nucleosomes even in such "open chromatin" regions, it would be difficult to directly detect changes in histone levels at replication origins

of the APC/C ubiquitin ligase and is destabilized during the G1 phase and accumulates during the S, G2, and M phases (McGarry and Kirschner 1998; Wohlschlegel et al. 2000; Tada et al. 2001; Vodermaier 2004). The protein level of Cdt1 is also strictly controlled during the cell cycle, being high in G1 phase, low in S phase, and high again at the M-to-G1 transition (Nishitani et al. 2001). In human cells, two E3 ubiquitin ligases, $SCF^{Skp2}$ and $CUL4\text{-}DDB1^{Cdt2}$, redundantly mediate proteolysis of Cdt1 (Fujita 2006; Arias and Walter 2007; Coleman et al. 2015). Human Cdt1

interacts with the $SCF^{Skp2}$ ubiquitin ligase when phosphorylated by cyclin A-dependent kinases and is then degraded (Li et al. 2003; Liu et al. 2004; Sugimoto et al. 2004; Takeda et al. 2005). By contrast, the binding of $CUL4\text{-}DDB1^{Cdt2}$ to Cdt1 is dependent on prior interaction with chromatin-bound PCNA (Arias and Walter 2006; Jin et al. 2006; Nishitani et al. 2006; Ishii et al. 2010). These tight regulations of Cdt1 ensure that MCM is recruited only in G1 phase and that replication occurs only once during the cell cycle.

## 3.4 Cdt1 Interacts with Three Chromatin-Handling Factors to Promote Pre-RC Formation

How does Cdt1 promote MCM loading so strongly in human cells? One explanation is that Cdt1 might interact with other factors that enhance MCM recruitment. In human cells, Cdt1 interacts with histone acetyltransferase HBO1, ATP-dependent chromatin remodeler SNF2H, and novel histone chaperone GRWD1 (Miotto and Struhl 2008; Sugimoto et al. 2008, 2011, 2015; Miotto and Struhl 2010; Aizawa et al. 2016). As mentioned above, the regulation of local nucleosomal structure during replication licensing may be important. Thus, Cdt1 may recruit these chromatin-handling factors to facilitate MCM loading by altering chromatin accessibility. In support of this notion, LacI-Cdt1 tethered to LacO induces large-scale chromatin decondensation that may be required for MCM recruitment in G1 phase (Wong et al. 2010).

### *3.4.1 Histone Acetylation and HBO1 (Also Known as MYST2 or KAT7)*

Highly conserved lysine residues present on all four core histones serve as the targets of acetylation. When histone tails are acetylated, inter-nucleosomal interactions are reduced, resulting in unfolding of the chromatin fiber (Tse et al. 1998; Annunziato and Hansen 2000; Shogren-Knaak et al. 2006; Robinson et al. 2008).

The acetylation of histone lysine residues has been implicated in origin activation during early development in *Xenopus* and at the chorion gene loci in *Drosophila* follicle cells (Aggarwal and Calvi 2004; Danis et al. 2004; Hartl et al. 2007). In *S. cerevisiae*, histone deacetylases Sir2 and Rpd3 control replication timing by regulating silencing at the rDNA array (Yoshida et al. 2014). In *Drosophila*, Rpd3 suppresses origin activity, whereas histone acetyltransferase Chameau promotes origin activation (Aggarwal and Calvi 2004). HBO1 is the mammalian homolog of Chameau and was originally identified through its physical interactions with human ORC1 (Iizuka and Stillman 1999). Subsequent work has shown that HBO1 interacts with Cdt1 and acetylates histone H4 tails at origin regions during G1, and this is

required for efficient MCM loading (Miotto and Struhl 2008, 2010). In addition, HBO1 stimulates Cdt1-dependent re-replication (Miotto and Struhl 2008) and interacts with Kaposi's sarcoma-associated herpes virus terminal repeats and promotes replication of the viral genome (Stedman et al. 2004). It was recently reported that the HBO1-BRPF3 complex regulates origin activation through H3K14 acetylation and CDC45 loading (Feng et al. 2016). Therefore, these findings suggest that HBO1 has two distinct functions in promoting replication; during G1 phase, it promotes MCM2-7 loading, whereas during S phase, it promotes CDC45 loading. However, whether HBO1 is essential for replication remains to be clarified (see below).

### *3.4.2 SNF2H, an ATP-Dependent Chromatin Remodeler*

Chromatin remodeling complexes that use energy derived from ATP hydrolysis alter chromatin structure by sliding, evicting, and/or modifying nucleosomes. This large group of complexes can be subdivided into four subfamilies that include the SWI/SNF-type complex, the ISWI-type complex, the INO80-type complex, and the CHD-type complex (Varga-Weisz 2001; Tsukiyama 2002; Corona and Tamkun 2004; Dirscherl and Krebs 2004; Eberharter and Becker 2004). In yeast, the function of the SWI/SNF remodeling complex is not limited to transcriptional regulation. For example, the SWI/SNF complex is required for efficient maintenance of a minichromosome containing ARS121, a yeast replication origin (Flanagan and Peterson 1999). In addition, ISW2 and INO80 facilitate replication fork progression in the presence of replication stress (Vincent et al. 2008; Au et al. 2011).

In human cells, SNF2H, an ISWI-type factor, has been implicated in regulation of DNA replication. The dyad symmetry (DS) region of EB viral origin of plasmid replication (*oriP*) is flanked by nucleosomes that undergo chromatin remodeling by SNF2H (Zhou et al. 2005). Furthermore, SNF2H depletion by siRNAs reduces MCM3 loading and replication at *oriP* (Zhou et al. 2005). It has been also reported that SNF2H is enriched at two genomic replication origins in G1 phase, and silencing of SNF2H suppresses MCM7 and MCM3 loading at the origins (Sugimoto et al. 2011). These studies provide evidence that chromatin remodeling may be required to move nucleosomes around the replication origin to unmask the pre-RC formation site. If chromatin remodeling complexes are required to promote pre-RC formation at the replication origins, a mechanism must exist to ensure they are recruited there. One mechanism may be through interaction with pre-RC components. In this regard, SNF2H and WSTF are identified as Cdt1-binding proteins and are recruited to origins through interaction with Cdt1 (Sugimoto et al. 2008, 2011). Another potential mechanism is the direct binding of chromatin remodeling complexes to origins, which could be mediated either through DNA binding or by recognition of replication licensing-coupled histone code(s) such as acetylation (see below). The involvement of other classes of ATP-dependent chromatin remodeling complexes in licensing remains to be clarified.

### 3.4.3 *Novel Histone Chaperone GRWD1*

Histone chaperones interact with histones and play crucial roles in mediating nucleosome assembly and disassembly. In *S. cerevisiae*, the histone chaperone FACT is ubiquitinated by E3 ubiquitin ligase Rtt101 and promotes MCM loading (Han et al. 2010). However, the mechanism of FACT recruitment to origins remains unclear. In human cells, Cdt1-binding protein GRWD1 is a novel histone chaperone that controls chromatin openness at pre-RC sites (Sugimoto et al. 2008, 2015; Aizawa et al. 2016) (Fig. 3.2). GRWD1 is recruited to replication origins in a CDC6- and Cdt1-dependent manner in G1 phase and promotes MCM loading (Sugimoto et al. 2015) (Fig. 3.2). Although GRWD1 is highly conserved among species (Gratenstein et al. 2005), it is not clear whether GRWD1 homologs in other species have a similar function. However, several findings link the budding yeast homolog of GRWD1, Rrb1, to replication licensing. Rrb1 is essential for growth, is involved in early ribosome assembly, and genetically interacts with Orc6 (Iouk et al. 2001; Schaper et al. 2001; Killian et al. 2004). In addition, Rrb1 interacts with Yph1, which functions cooperatively with ORC and MCM (Du and Stillman 2002). Taken together, these observations suggest that GRWD1 plays an important role in pre-RC formation at DNA replication origins.

GRWD1 has an acidic domain and promotes chromatin openness at replication origins and efficient MCM loading (Sugimoto et al. 2015). Recently, we used an in vitro reconstituted system to further show that GRWD1 can evict H2A-H2B dimers from nucleosomes (Aizawa et al. 2016). Interestingly, the acidic domain of GRWD1 is important for these activities (Aizawa et al. 2016), consistent with previous reports that acidic domains are found in numerous histone chaperones and play roles in histone chaperone activity (De Koning et al. 2007). In vitro studies have also shown that many acidic transcriptional activators can stimulate DNA replication by competing with the repressive effects of nucleosomes (Cheng and Kelly 1989; Cheng et al. 1992; Li and Botchan 1994). The acidic domain of GRWD1 is conserved among its homologs, including those in mouse, *Drosophila*, *C. elegans*, and *S. cerevisiae* (Gratenstein et al. 2005). Thus, this domain could be functionally important in other species.

## 3.5 Are HBO1, SNF2H, and GRWD1 Essential for Minimal Licensing to Support Normal Cell Growth?

Although H3K14 acetylation is reduced in HBO1 knockout mouse cells, both DNA replication and cell proliferation proceeded normally (Kueh et al. 2011). Also in flies, proliferation occurs with reduced levels of HBO1 (Grienenberger et al. 2002). Yeasts do not have obvious structural homologs of HBO1 (Avvakumov and Cote 2007; Lafon et al. 2007). Regarding SNF2H, DNA replication appears not to be affected by immunodepletion of SNF2H-WSTF complexes in *Xenopus* egg extract system (MacCallum et al. 2002). In addition, in human cells, the siRNA-mediated

silencing of SNF2H, HBO1, or GRWD1 does not impede re-replication induced by overexpression of the degradation-resistant Cdt1 mutant Cy+D1m (Sugimoto et al. 2008, 2009, 2011; our unpublished data). How can these findings be reconciled with the observations suggesting that these chromatin-handling factors play important roles in promotion of MCM loading? It is possible that SNF2H-, HBO1-, and GRWD1-mediated chromatin regulation plays an important role in efficient MCM loading in human cells, but is not essential for minimal origin firing. Alternatively, SNF2H-, HBO1-, and GRWD1-mediated enhancement of licensing may be species or cell type specific. Since HBO1, SNF2H, and GRWD1 are overexpressed in cancer cell lines (Iizuka et al. 2009; Jin et al. 2015; Sugimoto et al. 2015), regulations of DNA replication by these factors could be important in cancer cell growth.

It is important to consider the fact that excess MCM loading is crucial for maintaining genome stability. Even if the number of MCM2-7 molecules loaded is reduced, normal replication rates are maintained (Edwards et al. 2002; Cortez et al. 2004; Tsao et al. 2004; Ge et al. 2007; Ibarra et al. 2008). However, depletion of MCM causes hypersensitivity to replicative stress and a defect in Rad17-dependent ATR-mediated checkpoint activation (Tsao et al. 2004; Woodward et al. 2006; Ge et al. 2007). A hypomorphic mutation in MCM4 termed *Chaos3* (chromosome aberrations occurring spontaneously 3) causes severe genomic instability, and Chaos3 females are highly prone to mammary adenocarcinoma (Shima et al. 2007). Although mutant mice expressing low levels of MCM2 grow normally, they develop T- and B-cell lymphomas (Pruitt et al. 2007). A reduction of MCM levels causes DNA damage involving ATR and ATM activation (Orr et al. 2010). These studies demonstrate that excess MCM loading is critical for toleration of replication stress and activation of the checkpoint. Therefore, the recruitment of HBO1, SNF2H, and GRWD1 by Cdt1 and subsequent enhancement of MCM loading may be important for the maintenance of genome stability. On the other hand, Das et al. suggest that the number of loaded MCMs at origins may regulate replication timing in budding yeast (Das et al. 2015).

## 3.6 Relationship Between HBO1, SNF2H, and GRWD1 in Nucleosome Regulation and Its Involvement in the Promotion of MCM Loading

We now favor a model in which HBO1, SNF2H, and GRWD1 function cooperatively in the promotion of pre-RC formation (Fig. 3.3). However, each factor may act individually on specific origins or under specific conditions. SNF2H-WSTF complexes bind to acetylated histones in chromatin (Hakimi et al. 2002). Therefore, it is possible that HBO1 is first recruited to replication origins via interaction with Cdt1 and acetylates histone H4, and then SNF2H is recruited to the origin through interaction with both Cdt1 and acetylated histone H4, where these factors together with GRWD1 alter chromatin plasticity (Fig. 3.3). In contrast to this model, SNF2H, MCM, and HDAC1/2 (histone deacetylase) co-localize at EB virus *OriP* (Zhou et al. 2005). Also, in an in vitro system, SNF2H preferentially interacts with

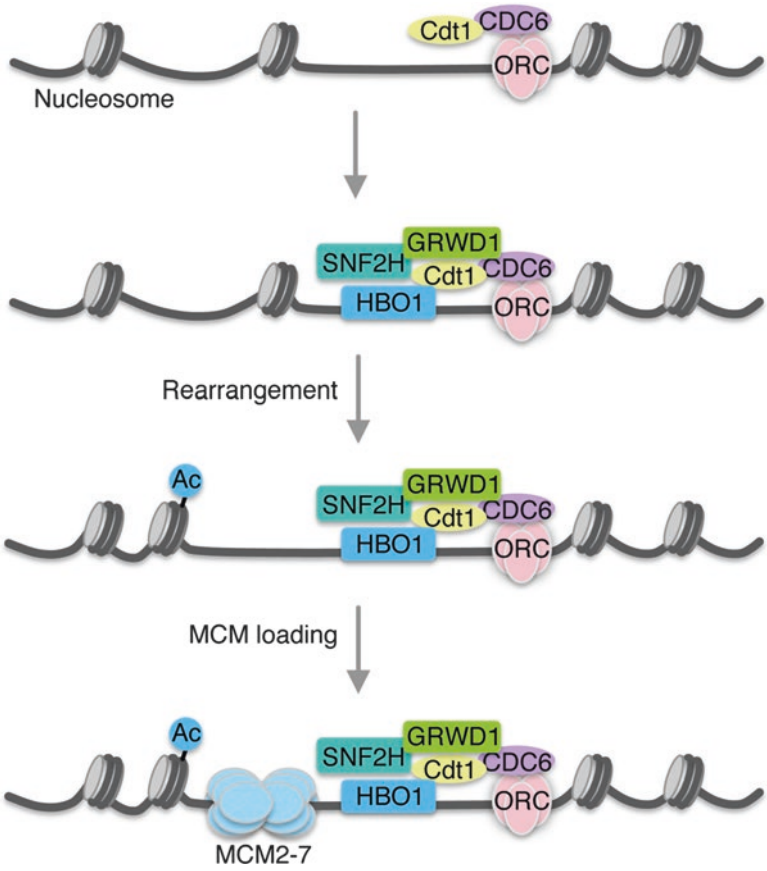


**Fig. 3.3** A model for enhancement of MCM loading by the three chromatin-handling factors in the context of chromatin. Since nucleosome structure interferes with MCM loading, it should be rearranged during this process. Histone acetyltransferase HBO1, chromatin remodeler SNF2H, and histone chaperone GRWD1 are involved in this reaction. These factors are recruited to origins in a Cdt1-dependent manner. It is currently unknown whether they are recruited interdependently

unacetylated histone H4 tails (Alenghat et al. 2006). The reason(s) for this discrepancy is unknown. Nucleosome eviction activity of SWI/SNF is enhanced by transcription factors (Gutierrez et al. 2007). Thus, other cellular cofactors could also be required to effectively load MCM complexes.

There is a strong association between promotion of replication licensing and transcriptional activation. For example, HBO1 also acts as a transcriptional coactivator for hormone receptors and AP-1 proteins (Georgiakaki et al. 2006; Miotto and Struhl 2006; Miotto et al. 2006), and many studies have shown that SNF2H is involved in transcriptional regulation (Varga-Weisz 2001; Tsukiyama 2002; Corona and Tamkun 2004; Dirscherl and Krebs 2004; Eberharter and Becker 2004). Our recent results suggest that GRWD1 functions not only in replication licensing but

also in transcription and other chromosome transactions (Sugimoto et al. 2015; our unpublished data). Furthermore, chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq) in mammalian cells shows that HBO1, SNF2H, and GRWD1 are highly enriched near the transcription start sites (Morris et al. 2014; Sugimoto et al. 2015; Feng et al. 2016). These results indicate that the Cdt1-interacting chromatin-handling proteins also act in transcriptional control. The dual roles of HBO1, SNF2H, and GRWD1 as coactivators for transcriptional regulation and for DNA licensing suggest the possibility that these factors might integrate internal and external stimuli to coordinate transcriptional responses with initiation of DNA replication.

## 3.7 Conclusion and Perspectives

Although increasing evidence suggests that HBO1, SNF2H, and GRWD1 promote MCM loading by regulating nucleosome structure in human cells, the detailed molecular mechanisms remain unclear, mainly because of the lack of useful cell-free in vitro reconstitution systems. With budding yeast proteins, pre-RC formation has been reconstituted on naked DNA with ARSs (Seki and Diffley 2000; Bowers et al. 2004; Kawasaki et al. 2006) or on the fully chromatinized templates (Kurat et al. 2017). In human cells, there is no sequence specificity for assembly of pre-RC, and numerous factors may provide either backup or fine-tuning mechanisms for the regulation of pre-RC assembly. Therefore, it may take some more time and effort if in vitro pre-RC reconstitution will be established using human proteins. Nevertheless, it should be examined whether the Cdt1-binding chromatin-handling factors have synergistic capabilities to efficiently remodel nucleosomes and/or evict histones in in vitro reconstitution assays. In the future, it will be very tempting to combine these factors with pre-RC reconstitution on nucleosome templates. Under these conditions, the histone-handling activity of MCM2 might also have a crucial role.

Finally, whereas it seems clear that “open chromatin” structure facilitates DNA replication, it remains unclear whether chromatin openness mainly promotes efficient MCM “loading” or efficient MCM “activation” by enhancing recruitment of CDC45 and GINS. Some suggest that efficient pre-RC formation leads to efficient DNA replication initiation (Das et al. 2015). However, it has also been suggested that “open chromatin” more strongly stimulates the latter process (Feng et al. 2016; our unpublished data). It will be interesting to address this important issue in human cells.

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# Chapter 4
# Initiation of DNA Replication at the Chromosomal Origin of *E. coli*, *oriC*

**Tsutomu Katayama**

**Abstract** The *Escherichia coli* chromosomal origin consists of a duplex-unwinding region and a region bearing a DNA-bending protein, IHF-binding site, and clusters of binding sites for the initiator protein DnaA. ATP-DnaA molecules form highly organized oligomers in a process stimulated by DiaA, a DnaA-binding protein. The resultant ATP-DnaA complexes promote local unwinding of *oriC* with the aid of IHF, for which specific interaction of DnaA with the single-stranded DNA is crucial. DnaA complexes also interact with DnaB helicases bound to DnaC loaders, promoting loading of DnaB onto the unwound DNA strands for bidirectional replication. Initiation of replication is strictly regulated during the cell cycle by multiple regulatory systems for *oriC* and DnaA. The activity of *oriC* is regulated by its methylation state, whereas that of DnaA depends on the form of the bound nucleotide. ATP-DnaA can be yielded from initiation-inactive ADP-DnaA in a timely manner depending on specific chromosomal DNA elements termed *DARS* (DnaA-reactivating sequences). After initiation, DnaA-bound ATP is hydrolyzed by two systems, yielding ADP-DnaA. In this review, these and other mechanisms of initiation and its regulation in *E. coli* are described.

**Keywords** *oriC* • DnaA • IHF • DiaA • Hda • *DARS* • *datA* • Methylation • AAA+ • In vitro reconstitution

## 4.1 Introduction

*Escherichia coli* is a model bacterium for many aspects of molecular biology. Replication initiation of the *E. coli* chromosome occurs in a complex consisting of the initiator protein DnaA, the DnaA-binding protein DiaA, the DNA-bending protein IHF, and the chromosomal origin *oriC* (Costa et al. 2013; Kaguni 2011;

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Katayama et al. 2010; Leonard and Grimwade 2015; Wolański et al. 2015). DnaA tightly binds ATP or ADP, and ATP-DnaA rather than ADP-DnaA efficiently assembles specific oligomers on *oriC*, forming an initiation complex that is competent for localized DNA unwinding. DiaA is thought to be dissociated from DnaA to enable DnaB helicase-DnaC loader complexes to bind to the initiation complex (Katayama et al. 2010). For bidirectional replication, a pair of DnaB helicases is loaded onto the single-stranded DNA region via dynamic interactions with DnaC and DnaA. The loaded DnaB helicases form mobile complexes with DnaG primases, and the DnaB-DnaG complexes proceed on the single-stranded DNA in a 5–3′ direction, with duplex DNA unwinding and synthesis of primer RNAs for loading of DNA polymerase III holoenzymes (O'Donnell et al. 2013).

Replication initiation at *oriC* is highly regulated so that it occurs only once per *oriC* at a specific time in the cell cycle. This strict regulation is sustained by positive and negative regulatory systems for DnaA and *oriC* (Katayama et al. 2010; Wolański et al. 2015). In this review, we give an overall picture of the mechanisms of initiation as well as the multiple coordinated systems for regulating initiation in *E. coli*.

## 4.2 Basic Structure of *oriC*

The 245 bp *oriC* sequence has two functional domains, the duplex-unwinding element (DUE) and DnaA-oligomerization region (DOR) (Leonard and Grimwade 2015; Wolański et al. 2015) (Fig. 4.1a). The DUE contains three AT-rich repeats of a 13-mer sequence with the consensus GATCTnTTnTTTT. It should be noted that this consensus includes a GATC sequence and a T-stretch in one strand and an A-stretch in the other strand (see below). The DOR contains a specific binding site for the DNA-bending protein IHF (IHF-binding site; IBS) with the consensus (A/T) ATCAAnnnnTT(A/G). IHF binding effectively stimulates DUE unwinding in vitro (Hwang and Kornberg 1992; Ozaki and Katayama 2012). HU protein, a structural homolog of IHF, can substitute for IHF in this role in vitro (Hwang and Kornberg 1992). In vivo, a single mutation in IHF or HU causes moderate inhibition of initiation, whereas double mutations of IHF and HU are lethal (Kano and Imamoto 1990), consistent with the roles of these proteins identified in vitro.

The DOR is subdivided into at least two regions, the left-half and middle–right-half regions, which contain sets of DnaA boxes with opposite orientations (Rozgaja et al. 2011; Ozaki and Katayama 2012; Ozaki et al. 2012a; Noguchi et al. 2015; Shimizu et al. 2016) (Fig. 4.1a). DnaA box R1 and box R4, which reside at opposite ends of DOR, have high affinity for DnaA. DnaA box R2 has moderate affinity and comprises the middle region (Rozgaja et al. 2011; Shimizu et al. 2016). Other DnaA boxes are low-affinity sites and form clusters: τ1-I2 in the left half and C3-C1 in the right half (McGarry et al. 2004; Kawakami et al. 2005; Rozgaja et al. 2011; Shimizu et al. 2016). These low-affinity sites bear moderate similarities to the DnaA box consensus. DnaA binding to these low-affinity sites is supported by cooperative binding of ATP-DnaA

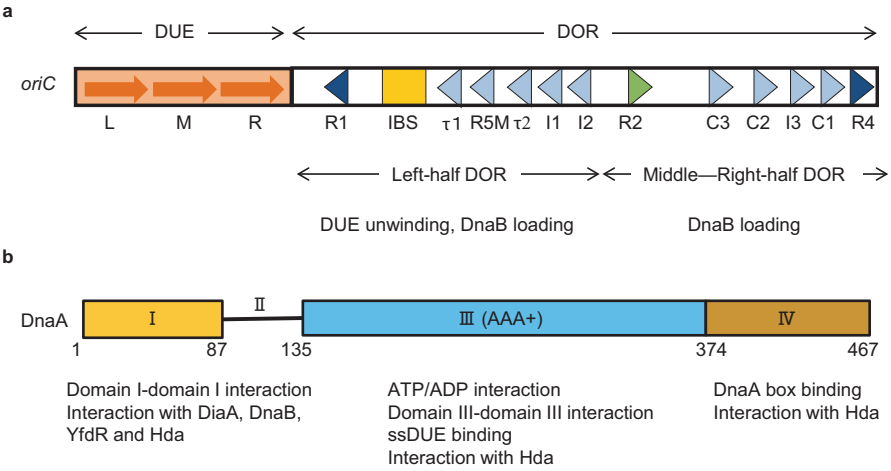


**Fig. 4.1** Basic structures of *E. coli oriC* and DnaA. (**a**) *E. coli oriC* consists of the duplex-unwinding element (*DUE*) and DnaA-oligomerization region (*DOR*) domains. The DUE includes 13-mer repeats (L, M, and R). DnaA boxes (*triangles*) are shown with their directionality. Functions of the left-half and middle–right-half DOR are indicated (the middle region includes only the DnaA box R2). *IBS* IHF-binding sequence. (**b**) *E. coli* DnaA consists of four domains. Amino acid numbers are indicated for each domain. See text for details

(see below). ATP-DnaA binding to τ1 might be specific for linear form *oriC* and might not occur in supercoiled *oriC* (Kawakami et al. 2005; Rozgaja et al. 2011).

Functional differences exist between the left-half DOR and the middle–right-half DOR (Fig. 4.1a). When the left-half DOR binds IHF and ATP-DnaA in vitro, the flanking DUE region is unwound even without the middle–right-half DOR (Ozaki and Katayama 2012). Efficient DnaB loading requires both subregions of the DOR; the DUE together with the left-half DOR has only moderate activity for DnaB loading (Ozaki and Katayama 2012). Consistently, in vivo, cells bearing deletion of the DnaA box R4 in the chromosomal *oriC* initiate replication but with a moderate inhibition (Bates et al. 1987).

## 4.3 Basic Structure of DnaA

DnaA protein consists of 473 amino acid residues, which comprise four functional domains (Ozaki and Katayama 2009; Kaguni 2011) (Figs. 4.1b and 4.2a). The N-terminal domain I has at least two specific sites for protein binding. The first site contains Trp6 and supports DnaA domain I-domain I interaction with weak affinity (Felczak et al. 2005) (Table 4.1). The second site, containing Phe46, is used for binding to at least three proteins, DnaB helicase, DiaA protein, and YfdR protein (Abe et al. 2007; Keyamura et al. 2009; Noguchi and Katayama 2016) (Table 4.1, Fig. 4.2a, b). This residue forms a patch with Glu21, Trp25, and Trp50 on the

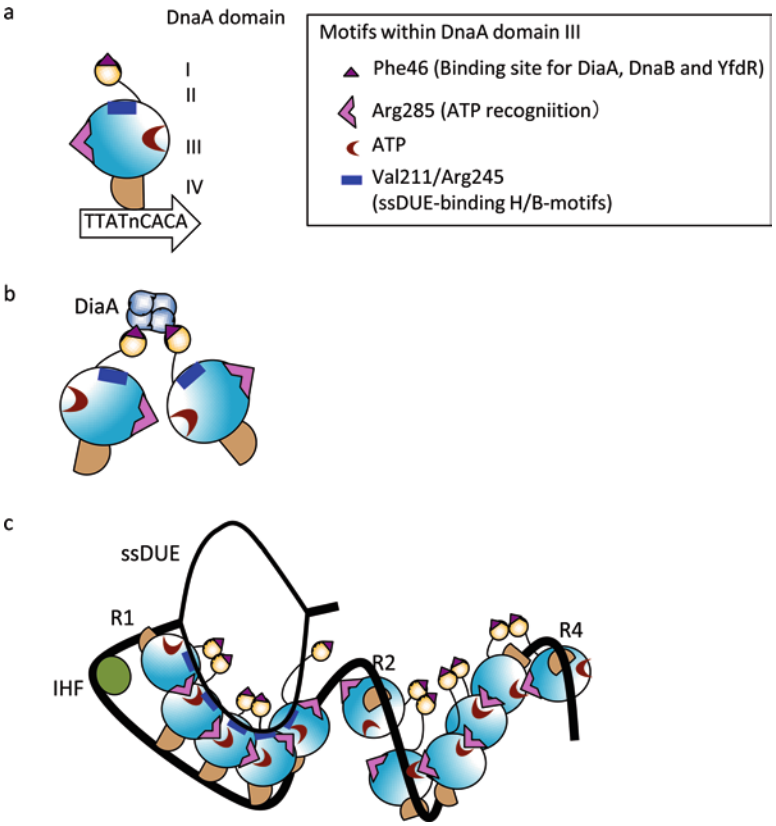


**Fig. 4.2** Binding structures of DnaA. (**a**) DnaA-DnaA box binding. The arginine finger side of DnaA orients to the 5′ end of the DnaA box consensus. Important motifs are indicated. (**b**) DnaA-DiaA binding. Each protomer of a DiaA homotetramer can bind DnaA. For simplicity, only two DnaA molecules are shown. (**c**) A model for DUE unwinding by a DnaA-*oriC* complex. ATP-DnaA molecules and IHF bind to the DOR and can unwind the DUE. In the left-half DnaA sub-complex, DnaA boxes R1, R5M, τ2, I1, and I2 are occupied with DnaA, and in the ssDUE-recruitment model, DnaA bound to these sites bind the ssDUE. Even if ADP-DnaA binds to the R1 and R4 boxes, unwinding activity is sustained. See text for details

surface of domain I, which should interact with a specific site of DiaA (Keyamura et al. 2007, 2009). Affinity of DnaA domain I for DiaA is high, but affinity for DnaB and YfdR is weaker. DiaA is a stimulator of DnaA assembly on *oriC* (see below) (Fig. 4.2b). YfdR is encoded by a cryptic prophage (termed CPS-53) in the *E. coli* K12 chromosome and inhibits the DnaA-DnaB interaction. YfdR might be expressed under specific growth conditions to inhibit initiation at *ori*C (Noguchi and Katayama 2016). In addition, HU protein interacts with domain I, but its specific binding site has not been determined (Chodavarapu et al. 2008). Domain II is a flexible linker (Abe et al. 2007; Nozaki and Ogawa 2008).

**Table 4.1** Important functional residues in DnaA

| Residue | Domain | Function |
|---|---|---|
| Trp6 | I | Domain I-domain I interaction |
| Asn44 | I | Interaction with Hda |
| Phe46 | I | Binding to DnaB, DiaA, and YfdR |
| Glu143 | III | AAA+ N-linker, stable ATP/ADP binding |
| Glu173–Thr179 | III | Walker A motif |
| Val211 | III | ssDUE-binding H motif |
| Asp236–Asp237 | III | Walker B motif |
| Arg227 | III | AID1 motif, domain III-domain III interaction |
| Arg245 | III | ssDUE-binding B motif |
| Asp269 | III | AAA+ sensor I, stable ATP/ADP binding |
| Arg281 | III | AAA+ box VII, domain III-domain III interaction |
| Arg285 | III | AAA+ Arg finger, recognition of ATP, domain III-domain III interaction |
| Leu290 | III | AID2 motif, domain III-domain III interaction |
| Arg334 | III | AAA+ sensor II, ATP hydrolysis in RIDA, and DDAH |
| Lys366 | III | Modulation of DnaA complex |
| Arg399–Lys443 | IV | DNA-binding HTH motif |
| Leu422, Pro423 | IV | Interaction with Hda |

Domain III consists of the AAA+ domain that contains the Walker-type ATP(ADP)-binding sites and specific motifs for ATP binding/hydrolysis and for domain III-domain III interactions (Neuwald et al. 1999; Erzberger et al. 2002; Nishida et al. 2002; Iyer et al. 2004; Katayama et al. 2010) (Figs. 4.1b and 4.2a, Table 4.1). In addition to the typical Walker A motif (the P-loop), the AAA+ sensor 1 and N-linker motifs of DnaA support high-affinity ATP(ADP) binding (Kawakami et al. 2006; Ozaki et al. 2012b) (Table 4.1). ATP binding induces a conformational change in domains II–III (Saxena et al. 2015).

The sensor 2 Arg334 residue specifically supports ATP hydrolysis and is not required for the replication-initiation activity of DnaA (Nishida et al. 2002) (Table 4.1). The arginine finger motif Arg285 is the key residue in ATP activation of DnaA for initiation at *oriC* (Fig. 4.2a, Table 4.1); in common with typical AAA+ family proteins, DnaA domain III forms homo-oligomers by head-to-tail interaction, and at the interface, Arg285 of one protomer interacts with ATP bound to the flanking protomer (Neuwald et al. 1999; Iyer et al. 2004; Kawakami et al. 2005; Erzberger et al. 2006; Noguchi et al. 2015). DnaA is monomeric in solution, and these specific domain III-domain III interactions occur when multiple ATP-DnaA molecules are bound to *oriC*, which results in ATP-DnaA-specific, initiation-competent complexes (Felczak and Kaguni 2004; Kawakami et al. 2005; Noguchi et al. 2015; Shimizu et al. 2016) (Fig. 4.2c). In addition, AID (ATP-DnaA-specific interactive locus for DUE unwinding) motifs corresponding to Arg227 and Leu290 in domain III support specific DnaA-DnaA interaction for construction of DUE unwinding-competent DnaA oligomers (Ozaki et al. 2012a) (Table 4.1).

The single-stranded DUE (ssDUE)-binding H (hydrophobic)/B (basic) motifs of domain III correspond to Val211 and Arg245 residues and have a crucial role in DUE unwinding (Ozaki et al. 2008) (Fig. 4.2a, c, Table 4.1). These residues would be exposed on the surface of the central pore when DnaA domain III oligomers form a helical configuration as in typical AAA+ proteins (Ozaki et al. 2008; Ozaki and Katayama 2009; Duderstadt et al. 2011) (see below). The C-terminus of domain III is an amphipathic α-helix, in which Lys366 is important for modulation of *oriC*-DnaA complexes (Garner and Crooke 1996; Saxena et al. 2011) (Table 4.1). Also, a short loop connecting to the N-terminus of this α-helix allows domain IV to rotate at a certain extent (Erzberger et al. 2002; Shimizu et al. 2016).

Domain IV contains the sequence-specific DNA-binding helix-turn-helix motif (Erzberger et al. 2002; Obita et al. 2002; Yoshida et al. 2003; Fujikawa et al. 2003) (Figs. 4.1b and 4.2a, Table 4.1). The DnaA box consensus sequence is the nonamer TTATNCACA. As this sequence is asymmetric, the orientation of bound DnaA is determined by the directionality of the sequence; the arginine finger side of DnaA is oriented toward the 5′ end of the nonamer, whereas the ATP/ADP-binding site is oriented toward the 3′ end (Noguchi et al. 2015) (Fig. 4.2a).

## 4.4 Basic Structure of DiaA

The 196 amino acid residue protein DiaA forms homotetramers with structural symmetry (Keyamura et al. 2007). Each protomer contains a DnaA-binding site that includes Leu190. This site binds tightly to the DnaA domain I Phe46 region, and, theoretically, four DnaA molecules can simultaneously bind to a single DiaA molecule (Fig. 4.2b). Experimentally, binding of at least three DnaA molecules has been demonstrated (Keyamura et al. 2009). As such, DiaA acts as a bridge between DnaA molecules, exerting the so-called linkage effect, which drastically enhances cooperative binding of ATP-DnaA molecules (Stauffer and Chazin 2004; Katayama 2008). Thus, DiaA enhances specific assembly of ATP-DnaA molecules on *oriC* and DUE unwinding (Ishida et al. 2004; Keyamura et al. 2007). Even in the presence of DiaA, the requirement for ATP-DnaA (not ADP-DnaA) in construction of active *oriC*-DnaA complexes is preserved (Keyamura et al. 2007, 2009).

As DiaA binds tightly to the DnaA domain I Phe46 site, and this site is also the primary, but weak, binding site of the DnaB helicase, DiaA binding to DnaA results in inhibition of DnaA-DnaB binding and DnaB loading onto unwound DUE strands (Keyamura et al. 2009). The question of how this inhibition is resolved has to be explored in the future.

DiaA deletion mutations, as well as a DiaA L190A mutation, cause moderate inhibition of the initiation of chromosome replication, consistent with the role for this protein in the stimulation of initiation that was revealed in vitro (Ishida et al. 2004; Keyamura et al. 2007). Oversupply of DiaA also causes moderate inhibition of initiation (Ishida et al. 2004; Flåtten et al. 2015), which might be caused by inhibition of DnaA-DnaB interaction.

## 4.5 Structure and Dynamics of the Initiation Complex

### *4.5.1 Assembly of DnaA on* oriC

Assembly of DnaA can be subdivided into several stages, although further analyses are required to fully understand this process. The cellular level of ATP-DnaA fluctuates during the cell cycle, with a peak at the time of replication initiation (Kurokawa et al. 1999). For a certain period before replication initiation, ADP-DnaA predominates, and only high-affinity DnaA boxes R1 and R4 and moderate-affinity DnaA box R2 bind DnaA, according to the results of in vivo footprinting experiments (Samitt et al. 1989; Miller et al. 2009). As the DiaA-DnaA interaction is stable, DnaA molecules bound at the R1, R4, and R2 boxes might be accompanied by DiaA (Fig. 4.2b), which would enhance the next step of ATP-DnaA assembly on the clusters of low-affinity DnaA boxes. As the ATP/ADP-binding sites of DnaA protomers that are bound to the R1 and R4 boxes orient toward the outer edges of *oriC* (Fig. 4.2c), whether ADP-DnaA or ATP-DnaA binds at these sites is not important for the assembly of ATP-DnaA to the low-affinity regions (Noguchi et al. 2015).

When the level of ATP-DnaA increases and becomes predominant, ATP-DnaA cooperatively binds to the low-affinity DOR sites, with head-to-tail domain III-domain III interactions (Fig. 4.2c). This process depends on the ATP-arginine finger intermolecular interaction and is enhanced by DiaA, as described above. In addition, IHF binding also enhances ATP-DnaA binding to the I1–3 sites (Grimwade et al. 2000; McGarry et al. 2004), which may be an indirect consequence of IHF-dependent DNA bending and the resultant DnaA-DnaA interactions. Results from studies of structural biology suggest that domain III homo-oligomers have a helical configuration, which is a common structural feature in the AAA+ family proteins (Erzberger et al. 2006; Duderstadt et al. 2011) (Fig. 4.2c).

Finally, three DnaA subcomplexes are constructed on *oriC* (Rozgaja et al. 2011; Ozaki and Katayama 2012; Ozaki et al. 2012a; Noguchi et al. 2015; Shimizu et al. 2016). The subcomplex that binds to the left-half *oriC* sustains the full activity in DUE unwinding and basal activity for DnaB loading, whereas the subcomplexes that bind to the R2 site and the region from the R4 site to the C3 site in the middle–right-half *oriC* is not required for DUE unwinding but enhances the DnaB loading activity (Fig. 4.2c).

### *4.5.2 DUE Unwinding*

DUE unwinding is enhanced by IHF binding to *oriC* (Hwang and Kornberg 1992). The possibility that modulation of superhelicity by IHF is the main cause of this stimulation has been considered. However, it has now been demonstrated that even in the absence of superhelicity, IHF binding drastically stimulates DUE unwinding in the presence of ATP-DnaA (Ozaki and Katayama 2012). This observation supports the idea that local structural change, in the form of sharp DNA bending

between DUE and DnaA box R1, is crucial for stimulation of DUE unwinding (see below) (Fig. 4.2c).

DUE unwinding requires binding of DnaA to the ssDUE. ATP-DnaA complexes (but not ADP-DnaA complexes) assembled on the DOR bind specifically to the ssDUE T-rich strand, but not the A-rich strand (Ozaki et al. 2008). In the absence of DOR binding, DnaA does not bind ssDUE efficiently. The DnaA Val211 and Arg245 residues (H/B motifs) located in domain III are required for DUE unwinding as well as binding to the ssDUE T-rich strand (Ozaki et al. 2008). As the corresponding residues are conserved in DnaA orthologs, they are named ssDUE-binding H (hydrophobic) motif (Val211 in *E. coli*) and B (basic) motif (Arg245 in *E. coli*) (Table 4.1). These motifs are exposed on the inner surface of the central pore of the DnaA domain III helical oligomer in a model structure (Fig. 4.2c). A study of the crystal structure of a hyperthermophilic bacterial DnaA ortholog domains III–IV supports the specific structure and role of these residues (Duderstadt et al. 2011).

Overall structure models of DnaA complexes binding the ssDUE have been proposed. In the ssDUE-recruitment model (Fig. 4.2c), the DnaA subcomplex constructed on the left-half DOR binds the ssDUE T-rich strand via DNA bending by IHF (Ozaki et al. 2008; Ozaki and Katayama 2009; Noguchi et al. 2015). This model explains the importance of IHF binding, ATP-DnaA complex formation on the DOR and structural features of *oriC*, such as strict conservation of the spacing between the DUE and the DnaA box R1 (Ozaki and Katayama 2009). Another model postulates formation of a continuous DnaA fiber from DnaA box R1 to the DUE region (Duderstadt et al. 2011). This model also supports the importance of H/B motifs but is less able to explain the roles of IHF and specific DnaA complex formation on the DOR in the unwinding mechanism. In addition, when ADP-DnaA is bound to box R1 (the origin of the postulated DnaA fiber that expands to the DUE in the continuous-fiber model), activity in DUE unwinding is sustained similarly to the case of ATP-DnaA binding to R1 (Noguchi et al. 2015). This fact is consistent with the ssDUE-recruitment model, but not with the continuous DnaA fiber model, as construction of a DnaA fiber requires the intermolecular ATP-arginine finger interaction, especially on DNA without high-affinity DnaA-binding sites. In addition, structures deduced from molecular dynamics simulation of *oriC*-IHF-DnaA complexes are consistent with the ssDUE-recruitment model (Shimizu et al. 2016).

### *4.5.3 Interaction with Helicase*

DnaA has specific binding sites for DnaB helicase; the primary site includes Phe46 (Abe et al. 2007; Keyamura et al. 2009) (Fig. 4.2a, Table 4.1). The secondary, weaker site is suggested to reside at a region spanning the domain II-C-terminus to the domain III-N-terminus but has not been determined at the amino acid residue level (Marszalek et al. 1996; Seitz et al. 2000). Because affinity between DnaA monomers and DnaB is low (Sutton et al. 1998), formation of DnaA oligomers is required for functional interaction with DnaB helicase. DnaB helicase is a

homo-hexamer, so oligomerization of DnaA would provide multiple binding sites for a single DnaB hexamer, stabilizing DnaA-DnaB binding by the linkage effect (Abe et al. 2007; Keyamura et al. 2009). A pair of DnaB helicases is proposed to bind to the two DnaA oligomers assembled on the DOR in opposite directions, enabling bidirectional loading on the single-stranded DNA region (Ozaki and Katayama 2009; Noguchi et al. 2015) (Fig. 4.2c). This hypothesis is consistent with structures deduced from molecular dynamics simulation of *oriC*-IHF-DnaA complexes (Shimizu et al. 2016). The mechanism of DnaB loading onto the single-stranded DNA has yet to be determined.

## 4.6 Regulation Systems for DnaA and *oriC* in *E. coli*

### *4.6.1 Overview*

Regulation of initiation during the *E. coli* cell cycle is achieved by multiple systems targeting DnaA or *oriC* (Katayama et al. 2010; Saxena et al. 2013; Skarstad and Katayama 2013; Riber et al. 2016). These systems operate in different or overlapping periods of the replication cycle, ensuring robust regulation of initiation. For DnaA, multiple systems promote timely inactivation of DnaA by DnaA-ATP hydrolysis or reactivation of DnaA by exchange of bound ADP for ATP. Specific protein and DNA factors support these systems as key elements. The *dnaA* transcription is cell cycle dependent. For *oriC*, a specific protein binds to the nascent *oriC* copies, inhibiting reinitiation.

In addition, specific acetylation of DnaA occurs in the stationary phase, which might be important to regulate replication (Zhang et al. 2016). Interaction of DnaA with acidic phospholipids can stimulate exchange of bound nucleotide of DnaA in vitro, and changes in components of membrane phospholipids in cells influence initiation of replication (Sekimizu and Kornberg 1988; Yung and Kornberg 1988; Crooke et al. 1992; Aranovich et al. 2006; Fingland et al. 2012; Saxena et al. 2013). DnaA-phospholipid interaction also might participate in regulation of replication; a possibility that phospholipids affect initiation in vivo in an indirect unknown manner is not mutually exclusive.

### *4.6.2 The Replicative Clamp-Dependent, Negative Feedback for DnaA*

This system, termed RIDA (regulatory inactivation of DnaA), depends on the DNA-loaded form of the replicative clamp (i.e., the β-subunit of the DNA polymerase III holoenzyme) as well as ADP-Hda protein (Katayama et al. 1998; Kurokawa et al.1999; Kato and Katayama 2001; Su'etsugu et al. 2008). As DNA-free clamps are

inactive in RIDA, this system is activated upon replication initiation and loading of DNA polymerase III holoenzyme. During lagging-strand replication, Okazaki fragments are repeatedly synthesized, which leaves the used clamps on the nascent DNA regions. These DNA-bound clamps interact with ADP-Hda protein, and the resultant complex then interacts with ATP-DnaA, promoting ATP hydrolysis. RIDA operates throughout chromosomal replication.

Hda consists of a short N-terminal region bearing a clamp-binding motif and an AAA+ domain (Kato and Katayama 2001; Su'etsugu et al. 2005, 2008). Hda binds ADP, but not ATP, resulting in RIDA-active, monomeric Hda. The arginine finger motif of the Hda AAA+ domain is required for DnaA-ATP hydrolysis. A head-to-tail complex of DnaA domain III and the Hda AAA+ domain assembles. In addition, Hda interacts with DnaA domain I and domain IV with low affinity (Keyamura and Katayama 2011; Su'etsugu et al. 2013) (Fig. 4.1b, Table 4.1). These multiple weak interactions would support repeated interaction of a single ADP-Hda-clamp-DNA complex with ATP-DnaA molecules, catalytically promoting ATP hydrolysis. Whereas the cellular level of DnaA is about 2000 monomers per cell (Sekimizu et al. 1988), that of Hda is only about 100 monomers, and oversupply of Hda is very toxic to cells (Su'etsugu et al. 2005; Baxter and Sutton 2012).

RIDA is the predominant system for inactivating DnaA (Camara et al. 2005; Kasho and Katayama 2013). *Hda* mutations increase the cellular level of ATP-DnaA and cause over-initiation, which results in inhibition of cell division and cell growth (Kato and Katayama 2001; Fujimitsu et al. 2008; Charbon et al. 2014), although suppressor mutations frequently occur. It should also be noted that, in the presence of RIDA, an increase in the total cellular amount of DnaA causes only slight over-initiation (Atlung et al. 1987; Flåtten et al. 2015). By contrast, unlike expression of wild-type DnaA, expression of a RIDA-insensitive DnaA variant, which substantially constitutively takes on the ATP form, causes severe over-initiation, leading to inhibition of cell growth (Nishida et al. 2002; Keyamura and Katayama 2011).

### *4.6.3 The Specific DNA Element (*datA*)-Dependent Timely Inactivation of DnaA*

The *datA* locus of the *E. coli* chromosome also has a role in repressing extra initiations (Kitagawa et al. 1998) (Fig. 4.3). This locus contains a DnaA box cluster, in which three boxes with the same direction (DnaA boxes 7, 2, and 3) are essential for function, and one box with the opposite direction (DnaA box 4) is stimulatory (Ogawa et al. 2002; Kasho and Katayama 2013; Kasho et al. 2017) (Fig. 4.3b). In addition, this locus contains a single IHF-binding site, which is essential for *datA* function (Nozaki et al. 2009b).

The IHF-*datA* complex promotes DnaA-ATP hydrolysis, yielding ADP-DnaA (Kasho and Katayama 2013). This function is termed DDAH (*datA*-dependent DnaA-ATP hydrolysis). The detailed mechanism of DDAH is under investigation, but specific DnaA domain III-domain III interaction is important for activating the

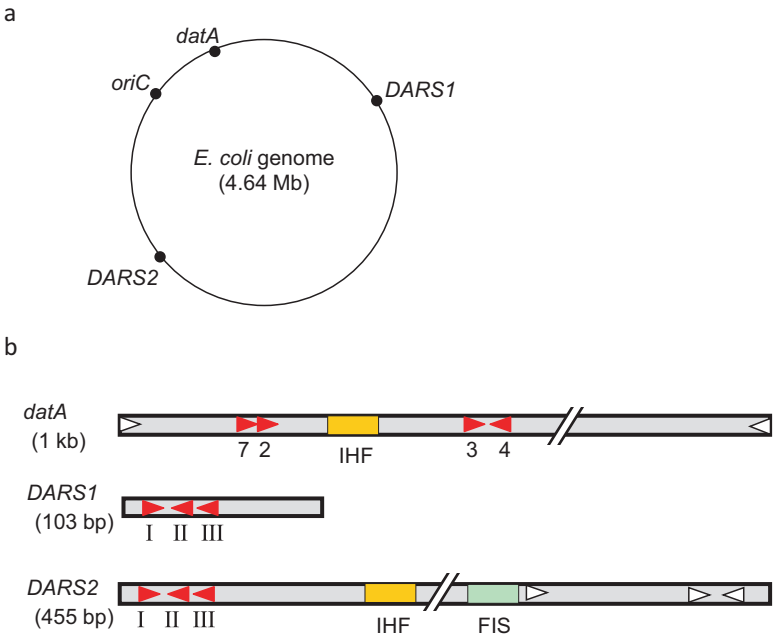


**Fig. 4.3** Basic structures of *datA*, *DARS1*, and *DARS2*. (**a**) Chromosomal positions of *oriC*, *datA*, *DARS1*, and *DARS2*. (**b**) Overall structures of *datA*, *DARS1*, and *DARS2* are shown. Important DnaA boxes are shown by filled *triangles*. Those are DnaA boxes 2, 3, 4, and 7 of *datA*, and DnaA boxes I, II, and III of *DARS1* and *DARS2*. IHF and Fis binding sites are also indicated

ATPase activity of DnaA (Table 4.1). Supercoiled structure of the *datA* region stimulates assembly of DnaA and IHF, thereby enhancing DDAH (Kasho et al. 2017). It might be important to investigate if the chromosomal *datA* locus is regulated specifically in superhelicity.

Binding of IHF to *datA* is cell cycle specific; whereas binding is repressed before initiation, it increases after initiation, peaking 15 min after initiation (Kasho and Katayama 2013). DDAH is independent of RIDA and assists in repressing untimely initiations. *datA*-deleted cells can grow but experience a moderate level of untimely initiations (Kitagawa et al. 1998; Morigen et al. 2003, 2005; Nozaki et al. 2009b).

Fluorescent labeling experiments and genome conformation analysis suggest that in a cell, *oriC* is colocalized with *datA* (Nozaki et al. 2009a; Cagliero et al. 2013). The genomic locus of *datA* is near *oriC* (i.e., distance between the two is ~470 kb) (Fig. 4.3a), and both two are included in a compactly folded domain (i.e., Ori macrodomain) of the chromosome (Niki et al. 2000; Valens et al. 2004). Biological significance of the colocalization of *oriC* and *datA* remains unclear. However, relocation of *datA* to a site near *terC* (the replication termination site of the chromosome) inhibits function of *datA* in rapidly growing cells, which could be caused by delay of duplication of *datA* in a replication cycle or enlarged spacing itself between the two in a cell (Kitagawa et al. 1998; Frimodt-Møller et al. 2016).

### 4.6.4 *Sequestration:* oriC *DNA Methylation-Dependent Regulation*

This system depends on the Dam (DNA adenine methyltransferase) and SeqA proteins (Waldminghaus and Skarstad 2009). Dam methylates the N-6 position of adenine in the sequence GATC. As this sequence is palindromic, both A residues in the duplex DNA sequence are methylated, resulting in a fully methylated state. When this sequence is replicated, a hemi-methylated state (where the nascent strand is not yet methylated) exists before further action of Dam. The hemi-methylated GATC sites are binding targets of SeqA (Lu et al. 1994; Slater et al. 1995). *SeqA*-deleted cells can grow but experience a moderate level of untimely initiations.

SeqA contains GATC-binding and self-oligomerization domains (Fujikawa et al. 2004; Odsbu et al. 2005; Guarné et al. 2005; Chung et al. 2009). The *oriC* region includes a cluster of GATC sites (11 in total), and hemi-methylated *oriC* is bound by SeqA oligomers; this binding is maintained for about 10 min after initiation in cells with a doubling time of 30 min (Lu et al. 1994). Cooperative SeqA binding to the supercoiled DNA causes DNA topological changes (Torheim and Skarstad 1999; Kang et al. 2003), and within *oriC* it inhibits DnaA binding to the low-affinity boxes, thereby inhibiting initiation (Nievera et al. 2006). Subcellular localization of SeqA is dynamically changed during the replication cycle: observation of fluorescence-labeled SeqA in live cells suggests that SeqA molecules are assembled on *oriC* during the sequestration period, and thereafter those molecules are dissociated from *oriC* and assembled on the nascent DNA regions flanking the replication forks (Fossum-Raunehaug et al. 2014; Helgesen et al. 2015). Dissociation of SeqA from a hemi-methylated *oriC* region spontaneously occurs independently of Dam in vitro (Kang et al. 1999).

### 4.6.5 *Specific DNA Element (*DARS1 *and* DARS2*)-Dependent Timely Reactivation of DnaA*

The *E. coli* chromosome contains two sites which specifically interact with ADP-DnaA and reactivate it by exchanging ADP to ATP and producing ATP-DnaA (Fujimitsu et al. 2009). These DnaA-reactivating sequence sites are termed *DARS1* and *DARS2* (Fig. 4.3a). These sites both include a cluster of three DnaA boxes (Fig. 4.3b), which promote specific domain III-dependent DnaA-DnaA interactions, thereby enhancing dissociation of DnaA-bound nucleotide. These trios of DnaA boxes are connected to regulatory regions which structurally differ between *DARS1* and *DARS2*. The regulatory region of *DARS2* bears specific binding sites for IHF and Fis, which are required for activating *DARS2* function (Kasho et al. 2014) (Fig. 4.3b; also see below). However, the mechanisms by which the regulatory regions stimulate the DnaA-reactivating function are not yet known.

Both *DARS1* and *DARS2* are required for timely initiation during the cell cycle (Fujimitsu et al. 2009). In vivo, *DARS2* has a predominant role and *DARS1* has a supporting role. Activation of *DARS2* is regulated by timely binding of Fis and IHF; Fis binds to *DARS2* in log-phase cells, but not in stationary-phase cells, whereas IHF binds to *DARS2* in a pre-initiation stage of the cell cycle (Kasho et al. 2014). At the time of initiation, the ATP-DnaA level reaches its maximum, and IHF is dissociated from *DARS2*. Mechanisms involved in the timely binding/dissociation of IHF to/from *DARS2* should be explored in the future. The chromosomal location of *DARS2*, in a central region between *oriC* and the replication termination site (Fig. 4.3a), is important for regulating timely initiation in cells growing at 42 °C in LB medium (Inoue et al. 2016; Frimodt-Møller et al. 2016). In addition, genomic conformation analysis suggests that *oriC* is colocalized with *DARS2* in a cell (Cagliero et al. 2013). The biological significance of this colocalization remains to be investigated.

### *4.6.6 Cell Cycle-Dependent Transcription of the* dnaA *Gene*

Transcription of the *dnaA* gene is regulated in a cell cycle-specific manner; it increases before initiation and decreases after initiation (Theisen et al. 1993). This fluctuation depends on Dam and SeqA (Bogan and Helmstetter 1997) and is important to sustain timely initiation (Riber and Løbner-Olesen 2005), probably because de novo-synthesized DnaA will bind ATP (which is much more abundant in the cell than ADP) to form ATP-DnaA. In addition, *dnaA* gene transcription is autoregulated (Speck et al. 1999).

*oriC* is flanked by two genes, *gidA* and *mioC*, which are transcribed in the same direction (*oriC* is downstream of *mioC*). The pattern of change of *gidA* transcription is similar to that of *dnaA*, whereas fluctuation of *mioC* transcription is opposite to that of *gidA* and *dnaA* (Theisen et al. 1993; Ogawa and Okazaki 1994). However, transcription of *gidA* and *mioC* is not required for regulation of initiation (Bates et al. 1987), although constitutive transcription of *mioC* moderately inhibits initiation (Su'etsugu et al. 2003).

## 4.7 Conservation in Eubacterial Species

This review focuses on mechanisms and regulation of replication in *E. coli*. However, many important studies on DNA replication initiation have been conducted in other bacteria, phages, and plasmids. Evidence from these studies indicates that the overall structure of *oriC* might be fundamentally similar in relation to the locations of the DUE and DOR, although the detailed structures vary in different species (Wolański et al. 2015). ssDUE-binding activity of DnaA has been reported

in *Bacillus subtilis* (Richardson et al. 2016). In the hyperthermophilic eubacterium *Thermotoga maritima*, DnaA specifically binds to its cognate ssDUE when it forms homo-oligomers on its cognate DOR (Ozaki and Katayama 2012). In plasmid RK2, the origin includes iterons, which are repeats of the initiator protein TrfA-binding sequence and its flanking DUE region. TrfA forms a homo-oligomer on the iteron region, and the resultant complex binds the ssDUE-bottom (but not the DUE-top) strand, which is followed by loading of replisomes (Wegrzyn et al. 2014; Wawrzycka et al. 2015). This mechanism might be similar to the ssDUE-recruitment mechanism of *E. coli oriC*.

In *Helicobacter pylori*, HobA, the DiaA functional homolog, has a crucial role in assembly of the cognate DnaA at the replication origin (Zawilak-Pawlik et al. 2007; Natrajan et al. 2009). In *Caulobacter crescentus*, HdaA, a structural and functional homolog of *E. coli* Hda, is important for regulation of the cognate DnaA and replication initiation (Collier and Shapiro 2009; Fernandez-Fernandez et al. 2011). In addition, *C. crescentus* CtrA binds to the cognate *oriC* in a timely manner, inhibiting extra initiation. In *B. subtilis*, YabA, the functional counterpart of Hda, binds both the replicative clamp and DnaA, sequestrating DnaA from *oriC* and repressing untimely initiation (Noirot-Gros et al. 2006; Soufo et al. 2008). In addition, in *B. subtilis*, binding of DnaA domain I by SirA and domain III by Soj/ParA, is important for the repression of untimely initiation for sporulation (Jameson et al. 2014; Scholefield et al. 2012).

The *B. subtilis* and *Streptomyces coelicolor* chromosomes have specific DnaA box clusters that can repress untimely initiations (Smulczyk-Krawczyszyn et al. 2006; Okumura et al. 2012). Sequences corresponding to *DARS* are highly conserved in *Gammaproteobacteria* (Fujimitsu et al. 2009), but except for *E. coli*, these sequences have not yet been functionally analyzed.

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# Chapter 5
# Initiation of DNA Replication in the Archaea

**Stephen D. Bell**

**Abstract** Organisms within the archaeal domain of life possess a simplified version of the eukaryotic DNA replication machinery. While some archaea possess a bacterial-like mode of DNA replication with single origins of replication per chromosome, the majority of species characterized to date possess chromosomes with multiple replication origins. Genetic, structural, and biochemical studies have revealed the nature of archaeal origin specification. Recent work has begun to shed light on the mechanisms of replication initiation in these organisms.

**Keywords** DNA replication • Initiator protein • Helicase • Replication origin • Archaea • *Sulfolobus*

The archaea are a diverse range of microorganisms that share more recent evolutionary history with eukaryotes than do the bacteria (Woese and Fox 1977). The precise timing of the divergence of the archaeal and eukaryotic lineages is the subject of considerable debate, with some studies even suggesting that eukaryotes arose from within the archaeal domain of life (Williams et al. 2013; Rivera and Lake 2004; Forterre 2015). A number of phyla have been identified within the Archaea; again controversy exists regarding the precise nature of the taxonomic divisions between archaeal phyla. With increased sampling, particularly at the metagenomic level, some degree of consensus is being established. It is generally accepted that there is a broad divide between the phylum of the Euryarchaea and those of the Thaumarchaea, Aigarchaea, Crenarchaea, and Korarchaea. The latter four taxonomic groupings appear more closely related to one another and have been termed the "TACK superphylum" (Guy and Ettema 2011; Brochier-Armanet et al. 2008; Forterre 2015). At the morphological level, archaea are prokaryotes; most species have a single cell membrane and are devoid of any organellar structures.

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**Table 5.1** Taxonomic distribution of archaeal species described in the text

| Phylum | Species | Origins in main chromosome | Copy number |
|---|---|---|---|
| Crenarchaea | *Sulfolobus islandicus* | 3 | 1C-2C |
| | *Sulfolobus solfataricus* | 3 | 1C-2C |
| | *Aeropyrum pernix* | 2 | 1C-2C |
| | *Pyrobaculum calidifontis* | 4 | 1C-2C |
| Euryarchaea | *Pyrococcus abyssi* | 1 | Polyploid |
| | *Haloferax volcanii* | 3 (or 4) | Polyploid |
| | *Haloferax mediterranei* | 3 | Polyploid |
| | *Methanothermobacter thermautotrophicum* | one mapped thus far | Polyploid |
| | *Thermococcus kodakarensis* | ND | Polyploid |

The ambiguity in origin number in *Haloferax volcanii* lies in the description of the integration of an extrachromosomal element into the 3-origin main chromosome in a lab-adapted strain (Hawkins et al. 2013). While one origin has been mapped in *M. thermautotrophicum* (Majernik and Chong 2008), no genome-wide repication profiling has been performed on this organism. It is possible, therefore, that additional origins exist in this species
*ND* not determined

Thus far, all archaea characterized have circular chromosomes; however, the chromosome copy number shows considerable variation across taxonomic divides. To a broad approximation, euryarchaea appear to be generally oligoploid or polyploid, while the members of TACK that have been studied have cell cycles that oscillate between one and two copies of their chromosome (Table 5.1) (Samson and Bell 2014; Breuert et al. 2006; Hildenbrand et al. 2011). Flow cytometry studies have revealed that the TACK superphylum organisms, such as members of the *Sulfolobus* genus of hyperthermophilic acidophiles, have cell cycles that contain defined gap phases separating DNA replication and cell division (Lundgren et al. 2008; Pelve et al. 2013). These observations have led to the adoption of the G1, S, G2, and M phase nomenclatures established in studies of the eukaryotic cell cycle to describe the analogous stages of archaeal cell cycle progression. It must be emphasized, however, that there is no evidence that archaeal chromosome segregation is in any way related to eukaryotic mitosis. Interestingly, in marked contrast to the orchestrated cell cycles of crenarchaea, the euryarchaea that have been studied appear to lack obvious gap phases, perhaps hinting that cell division can occur during ongoing rounds of replication of the multiple copies of the chromosome, in a manner somewhat reminiscent of fast-growing *E. coli* (Sherratt 2003).

## 5.1 The Replication Machinery of Archaea

With the availability of whole genome sequences of archaeal species in the 1990s, it became apparent that archaea possess clear orthologs of eukaryotic DNA replication-associated proteins (Edgell and Doolittle 1997). In general, and in keeping with the organizational simplicity of the organisms, the archaeal replication proteins are

simplified versions of their eukaryotic counterparts (Barry and Bell 2006; Kelman and Kelman 2014). For example, the eukaryotic MCM(2-7) replicative helicase has six distinct subunits. However, all six subunits are related to each other in sequence, suggesting derivation from a common ancestor. Indeed, the majority of present-day archaea encode a single *mcm* gene the product of which homo-multimerizes to form a homohexameric assembly (Costa and Onesti 2009; Bochman and Schwacha 2009). Similarly, almost all archaea encode a protein that is related to both Cdc6 and Orc1 component of eukaryotic origin recognition complex, ORC (Bell 2012). Interestingly, early branching eukaryotes, such as trypanosomes, also encode an archaeal-like Orc1/Cdc6 protein, suggesting that the gene duplication and sequence diversification leading to "higher" eukaryotic Orc1 and Cdc6 occurred within the eukaryotic lineage (Samson and Bell 2016; Tiengwe et al. 2012). Importantly, the bacterial replication machinery, although, ultimately, performing the same function, is largely non-orthologous to the shared archaeal/eukaryotic replication apparatus. The key exceptions lie in the clamp/loader and sliding clamp that facilitate DNA polymerization, leading to the proposal that the elongation machinery is fundamentally conserved and thus ancestral, even though the rest of the replisome components are not conserved between bacteria and archaea/eukarya (Yao and O'Donnell 2016).

## 5.2 Archaeal Replication Initiation

The first archaeon in which the replication mode was experimentally determined, a euryarchaeon *Pyrococcus abyssi*, revealed a single origin of replication. The origin, *oriC*, is located in a gene environment that contained genes for several replication-associated proteins, including the candidate initiator protein *orc1/cdc6* gene (Myllykallio et al. 2000; Bell 2012). The *orc1/cdc6* nomenclature is cumbersome, and orthologs in archaeal genomes have been variously annotated as *orc1* or *cdc6* on an apparently random basis. In this chapter, for simplicity's sake, I will refer to these genes as *orc1*. Many archaea encode multiple Orc1 paralogs, and I will refer to these as Orc1-1, Orc1-2, etc.

Interestingly, the single-origin paradigm in *Pyrococcus* species actually appears to be atypical among the archaea, and it is now known that many archaea from both euryarchaea and TACK species have multiple replication origins per chromosome (Robinson and Bell 2007; Robinson et al. 2004; Robinson et al. 2007; Lundgren et al. 2004; Norais et al. 2007; Hawkins et al. 2013; Yang et al. 2015; Pelve et al. 2012). The highest number of origins reported is four per chromosome for lab strains of the euryarchaeon *Haloferax volcanii* and also the crenarchaeon *Pyrobaculum calidifontis* (Pelve et al. 2012; Hawkins et al. 2013). For most species, while origin number and location have been established, the extent to which each origin is used remains poorly resolved. The exception to this lies in *Sulfolobus* species where three origins have been mapped, and these have been experimentally determined to fire once per cell cycle (Duggin et al. 2008). Studies with synchronized cell populations have revealed that two of the origins, *oriC1* and *oriC3*, fire

synchronously, thereby defining the start of S phase. Notably, *oriC2* fires a few minutes later. As will be discussed below, this temporal delay is likely linked to the expression of the initiator protein that defines this origin.

Many archaea encode multiple Orc1 paralogs. In the case of *Sulfolobus*, three such proteins, Orc1-1, Orc1-2, and Orc1-3, are encoded in the 2.2–3 megabase-pair genome. *Sulfolobus* also encodes a further candidate initiator protein, WhiP, that is a distant homolog of another eukaryotic replication initiation protein, the helicase co-loader, Cdt1 (Robinson and Bell 2007).

## 5.3 Origin Specification

Genetic studies in *Sulfolobus islandicus* have revealed a simple one-to-one relationship between the location of initiator protein genes (Fig. 5.1) and the origins that they specify (Samson et al. 2013). More specifically, Orc1-1 is encoded adjacent to, and specifies origin function at, *oriC1*; Orc1-3 is adjacent to *oriC2* and is required for function at that origin, and finally the gene for WhiP is beside *oriC3*, and the WhiP gene product is necessary for *oriC3* function. Furthermore, the initiator protein encoded adjacent to each origin is both necessary and sufficient for its cognate origin function. What then of Orc1-2? The *orc1-2* gene is not encoded immediately adjacent to any of the three origins, and deletion of *orc1-2* does not affect firing at any of the three origins. A range of biochemical and transcriptomic analyses have implicated Orc1-2 as a negative regulator of replication (Robinson et al. 2004; Maaty et al. 2009; Frols et al. 2007; Gotz et al. 2007). However, its role in this regard remains to be firmly established. Thus, the *Sulfolobus islandicus* chromosome is a mosaic of three distinct replicons, each origin having its own specific

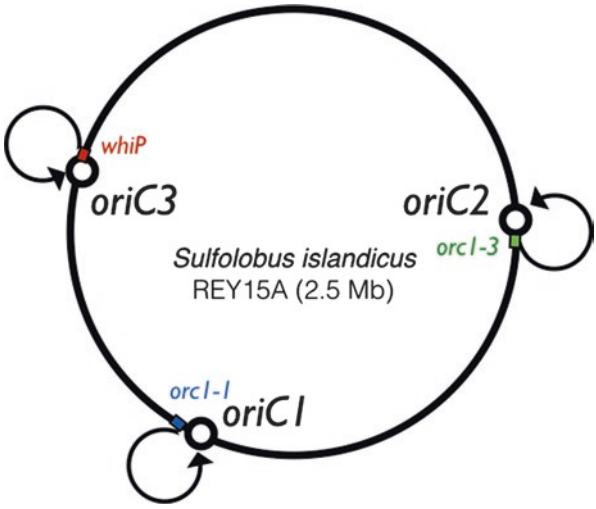


**Fig. 5.1** Diagram of the organization of the 2.5 Mb chromosome of *Sulfolobus islandicus*. The relative positions of the three origins are indicated along with their cognate initiator proteins (Samson et al. 2013). Genetic dependence of the origin upon initiators is indicated by the circular arrows

initiator. Analyses of the phyletic distribution of the initiator proteins reveal that Orc1-1 is highly conserved across a broad range of archaeal species. For example, the single *orc1* gene encoded by *Pyrococcus* is most closely related to *Sulfolobus* Orc1-1. Indeed, it was demonstrated that *Sulfolobus solfataricus* Orc1-1 can bind specifically to conserved sequence elements, termed ORB (origin recognition box), in the *Pyrococcus oriC* in vitro (Robinson et al. 2004). ORB elements are conserved across the archaeal domain of life and possess a dyad symmetric element flanked uniquely on one side by a G-rich element. Interestingly, all characterized *oriC1* origins in archaea possess at least two ORB elements in inverted orientation and separated by an AT-rich candidate duplex unwinding element (see Samson and Bell 2016 for a review). The nature of Orc1-1 interaction with ORB elements is discussed below.

In contrast to the near universality of Orc1-1, Orc1-3 appears to be restricted to the Sulfolobales, and WhiP is found in both Sulfolobales and Desulfurococcales. This patchy distribution of the initiators suggests that the *oriC2* and *oriC3* origin/initiator cassettes are relatively recent acquisitions, and it has been proposed that they have been acquired by incorporation of extrachromosomal elements into an ancestral *oriC1*/Orc1-1 containing chromosome (McGeoch and Bell 2008; Robinson and Bell 2007; Samson and Bell 2014).

Direct evidence for functional incorporation of extrachromosomal origins has been documented in the halophilic euryarchaeon *Haloferax volcanii* where a lab strain differs from the parental strain by incorporation of a large plasmid, pHV4, into the host chromosome (Hawkins et al. 2013). Importantly, the origin on the plasmid remains functional in its new integrated location. The malleability of the replicon architecture of *H. volcanii* main chromosome is underscored by the remarkable observation that its replication can be maintained even in the apparent absence of active replication origins. More specifically, experiments to delete all four origins in the lab strain of *H. volcanii*'s main chromosome were successful, and, very strangely, the resultant "zero origin" strain actually outcompeted the wild type in coculture experiments. The zero origin strain was highly dependent on the RAD51/RecA ortholog, RadA, suggesting a recombination-based mechanism was able to drive genome duplication (Hawkins et al. 2013). How universal this remarkable observation is is not yet clear (Michel and Bernander 2014). When similar experiments were performed in the closely related *H. mediterranei*, the main chromosome of which normally has three active origins (Fig. 5.2), deletion of the three origins led to activation of a cryptic novel origin of replication (Yang et al. 2015). It is possible that the high ploidy, sexual promiscuity (as manifested by high levels of intraspecies and even interspecies genetic exchange mediated by this organism), and natural competence, i.e., ability to uptake DNA from the media, may be contributory to *H. volcanii*'s remarkable genomic plasticity (Zerulla et al. 2014; Zerulla and Soppa 2014; Naor et al. 2012).

Genetic studies in *Sulfolobus islandicus* (Sis) reveal that at least one replication origin is essential for viability and that each origin has a unique initiator protein. Intriguingly, this simple binary relationship of origin and initiator is not conserved across the *Sulfolobus* genus. Studies in *Sulfolobus solfataricus* (Sso) have revealed

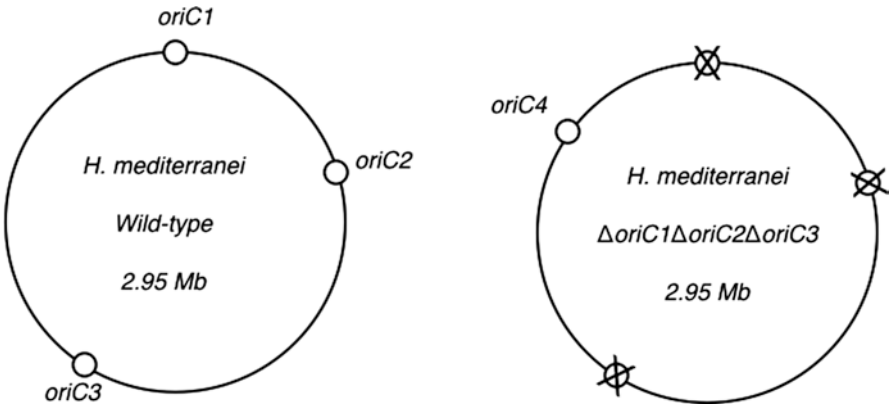


**Fig. 5.2** Diagram of the organization of the 2.95 Mb main chromosome of *Haloferax mediterranei*. The locations of the three active origins in wild-type cells are shown in the left-hand panel. The right-hand panel indicated that, upon deletion *of oriC1*, *oriC2*, and *oriC3*, cell viability is maintained by activation of a novel cryptic origin, *oriC4*. For details see Yang et al. (2015)

that *oriC2* in that species is bound by both Orc1-1 and Orc1-3. While the genetic dependence of this origin on both initiators has not been tested, a range of chromatin immunoprecipitation and biochemical and structural studies have demonstrated that this origin is bound by both Orc1-1 and Orc1-3 (Robinson et al. 2004; Dueber et al. 2007; Dueber et al. 2011). The Orc1-1 and Orc1-3 binding sites at this origin are immediately adjacent, and the two proteins have a 360 $Å^2$ protein-protein interface (Dueber et al. 2007). A biochemical comparison of Orc1-1 and *oriC2* between *S. islandicus* and *S. solfataricus* revealed that both origin sequence and protein sequence have evolved to allow the binding of *S. solfataricus* Orc1-1 to *oriC2* in that species (Samson et al. 2013). This enhanced complexity of origin specification may give insight into the evolutionary transitions that drove the evolution of the multi-subunit present-day ORC complex found in eukaryotes.

## 5.4 Orc1 Protein Structure and Function

The structural studies of *Sulfolobus* Orc1-1 and Orc1-3 bound to *oriC2*, in conjunction with the work from the Wigley lab on *Aeropyrum pernix* Orc1-1 bound to its cognate *oriC1*, revealed some general principles of Orc1 protein/DNA interactions (Dueber et al. 2007; Gaudier et al. 2007). The archaeal Orc1 proteins are approximately 43 kDa in size and possess an N-terminal AAA+ domain and a C-terminal winged-helix (wH) DNA-binding domain (Fig. 5.3). While mutational studies had demonstrated the importance of the wH domain in DNA binding, the structural studies revealed that the AAA+ domain also contacted the DNA (Gaudier et al. 2007; Robinson et al. 2004; Dueber et al. 2007; Dueber et al. 2011). The contact between the AAA+ domain and DNA is mediated by a signature embellishment to

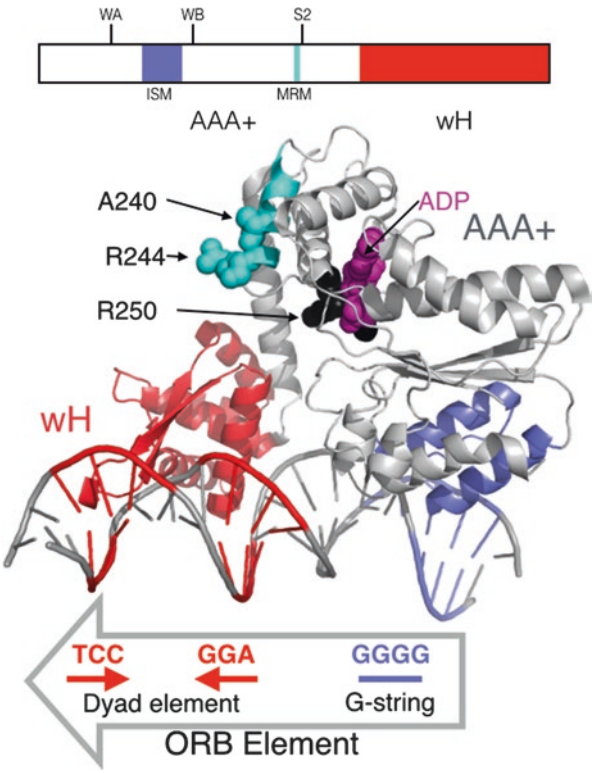


**Fig. 5.3** Structure of the Orc1 proteins. The upper panel is a linear representation of the protein. The N-terminal two-thirds are a AAA+ domain, and the positions of the Walker A (WA), Walker B (WB), and Sensor 2 (S2) motifs are indicated. The ISM is the signature initiator-specific motif embellishment to the AAA+ fold found in the initiator clade of AAA+ proteins. MRM indicates the location of the MCM recruitment motif. The C-terminal third of the protein forms a winged-helix (wH) fold. The crystal structure shown below is of Orc1-1 bound to an ORB element (PDB Accession Number 2V1U). The ORB element is shown by a large gray arrow with internal dyad element and G-string element indicated. The orientation of the arrow is the same as that in Fig. 5.5. The wH domain in red interacts with the dyad symmetric element of the DNA. The ISM, in blue, mediates contacts a G-rich element, the so-called G-string. ADP is present in the active site of the AAA+ domain and is shown in magenta. The residues highlighted in cyan have been demonstrated to be essential for recruitment of MCM by Orc1-1 (residue numbering based on the *S. islandicus* Orc1-1 protein)

the classical AAA+ fold found in the initiator clade of AAA+ proteins, termed the initiator-specific motif (ISM). Thus, the *orc1* proteins make extended bipartite interactions with the origin DNA (Fig. 5.3). It had been demonstrated that Orc1-1 bound to conserved sequence elements, termed ORBs, at *oriC1* (Robinson et al. 2004). ORB elements contain a dyad symmetric element flanked on one side only by a string of G-C base pairs. The wH domain recognizes the dyad element, and the G-string interacts with the ISM (Fig. 5.3). Despite the presence of the dyad element, only a single Orc1-1 molecule binds per ORB element. The structural studies revealed that binding of Orc1-1 substantially distorts and underwinds the DNA to

the extent that a second Orc1-1 molecule is unable to recognize the symmetry-related binding site (Gaudier et al. 2007). The preferred orientation of Orc1-1 on an ORB element is presumably defined by the unique ISM-G-string interaction.

Thus, at *oriC1*, Orc1-1 binds to ORB elements as a monomer. Another key feature in the structural studies was that the active site of the AAA+ ATPase domain was occupied by ADP. As no nucleotide was supplemented during purification and crystallization, this presumably reflects ATP bound during expression in *E. coli* and hydrolyzed during the expression and purification processes. Biochemical studies have confirmed that ADP is extremely stably bound to Orc1 proteins. Indeed, protein denaturation and extensive and subsequent re-folding are required to obtain nucleotide-free protein with which to perform ATPase studies (Grainge et al. 2006; Samson et al. 2013). Such studies have revealed that Orc1-1 undergoes a single-turnover ATP hydrolysis event leaving ADP stably bound in the active site. While bacterial DnaA is also active in its ATP-bound state, this activation is manifested in a fundamentally distinct manner from that of Orc1-1. ATP facilitates multimerization of DnaA, ultimately resulting in direct remodeling and melting of the origin DNA (see Bleichert et al. 2017 for a review). In contrast, Orc1-1 remains monomeric when ATP bound and undergoes a subtle conformational change (Samson et al. 2013) that facilitates interaction with MCM, as described below.

Studies using mutated versions of Orc1-1 in vivo and in vitro have revealed that stabilization of the ATP-bound form of the protein by substitution of the so-called Walker B glutamic acid residue by alanine results in a highly active form of the protein (Samson et al. 2013; Samson et al. 2016). In contrast, the ADP-bound form of Orc1-1 is inactive in MCM loading in vitro. On the biochemical level, ATP binding did not alter either the affinity or stoichiometry of Orc1-1 binding to DNA. Rather, ATP binding simply induced a modest conformational change in the protein, as detected by analytical ultracentrifugation and protease sensitivity assays. Despite these modest changes, the constitutively ATP-bound form of the protein was far more active in vitro than the ADP form (Samson et al. 2013). Thus, it appears that ATP binding and not hydrolysis is required for Orc1-1 function. Importantly, expression of the Walker B mutant form of the protein in vivo resulted in an overreplication phenotype, suggesting that ATP hydrolysis serves as an off switch. In this regard, it is significant that the *orc1-1* gene shows cell cycle-dependent regulation of its expression with transcript levels highest in cells about to enter G1 (Samson et al. 2013). Thus, the cell cycle dependence of *orc1-1* expression, coupled with the single-turnover ATP hydrolysis activity, indicates that Orc1-1 is acting as a molecular switch, permitting MCM recruitment in the Orc1-1•ATP state and inhibiting it in the Orc1-1•ADP state. Such a binary switch behavior is likely important for ensuring once-per-cell cycle regulation of origin activity (Fig. 5.4). The timing of expression of the initiator protein gene thus helps define a permissive window for initiator function. As mentioned above, *oriC2* fires a few minutes later in the cell cycle than does *oriC1*. This is reflected in the later peak of transcription of the Orc1-3 mRNA, relative to that for Orc1-1 (Samson et al. 2013). How the ADP-bound form of the initiator is removed from the origin at the end of the cell cycle is currently unknown. Possible

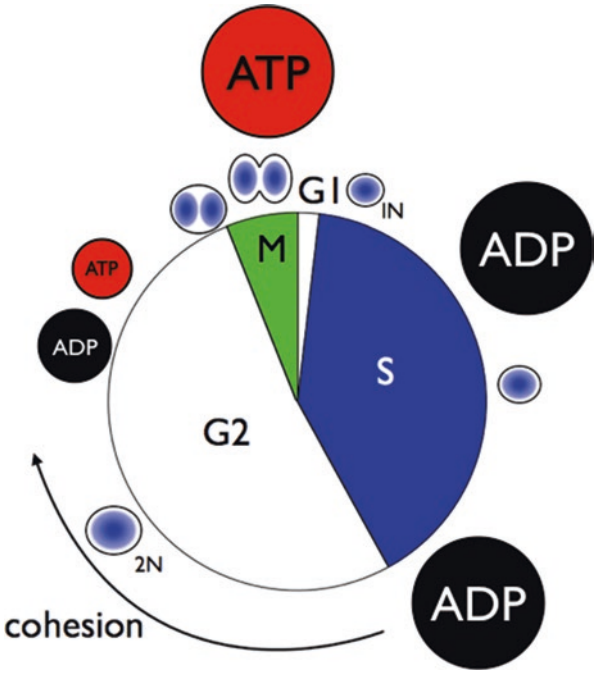


**Fig. 5.4** Cartoon of the *Sulfolobus* cell cycle. The red and black spheres labeled ATP and ADP represent the nucleotide status of Orc1 proteins. A pulse of transcription of *orc1-1* at the time of cell division will produce Orc1-1 associated with ATP; subsequently ATP will be hydrolyzed to ADP in a single-turnover event. ADP will thus remain stably associated with Orc1-1 for the rest of the cell cycle

explanations include an ATP exchange factor or, perhaps more likely given the new wave of *orc1-1* transcription, targeted destruction of Orc1-1•ADP at cell division.

## 5.5 MCM Recruitment to Archaeal Replication Origins

As alluded to above, Orc1-1 is able to recruit MCM to *oriC1* in a defined reaction using recombinant proteins purified from *E. coli* (Samson et al. 2016). These experiments reveal that, in addition to Orc1-1 sharing sequence homology with Orc1 and Cdc6 of eukaryotes, Orc1-1 also shares Orc1 and Cdc6's respective functions of origin binding and helicase recruitment. Orc1-1•ATP was shown to contact MCM's C-terminal wH domain via a conserved motif in the lid domain of the AAA+ domain (the MRM – MCM recruitment motif; see Fig. 5.3). The basis of the ATP dependence of Orc1-1's functionality was ascribed to the Sensor 2 motif. This conserved arginine residue has the capacity to coordinate the gamma phosphate of ATP and in doing so modulate the relative positions of the two subdomains of the AAA+ module. Importantly, mutation of the Sensor 2 residue led to a protein that bound ATP but had substantially reduced ATPase activity. However, unlike the Walker B mutant that has similar ATPase-null behavior, the Sensor 2 mutant Orc1-1 was unable to recruit MCM to the origin in vitro and did not support origin firing in vivo. Thus, the

Sensor 2 residue may act to transduce the information of the nucleotide status of Orc1-1 to the conformation of the MCM recruitment site (Samson et al. 2016).

## 5.6 Active Loading or Passive Recruitment of MCM?

Classical views of the MCM helicase portray it as a ring-shaped hexamer (Costa and Onesti 2009). However, structural studies of both eukaryotic MCM2-7 and archaeal MCM have revealed a range of conformations. With regard to the archaeal MCMs, single and double hexamers and heptamers have been described, as have open-ring and even filamentous forms of the protein (Chen et al. 2005; Pape et al. 2003; Slaymaker et al. 2013; Samson and Bell 2016; Samson et al. 2016). There has been considerable debate about how the MCM ring might be opened to allow loading onto DNA (Yardimci and Walter 2014; Sakakibara et al. 2009). With regard to the archaeal protein, a notable electron microscopy study demonstrated that simply heating the MCM of *Methanothermobacter thermautotrophicum* to its normal physiological growth temperature resulted in greater than half of the particles adopting an open-ring conformation (Chen et al. 2005). Similarly, heat treatment of *Sulfolobus* MCM resulted in substantial elevation of recruitment of MCM by Orc1-1 to *oriC1* in vitro (Samson et al. 2016). Thus, based on Orc1-1's monomeric behavior, single-turnover ATP hydrolysis, activity when ATP bound, switch-off upon ATP hydrolysis, and the thermodynamically favored opening of MCM, we have proposed that Orc1-1 is acting as a conditional platform for MCM recruitment to replication origins. Importantly, *oriC1* possesses ORB elements aligned in inverted orientation flanking a ~90 bp AT-rich region. Replication initiation has been mapped at the boundary of this candidate duplex unwinding element, and so it is believed that two hexamers of MCM are loaded into this region by Orc1-1 bound to the flanking ORB elements (Fig. 5.5).

## 5.7 The Archaeal CMG Complex

The molecular basis of how initial DNA unwinding at replication origins is effected remains unknown at this time in both archaeal and eukaryotic systems. In eukaryotes, it is well established that the ultimate activation of the MCM helicase is tightly regulated and involves the facilitated recruitment of Cdc45 and GINS to form an active helicase assembly, termed CMG, that is capable of driving replication fork progression (Bell and Labib 2016).

Eukaryotic GINS is composed of four distinct subunits, Psf1, Psf2, Psf3, and Sld5 (Labib and Gambus 2007; MacNeill 2010). The subunits fall into two classes, related to each other by circular permutation. Psf2 and Psf3 have a domain order BA with a beta-strand domain followed by an alpha-helical domain. In Psf1 and Sld5, the order of the domains is switched to AB. The archaeal orthologs were initially

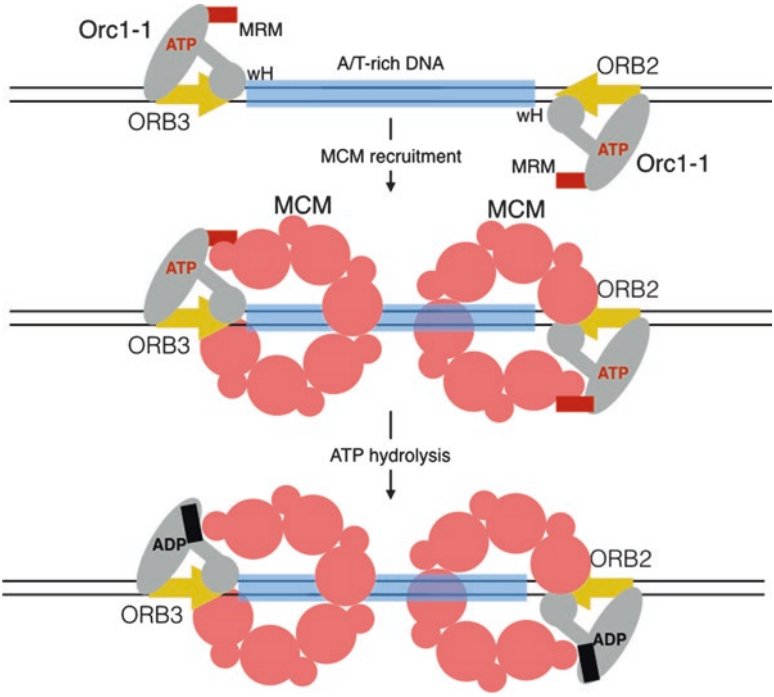


**Fig. 5.5** Model of the ATP-dependent recruitment of MCM by Orc1 proteins. ATP-bound Orc1-1 associates with inverted ORB elements at *oriC1*. As illustrated in Fig. 5.3, Orc1-1 binds to ORB elements as a monomer with a defined polarity – the AAA+ module contacting a G-rich element and the wH domain binding a short inverted repeat. The region between the inverted ORB elements, colored in blue, is highly AT rich. The MRM is positioned such that it can interact with MCM, leading to MCM's recruitment to the origin with both hexamers encircling double-stranded DNA. Subsequent hydrolysis of ATP to ADP repositions the MRM (shown in black in the "off" state), preventing further rounds of MCM recruitment

identified by virtue of their ability to interact with the N-terminal domains of MCM in a yeast two-hybrid screen. The first archaeal GINS ortholog identified was shown to be related to both Psf2 and Psf3 and was thus named Gins23 (Marinsek et al. 2006). Interestingly, the *gins23* gene is encoded within a bi-cistronic operon with *mcm*. Biochemical studies revealed that Gins23 co-purified with another small protein that was revealed to be related to Psf1 and Sld5 and thus named Gins15. The archaeal GINS assembly was shown to be a tetramer, containing two copies each of Gins15 and Gins23 (Marinsek et al. 2006). While the initial work was performed in *Sulfolobus*, the archaeal GINS complex is now known to be conserved across the archaeal domain of life (MacNeill 2010; Oyama et al. 2011; Yoshimochi et al. 2008; Oyama et al. 2016). During the biochemical isolation of *Sulfolobus* GINS, a further polypeptide co-purified over eight steps and was identified as being related to the DNA-binding fold of the RecJ superfamily of proteins, leading to its initial name of RecJdbh (Marinsek et al. 2006). Subsequent work has revealed an unambiguous

relationship between RecJ and eukaryotic Cdc45, and so RecJdbh has been renamed as Cdc45 (Sanchez-Pulido and Ponting 2011; Xu et al. 2016). Interestingly, Cdc45-related proteins have been identified across the archaeal domain of life but appear phylogenetically diverse (Makarova et al. 2012). One such protein, termed GAN, has been shown to be capable of association with GINS in the organism *Thermococcus kodakarensis* and, intriguingly, appears to be active as a nuclease (Li et al. 2011; Oyama et al. 2016). Recent structural studies have confirmed the GAN•GINS interaction and revealed the basis of the interaction between the GAN and the C-terminal domain of Gins15 (Oyama et al. 2016). Notably, in eukaryotes, an analogous interaction is observed between Psf1's CTD and Cdc45 (Costa et al. 2011).

In *Sulfolobus*, Cdc45 appears to be very tightly associated with GINS as evidenced by their co-purification over multiple steps (Marinsek et al. 2006). Furthermore, experiments with recombinant GINS and Cdc45 have revealed that the Cdc45•GINS complex (termed CG) is resistant to up to 8 M urea (Xu et al. 2016). Chromatin immunoprecipitation experiments have demonstrated that Cdc45 (and by inference, GINS) associates with MCM at replication origins and proceeds with the helicase during DNA synthesis. At the biochemical level, association of CG with MCM leads to a robust stimulation of helicase activity. Importantly, neither Cdc45 nor GINS when individually added to MCM results in detectable stimulation of helicase activity (Xu et al. 2016). While this latter observation agrees with initial reports that *Sulfolobus* GINS did not stimulate MCM's helicase activity (Marinsek et al. 2006), a report from the Huang laboratory has suggested that *Sulfolobus* GINS alone could stimulate MCM (Lang and Huang 2015).

One important difference between the archaeal and eukaryotic Cdc45 and GINS association lies in the composition of the assembly. While both eukaryotic Psf1 and Sld5 possess the AB domain organization, only Psf1 interacts with Cdc45 (Costa et al. 2011). This enforces a stoichiometry of one Cdc45 per GINS complex. In contrast, in the archaeal GINS, two identical copies of Gins15 are present, thus conferring the potential to interact with two Cdc45 molecules per GINS complex. Native electrospray ionization mass spectrometry experiments on the reconstituted *Sulfolobus* CG complex revealed that this was indeed the case, revealing a mass compatible with two copies each of Cdc45, Gins15, and Gins23 (Xu et al. 2016). While it has not been directly determined, it seems likely that this organization will also apply to the euryarchaeal *Thermococcus* GINS•GAN assembly (Oyama et al. 2016). Although this observation suggests a distinct difference between archaeal and eukaryotic CMG, hidden Markov modeling of the predicted structure of *Sulfolobus* Cdc45 revealed a hitherto undocumented similarity with an unanticipated region of eukaryotic Cdc45 (Xu et al. 2016). More specifically, the RecJ fold of eukaryotic Cdc45 is interrupted by a so-called CID domain (Simon et al. 2016). Surprisingly, *Sulfolobus* Cdc45 was predicted to form a structure related to this CID domain. As it had already been documented that *Sulfolobus* Cdc45 has similarities to the RecJ fold, this observation suggests that eukaryotic Cdc45 may have arisen via a gene duplication and internal fusion event, yielding a Russian doll-like organization (Fig. 5.6a). Thus, eukaryotic Cdc45 can be viewed as a pseudodimer when compared to its archaeal antecedents.

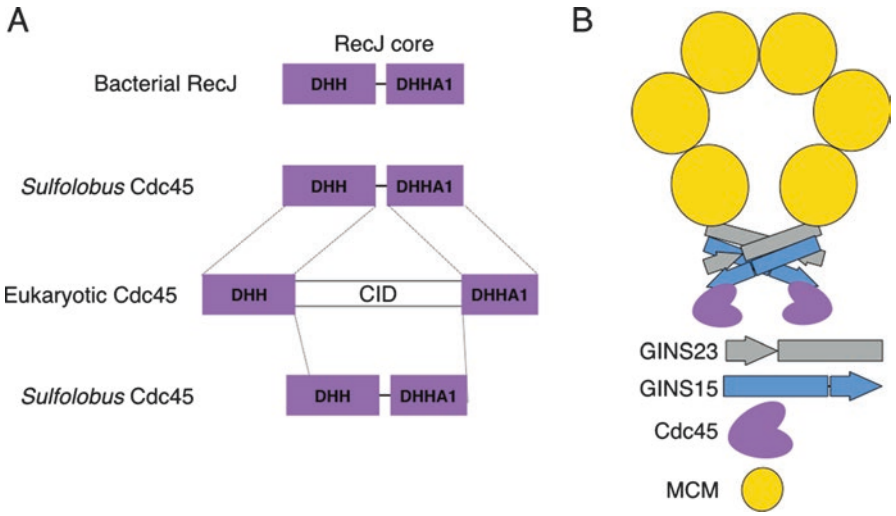


**Fig. 5.6** The archaeal CMG complex. (**a**) Relationship between bacterial RecJ and archaeal and eukaryotic Cdc45. The *Sulfolobus* Cdc45 corresponds to the core fold of RecJ – comprised of the DHH and DHHA1 domains. Eukaryotic Cdc45 has these two domains separated by the "CID" domain. Hidden Markov modeling revealed that the CID may have evolved from a partial copy of a core RecJ fold. See Xu et al. (2016) for details. (**b**) Speculative model for the architecture of the archaeal CMG complex. Gins23 and Gins15 are shown in gray and blue, respectively. Their beta-strand-rich domains are shown as arrows and their alpha-helical domains as rectangles. Gins15 and Gins23 form a 2:2 complex. Further, Gins15 interacts with Cdc45, and Gins 23 interacts with MCM. An open-ring form of MCM, such as that loaded on the replication origins, is depicted

Electron microscopy studies of the eukaryotic CMG complex reveal that GINS and Cdc45 interact over the interface between MCM2 and MCM5 subunits (Costa et al. 2011). This interface serves as a gate in the MCM ring, and elegant cross-linking studies have revealed that the ability of this gate to open is key to loading eukaryotic MCM(2-7) at replication origins (Samel et al. 2014). The innate asymmetry of the eukaryotic heterohexameric MCM(2-7) makes it easy to understand how the location and stoichiometry of Cdc45 and GINS association are imposed. This contrasts with the situation in archaea where the MCM is composed of six identical subunits. However, the available data indicate that MCM is recruited to origins in an open-ring form (Samson et al. 2016). It is possible that the nature of the opening between MCM subunits is such that it favors association of CG with that locus on the MCM complex (Fig. 5.6b). It may be significant that CG interacts with MCM's N-terminal domains via the Gins23 subunit (Marinsek et al. 2006). It is conceivable that the presence of two identical MCM-interaction interfaces on archaeal CG favors interactions between MCM N-terminal domains juxtaposed across the opening in the MCM ring.

In eukaryotes, the sequential and regulated associations of first Cdc45 and then GINS with loaded MCM are pivotal events in the control of the initiation of DNA replication (Siddiqui et al. 2013; Tanaka and Araki 2013; Bell and Labib 2016).

Interestingly, the so-called firing factors that facilitate this process (e.g., Sld2, Sld3, Sld7, Dpb11) are eukaryotic innovations with no discernable homologs in the archaea. Furthermore, the CDK and DDK kinases that in turn govern the behavior of the firing factors are also absent from archaea. The tight association of Cdc45 and GINS in archaeal cell extracts might imply that these factors interact en bloc with origin-associated MCM, leading to activation of MCM's helicase activity. Whether this step in archaeal DNA replication initiation is subject to regulatory control is currently unknown. However, in species such as *Sulfolobus* where multiple replication origins are coordinately regulated to trigger a single initiation event per cell cycle, it is very tempting to speculate that MCM activation by CG could be a key and committing step in regulating replication initiation.

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# Chapter 6
# Mechanism of Lagging-Strand DNA Replication in Eukaryotes

**Joseph L. Stodola and Peter M. Burgers**

**Abstract** This chapter focuses on the enzymes and mechanisms involved in lagging-strand DNA replication in eukaryotic cells. Recent structural and biochemical progress with DNA polymerase α-primase (Pol α) provides insights how each of the millions of Okazaki fragments in a mammalian cell is primed by the primase subunit and further extended by its polymerase subunit. Rapid kinetic studies of Okazaki fragment elongation by Pol δ illuminate events when the polymerase encounters the double-stranded RNA-DNA block of the preceding Okazaki fragment. This block acts as a progressive molecular break that provides both time and opportunity for the flap endonuclease 1 (FEN1) to access the nascent flap and cut it. The iterative action of Pol δ and FEN1 is coordinated by the replication clamp PCNA and produces a regulated degradation of the RNA primer, thereby preventing the formation of long-strand displacement flaps. Occasional long flaps are further processed by backup nucleases including Dna2.

**Keywords** DNA replication • Lagging strand • Okazaki fragment maturation • DNA polymerase α-primase • DNA polymerase δ • Flap endonuclease 1 • Dna2

## 6.1 Introduction

Three DNA polymerases are responsible for the bulk of genomic DNA replication, Pol α, Pol δ, and Pol ε. A preponderance of evidence supports the following division of labor at the replication fork: The Pol α-primase complex primes synthesis on both the leading and lagging strands, with Pol ε synthesizing the leading strand and Pol δ synthesizing the discontinuous Okazaki fragments that make up the lagging strand (Burgers 2009). This model has been supported by analysis of replication errors (Pursell et al. 2007; Nick McElhinny et al. 2008; Larrea et al. 2010), studies of

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polymerase localization on replication forks (Yu et al. 2014), and genomic rNMP incorporation studies (Nick McElhinny et al. 2010a; Miyabe et al. 2011; Reijns et al. 2015; Daigaku et al. 2015; Koh et al. 2015; Clausen et al. 2015). Biochemical studies have shown that Pols ε and δ replicate their respective strands spontaneously in the presence of purified CMG helicase (Cdc45-$Mcm_{2-7}$-GINS) complex (Georgescu et al. 2014a, 2015) and are excluded from the incorrect strand (Schauer and O'Donnell 2017). For these reasons, the model placing Pol ε on the leading strand and Pol δ on the lagging strand has become widely accepted.

A recent study has suggested an alternate arrangement of polymerases at the replication fork (Johnson et al. 2015), concluding that Pol δ replicates both strands of the replication fork. These conclusions have become a matter of debate in the field (Stillman 2015; Johnson et al. 2016; Burgers et al. 2016), and some very recent biochemical data support a very limited engagement of Pol δ during the initiation of leading-strand DNA replication (Yeeles et al. 2017). However, no study disputes the current model of lagging-strand DNA replication involving the synthetic activities of Pol α-primase and Pol δ, which will be the primary focus of this review.

## 6.2 Priming by Pol α-Primase

DNA synthesis on both strands of the fork is initiated by the synthesis of RNA primers by the Pol α-primase complex. Pol α and its associated primase each contain one accessory subunit, forming a hetero-tetrameric complex overall, often designated as the eukaryotic primosome. The polymerase catalytic and accessory subunits are Pol1 and Pol12, respectively, in budding yeast and p180 and p70 in human cells (Johansson and Dixon 2013). The catalytic subunit is one of the four, eukaryotic B-family polymerases, which comprises a conserved polymerase domain and a separate C-terminal domain that is connected to the polymerase domain by a flexible linker (Klinge et al. 2009; Suwa et al. 2015; Kilkenny et al. 2012; Baranovskiy et al. 2016a). Interactions between the catalytic and the accessory subunit are made through this C-terminal domain (denoted $p180_C$ below). Similarly, the primase contains a catalytic and an accessory subunit: Pri1 and Pri2, respectively, in yeast, and p49 and p58 in humans. Integral to the mechanism described below, the primase accessory subunit contains two domains (N-terminal and C-terminal, denoted $p58_N$ and $p58_C$ below) connected by a flexible linker (Baranovskiy et al. 2015, 2016b).

The primase initiates RNA synthesis de novo, synthesizing an 8–10-nucleotide primer that is transferred to the polymerase subunit of the Pol α-primase complex for extension with dNTPs (Baranovskiy et al. 2016b; Singh et al. 1986; Kuchta et al. 1990; Kuchta and Stengel 2010) and then creating an ~30-nucleotide hybrid primer that becomes the substrate for Pol δ (Bullock et al. 1991; Murakami and Hurwitz 1993). The mechanism by which Pol α-primase makes uniformly sized RNA primers has long been unclear. Recent structural and biochemical studies with the human and yeast primosome have contributed to the proposal of a new model for primer synthesis (Klinge et al. 2009; Baranovskiy et al. 2015; Vaithiyalingam et al. 2014;

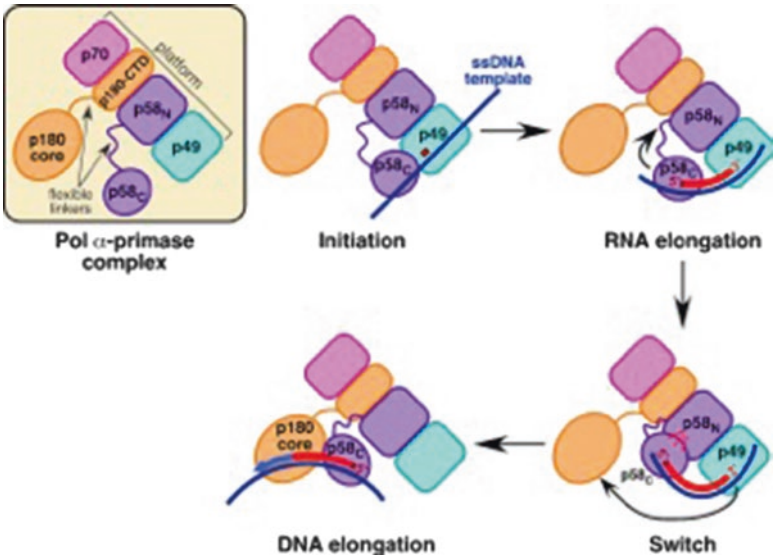


**Fig. 6.1** Priming of DNA synthesis by Pol α-primase. The sequential steps in the initiation of RNA priming, the elongation of the RNA primer, and the switch to DNA synthesis are shown. The model of Pol α-primase is based on Baranovskiy et al. (2016a)

Agarkar et al. 2011; Nunez-Ramirez et al. 2011; Sauguet et al. 2010; Kilkenny et al. 2013; Perera et al. 2013). This model is outlined by Baranovskiy et al. and described below (Baranovskiy et al. 2016a).

The crystal structure of the apo form of the primosome (not bound to DNA) indicates that the entire complex is built upon a stable platform with a p49-p58$_N$-p180$_C$-p70 arrangement (human subunit designations). Flexible linkers connect the polymerase (p180$_{core}$) and the C-terminal half of the primase accessory subunit (p58$_C$) to this platform (Fig. 6.1). Large conformational changes of p180$_{core}$ and p58$_C$ with respect to the primosome platform enable the substrate exchanges necessary for priming and extension. De novo RNA synthesis occurs at the interface of the primase catalytic and accessory subunits (p49-p58$_C$ interface) (Zerbe and Kuchta 2002). As the new primer grows, p58$_C$ retains interactions with its 5′-terminus and rotates away from p49. Eventually, this rotation brings on steric clashes between p58$_C$ and p58$_N$ (Fig. 6.1). Molecular modeling predicts that these clashes would occur when the RNA primer had reached about ten nucleotides in length, providing an explanation for why RNA primers longer than ten nucleotides are rarely produced (Baranovskiy et al. 2016a).

After further RNA synthesis is inhibited, the DNA/RNA duplex is intramolecularly transferred from the primase to the polymerase subunit (Fig. 6.1). Since p58$_C$ makes extensive contacts with the duplex and p49 is only weakly bound, it is predicted that p58$_C$ delivers the primer terminus to the polymerase. Molecular modeling of the potential transfer complex predicts that the polymerase is only able to access the 3′-primer terminus when the primer is at least nine nucleotides in length,

consistent with biochemical data (Baranovskiy et al. 2016a). Pol α extends the RNA primers for an additional 20–30 nucleotides with dNTPs, yielding a 30–40-nucleotide-long primer. This estimate dates back to early in vitro SV40 replication studies (Bullock et al. 1991; Murakami and Hurwitz 1993).

## 6.3 Polymerase Exchanges at Pol α-Synthesized Primers

After primer synthesis, Pol α is exchanged for Pol ε or Pol δ for further high-fidelity DNA replication. It is still unclear how DNA synthesis by Pol α remains so precisely limited and how polymerase exchange occurs. Several mechanisms have been proposed. First, it has been hypothesized that the different helical characteristics of the RNA/DNA duplex and double-stranded DNA may be sensed by the Pol α active site. Pol α has been shown to bind more tightly to RNA/DNA duplexes, which adopt an A-form helix, than to the B-form DNA helix (Perera et al. 2013). As Pol α extends the RNA primer with dNTPs, the A-form helix initially present will be converted to a B-form helix. It has been proposed that the formation of the B-form structure inhibits further synthesis by Pol α (Perera et al. 2013). However, the biochemical experiments supporting this hypothesis were performed using poly(dT) templates, where formation of triplex structures (dT-dA-dT) after limited replication causes inhibition of DNA synthesis by most DNA polymerases, and not just Pol α (Mikhailov and Bogenhagen 1996; Zhang et al. 2016). As a result, the extent to which Pol α extended these homopolymeric templates was artificially low.

Alternately, it has been suggested that the switch from Pol α to Pol δ is mediated by loading of PCNA onto 3′-primer termini by the RFC complex (Schauer and O'Donnell 2017; Tsurimoto and Stillman 1991; Eki et al. 1992; Yuzhakov et al. 1999; Maga et al. 2000; Mossi et al. 2000). In the absence of PCNA, the RFC complex has been shown to inhibit Pol α activity when present at high concentrations (Yuzhakov et al. 1999; Maga et al. 2000). However, Pol α inhibition is greatly enhanced when both RFC and PCNA are both present, suggesting that clamp loading is integral to polymerase switching (Schauer and O'Donnell 2017; Tsurimoto and Stillman 1991). Polymerase switching has also been shown to be stimulated by the presence of the single-stranded binding protein RPA at the template-primer junction. RPA directly binds RFC, providing specificity of PCNA loading and the displacement of Pol α (Yuzhakov et al. 1999; Gomes and Burgers 2001). Regardless of the exact details of the mechanism of Pol α ejection, the preponderance of evidence points to PCNA loading by RFC as essential to the recruitment of Pol δ, which prevents rebinding of Pol α.

CMG helicase-dependent leading- and lagging-strand synthesis has recently been reconstituted in vitro using the budding yeast replication system (Georgescu et al. 2015, 2014b; Yeeles et al. 2017; Devbhandari et al. 2017). These studies have provided biochemical support for the current model of the eukaryotic replication fork, with Pol ε replicating the leading strand and Pol δ the lagging strand, and Pol

α priming synthesis on both strands. It appears from these studies that replicating a bidirectional fork in the presence of the CMG helicase complex enforces the division of labor of the replication machinery; i.e., Pol ε is suppressed on the lagging strand, and Pol δ is suppressed on the leading strand (Schauer and O'Donnell 2017). Interestingly, Diffley and coworkers found that leading-strand replication proceeded more efficiently if the initial elongation of the leading-strand primer was carried out by Pol δ, followed by a second exchange from Pol δ to Pol ε (Yeeles et al. 2017). Presumably, the latter polymerase exchange occurs when the elongating PCNA-Pol δ complex collides with the leading CMG complex ahead of it and GINS enforces the exchange to Pol ε.

## 6.4 Strand Displacement Synthesis and Nick Translation

Pol δ extends primers on the lagging strand until it reaches the 5′-end of the preceding Okazaki fragment. Before ligation, however, the initiator RNA at the 5′-terminus of the primer must be removed. Biochemical and genetic studies support a model in which the initiator RNA is predominantly removed through the joint action of Pol δ and flap endonuclease 1 (FEN1), a structure-specific nuclease (Grasby et al. 2012; Balakrishnan and Bambara 2013). When Pol δ collides with the previous Okazaki fragment, it continues replicating through limited displacement of the RNA primer, forming a short 5′-flap. This flap is the substrate for FEN1; repetition of strand displacement synthesis by Pol δ followed by FEN1 cleavage removes the initiator RNA (Garg et al. 2004; Rossi and Bambara 2006; Stodola and Burgers 2016). Most frequently, one- or two-nucleotide products are liberated by FEN1 (Stodola and Burgers 2016; Stith et al. 2008). After removal of the RNA through these iterative actions of Pol δ and FEN1, a process termed "nick translation," the nick can be sealed by DNA ligase. These basic steps are sufficient to process the vast majority of Okazaki fragments. On rare occasions, strand displacement synthesis may become decoupled from flap cutting, and flaps can grow to lengths that cannot be processed by FEN1 (Murante et al. 1995; Bae et al. 2001a). Backup mechanisms, described below, are required to cleave these flaps so that they do not lead to DNA damage.

Pol δ possesses two enzymatic activities, a DNA polymerase activity and a 3–5′-exonuclease activity. The exonuclease is required for proofreading of misincorporated nucleotides during DNA replication but also plays an important role in Okazaki fragment maturation (Jin et al. 2001). After Pol δ initiates strand displacement synthesis, the forward, flap-generating movement of the polymerase is countered by the exonucleolytic activity of the polymerase. After formation of a short flap, the exonuclease activity of Pol δ cuts out the nucleotides that the polymerase had inserted, with the release of dNMPs. Repeated short-flap formation followed by exonucleolytic cleavage back to the nick position has been termed "polymerase idling." This activity appears to be unique to the lagging-strand polymerase; Pol ε exhibits

very weak strand displacement and idling activities (Garg et al. 2004; Ganai et al. 2016). In most sequence contexts, idling is sufficient to restrict forward movement of Pol δ to within three nucleotides of the nick position, and most frequently, one- or two-nucleotide products are liberated by FEN1 (Garg et al. 2004; Stodola and Burgers 2016; Stith et al. 2008).

Polymerase idling is not the only restraint placed on strand displacement. As Pol δ initiates strand displacement, the rate of forward polymerase movement slows down progressively as the 5′-flap grows longer (Stodola and Burgers 2016), i.e., the growing flap inhibits further synthesis in a length-dependent manner. Thus, the nascent flap acts as a "molecular brake" on the polymerase. Idling and flap inhibition allow Pol δ to produce a substrate with a short 5′-flap for FEN1 while simultaneously limiting extensive strand displacement synthesis. This cooperation is necessary. If the rate of strand displacement remained constant irrespective of flap length, idling alone would be insufficient to constrain the polymerase near the nick.

Surprisingly, the ability of Pol δ to displace the duplex region of the preceding Okazaki fragment is not dependent on the nature of the block, i.e., RNA versus DNA, but solely on the stability of the duplex (Stodola and Burgers 2016; Stith et al. 2008). Extensive strand displacement synthesis is favored in sequence contexts with low duplex stability such as AT-rich sequences. Furthermore, when flaps reach a critical length, Pol δ continues strand displacement synthesis in a manner that is decoupled from its regulatory mechanisms, generating long flaps (Ayyagari et al. 2003). This "critical length" remains poorly defined. It is possible that the "molecular brake" exerted on Pol δ only applies in situations where flaps are very short, perhaps due to interactions between the polymerase and the 5′-end of the flap (Koc et al. 2015). Perhaps, the failure of the flap-controlling mechanisms could be caused by a failure of very long flaps to interact with the enzyme. Further investigation is required to more fully examine this phenomenon.

## 6.5 Short-Flap Processing by FEN1

The iterative action of Pol δ and FEN1 removes initiator RNA so that nick ligation can occur. In vitro, these enzymes together comprise an efficient maturation machine, rapidly degrading either RNA or DNA annealed downstream of Pol δ (Stodola and Burgers 2016; Stith et al. 2008; Lin et al. 2013). In the absence of DNA ligase, nick translation can continue indefinitely unless it is blocked by other DNA-binding proteins, as observed in yeast (Smith and Whitehouse 2012). Much effort has been dedicated to determine the structure of FEN1's optimal substrate. The consensus model is that FEN1 most efficiently cuts double-flap structures with a single-nucleotide 3′-flap and a variable length 5′-flap (Kao et al. 2002; Tsutakawa et al. 2011, 2014). Irrespective of the length of the 5′-flap, FEN1 cuts a single base into the 5′-duplex region, yielding a ligatable nick when the single-nt

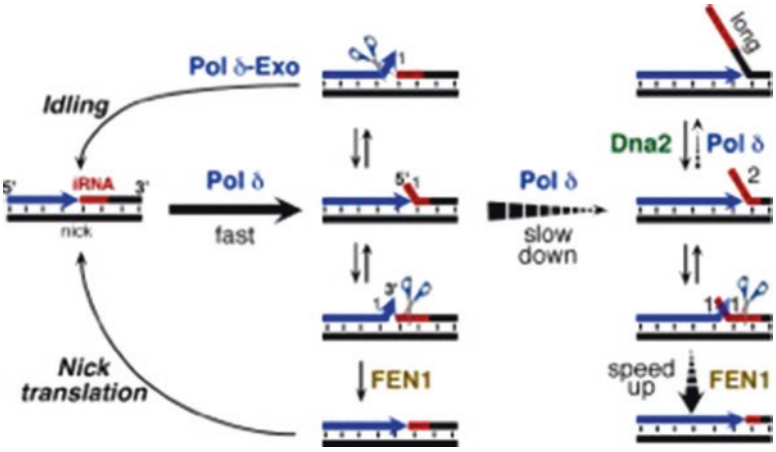


**Fig. 6.2** Regulatory steps that limit strand displacement synthesis by Pol δ. The formation of long flaps is restricted by the 3′-exonuclease activity of Pol δ (*idling*), by a progressive slowdown of strand displacement synthesis as the flap grows, and by cutting of the nascent flap by FEN1. FEN1 cleavage may be accelerated when double-flap structures are formed as flaps grow in size, thereby further limiting their length. Long flaps that still do occur are trimmed by Dna2

3′-flap reanneals to the template (Fig. 6.2) (Kao et al. 2002; Kaiser et al. 1999; Xie et al. 2001).

Whether this optimal substrate, requiring at least two unpaired nucleotides, represents the substrate that is *most often* cut during nick translation has recently been addressed. Data suggest that the major FEN1 substrate in nick translation results from a single-nucleotide flap (Stodola and Burgers 2016). Since a 3′-flap is required for FEN1 activity, we hypothesize that the single-nucleotide 5′-flap formed by Pol δ strand displacement must re-equilibrate into a single-nucleotide 3′-flap and a fully base-paired 5′-junction before cutting (Fig. 6.2). Although shown to be efficiently cut by FEN1, this single-flap structure is not processed as avidly as double-flap structures. Thus, these data suggests that in most contexts, the major FEN1 substrate is not actually the optimal substrate (Stodola and Burgers 2016; Kao et al. 2002). These observations could be interpreted as a contradiction, but it may in fact represent another layer of regulation limiting the formation of long flaps. It is likely that during nick translation, FEN1 binds and cuts double-flap structures more avidly than single 3′-flap structures. The higher-affinity binding of FEN1 to these double-flap intermediates would aid in the preferential recruitment of FEN1 to longer flaps in the case that the enzyme was not associated with PCNA-Pol δ at the start of strand displacement synthesis. Such a mechanism would ensure that flaps longer than a single nucleotide are processed before Pol δ strand displacement extends too far.

## 6.6 Alternative and Long-Flap Processing

The occurrence of long flaps in the cell was initially inferred from genetic studies in *S. cerevisiae*. Deletion of *RAD27*, which encodes FEN1, is associated with a dramatic increase in the occurrence of duplications between direct repeats, up to ~100 nt in length, as it could result from slippage mispairing of long 5′-flaps (Tishkoff et al. 1997a). The related exonuclease 1 (Exo1) also shows flap processing activity and can process nascent flaps generated during strand displacement synthesis by Pol δ, although less efficiently than FEN1 (Tran et al. 2002; Sparks et al. 2012). However, the spectrum of mutations observed in an *exo1Δ* strain is most consistent with a defect in mismatch repair rather than in Okazaki fragment maturation (Tran et al. 2001). Because *rad27 exo1* double mutants are lethal, the model has been proposed that Exo1 serves as a backup nuclease for FEN1, and in the absence of both enzymes, the burden of long flaps overwhelms the ability of the cell to process them (Stith et al. 2008; Tishkoff et al. 1997b). Further genetic studies have highlighted Dna2 as the principal enzyme responsible for processing long flaps. For instance, the conditional lethality of *DNA2* mutations is suppressed by overexpression of *RAD27*, and the temperature sensitivity of *rad27Δ* is suppressed by *DNA2* overexpression (Budd and Campbell 1997).

Based on biochemical studies, FEN1 has been apportioned the task of processing short flaps and Dna2 that of long flaps (reviewed in Burgers 2009; Kang et al. 2010; Balakrishnan and Bambara 2010; Burgers and Kunkel 2017). Long flaps are operationally defined as those longer than ~20 nucleotides, the length at which RPA stably binds flaps (Kumaran et al. 2006). FEN1 itself can cleave long flaps in vitro, but when the 5′-flap is coated with RPA or assumes a secondary structure, FEN1 cutting is abrogated (Murante et al. 1995). In wild-type cells, long flaps could be formed in certain sequence environments, such as AT-rich sequences, where strand displacement synthesis by Pol δ is predicted to be very rapid (Stodola and Burgers 2016). Alternatively, Pol δ strand displacement could become decoupled from flap cutting for other reasons, e.g., if FEN1 and Exo1 were absent from the replisome. In addition, the generation of long flaps is enhanced by the action of Pif1 helicase (Budd et al. 2006; Rossi et al. 2008) or by a defect in the proofreading activity of Pol δ (Jin et al. 2003). Therefore, backup mechanisms are required to process long flaps and rescue replication forks (Stith et al. 2008; Jin et al. 2001, 2005).

*S. cerevisiae* Dna2 is a multifunctional enzyme with nuclease, helicase, and cell-cycle checkpoint activities (Lee et al. 2000; Budd et al. 2000; Bae et al. 2001b; Kumar and Burgers 2013). Of these activities, the nuclease is most critical to Okazaki fragment maturation. Dna2 nuclease threads onto the 5′-end of flaps, displacing RPA before cutting DNA (Stewart et al. 2010; Zhou et al. 2015). In several reports, Dna2 was observed to cleave flaps several nucleotides away from their base, leaving behind a ~5–8-nucleotide 5′-flap (Bae et al. 2001a, 1998; Kao et al. 2004). Additionally, in one report, cutting at the base of the flap was also observed (Levikova and Cejka 2015). However, regardless of the exact cleavage accuracy of Dna2, efficient Okazaki fragment maturation of long-flap intermediates requires additional

nucleolytic processing beyond that by Dna2. Either additional 5′-flap cutting by FEN1 or 3′-exonucleolytic processing by the proofreading activity of Pol δ is required to produce ligatable nicks with high efficiency. When in biochemical studies, Dna2 was the only 5′-nuclease provided, the maturation of long flaps carried out with a proofreading-defective form of Pol δ produced ligatable nicks very inefficiently (Jin et al. 2003; Levikova and Cejka 2015). Consistent with these biochemical results is the observation that yeast mutants defective for the Pol δ 3′-exonuclease activity are exquisitely sensitive to additional defects in FEN1 (Jin et al. 2001).

A recent electron-microscopic study of isolated fission yeast replication forks provides structural support for the existence of the long-flap pathway (Liu et al. 2017). In wild-type cells, 10% of the isolated forks had associated with it a 40–50-nt-long flap. Often, these long flaps were detected kilobases distant from the fork. Because the EM methodology cannot detect very short flaps and nicks that are normally generated during short-flap processing, one conclusion from these data is that long flaps are rare. The frequency of long flaps increased in *rad2* (*S. pombe* FEN1) mutants as well as in *dna2* mutants. These results are consistent with the model, because FEN1 defects are expected to generate more long flaps while Dna2 defects are expected to abrogate their resolution. Accordingly, quantification of the frequency of long flaps in the *dna2*$^{-}$ mutant should give a good estimate of their normal occurrence during Okazaki fragment maturation. In *dna2*$^{-}$ cells, 32% of the forks showed long flaps, and the average distance between long flaps was about 6.5 kb. If one assumes that an Okazaki fragment is ~150 nt in length, one can estimate that long flaps are generated at a frequency of 1–2%. With about 50,000 Okazaki fragments being generated per fission yeast cell cycle, this amounts to as many as 500–1000 long flaps, which makes it unsurprising that *dna2* is essential for cell growth in *S. pombe*, as it is in *S. cerevisiae* (Kang et al. 2000; Budd et al. 1995).

The same EM study also determined the role of fission yeast RNase H2 and Exo1 in Okazaki fragment maturation (Liu et al. 2017). Defects in RNase H2 (*rnh201Δ)* did not result in a significant increase in the frequency of long flaps, suggesting that this enzyme does not participate in the degradation of the RNA primers during Okazaki fragment maturation. However, an *exo1Δ* mutant showed a clear increase in the frequency of long flaps, suggesting that Exo1 participates in Okazaki fragment maturation in wild-type cells. When compared with the known phenotypes of *S. cerevisiae exo1Δ* (see above), it appears that in *S. pombe*, Exo1 plays a more prominent role in Okazaki fragment maturation.

While there is strong evidence in both yeasts for the processing of long flaps by Dna2, the situation is less clear in human cells. Human Dna2 has been shown to play a role in nuclear genome maintenance, specifically promoting the rescue of stalled replication forks (Thangavel et al. 2015). However, currently there is no strong evidence for a role for Dna2 in Okazaki fragment maturation analogous to its role in both yeasts (Duxin et al. 2009, 2012). It is unknown whether human Okazaki fragment maturation can be accomplished by just FEN1 and Exo1 or whether long flaps are processed by additional nucleases redundant with Dna2, or different nucleases.

## 6.7 DNA Ligation

Following the removal of initiator RNA, nicks are sealed by DNA ligase I (*cdc9* in budding yeast and *LIG1* in human cells) (Howes and Tomkinson 2012). The eukaryotic ligase contains a conserved PCNA-interacting protein motif that binds PCNA in the interdomain connection loop (Vijayakumar et al. 2007). This interaction is important for localizing ligase to replication foci and for completing Okazaki fragment maturation in mammalian cells (Montecucco et al. 1998; Levin et al. 2000). PCNA has also been shown to stimulate ligase activity on nicked DNA substrates (Tom et al. 2001). Despite these effects, it is unclear whether the ligase is actually a stable component of the PCNA-mediated maturation complex like Pol δ and FEN1. During in vitro Okazaki fragment maturation, yeast ligase acts distributively, with the position of ligation following RNA removal dependent on the ligase concentration (Ayyagari et al. 2003). The cause of this observation remains unclear, but it is possible that when Pol δ and FEN1 are bound to PCNA, ligase cannot gain access to the PCNA ring, resulting in distributive ligation.

## 6.8 Limits to Nick Translation and the Size of Okazaki Fragments

The transient nature of Okazaki fragments has made the study of their properties in vivo difficult. However, advances have been made in recent years in isolating and examining Okazaki fragments in vivo and also in reconstituting lagging-strand replication in vitro. Both approaches have yielded new insights into the controls placed on Okazaki fragment synthesis and maturation.

Recent in vitro replication studies showed that, when lagging-strand replication was coupled to leading-strand synthesis by CMG-Pol ε, Pol α spontaneously primed on the lagging strand (Georgescu et al. 2015; Yeeles et al. 2017). The distance between priming events decreased as the concentration of Pol α in the assay was raised, indicating that priming itself is stochastic (Yeeles et al. 2017). PCNA-Pol δ spontaneously extended these Pol α-synthesized primers, producing Okazaki fragments that ranged from 100 to 500 nucleotides (Georgescu et al. 2015; Yeeles et al. 2017). Chromatin structure further modulated the size distribution of Okazaki fragments (Devbhandari et al. 2017; Kurat et al. 2017).

Maturation of these synthesized Okazaki fragments minimally requires FEN1 and DNA ligase as well as PCNA-Pol δ. In the absence of ligase, Pol δ and FEN1 could perform nick translation indefinitely (Ayyagari et al. 2003), although this would represent a major inefficiency in lagging-strand DNA replication. There is strong evidence that the chromatin context of the cell places a limit on the amount of nick translation synthesis that can be performed by Pol δ FEN1 (Smith and Whitehouse 2012). By purifying Okazaki fragments from a budding yeast strain deficient for DNA ligase, the Whitehouse Group found that the size distribution of Okazaki fragments

was strongly influenced by the placement of nucleosomes, with Okazaki fragment termini preferentially located at nucleosome dyads (Smith and Whitehouse 2012). Thus, it appears that a bound nucleosome upstream of the nick translation machinery is enough to block its further movement. This phenomenon has been extended to other DNA-binding proteins that bind the double-stranded DNA downstream of the migrating nick; transcription factor binding sites have been shown to be correlated with Okazaki fragment termination sites (Reijns et al. 2015; Smith and Whitehouse 2012). The lagging-strand replication machinery has also been shown to be blocked by double-stranded DNA-binding proteins in vitro (Koc et al. 2016).

It is currently unknown whether nick translation regularly extends to where nucleosomes or protein blocks are positioned or whether DNA is ligated before PCNA-Pol δ and FEN1 reach these blocks. Since the observations discussed above were generated in a yeast strain deficient for ligase, this data may report more on the limits placed on the maturation machinery rather than representing true Okazaki fragments (Smith and Whitehouse 2012; Smith et al. 2015). Since ligase acts distributively, in some situations ligation could leave some Pol α-synthesized DNA in the mature genome, despite the fact that more extensive nick translation would replace the lower-fidelity DNA produced by Pol α with that of the higher-fidelity Pol δ (Kadyrov et al. 2009; Liu et al. 2015). Indeed, several studies have shown that a significant amount of Pol α-synthesized DNA remains in the mature yeast genome (Reijns et al. 2015; Nick McElhinny et al. 2010b; Lujan et al. 2014). It remains to be determined to what extent nucleosome positioning directly influences the retention of Pol α-synthesized DNA.

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# Chapter 7
# Functions of Multiple Clamp and Clamp-Loader Complexes in Eukaryotic DNA Replication

**Eiji Ohashi and Toshiki Tsurimoto**

**Abstract** Proliferating cell nuclear antigen (PCNA) and replication factor C (RFC) were identified in the late 1980s as essential factors for replication of simian virus 40 DNA in human cells, by reconstitution of the reaction in vitro. Initially, they were only thought to be involved in the elongation stage of DNA replication. Subsequent studies have demonstrated that PCNA functions as more than a replication factor, through its involvement in multiple protein-protein interactions. PCNA appears as a functional hub on replicating and replicated chromosomal DNA and has an essential role in the maintenance genome integrity in proliferating cells.

Eukaryotes have multiple paralogues of sliding clamp, PCNA and its loader, RFC. The PCNA paralogues, RAD9, HUS1, and RAD1 form the heterotrimeric 9-1-1 ring that is similar to the PCNA homotrimeric ring, and the 9-1-1 clamp complex is loaded onto sites of DNA damage by its specific loader RAD17-RFC. This alternative clamp-loader system transmits DNA-damage signals in genomic DNA to the checkpoint-activation network and the DNA-repair apparatus.

Another two alternative loader complexes, CTF18-RFC and ELG1-RFC, have roles that are distinguishable from the role of the canonical loader, RFC. CTF18-RFC interacts with one of the replicative DNA polymerases, Polε, and loads PCNA onto leading-strand DNA, and ELG1-RFC unloads PCNA after ligation of lagging-strand DNA. In the progression of S phase, these alternative PCNA loaders maintain appropriate amounts of PCNA on the replicating sister DNAs to ensure that specific enzymes are tethered at specific chromosomal locations.

**Keywords** DNA polymerase • PCNA • RFC • PIP box • Leading strand • Lagging strand • Chromatin • Cohesion • Unloading

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## 7.1 Overview of the Clamp-Loader System

### *7.1.1 PCNA and RFC*

Proliferating cell nuclear antigen (PCNA) was originally identified as an autoimmune-disease antigen, which specifically accumulated in S-phase nuclei; it was later rediscovered as an essential replication factor in human cells (Prelich et al. 1987). Subsequent studies demonstrated that PCNA functions as the processivity factor for DNA polymerase δ (Polδ) through its activity as a sliding clamp on DNA (Tinker et al. 1994). Sliding clamps are highly conserved in all organisms; examples include β-clamp in *Escherichia coli*, the phage T4 gene 45 product (gp45), and PCNA in archaea and eukaryotes (Georgescu et al. 2015b). Clamps have dimeric or trimeric ring structures, which enable them to bind double-stranded DNA (dsDNA) topologically and slide along it freely. Binding requires temporary opening of the closed ring by the action of specific loader complexes. RFC, the loader for PCNA, consists of one large and four small subunits (RFC1 and RFC2-5, respectively), which are highly related proteins of the AAA+ ATPase family (Majka and Burgers 2004; Kelch et al. 2012; Yao and O'Donnell 2012). PCNA and RFC are involved in the elongation stage of DNA replication and are essential for the coordinated synthesis of leading-strand and lagging-strand DNA (Prelich and Stillman 1988; Tsurimoto and Stillman 1989).

Because of the essential role of sliding clamps in DNA replication, PCNA-loading mechanisms have been intensively studied by biochemical and structural approaches (Indiani and O'Donnell 2006; Yao and O'Donnell 2012). The PCNA ring consists of three identical subunits that connect in a head-to-tail manner. The ring has asymmetric surfaces, known as the N face and C face (or the back face and front face), because of the presence of N termini and C termini, respectively (Fig. 7.1). The PCNA protomer has two repetitive domains, one and two, each of which consists of a framework of β-sheet arrays and two α-helices facing the hole of the ring. These two domains are bridged by the interdomain connecting loop (IDCL), which locates on the C face and provides a structure that interacts with many PCNA-binding proteins (Tsurimoto 1999; Moldovan et al. 2007; Park et al. 2016; Choe and Moldovan 2017).

A sophisticated process has been demonstrated, in which RFC opens the ring structure to enable loading of PCNA on a DNA substrate (Fig. 7.1). In the presence of ATP, RFC attaches to the C face of PCNA, opens one interface between subunits, and binds to DNA at a 3′-terminal primer-template junction. Subsequent ATP hydrolysis induces a structural change in RFC and dissociation from PCNA and DNA. The closed PCNA ring remains on the dsDNA and is "loaded" with its C face directed to the 3′ end of the primer. The loaded PCNA recruits Polδ to the 3′ primer end, using the C face as the docking surface. Polδ is thereby bound to DNA in the correct orientation, enabling processive DNA synthesis by the Polδ-PCNA complex.

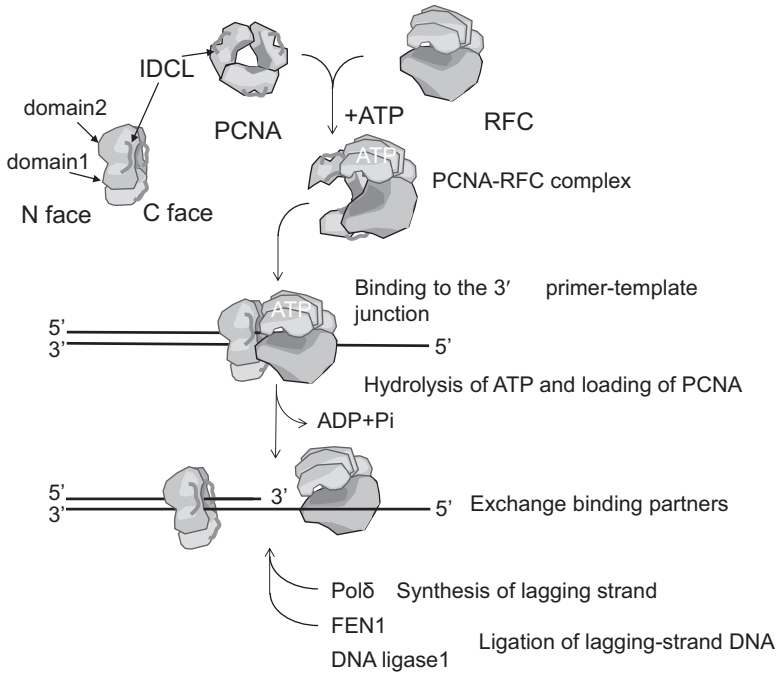


**Fig. 7.1** A schematic model of PCNA loading by RFC. The homotrimeric ring of PCNA has a head-to-tail configuration of subunits. The ring has asymmetric side surfaces known as the N face and C face. PCNA protomer has two repetitive domains, 1 and 2 that are bridged by IDCL, which is located on the C face. In the presence of ATP, RFC attaches to the C face, opens one interface between the subunits, and binds to the 3′ primer-template junction. Upon ATP hydrolysis, the structure of RFC changes to dissociate from PCNA and DNA, leaving a closed PCNA ring that is loaded on the duplex DNA with the C face directed to the 3′ end of the primer. Polδ then binds to the 3′ primer end using the C face of PCNA as its docking surface and synthesizes lagging-strand DNA processively. After completion of the DNA elongation, FEN1 and DNA ligase 1 are tethered sequentially to ligate the lagging strands

The dynamic status of PCNA on dsDNA was proposed by structural analyses, single-molecule imaging, and molecular-dynamics simulations (Kochaniak et al. 2009; De March et al. 2017). PCNA moves along dsDNA in a diffusive fashion in both directions. Most of the time, PCNA tracks rotationally the helical pitch of dsDNA by tilting with the DNA axis. This rotational motion of the tilted PCNA on DNA facilitates formation of a large number of electrostatic interactions between DNA backbone and the positively charged residues lining the PCNA inner surface, and it may provide a structure that captures a proper PCNA-binding partner.

### 7.1.2 PCNA and Replicative DNA Polymerases

Eukaryotes have three distinct replicative DNA polymerases: Polα, Polδ, and Polε (Waga and Stillman 1998, Burgers 2009). Polα DNA synthesis does not depend on PCNA; instead, Polα tightly associates with primase (pri) subunits. The Polα-pri complex synthesizes a 20–30 nucleotides-long RNA-DNA hybrid that acts as a primer. The 3′ end of this primer is a target of PCNA loading and efficient switching to Polδ, resulting in DNA synthesis for lagging strands. The Polδ-PCNA complex has a major role in lagging-strand DNA synthesis in the eukaryotic replication fork, and in repair DNA synthesis, at the late stage of various repair reactions (Prindle and Loeb 2012). The importance of PCNA for Polδ-mediated DNA synthesis is apparent, as Polδ alone can only incorporate several nucleotides at the primer end; whereas in the presence of PCNA, it can produce DNA strands longer than 200–300 nucleotides. The PCNA-RFC-Polδ system can efficiently fill DNA gaps from short patches to lagging-strand sizes.

The largest subunit of Polε consists of a catalytic N-terminal half and a C-terminal half that is involved in the protein-protein interaction necessary for initiation of DNA replication (Dua et al. 1999). The catalytic half is not essential for viability of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Kesti et al. 1999; Feng and D'Urso 2001), but it has a major role in leading-strand synthesis (Pursell et al. 2007). Polε alone has polymerase activity, but, for its full activity in leading-strands synthesis, other replication-fork components are necessary, such as CMG helicase (see previous chapters) and PCNA (Georgescu et al. 2014). The requirement for PCNA for full activity of Polδ and Polε indicates that the synthesis of both leading and lagging strands in eukaryotes is dependent on PCNA.

### 7.1.3 Alternative Clamps and Loaders

Three *RFC1* paralogues, *RAD17* (*RAD24* in *S. cerevisiae*), *CTF18* (chromosome transmission fidelity 18), and *ELG1* (enhanced level of genome instability 1; in human, also called as *ATAD5*, ATPase family, AAA domain containing 5 or *FRAG1*, FGF receptor activating protein 1) have been identified in eukaryotes. Proteins from these genes form the alternative clamp-loader-type complexes RAD17-RFC, CTF18-RFC, and ELG1-RFC in association with RFC2-5, (reviewed in Kim and MacNeill 2003, Majka and Burgers 2004, Kubota et al. 2013b, Shiomi and Nishitani 2017) (Fig. 7.2). The functions of these complexes have mainly been analyzed in yeast, where they are involved in checkpoint responses, sister chromatid cohesion, and maintenance of chromosome stability, respectively. Furthermore, three proteins that share significant amino acid sequence similarities with PCNA (RAD9, RAD1 and HUS1 in humans and *S. pombe*, and DDC1, RAD17 and MEC3 in *S. cerevisiae*) are necessary for the checkpoint-response pathway, along with RAD17-RFC (Kondo et al. 1999; Volkmer and Karnitz 1999; St Onge et al. 1999; Caspari et al.

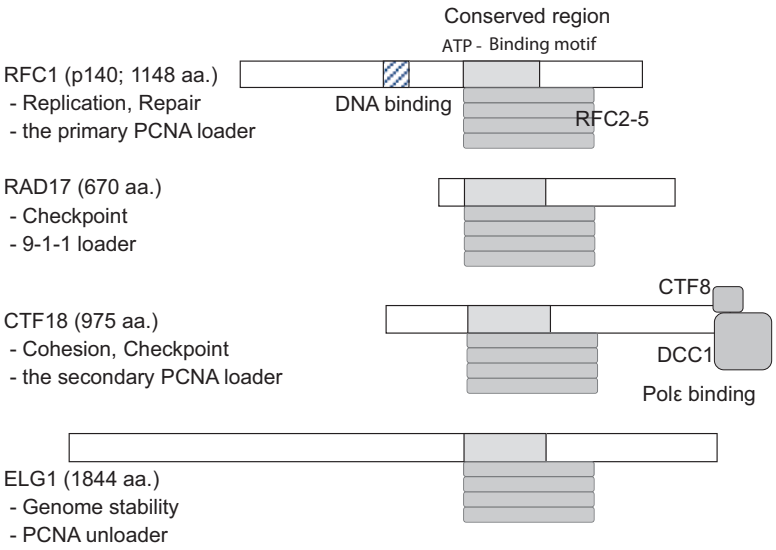


**Fig. 7.2** Structures and functions of multiple clamp-loader complexes in humans. Schematic structures and representative functions of four clamp-loader complexes in human cells are shown

2000). The three proteins form a heterotrimeric complex RAD9-HUS1-RAD1 (9-1-1) with a ring structure similar to that of PCNA (Burtelow et al. 2001; Kaur et al. 2001; Lindsey-Boltz et al. 2001; Shiomi et al. 2002; Griffith et al. 2002). Thus, eukaryotes have two clamps and four clamp-loader complexes, which are functionally distinct from each other.

## 7.2 PCNA Is a Hub Protein That Connects DNA Replication and Peripheral Chromosomal Reactions

### *7.2.1 During DNA Synthesis*

Nearly 50 PCNA-interacting proteins have been identified, and most are involved in DNA replication, repair, and cell cycle control (reviewed in Moldovan et al. 2007, Park et al. 2016). Many of these proteins have a conserved motif, known as the PCNA-interacting protein (PIP) box, at their N-termini or C-termini (Mailand et al. 2013; Boehm and Washington 2016). The PIP box motif is defined by the sequence Q-X-X-Ψ-X-X-Θ-Θ (where Ψ is I/L/M/V, and Θ is F/Y), and it usually protrudes from the main body of a protein and associates with IDCL in PCNA. Studies with PCNA mutants have demonstrated that PCNA-partner interactions have coevolved during evolution, and are not optimized for affinity, but rather may be designed for rapid exchangeability (Zamir et al. 2012; Fridman et al. 2013).

In eukaryotes, the interactions between PCNA, RFC, and Polδ are essential for chromosomal DNA replication. Several other enzymes that interact with DNA, such as FEN1 nuclease and DNA ligase 1, also bind PCNA via PIP boxes and thereby facilitate efficient lagging-strand processing (as described in the previous chapter). Similarly, DNA polymerases, Polη, Polι, Polκ, Polλ, REV1, and Polζ involved in translesion synthesis (TLS) can interact with PCNA via the PIP box or its variants (Mailand et al. 2013). PCNA provides a common platform, enabling rapid exchange of polymerases at 3′ DNA ends and resulting in loading of enzymes that are compatible with DNA structures at stalled forks (Maga and Hübscher 2003).

The clamp-based switching of DNA polymerases and related enzymes is an important feature of DNA replication and is highly conserved among organisms. The *E. coli* β-clamp, like PCNA, interacts with lagging-strand-processing enzymes DNA polymerase I (Pol I) and DNA ligase, and also with TLS polymerases Pol II, PolIV, and Pol V, in addition to the replicative DNA polymerase Pol III (Indiani et al. 2005).

### *7.2.2 After DNA Synthesis*

Even with the proofreading activity of replicative DNA polymerases, a number of mis-incorporated nucleotides occur in replicated DNA. The mismatch repair (MMR) reaction corrects such errors after DNA replication, thereby avoiding mutation. MutS in bacteria (Iyer et al. 2006), and two MutS-related heterodimer complexes (MutSα (MSH2-MSH6) and MutSβ (MSH2-MSH3) in eukaryotes) (Marsischky et al. 1996), function as sliding clamps in MMR and have essential roles in the recognition of unmatched bases. ATP-dependent mismatch recognition by MutS discriminates between DNA strands to specifically repair mis-incorporated nucleotides in newly synthesized DNA. In *E. coli*, the hemi-methylated structures produced by DNA replication of A-methylated GATC sites enable this discrimination. A major mechanism for discrimination in eukaryotes involves the targeting of single-strand breaks in the nascent DNA strands by the MutS complexes (Iyer et al. 2006; Kunkel and Erie 2005). However, although these mechanisms differ, the interactions of MMR proteins with clamp molecules are widely conserved from *E. coli* to humans, indicating the importance of PCNA in the coordination between replication and MMR (López de Saro and O'Donnell 2001). Subunits of eukaryotic MutS complexes, MSH3 and MSH6, interact with PCNA for efficient mismatch recognition (Flores-Rozas et al. 2000; Kleczkowska et al. 2001). After replication, PCNA remaining on the DNA is positioned with a specific orientation in the direction of synthesis, and it functions as the strand-discrimination marker for MutS complexes (Pluciennik et al. 2010; Georgescu et al. 2015a; Kawasoe et al. 2016). In the processing of mismatched DNA, the endonuclease MutL and an exonuclease EXO1 also interact with PCNA (Kadyrov et al. 2006, 2007; Pluciennik et al. 2010; Chen et al. 2013; Goellner et al. 2014). MutL complex that consists of MLH1 and

PMS2 interacts with MutS complexes and incises DNA strands near mismatched bases, and EXO1 excises the mismatched DNA region. Subsequently, PCNA-RFC-Polδ repairs the processed DNA gap. Thus, PCNA functions in the sequential steps of MMR by tethering MMR proteins, discriminating the newly synthesized DNA strands, and facilitating processing of the mismatched DNA region (López de Saro and O'Donnell 2001).

Following DNA synthesis at the replication fork, various chromosomal structures including epigenetic information encoded by covalent modifications of DNA and histones must be reconstituted. DNA methyltransferase 1 (DNMT1) has a PIP box in its N-terminal region and physically interacts with PCNA (Chuang et al. 1997). This interaction is not essential for the DNMT1 action, but it facilitates efficient methylation of hemi-methylated DNA in vitro and may help to maintain the methylation status of replicated DNA in S phase (Iida et al. 2002). Laser microirradiation experiments with mammalian cells demonstrated that interaction with PCNA is required for efficient recruitment of DNMT1 to DNA-repair sites, suggesting its importance for restoration of epigenetic information during DNA repair (Mortusewicz et al. 2005).

Chromatin assembly factor-1 is an evolutionarily conserved histone-chaperone complex that is capable of chromatin assembly in newly replicated DNA in vitro. This complex associates histones H3 and H4 with replicated DNA through its interaction with PCNA (Shibahara and Stillman 1999) and is required for the maintenance of epigenetic information in *S. cerevisiae* (Zhang et al. 2000). Similarly, several regulators of chromatin, such as histone deacetylase 1, histone acetyltransferase p300, and the WSTF-SNF2H chromatin remodeling complex, bind to PCNA, indicating its pleiotropic significance for the maintenance of chromatin structures in replicated DNA (Moldovan et al. 2007).

Cohesion of sister chromosomes is essential for precise segregation of replicated chromosomes to daughter cells (Skibbens 2009). This physical coupling of the replicated DNA is mediated by a protein ring, known as cohesin, which consists of SMC1, SMC3, SCC1, and SCC3 proteins. Cohesion is established in S phase simultaneously with the passage of the replication fork. The N-acetyltransferase ECO1 of *S. cerevisiae* (ESCO1 and ESCO2 in humans), which acetylates SMC3, has an essential role in this step. Replication-fork components, FEN1, RFC, and PCNA interact with ECO1-family proteins and couple cohesion establishment with replication (Rudra and Skibbens 2013). Indeed, ECO1-family proteins contain PIP-box motifs that are essential for PCNA binding and establishment of cohesion in S phase (Moldovan et al. 2006).

### *7.2.3 Repair of Damaged DNA*

Genomic DNA is exposed to various damaging events, such as ionizing and ultraviolet radiation, exposures to chemicals, and oxidation. Various repair systems have evolved that utilize PCNA as the assembly target and as a component of the

resynthesis machinery after processing of damaged DNA, as in MMR. Base excision repair (BER) proteins correct DNA with damaged bases. This reaction is initiated by the recognition and removal of damaged bases from nucleotides by specific DNA glycosylases. The phosphodiester bond of the 5′ abasic site is then cleaved by apurinic/apyrimidinic (AP) endonucleases (Krokan and Bjørås 2013). PCNA binds to four DNA glycosylases (UNG2, MPG, NTH1, and hMYH) and two AP endonucleases (APE1 and APE2) to colocalize with them and stimulate their activities (Moldovan et al. 2007). In the reaction that follows, short-patch repair reaction using Polβ and XRCC1-DNA ligase 3 complex seals the gap (reviewed in Dianov et al. 2003). Polβ and XRCC1 also interact with PCNA (Kedar et al. 2002; Fan et al. 2004). Thus, PCNA may recruit these BER proteins sequentially to the repair sites.

Nucleotide excision repair (NER) removes bulky DNA lesions, and as with other types of repair, it involves PCNA (Sancar et al. 2004). Seven xeroderma pigmentosum (XP) proteins engage in detection of lesions, unwinding of damaged duplex DNA, and removal of the damaged DNA strand by endonuclease and exonuclease activities. Among XP proteins, the endonuclease XPG has a PIP box in its C-terminal region, which is required for PCNA binding and NER activity. This interaction may facilitate DNA resynthesis after DNA excision by XPG (Gary et al. 1997).

### *7.2.4 Cell-Cycle Regulation*

PCNA was characterized as a marker protein of S-phase cells; it is involved in the activities of these cells and in cell proliferation. PCNA interacts with proteins that regulate cell-cycle progression, such as the cyclin-dependent kinases (CDKs) (Zhang et al. 1993) and the tumor-suppressor protein p21 (Waga et al. 1994), which binds to CDK and inhibits its activity (Xiong et al. 1993). These interactions produce a quaternary complex, PCNA–p21/CDK-cyclin, the function of which is unclear. The interaction of PCNA with CDK-cyclin may locate CDK activity to the replisome and result in phosphorylation of PCNA-binding partners, such as RFC, DNA ligase 1 (Koundrioukoff et al. 2000), and FEN1 (Henneke et al. 2003). p21 has a typical PIP box at the C-terminus and binds stably to PCNA (Warbrick et al. 1995). This binding results in competitive inhibition of PCNA binding to its partners, including Polδ, and inhibits DNA replication in vitro and in vivo (Rousseau et al. 1999; Waga et al. 1994). Thus, p21 may regulate PCNA-stimulated reactions competitively in accordance with cell-cycle progression. p21 is in turn regulated by PCNA-dependent proteolysis (Chuang and Yew 2001; Kim et al. 2008; Nishitani et al. 2008; Abbas et al. 2008). p21 has a specialized PIP box, the “PIP degron,” which is characterized by the sequence Q-X-X-Ψ-T-D-Θ-Θ-X-X-X-B (B: K/R) (Havens and Walter 2009). An ubiquitin ligase ($CRL4^{Cdt2}$) is activated by chromatin-loaded PCNA, and poly-ubiquitinates the PCNA-associated PIP degron proteins, such as p21. Because chromatin-loaded PCNA is maintained during DNA replication and repair, this PCNA-dependent proteolysis is responsible for degradation of p21 from G1/S transition to S phase and upon UV irradiation (Abbas et al. 2008).

One of the licensing factors, CDT1 (*cdc10* dependent transcript 1) (Hofmann and Beach 1994), which is required in G1 phase to commit replication origins to initiation of DNA synthesis, is the first protein that is characterized to have a PIP degron (Arias and Walter 2005; Nishitani et al. 2006). CDT1 has a PIP degron at its N-terminus and exhibits similar cellular behavior to p21, accumulating in G1 phase and degrading from G1/S transition to S phase, concomitantly with loading of PCNA to chromatin. CDT1 also degrades upon DNA damage. PIP-degron-dependent CDT1 degradation is the major cause of the temporal alteration of chromosomal CDT1, and it ensures the initiation of DNA replication once per cell cycle. Similar PIP-degron-dependent proteolysis has been reported for the transcription factor E2F, the histone H4K20 mono methyltransferase Set8 and Polη (Shibutani et al. 2008, Kim and Michael 2008, Abbas et al. 2010), which also exhibit S-phase and DNA-damage-dependent degradation.

## 7.3 PCNA Dynamics

When 3′ DNA ends are produced during DNA replication and repair, PCNA is loaded on the sites mainly by RFC, setting the stage for the binding of other DNA-interacting proteins (Leonhardt et al. 2000; Kim and Lee 2008). The topologically bound PCNA remains on the DNA until a specific unloading process takes place. Important reactions occur sequentially on replicated chromosomes, with PCNA as a platform, and the timing and preference of target sites for PCNA loading and unloading, along with the life-span of loaded PCNA, are essential factors in the maintenance of chromosomal structure and progression of the cell cycle (Fig. 7.3). PCNA and its loading proteins participate in a multilayered regulatory network to maintain genome integrity.

### *7.3.1 Primary PCNA Loading by RFC*

Biochemical analyses showed that RFC recognizes the 3′ end of primer without any preference for the DNA sequence. RFC will load PCNA on any primer end to synthesize lagging strands in a replication fork. Coordination of clamp-loading activity with replication forks is likely to require physical association of the clamp loader with the replisome complex. The γ clamp-loader complex of *E. coli* is physically integrated in the replisome (McInerney et al. 2007). The γ complex consists of five subunits (δ, δ', and three subunits of γ and/or τ) and associates with Pol III and DnaB DNA helicase via the C-terminal domain of τ subunits. The γ complex is the core protein for assembly of the multiple DNA polymerases and DNA helicase in the *E. coli* replisome. However, unlike the *E. coli* loader, direct connections of RFC and Polδ to replisome components apart from PCNA are currently unknown. Instead, Polε and Polα-pri are integrated in the eukaryotic replisome through direct

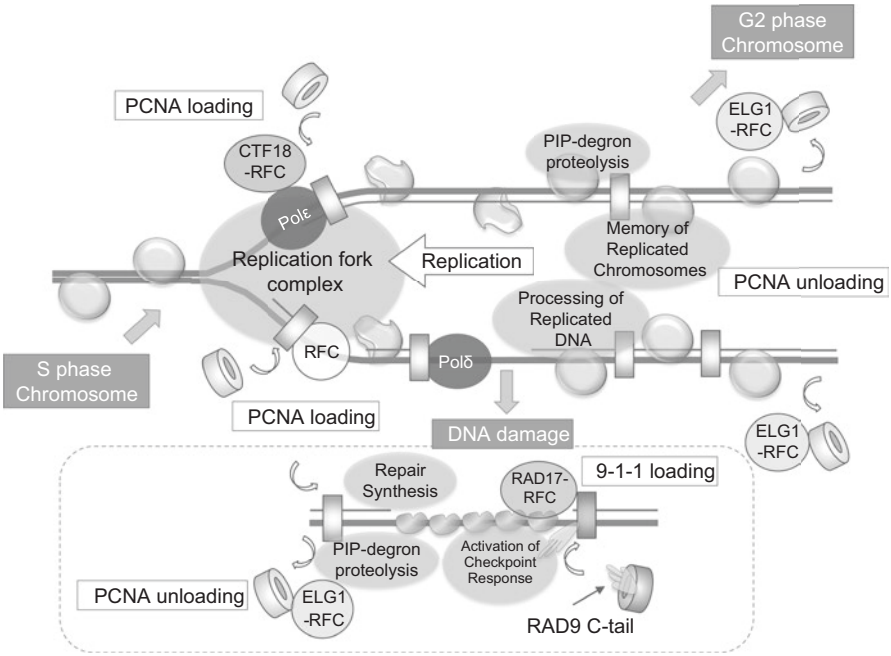


**Fig. 7.3** PCNA dynamics (loading and unloading) on replicating and post-replication chromosomes. PCNA is loaded on lagging-strand DNA by RFC and on leading-strand DNA probably by Polε–CTF18-RFC complex. This PCNA loading makes the chromosome region in S-phase mode, in which various DNA processing reactions take place. After completion of these reactions, ELG1-RFC unloads PCNA from chromosomes and switches to the G2-phase mode. Proper switching of the chromosome modes is crucial for precise maintenance of genomic integrity and transmission of epigenetic information. Similar PCNA dynamics, along with ATR checkpoint-signal activation by 9-1-1 and RAD17-RFC, occur at sites of DNA damage. To simplify this model, several essential components, such as CMG helicase, Polα/primase, and most of PCNA-interacting proteins are represented by unlabeled markers. Ubiquitination and SUMOylation that occur on DNA-bound PCNA are also not indicated

or indirect interactions with the replicative helicase CMG complex (O'Donnell and Li 2016). This observation suggests that the action of RFC-PCNA-Polδ during DNA replication will be distributive with CMG complex in the DNA area proximal to the replication fork. RFC-PCNA-Polδ associates distributively with gapped DNA regions between short RNA-DNA hybrid primers and the previously synthesized lagging strands, and fills a few hundred nucleotides in the gaps, as it does in the resynthesis of excised damaged DNAs.

In one particular situation, DNA-sequence-specific PCNA loading occurs via interaction of RFC with a sequence-specific DNA-binding protein. Kaposi's sarcoma-associated herpesvirus (KSHV) latency-associated nuclear antigen mediates viral DNA replication and persistence, binding specifically to the viral terminal-repeat sequence that contains the viral replication origin and interacting with RFC. Through this interaction, RFC loads PCNA to the terminal-repeat DNA

efficiently in vitro (Sun et al. 2014). Thus, the viral antigen facilitates efficient virus replication by recruiting host-cell replication machinery, such as the PCNA-loading system, to its replication origin.

### *7.3.2 Secondary PCNA Loading by CTF18-RFC*

The heptameric complex, CTF18-RFC, in addition to CTF18 and RFC2–5, contains the subunits DCC1 and CTF8 (Mayer et al. 2001; Merkle et al. 2003) (Fig. 7.2) and is involved in replication-fork progression, DNA-damage response, and proper establishment of cohesion (Hanna et al. 2001; Naiki et al. 2001; Terret et al. 2009; Crabbé et al. 2010). In DNA replication, CTF18 localizes at the replication fork in *S. cerevisiae* and humans (Lengronne et al. 2006; Sirbu et al. 2013; Alabert et al. 2014). Biochemical studies demonstrated that CTF18-RFC functions as a second PCNA loader, loading PCNA at the 3′ end of a primer-template junction through ATP hydrolysis and stimulating Polδ-mediated DNA synthesis (Bermudez et al. 2003; Shiomi et al. 2004). However, PCNA-unloading activity has also been reported for CTF18-RFC in *S. cerevisiae* (Bylund and Burgers 2005). Purified CTF18-RFC unloads PCNA in the presence of a sufficient amount of replication protein A (RPA), although the physiological relevance of this unloading has not been fully addressed.

CTF18-RFC directly interacts with Polε via a trimeric assembly consisting of CTF18, DCC1, and CTF8 (Murakami et al. 2010; García-Rodríguez et al. 2015). The results of a recent study (Fujisawa et al. 2017) demonstrated that PCNA-loading activity by CTF18-RFC alone is intrinsically weak and almost inactive at a near-physiological salt concentration in the presence of RPA. However, if CTF18-RFC is associated with Polε, its PCNA-loading activity is restored even in these conditions. This result indicates that the active status of Ctf18-RFC has to be in a complex with Polε, and it suggests the involvement of the secondary PCNA loader CTF18-RFC in the leading strand DNA-polymerase complex. PCNA loading by the CTF18-RFC–Polε complex occurs when Polε is in non-synthesis mode, and placing of Polε in the synthesis mode greatly suppressed the loading. PCNA loading forms the novel complex of CTF18-RFC–Polε–PCNA, which synthesizes DNA processively. From these results, two roles of PCNA loading by CTF18-RFC–Polε for DNA replication have been proposed. One role is the maintenance of leading-strand synthesis at template DNA structures that disrupt Polε progression. The second role involves active PCNA loading on a routine basis by CTF18-RFC–Polε during replication, which balances PCNA dosage between the leading and lagging DNA strands, enabling PCNA-binding proteins to function properly on both strands. Accordingly, it was reported that CTF18-RFC has a key role in the PCNA-degron-dependent CDT1 degradation in S phase, whereas RFC is required for CDT1 degradation after UV irradiation (Shiomi et al. 2012). These results suggest that the ubiquitin ligase $CRL4^{Cdt2}$ may be activated by PCNA on the leading strand via an unknown mechanism.

### 7.3.3 Unloading via ELG1-RFC, and PCNA Memory

Defects of *ELG1* exhibit various genomic instabilities (Kubota et al. 2013b; Ulrich 2013; Shiomi and Nishitani 2017). Control of the life-span of PCNA loaded on replicated chromosome is necessary to adjust enzymatic activity on the chromosomes from S phase to G2 phase (Fig. 7.3). ELG1-RFC unloads PCNA from chromosome in yeast and mammalian cells (Lee et al. 2013; Kubota et al. 2013a; Shiomi and Nishitani 2013). Depletion of ELG1 from *S. cerevisiae* or human cells results in an accumulation of chromatin-bound PCNA, which is removed upon expression of ELG1 in vivo. Similarly, treatment of isolated chromatin with partially purified ELG1-RFC causes PCNA unloading in vitro (Kubota et al. 2013a). Recent analyses by eSPAN (enrichment and sequencing of protein-associated nascent DNA) showed that the ratio of PCNA on the lagging strand to that on the leading strand in *S. cerevisiae* is about two to one, but PCNA becomes preferentially associated with the leading strand in a stalled replication fork (Yu et al. 2014). ELG1-RFC is required for this shift of PCNA distribution and has a role as an unloader of PCNA from the lagging strand in a stalled replication fork. PCNA unloading by ELG1-RFC will shorten the life-span of PCNA on S-phase chromatin.

*ELG1* is not essential for cell viability in *S. cerevisiae*, suggesting that other loader complexes can complement its activity during normal DNA replication. Indeed, as RFC and CTF18-RFC unload PCNA from DNA with nicks or gaps in vitro (Yao et al. 1996; Shibahara and Stillman 1999; Bylund and Burgers 2005), their activities may provide the unloading function in the absence of ELG1.

Timing of unloading is another important aspect of PCNA functionality. S-phase-specific PCNA modifications, such as SUMOylation (addition of small ubiquitin-like modifier protein (SUMO)), may be involved in this timing. *S. cerevisiae* ELG1 has putative SUMO-interaction motifs (SIMs) within its N-terminal domain, and ELG1-RFC preferentially interacts with SUMOylated PCNA through these motifs (Parnas et al. 2010). Furthermore, SUMOylated PCNA accumulates on chromatin in an *elg1*Δ mutant, especially in the presence of methyl methanesulfonate. These results suggest that *S. cerevisiae* ELG1-RFC is an unloader that is specific to SUMOylated PCNA. However, SUMOylation is not absolutely required for PCNA unloading by ELG1-RFC (Kubota et al. 2013a), suggesting that accumulation of SUMOylated PCNA in an *elg1*Δ mutant is probably the result of prolonged retention of PCNA on chromatin, caused by the absence of its unloader.

Although a PCNA-unloading reaction with purified ELG1-RFC has not been reconstituted, partially purified yeast ELG1-RFC unloads PCNA from chromatin of an *elg1*Δ mutant in vitro in an ATP-dependent manner (Kubota et al. 2013a). This reaction also demonstrates that PCNA unloading by ELG1-RFC is replication-coupled, but inactive with a ligase-deficient cell extract, although it can be restored by addition of an exogenous ligase (Kubota et al. 2015). Thus, ELG1-RFC acts as a PCNA unloader on dsDNA, after lagging-strand ligation. This property may distinguish the active timing of ELG1-RFC from that of RFC and CTF18-RFC, which

require unligated 3′ ends for their activity (Yao et al. 1996, Shibahara and Stillman 1999, Bylund and Burgers 2005).

DNA-bound PCNA will transmit various signals to DNA-transaction enzymes; the presence of PCNA represents the "memory" of specific events related to newly synthesized DNA strands, sister DNAs, de novo replicated DNA regions, and incompletely replicated or repaired regions (Georgescu et al. 2015b). DNA-bound PCNA induces strand-specific MMR in *Xenopus*-egg extracts through the interaction of MutSα with PCNA (Kawasoe et al. 2016). MutSα that recognizes unmatched bases prevents PCNA from being removed from the DNA. This interaction between MutSα and PCNA keeps the memory of the new DNA strand that is contained in the orientation of PCNA, through the inhibition of PCNA unloading, until MMR has taken place. In vivo, PCNA unloading during S phase largely depends on ELG1-RFC, suggesting that the PIP box in MutSα may limit the access of ELG1-RFC to DNA-bound PCNA, thereby blocking the unloading.

If PCNA functions as a memory molecule on replicated chromosomes, ELG1-RFC acts as an eraser of PCNA memory. PCNA-binding proteins may also regulate the period of memory mediated by DNA-bound PCNA. The proper duration of this memory is crucial to the maintenance of genome integrity after DNA replication, and functional defects in the loader and unloader complexes lead to chromosomal abnormalities (Johnson et al. 2016).

### *7.3.4 Post-translational Modifications*

Quantitative regulation of PCNA levels on chromosomes is essential to maintain genome stability in S phase and after DNA damages. In addition, qualitative alterations to PCNA, in the form of several post-translational modifications (PTMs), affect interactions with various partners, and function as "PCNA code" to select these partners and thereby regulate PCNA functions. Among various PTMs, we focus on two modifications, ubiquitination and SUMOylation (Hoege et al. 2002; Stelter and Ulrich 2003), which sort the two DNA-damage-tolerance pathways during S phase.

The highly conserved lysine 164 (K164) of PCNA is the target site of the PTMs (PCNA in *S. cerevisiae* has a secondary SUMOylation site at lysine 127). Mono-ubiquitination of K164 occurs by Rad6-Rad18 E2/E3 ligase upon DNA damage in S phase and promotes the TLS pathway. Mono-ubiquitination of K164 also occurs in human cells, via $CRL4^{Cdt2}$ ubiquitin ligase. Unlike yeast, a temporal-negative regulation of a ubiqutin-specific protease, USP1, results in the accumulation of mono-ubiquitinated PCNA in S phase and upon DNA damage in human cells (Huang et al. 2006; Terai et al. 2010). Most TLS polymerases have ubiquitin-binding domains that are necessary for their activities, in addition to the PCNA binding motifs (Bienko et al. 2005; Guo et al. 2006). At stalled replication forks, mono-ubiquitination of K164 switches the affinity of PCNA from high-fidelity replicative

polymerases to low-fidelity TLS polymerases, to bypass the lesions and continue the replication (Masuda et al. 2010; Edmunds et al. 2008).

Mono-ubiquitination of K164 also links with poly-ubiquitination at the same site by MMS2-UBC13 and RAD5, another E2-E3 ligase (Ulrich and Jentsch 2000). The poly-ubiquitinated PCNA leads an alternative, error-free lesion bypass through template switching between de novo synthesized sister DNAs by recombination-like processes. The different ubiquitination modifications can switch between error-prone and error-free pathways at stalled replication forks, to bypass DNA lesions and to restart the forks. However, the mechanism by which poly-ubiquitinated PCNA promotes template switching is not yet understood.

SUMO modification of PCNA is more prominent in *S. cerevisiae* than other species. In *S. cerevisiae*, this modification occurs at K164 in DNA-bound PCNA constitutively during S phase by UBC9-SIZ1 SUMO ligase (Hoege et al. 2002) and results in the recruitment of the anti-recombination helicase SRS2 via its PIP box and SIM. K164 SUMOylation is also detectable in human cells, and a functional orthologue of SRS2, PCNA-associated recombination inhibitor PARI (which contains a PIP box and SIM), has been identified (Gali et al. 2012; Moldovan et al. 2012). These results show that PCNA SUMOylation has a role in the inhibition of homologous recombination, and may suppress recombination-based restart of stalled replication forks, which would cause potential deleterious recombination events between newly synthesized sister chromatids, leading to chromosome rearrangements (Carr and Lambert 2013). SUMOylated PCNA is also a target of ELG1-RFC, and may have a role in regulation of the unloading of DNA-bound PCNA, although an active role for the unloading remains to be demonstrated.

## 7.4 Checkpoint Clamp and Loader

### *7.4.1 Loading of the 9-1-1 Clamp onto DNA by RAD17-RFC*

The heterotrimer clamp 9-1-1 has the characteristic RAD9 C-terminal extension ("C-tail") that is intrinsically disordered, in addition to its PCNA-like ring structure. 9-1-1 and RAD17-RFC are thought to be the specialized clamp and clamp loader for "emergency" situations. When DNA replication is perturbed by DNA damage, uncoupling of replicative DNA helicase and DNA polymerase occurs, and single-stranded DNA (ssDNA) regions are exposed and covered by the ssDNA-binding protein, RPA (Byun et al. 2005). Nucleolytic degradation after generation DNA double-strand breaks (DSBs), for example, by ionizing radiation, also leads to exposure of ssDNA regions. Whereas RFC loads PCNA at 3′-recessed ends of DNA, human RAD17-RFC and its *S. cerevisiae* orthologue load 9-1-1 onto 5′-recessed ends, in a reaction that is dependent on ATP and RPA (Ellison and Stillman 2003, Majka et al. 2006).

DNA-damaging treatments result in increased chromatin association of 9-1-1 and nuclear-foci formation of its components (Caspari et al. 2000; Burtelow et al. 2000; Zou et al. 2002; You et al. 2002). These responses are dependent on RAD17 and are thought to correspond to the loading of 9-1-1 onto DNA by RAD17-RFC. RPA has an important role in the localization of 9-1-1 to damaged chromatin, probably through the interaction between RPA and the RPA-binding motif in the C-tail of RAD9 (Wu et al. 2005; Xu et al. 2008). RAD17 is also recruited to the site of DNA damage through the interaction with RPA (Lee et al. 2003a; Zou et al. 2003). Phosphorylation of RAD17 at two serine residues (S635 and S645) is induced by DNA-damaging treatments and is required for the checkpoint responses, probably through stimulation of its 9-1-1 loading (Bao et al. 2001; Wang et al. 2006). S635 and S645 are the targets of two major checkpoint kinases, ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related) (Kim et al. 1999, O'Neill et al. 2000, Bao et al. 2001, Matsuoka et al. 2007). Activated ATR phosphorylates RAD17, which stimulates 9-1-1 loading that in turn contributes to ATR activation, implying a positive-feedback loop to maintain activation of ATR until the DNA damage is removed. The 9-1-1 complex is also phosphorylated by ATR or ATM in the presence of DNA damage (Chen et al. 2001; Roos-Mattjus et al. 2002, 2003), but the biological significance of this phosphorylation is unclear.

## *7.4.2 Activation of the ATR-Dependent DNA-Damage-Checkpoint Pathway*

### 7.4.2.1 From Loading of 9-1-1 to Activation of ATR

ATM and ATR respond mainly to exposure to DSBs and ssDNA, respectively (reviewed in Lavin 2008, Cimprich and Cortez 2008, Maréchal and Zou 2013, Awasthi et al. 2015). The activated kinases phosphorylate and activate their downstream kinases, CHK2 (for ATM) and CHK1 (for ATR). All the activated kinases phosphorylate and regulate the functions of a range of proteins involved in DNA repair, cell-cycle regulation, and apoptosis. RAD17-RFC and 9-1-1 are involved in an early stage of DNA-damage response, in activation of the ATR–CHK1 pathway (Fig. 7.4). 9-1-1 is loaded by RAD17-RFC at the junction of RPA-bound ssDNA and dsDNA. ATR complexed with ATRIP (ATR-interacting protein) is also recruited to the RPA-bound ssDNA via an interaction between ATRIP and RPA. Thus, ATR–ATRIP and 9-1-1 complexes are recruited to the damaged DNA independently (Kondo et al. 2001; Melo et al. 2001; Zou et al. 2002; You et al. 2002). The DNA topoisomerase 2-binding protein 1(TopBP1) binds to 9-1-1 and to ATR-ATRIP complexes, and both of these interactions are involved in the activation of ATR (Kumagai et al. 2006; Mordes et al. 2008; Navadgi-Patil and Burgers 2008).

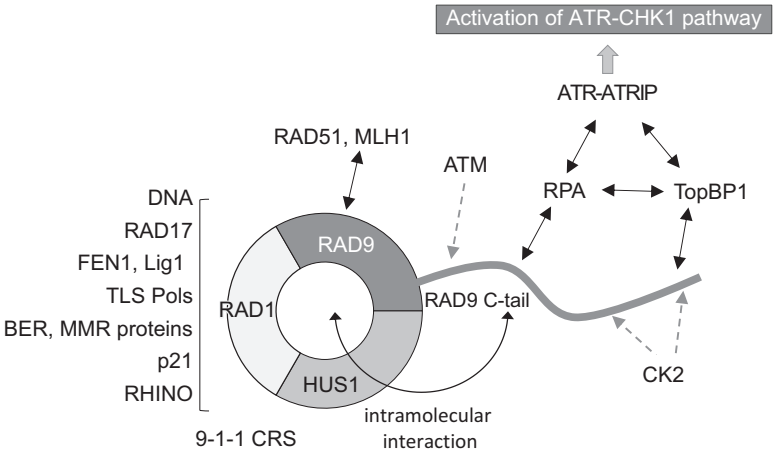


**Fig. 7.4** Activation of the ATR–CHK1 pathway and interaction of 9-1-1 with factors involved in DNA-damage responses. Proteins interacting with 9-1-1 and RAD9 described in this review are shown. Double-headed arrows indicate interactions. Single-headed arrows with broken lines indicate phosphorylations on 9-1-1. CRS, core ring structure; Lig1, DNA ligase 1; TLS pols, translesion synthesis polymerases

### 7.4.2.2 Phosphorylation of the C-Tail and Activation of ATR

Phosphorylation of S387 in the RAD9 C-tail is required for the interaction of 9-1-1 with TopBP1 and the subsequent activation of ATR (St Onge et al. 2003; Delacroix et al. 2007; Lee et al. 2007). The C-tail has multiple phosphorylation sites (10 in humans: S272, S277, T292, S328, S336, S341, T355, S375, S380, and S387). Among these sites, S272 is known to be phosphorylated by ATM in response to DNA damage, although the phosphorylation is not involved in the interaction with TopBP1 (Longhese et al. 1997; Furuya et al. 2004; St Onge et al. 2001; St Onge et al. 2003; Roos-Mattjus et al. 2003). S387 and the internal S341 are typical targets of casein kinase 2 (CK2), a ubiquitous, pleiotropic, and constitutively active kinase (Fig. 7.4). The constitutive phosphorylation of S387 and S341 promotes binding of 9-1-1 to TopBP1 (Takeishi et al. 2010; Rappas et al. 2011) and is required for efficient phosphorylation of CHK1 (Ueda et al. 2012).

As the CK2-dependent interaction between 9-1-1 and TopBP1 is constitutive, a pathway should exist for immediate ATR activation upon DNA damage. Indeed, the interaction of the counterparts of 9-1-1 and TopBP1 in *S. pombe* is damage-inducible, and the phosphorylation sites responsible for their interaction in both *S. cerevisiae* and *S. pombe* are damage-inducible using ATM/ATR kinase activities (Furuya et al. 2004; Puddu et al. 2008; Navadgi-Patil and Burgers 2009). In addition, the C-tail is also able to stimulate ATR (Mec1), in the absence of the TopBP1 (Dpb11) in *S. cerevisiae* (Navadgi-Patil and Burgers 2009). Different models for the binding of 9-1-1 and TopBP1 to damaged chromatin have been reported. One suggestion is that 9-1-1 recruits TopBP1 in the vicinity of ATR-ATRIP complex (Greer

et al. 2003; Delacroix et al. 2007; Lee et al. 2007), whereas an alternative is that 9-1-1 is recruited to sites of replication stress via the pre-assigned TopBP1 (Yan and Michael 2009). However, recent studies with *Xenopus*-egg extract and human cells demonstrated that 9-1-1, TopBP1, and ATR-ATRIP can be recruited to sites of stress independently of each other (Duursma et al. 2013; Lee and Dunphy 2013; Gong et al. 2010; Ohashi et al. 2014; Acevedo et al. 2016). The RAD9, HUS1, and RAD1-interacting nuclear orphan protein 1 (RHINO) has been identified in human cells as a co-binding factor to TopBP1 and 9-1-1 (Cotta-Ramusino et al. 2011). Recruitment of this protein to the site of DNA damage is dependent on the 9-1-1 complex and may have a role in damage-dependent activation of the 9-1-1–TopBP1 pathway in vertebrates (Lindsey-Boltz et al. 2015).

#### 7.4.2.3 The Intramolecular Interaction of the C-Tail

The RAD9 C-tail and 9-1-1 core ring structure (CRS) interact in the regulation of 9-1-1 function. The C-tail binds to the 9-1-1 CRS, and this intramolecular interaction interferes with the DNA binding of 9-1-1 (Fig. 7.4) (Takeishi et al. 2015). The region in the C-tail that is necessary for binding to the CRS partially overlaps with the region for binding to TopBP1. When TopBP1 binds to the C-tail, the C-tail unfolds and its binding motif(s) on the CRS are exposed. Many proteins interact with 9-1-1, and some of them may bind through the same binding motif(s) on the CRS as the C-tail does. The intramolecular interaction of the C-tail with the CRS may alter the interaction of 9-1-1 with DNA, TopBP1, and other proteins and consequently may have a role in genome maintenance following DNA damage.

### *7.4.3 Roles in Other Pathways*

#### 7.4.3.1 The Response to DSBs and Homologous Recombination

The results of early studies suggested that 9-1-1 and RAD17-RFC are involved in ATR activation, but are not required for phosphorylation of CHK2, a mediator kinase of the ATM pathway for response to DSBs (Weiss et al. 2002; Roos-Mattjus et al. 2003; Wang et al. 2003). However, results from a number of studies involving knockout of genes encoding 9-1-1 and RAD17-RFC in *S. cerevisiae*, chicken DT40 cells, and mouse cells demonstrated that these complexes have direct roles in DSB repair and homologous recombination. Compared with wild-type cells, the mutants show higher sensitivity to ionizing radiation, as well as inefficient gene targeting and gene conversion (Aylon and Kupiec 2003, Kobayashi et al. 2004, Saberi et al. 2008, Nishino et al. 2008). In humans, RAD9 interacts with RAD51 recombinase and influences the DSB repair activity through homologous recombination (Pandita et al. 2006).

#### 7.4.3.2 TLS and Template Switching

The 9-1-1 complex is involved in the two DNA damage-tolerance pathways (TLS and template switching), as is ubiquitinated PCNA. In *S. cerevisiae*, the 9-1-1 complex interacts with the TLS polymerase Polζ, and enhances efficiency of Polζ-dependent mutagenesis (Paulovich et al. 1998; Sabbioneda et al. 2005). The *S. pombe* checkpoint protein Rad17 is required for the enhanced chromatin association of Polκ (Kai and Wang 2003). Human 9-1-1 complex stimulates the activity of Polβ and Polι (Smith et al. 2012). Evidence suggests that 9-1-1 is also involved in template switching. The DNA-damage-inducible phosphorylation of Rad9 at Thr-225 by ATR in *S. pombe* promotes interaction with Mms2, which is involved in poly-ubiquitylation of PCNA, and represses the error-prone TLS pathway (Kai et al. 2007). In addition, 9-1-1 and RAD17-RFC promote the template-switching pathway independently of their canonical checkpoint-signaling pathway in *S. cerevisiae* (Karras et al. 2013).

#### 7.4.3.3 DNA Repair Pathways

9-1-1 also interacts with many proteins that are involved in BER and MMR, such as Polβ, FEN1, DNA ligase 1, several DNA glycosylases, AP endonuclease 1, MLH1, MSH2, MSH3, and MSH6, and enhances their activities (reviewed in Li et al. 2016). Thus, 9-1-1 may have a direct role for efficient DNA-repair reactions. Because these BER and MMR proteins have also been reported to interact with PCNA, it is unclear which clamp is more important for the repair pathways (Gembka et al. 2007; Balakrishnan et al. 2009). Notably, because 9-1-1 is involved in the DNA-damage-checkpoint pathway, interaction of these proteins with 9-1-1 could provide signals to activate the ATR pathway. 9-1-1 also interacts with p21 as PCNA does (Doré et al. 2009). p21 binding to 9-1-1 was suggested to competitively block the interaction between 9-1-1 and these repair enzymes.

#### 7.4.3.4 Meiosis

DSBs occur during meiosis and are required for meiotic recombination and segregation of homologous chromosomes. To ensure the appropriate resolution of meiosis, many canonical DNA-damage-response proteins are involved (reviewed in Hochwagen and Amon 2006, Subramanian and Hochwagen 2014). RAD17-RFC and 9-1-1 participate in meiosis through regulation of both meiotic checkpoint and meiotic recombination. 9-1-1 is loaded to the ssDNA region close to the resected meiotic DSB sites, and this loading can induce delays in the entry into meiosis I (Lydall et al. 1996). In addition, RAD17-RFC and 9-1-1 have a direct role in meiotic recombination (Grushcow et al. 1999; Thompson and Stahl 1999; Shinohara et al. 2003).

## 7.5 Conclusion

PCNA has important roles, both as a component of the replisome and as a memory molecule marking newly synthesized DNA strands to recruit specific DNA transaction enzymes under a defined spatiotemporal regulation. The dynamics of PCNA binding to DNA are involved in switching from G1 phase to S phase and from S phase to G2 phase (Fig. 7.3). PCNA is, therefore, essential for maintenance of genome integrity and epigenetic programming. We have a growing understanding of the molecular mechanisms that determine how, where, and when PCNA loading and unloading occur. However, many questions remain to be answered. The mechanism that determines whether PCNA is loaded by RFC or CTF18-RFC is not yet known. We have not yet discovered how PCNA loading discriminate between leading and lagging strands and how the loading process "determines" the relative frequencies in a certain DNA region. Whether particular DNA sequences, chromatin structures, stages in S phase, and cell-cycle progression events are able to switch PCNA dynamics actively through effects on the interacting proteins and PTMs is also not known. Furthermore, the mechanisms that determine the order and the specificity of the actions of PCNA-binding proteins remain to be determined. Approaches to address these questions should clarify the dynamic features of multiple clamps and loaders in eukaryotes, and how they change from fork to fork. Factors that can influence these events include chromatin structure, DNA sequence, and replication timing. Progress in the development of analytical technologies for single molecules and single cells, along with data mining, should help to elucidate the biological significance of multiple clamps and loaders in replicating chromosomes.

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# Chapter 8
# Termination of Eukaryotic Replication Forks

**Agnieszka Gambus**

**Abstract** Termination of DNA replication forks takes place when two replication forks coming from neighbouring origins meet each other usually in the midpoint of the replicon. At this stage, the remaining fragments of DNA have to be unwound, all remaining DNA replicated and newly synthesised strands ligated to produce continuous sister chromatids. Finally, the replication machinery has to be taken off, chromatin re-assembled, and entwisted sister chromatids resolved topologically.

Over the last few decades, we have learned a lot about the assembly of the helicase and replisome and the initiation stage of DNA replication. We also know much more about the ability of forks to cope with replication stress. However, only within recent years we have gained the first glimpse of the mechanism of replication fork termination. In this chapter I will summarise the recent findings on replication termination, weigh this against the past literature and discuss relevant consequences and views for the future.

**Keywords** Eukaryotic DNA replication • Termination of DNA replication • Ubiquitin • Cdc48 p97 segregase • Cullins

## 8.1 Introduction

To maintain genomic stability, it is essential that every step of DNA replication is faultlessly executed. Mistakes during eukaryotic replication that are not efficiently repaired can lead to mutations and genome rearrangements that promote changes leading to development of cancer and other disorders.

DNA replication can be divided into three stages: initiation, elongation and termination. Initiation of DNA replication happens when licenced origins of replication fire creating two DNA replication forks, which move in opposite directions. The elongation stage involves the progression of replication forks as they unwind and replicate DNA. Finally, termination happens when two replication forks from

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neighbouring origins converge and the duplication of remaining fragment of DNA is neatly completed. Over the years we have learnt a lot about the mechanisms of DNA replication initiation and elongation (briefly explained below), but until recently our knowledge of replication termination was very restricted. The last few years have brought a breakthrough in our understanding of mechanisms of replication termination: we have learnt that converging replication forks can pass each other when terminating, and we have also unravelled the workings of disassembly of terminated replisomes.

## 8.2 Replication Fork Termination Occurs Throughout S-Phase

When does replication termination take place? In our mind replication termination should happen mostly at the end of the whole replication process, so in terms of cell cycle stages – at the end of S-phase. In reality, however, DNA replication forks encounter forks from neighbouring origins throughout the entire S-phase. Forks emanating from origin clusters firing in early S-phase will also terminate in early S-phase; with average replicon size of 31 kbp (Moreno et al. 2016; Picard et al. 2014) and an average fork speed of 1.5 kb/min (Conti et al. 2007), it takes about 10 min for the two neighbouring forks to reach one another. In fact there is likely more termination occurring in mid S-phase than in late S-phase as the strict replication timing programme driving replication in each cell means that only difficult-to-replicate regions are replicated in late S-phase (Gilbert 2010).

## 8.3 Replication Initiation and Elongation

To ensure that all of the large eukaryotic genomes are duplicated in full before each cell division, eukaryotic DNA replication starts from multiple origins of replication. Human cells have on average about 50,000 of them spread throughout the genome. It is also essential that DNA is replicated just once per cell cycle as re-replication of parts of the genome is a threat to the maintenance of genome integrity. To achieve this, the replicative helicase (protein complex, which unwinds double-stranded DNA during replication) can be loaded onto DNA only before the onset of S-phase when CDK activity is low and can be activated only during S-phase when CDK activity is high. Origins of replication are therefore "licenced" in late M and G1 stages of the cell cycle, by loading of the core of the replicative helicase: Mcm2–7 (Minichromosome maintenance 2,3,4,5,6,7) complexes. Double hexamers of the Mcm2–7 complexes are loaded onto origins through the concerted action of ORC (origin recognition complex), Cdc6 and Cdt1 factors. These double hexamers encircle the double-stranded DNA and are arranged in N-terminus to N-terminus

orientation with the C-terminal helicase domains on the outside. There are multiple Mcm2–7 double hexamers loaded around each origin of replication, which may be facilitated by their ability to slide on double-stranded DNA (Evrin et al. 2009; Gambus et al. 2011; Remus et al. 2009).

The initiation of DNA replication requires the activity of two S-phase kinases: Cdc7/Dbf4 (DDK – Dbf4-dependent kinase) and Cdk/cyclin (CDK – Cyclin-dependent kinase). DDK phosphorylates double hexamers of Mcm2–7, while CDK drives association of GINS (Go-Ichi-Ni-San, complex of Sld5, Psf1, Psf2 and Psf3 or GINS1,2,3,4) and Cdc45 with the Mcm2–7 complexes, forming the CMG complex (**C**dc45/**M**cm2–7/**G**INS), which is an active replicative helicase (Ilves et al. 2010; Moyer et al. 2006; Simon et al. 2016). The initiation process leads to rearrangement of Mcm2–7 complexes: the double hexamers split into two CMGs, and each of them now likely encircles just single-stranded DNA (Costa et al. 2011; Gambus et al. 2006; Yardimci et al. 2010).

During the elongation stage of DNA replication, the helicase (CMG complex) travels at the tip of the replication fork, unwinding the double-stranded DNA and exposing single strands that can act as a template for DNA synthesis by DNA replication polymerases. The MCM motor of CMG belongs to the superfamily of AAA+ ATPases and is a 3–5′ DNA translocase, which encircles the leading strand of the replication fork (reviewed in Pellegrini and Costa (2016)). The Polα-primase complex initiates DNA synthesis with a short RNA primer that is then elongated for another 20-nt by Polα polymerase activity. The leading strand is believed to be synthesised mainly by DNA Polε (DNA polymerase epsilon) in a continuous manner, while the lagging strand is thought to be completed by DNA Polδ (DNA polymerase delta) (Daigaku et al. 2015; Georgescu et al. 2015; Pavlov et al. 2006). The latter synthesises short Okazaki fragments in the opposite direction to the movement of the fork, and these fragments need to therefore be processed and ligated to produce the continuous DNA strand (maturation of Okazaki fragments). DNA Polε is therefore following the helicase and indeed has a number of connections linking it directly to the helicase to facilitate the smooth progression of the fork (see below and reviewed in Pellegrini and Costa 2016).

DNA unwinding generates a compensatory increase in the intertwining of parental strands, which can be converted into helical overwinding (positive supercoiling) of the unreplicated portions of the DNA ahead of the forks (Postow et al. 2001; Wang 2002). This mechanical strain can be transmitted to replicated DNA by rotation at the branching point of the replication fork, thus generating intertwining of the daughter duplexes (known as precatenates) (Been and Champoux 1980) (Fig. 8.1). Recent research in budding yeast has shown, however, that during normal progression of replication forks, fork rotation and precatenation are actively inhibited by components of the replisome Timeless/Tof1 and Tipin/Csm3 (Schalbetter et al. 2015). Instead, supercoils generated during replication elongation can be relaxed by both type I and type II topoisomerases (Wang 2002). Indeed, the current view assumes that positive supercoiling is mainly relaxed by type I enzymes (Topo I, *S. cerevisiae* Top1) anywhere in the unreplicated region (Postow et al. 2001). The replisome progression complex (RPC) built around CMG at the tip of the fork

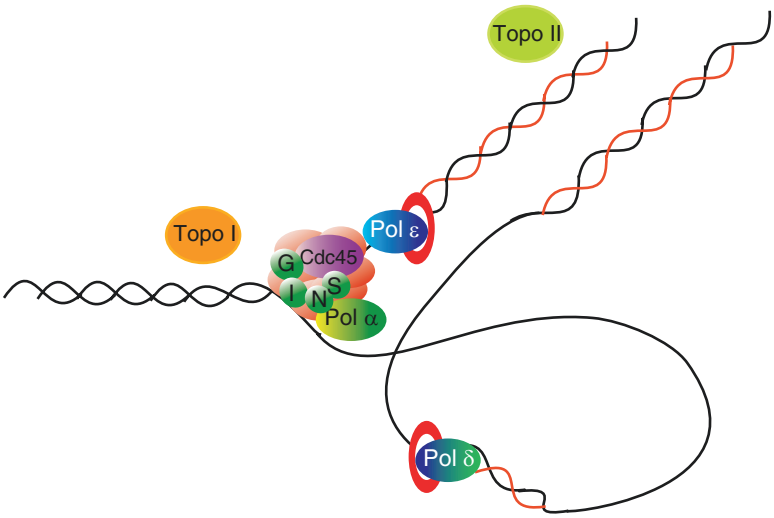


**Fig. 8.1** Topoisomerases at the replication fork. Topo I relaxes the positive supercoiling building up ahead of the fork. Sometimes this supercoiling can lead to rotation of the fork and intertwining of the daughter strands of DNA behind the fork (precatenates). These are resolved by Topo II

contains Top1, positioning it perfectly for its function ahead of the fork (Gambus et al. 2006) (Fig. 8.1). Interestingly, yeast cells without Top1 and also *top2* mutants can replicate DNA, but replication is not possible when both proteins are defective (Bermejo et al. 2007; Brill et al. 1987).

Importantly, replication forks do not move through naked DNA but through a chromatin structure. Nucleosomes therefore need to be dismantled ahead of the forks and rebuilt behind the forks. The efficient repositioning of parental histones is essential for full reconstitution of epigenetic markings throughout the replicating genome. Studies of SV40 replication forks provided evidence for the existence of only 200–300 bp of apparently nucleosome-free DNA behind the replication fork (Gasser et al. 1996), and the nucleosomes in yeast were shown recently to be positioned immediately after the fork passage and restrict Okazaki fragments sizes (Smith and Whitehouse 2012). Progressing replication forks need also to remove other proteins attached to DNA, for example, the unfired Mcm2–7 double hexamers, which, loaded in excess, serve as dormant origins ready to rescue collapsed forks. Finally, sister chromatids are topologically embraced and held together until mitosis by cohesin ring complexes. This cohesion is established during DNA replication as forks progress (reviewed by Uhlmann 2009).

## 8.4 Where Does Termination of Eukaryotic Replication Forks Happen?

The simplest answer to this question is wherever the two neighbouring forks meet each other. Recent analysis of genome-wide replication profiles in budding yeast, both through high-resolution replication profiling (Hawkins et al. 2013) and through deep sequencing of Okazaki fragments (McGuffee et al. 2013), showed that termination generally occurs midway between two adjacent replication origins. The precise position of termination depends on the relative activation time of each of the origins and their variable efficiency. Okazaki fragment mapping in human cells (HeLa and GM06990) also confirmed such midpoint localisation (Petryk et al. 2016).

Eukaryotes not only have specific spatial patterns but also possess temporal patterns of genome replication, which are executed by regulated activation of replication origins throughout S-phase. High-throughput experiments allowed the identification of a genome-wide temporal order of replication (Gilbert 2010). In early S-phase, "active" chromatin is replicated with origins of replication located in general in-between the genes. Not surprisingly, therefore, many termination events in early S-phase were found to overlap with transcribed genes. In late S-phase, however, when heterochromatin is replicated, many termination zones were found in large non-expressed regions of DNA (Petryk et al. 2016).

This sequence independent localisation of termination sites is in sharp contrast to the organisation of termination events in *E. coli* chromosome where termination takes place within a broad region containing several specialised fork barriers, i.e. Tus-TER complexes, which confine fork fusion to a site of 270 kb (reviewed in Dimude et al. (2016)). Due to these defined prokaryotic termination regions (TER), for a number of years, termination of eukaryotic replication forks was studied only at the existing few loci within the eukaryotic genome, which contain specialised replication fork barriers (RFBs). The best characterised of such sites are: the RTS1 site in *S. pombe*, which regulates mating type switching (Brewer and Fangman 1988), and the rDNA locus within ribosomal DNA repeats of metazoa and yeasts (Dalgaard and Klar 2000). The RFB barriers are able to arrest one of the two neighbouring forks and therefore create specific termination sites (Bastia and Zaman 2014; Dalgaard et al. 2009). To minimise fork pausing at RFBs, the protein displacement helicase Rrm3 helps to displace the barriers to allow replication passage and is required for fork termination at these sites. In yeast lacking Rrm3, tenfold accumulation of termination structures ("X"-shaped DNA structures in 2D DNA gels) was observed, while only twofold accumulation of paused forks at the barrier (Ivessa et al. 2000, 2003). Despite this, Rrm3 is not required for bulk replisome unloading during normal termination (Maric et al. 2014), and it is needed only for fork convergence at rare situations when one fork is paused.

In 2010, Fachinetti et al. identified 71 termination regions (TERs) in budding yeast, through a combination of chromatin immunoprecipitation (ChiP) and BrdU incorporation experiments. Their work found that the majority of these regions contain fork-pausing elements, such as transcription clusters, and that efficient ter-

mination at the identified sites requires activity of Rrm3 and Top2 (Fachinetti et al. 2010). However, the more recent high-resolution approaches suggest that these TERs actually represent sites with a higher than average probability of termination as they are flanked by early-firing efficient origins. Importantly, changes of origin-firing pattern moved the termination positioning both in non-TER and TER replicons, indicating that it is the timing and efficiency of origin firing and not fork-pausing elements that dictate the precise place of replication fork convergence (Hawkins et al. 2013; McGuffee et al. 2013).

## 8.5 How Do Replication Forks Converge?

Figure 8.2 summarises our current model of replication fork termination. To allow convergence of two approaching DNA replication forks, all of the proteins bound to DNA between them must be evicted (Fig. 8.2a). Unwinding of final stretches of DNA can present a problem for the forks as the torsional stress created ahead of the fork cannot be easily released due to lack of access for Top I (see below for more details) and has to be translated into precatenates, which accumulate behind the fork (Fig. 8.2b). Two converging forks present two large protein machineries approaching one another and heading for head-on collision while unwinding the remaining DNA between them (Fig. 8.2c). After forks converge, all of the remaining DNA needs to be replicated, and the RNA-DNA primer of the last Okazaki fragment on the lagging strand needs to be processed (Fig. 8.2d, e). Once this is complete, DNA needs to be ligated into a continuous strand, and replisomes need to be disassembled (Fig. 8.2e). Finally, the entangled sister chromatids need to be resolved into two separate strands (Fig. 8.2g).

Recent years have brought a breakthrough in our understanding of the above processes. Beautiful work from Prof. Johannes Walter's lab shed light on the mechanism by which forks converge and termination is resolved (Dewar et al. 2015). To synchronise termination events and facilitate their analysis, they constructed plasmids with an array of *lac* repressors (LacRs) bound to *lac* operators (LacOs), which can be disrupted by IPTG. Such plasmids replicated in cell-free *Xenopus laevis* egg extract accumulated blocked forks at the edges of the array. The blocked forks were then released by addition of IPTG and proceeded to terminate within the DNA fragment comprising the array. Using this system, Dewar et al. could monitor unwinding of DNA as forks approach each other, synthesis of DNA, ligation of the replicated DNA and decatenation of daughter molecules. Strikingly, the rate of DNA synthesis within the array was almost perfectly linear after IPTG addition and resembled the fork progression speed reported in the same extracts. It suggests therefore that converging forks do not slow significantly before they meet; they do not collide with each other or stall but rather pass each other (Dewar et al. 2015) (Fig. 8.2c, d). Such passage can be possible as CMGs encircle the leading strand of the replication fork and therefore approach each other on opposite strands when converging at termination (Ali et al. 2016; Costa et al. 2011; Fu et al. 2011). Interestingly, how-

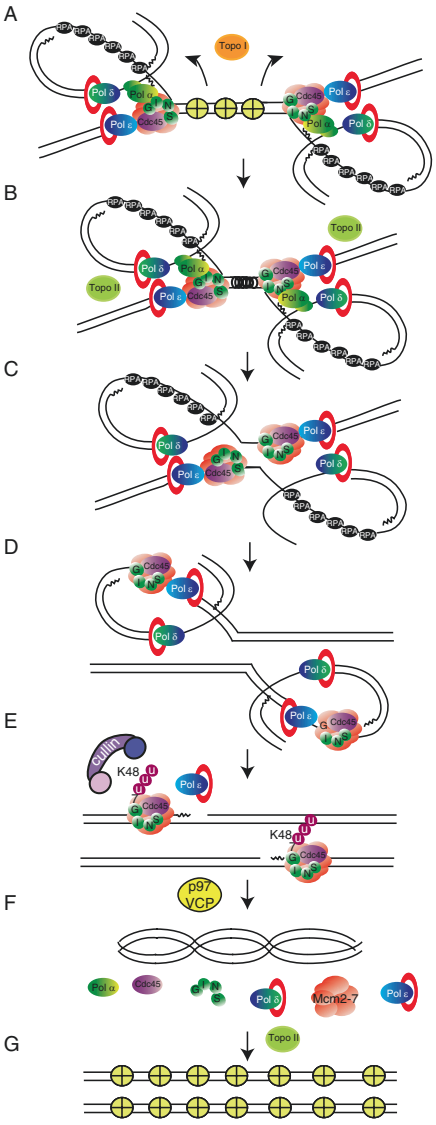


**Fig. 8.2** Model of termination of eukaryotic replication forks. When two neighbouring replication forks approach each other from opposite directions, all of the proteins organising DNA in between the forks (nucleosomes and others) have to be removed, while Topo I relaxes the torsional stress (positive supercoiling) (**a**). When two terminating forks converge, the supercoiling of DNA between them cannot be resolved by Topo I due to lack of space for it to act. Instead, terminating forks depend on transmission of this torsional stress behind the forks creating precatenates resolved by Topo II (**b**). During convergence two replisomes approach each other moving on opposite strands of DNA (leading strand of each fork) (**c**). The replisomes can pass each other and most likely CMG slides onto the double-stranded DNA of last Okazaki fragment (**d**). The synthesis of DNA is completed, the last Okazaki fragment matured and the DNA is ligated. Helicase is then ubiquitylated and removed by p97/VCP/Cdc48 segregase (**e**). Intertwined sister chromatids need to be resolved by Topo II (**f**). The final product: two individual sister chromatids with reconstituted chromatin structure (**g**)

ever, recent reports suggest that large protein barriers on the lagging strand can indeed slow down progression of the fork (Duxin et al. 2014; Langston and O'Donnell 2017). Does the approaching neighbour replisome, which is on the lagging strand, not present such a barrier? Is there an active mechanism regulating the smooth passage of the replisomes? Or are the replisomes idling at the edge of the barrier, in the attempt to unwind it, especially prepared to deal with barriers laying ahead and hence better at passing each other smoothly? More work is needed to answer these questions.

The results presented by Dewar et al. also suggest, at least in the context of the plasmid template, that torsional stress building up ahead of the forks does not slow down fork convergence (Dewar et al. 2015) (see also below for role of topoisomerases) (Fig. 8.2b). The removal of proteins (nucleosomes) ahead of the fork could not be directly addressed in this setup due to the artificial "clearing up" of chromatin ahead of the fork due to removal of the *lac* array. Interestingly, the in vitro reconstitution of eukaryotic DNA replication with purified budding yeast proteins revealed that nucleosomal packaging does appear to inhibit replication termination. As the elongation stage of the reaction was efficient, but termination alone was blocked, it suggests that the termination stage may be especially sensitive to the presence of chromatin structure (Devbhandari et al. 2017) (Fig. 8.2a).

## 8.6 The Completion of DNA Synthesis

Data provided by Dewar et al. suggest that leading-strand DNA is replicated up to a few bases away from the end of the last Okazaki fragment of the encountered lagging strand (Fig. 8.2d, e). There is no evidence for persistent gaps between these strands upon termination (Dewar et al. 2015). These data, however, do not explain which polymerase carries on synthesis of last fragments of DNA and maturation of the last Okazaki fragment. The RNA-DNA primer of each Okazaki fragment on the lagging strand is removed by concerted action of DNA Polδ and Fen1 endonuclease (reviewed in Balakrishnan and Bambara (2013)). DNA Polδ can support strand displacement resynthesis of the DNA previously synthesised by Polα and in doing so can progress until it encounters the nucleosome or another DNA-binding protein, both of which are efficiently repositioned behind the replication fork (Smith and Whitehouse 2012). Interestingly, fragments of DNA synthesised by Polα can be detected in mature genome mostly at the junctions of Okazaki fragments, usually at the nucleosome midpoint (dyad position). In total about 1.5% of mature genome was shown to be synthesised by Polα (Reijns et al. 2015).

Is the last Okazaki fragment matured by DNA Polε? The holoenzyme of DNA Polε is unable to carry on extended strand displacement synthesis in in vitro reconstitution experiments, unless its 3′–5′ exonuclease activity is removed, and it cannot mature Okazaki fragments on lagging strand (Devbhandari et al. 2017; Ganai et al. 2016). However, DNA Polε in the context of the replisome tightly associates with the CMG complex through the Dpb2 subunit of Polε and GINS and forms a func-

tional unit (Langston et al. 2014; Muramatsu et al. 2010; Sengupta et al. 2013). A recent negative stain electron microscopy reconstruction of a CMG-Polε complex visualised the close association of this complex (Pellegrini and Costa 2016; Sun et al. 2015), and we found that the post-replication replisome in both *C. elegans* and *X. laevis* interacts with Polε and not Polδ (Sonneville et al. 2017). This interaction of Polε with the replisome likely acts as additional processivity factor for Polε, in addition to action of PCNA (Kang et al. 2012; Langston et al. 2014; Yeeles et al. 2017). It would be interesting to investigate Polε strand displacement activity in the context of the replisome. In support of the Polε role at termination, analysis of the genome-wide location of ribonucleotides incorporated into DNA by mutants of Polδ and Polε especially prone to such misincorporations discovered a substantial bias towards Polδ proximal to origins which declined towards the centre of the replicons where Polε synthesis was more evident (Daigaku et al. 2015). This would suggest that Polε carries out the replication at sites of termination.

Can Polε mature the last Okazaki fragment? Can it sustain strand displacement synthesis when supported by both PCNA and the CMG? It remains to be unravelled. Importantly, DNA Polε on its own does not interact with Fen1 (Garg et al. 2004); therefore, another processing mechanism would be required to complete maturation of the last Okazaki fragment, unless Fen1 is brought in by a different component of the terminating replisome. Alternatively, Polε can slide along the last Okazaki fragment together with the post-termination replisome, making room for Polδ to displace and mature the last RNA-DNA primer. Much is to be discovered about the ability of the terminated CMG to move away from the termination site especially in the context of re-established nucleosomes. However, Polδ has been shown previously to play a role in leading-strand synthesis in vivo (Daigaku et al. 2015; Johnson et al. 2015; Waga et al. 2001). Moreover, recent data obtained from the budding yeast in vitro reconstitution system of replication revealed that polymerase switching may be more common than expected. Polδ can play an important role in establishing leading-strand synthesis (Yeeles et al. 2017), and Polδ assembled at the leading strand was shown to be displaced if Polε was added after DNA synthesis has initiated (Georgescu et al. 2014). More research is required to show which of the polymerases finishes the replication job.

## 8.7 Role of Topoisomerases During DNA Replication Termination

The ability of topoisomerases to act ahead of the replication forks becomes very limited as two replication forks converge (Sundin and Varshavsky 1980). In this circumstance, fork rotation and precatenation become the primary pathway of DNA relaxing ahead of the fork. Catenated, double-stranded DNA (intertwined sister chromatids) can only be resolved by type II topoisomerases (Topo II, *S. cerevisiae* Top2) (Fig. 8.2b). Experiments with Topo II inhibitors in *Xenopus* egg extract

showed that Topo II can be trapped behind, but not in front of the forks, and resolves replication intermediates in a nonredundant manner with Topo I (Hyrien 2009; Lucas et al. 2001). Interestingly, Top2 depletion in yeast does not stop cells from completing DNA replication, nor passing through mitosis, although they do dramatically mis-segregate and break their chromosomes due to sister chromatid catenation. On the other hand, inhibition of Top2 enzymatic activity in a way that Top2 is still able to bind DNA but unable to catalyse strand breakage causes incomplete DNA replication and induces G2/M cell cycle arrest (Baxter and Diffley 2008). Similarly, inhibition of Topo II activity in higher eukaryotes with the small molecule inhibitor ICRF-193 was shown to block termination of DNA replication in *Xenopus* egg extract and induce G2 arrest in human cells without the high level of DNA strand breaks associated with Topo II poisons (Cuvier et al. 2008; Downes et al. 1994; Skoufias et al. 2004). ICRF-193 traps Topo II on the DNA in the form of a non-covalent intermediate named the closed clamp (Roca et al. 1994). It is unclear therefore whether replication termination defects observed upon addition of ICRF-193 to *Xenopus* egg extracts are due to inhibition of Topo II activity or some other effect of the closed clamps, such as changes to nucleosome spacing and chromatin structure (Gaggioli et al. 2013; Germe and Hyrien 2005).

In agreement with the role of Topo II in replication fork termination, post-termination replisomes from *C. elegans* and *X. laevis* contain Topo II, unlike the budding yeast replisome progression complex, which represents active helicase and contains Top1 (Gambus et al. 2006; Sonneville et al. 2017). Moreover, Dewar et al. reported that site-specific termination plasmids (described above) require Topo II for decatenation of daughter plasmids, but Topo II activity is not needed for fork convergence and DNA ligation (Dewar et al. 2015) (Fig. 8.2b, f).

## 8.8 Replisome Disassembly

The data presented by Dewar et al. suggest that the dissolution of the replisome in the plasmid-based system is the last stage of replication fork termination, executed after ligation of leading and lagging strands (Dewar et al. 2015) (Fig. 8.2e). Work in budding yeast and *Xenopus laevis* egg extract discovered the first elements of this dissolution mechanism, which was found to be a highly evolutionary conserved process (Maric et al. 2014; Moreno et al. 2014) (Fig. 8.3a, b). In both model organisms, the Mcm7 subunit of the CMG complex becomes polyubiquitylated when forks terminate. The ubiquitin chains attached to Mcm7 are linked through lysine 48 (K48), but ubiquitylated Mcm7 is not degraded directly on chromatin as inhibition of proteasomal activity does not inhibit CMG disassembly. Instead, a protein remodeller Cdc48 (p97, VCP, segregase) recognises the ubiquitylated CMG and through its ATPase activity removes the CMG complexes from chromatin (Maric et al. 2014; Moreno et al. 2014). It is unclear at present whether the ubiquitylated Mcm7 is degraded upon removal from chromatin or de-ubiquitylated. A recent report by Fullbright et al. suggests that during unperturbed DNA replication in *Xenopus* egg

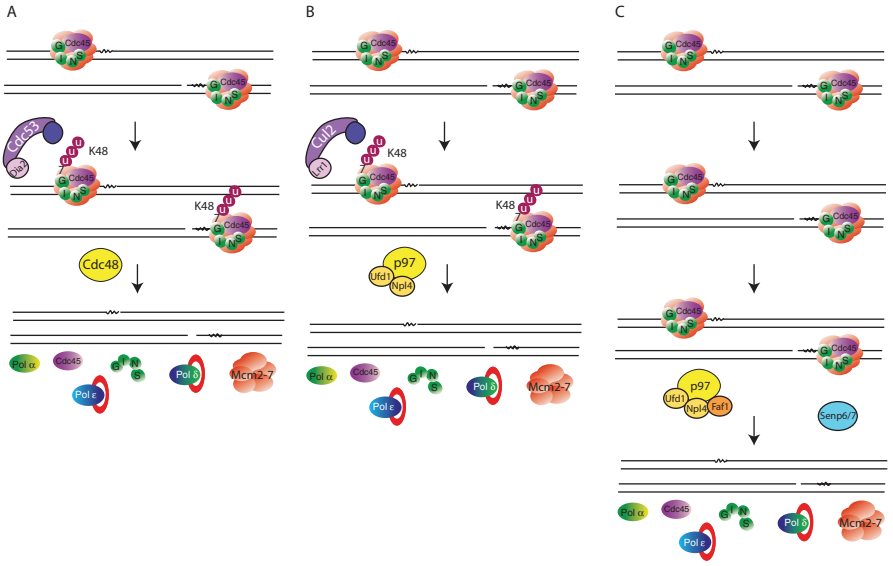


**Fig. 8.3** Model of replisome disassembly at the termination of replication forks. In budding yeast *S. cerevisiae*, the Mcm7 subunit of the terminating replisome is ubiquitylated by $SCF^{Dia2}$ and removed from chromatin by Cdc48 segregase (**a**). In *Xenopus* egg extract and *C. elegans* embryos, $CRL2^{Lrr1}$ ubiquitylates Mcm7 during termination of replication forks, and CDC-48/p97 segregase removes it from chromatin with help of Ufd1/Npl4 cofactors (**b**). If the mechanism of removal of the replisome during termination of forks in S-phase does not work, *C. elegans* embryos have a backup mechanism removing replisomes in prophase in mitosis. This mechanism requires CDC-48/p97 and Npl4/Ufd1 but also UBXN-3/FAF1 cofactor and is regulated by ULP-4/Senp6,7 (**c**)

extract, ubiquitylated Mcm7 is likely to be de-ubiquitylated (Fullbright et al. 2016). Interestingly, ubiquitylation of human Mcm7 (both endogenous and exogenously expressed in cells) was reported in the past, but the fate of the ubiquitylated form of Mcm7 and the function of the ubiquitylation were not clear (Buchsbaum et al. 2007; Kuhne and Banks 1998).

### *8.8.1 $SCF^{Dia2}$ Ubiquitin Ligase in Budding Yeast*

In budding yeast the ubiquitin ligase, which ubiquitylates Mcm7, is $SCF^{Dia2}$ (Maric et al. 2014). $SCF^{Dia2}$ is a multisubunit ligase built around a Cdc53 cullin scaffold (homologue of Cullin 1 in higher eukaryotes) (Fig. 8.3a). Dia2 is the substrate-specific receptor, F-box protein, which binds through the substrate adaptor (Skp1) to the N-terminal part of Cdc53. The C-terminus of Cdc53, on the other hand, binds RING domain factor Hrt1, connecting the ligase to the ubiquitin-conjugating enzyme (E2) Cdc34 (SCF = **S**kp1 + **C**ullin1 + **F**-box) (Fig. 8.4b). $SCF^{Dia2}$ was shown to be essential for Mcm7 ubiquitylation, specifically in the context of CMG

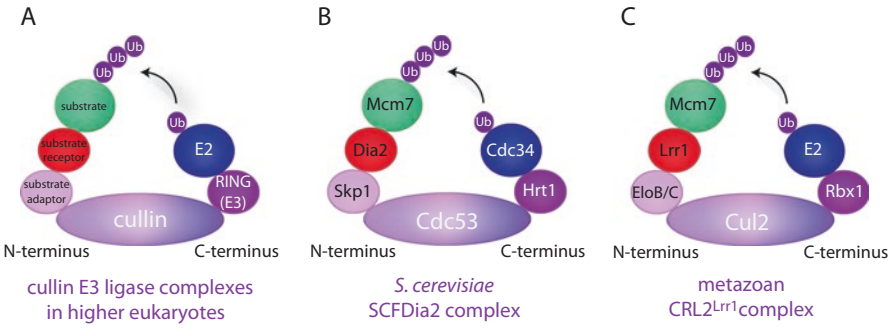


**Fig. 8.4** Model of cullin ligases ubiquitylating Mcm7 during termination of replication forks. General model of organisation of cullin family members (**a**). Model of $SCF^{Dia2}$ ubiquitylating Mcm7 in *S. cerevisiae* (**b**). Model of $CRL2^{Lrr1}$ ubiquitylating Mcm7 in *C. elegans* embryos and *Xenopus* egg extract (**c**)

during S-phase – both in vitro and in vivo. Moreover cells lacking Dia2 (*dia2Δ*) retain CMG complexes on chromatin after S-phase until the next G1 stage of the cell cycle (Maric et al. 2014). Not surprisingly budding yeast cells lacking Dia2, although viable, are defective in S-phase progression and present high rates of endogenous DNA damage and genome instability. They are also unable to grow at low temperatures and are sensitive to DNA-damaging agents that affect replication fork progression (Blake et al. 2006; Koepp et al. 2006).

Dia2 contains a protein-protein interaction N-terminal tetratricopeptide repeat (TPR) domain, a nuclear localisation signal (NLS), an F-box that connects it to the rest of the SCF ligase, and a C-terminal substrate recognition domain comprising of leucine-rich repeats (LRR). The TPR domain of Dia2 was shown to interact with Mrc1 and Tof1 components of the replisome progression complex (RPC) built around the CMG helicase (Gambus et al. 2006; Morohashi et al. 2009). As a result, Dia2 was detected interacting with RPC in S-phase, and this interaction was preserved when cells were treated with hydroxyurea (HU) to stall progressing replication forks (Morohashi et al. 2009). Interestingly cells lacking the TPR domain within Dia2 (*dia2-ΔTPR*) do not present the severe phenotype of *dia2Δ* cells – with the exception of synthetic lethality with rrm3Δ (a helicase supporting passage of forks past protein-DNA barriers). Cells lacking the TPR domain in Dia2 were, however, shown consequently to have a partial defect in Mcm7 ubiquitylation and CMG disassembly (Maculins et al. 2015). It seems that attaching $SCF^{Dia2}$ to the replisome via the TPR domain increases the efficiency of CMG ubiquitylation. It may not be essential for normal CMG disassembly as the LRR domain can still recognise its substrate even without the tethering, but there may be situations when this stabilised interaction with the replisome is more vital – for example, when forks struggle to pass DNA-protein barriers in the absence of Rrm3.

### 8.8.2 *CRL2$^{Lrr1}$ Ubiquitin Ligase in Higher Eukaryotes*

Recent research from our and two other groups discovered that in higher eukaryotes the ubiquitin ligase ubiquitylating Mcm7 at termination of replication forks is not an SCF but a Cullin2-based ubiquitin ligase with a leucine-rich repeat 1 protein (Lrr1) as a substrate receptor (**C**ullin-**R**ing **L**igase 2 with Lrr1 = CRL2$^{Lrr1}$) (Dewar et al. 2017; Sonneville et al. 2017) (Fig. 8.4c). Both in *Xenopus* egg extract and in *C. elegans* embryos, inhibition or downregulation of Cullin 1 ligase activity did not influence Mcm7 ubiquitylation nor helicase disassembly during S-phase (Sonneville et al. 2017 and our unpublished data). On the other hand, siRNA downregulation of CUL-2/LRR-1 complex in *C. elegans* embryos and immunodepletion of CRL2$^{Lrr1}$ in egg extract blocked both phenotypes (Dewar et al. 2017; Sonneville et al. 2017) (Fig. 8.3b). CRL2$^{Lrr1}$ was also shown to be the only cullin-type ubiquitin ligase that interacts with post-termination replisomes in *Xenopus* egg extract and *C. elegans* embryos and accumulates at the sites of termination in plasmid-based termination system described above (Dewar et al. 2017; Sonneville et al. 2017). Importantly, both studies found that CRL2$^{Lrr1}$ interacts specifically with terminating CMG and not with actively unwinding helicase nor double Mcm2–7 hexamers of dormant origins. The regulated binding of CRL2$^{Lrr1}$ to post-termination replisome represents therefore the first known step of replisome disassembly. Finally, the ubiquitin ligase activity of CRL2$^{Lrr1}$ is necessary for the Mcm7 ubiquitylation and helicase disassembly, as a mutant of Cul2-Rbx1 complex, which cannot be activated by neddylation, is unable to rescue the CRL2$^{Lrr1}$-immunodepleted egg extract unlike a wild-type fully functioning complex (Sonneville et al. 2017).

What is CRL2$^{Lrr1}$? Previous work has shown that *C. elegans* LRR-1 is an essential gene (Piano et al. 2002). LRR-1 is required for embryonic development, but maternal rescue allows analysis of *lrr-1* loss of function in adult tissues. *Lrr-1* mutants are sterile owing to severe defects in germ cell proliferation (Merlet et al. 2010; Starostina et al. 2010). Inactivation of *lrr-1* induces DNA damage, which may arise due to DNA re-replication problems (ssDNA/RPA-1 foci accumulate in *lrr-1* germ cells, which also contain greater than 4 N DNA content). This in turn leads to hyperactivation of ATL-1/CHK-1 pathway (ATR/Chk1 pathway in vertebrates), which delays mitotic entry and results in embryonic lethality. Inactivation of ATL-1/CHK-1 checkpoint components supresses the proliferation defect and fully restores *lrr-1* mutant fertility (Burger et al. 2013; Merlet et al. 2010). How the re-replication/DNA damage is induced in *lrr-1* worms is not as yet determined. Interestingly, an RNAi-based suppressor screen of *lrr-1* and *cul-2* mutants identified two genes encoding components of the GINS complex, as well as CDC-7 and MUS-101, which are needed for CMG activation (Ossareh-Nazari et al. 2016). These data suggest that reducing CMG levels on chromatin can supress the DNA damage created in *lrr-1* mutants and supress their lethality. This is in agreement with LRR-1's role in Mcm7 ubiquitylation as lower levels of CMG on chromatin would compensate for a defect in CMG unloading.

On the other hand, another study found that *C. elegans lrr-1* mutants germ cells arrest with 2C DNA content, which may be due to accumulation of CDK inhibitor CKI-1 as deletion of one copy of CKI-1 or *cki-1* RNAi treatment can rescue *lrr-1* mutant germ cells numbers. In support of the CUL-2/LRR-1 role in targeting CKI-1 for degradation, study in human cells found that overexpressed CKI-1 was degraded faster when LRR-1 was also overexpressed (Starostina et al. 2010). Interestingly, LRR1 or CUL2 knockdown in HeLa cells did not induce a strong cell cycle arrest, and LRR1 was shown to be important to regulate levels of cytoplasmic p21 (human CKI) to control actin cytoskeleton remodelling (Starostina et al. 2010). Further studies are required to analyse in depth the role of LRR1 in human cells and the interplay between different substrates of this ubiquitin ligase.

Several questions remain – what is the signal for polyubiquitylation of Mcm7 and removal of helicase? How are CMGs protected from ubiquitylation during elongation and efficiently ubiquitylated at termination (Fig. 8.2)? Dewar et al. hypothesise that it may be conformational changes within CMG upon transition from encircling single-stranded DNA to double-stranded DNA of last Okazaki fragment that provide this post-termination specificity (Dewar et al. 2015). In support of this hypothesis, it was shown that CMG is indeed able to slide on double-stranded DNA (Kang et al. 2012).

We should also keep in mind that many substrate-specific receptors of CRLs recognise their substrates only when they are post-translationally modified; e.g. F-box receptors of SCF often recognise phosphorylated proteins, and VHL interacting with CRL2 recognises Hif1α upon its hydroxylation. It is possible therefore that terminating CMG is first modified in a yet undiscovered manner before being ubiquitylated. Budding yeast Mcm2–7 complex has been recently shown to be SUMOylated upon loading at origins in G1 stage of cell cycle before Mcm2–7 phosphorylation. The level of Mcm2–6 SUMOylation decreases during S-phase as MCM becomes phosphorylated and activated, with exception of Mcm7, which SUMOylation was retained during S-phase (Wei and Zhao 2016). Additionally, deubiquitylating enzyme Usp7 was described recently as a SUMO-specific DUB, removing ubiquitin from SUMOylated proteins and maintaining high SUMO/low ubiquitin ratio at replication forks (Lecona et al. 2016; Lopez-Contreras et al. 2013). A theory was therefore proposed that SUMO-driven ubiquitylation could act as a signal for the termination of DNA replication (Lecona and Fernandez-Capetillo 2016). Usp7 was also previously shown to interact with MCM-binding protein MCM-BP and to cooperate with it to unload the Mcm2-7 complexes from chromatin at the end of S-phase (Jagannathan et al. 2014; Nishiyama et al. 2011). Is Usp7 DUB activity for SUMOylated proteins linked with its MCM-BP interaction? Is Mcm7 in higher eukaryotes modified by SUMO? Is SUMOylation of Mcm7 regulating its ubiquitylation at termination events? More work is needed to understand fully this complex process.

Another possibility in need of investigation is involvement of priming ubiquitin ligase. Indeed ARIH1, an Ariadne family Ring-Between-Ring (RBR) ubiquitin ligase, was shown recently to interact with a number of CRLs including CRL2s and prime their substrates (Scott et al. 2016). It is probable, therefore, that such a priming

ligase recognises the terminating helicase and $CRL2^{Lrr1}$ only acts on primed substrate.

### 8.8.3 The Role of p97 Segregase in Replisome Disassembly

p97, also known as VCP in metazoans, CDC-48 in *C. elegans*, Cdc48 in yeast and Ter94 in insects, is a ubiquitin-dependent segregase that plays a central role in the regulation of protein homeostasis. Once bound to ubiquitylated substrates, this conserved hexameric AAA+ ATPase utilises the energy released from ATP hydrolysis to undergo a conformational change across its hexamer structure called interprotomer motion transmission (Huang et al. 2012; Li et al. 2012). This movement allows p97 to remove substrates from different cellular locations and complexes, likely by substrate translocation through p97's narrow central pore (Tonddast-Navaei and Stan 2013). The separated or unfolded substrates can then be directed to the proteasome and degraded or de-ubiquitylated and recycled with the help of DUBs associating with p97. p97 carries on this segregase/unfoldase activity on a myriad of substrates participating in a large variety of cellular processes. Not surprisingly, knockdown of both p97 alleles causes early embryonic lethality in mice, and siRNA-depletion of p97 in cells causes apoptosis (Muller et al. 2007; Wojcik et al. 2004).

The interaction of p97 with its many different substrates is mediated by a group of about 30 adaptor proteins that specifically recruit ubiquitylated proteins (Meyer et al. 2012; Yeung et al. 2008). The cofactors usually bind to the N-terminal domain of p97 using p97 interacting motifs. The best characterised major p97 cofactors include Ufd1/Npl4 heterodimer and p47, which bind to the p97 in mutually exclusive manner (Bruderer et al. 2004). Further, minor cofactors such as FAF1 or UBXD7 can then associate to the p97 complex with a major cofactor (Hanzelmann et al. 2011). Some of the cofactors, such as UBXD7, can also interact with various ubiquitin ligases and streamline the process of ubiquitin-dependent substrate removal/degradation (reviewed in Meyer et al. 2012).

The role of p97 during DNA replication was first suggested in *C. elegans* embryos. RNAi-mediated depletion of the CDC-48 complex leads to a defect in cell division: mitotic entry was delayed as a result of the activation of the DNA damage checkpoint. The severe chromatin defects observed in embryos as well as mitotic cells of the gonads included mitotic bridges and accumulated foci of RAD-51 DNA repair protein. Moreover, embryos lacking CDC-48, UFD-1 or NPL-4 are strongly reduced in DNA content (Deichsel et al. 2009; Mouysset et al. 2008). It was subsequently shown that embryos lacking CDC-48 or UFD1/NPL-4 cofactors accumulate origin licencing factor CDT-1 on mitotic chromatin and present persistent chromatin association of CDC-45/GINS after S-phase is completed (Franz et al. 2011). This process involves another p97 cofactor UBXN-3/FAF1 (Franz et al. 2016). Interestingly, inhibition of CDT-1 degradation and its accumulation on chromatin in

embryos lacking CDC-48 or UFD1/NPL-4 does not lead to re-replication phenotype in these embryos but rather a strong reduction in their DNA content.

In the case of replisome disassembly, the segregase function was shown to be essential to disassemble ubiquitylated post-termination CMG in budding yeast, *C. elegans* embryos and *Xenopus* egg extract (Maric et al. 2014; Moreno et al. 2014; Sonneville et al. 2017). The ATPase activity of p97 is essential for this disassembly function as the replisome can be blocked on chromatin when two ATPase domains of p97 (D1 and D2) are mutated or the activity of p97 is blocked with a small molecule inhibitor NMS973 (Dewar et al. 2017; Moreno et al. 2014; Sonneville et al. 2017). This replisome disassembly defect phenotype is not driven through Cdt1 deregulation nor represents novel binding of GINS/Cdc45 to mitotic chromosomes (Moreno et al. 2014; Sonneville et al. 2017). In worm embryos, RNAi-directed inactivation of *ufd-1* and *npl-4* leads to a defect in replisome unloading, and the Ufd1/Npl4 heterodimer is found to interact with the post-termination replisome in *Xenopus* egg extracts (Dewar et al. 2017; Sonneville et al. 2017). Moreover, plasmids with accumulated terminating forks contain enriched Ubxn7 and Dvc1/SPRTN bound to them (Dewar et al. 2017). Future work will show whether these additional cofactors play a role in replisome disassembly.

### *8.8.4 Backup Pathway for Replisome Disassembly*

Importantly, work in *C. elegans* embryos revealed that if the removal of CMG complexes is not accomplished during S-phase due to defective $CRL2^{Lrr1}$, then they can be removed from chromatin at the beginning of mitosis, in late prophase (Sonneville et al. 2017) (Fig. 8.3c). This backup mitotic pathway of replisome disassembly also requires p97/Ufd1/Npl4 (worm CDC-48/UFD-1/NPL-4) segregase, but to accomplish it p97 requires yet another cofactor: Fas-associated factor 1 FAF1 (worm UBXN-3) (Sonneville et al. 2017). FAF1 is an evolutionarily conserved proapoptotic factor that contains multiple protein interaction domains: ubiquitin-associated UBA, ubiquitin-like UBL1 and UBL2, Fas-interacting domain FID, death effector domain-interacting domain DEDID, Ubiquitin-associated UAS and ubiquitin regulatory X UBX (Lee et al. 2013; Menges et al. 2009). FAF1 is an essential gene (Adham et al. 2008), an established modulator of apoptosis, which regulates NFκB and is involved in ubiquitin-mediated protein turnover (reviewed in Menges et al. (2009)). FAF1 was also shown to bind to p97-Ufd1-Npl4 complex via the UBX domain and polyubiquitylated proteins via the UBA domain to promote endoplasmic reticulum-associated degradation ERAD (Lee et al. 2013). Finally, recent work from the Thorsten Hoppe lab showed that FAF-1/UBXN-3 is required for cell cycle progression in *C. elegans* embryo due to the problem with CDT-1 degradation and its inappropriate maintenance on chromatin during mitosis, together with CDC-45 and GINS (Franz et al. 2016). Moreover, Franz et al. have shown that downregulation of FAF1 by siRNA in human cells causes a pronounced replication stress phenotype: defective fork progression, fork stalling, dormant origin firing and activation

of both S-phase checkpoint (ATR/Chk1) and DNA damage checkpoint (ATM/Chk2) (Franz et al. 2016). It remains to be investigated whether this observed replication stress is the result of Cdt1-induced re-replication, a defect in unloading of the post-termination replisomes or one of the many other FAF1 functions.

Intriguingly, the backup mitotic pathway of replisome disassembly in *C. elegans* embryos is modulated by the activity of the SUMO protease ULP-4: co-depletion of ULP-4 with LRR-1 delayed the release of CMG components from chromatin (Sonneville et al. 2017). ULP-4 is a major mitotic SUMO protease in worms and is present at mitotic chromosomes and at the spindle midzone (Pelisch et al. 2014). The ULP-4 analogous proteases in human cells are SENP6–7. It remains to be unravelled whether SUMO plays a regulatory role in the backup process or whether ULP-4 functions in another way, e.g. by bridging some important interactions and allowing p97 complex recruitment. It would be very interesting to investigate the existence of such a potential backup pathway in human somatic cells.

## 8.9 The Importance of Faultless Termination

Does deregulation of termination contribute to genomic instability and human disease? Cancer chromosomal instability (CIN) is observed in most solid tumours and is associated with poor prognosis and drug resistance (McGranahan et al. 2012). CIN leads to increased rate of changes in chromosomal numbers and structure and generates intra-tumour heterogeneity. Recent data implicate a central role for replication stress in the generation of CIN (Burrell et al. 2013). Can faulty termination provide a source of replication stress, which then contributes to the generation of genomic instability and CIN? What are the ways in which problems during replication fork termination could lead to genomic instability? At present we have limited experimental data on consequences of problems with replication fork termination, but we can speculate based on what we know.

We know that failure to decatenate newly replicated sister chromatids upon termination of replication forks does not tend to be detected by G2/M checkpoint but leads to dramatic mis-segregation of chromosomes during mitosis (Baxter and Diffley 2008). What about other stages of the termination process?

What would happen if forks cannot converge properly? What if their passing each other at the termination stage is blocked? We can imagine that problems during convergence of replication forks could lead to similar torsional stresses as these created by lack of topoisomerase I during elongation. Inhibition of Topo I activity in human cells, mouse embryonic fibroblasts and *Xenopus laevis* egg extract frequently induces replication fork reversal (reviewed in Neelsen and Lopes 2015). Fork reversal can have physiological roles during replication but can also have pathological consequences, contribute to genome instability in neurodegenerative syndromes and cancer. A small but reproducible number of reversed forks were detected also in various unchallenged human cell lines, while deregulation of poly (ADP-ribose) metabolism, which regulates fork reversal and restart, induces high level of reversed

forks even in the absence of genotoxic replication stress (reviewed in Neelsen and Lopes 2015). Fork reversal is also very frequent in mouse embryonic stem cells (Ahuja et al. 2016). Where do these reversed forks come from? Could problems with termination of replication forks be one of the sources of such reversed forks? Interestingly, transient over-replication, fork reversal and end-processing by exonucleases were recently associated with completion of replication termination in *E. coli* (Wendel et al. 2014). More research and visualisation of converging forks either unchallenged or upon termination perturbations are needed to elucidate the possibility of fork reversal at sites of troubled replication fork termination.

Can failure to complete DNA synthesis at termination sites create genome instabilities? It has been shown recently that not all of the DNA is always replicated in human cells during S-phase – unreplicated segments resulting from double fork stalling in large replicons are frequently present in G2. They can be partially resolved during mitosis, create ultrafine bridges during segregation in mitosis and are subsequently recognised in the G1 stage of the cell cycle by DNA repair protein p53-binding protein 1 (53BP1) to be resolved in this new cell cycle (Moreno et al. 2016). Failure to complete DNA synthesis at termination sites would likely lead to a similar scenario.

What about inhibiting disassembly of the replisome? This is the part of the termination process that we understand best at present. If disassembly of the replisome constitutes the last step of replication termination, then the failure to remove it should not leave unligated DNA nor unusual DNA structures (Dewar et al. 2015). It would however leave a DNA helicase on a DNA substrate. Tested on synthetic in vitro substrates CMG can translocate on double-stranded DNA and then start unwinding DNA if a fork structure is present (Kang et al. 2012). One can imagine therefore that the second to the last Okazaki fragment, which may be in a mid-maturation stage with a flap created by Polδ, could be such a substrate for the approaching post-termination CMG to start de novo unwinding. In bacteria, recent data suggest that in termination zones 3′ ssDNA flaps are created that, if not removed by RecG nuclease (in RecG mutants), can provide substrates for de novo replication, leading to re-replication and creating pathological DNA structures. Tus termination sequences limit the extent of such re-replication initiated in termination zones (Rudolph et al. 2013). What about eukaryotic cells? They do not have Tus termination sequences. Can faulty termination of replication forks initiate re-replication?

Moreover, CMG complexes left behind on chromatin would disturb proper chromatin re-establishment and pose a problem to processes for which DNA is a substrate, such as transcription and next replication. As mentioned above, CMGs can translocate on double-stranded DNA (Kang et al. 2012), and by moving along DNA, they could displace other proteins bound to DNA. At present we do not know whether CMG sliding on dsDNA can displace nucleosomes or if they will be trapped by them.

A final potential problem arising from lack of efficient disassembly of the CMG complexes at the termination of replication forks is entrapment of Cdc45 and GINS within these post-termination complexes. Cdc45 was shown to be a rate limiting

factor for DNA replication in mammalian cells. It was proposed that regulated expression levels of Cdc45 enforce reutilisation of existing Cdc45 during S-phase, which in turn can limit and stagger origin activation throughout the S-phase (Kohler et al. 2016; Wong et al. 2011). A lack of Cdc45 available for recycling can therefore potentially slow S-phase progression and inhibit DNA synthesis. Primary untransformed human cells with reduced levels of GINS components present all the phenotypes of replication stress and accumulation of DNA damage (Barkley et al. 2009). Future studies of replisome disassembly in human somatic cells are essential to shed light at this possibility as so far this process was investigated only in embryonic systems (*Xenopus laevis* egg extract and *C. elegans* embryos) which have higher levels of Cdc45 and GINS.

Is there experimental evidence that faulty disassembly of the replisome can lead to genome instability? *S. cerevisiae* cells lacking Dia2, which are unable to remove post-termination CMG from chromatin, are viable but present very high levels of genomic instabilities (described above). LRR-1 – the Mcm7-specific substrate receptor in higher eukaryotes – is an essential gene in *C. elegans*. LRR1 may have other than Mcm7 substrates, as CMG becomes unloaded by a backup system in *lrr-1* embryos. However, partial downregulation of LRR-1 together with downregulation of the backup pathway factors FAF-1/UBXN-3 or ULP4 results in synthetic lethality, suggesting that inhibition of CMG removal by partial blocking of both pathways results in non-viable worms (Sonneville et al. 2017). FAF1 itself is a factor often downregulated or mutated in multiple cancers. It may be its proapoptotic function that drives this downregulation, but in consequence these cancers could exhibit higher levels of genomic instability due to their replication fork termination problems. It is crucial therefore that we investigate the process of replisome disassembly in human cells to confirm its analogy.

Factors that drive replication initiation and the assembly of CMG, such as Cdc7 kinase and TopBP1 (Cut5) initiation factor, are currently being explored as potential anticancer therapy targets in tumours that present defects in chromosome replication (Chowdhury et al. 2014; Montagnoli et al. 2010). Can CMG disassembly also serve as a potential target for future therapies? Could we target the S-phase pathway of CMG disassembly in cancers with mutated or downregulated FAF1? For this we need to understand the CMG disassembly process in much more detail and crucially confirm its conservation in human cells. It seems likely that ubiquitylation is rate limiting for CMG disassembly, although it needs to be demonstrated by mapping the ubiquitylation sites and creating an unmodifiable mutant. It is clear, however, that Mcm7 ubiquitylation is regulated in a precise fashion on many levels, both spatially and temporally.

Finally, many factors implicated in DNA replication fork termination and replisome disassembly, such as p97 segregase and Usp7, are also targets of small molecule inhibitors used or being tested for antitumour therapies (Magnaghi et al. 2013; Reverdy et al. 2012). A better understanding of CMG disassembly pathway and replication fork termination in human cells might help us to explain the mode of action of these inhibitors in clinic.

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# Chapter 9
# Structure of the MCM2-7 Double Hexamer and Its Implications for the Mechanistic Functions of the Mcm2-7 Complex

**Yuanliang Zhai and Bik-Kwoon Tye**

**Abstract** The eukaryotic minichromosome maintenance 2–7 complex is the core of the inactive MCM replication licensing complex and the catalytic core of the Cdc45-MCM-GINS replicative helicase. The years of effort to determine the structure of parts or the whole of the heterohexameric complex by X-ray crystallography and conventional cryo-EM produced limited success. Modern cryo-EM technology ushered in a new era of structural biology that allowed the determination of the structure of the inactive double hexamer at an unprecedented resolution of 3.8 Å. This review will focus on the fine details observed in the Mcm2-7 double hexameric complex and their implications for the function of the Mcm2-7 hexamer in its different roles during DNA replication.

**Keywords** DNA replication • Replication licensing • MCM2-7 • Cryo-EM structure

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## 9.1 Introduction

Of the three macromolecule-synthesizing machines that synthesize proteins, mRNAs and DNA, respectively, the crystal structures of the ribosome (Ramakrishnan 2010; Steitz 2010; Yonath 2010) and RNA polymerase (Kornberg 2007) have been determined at atomic resolution, and Nobel prizes were even awarded for these achievements. However, the replisome and many of its integral components have been recalcitrant to crystallization, and structural determination of the replisome has lagged behind. The replisome is a complex machine with multiple engines including a DNA-unwinding helicase at the front end and one leading-strand and two lagging-strand DNA polymerases chugging along at the back end. The size, asymmetry, flexibility, and multipartite nature of the replisome have posed difficult challenges for structural biologists on the mission to unravel its anatomical secrets. However, the prospects for unraveling the atomic structure of the replisome soon will change because of the advent of the cryo-EM revolution that took place in 2013 (Fernandez-Leiro and Scheres 2016). To date, two major structures that are critical for unraveling the anatomy of the helicase component of the replisome have been determined at near-atomic resolution. The first is the inactive MCM double hexamer determined at an overall resolution of 3.8 Å (Li et al. 2015). The unprecedented resolution achieved in this study lays a strong foundation for structural work on the helicase component of the replisome and will serve as a template for subsequent atomic modeling of all MCM-associated DNA replication complexes. The second is the CMG helicase resolved at a slightly lower resolution by two groups (Abid Ali et al. 2016; Yuan et al. 2016). In this chapter, we will focus on the structure and structure-informed functions of the MCM double hexamer. In the following chapter, the structure of the CMG helicase and the architecture of the replisome will be reviewed.

The Mcm2-7 hexameric complex is the core of the inactive MCM replication licensing complex (Tye 1999a; Donovan et al. 1997; Chong et al. 1995; Thommes et al. 1997) and the catalytic core of the Cdc45-MCM-GINS replicative helicase (Labib et al. 2000; Moyer et al. 2006; Gambus et al. 2006; Ilves et al. 2010). Three of the MCM subunits (Mcm2, Mcm3, and Mcm5) were initially identified in a yeast mutant hunt for proteins that regulate the initiation step of DNA replication (Tye 1999a). This screen utilized the maintenance of minichromosomes as an assay and hence the name (Maine et al. 1984; Tye 1999b). Paralogs of these Mcm proteins were subsequently identified in the yeasts and other eukaryotes from screens not necessarily related to DNA replication functions (Moir et al. 1982; Johnston and Thomas 1982; Bae et al. 2009). There is a total of eight Mcm paralogs. Mcm8 and Mcm9 are only found in metazoans, and their functions are less well known (Maiorano et al. 2005; Gambus and Blow 2013; Lutzmann et al. 2005; Nishimura et al. 2012; Traver et al. 2015). Mcm2, Mcm3, Mcm4, Mcm5, Mcm6, and Mcm7 form a hexameric complex that is involved in replication initiation and elongation in all eukaryotes (Bochman and Schwacha 2009). However, purified Mcm2-7 complexes show little or no helicase activity unless provided with the accessory factors, the tetrameric GINS and Cdc45 (Bochman and Schwacha 2008; Ilves et al. 2010).

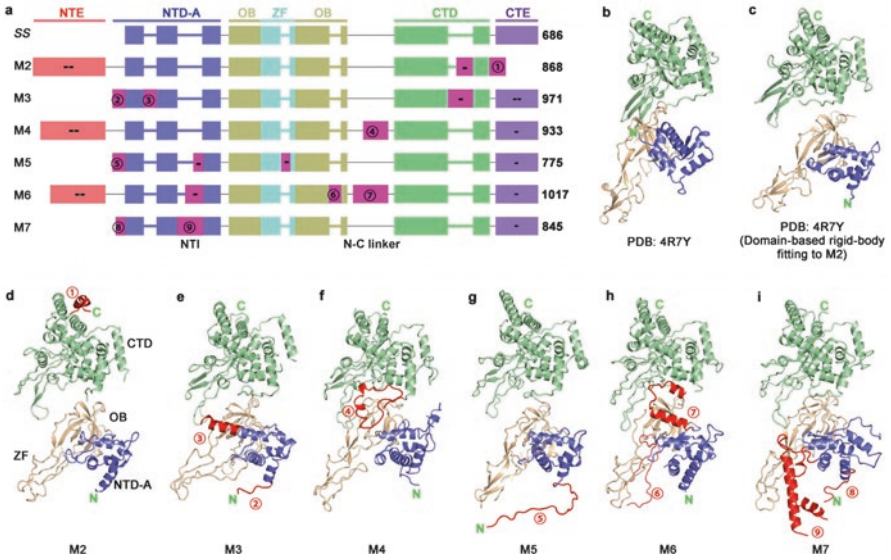


**Fig. 9.1** Structural organization of the MCM2-7 subunits. **a** Schematic illustration of domain organization and subunit-specific features of MCM2-7 subunits, with comparison to the archaeal MCM (SS, *Sulfolobus solfataricus*). Numbered regions correspond to numbered extensions and insertions highlighted in (**d–i**). "-" symbols denote corresponding regions with reliable densities to trace the main-chain direction, but not sufficient for atomic modeling. "--" symbols denote sequences with highly disordered densities. (**b, c**) A protomer of the crystal structure of a chimeric archaeal MCM hexamer structure (PDB code 4R7Y) was used as the template for modeling with its subdomains divided and colored. The archaeal MCM was aligned globally (**b**) or domain-based flexibly fitted (**c**) to the atomic model of Mcm2. (**d–i**) Side-by-side structural comparison of MCM2-7 proteins, with Mcm3-7 globally aligned to the atomic model of Mcm2. The well-resolved insertions and extensions of each MCM subunit (**d–i**) are numbered and colored in *red* (Reproduced from Li et al. 2015)

In contrast, in archaebacteria, a single Mcm protein forms a homohexameric ring that displays robust helicase activity (Kelman et al. 1999; Chong et al. 2000). An alignment of the Mcm2, 3, 4, 5, 6, 7 amino acid sequence with their archaeal homologue shows that the CTD catalytic core and NTD core are highly conserved (Fig. 9.1a). Each of the Mcm2-7 subunit has its own characteristic CTE, NTI, and NTE that suggest subunit-specific functions.

The assembly and activation of Mcm2-7 helicases at replication origins are strictly regulated during each cell division cycle, ensuring replication initiation to occur at each origin no more than once (Diffley et al. 1994; Siddiqui et al. 2013). Several laboratories have been able to reconstitute the DNA replication system using purified yeast proteins (Georgescu et al. 2015; Yeeles et al. 2015). These laboratories have taken the approach to look at in vitro assembled replication complexes by negative staining EM and cryo-EM (Remus et al. 2009; Costa et al. 2011, 2014; Sun et al. 2013, 2014, 2015; Yuan et al. 2016; Abid Ali et al. 2016). An alternative approach is

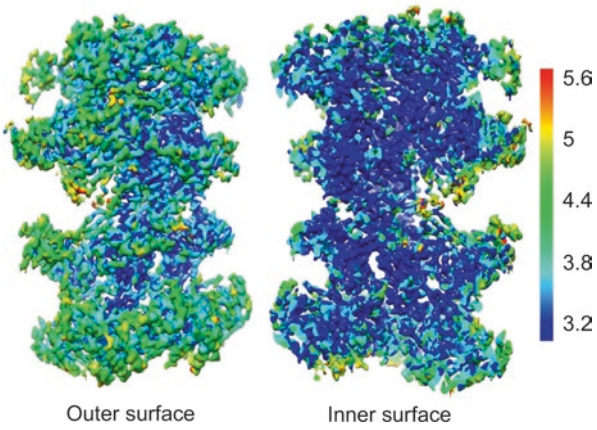


**Fig. 9.2** The density map of the MCM2-7 double hexamer (sharpened) is shown in two views for the outer and inner surfaces. The map is color-coded to indicate the range of the local resolution

to purify endogenously assembled DNA replication complexes that may have a better chance of preserving fidelity but may compromise on the yield of such preparations.

In yeast, the soluble form of the Mcm2-7 complex is a single hexamer, and the chromatin-bound form of the complex is a double hexamer. Tye and collaborators purified the soluble single hexamer (SH) from an overexpression strain and the double hexamer (DH) from native chromatin and examined their structures by cryo-EM (Li et al. 2015). After much effort, they were unable to get a respectable 3-D structure of the SH because of its flexibility and instability. In contrast, the double hexamer was clearly much more stable. After only two sessions of data collection, a refined 3-D structure of the DH was obtained with a resolution of about 4.4 Å for the more flexible exterior and a better than 3.2 Å for the interior core (Fig. 9.2). The high quality of this EM density map allowed the assignment of each of the MCM subunits unambiguously without the help of conventional tagging strategy (Figs. 9.1d–i and 9.3c–e). The crystal structure of the conserved core region of the chimera of *Sulfolobus solfataricus* NTD and *Pyrococcus furiosus* CTD (Fig. 9.1b) (Miller et al. 2014) was used for the atomic modeling. Rigid body domains of the chimera were divided into four segments (NTD-A, OB-fold, ZF, and CTD) and fitted onto the electron density map with manual adjustments according to predicted secondary structures (Fig. 9.1b–i). For regions without a known template, the model relied on predicted secondary structure and tracing of the main chain based solely on densities. As a result, an atomic model of the DH for ~80% of its sequence, including most of the subunit-specific extensions and insertions, was built.

## 9.2 Asymmetry of the Double Hexamer

The double hexamer is a rigid, head-to-head double ring structure that is slightly tilted, twisted, and offset with respect to each other, similar to that described previously for in vitro purified DH (Sun et al. 2014). The relative positions of the subunits

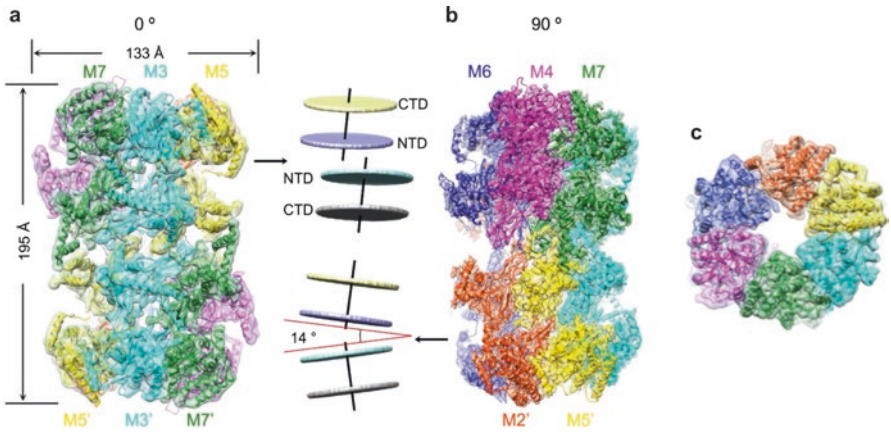


**Fig. 9.3** Overall structure of the MCM2-7 double hexamer. (**a–c**) Side (**a, b**) and *top* (**c**) views of the cryo-EM map of the MCM2-7 DH purified from native chromatin (Reproduced from Li et al. 2015). The map is superimposed with the atomic model. Unsharpened map (**a**) is displayed from the twofold axis, and sharpened map (**b**) is displayed with indicated rotations relative to **a** along the cylinder axis. The tilted and twisted arrangements of the two single hexamers are illustrated in the side panels of (**a**, **b**)

were also in the precise predicted order determined by low-resolution cryo-EM except that the assignment of the CTD relative to the NTD was different. In the atomic model, the CTD is almost vertically aligned with respect to the NTD (Fig. 9.3) rather than the sharp anticlockwise displacement in the earlier model. The top view from the CTD shows a compact closed ring with an open channel wide enough for dsDNA to pass through without the obstructions of the CTE reported in Sun et al. (2014). These observed differences could result from in vitro versus in vivo assembly.

The tilted arrangement of the two SHs forms a 14° wedge in between (Fig. 9.3). The Mcm2/Mcm6/Mcm4 subunits from both SHs are all positioned at the thick edge. Although the very long NTEs from Mcm2, Mcm4, and Mcm6 (Fig. 9.1a) appear too disordered to produce observable electron density, their bulkiness is believed to be the cause of the tilted conformation of the DH. The localization of these three subunits at the thick edge of the DH wedged site allows an increased opening between the two hexamers and thus a solvent exposure of their NTEs. Notably, they are side by side on one SH and head-to-head on the DH (Fig. 9.3), and hence, a large surface area on top of six NTEs from two hexamers virtually forms a docking platform for regulatory kinases such as DDK during helicase activation. Consistent with this notion, the NTEs of Mcm2 (Lei et al. 1997), Mcm4, and Mcm6 (Sheu and Stillman 2006; Randell et al. 2010) are known substrates of DDK, and the DH, but not the SH, is the preferred substrate for DDK (Sun et al. 2014). In vitro assembly assays indicate that phosphorylated DH supports initial recruitment of Sld3 and Cdc45 (Deegan et al. 2016), followed by GINS and further phosphorylation by CDK, to form a functional CMG helicase for DNA unwinding (Yeeles et al. 2015; Heller et al. 2011). Low-resolution EM studies showed that DDK phosphorylation neither triggers the

uncoupling of the DH nor causes conformational change in its overall structure (On et al. 2014; Sun et al. 2014). However, subtle alterations in DH structure induced by DDK may be critical for the transformation of the inactive DH into the active CMG helicases via Mcm2/Mcm5 gate opening and/or DH uncoupling. High-resolution structure determination of the phosphorylated DH using advanced cryo-EM analysis may provide insight into this intricate pivotal mechanism.

## 9.3 Tight Junction at the NTDs

Archaeal homohexameric MCM complex can form DH in solution directly through head-to-head interactions between zinc finger motifs from opposite hexamers (Chong et al. 2000; Brewster et al. 2008). In contrast, the eukaryotic heterohexameric Mcm2-7 can only be assembled into a dimer of hexamers on duplex origin DNA (Remus et al. 2009; Evrin et al. 2009). Furthermore, this assembly process is tightly controlled in a stepwise manner. The Mcm2-7 hexamer is recruited to the ORC-Cdc6-binding site during the G1 phase one at a time (Ticau et al. 2015; Sun et al. 2014). Once assembled, the Mcm2-7 hexamers form a topologically stable, inactive head-to-head double hexamer that encircles duplex origin DNA (Remus et al. 2009). The assembly of the double hexamer is an energy-consuming process requiring the hydrolysis of ATP by both ORC-Cdc6 and Mcm2-7 during the loading of the first and the second hexamer (Coster et al. 2014; Kang et al. 2014). Similar to archaeal DH formation, interactions among ZF motifs from Mcm2-7 subunits contribute to the stabilization of DH. However, unlike the simple head-on interactions between ZFs from two archaeal SHs, the inter-ZF interactions of eukaryotic Mcm2-7 appear to be more versatile, forming additional interactions with NTEs and β turns of the opposing hexamer in some cases (Fig. 9.4). This elaborate intertwining and extensions of NTEs into the opposite hexamers suggest that formation of the head-to-head tight junction is an ATP-consuming process that requires partial unfolding of the NTDs before forming the rigid interlocked structure.

Due to the tilted and twisted arrangement of the two SHs, the orientations of ZFs are completely different at two sides of the wedged interface of the DH: vertical at the thick Mcm2/Mcm6/Mcm4 side and horizontal at the thin Mcm5/3/7 side. This unique arrangement ensures intimate contacts among ZFs. In addition, according to the buried surface calculated at the hexamer interface, NTDs, NTEs, and NTIs from Mcm5/3/7 play significant roles in contributing to a stable DH (Fig. 9.4), which is even greater than ZF interactions. The involvement of NTEs of Mcm2/Mcm6/Mcm4 in stabilizing DH remains unknown because of their highly disordered feature in the structure. More importantly, although N-terminal sequences of each MCM subunits are less conserved in higher eukaryotes, most of the NTDs, NTEs, and NTIs (mainly NTI of M7, NTE of M5, β-turn of M6) involved in inter-hexamer interactions are conserved, suggesting the inter-subunit interactions are most likely maintained in metazoans as well.

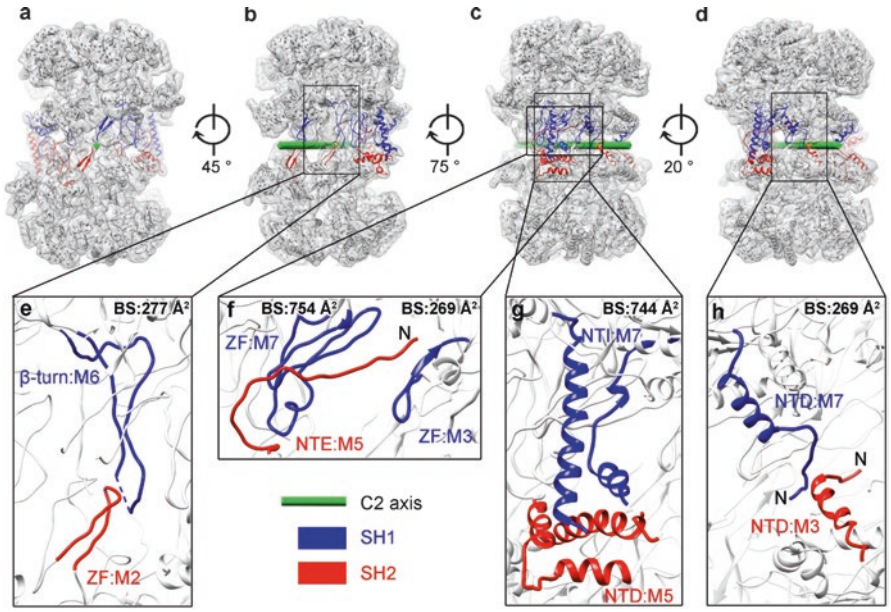


**Fig. 9.4** Stabilization of MCM2-7 double hexamer contributed by NTEs and NTIs. (**a–d**) Side views of the MCM2-7 DH, with indicated rotations around the cylinder axis. Atomic structure is superimposed with the unsharpened map. The sequence elements involved in inter-hexamer interactions are highlighted. (**e–h**) Zoomed-in views of the boxed regions from (**b–d**). Buried areas (Å$^2$) of these interfaces in (**e–h**) are labeled. BS, buried surface (Reproduced from Li et al. 2015)

The tight coupling of the double hexamer at the NTD raises the question of how the two hexamers uncouple upon helicase activation. In vitro reconstitution and genetic analysis show that separation of MCM DH in S phase involves the engagement of other initiation factors such as Cdc45, GINS, and Mcm10 to unravel the entanglement between the NTDs and NTIs of M5, M3, and M7. Consistent with this idea, recent CMG structure showed that two α-helices of Psf2 interact with NTD of Mcm5 (Abid Ali et al. 2016; Yuan et al. 2016) which is contacted by the NTE of the opposite Mcm7 in the DH. Previous study also showed that Cdc45 can interact with Mcm5-N (N-terminus) and Mcm2-N and Psf2 with Mcm5-N and Psf3 with Mcm3-N(Costa et al. 2011; Yuan et al. 2016; Abid Ali et al. 2016); in addition, Mcm10 has been shown to interact physically with MCM6 (van Deursen et al. 2012) and MCM7 (Homesley et al. 2000). Presumably these factors work cooperatively to uncouple the MCM DH. The recruitment of all these factors requires the action of DDK and CDKs during S phase to ensure the sequential coupling and uncoupling of the MCM hexamers at separate stages of each cell division cycle (Heller et al. 2011; Yeeles et al. 2015). Again, the role of DDK phosphorylation in triggering the DH uncoupling is still unclear. A better understanding of this process will rely on the determination of the atomic structures of the intermediate replication complexes during helicase activation and the delineation of conformational changes induced by the uncoupling factors.

## 9.4 A Unique Side Channel at the Mcm2-Mcm6 Interface

The archaeal MCM helicase is made up of six identical subunits, which form a ring with six identical interfaces (Bochman and Schwacha 2009). The atomic model of the archaeal MCM helicase built from the crystal structure of the near-full-length protomer of the ssoMCM by applying a sixfold symmetry showed six side channels at the neck region of the ring (Brewster et al. 2008). The size of the side channels large enough for ssDNA to pass through raised speculations that dsDNA may pass through the central channel at the CTD and unwound by extrusion of one strand laterally at the side channel. This configuration would favor the strand extrusion model for DNA unwinding. Throughout the years, this model has been disfavored based on in vitro biochemical studies using bulky chemical crosslinks on the leading and lagging strands (Fu et al. 2011). The CMG helicase efficiently bypasses a roadblock embedded on the lagging strand more efficiently than that embedded on the leading strand, arguing against the translocation of CMG helicase on dsDNA. Most recently, the structure of the apo CMG helicase has been determined by cryo-EM (Yuan et al. 2016). The WH domain of Mcm5 restricts the main channel of the CMG helicase to the extent that only ssDNA can be accommodated. The positions of the ssDNA associated with the CMG are consistent with the steric exclusion model in which duplex DNA is unwound by the translocation of the CMG on ssDNA.

The observation that the yeast MCM double hexamer contains a central channel that can accommodate dsDNA and a side channel between Mcm2 and Mcm6 wide enough to accommodate ssDNA (Fig. 9.5a) encourages a revisit of the strand extrusion model. Despite all the evidence against this model, it is worthwhile to reevaluate the evidence. First, the in vitro roadblock bypass experiment showed only a bias in the efficiency of bypass rather than a clear-cut bypass on the lagging strand but not the leading strand. Also, the latching of the Mcm2-Mcm5 gate by GINS and Cdc45 in the CMG helicase structure suggests that the gate is only loosely fastened and may not be closed shut or irreversibly latched on during translocation (Costa et al. 2011, 2014). As for the threading of the artificial Y-shaped DNA substrate through the in vitro assembled CMG helicase, it may not correctly represent how DNA is threaded in a CMG helicase assembled de novo at replication origins. Most interestingly, a recent model for the architecture of the in vitro assembled core replisome suggests that DNA polymerase epsilon is placed ahead of the helicase on the leading strand (Sun et al. 2015). Although the path of the DNA could not be traced, the position of Polε would require a sharp U-turn in the threading of the leading strand from the NTD of the CMG helicase to the DNA polymerase if the steric exclusion model is correct (Fig. 9.5b). Indeed, without the evidence of an atomic structure of an endogenous replisome, one can easily come up with radical models that are more compatible with the positioning of Polε by using the unique side channel (Fig. 9.5c). In this model, the side channel would act like a plowshare to force apart the two strands as the helicase plows ahead. Whether the CMG helicase unwinds DNA by strand extrusion, steric exclusion or some other mechanisms remains to be verified by cryo-EM structures of CMG helicase assembled from replication origins.

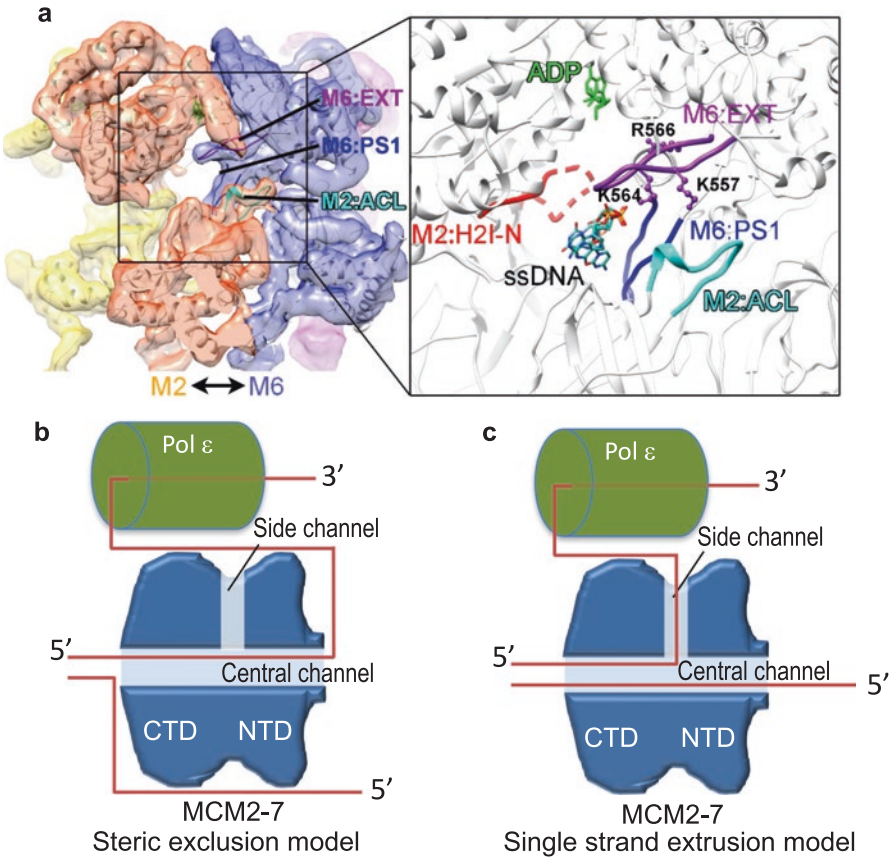


**Fig. 9.5** A unique side channel between Mcm2 and Mcm6. **a** Outer surface representation of the Mcm2/Mcm6 interface. The boxed region is shown in a zoomed-in view (*right*) with individual components (H2I-N, EXT, PS1, and ACL) colored individually. A short piece of ssDNA is modeled in the structure to indicate the size of this side channel large enough to act as a pore for strand extrusion from the central channel during DNA unwinding along with basic residues (Arg566, Lys557, and Lys564) of the EXT hairpin from MCM6. The H2I-N is partially disordered. (**b, c**) Threading paths proposed for leading- and lagging-strand DNA based on the architecture of the replisome (Sun et al. 2015) in the steric exclusion model (**b**) versus the strand extrusion model where the 3′-5′ strand extrudes through Mcm2/Mcm6 side channel (**c**)

## 9.5 ATPase Pockets in the Double Hexamer

Mcm2-7 forms the core of the replication-licensing complex that assembles the replication initiation complex at replication origins and the core of the replicative helicase that unwinds dsDNA at replication forks (Bochman and Schwacha 2009). In both capacities, ATP hydrolysis by the Mcm2-7 catalytic core is required to fuel the energy-consuming functions that it performs.

Heterohexameric Mcm2-7 belongs to the AAA+ family of ATPases, whose ATPase active sites are formed at inter-subunit interfaces. One subunit contributes the Walker A and B motifs, while the other contributes the arginine finger. Thus, each Mcm subunit participates in two ATPase active sites, resulting in six distinct ATPase pockets. At each step of the assembly, starting from the single hexamers to the pre-RC and then from the pre-RC to the pre-IC, a different set of ATPases is called into action. Exhaustive mutant and biochemical analysis combined with in vitro reconstitution studies was able to identify which ATPase pocket is required for activity at each assembly step (Bochman and Schwacha 2008; Coster et al. 2014; Kang et al. 2014). Figure 9.6g (modified from Kang et al. 2014) summarizes the results of these elegant and intricate studies that show which ATPase pocket(s) is required at each stage. During this process, the 2:6 ATPase appears to play a role in the loading of the first SH. The 4:7, 6:4, 5:2, and 3:5 ATPases are required for the loading and head-to-head fusion of the second SH to form the DH. Up to this point, the only ATPase pocket that is not required for activity is the 7:3 pocket as if all of the energy consumed up to this point is to assemble this high-energy structure that is stable throughout G1 phase. Interestingly, in the high-resolution structure of the inactive DH, only the 7:3 ATPase pocket shows extra density indicating the possibility of ATP bound (Fig. 9.6a–f) (Li et al. 2015). It would be interesting to find out what exactly happens in the steps that follow. For example, is ATP hydrolysis by the 7:3 pocket the first responder of DDK or CDK phosphorylation to initiate DNA melting at the G1-S phase transition, which in turn recruits Sld3 and Cdc45? According to the in vitro reconstitution study, all of the ATPases except 6:4 act immediately after the CDK action to recruit GINS and RPA (Kang et al. 2014). What seems remarkable is how the asymmetry of the MCM ATPase pockets directs the sequential assembly of the replication initiation complex to form the helicase engine of the replisome. However, to unravel which ATPase is called into action at which specific step would require the high-resolution structure of each staged assembly that shows the nucleotide-binding state of each ATPase pocket as achieved for the double hexamer (Li et al. 2015). The double hexamer is an unusually stable structure that can withstand washing by 0.5 M salt (Bowers et al. 2004; Remus et al. 2009; Evrin et al. 2009) and therefore more amenable to structural analysis. It may be difficult to find stable conditions for each of the intermediate assembly structures for high-resolution structural analysis.

As a footnote to the importance of high-resolution structures, a previous study using negative staining EM suggested that the inactivation of the ATPase pocket in the DH is due to the uncoupling of the ATP hydrolysis motifs by the staggering of the CTD and NTD of each subunit (Sun et al. 2014). It should be pointed out that the model presented was based on the misassignment of the CTDs of the subunits that led the authors to these conclusions.

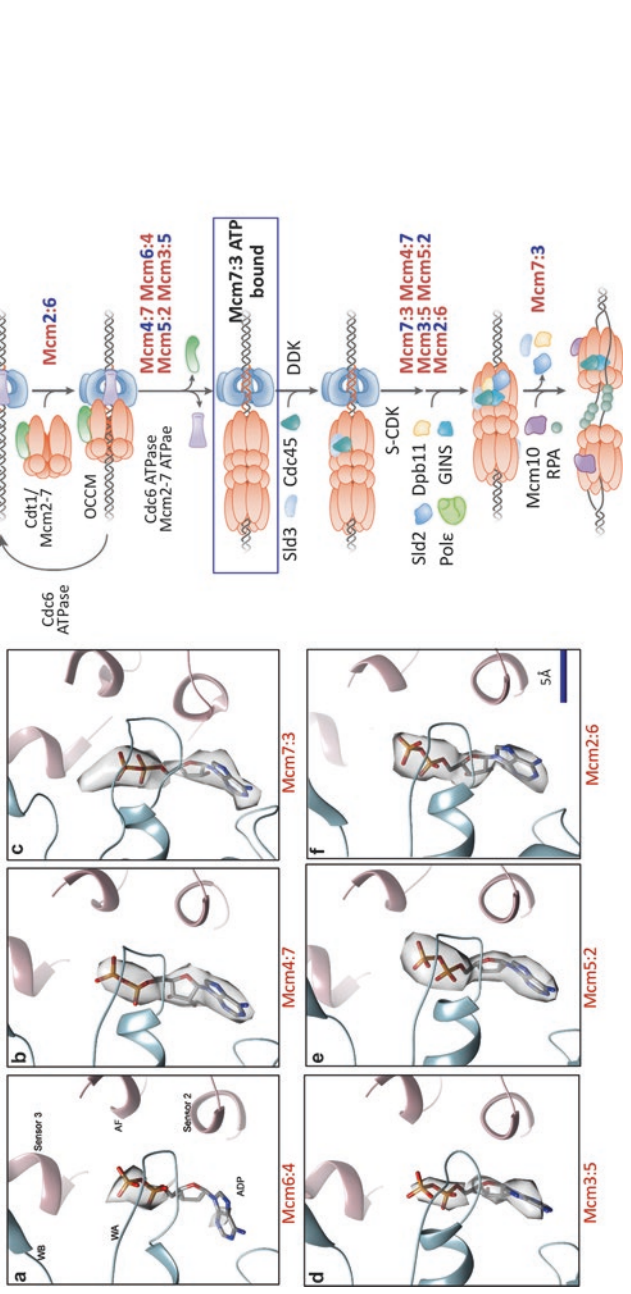


**Fig. 9.6** MCM2-7 ATPase pockets involved in different stages of helicase loading and activation. (**a**–**f**) Zoomed-in views of nucleotide occupancy at the six ATPase centers of MCM2-7 double hexamer. The conserved ATPase elements of the active centers are labeled as in (**a**). Segmented nucleotide densities at a contour level of 5.5σ were superimposed (transparent gray). Nucleotides were modeled using ADP (Reproduced from Li et al. 2015). (**g**) Kang et al. (2014) purified a series of Cdt1-MCM2-7 heptamers to examine the role of each MCM2-7 ATPase active site during DNA replication initiation. Each complex contains a single mutation in either Walker A (K to A) or arginine finger (R to A) motif of a particular Mcm subunit. The identified ATPase active sites are summarized in this cartoon. McmX:X in which number highlighted in blue represents the subunit location of that single mutation: left, Walker A motif, and right, arginine finger motif (Adapted from Kang et al. (2014) Fig. 7 by permission from Elsevier publisher Inc. copyright 2014). The unique ATP-bound state of the 7:3 ATPase in the DH atomic model is highlighted by a *boxed* frame

## 9.6 Central Channel and Model of Initial Origin Melting

Like many hexameric AAA+ machineries (Brewster et al. 2010; Enemark and Joshua-Tor 2006), Mcm2-7 complex also has a central-pored chamber decorated by multiple layers of hairpin loops formed by the H2Is and β-turn motifs from the OB domains. These motifs are placed in axially staggered positions, resembling archaeal MCM structures in contacting with ssDNA and dsDNA (Miller et al. 2014). The atomic model for the DH suggests that MCM2-7 helicase employs a similar mechanism in controlling the axial displacement of these loops to facilitate DNA translocation and unwinding (Fig. 9.7a, b).

The most striking feature of the MCM2-7 DH structure is that the central channel, formed by these two staggered MCM rings, has four constriction points in the channel and a kink at the interface of the two rings (Fig. 9.7a, b). Modeling with dsDNA shows that these constriction points interact with the major and minor grooves of dsDNA consistent with observations that the DH complexes stably associate with linear dsDNA under physiological condition and only becomes mobile on dsDNA in high-salt buffer (Remus et al. 2009; Kumar and Remus 2016). These fine structures of the MCM2-7 DH provide insight into its function in DNA melting (Fig. 9.8). First, the kinked interface of the two rings would deform dsDNA (Fig. 9.7e), which could serve as a nucleation center for DNA destabilization. It has been shown that DNA bending that distorts hydrogen bonds between base pairs causes local DNA melting and facilitates DNA unwinding during transcription (Tang and Patel 2006). Second, the tight grip of duplex DNA by the two restriction points in the two SH chambers would further deform DNA at the nucleation point if rotated against each other. Third, possible rotations between ring structures formed by subdomains of each hexamer would lower the activation energy for DNA destabilization even fur-

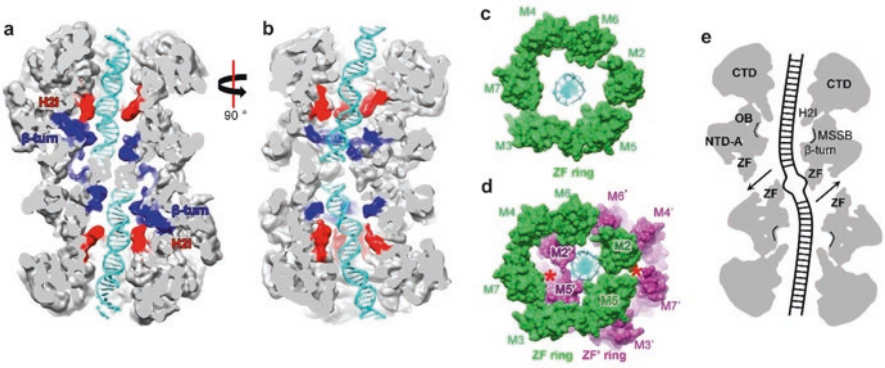


**Fig. 9.7** Central channel and its implication in origin melting. (**a, b**) Cutaway views of the density map (unsharpened) with two dsDNA fragments fitted in the central channel. Surface representation of the atomic models of ZFs from one single hexamer (**c**) and ZFs from both single hexamers (**d**). (**e**) A simplified diagram of the cryo-EM map of the MCM2-7 DH. The structural features that may involve in the initial melting step are labeled as indicated (Reproduced from Li et al. 2015)

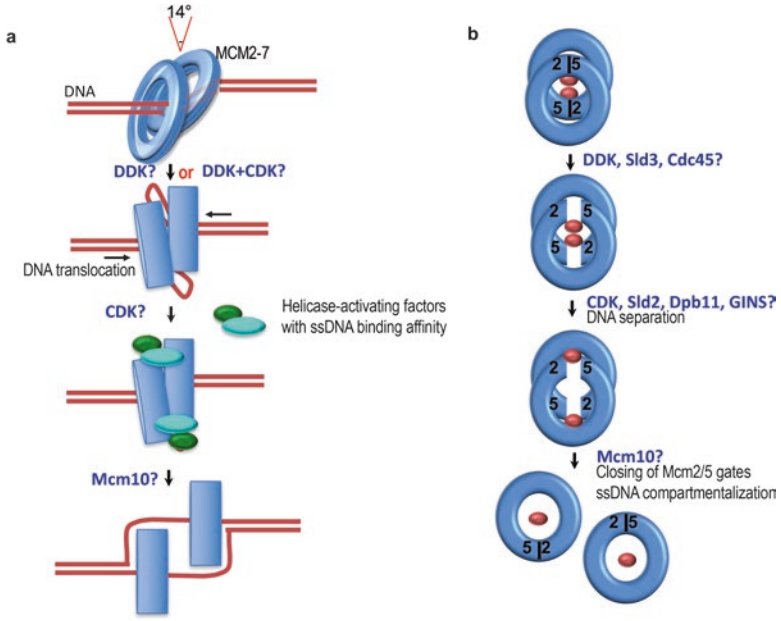


**Fig. 9.8** A model for gate opening and initial DNA melting during helicase activation. Although many factors have been shown to be required for this process, it is unclear when and how they are involved in the actual gate opening and DNA melting events. See text for details

ther. Recent single-molecule analysis of *Dm*CMG helicase showed that the CTD and NTD of the DmCMG helicase could rotate in opposite directions, clockwise and anticlockwise, during its engagement with DNA (Abid Ali et al. 2016). These special features of the atomic model of the DH suggest that allosteric conformational changes following the activation of the MCM2-7 complex by cell cycle-regulated kinases might bring about DNA destabilization for initial DNA melting.

Another notable feature about the DH structure is that the overlapping of two central channels at the interface forms a narrowed main channel and two minor or exit channels. Interestingly, the main channel is formed mostly by two ZF pairs from the gate-forming subunits Mcm2 and Mcm5 (Fig. 9.7c, d). The size of the main channel is just wide enough to fit dsDNA. However, once the Mcm2/Mcm5 gates are opened at the interface region, the three channels would join together to form a space large enough for strand separation (Fig. 9.8b). Strand separation is likely assisted by the MSSB motifs conveniently located on opposite sides of the enlarged chamber (Fig. 9.7e) (Miller et al. 2014). During this DNA melting step, if the two MCM hexamers fused at the NTDs translocate along dsDNA in opposite directions, they will effectively pump in dsDNA from both ends. The already deformed DNA at the kink region would be subsequently separated (Fig. 9.8b). The melted single-DNA strands that become accessible to the outside by looping out from the exit channels could be captured by helicase-activating factors to secure their separation (Fig. 9.8a). Several of the helicase-activating factors, such as Sld2,

Sld3, Cdc45, and Mcm10, have been shown to have ssDNA-binding property (Bruck and Kaplan 2011, 2013; Costa et al. 2014; Homesley et al. 2000; Eisenberg et al. 2009). At the final step, which is yet to be understood, the DH is uncoupled, and the lagging-strand DNA is excluded from each of the MCM hexameric ring (Yardimci et al. 2010; Fu et al. 2011).

## 9.7 Looking Ahead

Despite the many hurdles that have held back fine-detailed structural studies of the DNA replication machinery, there is reason for optimism for unraveling this last macromolecule-synthesizing machine in the near future. Determining the near-atomic resolution structure of the inactive MCM double hexamer is a crucial start of this endeavor. The Mcm2-7 complex is a major player throughout the entire process of DNA replication, from replication licensing to initial melting of origin DNA, progression of bidirectional forks, and finally replication termination (Bell and Labib 2016). In each step, the MCM complex appears to play a somewhat different role first as an inert assembly platform, and then a duplex DNA pump, a translocator on ssDNA, and finally, a disbander of the replisome. The recent advances in cryo-EM promise that the determination of many of these MCM-associated structures is at hand. Combined with single-molecule studies and the in vitro reconstitution of the stepwise-assembled intermediates of the replisome, the next decade will witness the visualization of the workings of the replisome that is so vividly portrayed in textbooks from elegant biochemical and genetic studies of decades before.

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# Chapter 10
# Architecture of the *Saccharomyces cerevisiae* Replisome

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**Abstract** Eukaryotic replication proteins are highly conserved, and thus study of *Saccharomyces cerevisiae* replication can inform about this central process in higher eukaryotes including humans. The *S. cerevisiae* replisome is a large and dynamic assembly comprised of ~50 proteins. The core of the replisome is composed of 31 different proteins including the 11-subunit CMG helicase; RFC clamp loader pentamer; PCNA clamp; the heteroligomeric DNA polymerases ε, δ, and α-primase; and the RPA heterotrimeric single strand binding protein. Many additional protein factors either travel with or transiently associate with these replisome proteins at particular times during replication. In this chapter, we summarize several recent structural studies on the *S. cerevisiae* replisome and its subassemblies using single particle electron microscopy and X-ray crystallography. These recent structural studies have outlined the overall architecture of a core replisome subassembly and shed new light on the mechanism of eukaryotic replication.

**Keywords** Eukaryotic DNA replication • Replisome • Replicative helicase • Cryo-EM • Mcm • CMG • DNA polymerase • Structural biology

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## 10.1 Introduction

The DNA genome is duplicated in semiconservative fashion wherein each strand serves as the template for synthesis of its complementary strand (Meselson and Stahl 1958; Watson and Crick 1953a, b). This process may seem simple, but nothing could be further from the truth; it requires >50 different proteins in eukaryotes, and many of their individual functions remain unknown, much less how they work together to accomplish high-fidelity genome duplication (Costa et al. 2013; Li and Araki 2013; MacNeill 2012). Unlike protein synthesis and mRNA synthesis that are well conserved across the three domains of life, DNA synthesis is not well conserved. Many key replication proteins, such as the replicative helicase, DNA polymerases, primase, and single strand binding protein, are evolutionarily unrelated between bacteria and archaea/eukarya (Forterre et al. 2004; Leipe et al. 1999). Thus one cannot rely on the wealth of bacterial studies to understand eukaryotic replication, and detailed studies of eukaryotic replication mechanisms are warranted. Fortunately, the replication machinery within eukaryotes is highly conserved, and thus the relatively simple *Saccharomyces cerevisiae* can be used as a reliable model for replication in higher eukaryotes (Leman and Noguchi 2013).

The "core replisome" encompasses proteins that are necessary to propagate the replication fork (Zhang and O'Donnell 2016). The eukaryotic replisome core is composed of CMG helicase, the leading strand DNA polymerase (Pol) ε, lagging strand Pol δ, Pol α-primase, along with the RFC clamp loader, PCNA clamp, and RPA single strand (ss) DNA-binding protein. Many other components, such as Ctf4, Mcm10, Topo I, the checkpoint response factors Mrc1, Tof1, Csm3, and the FACT nucleosome mobility factor, either travel with the replisome or hop on and off at different points during replication (Bell and Labib 2016).

The eukaryotic helicase is an 11-protein machine composed of *C*dc45, the *M*cm2-7 heterohexamer, and the four-protein *G*INS complex (Psf1-3, and Sld5) referred to as CMG (Bochman et al. 2008; Ilves et al. 2010; Moyer et al. 2006). Eukaryotic CMG helicase is assembled through a series of replication initiation events (reviewed in (Costa et al. 2013; O'Donnell et al. 2013; Tanaka and Araki 2013; Tognetti et al. 2015)). Briefly, the origin recognition complex (ORC) along with Cdc6 loads onto replication origins in the G1 phase of the cell cycle (Bell and Stillman 1992) and then recruits the helicase core Mcm2-7 heterohexamer with the help of Cdt1 (Cocker et al. 1996; Liang et al. 1995; Mimura et al. 2004; Nishitani et al. 2000; Santocanale and Diffley 1996; Sun et al. 2013; Tanaka and Diffley 2002). Subsequently, another Mcm2-7 is recruited onto double-stranded (ds) DNA forming a double hexamer of Mcm2-7 that is inactive (Duzdevich et al. 2015; Evrin et al. 2009; Remus et al. 2009; Sun et al. 2014; Ticau et al. 2015). Finally, at the G1-to-S transition, the double hexamers are converted to two active helicases comprised of Cdc45-Mcm2-7-GINS (CMG) that each encircle opposite strands of single-stranded (ss) DNA to carry out bidirectional separation of the DNA duplex (Bell and Botchan 2013; Duzdevich et al. 2015).

Eukaryotic replication requires at least three DNA polymerases to propagate fork movement (Burgers 2009). These include Pol α-primase, Pol ε, and Pol δ. Pols α, ε, and δ are all members of the B family of DNA polymerases (Steitz 1999). Pol α initiates DNA synthesis on both the leading and lagging strands by synthesizing a RNA/DNA hybrid primer (Conaway and Lehman 1982). Numerous genetic, cell biology, and biochemical studies concur that Pol ε and Pol δ extend primers on the leading and lagging strands, respectively (Burgers et al. 2016; Clausen et al. 2015; Kunkel and Burgers 2008; Miyabe et al. 2011; Nick McElhinny et al. 2008; Pursell et al. 2007), although one report suggests that Pol δ functions on both strands leaving some uncertainty for future studies to resolve (Johnson et al. 2015).

The antiparallel structure of dsDNA, combined with unidirectional synthesis by DNA polymerases, results in continuous synthesis on the leading strand that travels in the direction of DNA unwinding and discontinuous synthesis on the lagging strand that is extended as multiple Okazaki fragments in the direction opposite DNA unwinding. A preformed primed site is required for DNA polymerase function, and both strands are primed by Pol α-primase. However, Pol α-primase is needed repeatedly on the lagging strand, once for each 100–200 bp Okazaki fragment. Pol α-primase is a four-subunit enzyme; the largest subunit is a DNA polymerase that lacks a proofreading 3′–5′ exonuclease, the second largest is the B subunit (unknown function), and the two small subunits, PriL and PriS, contain the priming activity (Banks et al. 1979; Conaway and Lehman 1982; Kaguni et al. 1983a, b).

There have been numerous important advances on the structure of components of the eukaryotic replisome in the last decade. In this chapter, we focus mainly on structures that have been solved during the past 3 years, specifically of proteins directly involved in moving replication forks. We first review the cryo-EM structure of the CMG helicase and compare the structures of CMG components GINS and Cdc45 of yeast and human. We then review the relative positions of Pol ε, Ctf4, and Pol α in the context of the CMG helicase and the possible replisome architecture at the replication fork. We end our chapter by providing a brief perspective on key missing information and what to expect in the coming years.

## 10.2 The CMG Structure

The first structure of eukaryotic CMG was determined in the *Drosophila melanogaster* system by negative stain EM at low resolution (Costa et al. 2011). The structure revealed a Mcm2-7 ring braced on the side by Cdc45 and GINS that interacted mainly with Mcm2, Mcm3, and Mcm5, apparently forming a secondary pore. However, this secondary pore is essentially filled in by side chains in the higher resolution cryo-EM map of the *S. cerevisiae* CMG at a resolution of 3.7–4.8 Å (Fig. 10.1a–c) (Yuan et al. 2016). The secondary pore also disappears in a medium resolution cryo-EM map of *Drosophila* CMG at 7–10 Å resolution (Abid Ali et al. 2016). The Mcm2-7 core in the CMG forms a two-tiered ring structure: an N-terminal domain (NTD) tier composed of a helical subdomain, a Zn-binding

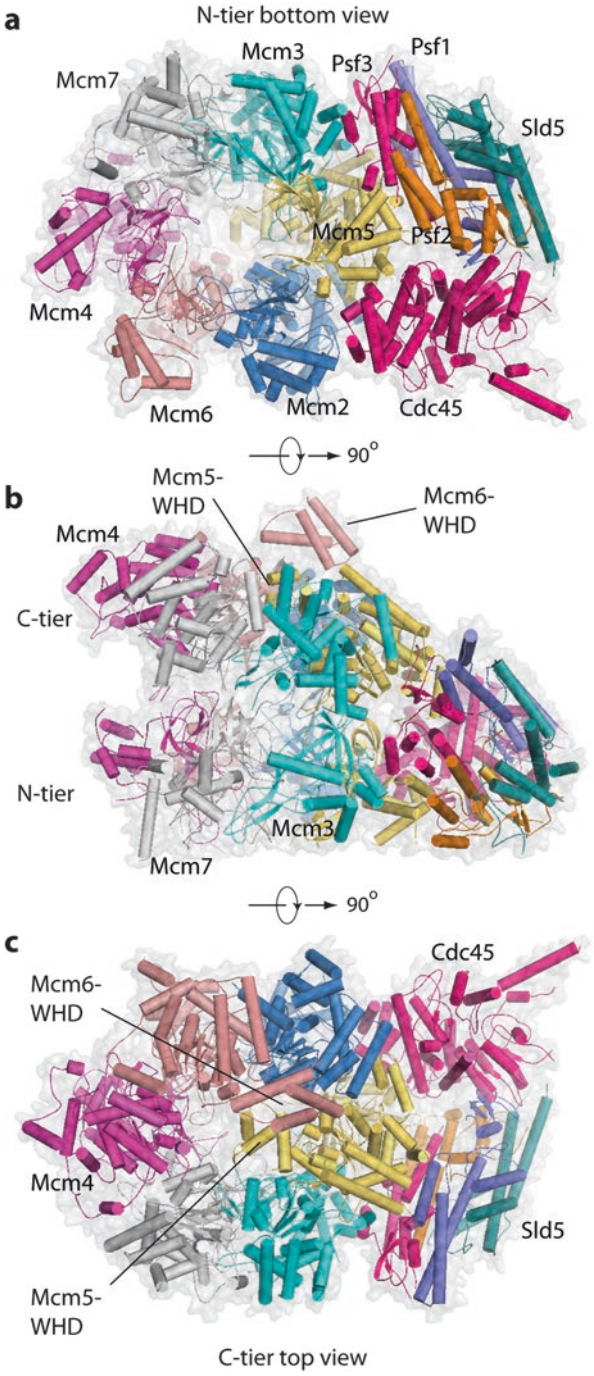


**Fig. 10.1** The CMG structure. The CMG structure is shown in cartoon view with each subunit a different color (PDB 3JC7). The surface representation of the structure is superimposed as a dim gray surface view. The structure is shown from the following angles: (**a**) top CTD ring view, (**b**) a side view, and (**c**) a bottom NTD view

motif, and an oligonucleotide/oligosaccharide-binding (OB) motif, while the C-terminal domain (CTD) tier contains the AAA+ motors. This domain architecture is similar to that of other hexameric helicases including the archaeal MCM hexamer and the inactive yeast Mcm2-7 in the double hexamer (Brewster et al. 2008; Enemark and Joshua-Tor 2006; Li et al. 2015; Miller et al. 2014; Singleton et al. 2000; Slaymaker and Chen 2012). Cdc45 and GINS mainly bind the NTD-tier ring of Mcm2-7, forming a rigid unit of Cdc45-GINS-Mcm2-7 NTD. More precisely, Cdc45 contacts only the NTDs of Mcm2 and Mcm5, and GINS contacts only the NTDs of Mcm3 and Mcm5 (GINS and Cdc45 also contact one another). It is the tight interaction between the NTD of Mcm5 and Cdc45-GINS that occludes the secondary pore.

In the medium resolution cryo-EM structures of the *Drosophila* CMG, density corresponding to six nucleotides of ssDNA was observed either inside the CTD-tier motor ring or inside the NTD-tier ring (Abid Ali et al. 2016). This observation is consistent with CMG acting as an ssDNA translocase. Interestingly, in an archaeal MCM hexamer, ssDNA was found to bind the NTD-tier ring as a circle perpendicular to the MCM channel axis and was proposed to be an intermediate in origin initiation (Froelich et al. 2014). How a CMG engages DNA at a replication fork remains an important issue in the field.

## 10.3 The Mcm2-7 Hexamer Undergoes Large Conformational Changes from the Inactive Double Hexamer to the Active CMG Helicase

Recent studies have provided two yeast Mcm2-7 hexamer structures: one determined in the context of the inactive Mcm2-7 double hexamer and the other in the context of active CMG as described above (Li et al. 2015; Yuan et al. 2016). This allows a comparison of the structures to gain insight into the mechanism by which the helicase is activated. To transition from the inactive Mcm2-7 double hexamer to the single Mcm2-7 in CMG requires large domain movements of up to 20 Å and rotations of up to 30° (Yuan et al. 2016). Movements in the CTD-tier motor domains are more extensive than the NTD-tier ring. As an example, we compare Mcm2-Mcm5 in the two structures (Fig. 10.2a–c). Notably, the interface between the CTDs of Mcm2 and Mcm5 is looser in the CMG structure than in the double hexamer. Activation of CMG requires it to encircle the leading strand and exclude the lagging strand. The looser Mcm2/5 interface at the C-tier ring may have allowed the extrusion of the lagging strand to the outside surface during activation of the CMG helicase. Another major change is the insertion of the Mcm5 C-terminal winged helix domain (WHD) into the interior channel in the active helicase, while the Mcm5 WHD of the inactive Mcm2-7 hexamer is visualized as weak density outside the central channel (Li et al. 2015; Yuan et al. 2016). The Mcm6 C-terminal WHD in the active and inactive Mcm2-7 was also reconfigured. Considering that the WHD is

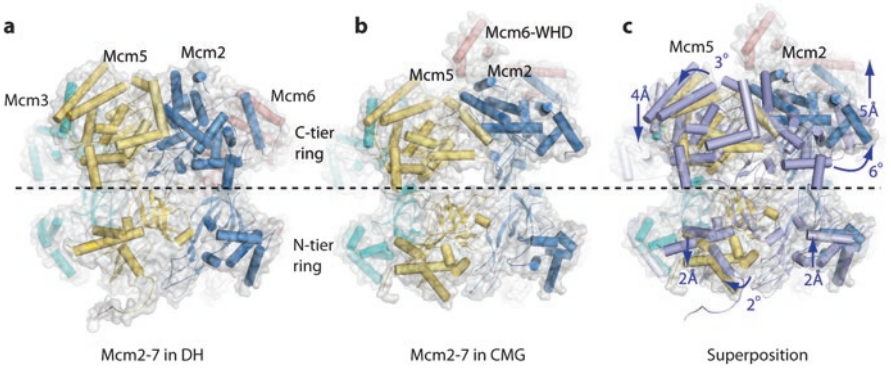


**Fig. 10.2** Conformation changes proceeding from the inactive Mcm2-7 at an origin to Mcm2-7 in the active CMG helicase. The figure focuses on a side view of the Mcm2 and Mcm5 subunits in the Mcm2-7 hexamer in which one Mcm2-7 hexamer has been extracted from the inactive double hexamer (PDB 3JA8) (left side) and the Mcm2-7 hexamer has been extracted from the active CMG structure (PDB 3JC7) (middle). The two Mcm2-7 structures are then superimposed (right side). Structures are in cartoon view wrapped in a dim gray surface view. Mcm5 is gold and Mcm2 is blue. The Mcm2/5 interface is nearly opened in Mcm2-7 within CMG

usually involved in DNA binding or interaction with other proteins, the observed movement of these WHDs may have important consequences for helicase activation and replisome function during fork progression.

## 10.4 GINS Is a Protein-Binding Hub of the Replisome but Does Not Contain a Central Channel for ssDNA

The yeast CMG structure reveals for the first time the complete structure of the GINS heterotetramer (Yuan et al. 2016). The GINS subunits are related in evolution and are composed of two domains each (A and B) (Chang et al. 2007; Choi et al. 2007; Kamada et al. 2007). The B domain of the Psf1 subunit was missing in all previously reported GINS structures (Chang et al. 2007; Choi et al. 2007; Kamada et al. 2007). The Psf1 B domain interacts with Cdc45 by forming an interfacial cross-molecule β-sheet (Yuan et al. 2016). Perhaps the Psf1 B domain is disordered in the absence of Cdc45, explaining why it was not resolved in the crystal structures of the GINS alone.

Earlier low-resolution EM observations as well as the crystal structure of the human GINS suggested a potential hole in the middle of GINS (Boskovic et al. 2007; Chang et al. 2007; Kubota et al. 2003). It was further suggested that ssDNA might thread through the central hole of the GINS. The structure of the full-length yeast GINS contains only a small hole of ~7 Å diameter, too small to thread ssDNA (Fig. 10.3) (Yuan et al. 2016). Importantly, although the GINS structures are very similar between human and yeast, structural features surrounding the central region

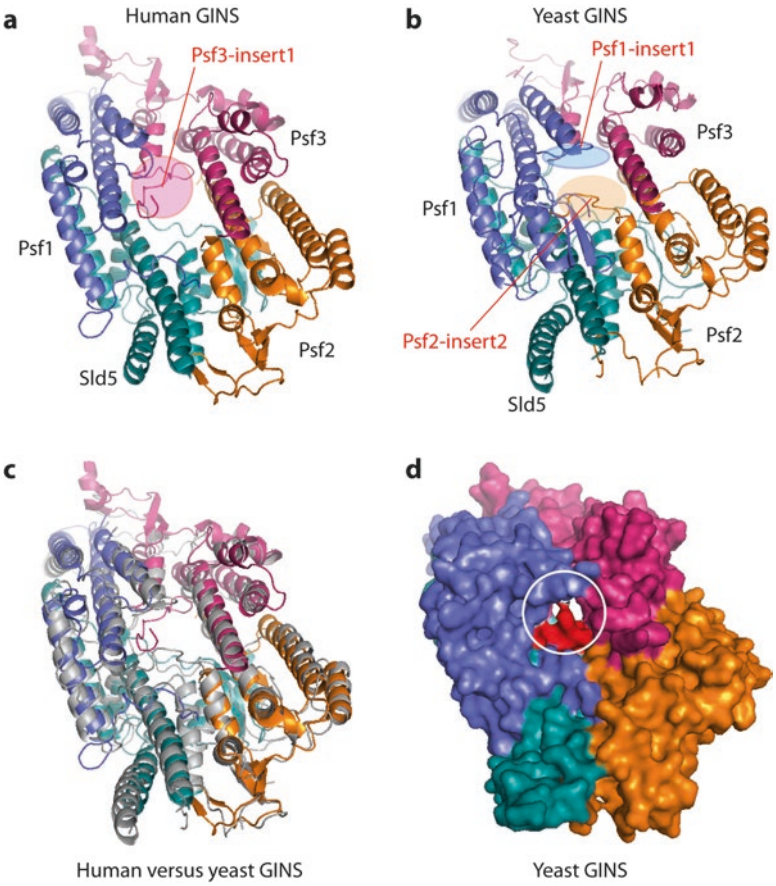


**Fig. 10.3** Comparison of the human and the yeast GINS structures. (**a**) Human GINS with each subunit shown in a different color (PDB 2Q9Q). An N-terminal insertion loop in Psf3 is highlighted by a semitransparent circle near the central pore region. (**b**) The yeast GINS structure, extracted from the CMG structure (PDB 3JC6), is shown in the same color scheme as in (**a**). (**c**) Superimposition of the human and the yeast GINS structures, revealing that the structural variation is focused in the central region. (**d**) Surface representation of the yeast GINS structure. The central pore is too small to thread ssDNA

vary (Chang et al. 2007; Choi et al. 2007; Kamada et al. 2007; Yuan et al. 2016). The yeast GINS has two insertions in Psf1 and Psf2 that are absent in human GINS, and the human GINS has a Psf3 insertion loop that is absent in the yeast GINS. The structural variation, together with the small size, argues against ssDNA threading through the central hole. Instead, the GINS functions mainly to scaffold the replisome by recruiting other proteins. The CMG structure appears to bear this out. Thus GINS interacts extensively with Mcm3/5 and Cdc45 (Yuan et al. 2016). The Psf1 B domain forms the main connection to Cdc45, and the Sld5 subunit binds the Ctf4 trimer scaffold protein that in turn binds to Pol α-primase (Simon et al. 2014). An

interaction between Psf1 and the B subunit of Pol ε has also been documented by biochemical studies (Gambus et al. 2009), and recently, the direct interaction between GINS and Pol ε was visualized by negative stain EM (Sun et al. 2015).

## 10.5 Cdc45 Has Two RecJ-Like Domains and a Protruding Helical Motif

Cdc45 has been predicted to have a RecJ-like fold (Krastanova et al. 2012; Onesti and MacNeill 2013; Sanchez-Pulido and Ponting 2011). The structure of yeast and human Cdc45 confirmed this prediction and further revealed that there are actually two RecJ-like α-/β-domains that are separated by a small helical inter-domain (ID) (Fig. 10.4a–c) (Simon et al. 2016; Yuan et al. 2016). Human and yeast Cdc45 are highly conserved at the structure level and superimpose with a root-mean-square deviation of <2 Å (Fig. 10.4c). Cdc45 has a protruding helical motif (PHM) that contains a highly negatively charged and disordered loop (D166 – R217 in yeast). The protrusion may interact and support the largely flexible N-terminal catalytic domain of Pol2 (see below), because a lysine at the tip of the PHM helix (K222) cross-links to the N-terminal catalytic domain of Pol2 (Yuan et al. 2016). The helical ID contacts and stabilizes the NTDs of Mcm2 and Mcm5 (Fig. 10.1a, c).

The fact that Cdc45 faces the neighboring Mcm2 and Mcm5 subunits in the CMG helicase is suggested to poise Cdc45 to capture DNA if the Mcm2/5 gate breaches open (Petojevic et al. 2015). Although Cdc45 does not have a nuclease activity, there is evidence that Cdc45 binds DNA weakly and nonspecifically (Krastanova et al. 2012; Simon et al. 2016; Bruck and Kaplan 2013). RecJ forms an O-like structure with a pore in the middle to serve as a ssDNA-binding groove, but unlike RecJ the yeast and human Cdc45 do not have a central pore. Therefore, Cdc45 does not contain an internal DNA-binding groove. It is possible that the exterior surface of Cdc45 may help to coordinate DNA in the context of CMG, but the nature of the DNA contact is currently unknown.

Meier-Gorlin syndrome (MGS) is a rare autosomal recessive disorder characterized by short stature (dwarfism) (Klingseisen and Jackson 2011). Previous work has found that mutations in genes involved in establishing the pre-replication complex, such as ORC1, ORC4, ORC6, CDT1, and CDC6, can cause MGS (Hossain and Stillman 2016). More recently, eight point mutations in *CDC45* have been identified in MGS patients (Fenwick et al. 2016). The mutations are scattered across the protein structure (Fig. 10.4d). Because Cdc45 is involved in both replication initiation and elongation, it is unclear whether initiation or ongoing fork movement, or both, is the underlying cause of the disease.

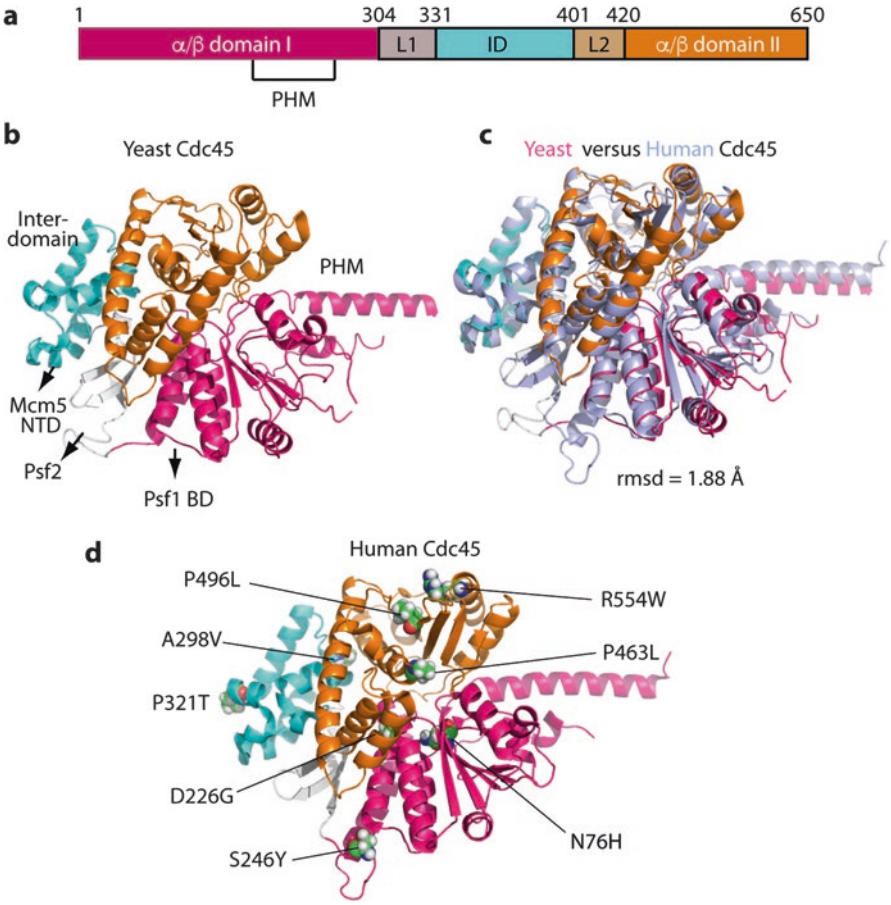


**Fig. 10.4** Comparison of the yeast and human Cdc45 structures. (**a**) The domain structure of Cdc45. (**b**) Cartoon view of the yeast Cdc45 structure extracted from the CMG structure (PDB 3JC6), with each domain colored according to the domain sketch in (**a**). PHM refers to protruding helical motif. (**c**) Superimposition of the yeast and human Cdc45 structures. Human Cdc45 is shown in light purple (PDB 5DGO). (**d**) Human Cdc45 point mutations identified in patients with Meier-Gorlin syndrome are highlighted as spheres

## 10.6 CMG Helicase May Function as an Oil Rig Pump Jack to Inchworm Along DNA

Cryo-EM analysis of CMG particles embedded in vitreous ice revealed two conformations, an extended structure (conformer I) and a compact structure (conformer II) at 4.7 Å and 4.8 Å resolution, respectively (Fig. 10.5a, b) (Yuan et al. 2016). In conformer I, when viewed from the Cdc45-GINS side of the structure, the CTD ring of Mcm2-7 containing the AAA+ motors is tilted by ~10° with respect to the NTD ring, leading to an approximate spiral arrangement of motor domains comprising

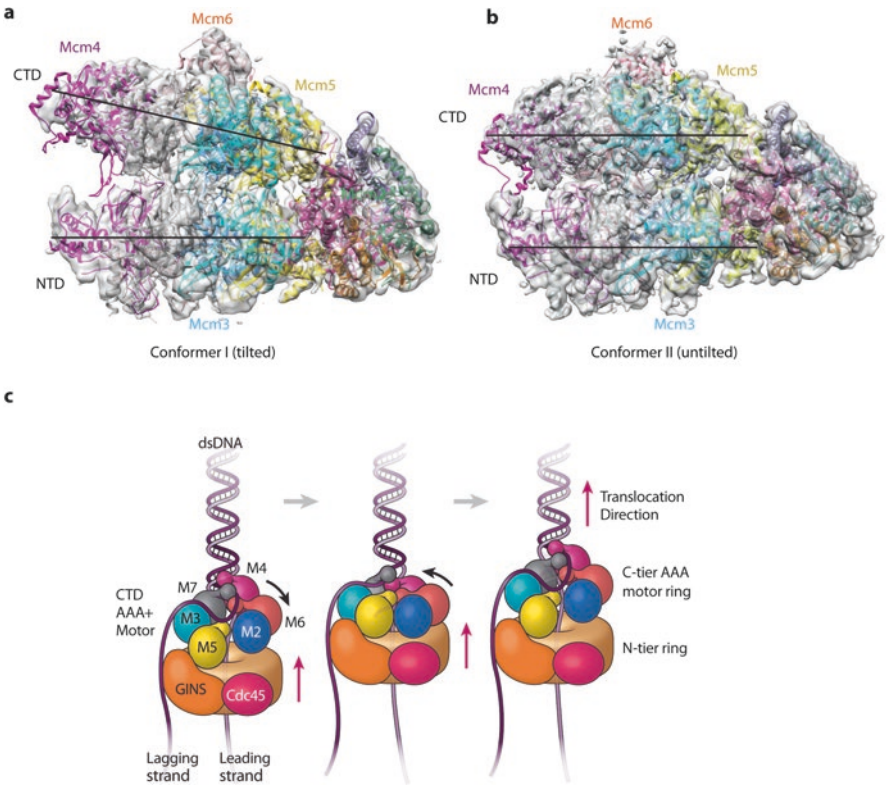


**Fig. 10.5** CMG helicase alternates between tilted (extended) and untilted (compact) conformations. (**a**) Side view of CMG conformer I (extended) in which the CTD motor ring is tilted relative to the NTD ring. (**b**) Side view of the CMG in conformer II (compact) with an untilted CTD ring. In panels (**a**, **b**), the cryo-EM density map is shown as a semitransparent surface rendering, and the atomic model is shown in cartoon (EMD-6535, EMD-6536, PDB 3JC5, and 3JC7). (**c**) An oil rig-like pump jack DNA unwinding model. Panel (**c**) is reproduced in part from Figure 7 in Yuan et al. (2016) with permission. Note that the CTD-tier ring pushing on the dsDNA is only for the purpose of illustration. The pump jack model would still function as a translocase if CMG were oriented with the NTD-tier ring pushing on the dsDNA

the CTD ring. In conformer II, the CTD motor ring is approximately parallel to the NTD ring, and CMG is more compact than conformer I. In both conformers, the Mcm2-7 NTD ring-GINS-Cdc45 unit appears to be a rigid platform upon which the CTD AAA+ motor domains switch between extended and compact states during cycles of ATP hydrolysis. These structures suggest that CMG may function like an oil rig pump jack attached to a stable platform, nodding up and down to inchworm along ssDNA and unwind dsDNA at a forked junction (Fig. 10.5c) (Yuan et al. 2016). This linear inchworm ratchet mechanism is distinct from the sequential rotary ATP hydrolysis unwinding mechanism proposed for the homo-hexameric helicases such as *E. coli* DnaB, *E. coli* Rho transcription terminator, and bovine

papillomavirus (BPV) E1 (Enemark and Joshua-Tor 2006; Itsathitphaisarn et al. 2012; Thomsen and Berger 2009). Additionally, single point mutants of the ATP sites in the Mcm2-7 subunits of *Drosophila* CMG show major helicase defects in only two of the six mutants, inconsistent with a sequential rotary hydrolysis mechanism that predicts each ATP site would be important to helicase activity (Ilves et al. 2010). Similar conclusions of nonequivalent ATP sites have been made by mutational studies of yeast Mcm2-7 (Schwacha and Bell 2001).

Aside from its main function in unwinding parental DNA, CMG also functions as a scaffold for assembly of the replisome. Indeed, the CMG helicase interacts with many other replicative factors, chief among them are the leading strand Pol ε (Langston et al. 2014) and the Pol α-primase that is required repeatedly on the lagging strand (Gambus et al. 2006).

## 10.7 Pol ε Binds to the CTD Motor Side of CMG

In 1990 Pol ε was identified as a third DNA polymerase in budding yeast that is essential for cellular replication (Morrison et al. 1990). Subsequent studies revealed that Pol ε is also involved in many other pathways such as the DNA damage checkpoint response, epigenetic silencing, sister chromatid cohesion, and possibly DNA recombination during repair of DNA lesions (Pursell and Kunkel 2008). Nearly two decades after its discovery, Pol ε was assigned as the major leading strand polymerase (Clausen et al. 2015; Kunkel and Burgers 2008; Miyabe et al. 2011; Nick McElhinny et al. 2008; Pursell et al. 2007). In all eukaryotes studied to date, Pol ε consists of four proteins; the largest is the DNA polymerase, followed by the B subunit and two small histone-fold subunits (Pursell and Kunkel 2008). The Pol ε of *Saccharomyces cerevisiae* consists of Pol2, Dpb2 (DNA polymerase-binding protein 2), Dpb3, and Dpb4. Pol2 is composed of two subdomains: a N-terminal catalytic domain that contains the polymerase and exonuclease active sites and an inactive C-terminal domain that shares homology to B-family DNA polymerases (Fig. 10.6a) (Tahirov et al. 2009). The ternary crystal structure of the N-terminal catalytic subdomain of Pol2 in complex with primer-template DNA and incoming dNTP was recently determined, and it shares many features of other B-family DNA polymerases such as Pol δ (Hogg et al. 2014). However, a P domain, new to the B-family polymerases, enables Pol ε to encircle the nascent dsDNA and enhances the processivity of DNA synthesis. This may explain why Pol ε has the highest fidelity among B-family polymerases despite the absence of an extended β-hairpin loop that is required for high-fidelity replication by other B-family polymerases (Fortune et al. 2005; Hogg and Johansson 2012; Hogg et al. 2014).

Cryo-EM analysis of yeast Pol ε revealed a bilobed overall architecture (Asturias et al. 2006). Pol ε has also recently been shown to bind directly to CMG helicase, independent of DNA, and the 15 protein complex is referred to as CMGE (Langston et al. 2014). Negative stain EM analysis of CMGE provided a 16 Å 3D map

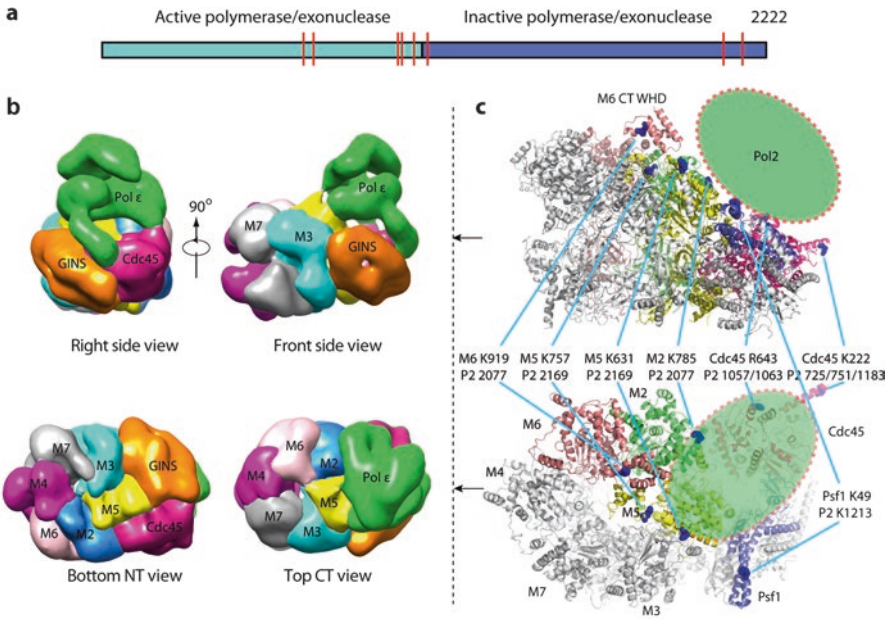


**Fig. 10.6** Architecture of the CMGE complex. (**a**) The Pol2 (P2) subunit of Pol ε is comprised of a catalytic N-terminal subdomain and an inactive B-family Pol in the C-terminal subdomain. (**b**) Negative stain 3D EM map of the CMGE in four different views (EMD-6465). The EM density is segmented and colored according to individual subunits except for the Pol ε where the complex is shown in green. Part of the Pol ε density is not visible in the EM and may be the N-terminal subdomain of Pol2 as proposed in Sun et al. (2015). (**c**) Cross-linking mass spectrometry identified multiple contacts between Pol2 and GINS, Cdc45, and the CTD regions of Mcm2, Mcm5, and Mcm6 (This figure is modified with permission from Figure 6 of Yuan et al. 2016)

(Fig. 10.6b) (Sun et al. 2015). This low-resolution structure revealed that Pol ε is positioned on the CTD-tier side of CMG, sitting atop the CTDs of Mcm2 and Mcm5, and GINS and Cdc45. This Pol ε position was confirmed by extensive cross-linking mass spectrometry analysis (Fig. 10.6c). The visible density of Pol ε could only account for about 70 % the mass of Pol ε, and one suggestion for this missing density was that the catalytic Pol ε N-terminal subdomain was too mobile to visualize (Fig. S6 in ref. (Sun et al. 2015)). However, cross-linking mass spectrometry analysis of CMGE demonstrated that the Pol ε N-terminal subdomain was in proximity to the other subunits of Pol ε and thus located nearby.

## 10.8 Pol α and Ctf4 Are Located on the NTD-Tier Side of CMG, Opposite from Pol ε

DNA synthesis activity of Pol ε and Pol δ requires the primed site synthesized by the four-subunit Pol α-primase, which synthesizes a hybrid RNA/DNA primer (Conaway and Lehman 1982). A 7-9mer RNA is synthesized de novo by the Pri1/2 subunits followed by an internal transfer of the RNA primer to the DNA polymerase which adds a further 10–20 deoxyribonucleotides (Nethanel et al. 1992). Thus Pol α-primase "counts" the length of RNA and DNA and stops synthesis. The underlying mechanism of how this enzyme functions has been revealed by a series of elegant crystal structures and biochemical studies (Coloma et al. 2016; Kilkenny et al. 2013; Klinge et al. 2009; Perera et al. 2013).

Crystal structures of the yeast Pol α catalytic core (349–1258; 910 amino acids) in the apo form, bound to an RNA/DNA primer template (the binary complex), and in a ternary complex with RNA/DNA primer template and incoming dGTP show that Pol α specifically recognizes the A-form RNA/DNA helix for extension with dNTPs (Perera et al. 2013). But once a full turn of double-helix DNA has been synthesized, Pol α is no longer in direct contact with the A-form RNA/DNA helix, which causes its release from DNA. A recent crystal structure of human Pol α-polymerase subunit in complex with a DNA/DNA helix supports this view, but interestingly, the DNA/DNA helix in contact with the polymerase is in a hybrid A-B form (Coloma et al. 2016). It was suggested that the free energy cost of distorting DNA from B- to the A-B hybrid, rather than the loss of specific contacts, drives the termination of primer synthesis.

Pol α-primase is a bilobed structure in which Pol1 binds to the B subunit and Pri1-Pri2 dimer through its CTD, and the CTD of Pol1 is connected to the polymerase region by a flexible stalk (Nunez-Ramirez et al. 2011). Pol α-primase requires CMG for priming activity on both the leading and lagging strands during unwinding of the forked DNA in the presence of RPA (Georgescu et al. 2015b). Pol α-primase is anchored to CMG helicase by the Ctf4 homotrimer (Gambus et al. 2009; Simon et al. 2014; Tanaka et al. 2009). The C-terminal domain of Ctf4 self-associates into a disk-shaped trimer that binds to a peptide sequence in Sld5, a subunit of the GINS complex within CMG (Simon et al. 2014). The same region of Ctf4 also binds to a homologous peptide sequence within the catalytic subunit of Pol α (Simon et al. 2014). Therefore, Ctf4 acts as a scaffold that cross-links CMG and Pol α-primase, underlying the mechanism by which Ctf4 recruits Pol α-primase to the replisome (Gambus et al. 2009). These physical interactions were visualized in a recent EM analysis of combinations of CMG with Pol ε, Pol α-primase, and Ctf4 (Fig. 10.7) (Sun et al. 2015). Unexpectedly, Pol α-primase and Ctf4 were found to bind to the NTD-tier of CMG, opposite from the CTD-tier where Pol ε binds. This organization was novel because all previous illustrations of the replisome placed the polymerases and primase on the same side of the helicase, after DNA unwinding.

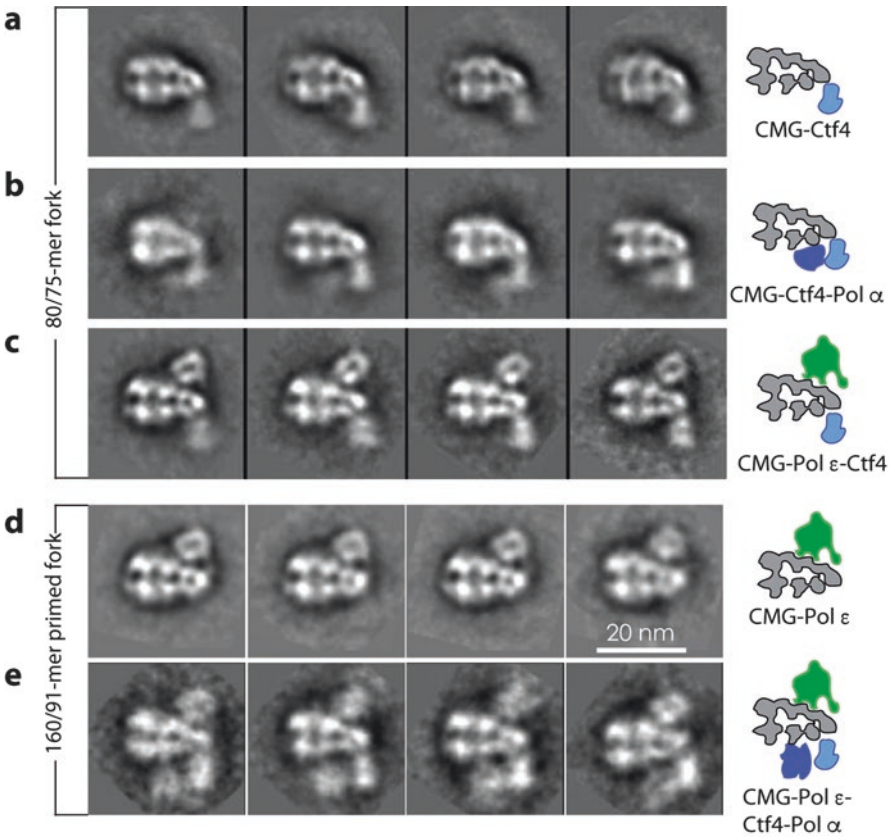


**Fig. 10.7** Selected 2D class averages of several replisome complexes. (**a**) The Ctf4 trimer binds to the GINS from the bottom NTD side of CMG. (**b**) Pol α-primase appears as a fuzzy density suggesting a flexible association with the Ctf4 just under the NTD ring of Mcm2-7. (**c**) Simultaneous binding of Pol ε to the CTD side of Mcm2-7 on top and the Ctf4 trimer to the NTD side of CMG. (**d**) Pol ε binds to the CTD side of CMG in the presence of a primed forked DNA. (**e**) Pol ε binds to the CTD side of CMG, while Ctf4 and Pol α bind to the NTD side of CMG (This figure is modified with permission from Figure 5 of Sun et al. 2015)

## 10.9 Future Perspectives

We have reviewed here just some of the numerous recent structural advances in the field of replication that have revealed the structures of replicative polymerases, the 11 subunit CMG helicase, and the overall architecture of the eukaryotic replisome core. There are numerous important questions that remain to be answered, in fact too many to list. One important question for future studies is to understand the DNA path through the replisome and how CMG translocates along DNA. Available biochemical data indicate that CMG functions by steric exclusion in which both the CTD and NTD rings of Mcm2-7 encircle and translocate along one strand of

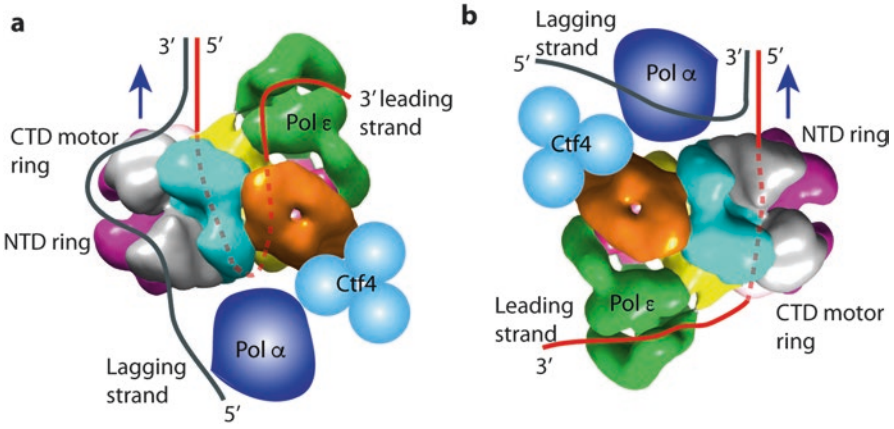


**Fig. 10.8** The eukaryotic replisome positions Pols ε and α on opposite sides of CMG. Depending on whether the CTD motor ring or the NTD ring pushes against the forked junction, the polymerases are positioned either: (**a**) with Pol ε in front and Pol α-primase in back of CMG or (**b**) with Pol α-primase in front and Pol ε in back of CMG

ssDNA, excluding the other strand to the outside of the ring and thus separating the strands (Fu et al. 2011). However, the atomic structure of the Mcm2-7 double hexamer reveals a possible side channel for one DNA strand that lies between the CTD and NTD rings, suggesting the DNA split point may be internal to CMG (Li et al. 2015). Also, the proposed inchworm pump jack mechanism of CMG translocation (Yuan et al. 2016) is a new alternative to the rotary sequential ATP hydrolysis mechanism of hexameric helicase translocation (Enemark and Joshua-Tor 2006; Itsathitphaisarn et al. 2012; Thomsen and Berger 2009). However, there is still very little evidence to support either model. Given the knowledge that CMG helicase moves on the leading strand in a 3′–5′ direction (Bochman and Schwacha 2008; Kang et al. 2012; Moyer et al. 2006), that the 3′ terminus of ssDNA enters CMG through the CTD of Mcm2-7 (Costa et al. 2014), and that Pol ε binds to the CTD side of CMG (Sun et al. 2015), the leading strand DNA may need to travel a long and winding path before reaching Pol ε. These facts, taken together, suggest that the leading strand ssDNA threads through the CTD ring of Mcm2-7, exits the NTD ring of Mcm2-7, and then needs to make a U-turn to reach Pol ε at the CTD side of CMG (Fig. 10.8a). This DNA path assumes that the Mcm2-7 CTD motor ring translocates ahead of the NTD ring and thus pushes against the dsDNA/forked junction, as indicated by EM studies using a biotinylated DNA-streptavidin bound to *Drosophila* CMG (Costa et al. 2014). Furthermore, in the steric exclusion model of unwinding, the DNA split point would be just before DNA entry into CMG, and thus the newly unwound lagging strand ssDNA would need to traverse the outside surface of the Mcm2-7 ring to reach Pol α-primase on the NTD-tier side of CMG. Why nature would thread DNA through the replisome in this fashion is not understood, considering the extensive ssDNA exposed in this orientation. However, one may propose

that this orientation enables Pol ε to be the first to interact with nucleosomes, as Pol ε is known to interact with nucleosomes (Foltman et al. 2013; Tackett et al. 2005) and to function in heterochromatin maintenance (Iida and Araki 2004; Tackett et al. 2005). On the other hand, it is interesting to note that BPV E1 helicase translocates on DNA such that the NTD ring faces the forked junction (Enemark and Joshua-Tor 2008; Lee et al. 2014). If the NTD ring of Mcm2-7 within CMG were to push against the forked junction, this orientation would place Pol α-primase at the top of the fork adjacent to the DNA split point, where Pol α-primase could prime the DNA template immediately after strand separation, and Pol ε would be behind CMG to extend the leading strand immediately after the DNA exits from the Mcm2-7 channel (Fig. 10.8b). We note that CMG helicase and the replisome architectures determined so far are based on in vitro assembled complexes on artificial substrates. It remains to be investigated if the in vivo assembled structures starting from origin DNA are the same especially in the threading of the DNA.

The DNA replication machinery must perform many additional tasks beyond DNA synthesis. For example, the replisome must deal with DNA lesions, transcribing RNA polymerase, DNA-bound proteins, recombination intermediates, nucleosomes in both euchromatin and heterochromatin, and cohesion rings that hold the sister chromosomes together after replication. Thus it may come as no surprise that there are numerous additional proteins that travel with or transiently interact with the replisome, and it is very likely that many new replisome interactive proteins will be identified in future studies. A first glimpse at the multitude of proteins that bind the replisome is provided by pull-outs using antibodies directed against CMG components followed by mass spectrometry to identify CMG-binding proteins (Gambus et al. 2006). This methodology identified a group of proteins referred to as the replisome progression complex (RPC) that contains CMG along with Ctf4, Mcm10, Tof1, Csm3, checkpoint mediator Mrc1, histone chaperone FACT (Spt16 and Pob3), and topoisomerase I (Gambus et al. 2006). The function of FACT in binding H2A/H2B and stimulating transcription through chromatin and of Topo I in relief of supercoiling is understood (Belotserkovskaya et al. 2003; Brill et al. 1987; Kim and Wang 1989; Orphanides et al. 1998; Orphanides et al. 1999; Schlesinger and Formosa 2000). However, most proteins of the RPC have nearly unknown functions. The Ctf4 trimer recognizes a peptide sequence to bind Pol α and Sld5 suggesting that Ctf4 acts as a platform for recruitment and exchange of different replication factors (Villa et al. 2016), in loose analogy to the many proteins that traffic on and off PCNA (Georgescu et al. 2015a). The Mrc1 checkpoint mediator is a stably attached replisome component that appears to aid normal replisome progression in addition to its checkpoint function (Lou et al. 2008), but how Mrc1 facilitates these activities and the spatial orientation of Mrc1 within the replisome are unknown. Tof1 and Csm3 are two proteins that ride with the replisome and appear to pause replication forks at protein-DNA barriers, but how they carry out this function is not understood (Calzada et al. 2005; Mohanty et al. 2006; Tourriere et al. 2005). Mcm10 is an essential replication factor that is reported to interact with ssDNA, dsDNA, Pol α-primase, the CMG helicase, and other factors (reviewed in Bielinsky 2016; Thu and Bielinsky 2013). Mcm10 appears to be the last initiation protein to act at an

origin, because two complete CMGs form at an origin yet cannot produce ssDNA until Mcm10 is present (Heller et al. 2011; Kanke et al. 2012; Watase et al. 2012; Yeeles et al. 2015). How Mcm10 fulfills this initiation role and whether Mcm10 moves with the replisome are presently unknown. Besides the RPC proteins, the flap endonuclease 1 (FEN1) and DNA ligase both interact with PCNA and function with Pol δ/PCNA on the lagging strand to remove the RNA primer and join the Okazaki fragments, respectively. How the Pol δ lagging strand polymerase and the RFC clamp loader associate with the replisome, if at all, is another ongoing mystery. Finally, the replisome must replicate through every nucleosome in the genome, including tightly packed heterochromatin without disrupting the epigenetic state of the cell. The CMG, Pol ε, and Pol α all bind nucleosomes or histone subassemblies (Foltman et al. 2013; Huang et al. 2015; Tackett et al. 2005; Wang et al. 2015), yet how the replisome handles nucleosomes and passes them onto the daughter strands is largely unexplored territory. Answers to these many important questions will require a multitude of experimental approaches, but one can be quite certain that cryo-EM structure determination will be essential to understanding the complete picture of these very large and complicated multiprotein assemblies.

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# Chapter 11
# Replication Domains: Genome Compartmentalization into Functional Replication Units

**Peiyao A. Zhao, Juan Carlos Rivera-Mulia, and David M. Gilbert**

**Abstract** DNA replication occurs in a defined temporal order during S phase, known as the replication timing programme, which is regulated not only during the cell cycle but also during the process of development and differentiation. The units of replication timing regulation, known as replication domains (RDs), frequently comprise several nearly synchronously firing replication origins. Replication domains correspond to topologically associating domains (TADs) mapped by chromatin conformation capture methods and are likely to be the molecular equivalents of replication foci observed using cytogenetic methods. Both TAD and replication foci are considered to be stable structural units of chromosomes, conserved through the cell cycle and development, and accordingly, the boundaries of RDs also appear to be stable in different cell types. During both normal development and progression of disease, distinct cell states are characterized by unique replication timing signatures, with approximately half of genomic RDs switching replication timing between these cell states. Advances in functional genomics provide hope that we can soon gain an understanding of the cause and consequence of the replication timing programme and its myriad correlations with chromatin context and gene regulation.

**Keywords** Replication timing (RT) • Replication foci • Replication domain (RD) • Topologically associating domain (TAD) • Chromatin • Nucleus • Cell cycle

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## 11.1 Introduction

The spatial organization of the genome is critical for proper cellular physiology; multiple nuclear processes and genome functions are linked to chromosome architecture (Cavalli and Misteli 2013; Cremer and Cremer 2010; Pombo and Dillon 2015). Initial cytogenetic characterization of genome organization unveiled the non-random arrangement of chromosomes into territories and revealed replication foci as stable chromosomal structural units or domains that arrange into distinct early and late replicating subnuclear compartments (Cremer et al. 2006; Dimitrova and Gilbert 1999; Ferreira et al. 1997; Jackson and Pombo 1998; Sparvoli et al. 1994). The advent of genome-wide methods enabled replication timing to be measured comprehensively in many different cell types and the identification of units of replication timing regulation, termed 'replication domains' (RDs), many of which could be seen to change replication timing during cell fate transitions. Recently developed chromatin conformation capture methods (e.g. Hi-C) that map long-range chromatin interactions confirmed the partitioning of the genome into distinct compartments within the nucleus that correspond to early and late replication timing compartments and also confirmed the existence of stable units of chromosome structure termed topologically associating domains (TADs) that correspond to RDs (Dixon et al. 2012; Lieberman-Aiden et al. 2009; Pope et al. 2014). Distinct genomic features are associated with early and late replicating compartments, such as GC content, gene density and transcriptional activity. Moreover, differences in chromosomal interactions have been observed between distinct cell types, and individual domains can be repositioned during development between the nuclear interior and periphery correlated with dynamic changes in their replication timing and transcriptional activation/repression (Hiratani et al. 2010; Takebayashi et al. 2012). Here we discuss historical and emerging evidences placing RDs in the context of global chromatin organization and providing a better understanding of the structural and functional properties and biological significance of nuclear organization.

## 11.2 Replication Foci and the Spatial Organization of the Genome

Duplication of the genome occurs in a defined temporal order known as the replication timing (RT) programme (Rhind and Gilbert 2013). Early cytogenetic studies of DNA replication that visualized DNA synthesis by incubating S phase mammalian cells with nucleotide homologues such as BrdU revealed distinct punctate structures in the nucleus termed replication foci that were found in different spatial patterns at distinct times during S phase (Nakamura et al. 1986; Nakayasu and Berezney 1989). Early in S phase, many small replication foci are associated with euchromatic regions interspersed throughout the nucleoplasm, while as S phase progresses, DNA synthesis moves to the heterochromatin-rich regions at nuclear and nucleolar

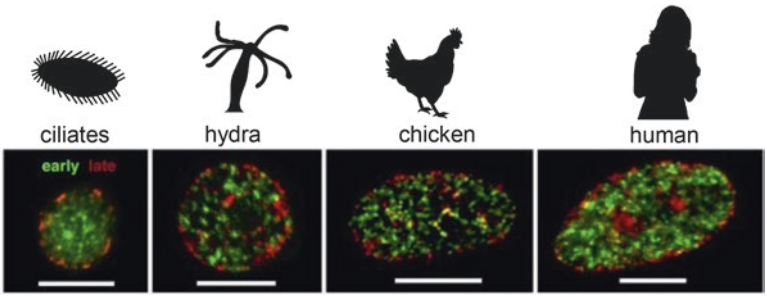


**Fig. 11.1** Evolutionary conservation of the spatio-temporal RT programme (Adapted from Solovei et al. 2016). Cells pulse labelled with CIdU, chased for approximately half of S phase, and then pulse labelled with IdU, followed by fixation and immunostaining for CldU (*green*) and IdU (*red*). Patterns are remarkably similar in a wide variety of species

peripheries forming what appeared under conventional light microscopy to be larger foci. Remarkably, this spatial segregation of chromatin into early and late replication foci is conserved in all eukaryotes (Fig. 11.1). Replication foci were estimated to contain 0.5–1 Mb of DNA and consist of clusters of replicons that replicate synchronously (Ma et al. 1998). Replication foci also co-localize with replication proteins, either by immunofluorescence or when visualized in living cells using GFP-tagged replication proteins, such as proliferating cell nuclear antigen (PCNA) and replication protein A (RPA) (Sporbert et al. 2002). In living cells, replication proteins can be seen to move from recently completed to spatially adjacent foci (Maya-Mendoza et al. 2010; Sporbert et al. 2002). Pulse-chase-pulse experiments in which cells were labelled with two different nucleotides separated by a variable chase time allowed for the study of the kinetics of replication foci patterns in different stages of S phase. With short chase times, the number of replication foci was found to remain constant and the two labels co-localized, while chase times of 60 minutes or more resulted in complete separation of the two pulse labels, suggesting that the replication of each focus was completed within that time (Dimitrova and Gilbert 1999; Jackson and Pombo 1998). Chase times of several hours resulted in differential labeling of the spatial patterns of replication (Fig. 11.1). Replication foci were further studied by chasing labelled cells for many generations, revealing that each focus retained its shape, size and intensity of label, demonstrating that the DNA that is synthesized together stays together as a structural and functional unit of chromosomes (Berezney et al. 2000; Dimitrova and Gilbert 1999; Ferreira et al. 1997; Jackson and Pombo 1998; Ma et al. 1998; Sadoni et al. 2004; Sparvoli et al. 1994). When spatially adjacent foci were labelled sequentially with different nucleotide analogues separated by an ~60 min chase and then further chased for multiple generations, spatially linked sequentially replicating foci remained adjacent demonstrating that they were genetically linked (Maya-Mendoza et al. 2012; Sporbert et al. 2002). Thus, DNA replication in large genomes is regulated both temporally and spatially.

## 11.3 Replication Origin Control at a Glance

Several mechanisms and cell cycle checkpoints are in place to ensure proper regulation of replication origin firing under normal vs. replication stress conditions (Fig. 11.2a). First, in telophase/early G1 phase, the pre-replication complex (pre-RC) is assembled at many potential origins under conditions that strictly prevent initiation in a process known as 'origin licencing' (Fragkos et al. 2015). Origin licencing initiates with the recruitment of the origin recognition complex (ORC; consisting of six subunits, ORC1–ORC6) followed by cell division cycle 6 (CDC6) and cdc10-dependent transcript 1 (CDT1), which assist the loading of the MCM helicase complex (MCM2–MCM7) (Deegan and Diffley 2016; Hyrien 2015). Then, at the G1-S phase transition, formation and activation of the pre-initiation complex (pre-IC) are triggered by DBF4-dependent kinase (DDK) and cyclin-dependent kinases (CDKs) (Fragkos et al. 2015). Upon activation, the MCM2-7 double hexamers separate and nucleate the replisome at each replication fork (Deegan and Diffley 2016). Activation of helicases to initiate DNA replication can only occur under conditions of high CDK and DDK, which are also conditions that strictly prevent the assembly of pre-RCs (Blow and Dutta 2005). This two-cycle engine, the temporal uncoupling of origin licencing and activation, is a crucial feature of eukaryotic replication, restricting replication to once-per-cell-cycle regardless of where or when replication initiates.

Only a small fraction of the pre-RCs that were assembled in telophase/early G1 get activated during S phase. The origins that are licenced without firing during one cell cycle are known as 'dormant' origins (Fragkos et al. 2015). It is speculated that assembly of an excess of pre-RCs and activation of a small fraction of potential origins might be a mechanism to ensure that genome is fully replicated, in such a way that in the case of fork stalling during conditions of replication stress, dormant origins fire to complete genome duplication within the immediate vicinity of the clusters of activated origins to rapidly complete replication in already initiated domains (Rivera-Mulia and Gilbert 2016b). At the same time, DNA damage response kinases, e.g. ATR and CHK1, suppress the formation of initiation in unreplicated RDs, restricting new initiation to the firing of only the dormant origins in the vicinity of the existing replication forks (Donzelli and Draetta 2003). In this manner, the cells are able to reduce the number of active replication forks rapidly to minimize reactive dangerous replication forks under conditions of replication stress (Rivera-Mulia and Gilbert 2016a, b).

## 11.4 Replication Origin Activation Within Replication Foci

The mechanism by which origins fire in clusters within foci remains unclear (Fig. 11.2b). The global RT programme can be seen as being determined at both the level of chromosomal structure when the RT programme is set up through elaborate

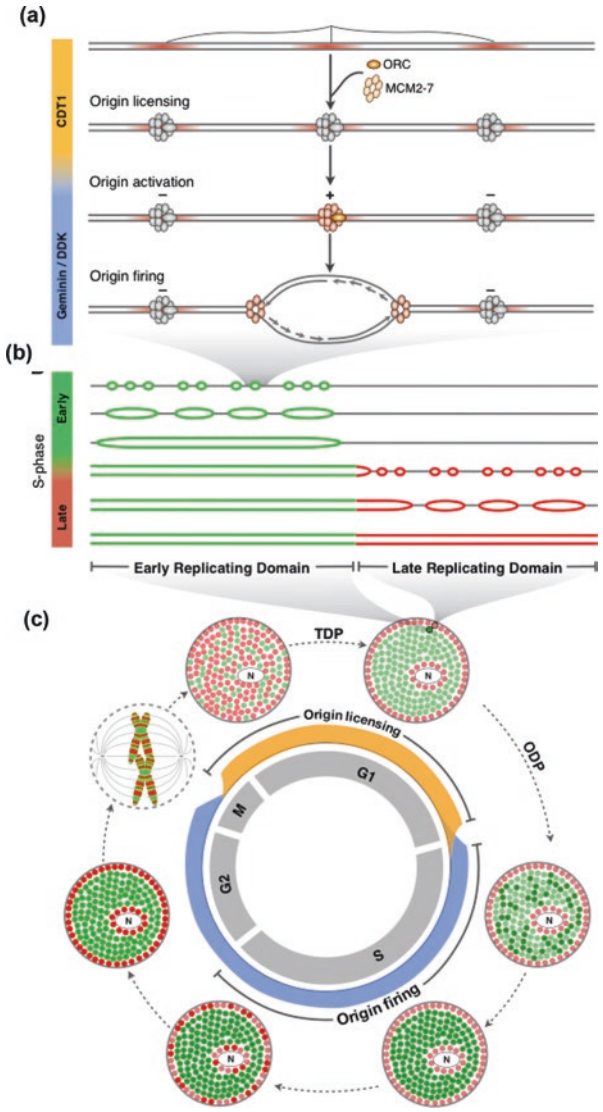


**Fig. 11.2** Replication is regulated at scales from origins to domains (Adapted from Rivera-Mulia and Gilbert 2016a). (**a**) Replication origin regulation. The ORC and other contributing proteins load the MCM2-7 double hexamer at origins in a step called origin licencing. A subset of licenced origins are subsequently activated, and origin firing is initiated by the bidirectional unwinding of the DNA strands by MCM2-7 double hexamers (see chapter by Araki in this issue). (**b**) Synchronized firing of clustered origins to form early and late replicating domains. (**c**) DNA replication and its regulation through the cell cycle. Origin licencing occurs from M through most of G1. The timing decision point (TDP) occurs early during G1 and precedes the origin decision point (ODP). RDs are compartmentalized in such a way that early RDs are located at the interior of the nucleus and late RDs are located at the nuclear and nucleolar periphery. Timing determinants are lost in G2 phase and regained at the TDP

mechanisms to commit origins to their characteristic firing times and the activation of individual origins at such times (Rivera-Mulia and Gilbert 2016b). Genome-wide determination of the RT programme will be discussed below. Here we discuss two alternative high-level views of origin regulation (Rhind et al. 2010). A deterministic model assumes a predetermined RT programme in which each origin is programmed to fire at specific times. The model is supported by some studies carried out in *S. cerevisiae*, which feature well-defined, site-specific origins not typically observed in metazoans (Ferguson et al. 1991; Newlon et al. 1991). A stochastic model posits that different origins have different probabilities of firing, giving rise to heterogeneous firing times for individual origins in a population of cells (Kaykov and Nurse 2015; Patel et al. 2006; Saner et al. 2013). Mathematical modelling of replication kinetics has shown that a stochastic firing model fits well with current experimental observations in various systems (Goldar et al. 2008, 2016; Lob et al. 2016; Norio et al. 2005; Yang et al. 2010). Moreover, DNA combing, a technique that allows the investigation of origin firing at the level of single DNA molecules (Kaykov and Nurse 2015) has experimentally demonstrated genome-wide stochastic origin firing in yeast. In other words, normally, early-firing origins have a chance to fire late or not at all, while normally late-firing origins occasionally fire early.

Similar single DNA molecule experiments on a whole genome are presently lacking for mammalian cells. Nonetheless, DNA fibre analyses carried out on the mouse *Igh locus* show different origins utilized on different DNA fibres, consistent with the stochastic usage vs. non-usage of origins in any given cell cycle but not sufficient evidence to demonstrate stochastic temporal firing (Demczuk et al. 2012). Additionally, studies in *Xenopus* egg extracts and mammalian cells have shown that the number of dormant origins firing can be modulated by manipulating the levels of cell cycle checkpoint proteins or depleting the concentration of MCM2-7 (Ge and Blow 2010; Woodward et al. 2006). It is important to emphasize that stochastic firing of origins is premised on a number of criteria regarding origin firing probability. One such criteria is an increase in firing probability as S phase progresses invoked in stochastic firing models to ensure that origins with relatively low firing probability can eventually fire later in S phase (Rhind et al. 2010). Several mechanisms accounting for the increased firing probability have been proposed (Rhind and Gilbert 2013), but all current models leave us with many unanswered questions: do active replication forks induce the firing of origins in their proximity? To what extent is the induction necessary for the timely completion of S phase? What factors regulate the initiation rate in various model systems? More data such as high-resolution genome-wide RT profiles and reliable maps of all potential origins and their frequencies of firing are needed to further refine the model.

There has been a long-standing correlation between transcription and origin selection and activation. However, the studies carried out so far are characterized by contradicting evidence, with some showing an enrichment of origins in active transcription start sites (TSSs) and CpG islands and others observing no significant overlap (Besnard et al. 2012; Karnani et al. 2010; Prioleau 2009; Sequeira-Mendes et al. 2009). The disparity between studies is primarily due to the lack of agreement in origin features and locations between the origin mapping techniques used or the cell

types under study (Hyrien 2015). Using nascent strand purification followed by microarray to map replication initiation sites in mouse embryonic stem cells (ESCs) (Sequeira-Mendes et al. 2009), a strong correlation between transcription initiation activity and origin firing efficiency was found with origins associated with CpG islands firing the most efficiently. On the other hand, replication origin sites identified through bubble-seq had surprisingly little overlap between the majority of origins and transcribed genes (Mesner et al. 2013). The lack of agreement between techniques used for origin mapping could potentially be due to heterogeneity in the molecular properties of the origin population that is detected using different methods. For this reason, the most parsimonious conclusion is that a positive correlation between transcription activity and early origin firing is restricted to a subset of origins.

Numerous additional genetic and epigenetic features correlate with origin licencing and firing. Attempts have been made to put origins into distinct classes based on their chromatin association and underlying genetic sequences (Cayrou et al. 2015). The structure known as a G-quadruplex (G4), which is a four-stranded nucleic acid structure with bonds formed between guanines, is a conserved feature that is associated with highly efficient origins in metazoan (Besnard et al. 2012; Cayrou et al. 2012; Valton et al. 2014). There is also evidence suggesting overrepresentation of the association of G4 structures with origins due to the inherent biases of the methods used for origin mapping (Foulk et al. 2015). The exact mechanisms of how G4s regulate origin firing remain unknown. Moreover, despite the strong link, the overlap between G4 structures and activated origins is far from perfect, and G4 structures cannot predict the locations of origin firing, suggesting the involvement of other origin specificity factors. In addition to underlying sequence features, nucleosomal organization near origins has been shown to influence both origin licencing and helicase activation steps of initiation (Azmi et al. 2017). Certain histone modifications and histone modifiers have been shown to be enriched near origins and be implicated in origin selection and activation (Feng et al. 2016; Liu et al. 2012; Pourkarimi et al. 2016; Rondinelli et al. 2015; Tardat et al. 2010; Wu et al. 2017). Acetylation of histones H3 and H4, the modification that is generally associated with open chromatin, has been shown in *Drosophila* to associate with active origins, and surprisingly acetylation of certain lysines is also dependent on ORC binding to the origins (Liu et al. 2012). Dynamic changes in H4K20me1 through the cell cycle have been shown to regulate origin licencing (Tardat et al. 2010). Early and late origins were also found to associate with different histone marks. Demethylation of H3K4me3 was shown to promote early origin firing, whereas the repressive chromatin mark H3K9me3 was found associated with late replicating origins (Rondinelli et al. 2015; Wang et al. 2016).

Unfortunately, the universal abundance of histone modifications across the genome makes any one modification unlikely to be sufficient for dictating origin selection and activation. Moreover, the intimate link between epigenetic landscape and transcription potential of the region further complicates investigation into the role of histone marks in origin regulation. There has been evidence suggesting cell cycle-dependent changes of histone modifications at one origin resulting in coordinated regulation of origin firing and transcription (Kylie et al. 2016). Several

transcription factors have also been shown to have the function as origin regulators with effects that can be either origin firing promoting or inhibiting (Bellelli et al. 2014; Knott et al. 2012; Ostrow et al. 2017). Altogether, evidence argues against a single genetic or epigenetic factor being necessary or sufficient for origin regulation. More likely complex combinations of genetic and chromatin features dictate the probability of origin firing. Genome-wide studies correlating dynamic changes in epigenetic landscape with cell cycle-dependent changes in transcription and origin regulation are needed to further evaluate the complex relationship between chromatin modifications, transcription and origin regulation. Most importantly, as discussed above, comprehensive and reliable lists of all origin sites and their probabilities of firing would be transformative for this field. Unfortunately, probabilities are only obtained through single-molecule studies, which to date have been low throughput.

## 11.5 Replication Factories

The spatial organization of replicons within replication foci has been the subject of much debate. An early electron microscopic study (Hozak et al. 1993) described ovoid-shaped bodies observed under an electron microscope that are ~150 nm in diameter. DNA synthesis first appeared within these 'replication factories' and after a brief chase was seen to extrude from them, leading to a model of a fixed and pre-existing protein complex, through which DNA replication forks are spooled during DNA synthesis. It had been proposed that neighbouring replicons are organized in close physical proximity in these factories with intermediate DNA sequences looped out forming rosette-like synthetic centres to facilitate synchronous firing (Vogelstein et al. 1980). Using time-lapse microscopy, Kitamura et al. (2006) and Meister et al. (2007) have visualized clusters of replication forks being co-ordinately processed during replication in live *S. cerevisiae* and *S. pombe*, respectively, supporting the model of replication factory. More recently, super resolution methods such as 3D-structured illumination microscopy (3D-SIM) and stimulated emission depletion (STED) can visualize structures 30 nm and smaller, providing the optical resolution to distinguish single replicons within foci in mammalian cells (Baddeley et al. 2010; Chagin et al. 2016). A recent study (Chagin et al. 2016) observed ~5000 simultaneously active replicons in human cells, finding that sites of labelling observed within the lower-resolution replication foci were clearly spatially separated, casting doubt on the 'replication factory' model of replicon clustering (Chagin et al. 2016; Lob et al. 2016). By simultaneously imaging PCNA replication foci and the chromosomal regions undergoing replication (Deng et al. 2016), DNA was observed to exit replication foci during replication and return without disturbing higher-order chromatin organization. Taken together, current evidence does not support the existence of fixed pre-existing replication factories but rather supports a model in which independent replicons within each focus are spatially clustered by virtue of their chromatin loop and higher-order chromatin organization.

## 11.6 Replication Domains and Large-Scale Chromosome Organization

Further investigation into the molecular properties of replication foci has been greatly facilitated by the development of methods to map RT on a genome-wide scale permitting genomic map coordinates to be placed into the context of cytogenetic findings. Several related methods to measure global RT have been developed (Gilbert 1986, 2010; Gilbert and Cohen 1987; Hiratani et al. 2008; Schubeler et al. 2002; Woodfine et al. 2004). RT profiles partition the genome into chromosome segments of uniform replication timing, known as constant timing regions (CTRs), punctuated by timing transition regions (TTRs). CTRs correspond to several tandem RDs that replicate at similar times, and so their boundaries cannot be detected because forks emanating from each RD fuse quickly. RDs have the potential to be independently regulated during development, and in cell types in which they replicate at different times, RD boundaries become revealed as TTRs (Pope et al. 2014; Rhind and Gilbert 2013). Genome-wide studies showed that these dynamic changes in RT occur at the level of sub-chromosomal units of 400–800 kb and that the boundaries of these units were at the same chromosomal map units regardless of the cell type in which they were observed to replicate discordantly with their neighbours. In fact, during cell fate commitment, a process called domain consolidation was found to occur where an increasing number of adjacent RDs replicate synchronously to form larger CTRs that are also consolidated spatially into the same subnuclear compartment coinciding with their re-localization within the nucleus (Fig. 11.3a) (Hiratani et al. 2008; Rivera-Mulia et al. 2015; Yue et al. 2014). From these observations emerged the concept of the replication domain model, that is, independent structural and functional units of chromosomes (RDs) that coalesce to form the variably sized CTRs observed in replication timing datasets (Fig. 11.3b). Although it has not been formally demonstrated that RDs are the molecular equivalents of the replication foci discussed above, their size and replication kinetics account for the observed behaviour of replication foci, and the fact that the boundaries of RDs are conserved in different cell types attests to their stability. Hence, genome-wide RT mapping has revealed RDs as a basic chromatin unit and provides the opportunity to correlate their structure to other chromosomal properties.

The advent of chromatin conformation capture-based methods such as Hi-C provided the opportunity to relate RDs to the chromatin context and genome coordinates. Hi-C identified stable structural units of chromosomes termed topologically self-associating domains (TADs) and detected the folding of TADs into multi-TAD compartments of active/open (A-compartments) or inactive/closed (B-compartments) chromatin. The comparison of RT profiles and Hi-C A/B compartments demonstrated that Hi-C confirms the physical compartmentalization of the genome revealed by prior replication labelling findings (discussed above), with A-compartments representing the early replicating internally localized foci and B-compartments replicating the late replicating and peripherally localized foci (Fig. 11.4a) (Dixon et al. 2012; Moindrot et al. 2012; Ryba et al. 2010; Yaffe et al. 2010). By mapping RT in

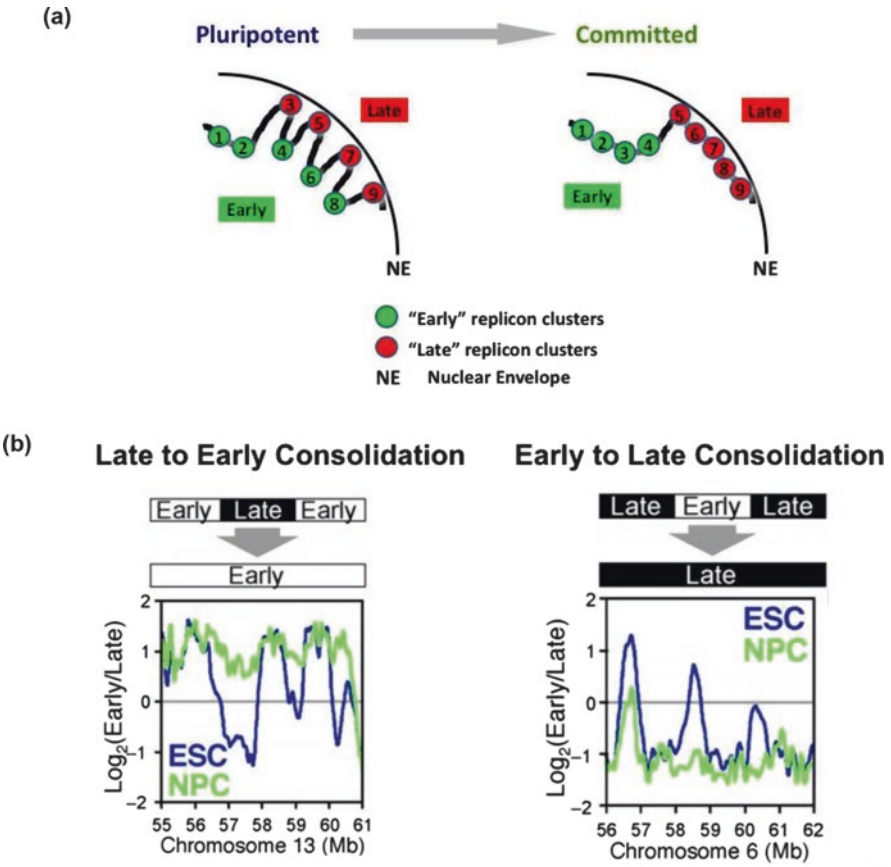


**Fig. 11.3** Consolidation of RDs upon differentiation from ESCs to neural progenitor cells (NPCs) (Adapted from Hiratani et al. 2008). (**a**) A model of domain reorganization during neural differentiation. The replicon clusters that replicate differentially in ESCs undergo spatial rearrangement and consolidate into larger co-ordinately replicating domains. (**b**) Exemplary late to early and early to late domain consolidation during differentiation

multiple cell types, the boundaries between RDs were determined and found to align to TAD boundaries, demonstrating that the units of RT regulation correspond to TADs (Fig. 11.4b) (Pope et al. 2014). Additionally, changes in RT during cellular differentiation were found to coincide with changes in inter-TAD interactions, with long-range chromatin interactions between RD/TAD shifting their RT such that they interact with other domains that replicate at similar times (Takebayashi et al. 2012). Together, evidence supports a unified model for large-scale chromosome organization known as the 'Replication Domain model' (Fig. 11.4b).

To better understand causal linkages in this intriguing relationship between genome organization (structure) and RT (function), new approaches to study large-scale chromosome architecture are necessary. One way to investigate causality has

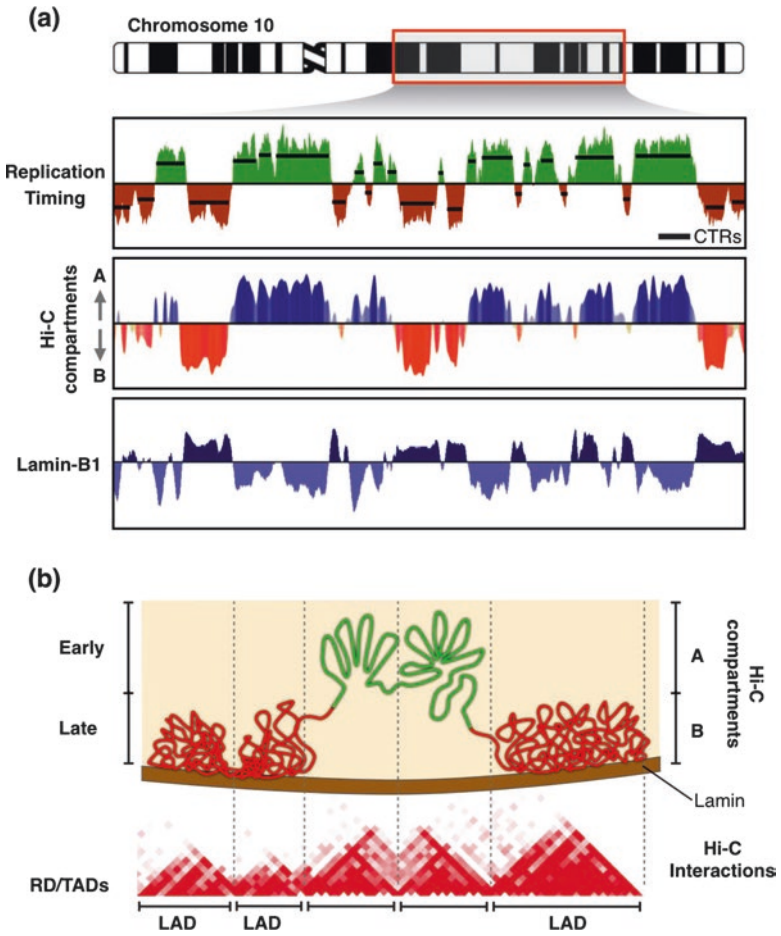


**Fig. 11.4** Replication timing reflects genome compartmentalization (Adapted from Rivera-Mulia and Gilbert 2016b). (**a**) Exemplary RT profile of 50 Mb of human Chr10 from IMR90 fibroblasts. The RT profile aligns with Hi-C A/B interaction compartments, and late replicating domains align with lamina-associated domains (LADs). Segments of chromosomes consisting of multiple RDs that replicate at similar times are marked with *horizontal lines* and are known as CTRs. (**b**) The Replication Domain Model: RDs are the equivalent of TADs, which fold in 3D space such that TADs in close proximity replicate at similar times

been to relate the formation of TADs and 3D compartment through the cell cycle to the establishment of RT. It has been shown that 3D structure is dismantled during mitosis, but both TAD structure and interaction compartments are re-established coincidentally with the re-establishment of RT in a very narrow time window during G1 referred to as the timing decision point (TDP) (Fig. 11.2c) (Dileep et al. 2015b). Interestingly, this same study found that, during G2 phase when chromatin has lost properties necessary to dictate a proper RT programme (Lu et al. 2010), TAD structure and inter-TAD interactions are retained, demonstrating that this 3D

organization is not sufficient for RT but may provide a scaffold on which other factors (such as Rif1; see chapter by Buonomo in this issue) can operate to define RT. Thanks to genome engineering tools such as CRISPR, we are now in a position to manipulate domain structure and determine the cis-elements necessary for autonomous regulation of TAD RT and eventually the contribution of the 3D chromosome scaffold to the establishment of RT.

In addition to TADs, RT also correlates with lamina-associated domains (LADs), which range in size from 10 kb to 100Mb and form physical contact with the nuclear periphery (Fig. 11.4a) (Amendola and van Steensel 2014; Pope et al. 2014). Consistent with the sequence dependence of RT with late RDs being mostly A/T rich and located at the nuclear periphery, LADs, which are also characterized by their high A/T content and high isochore concordance, are found to coincide with late RDs and timing transition regions (TTRs) that are located between early and late RDs (Meuleman et al. 2013; Pope et al. 2014). Hence, late domains and TTRs both contain origins with a low probability of firing and are enriched in LADs, and LADs tend to extend right to the boundaries of the locally earliest replicating RDs, which project into the nuclear interior. A subset of LADs known as facultative LADs exhibit cell type-specific lamina association, and their relocation towards or away from the nuclear lamina is often correlated with the transcription activation and repression of the genes contained within the domain (Kosak et al. 2002; Meuleman et al. 2013; Peric-Hupkes et al. 2010; Robson et al. 2016). Given the close correspondence between LADs and late RDs, the developmental regulation that facultative LADs are subjected to is often reflected in a switch in RT of the domain during cell differentiation in that the detachment of the domain from the nuclear lamina is often accompanied by a late to early switch in RT (Hiratani et al. 2008). Taken together the evidences suggest that RD and LAD organization are closely linked and RT is a genomic feature that reflects changes in chromatin organization through development.

RDs also share some properties with the sequence-based features of chromosomes known as isochores. DNA isochores are defined as homogenous DNA stretches of similar sequence composition. By assessing the segments' GC content, the human genome was found to be composed of ~3200 isochores divided into five families of increasing GC content (Costantini et al. 2007). Replication origins have been shown to enrich around GC-rich regions such as CpG islands and exhibit early firing in high origin density regions (Cayrou et al. 2011). RT programmes are found to correlate with isochore GC content, LINE density and gene density (Fig. 11.5a) (Costantini and Bernardi 2008; Woodfine et al. 2004). The sequence-based isochore features, however, are not sufficient to dictate RT as developmentally controlled regions residing in AT-rich/LINE-rich isochores have been shown to change their RT during ESC to NPC differentiation (Hiratani et al. 2004, 2008). Developmentally regulated RDs often exhibit an inverse correlation between GC content and gene density. We speculate that isochores that exhibit intermediate or mixed sequence features are more prone to change RT during development due to opposing effects from the underlying isochore sequence and gene density, possibly due to transcription or the regulatory features associated with transcription. It is clear that combina-

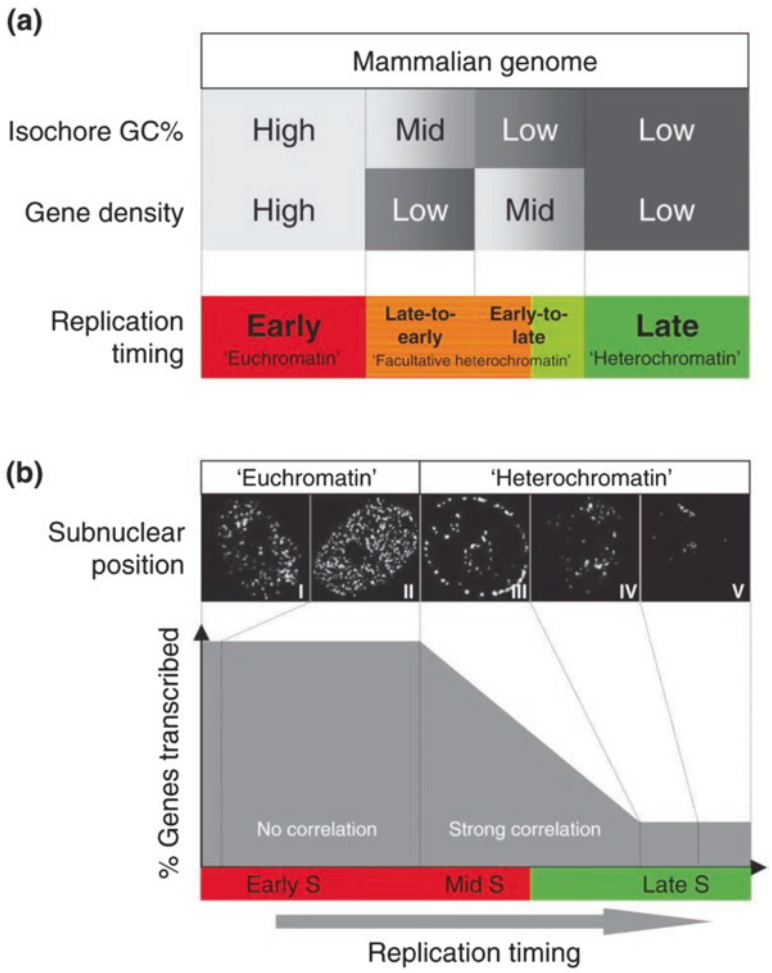


**Fig. 11.5** Relationship between RT regulation and isochore properties, subnuclear position and transcription (Adapted from Hiratani et al. 2009). (**a**) RT is correlated with properties of isochores. Mammalian genomes are composed of isochores of different GC content and gene density. Isochores that are high in both GC content and gene density are replicated early in S phase. Isochores that are low in both GC content and gene density are replicated late in S phase. Isochores that have intermediate GC content and gene density are subjected to developmentally regulated RT switches. (**b**) Changes in RT that traverse the middle to late S phase accompany changes in subnuclear position and transcriptional potential. Replication early in S phase (I and II) takes place at the euchromatin compartment located at the interior of the nucleus. Replication in mid- and late S phase (III, IV and V) takes place at the heterochromatin compartment located at the nuclear periphery (III), the nucleolar periphery (III) and interior heterochromatin (IV and V). Genes that are replicated in early S phase (I and II) have equally high probability of being expressed ('no correlation'); therefore, changes in RT for genes in this category are not necessarily accompanied by changes in nuclear position or transcriptional potential. Genes that are replicated in mid-late S phase exhibit a strong correlation between RT and transcription activity

tions of several factors mediate domain-level regulation of RT (discussed below). Isochores implicate the genetic sequence composition, LINE and gene density in RT determination.

## 11.7 Replication Domains, Gene Regulation and Chromatin Context

A correlation between RT, gene expression and nuclear positioning has been known for decades (Gilbert 2002; Goldman et al. 1984; Hatton et al. 1988). However, mechanisms and causal relationships remain elusive, and recent results suggest that there are likely to be many layers of context-specific complexity. Early studies found that transcriptionally silent heterochromatin generally replicates late during S phase and locates near the nuclear periphery, while active genes tend to replicate early and are preferentially located at the nuclear interior (Baer 1965; Himes 1967; Klevecz and Stubblefield 1967; Lima-De-Faria 1959; Pfeiffer 1968; Taylor 1960). Dynamic changes in RT are accompanied by subnuclear repositioning, further highlighting the importance of nuclear localization to RT (Hiratani et al. 2008). For instance, in the case of *Mash1*, a proneural regulator gene, it was shown that in the process of mouse ESC differentiation to neural cells, the gene switches from late replicating to early replicating accompanied by transcription activation and relocation from the periphery to the interior of the nucleus (Williams et al. 2006). Classical genetic characterization of position-effect variegation in fly demonstrated that changing the chromatin context of a gene influences its transcriptional activity, while studies in mammals showed that the insertion site of a transgene can influence the replication time of the inserted sequence (Elgin and Reuter 2013; Gilbert and Cohen 1990). The most recent development of the high-throughput approach known as thousands of reporters integrated in parallel (TRiP) in chromosomal loci with various chromatin context has further elucidated position effects on a genome-wide scale, and when performed in combination with RT profiling, the technique will provide us with a unique opportunity to investigate the relationship between transcription, chromatin context and RT in a high-throughput manner (Akhtar et al. 2013). Another example that illustrates the relationship between RT and chromatin context is provided by early studies on genomic imprinting in mammalian cells. Genomic imprinting is associated with silencing and delayed replication of the imprinted allele, and early RT is often seen on the monoallelically expressed genes (Farago et al. 2012; Kitsberg et al. 1993; Mostoslavsky et al. 2001; Simon et al. 1999). X chromosome inactivation in female mammals provides another example. The inactive chromosome X (Xi) in female mammals is tightly organized at the nuclear periphery in a nuclear compartment known as the Barr body and replicates late, while the expressed chromosome (Xa) is more interiorly located and replicates earlier than the Xi (Avner and Heard 2001; Koren et al. 2014). Moreover, when autosomes are translocated to the Xi, they become late replicating and genetically inactive suggesting that late replication might lead to stable gene silencing (Hiratani

et al. 2010; Keohane et al. 1996). Together these results suggest that the temporal order of replication of chromosomal segments is reflective of transcriptional potential, chromatin compaction and subnuclear position.

If replication timing is causally linked to transcriptional potential, it should also be subject to developmental control. Indeed, the temporal order of replication of chromosomal segments is reflective of cell type-specific chromatin organization and changes co-ordinately with differentiation state during development. Early studies of individual gene RT concluded that constitutively active genes replicate early while tissue-specific genes replicate early only in the cell type in which they are expressed (Ermakova et al. 1999; Gilbert 1986; Goldman et al. 1984; Guinta and Korn 1986; Holmquist 1987). These observations suggested that there might be coordinated changes in gene expression and the temporal order of replication of tissue-specific genes, but as these studies had compared established and often genetically unstable cell lines, they could not distinguish developmental control from genetic aberrations or epigenetic adaptations to cell culture. More recent studies with embryonic stem cells identified dynamically regulated RT switches with late to early switches coordinated with transcriptional activation (Hiratani et al. 2004; Perry et al. 2004). Later, genome-wide studies revealed that dynamic late to early or early to late RT changes are coordinated across hundreds of kb, often closely coincident with transcriptional activation or silencing of genes within the affected domain and the repositioning to the nuclear interior or periphery, respectively (Hiratani et al. 2008). Consistent with the global spatial patterns of replication discussed above, changes in subnuclear position only occurred in cases where the RT change traversed mid-late S phase, which was also the period when RT and transcription have the strongest correlation (Fig. 11.5b) (Hiratani et al. 2008). Ironically, these studies also revealed the existence of a small subset of genes that were transcribed when late replicating or even some that were activated when switching from early to late replication. These genes may be equivalent to genes identified in *Drosophila* that require heterochromatin to be expressed (Yasuhara et al. 2005). A more recent study showed that a strong transactivator targeted to an endogenous promoter to induce transcription resulted in both repositioning to the nuclear interior and a shift from late to early RT (Therizols et al. 2014). This observation led these authors to conclude that transcription was sufficient to advance the RT of a chromosomal domain. However, since there are natural examples of transcription within late replicating domains, this observation remains to be independently confirmed and the conclusions substantiated in a generalized manner.

Taken together, the experimental evidence linking RT, gene expression and genome organization has led to the construction of two alternative but not mutually exclusive models as to how they might be related. In chromatin biology, there is rarely a unidirectional cause and effect but more typically a series of positive feedback loops that reinforce a transcriptional state, and the links between transcription and RT appear to be no exception. First, based on studies in yeasts (see chapter by Masukata in this issue), it is clear that chromatin can influence the timing of replication. Unfortunately, homologues of most of these regulators have little effect on RT in mammalian cells (Dileep et al. 2015a). Moreover, replication initiation zones are

enriched for DNase I hypersensitive sites (Petryk et al. 2016), and computational model building has revealed DNase I hypersensitive sites to be the best predictor of RT (Gindin et al. 2014). Since changes in chromatin states are associated with transcriptional activity and the act of transcription can cause distinct components of chromatin to be disassembled, changes in transcriptional regulatory networks associated with cell fate changes and transcription itself are both consistent with an indirect relationship, with perhaps chromatin structure regulating RT (Belotserkovskaya et al. 2003; Bender and Fitzgerald 2002; Chakalova et al. 2005; Hogga and Karch 2002; Kireeva et al. 2002; Schmitt et al. 2005; Schwartz and Ahmad 2005). The alternative model is that RT controls chromatin structure. Since chromatin assembly takes place at the replication fork, it represents a window of opportunity to remodel chromatin structure across the entire chromosome domains by replicating DNA in a different time and place in the nucleus (Gilbert 2002, 2010). In fact, an artificial system in which bovine papilloma virus vectors replicate at different times in subsequent S phases has demonstrated that changes in RT are accompanied by the assembly of different chromatin composition, with earlier replication associated with a higher level of histone acetylation (Lande-Diner et al. 2009; Zhang et al. 2002). While direct evidence for both of these models is still wanting, the two models together would predict a strong positive feedback loop to maintain stable epigenetic states within a cell type and to rapidly alter those states under the conditions of changing transcriptional regulatory networks that occur during cell fate changes.

The results discussed above summarized decades of literature leading to a long-established dogma that early RT is correlated to transcriptional activity. However, recent studies have cast doubt even on this seemingly incontrovertible relationship (Mesner et al. 2013; Rivera-Mulia et al. 2015). In Rivera-Mulia et al. (2015), the authors generated RT profiles for 26 human cell types representing various intermediate stages of development and correlated RT with transcriptome data. The domains with developmentally regulated RT often lacked or exhibited weaker associations of RT to chromatin structure and transcription as compared to RT constitutive domains. Moreover, approximately two thirds of the genes that switch RT were found to be strongly expressed when late replicating in one or more cell types investigated. Despite their ability to be transcribed at high levels when late replicating, transcription of these genes was still coordinated with RT, being upregulated just prior to a late to early switch and downregulated just after an early to late switch. Such observations argue against a model in which active transcription is sufficient for early replication. Moreover, studies in *S. cerevisiae* have found that Fkh1/2-dependent origin and RT regulation are independent from the proteins' role as transcription factors but rather dependent on their structural roles in origin clustering and spatial organization of chromosomal elements, which is likely mediated through dimerization of Fkh1 and Fkh2 (Knott et al. 2012; Ostrow et al. 2017). Collectively, the evidence suggests that RT is related to features of chromatin, including modifications and nuclear architecture, rather than transcription per se. The complexity of behaviour and chromatin marks present on these domains suggests that different domains are likely to be regulated by distinct combinations of mechanisms resulting

in an intricate context-dependent interplay between factors such as chromatin modifications, accessibility and 3D conformation.

The connection between RT and chromatin modifications has been characterized extensively, and despite many correlations, no single modification has been found to significantly correlate it with RT on a genome-wide scale in mammalian cells (Petryk et al. 2016; Pope et al. 2014; Ryba et al. 2010). In addition, knockdown of histone-modifying enzymes resulted in no significant differences in global RT compared to wild-type control, suggesting the lack of causality between any single type of chromatin modification and global RT establishment (Pope et al. 2014; Ryba et al. 2010). In *Drosophila* cells the chromatin modification H3K16ac and the histone modifier heterochromatin protein 1 (HP1) have been shown to be implicated in genome-wide RT regulation suggesting potential fundamental differences in mechanisms underlying RT regulation by chromatin modifications between *Drosophila* and mammalian cells (Lubelsky et al. 2014; Schwaiger et al. 2009, 2010). However, at local level, there have been studies suggesting causal links between histone modifications and RT highlighting the possibility of isolated mechanisms at play in specific regions in addition to (or somehow translating into) domain-level regulation in mammalian system (Casas-Delucchi et al. 2011; Goren et al. 2008). These evidences taken together further highlight the context-dependent correlation between RT and chromatin modifications.

The replication domain model posits that replication begins within TADs that reside in the nuclear interior that are characterized by generally high transcriptional potential. Meanwhile, replication gradually advances into adjacent late replicating TADs that are located at the nuclear periphery (LADs) or other repressive compartments and contain features associated with repressed transcription. Upon differentiation, TADs that switch RT acquire features associated with their new nuclear localization, while new compartment boundaries are established with new interactions. Despite the many aspects of the model that require further experimental data to validate, it fits well with existing data that largely argues for a domain-wide RT regulation that is not solely reliant on transcription potential of the loci.

There is some evidence that proteins involved in controlling higher-order chromosome architecture may be important for RT as well. For instance, cohesin, a ring-shaped protein complex, has been shown to be necessary for the formation of large chromatin loops and may be able to limit the number of active origins and slow down S phase progression as a result (Guillou et al. 2010). However, it is difficult to evaluate the involvement of cohesion in DNA replication since cohesion depletion leads to severe cell cycle defects that confound the interpretation of results collected from cycling cells. CTCF is also implicated in the formation of chromatin loops, but loss of CTCF has no effect on RT (Sima and Gilbert, unpublished observations). One protein that may control chromatin architecture and RT is the Rap1-interacting factor (Rif1). (see chapter by Buonomo in this issue) Rif1 is a conserved telomere-binding factor that co-localizes with the mid-S replication foci and may participate in the formation of chromatin loop structures (Foti et al. 2016). Depletion of Rif1 results in loss of mid-S replication foci and enhancement of initiation events in early S phase and disruption of RT across a large portion of the

genome (Cornacchia et al. 2012; Yamazaki et al. 2012). Together with the results showing that 3D conformation of chromatin is established coincidentally with the establishment of an RT programme (Dimitrova and Gilbert 1999; Dileep et al. 2015a, b), results suggest RT is regulated by 3D chromatin conformation, but direct evidence and mechanistic insight are lacking.

## 11.8 Reshaping the Epigenome

DNA methylation, histone modifications and chromatin accessibility constitute the epigenomic landscape, which is critical for cell identity maintenance, gene regulation and proper cellular function. Hence, epigenetic information must be also faithfully transmitted after each cell division to maintain cell identity (Alabert and Groth 2012). Recently, genomic studies have witnessed great progress in the characterization of the epigenome in multiple cell types and its dynamic changes during cell fate specification (Gifford et al. 2013; Roadmap Epigenomics et al. 2015; Xie et al. 2013). However, how the epigenome is inherited and the remodelling of chromatin structure at replication forks has been a long-standing mystery (see chapter by Groth in this issue). During DNA replication, chromatin must be disassembled to allow the replication fork to progress and needs to be reassembled onto the duplicated strands (Ramachandran and Henikoff 2015). Moreover, essential replication proteins at the replication fork are directly involved in recycling of parental histones (Huang et al. 2015). Indeed, distinct types of chromatin are assembled at different times during S phase (Alabert et al. 2015; Lande-Diner et al. 2009), supporting the model that RT can shape the epigenome. By characterizing proteins associated with newly replicated DNA, it has been shown that levels of histone modifications decrease by ~50% on newly synthesized strands, due to a combination of recycled and new unmodified histones. These modifications are restored at different rates thereafter, with some requiring continuous remodelling of both parental and new histones across multiple cell cycles (Alabert et al. 2015). A question going forward will be how the epigenome landscape is remodelled as a result of DNA replication during the process of differentiation.

## 11.9 Altered RD Organization and Disease

RT is an exceptionally stable epigenetic property that is not easily disturbed by loss or misexpression of single master regulators of cell fate or chromatin regulatory factors (Dileep et al. 2015a; Pope et al. 2011) Nonetheless, every cell fate change has been associated with specific changes in RT. Since the RT profile is such a stable epigenetic characteristic of cell type, it stands to reason that features of the RT programme (i.e. RT of specific domains) would be altered in diseases including cancer. Indeed, a range of genetic disorders have been shown to be associated with aberrant

RT programmes. For instance, in fragile X syndrome, a genetic disorder commonly associated with an affected *FMR1* allele containing a hypermethylated CpG island and expanded CGG repeats, the mutant *FMR1* allele was found to replicate later the unaffected allele (Hansen et al. 1993). On the other hand, ICF immunodeficiency syndrome results from abnormal escape of X chromosome inactivation via hypomethylation and was shown to be dependent on a temporally advanced RT (Hansen et al. 2000). In addition, abnormal regulation of RT has been observed in every cancer investigated to date (Amiel et al. 1998a, b, 1999; Korenstein-Ilan et al. 2002). Several studies indicate malignancies are accompanied by disruption of the temporal order of allelic replication; most regions of the genome normally replicate synchronously, but some have been found to replicate asynchronously in malignant cells and associated with poor prognosis, suggesting that RT might be an attractive biomarker for diagnostic purposes (Donley and Thayer 2013). Moreover, a series of studies linking dysregulated RT with increased mutagenesis and chromosomal instability led to speculation that RT programmes may function as the driving force of mutagenesis during tumorigenesis and late replicating domains have a higher mutagenesis rate than early replicating domains (Lang and Murray 2011; Stamatoyannopoulos et al. 2009). The different mutation rates and patterns associated with early and late replicating regions are likely due to the differential DNA mismatch repair (MMR) efficiency at the regions (Koren et al. 2012; Liu et al. 2013; Supek and Lehner 2015). A chromosome-wide replication delay, which has been observed in many cancer types, was also shown to be associated with a range of tumorigenic abnormalities including delays in mitotic condensation, gain or loss of chromosomes and increased rate of chromosomal rearrangements (Breger et al. 2005; Chang et al. 2007; Smith et al. 2001). Alternatively, RT changes can also be induced by chromosomal rearrangements as a result of juxtaposing regions of differential RT (Breger et al. 2004; Pope et al. 2012; Ryba et al. 2012). Naturally, the interdependent nature of RT changes and chromosomal instability during tumorigenesis has made it a formidable task to delineate cause and consequence. However, these results strongly suggest that alterations in RT have implications in tumorigenesis and the development of various diseases.

The only systematic genome-wide analysis of RT in cancers to date explored paediatric B-cell precursor acute lymphoblastic leukaemia (BCP-ALL) (Ryba et al. 2012). This is an excellent model cancer due to the well-studied genetic subtypes linked to prognosis. The RT profiles of leukaemia exhibited more heterogeneity than non-leukaemic B-cell controls, suggesting either variable arrested/altered differentiation states of the different samples or a generalized epigenetic dysregulation. Several lines of evidence suggested that this heterogeneity was due to altered developmental programmes. First, using a previously developed algorithm to identify the statistically most significant RT differences between samples, the authors identified ‘RT fingerprints’ unique to genetic subtypes of BCP-ALL, suggesting that at least some of the fingerprints were causally linked to the particular subtype. Secondly, the differences that distinguished leukaemia aligned to the boundaries of developmentally regulated RT domains that distinguish normal cell types further supporting abnormal developmental control. Finally, in a follow-up study, BCP-

ALL samples were passaged serially in immunodeficient mice as patient-derived xenografts and the RT profiles were found to be stable characteristics of patient samples, demonstrating that RT profiling can reveal stable epigenetic features of specific cancer clones (Sasaki et al. 2017). Further research will be needed to reveal whether such features can function as prognostic markers and their mechanistic links to cancer phenotypes. Since different types of chromatin are assembled at replication forks that are active at different stages during S phase (discussed above), aberrations in RT likely contribute to large-scale changes in chromatin structure and epigenetic states that persist in malignant cells thereby having profound effects on their transcriptional programmes. These findings provide strong impetus for further exploration of the mechanisms by which RT aberrations arise and its role in determining the biological features of human cancer.

## 11.10 Conclusion and Perspectives

From cytogenetic observations of replication foci to genome-wide RT mapping and the discovery of an uncanny alignment between RD and TAD boundaries, the last decade has witnessed a conceptual leap in the linkage between RT and chromatin 3D structure. In what has come to be known as the 'Replication Domain model', RT is regulated at the level of TADs, within which 1–4 replication origins are selected stochastically from many potential sites to replicate the RD/TAD within 45–60 min, while at a higher scale, TADs are organized during nuclear reassembly in such a way that RDs/TADs in close proximity replicate at similar times to form distinct spatially separated nuclear compartments. RDs/TADs are stable chromatin units with profound implications on important aspects of chromosomal properties including transcription regulation (Fig. 11.6). Still, we are a long way from a complete understanding of how RT is regulated and its biological significance, particularly in organisms with large genomes. 3D organization is tightly associated with RT but is not sufficient to dictate the RT programme (Dileep et al. 2015b). Although most individual chromatin readers, writers and erasers are not necessary for the RT programme, there are certainly critical players such as Rif1 (Buonomo chapter in this issue) that interact with this scaffold and contribute to the creation of subnuclear domains of differing initiation potential, and these players must be identified. We need better and more reliable origin-mapping tools, particularly those that can measure single-molecule initiation events and single-cell RT so that the stochastic probabilities of origin firing and domain-scale RT can be related to the single-cell transcription and chromatin state measurements being currently developed (Liu and Trapnell 2016; Ramani et al. 2017). We need better means to unravel the enigmatic relationship between RT and transcription, which seems to correlate well in some contexts and not at all in others. Finally, we need to understand the mechanisms linking higher-order chromosome architecture and RT. The advent of novel robust gene editing tools should permit a genetic dissection of the structure and RT of chromosome domains.

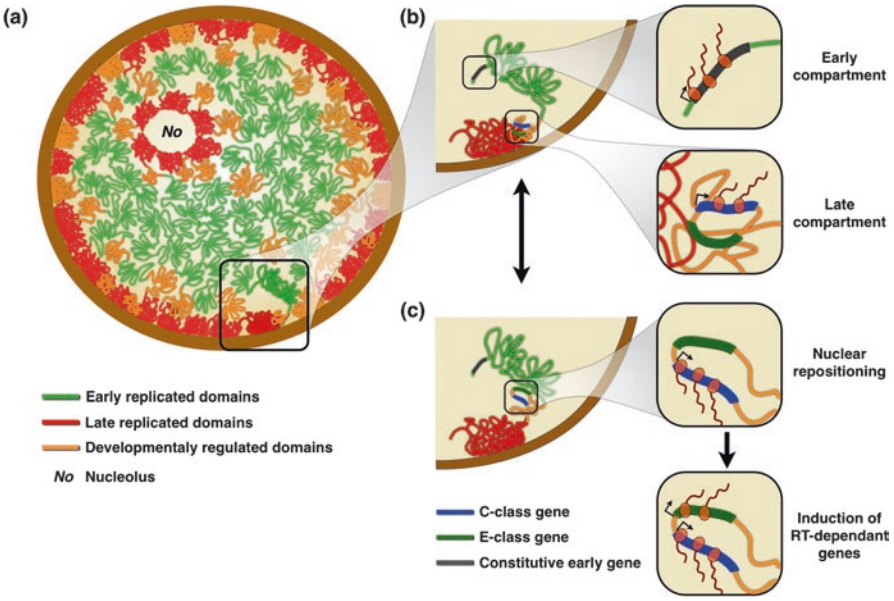


**Fig. 11.6** RD organization, RT and gene regulation in the nucleus during development (Adapted from Rivera-Mulia and Gilbert 2016b). Early RDs contain open chromatin at the nuclear interior, whereas late RDs are located at the lamina and nucleoli. During development, certain domains undergo a switch in RT from late to early replication while detaching from nuclear lamina. The RT switching domain harbours genes belonging to different classes based on their transcription potential during RT changes. There are genes (blue) that are induced only after the detachment from lamina and the RT change. There are also genes (green) that are expressed despite being at the periphery and late replicating

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# Chapter 12
# Rif1-Dependent Regulation of Genome Replication in Mammals

**Sara B.C. Buonomo**

**Abstract** Eukaryotic genomes are replicated starting from multiple origins of replication. Their usage is tightly regulated, and not all the potential origins are activated during a single cell cycle. In addition, the ones that are activated are activated in a sequential order. Why don't origins of replication normally all fire together? Is this important? And if so, why? Would any order of firing do, or does the specific sequence matter? How is this process regulated? These questions concern all eukaryotes but have proven extremely hard to address because replication timing is a process intricately connected with multiple aspects of nuclear function.

**Keywords** DNA replication timing • Nuclear architecture • Rif1 • Nuclear lamina • Nuclear organization • PP1 • Origin firing • Telomere replication

In 2012 the identification of the first genome-wide regulator of replication timing across evolution, a protein called Rif1 (Cornacchia et al. 2012; Hayano et al. 2012; Yamazaki et al. 2012), provided a long-awaited tool to start addressing these questions. However, Rif1 itself has also proven a very complex protein, confusingly involved in telomere length control (Gallardo et al. 2011; Hardy et al. 1992; Teixeira et al. 2004), DNA repair (Wang et al. 2009; Buonomo et al. 2009; Chapman et al. 2013; Daley and Sung 2013; Feng et al. 2013; Martina et al. 2014), DNA replication timing (Cornacchia et al. 2012; Dave et al. 2014; Hayano et al. 2012; Hiraga et al. 2014; Mattarocci et al. 2014; Peace et al. 2014; Sreesankar et al. 2015; Yamazaki et al. 2012), and nuclear architecture organization (Foti et al. 2016). Thus, the complexity of Rif1 biology reflects the intricacies of replication-timing control at a molecular level.

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## 12.1 Rif1 and DNA Replication Timing

Rif1 was originally discovered in budding yeast as a negative regulator of telomere length, where it is brought to the telomeric repeats through its interaction with Rap1 (Hardy et al. 1992), a sequence-specific DNA-binding protein that recognizes and directly binds telomeric repeats in yeast (Conrad et al. 1990; Lustig et al. 1990). Hence, the amount of Rif1 at telomeres is proportional to telomere length (Levy and Blackburn 2004). Interestingly, both sudden telomere shortening (Bianchi and Shore 2007) and Rif1 deletion-induced telomere elongation (Lian et al. 2011) correlate with a shift of telomere replication from late to early S phase. While it was originally hypothesized that telomere length per se might have affected the timing of firing of subtelomeric origins, these data suggested instead that Rif1 presence at budding yeast telomeres was responsible for their late replication (Hiraga et al. 2014). In mammalian cells, Rif1 is not detectable at normal telomeres (Silverman et al. 2004; Xu and Blackburn 2004) nor does it seem to be involved in telomere length regulation (Buonomo et al. 2009). This is consistent with the fact that mammalian telomeres do not specifically replicate in late S phase (Arnoult et al. 2010; Hultdin et al. 2001; Wright et al. 1999; Zou et al. 2004). Rif1 deficiency induces genome-wide changes in replication timing in *Schizosaccharomyces pombe* (Hayano et al. 2012), *Saccharomyces cerevisiae* (Peace et al. 2014), *Drosophila* (Sreesankar et al. 2015), mouse and human cells (Cornacchia et al. 2012; Yamazaki et al. 2012). Hence, Rif1-dependent control of replication timing is highly conserved throughout evolution.

In yeast, the control of replication timing via Rif1 requires its interaction with the Ser/Thr phosphatase PP1 (Dave et al. 2014; Hiraga et al. 2014; Mattarocci et al. 2014). Disruption of this interaction increases the amount of phosphorylated MCM4, thereby potentially promoting origin firing (Fu et al. 2011). According to the model drawn on the basis of these results, during the G1/S transition and the initial part of S phase, Rif1 recruits PP1 to the origins destined to fire later in S phase, acting locally to counteract the activating phosphorylation of MCM4 by Dbf4-dependent kinase (DDK). This hypothetical pathway places Rif1-dependent regulation of origin firing at the stage of execution of the replication-timing program. Recent data obtained also from human cell lines (Alver et al. 2017; Hiraga et al. 2017) support this hypothesis. However, there are also experimental results suggesting that there could be more to Rif1 function. The overlap between Rif1-bound origins and those whose timing of firing is dependent upon Rif1 is modest in yeast (Hayano et al. 2012; Peace et al. 2014), and in mouse embryonic stem cells (ESCs), there is no clear enrichment of Rif1 at the limited number of replication origins analyzed (Foti et al. 2016). This is puzzling in light of the model that sees Rif1 functioning locally at late origins as a PP1 adaptor, suggesting the possibility that Rif1 could control origin activation through additional mechanisms, acting on a genome-wide scale. In mammalian cells, there is evidence that this could be the case. Below, I am going to discuss some of the relevant data and what it tells us

about Rif1's role and the relationship between nuclear organization and regulation of replication timing.

## 12.2 Rif1, Nuclear Organization, and Replication Timing

The idea that spatial nuclear organization and sequential firing of replication origins are connected relies on evidence accumulated over the past several years. For example, based on the frequency of intra-domain chromatin interactions, the mammalian nucleus has been subdivided into two large compartments, A and B (Lieberman-Aiden et al. 2009), displaying a striking correlation with early and late replicating fractions of the genome (Ryba et al. 2010; Yaffe et al. 2010). More recently, it has been shown that the units that constitute the building blocks of these large nuclear compartments, the topologically associated domains (TADs) (Dixon et al. 2012), coincide with the units of replication-timing regulation, the developmentally defined replication domains (Pope et al. 2014). At the molecular level, it has recently been shown in budding yeast that Fkh1 and Fkh2 promote early firing of origins by mediating their clustering (Knott et al. 2012). On the other hand, the relationship between DNA localization at the nuclear periphery and its late replication is well established, with developmentally regulated loci switching replication from late to early S phase (LtoE) while also relocating toward the internal part of the nucleus and vice versa (Hiratani et al. 2008, 2010; Williams et al. 2006; Zhou et al. 2002). Similarly, the time at which replication timing is reestablished after mitosis (timing decision point, TDP), in early G1, coincides with chromosome repositioning, when domains destined to be late replicating occupy the nuclear periphery and peri-nucleolar areas (Dileep et al. 2015; Dimitrova and Gilbert 1999; Raghuraman et al. 1997). The peripheral position of late replicating domains is conserved throughout evolution.

Rif1 localizes to the nuclear periphery in a variety of cells, from yeast to mammals. In mammals roughly half of the pool of Rif1 is associated with the nuclear periphery and interacts with the lamina (Cornacchia et al. 2012; Foti et al. 2016; Roux et al. 2012; Yamazaki et al. 2012). In addition, Rif1 is distributed in large domains (Rif1-associated domains or RADs) strikingly overlapping with the late replicating genome (Foti et al. 2016). This distribution is highly reminiscent of the genome-binding profile of one of the major components of the nuclear lamina, Lamin B1. The nuclear lamina plays an important role in the organization of nuclear architecture (reviewed in Shimi et al. 2010). Lamin B1 covers large domains called Lamin B1-associated domains (LADs) (Peric-Hupkes et al. 2010) that also overlap with the late replicating genome (Peric-Hupkes et al. 2010), although not as extensively as RADs (our unpublished results). Finally, LADs and RADs display a substantial degree of overlap. Overall, these data suggest that Rif1 could have a role in three-dimensional (3D) organization of the genome in the nucleus. In agreement with this hypothesis, Rif1 deficiency in mammalian cells has been shown to alter chromatin loop size (Yamazaki et al. 2012) and chromocenter compaction (Cornacchia et al. 2012) and induce loss of temporal coordination of the replication

of neighboring as well as more distantly interacting replicons (Cornacchia et al. 2012; Foti et al. 2016). Nevertheless, these effects could be consequential to the changes of replication timing. However, we have recently shown that deletion of Rif1 in primary mouse embryonic fibroblasts arrested in G0 impacts the 3D organization of replication domains as early as the first G1 after Rif1 deletion (Foti et al. 2016). Such early alterations of chromatin architecture might be responsible for the changes in replication timing that appear in the following S phase. In fact, the constrains that normally limit domain interactions within either early or late replicating domains (Takebayashi et al. 2012) are lost in the absence of Rif1, with late domains promiscuously establishing numerous, low-frequency contacts with early domains and vice versa (Foti et al. 2016) (Fig. 12.1a, b). In agreement with this, we have observed by locus-specific 3D FISH that LtoE switches induced by Rif1 deletion are also accompanied by a tendency to relocate the corresponding portions of the genome from the periphery to the interior of the nucleus (our unpublished data). These observations indicate that in G1, Rif1 plays a role in the organization of nuclear architecture, possibly at two different levels: it orchestrates and spatially constrains contacts between different replication domains and ensures peripheral positioning of at least some late chromosomal domains. We suggest that these functions of Rif1 in nuclear architecture could be a first tier at which Rif1 instructs replication timing, as both of these aspects of nuclear organization have been related to replication timing.

## 12.3 Rif1 and PP1

In line with the identification of yeast Rif1 as a mediator of PP1 function at replication origins, mammalian and *Drosophila* Rif1s were identified in PP1-associated complexes (Guruharsha et al. 2011; Moorhead et al. 2008; Trinkle-Mulcahy et al. 2006). In addition, we have obtained structural and functional data classifying mouse Rif1 as a *bona fide* PP1 regulatory subunit (Sukackaite et al. 2017). It is therefore clear that one Rif1 function conserved throughout evolution is to act as a regulatory subunit of PP1 (Sreesankar et al. 2012). However, the idea that Rif1 could play a role in G1 during the establishment of replication timing is not mutually exclusive with its proposed function at late origins in G1/S. The spatial sequestration of late origins away from early ones (and from DNA replication's limiting factors? (Mantiero et al. 2011; Patel et al. 2006; Tanaka et al. 2011; Wu and Nurse 2009)) through the formation of RADs would also result in creation of large areas of high PP1 concentration which could facilitate PP1 function on late origins later in the cell cycle. In this scenario, Rif1 would act both in early G1 at the level of establishment (Fig. 12.2a, b) and later, at G1/S transition, at the execution step of the replication-timing program (Fig. 12.2c).

Alternatively, it is formally possible that the architectural function of Rif1 has no bearing on replication timing per se and that the common denominator between nuclear 3D organization and replication timing is Rif1 as a molecule, independently

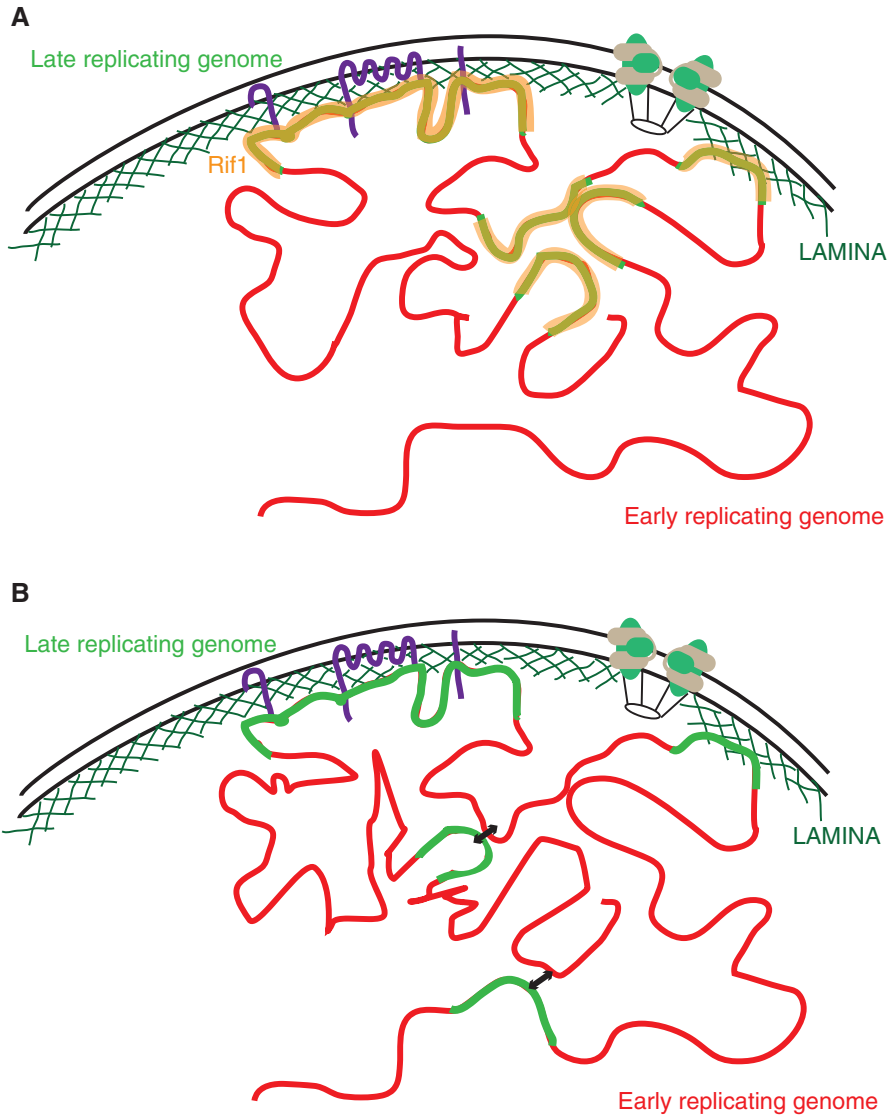


**Fig. 12.1** (**a**) Rif1 (orange) coats late replicating genome (green) and limits the interactions between regions with the same replication timing. (**b**) In absence of Rif1 late replicating regions of the genome not associated with Lamin B1 change replication timing as well as lose replication-timing specificity of interactions

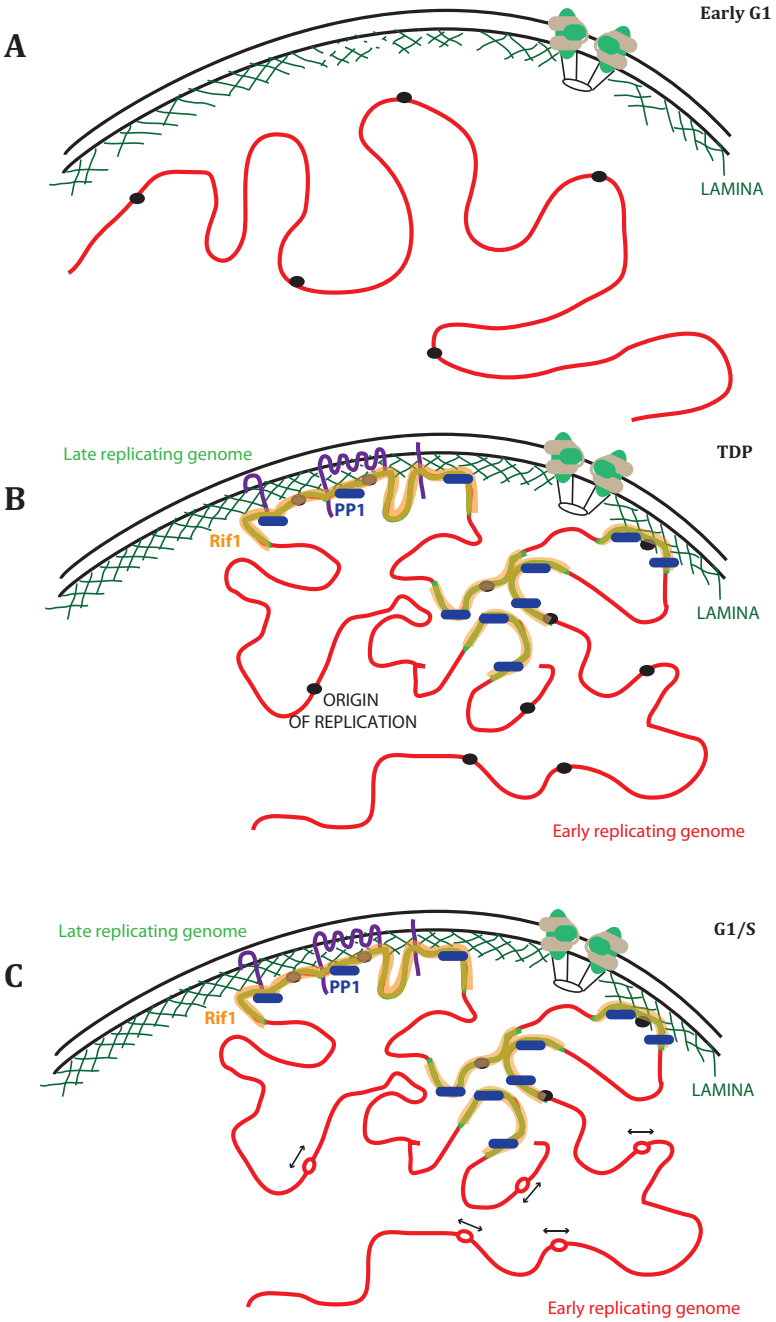


**Fig. 12.2** (**a**) At the end of mitosis, replication-timing determinants are lost together with chromatin-organized positions in the nucleus. (**b**) Reestablishment of replication timing takes place in G1. Rif1 associates with chromatin at the end of telophase-early G1 (Yamazaki et al. 2012), in time to enforce the restriction of 3D contacts between genomic regions within the same replication timing at the TDP. The result is the architectural compartmentalization of the late replicating genome and the consequent creation of domains of high PP1 density. (**c**) When DDK activity increases at G1/S, late origins are embedded in RADs and surrounded by high concentrations of PP1. Possible physical sequestration and high concentrations of phosphatase inhibit their firing

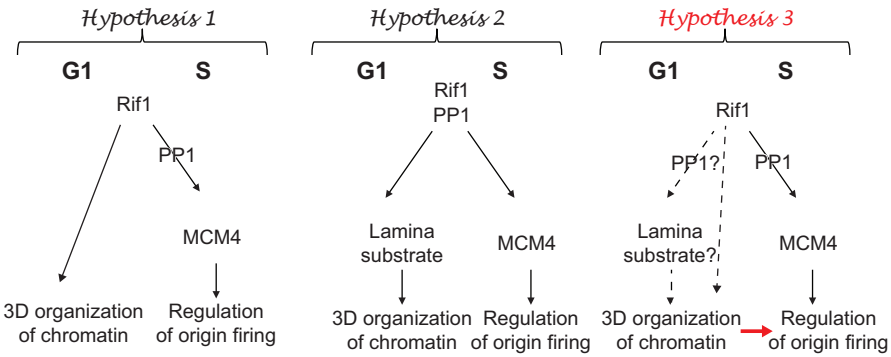


**Fig. 12.3** Nuclear architecture and DNA replication timing could be two independent processes connected only by their respective dependence upon Rif1 (Hypothesis 1 and 2). Alternatively, Rif1-dependent organization of nuclear architecture could also influence the firing of replication origins independently of Rif1 (Hypothesis 3)

involved in both processes. However, the loss of replication-timing-specific domain contacts observed in G1 in Rif1 null cells seems to contradict this hypothesis (Fig. 12.3).

Finally, direct transposition of functional data between distant organisms can be misleading. It cannot be excluded that in Metazoa, Rif1 has evolved an architectural function that is absent or not essential in yeast. This change could indeed have taken place concomitantly with the transition from a closed to an open mitosis, the evolution of the lamina, and the need to re-create an ordered nucleus after each mitosis (Sazer et al. 2014). In fact, it is not known whether the lamina or other nuclear architectural components also play a role in either the establishment or execution of the DNA replication-timing program. However, some preliminary observations suggest that they could. We have found that replication-timing regulation of the late replicating genome is indeed differentially sensitive to Rif1 function, depending on the concomitant stable presence of Lamin B1. The regions of the late replicating genome concurrently associated with Rif1 and Lamin B1 (RADs-LB$^+$) do not change their replication timing upon Rif1 deletion. In contrast, late replication of genomic regions associated with Rif1 only (RADs-LB$^-$) is entirely Rif1 dependent (Foti et al. 2016) (Fig. 12.1a, b). We have therefore unexpectedly revealed a further level of complexity in the regulation of replication timing, once again coinciding with some aspects of nuclear architecture organization. This finding also reinforces the question of the role of PP1 in Rif1-mediated control of replication timing. If PP1 is indeed the sole, ultimate effector of Rif1 function, another protein has to be responsible for recruiting PP1 at RADs-LB$^+$ in Rif1 null cells. This is in principle possible, as the PP1 phosphatase has been identified as a partner of different proteins associated with the nuclear envelope, such as AKAP149 (Steen et al. 2000) and LAP1β (Santos et al. 2013).

## 12.4 Telomere Length Regulation, DNA Repair, Replication Timing, and Nuclear Organization: Where Is the Connection?

Rif1 has been involved in processes other than replication timing and organization of nuclear architecture, namely, DNA repair (Wang et al. 2009; Buonomo et al. 2009; Chapman et al. 2013; Daley and Sung 2013; Feng et al. 2013; Martina et al. 2014) and telomere length regulation (Gallardo et al. 2011; Hardy et al. 1992; Teixeira et al. 2004). Such diversity of roles is puzzling and could either reflect multiple independent functions of different parts of this very large protein, multiple outcomes of a single molecular property of the protein applied in different contexts, or both.

The understanding of the functional organization of Rif1 domains is still rudimentary. The N terminus is highly conserved, composed of numerous HEAT repeats (Silverman et al. 2004; Sreesankar et al. 2012) required for localizing the protein to double-strand breaks in mouse cells, through binding of phosphorylated 53BP1 (Escribano-Diaz et al. 2013). The large middle region is non-conserved and predicted to be a non-structured polypeptide. Interestingly, in both mouse and human Rif1 genes, this region is encoded by a single, ~3000-bp-long exon, accounting for almost half of the entire coding region. This unusual gene organization might suggest acquisition of a mammalian-specific function. The C terminus of Rif1 contains three recognizable domains, indicated as conserved regions C, of which CI and CII are present from yeast to humans, while CIII is only conserved within vertebrates. In Metazoa, CI harbors PP1-interacting motifs, which are located at the N terminus of yeast Rif1. CII is a very intriguing portion of the protein. In Metazoa it contains a DNA-binding domain, preferentially recognizing cruciform structures (Sukackaite et al. 2014; Xu et al. 2010). However, the relevance of this interaction with DNA in vivo is yet to be determined, as Rif1 association with insoluble nuclear fractions renders this technically challenging to assess. Moreover, the in vivo distribution of cruciform DNA is unclear, having been associated with promoter melting, activated origins of replication, and DNA recombination intermediates (Brazda et al. 2011). In vivo DNA binding has been shown for fission yeast Rif1, which recognizes a consensus sequence capable of forming G-quadruplex (G4) (Kanoh et al. 2015). However, the specific domain mediating this interaction is yet to be mapped. A putative DNA-binding domain seems to be present in the budding yeast Rif1 but not in CII (Sreesankar et al. 2012), which is instead required for interaction with Rap1 (Shi et al. 2013), DDK (Hiraga et al. 2014), and for Rif1's tetramerization (Shi et al. 2013). The residues involved in the tetramerization of *S. cerevisiae* Rif1 largely overlap with the positions implicated in DNA binding in the human and mouse homologs, suggesting the possibility that the two functions are related. Multimerization has also been shown for the mammalian protein (Xu et al. 2010), supporting the idea that Rif1 could form patches of a lamina-associated protein network anchoring RADs to the nuclear periphery.

Clearly one of the conserved properties of Rif1 is its interaction with PP1. It is therefore reasonable to hypothesize that PP1 could also mediate Rif1 functions at

telomeres (Mattarocci et al. 2016) and/or during the DNA damage response (DDR), by ensuring the dephosphorylation of telomere or DDR-specific substrates. However, at least for budding yeast telomeres, there is an alternative hypothesis that Rif1-dependent control of late replication could be part of the mechanism directing telomerase-dependent telomere elongation. As telomerase-dependent telomere lengthening occurs after conventional replication (Diede and Gottschling 1999), telomere replication in late S phase would in turn limit the time available for telomerase-dependent telomere elongation (Bianchi and Shore 2007). This mechanism could explain how telomere length equilibrium is maintained via differential replication of longer and shorter telomeres: longer telomere length would translate into higher Rif1 occupancy at that specific telomere, and higher Rif1 occupancy would impose later subtelomeric origin firing and telomere replication. Late telomere replication will result in a shorter window of opportunity for telomerase to extend that telomere within a cell cycle. Therefore, longer telomeres are replicated later and are less likely to be extended by telomerase.

## 12.5 The Intricate Politics of Nuclear Function

The identification of Rif1 has provided an important gateway into understanding the genetic and molecular control of replication timing. However, it has also raised numerous questions, some unexpected, and uncovered a confusing network that interconnects several fundamental nuclear functions. For example, studying the impact of Rif1 deficiency on replication timing, gene expression, and nuclear organization in different cell types has revealed that nuclear architecture could be the common denominator between regulation of gene expression and replication timing. The relationship between these two aspects of nuclear function has been widely debated, as there are general correlations between genomic regions that are early replicating and those that are expressed and, reciprocally, between late replicating regions and heterochromatic, transcriptionally repressed domains. Changes of gene expression and replication timing during development often move in the same direction, with switches of replication from early to late S phase (EtoL) coinciding with reduced gene expression and vice versa. These data have prompted the idea that one aspect of nuclear function could determine the other. However, what controls what has been challenging to determine due to contradicting data (reviewed in Rivera-Mulia and Gilbert 2016). Recently, a detailed analysis of replication timing and gene expression changes at different stages of human ESC differentiation has in fact revealed that the temporal relationship between gene expression and replication-timing changes is different in different regions of the genome (Rivera-Mulia et al. 2015), indicating clearly that at least one important variable is still missing from the picture. Our data indicate that the missing component could be nuclear positioning. We found that “simply” changing replication timing is not sufficient to affect gene expression. Different cell types are either permissive or not for proliferation in the absence of Rif1, probably depending on the status of the DNA damage checkpoints.

For cells like primary mouse embryonic fibroblasts (pMEFs), where Rif1 deletion impairs proliferation, no gene expression changes can be detected as a consequence of the deletion, although chromatin organization and replication timing are affected (Cornacchia et al. 2012; Foti et al. 2016). However, in cells that proliferate upon Rif1 deletion, for example, immortalized MEFs or ESCs, a progressive deregulation of gene expression can be observed in time (Foti et al. 2016). These data suggest that an increasingly amplified deregulation of nuclear architecture could turn into transcriptional changes, in agreement with the evidence linking subnuclear positioning and gene expression (Andrulis et al. 1998; Finlan et al. 2008; Mattout et al. 2011; Peric-Hupkes et al. 2010; Reddy et al. 2008; Zullo et al. 2012). It remains unclear if the effect of nuclear architecture on gene expression is mediated through modification of the epigenetic landscape: we could not detect any major changes in the epigenome after Rif1 deletion, at least for the limited number of histone modifications we have examined (Foti et al. 2016).

## 12.6 Conclusions

The discovery of Rif1 and its multifaceted functions has represented an entry point into the molecular labyrinth of nuclear functions. Future work will have to span across different fields in order to unravel how specific to Rif1 this functional network is or if it is a general feature of nuclear structural components. Importantly, understanding to what extent PP1 mediates different aspects of Rif1's role bears the potential of creating separation of function mutants. Integrating different aspects of nuclear function is fundamental to dissect the complexity of cellular transitions such as aging, differentiation, and transformation.

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# Chapter 13
# G-Quadruplexes and DNA Replication Origins

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**Abstract** DNA replication ensures the accurate duplication of the genome at each cell cycle. During S phase, tens of thousands of replication origins throughout the vertebrate genome are activated according to a spatiotemporal program. The genome-wide mapping of origins in several model systems has identified G-quadruplexes—higher-order DNA structures formed from G-rich sequences—as potential key regulators of origin activity. Here, I describe genetic experiments demonstrating the role of G-quadruplexes in origin function. I discuss the different means by which G-quadruplexes might regulate origin function. Finally, comparisons of replicon organization in the three domains of life suggest that G-quadruplexes may have retained a conserved role in origin function during evolution.

**Keywords** Replication origin • G-quadruplex • Chromatin • Evolution

## 13.1 Main Text

In each cell cycle, tens of thousands of start sites for DNA replication are activated to ensure the faithful duplication of complex vertebrate genomes. The DNA replication program is responsible for guaranteeing the correct inheritance of genetic information, but replication origin selection and the timing of activation are flexible. This plasticity facilitates adaptation of the replication program to changes associated with the establishment of cell type specificity, which mostly involves transcriptional modifications. DNA sequence and chromosomal environment contribute to the selection and activation of mammalian replication origins through complex processes including epigenetic modifications, the binding of specific *trans*-acting factors and nuclear localization. These aspects are described in Chaps. 2 and 3 of this book. This more tightly focused section concentrates on the recent identification of a DNA motif common to most strong replication origins in vertebrates.

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This motif may be considered a "consensus" element within replication origins. We will discuss its potential role in controlling replication initiation in vertebrates and other species.

The distribution of replication origins throughout the genome defines the replicons: regions copied by the bidirectional progression of two replication forks arising from a single origin. Pioneering pulse radio-labeling experiments have estimated mean replicon size at about 150 kb in human cells (Berezney et al. 2000). The replicon model proposed by Jacob, Brenner, and Cuzin describes the regulation of bacterial chromosome replication. According to this model, a positive *trans*-acting factor known as the initiator binds to a specific *cis*-element, the replicator. This binding induces the bidirectional synthesis of the bacterial chromosome in response to a signal during S phase (Jacob et al. 1963). This model was validated by the identification of the DnaA protein as the initiator and of oriC as the replicator for replication of the *E. coli* chromosome. The large size of the genomes of eukaryotic cells results in a need for hundreds (yeast) to tens of thousands (vertebrates) of replicons. A spatiotemporal program of DNA replication is then established by the coordinated activation of these replicons.

Replication is initiated in a two-phase process, to ensure that each replicon is replicated once, and only once, in each cell cycle. During G1 phase, two copies of the MCM2-7 ATPases are loaded onto replication origins in a head-to-head orientation, to form the "pre-replicative complex" (pre-RC). This step requires three "licensing" factors, the hexameric ORC, Cdc6, and Cdt1. Pre-RC formation can occur only in late mitosis and G1 phase, because CDK (cyclin-dependent kinase), a potent inhibitor of pre-RC formation, is inhibited by APC/C and cyclin-dependent kinase inhibitors (CKIs) during these phases of the cell cycle (Diffley 2004). Many more origins than are actually used during S phase are licensed during this period, to ensure that the DNA is loaded with sufficiently large numbers of pre-RCs before the start of S phase. These "dormant" origins provide a backup if the fork stalls and may render the replication program more flexible and robust to perturbation (Ge et al. 2007; Ibarra et al. 2008). At the transition between the G1 and S phases, the APC/C complex and CKIs are inactivated, allowing S phase to be initiated through the cumulative effects of two kinases essential for pre-RC activation: S-CDK and DDK (a Dbf4-dependent kinase composed of Cdc7 kinase and its activator). However, not all replication origins fire at the same time. Instead, each cell type has its own precise temporal program, regulating the coordinated firing of groups of origins and defining replication timing domains with similar replication timing patterns (Hiratani et al. 2008). The activation of replication origins during S phase is regulated in part by limiting concentrations of key activating factors (Mantiero et al. 2011; Tanaka et al. 2011). These important findings led to the development of the hypothesis that there is a general mechanism for the correct temporal activation of origins during S phase. In early S phase, only early-firing origins can be activated, because these origins are the most "accessible" to the limiting firing factors. These limiting factors are released after firing and can then bind to and activate less

accessible origins and so on. This discovery led to studies aiming to identify the factors regulating the accessibility of specific pre-RCs to these limiting factors.

## 13.2 Vertebrate Replication Origins Are Enriched in Potential G4 Motifs

The highest-resolution method currently available for origin mapping in vertebrate cells is based on the purification and quantification of short nascent strands (SNS) (Prioleau and MacAlpine 2016). In this method, 1.5–2.5 kb nascent strands specific to replication origins are purified on the basis of the resistance of these strands to λ-exonuclease digestion due to the incorporation, by the primase, of small RNA primers at their 5′ ends (Bielinsky and Gerbi 1998) (Fig. 13.1a). This step is necessary as it results in digestion of the large excess of broken genomic DNA, which would generate a nonspecific signal if not correctly removed. The initial large-scale mapping of replication origins over a fraction of the genome (1%) in vertebrate cells provided important new insight into the potential mechanisms underlying origin firing (Cadoret et al. 2008; Sequeira-Mendes et al. 2009). These analyses revealed that replication origins were nonrandomly distributed, being particularly abundant in transcriptional regulatory elements, such as TSS and enhancer regions. CpG islands (CGI), which are associated with ~50% of TSS, were identified as the most efficient origins, and about 35% of all origins are of this type (Cadoret et al. 2008; Sequeira-Mendes et al. 2009). These analyses paved the way for genome-wide studies, which confirmed the observations made at a smaller scale. Moreover, these new studies showed that potential G4 motifs (pG4s) were particularly abundant within replication origins in mouse and human cells (Cayrou et al. 2011, 2012; Besnard et al. 2012).

PG4s consist of four tracts of at least three guanine residues separated by other bases (1–7), and they can fold to form a secondary structure known as a G-quadruplex (G4). The G4 is a four-stranded helical DNA structure constructed from G-quartet building blocks, square planar assemblies of four Hoogsteen-bonded guanine bases. These planar G-quartets stack on top of each another, forming four-stranded helical structures. Computational analyses have identified about 370,000 pG4s in the human genome (Huppert and Balasubramanian 2005). The distribution of pG4s is not random; they tend to accumulate in promoter regions and in the 5′ or 3′-untranslated regions of mRNA. There therefore seems to be a selection pressure driving the retention of these motifs at specific sites (Rhodes and Lipps 2015). They also cluster at telomeres, which, in humans, consist of tandemly arranged TTAGGG repeats. Several lines of indirect evidence have convincingly demonstrated the formation of G4 structures in vivo (Valton and Prioleau 2016). The development of specific G-quadruplex-interacting drugs has led to the identification of perturbed genomic regions in vivo that are prone to the formation of G4 structures. Pyridostatin, a specific G4 ligand, has been shown to induce transcription- and replication-dependent DNA damage, resulting in arrest of the cell cycle in the G2/M phase and activation

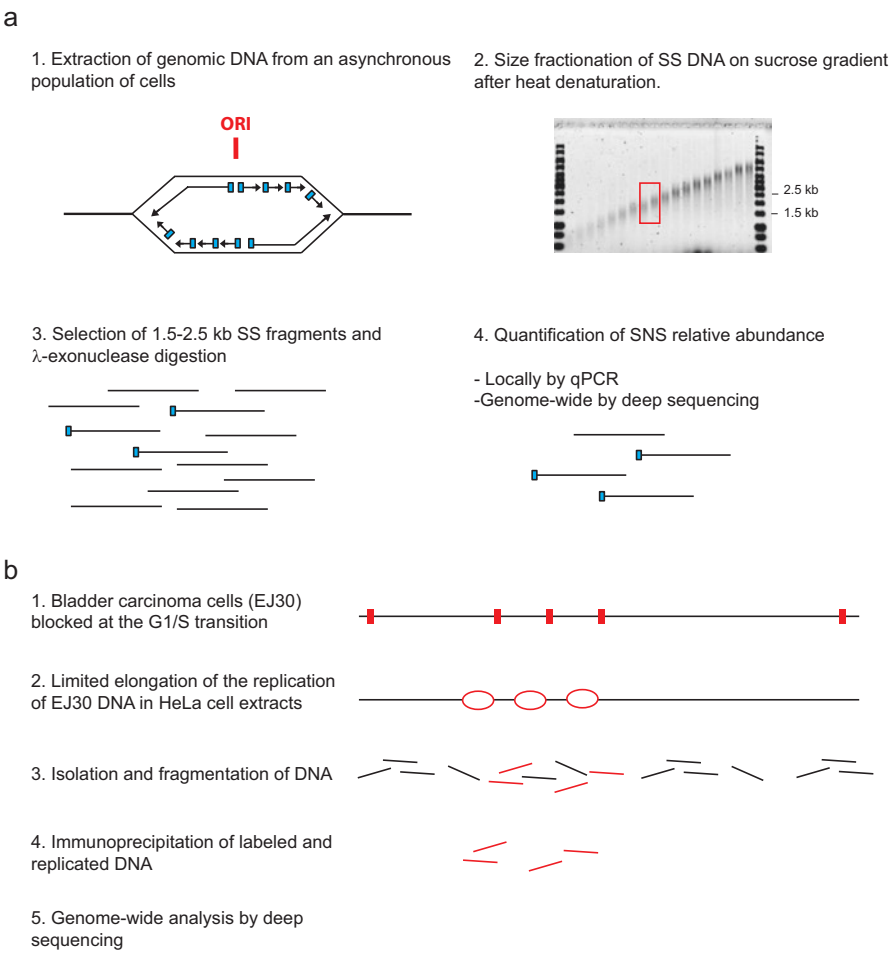



**Fig. 13.1** Methods for the genome-wide mapping of replication origins. (**a**) Description of the main steps in the purification of short nascent strands. A bubble located at a replication origin is represented, leading strands and Okazaki fragments synthesized on the lagging strands contain small RNA primers (blue rectangles). Genomic DNA is first gently extracted from an asynchronous population of cells. The purified DNA is then denatured by heating and run on a 5–30% linear sucrose gradient. Fractions containing SS DNA of 1.5–2.5 kb in size are selected. The DNA is phosphorylated and digested with a large excess of λ-exonuclease to remove excess nonspecific broken DNA; the SNSs are protected by their small RNA primers. Analyses may be carried out locally, by qPCR, or genome-wide, by deep sequencing the SNS-enriched preparation. (**b**) Genome-wide mapping of early-firing origins by ini-seq. Cells are synchronized by treatment with mimosine at the G1/S transition to allow origin licensing. Licensed origins are represented by red rectangles. The purified nuclei are then transferred into a HeLa S phase extract, to ensure limited elongation and the labeling of early-firing origins. These origins are indicated by red bubbles. DNA is then isolated and fragmented. Labeled DNA is purified and subjected to deep sequencing (Adapted form Langley et al. 2016)

of the ATR/Chk1 pathway (Rodriguez et al. 2012). Genome-wide mapping of γH2AX, a DNA damage marker, after the addition of pyridostatin showed that this drug mostly targeted gene-rich regions containing clusters of sequences capable of adopting a G-quadruplex conformation. These data suggested that pyridostatin interacted predominantly with non-telomeric regions at low concentration and demonstrated the formation of structured G4s throughout the human genome. The formation of G4 structures was recently captured directly in vivo (Hänsel-Hertsch et al. 2016). In this study, a G4 ChIP-seq protocol was developed with an engineered antibody specific for G4 structures, to map the location of these structures genome-wide in human epidermal keratinocyte HaCaT cells. About 10,000 G4 peaks were mapped, mostly in regulatory regions depleted of nucleosomes. G4 structures were particularly abundant in the promoters and 5′UTRs of highly transcribed genes. Moreover, enhanced G4 formation was found to be associated with stronger transcriptional activity, suggesting links between chromatin structure, gene expression, and transcription in the formation of G4 structures.

## 13.3 Does the Enrichment of SNSs in pG4 Reflect a Functional Role of G4 in Replication Origin Firing?

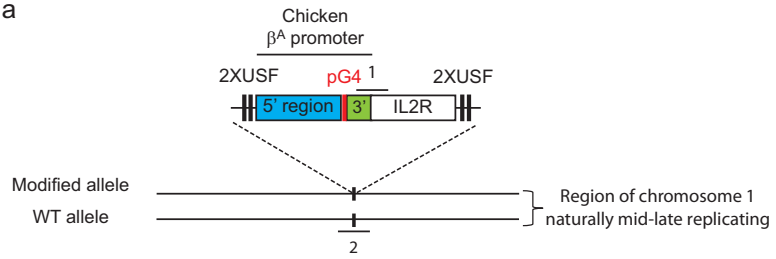
Studies in human and mouse cell lines have shown that ~80% of the origins mapped by the SNS method overlap with a pG4 (Cayrou et al. 2011, 2012; Besnard et al. 2012). However, not all the pG4s overlapped with a replication origin, suggesting that pG4s may be important determinants of origin specification but that they are not sufficient on their own (Picard et al. 2014). The discovery that pG4s might play a positive role in replication initiation came as a surprise, as pG4s were generally considered to impede the progression of replication forks (Valton and Prioleau 2016). It was suggested that the strong enrichment in pG4 of origins detected by SNS was a bias induced by the use of λ-exonuclease (Foulk et al. 2015). The authors of this study observed that the digestion of genomic DNA with low concentration of λ-exonuclease (one thousandth those used for SNS purification) tended to result in an enrichment in GC-rich and pG4-containing sequences, due to the lower efficiency of digestion for such sequences with this enzyme. However, the digestion of DNA from nonproliferating cells with a large excess of λ-exonuclease, as in classical SNS preparations, does not lead to specific peaks at pG4-rich sequences (Cayrou et al. 2011, 2015). It is therefore important to follow a very strict protocol for λ-exonuclease digestion. An orthogonal method with a resolution similar to that of SNS was recently developed, to exclude definitively any possible bias due to λ-exonuclease treatment (Langley et al. 2016). In this new method, initiation site sequencing (ini-seq), nuclei were synchronized at the G1/S border by treatment with mimosine and were then transferred to S phase extract (Fig. 13.1b). Newly synthesized DNA was directly labeled with digoxigenin-dUTP, with a short pulse corresponding to elongation by 1.5–2.5 kb. The labeled DNA was purified and analyzed by deep

sequencing. This method, which detects only early-firing origins, identified ~25,000 discrete peaks. About 50% of the activated origins identified by ini-seq were at TSS and contained pG4s. There was also a high level of concordance between the sites identified in this way and those identified by SNS-seq, validating the λ-exonuclease approach. Thus, λ-exonuclease, when used properly, is not subject to strong biases, contrary to the suggestions of Foulk et al. (2015).

Many potential initiation sites may be used stochastically within zones of initiation, but SNS preparations may be artificially enriched in sites containing pG4, due to transient pausing at G4 structures. This hypothesis is supported by the observation that SNS enrichment peaks are systematically located 3′ to a pG4, with a sharp decrease observed at the position of the pG4. This peak shape suggests that there is, indeed, a very transient pause at pG4s (Valton et al. 2014; Comoglio et al. 2015). However, the ini-seq method, which is based on very limited elongation, revealed a strong enrichment of TSS containing pG4s, suggesting that site-specific initiation occurs at these positions, with other regions not overrepresented.

A recent genetic study in the chicken DT40 cell line used a new functional origin assay to investigate the role of pG4s in replication initiation (Valton et al. 2014). This test is based on the ability of active origins to advance the replication timing of regions that are naturally replicated late (Fig. 13.2a). The well-characterized chicken $\beta^A$ origin, which contains a pG4, was inserted, by homologous recombination, into a region devoid of strong initiation events that normally replicates in mid-late S phase. The insertion of the chicken $\beta^A$ origin was sufficient to cause local SNS enrichment, demonstrating the ability of this origin to function in an ectopic position. However, this origin was not sufficient to advance replication timing. The flanking of this origin with binding sites for USF (upstream stimulating factor), a transcription factor, modified local histone H3 acetylation and H3K4me2, which advances replication timing in a $\beta^A$ origin-dependent manner (Hassan-Zadeh et al. 2012) (Fig. 13.2b, WT). This functional assay demonstrated that the $\beta^A$ promoter contained a bona fide origin. The role of the pG4 within the $\beta^A$ origin was also investigated in detail. Point mutations decreasing the stability of the G4 structure in vitro also reduced origin efficiency, as shown by SNS and timing assays (Fig. 13.2b). Thus, G-quadruplex structures play a critical positive role in replication initiation, independent of fork pausing (Valton et al. 2014). Furthermore, the orientation of the pG4 within the $\beta^A$ origin determines the position at which replication is initiated, consistent with genome-wide data showing a SNS enrichment peak ~220 bp 3′ to pG4 (Cayrou et al. 2012). Finally, cooperation between the pG4 of the $\beta^A$ model origin and a 250 bp module located 3′ to it was required for origin function (Fig. 13.2b, Δ3′). This finding is not consistent with SNS enrichment being due to a replication fork barrier induced by the pG4, because the pG4 is maintained in this construct. Deletion of the pG4 in a second model origin also abolished origin function. Overall, these results provide a formal demonstration that, at least in two model origins, pG4 is required for origin function.

a




b

| Construct | Relative SNS enrichment | Replication timing shift | PG4 probability |
|---|---|---|---|
| WT GGGGGGGGGGGGGCGGG | 100% | + | 1 |
| Δ5' | 62% | ND | 1 |
| Δ3' | 13% | - | 1 |
| ΔpG4 | 11% | - | 0 |
| m14 GGGGGGGGGGGGGAGGG | 95% | + | 1 |
| m4 GGGAGGGGGGGGGCGGG | 56% | ND | 0.6 |
| m9 GGGGGGGGAGGGGCGGG | 34% | ND | 0.6 |
| m6 GGGGGAGGGGGGGCGGG | 18% | - | 0.3 |
| m16 GGGGGGGGGGGGGCGAG | 25% | - | 0 |
| m12 GGGGGGGGGGGAGCGGG | 11% | - | 0.1 |

**Fig. 13.2** A G4 and a cooperating *cis*-module are required for the activity of the chicken $\beta^A$ globin origin. (**a**) Strategy for dissecting the role of *cis*-elements in origin activity. A construct containing a 1.1 kb fragment overlapping the chicken $\beta^A$ globin promoter fused to the reporter IL2R gene and flanked by two copies of binding sites for the transcription factor USF was inserted by homologous recombination. The $\beta^A$ promoter is represented by three fragments: a blue rectangle corresponding to an 850 bp region upstream from the pG4, a red rectangle corresponding to the pG4, and a green rectangle corresponding to a 250 bp region located 3′ to the pG4. Site-specific insertion was achieved in a 50 kb region of chromosome 1 devoid of strong initiation sites and replicated in mid-late S phase. Origin activity was tested in two independent assays. Relative SNS enrichment was quantified with primer pair 1 (black horizontal bar), systematically compared with a background signal located 5 kb away from the insertion site. SNS were also quantified at an endogenous origin (positive control). In the second method, the capacity of the transgene to advance replication timing was investigated with a pair of primers specific for the transgene (primer pair 1), with comparison to the WT allele analyzed with a primer pair overlapping the insertion site (primer pair 2). (**b**) Analyses of origin activity in different constructs (Adapted from Valton et al. 2014). As a reference, the WT ectopic $\beta^A$ origin was considered to display 100% relative enrichment. Constructs containing either deletions of part of the $\beta^A$ promoter (Δ5′, Δ3′ and ΔpG4) or single point mutations affecting the pG4 (indicated in *red*) were tested for origin function. Relative SNS enrichment is indicated for these mutants. The capacity of these constructs to advance replication timing was also analyzed for a fraction of constructs (data for Δ3′ is not published yet). + and – indicate transgenes that do and do not advance replication timing, respectively. The potential G4 probability is also shown in the table. The 17-mer $\beta^A$ G-rich motif can, in principle, fold into ten intramolecular G4 structures. For each mutant, we defined the PG4 probability as the number of possible G4 structures divided by the number of possible G4 structures for the WT sequence

## 13.4 To What Extent Have the Mechanisms Involved in Origin Selection Been Conserved During Evolution?

Studies of the nature and mode of operation of replicators and initiators in different model systems may reveal conserved mechanisms involved in origin selection during evolution, shedding light on the role of pG4 in origin selection in vertebrates. The replicator has two main functions, as a site for initiator recognition and as a region favoring the melting of DNA. Replication of the bacterial chromosome is initiated at a single origin of replication, oriC, and is regulated by the coordinated action of numerous *trans*-factors including DnaA, the initiator. All the replicators identified in bacteria to date consist of three functional modules: a cluster of binding sites for the initiator DnaA (DnaA boxes), the DNA unwinding element (DUE), and binding sites for regulatory proteins (Wolański et al. 2014). These elements control the opening up of the DNA double helix and regulate the initiation of bacterial chromosome replication. The DUE is a typical AT-rich stretch. DnaA boxes from different bacteria have a common core sequence motif (TTATCCACA) recognized by the replicator, DnaA. A similar organization is also found in many archaea, despite the use of multiple origins to replicate the genomes of these organisms. Studies in several strains have revealed that the basic structure of replication origins is conserved among archaea, with an AT-rich unwinding element and several conserved repeats (origin recognition box, ORB) (Kelman and Kelman 2014). Biochemical and structural approaches showed that the ORB elements act as recognition sites for the Orc/Cdc6 initiator protein. Interestingly, in one of the two origins (oriC1) found on the main chromosome of several haloarchaea, the conserved "G-string" motif (GGGG) found in most archaea origins is followed by another specific G-rich sequence (TGGGGGGG) (Norais et al. 2007). In *H. hispanica*, this long G-rich motif was shown to be necessary for efficient replication initiation (Wu et al. 2014). The capacity of this G-rich motif to form a G4 structure in vitro was not tested, but it will be important to determine whether or not the regulation of oriC1 in *H. hispanica* involves the formation of a G4 structure. The common G-string element found in most archaeal ORBs is recognized by the AAA+ domain of the replicator Orc/Cdc6 (Dueber et al. 2007; Gaudier et al. 2007). It has therefore been suggested that the extended halophile-specific G-stretches in haloarchaeal ORBs play an essential role in the interaction between the Orc/Cdc6 protein and its origin at high intracellular salt concentrations (Wu et al. 2014). Eukaryotic DNA replication initiates at multiple sites, from several hundred in yeast to tens of thousands in vertebrates. Replication origins have been extensively analyzed in the budding yeast *Saccharomyces cerevisiae*, in which small origin fragments can act as autonomously replicating sequences (ARSs), mediating the propagation of episomal plasmids. The signal includes a 17 bp ARS consensus sequence (ACS) required for recruitment of the ORC complex in a chromatin environment. In addition to the ACS, origin function requires flanking elements, including transcription factor binding sites and/or sequences known to exclude nucleosomes. It is therefore thought that the ACS increases the affinity of a subset of nucleosome-depleted regions (NDRs) for ORC,

thereby conferring origin activity on these loci (Eaton et al. 2010). A recent study on an industrially important methylotrophic budding yeast, *Pichia pastoris*, showed that this yeast used at least two types of replication origin: a GC-rich type associated with TSS and an AT-rich type resembling the canonical budding yeast origin (Liachko et al. 2014). GC-ARSs are mostly associated with early-replicating regions, whereas AT-ARSs tend to be associated with regions that replicate later. Moreover, a specific constrained motif was identified (TYGAAC) at GC-ARSs. This motif matches a binding site for human Hsf1 (heat shock factor 1) and could be recognized by the yeast HSF protein. It is possible that this GC-motif is involved in both origin activity and transcriptional regulation. The regions encompassing these origins also have a NDR region, although three nucleosomes are excluded, rather than just one, as for classical AT-rich yeast origins. Thus, this yeast, despite not having pG4 in its origins, is essentially similar to vertebrate origins, some of which have been shown to be GC-rich and associated with TSS. In the fission yeast *Schizosaccharomyces pombe*, origin positioning depends principally on the presence of sequences with a high adenine and thymine content (A+T-rich sequences), but no consensus elements have been identified (Segurado et al. 2003; Heichinger et al. 2006; Hayashi et al. 2007). The information is recognized by the Orc4 subunit of the ORC, which is unique among eukaryotes in that it contains a large domain harboring AT-hook subdomains that target the ORC to A+T-rich sequences (Chuang and Kelly 1999). A combined experimental and bioinformatics approach was developed for the characterization of origins in three distantly related fission yeasts: *S. pombe*, *S. octosporus*, *and S. japonicus* (Xu et al. 2012). A search for motifs predictive of origin function confirmed the key role of poly-A motifs in *S. pombe* and *S. octosporus*. However, *S. japonicus* origins were characterized by three highly abundant motifs, a 17 bp polyG motif, a head to tail repeat of CTCGCT, and the binding site for the Sap1 protein, a *trans*-acting factor known to exclude nucleosomes. It has been suggested that the polyG motif identified favors nucleosome exclusion, although this has not been definitively demonstrated. Origins mapped in a protozoan human parasite (*Leishmania major*) and in *Drosophila* (Cayrou et al. 2012; Comoglio et al. 2015; Lombraña et al. 2016) have also been shown to contain G-rich motifs. Thus, comparisons of the nature of replicators in different species have indicated that polyG motifs have been selected at replication origins several times during evolution. As suggested above, one potential role of polyGs is nucleosome exclusion. This motif has also been shown to be involved in initiator binding in haloarchaea. It would be of particular interest to determine whether G4 structures are involved in the regulation of origins in these species, as shown for the model origin from chicken.

## 13.5 How Do pG4s Regulate Origin Function?

Two phases of the cell cycle are particularly important for the regulation of DNA replication in eukaryotes. During G1, the chromatin is loaded with prereplication complexes (pre-RCs), but the absence of specific kinases prevents their activation at this time (Zegerman 2015). This phase is known as the licensing period. On entry into S phase, the activation of specific S phase kinases (CDKs and DDK) leads to the firing of pre-RCs, which are then released from the chromatin with no possibility of refolding onto it, thereby preventing re-replication. pG4s may be involved in licensing and/or firing. In one model of origin specification derived from the results of studies in *S. cerevisiae*, pre-RC formation is favored in nucleosome-depleted regions (NDRs) (Eaton et al. 2010; Berbenetz et al. 2010). NDRs containing ACSs have been shown to be the best substrates for pre-RC formation in vivo. It has been suggested that pG4s tend to exclude nucleosomes, thereby potentially favoring pre-RC formation (Fenouil et al. 2012) (Fig. 13.3). Alternatively, G4 formation may facilitate DNA melting and, therefore, the initiation of replication. Finally, pG4s may be recognized by specific factors involved in the formation of a functional origin (Fig. 13.3). Support for this hypothesis has recently been provided by the observation that the ORC binds preferentially to G4 structures formed on RNA or single-stranded DNA (Hoshina et al. 2013). The N-terminal part of human RecQL4, the ortholog of the essential firing factor Sld2 of *S. cerevisiae*, also binds G4 structures with high affinity (Keller et al. 2014). The observation that pG4 determines the location of the initiation site 3′ to the G4 structure suggests the involvement of a *trans*-acting factor able both to recognize the orientation of the G4 and to affect the position of the replication start site. Helicases, such as FANCJ and Pif1, which can interact with and unwind G4 structures in the 5–3′ direction, may control the direction of the process (Fig. 13.3). Genetic and genomic studies have shown that pG4s are not sufficient to define an origin of replication. A 250 bp *cis*-element located 3′ to the pG4 has been shown to cooperate with the pG4 in the chicken $\beta^A$ origin. This cooperating module may facilitate NDR formation, favor the formation of G4 structures, or facilitate the recruitment of licensing and/or firing factors. The identification of key motifs within the cooperating *cis*-module should improve our understanding of the functioning of pG4.

## 13.6 Concluding Remarks

The genome-wide mapping of replication origins in vertebrates has led to the identification of pG4s as motifs common to the most efficient, earliest, and constitutive origins. It has been demonstrated that a subset of pG4s adopt a G-quadruplex structure in vivo, particularly at actively transcribed promoters. The dynamics of G4 folding and unfolding may be so rapid at certain sites that G4 structures cannot be detected by existing methodologies. G4 structures are controlled by several

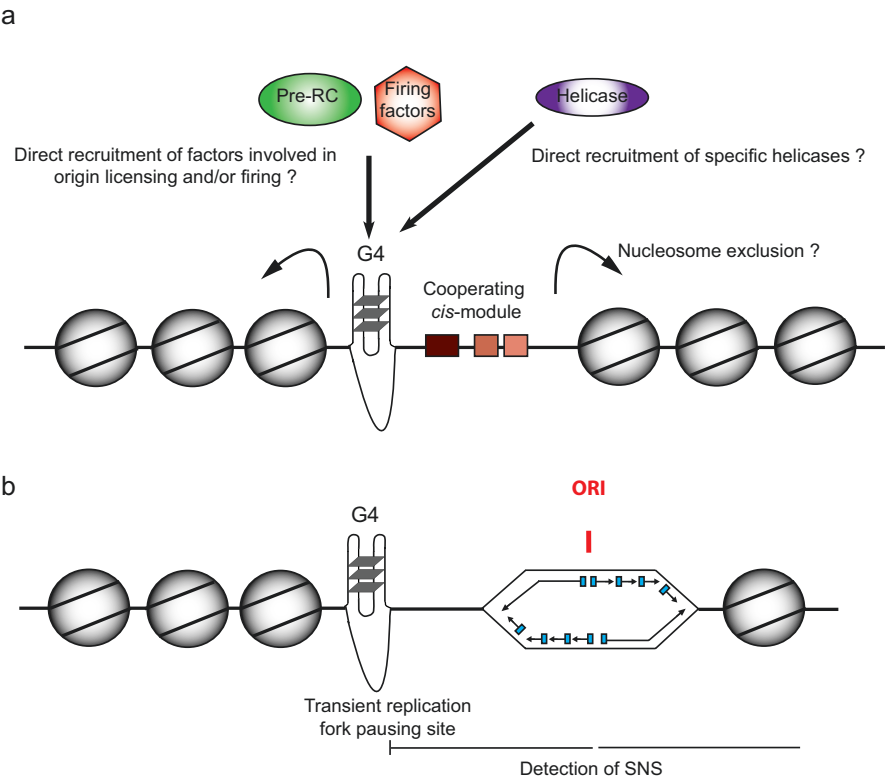



**Fig. 13.3** Potential mechanisms involving the action of G4 structures in origin selection and SNS profiles. (**a**) G4 structures can recruit pre-RC, firing components, and/or specific helicases. Alternatively, G4 may be involved in nucleosome exclusion and/or origin melting. A cooperative *cis*-module is required for origin function. The function of this module remains to be determined. (**b**) G4 structures at replication origins transiently block the progression of the leading strand initiated about 250 bp 3′ to the origin

mechanisms, including transcription, replication, and the action of specific helicases. Other mechanisms such as molecular crowding and the action of *trans*-acting factors capable of deforming DNA may also be involved. Surprisingly, these motifs have also been identified in replication origins in several other model systems (yeasts, archaea, protozoans, and *Drosophila*) suggesting a common mode of regulation. The development of the powerful CRISPR/Cas9 genome editing system should make it easier to dissect the nature of the *cis*-elements involved in origin selection. These studies should provide new clues to the molecular mechanisms involved in the regulation of origin firing and, more specifically, the role of pG4 in origin selection.

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# Chapter 14
# Interaction of Rif1 Protein with G-Quadruplex in Control of Chromosome Transactions

**Kenji Moriyama, Mong Sing Lai, and Hisao Masai**

**Abstract** Recent studies on G-quadruplex (G4) revealed crucial and conserved functions of G4 in various biological systems. We recently showed that Rif1, a conserved nuclear factor, binds to G4 present in the intergenic regions and plays a major role in spatiotemporal regulation of DNA replication. Rif1 may tether chromatin fibers through binding to G4, generating specific chromatin domains that dictate the replication timing. G4 and its various binding partners are now implicated in many other chromosome regulations, including transcription, replication initiation, recombination, gene rearrangement, and transposition.

**Keywords** Replication timing • G-quadruplex • Rif1 • Telomere • DSB repair • Nonhomologous end joining • Homologous recombination

## 14.1 Introduction

DNA with a specific sequence feature or base composition can adopt non-B-type forms of DNA under certain chemical conditions. For example, guanine-rich DNA has been known to take part in structures not only in the standard B-type DNA but also in other nonstandard forms of DNA including Z-DNA, hairpins, cruciforms, triplexes, and G-quadruplexes (Doluca et al. 2013). The presence of these unusual forms of DNA has been known for decades. It is only during the recent years that potential physiological importance of the non-B-type DNA starts to be realized. Among these structures, G-quadruplex (G4) has been a focus of intense research in the past several years. G4 can be formed under physiological conditions, and genetic and physical evidence points to its existence in the living organism. A number of

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recent studies strongly suggest that G4 or its related structures play crucial roles in various chromosome metabolism (reviewed in Maizels and Gray 2013; Murat and Balasubramanian 2014; Maizels 2015; Rhodes and Lipps 2015).

We have studied mechanisms of how DNA replication timing during S phase is regulated and have identified a crucial factor, Rif1, which plays a major, conserved role in regulation of spatiotemporal program of DNA replication (Hayano et al. 2012; Yamazaki et al. 2012; Cornacchia et al. 2012). Subsequent studies revealed that Rif1 regulates replication timing by binding to intergenic G4 structures (Kanoh et al. 2015). Other studies have suggested the potential role of G4 in the site selection for initiation of DNA replication in eukaryotic genomes (Besnard et al. 2012; Cayrou et al. 2011, 2012; Valton et al. 2014; Langley et al. 2016).

In this short article, we would like to first discuss Rif1 and its biological roles and then describe biochemical functions of Rif1 with particular emphasis on its ability to bind to G4. We will show a model on how Rif1 may regulate replication timing. Finally, we will introduce latest findings on the physiological functions of G4 structures in regulation of diverse chromosome transactions.

## 14.2 Rif1 Protein and Chromosome Regulation

### *14.2.1 Rif1 in Telomere Regulation*

Rif1 (Rap1-interacting factor 1) was first identified in budding yeast *Saccharomyces cerevisiae* (Sc) as a cofactor or mediator for Rap1 at silencers and telomeres (Hardy et al. 1992). Rap1 (repressor-activator protein 1) encodes an essential protein that binds extensively to telomeric regions, where its function is related to both transcriptional silencing and telomere maintenance (Marcand et al. 1997). Rif1 interacts with Rap1 at its carboxy-terminal domain (Fig. 14.1a). Cells lacking Rif1 are defective in transcriptional silencing and display an increase in the telomere length. Rap1 also recruits a second interacting factor Rif2 (Rap1-interacting factor 2) via its C-terminus. Rif1 and Rif2 form a functional complex that is essential for telomere length regulation (Wotton and Shore 1997). Recent studies using X-ray crystallography had revealed the architecture of Rap1-Rif1-Rif2 protein backbone at telomeres (Shi et al. 2013). Rif1 assists the essential telomere protection function of the CST (Cdc13-Stn1-Tel1) complex and inhibits the nucleolytic processing at telomeres (Anbalagan et al. 2011; Bonetti et al. 2010). Rif1 also possesses a telomeric anti-checkpoint activity and is required for protection of telomere-free chromosome ends which mimic double-strand breaks (Xue et al. 2011, 2016; Ribeyre and Shore 2012).

Rif1 was also identified in another yeast, fission yeast *Schizosaccharomyces pombe* (Sp) (Kanoh and Ishikawa 2001). However, unlike ScRif1, SpRif1 does not bind to SpRap1. It is recruited to telomeres through a different telomeric protein called Taz1, where it regulates the telomere length and telomere position effect (TPE). SpRap1 and SpRif1 have been shown to exert opposite effects in telomere

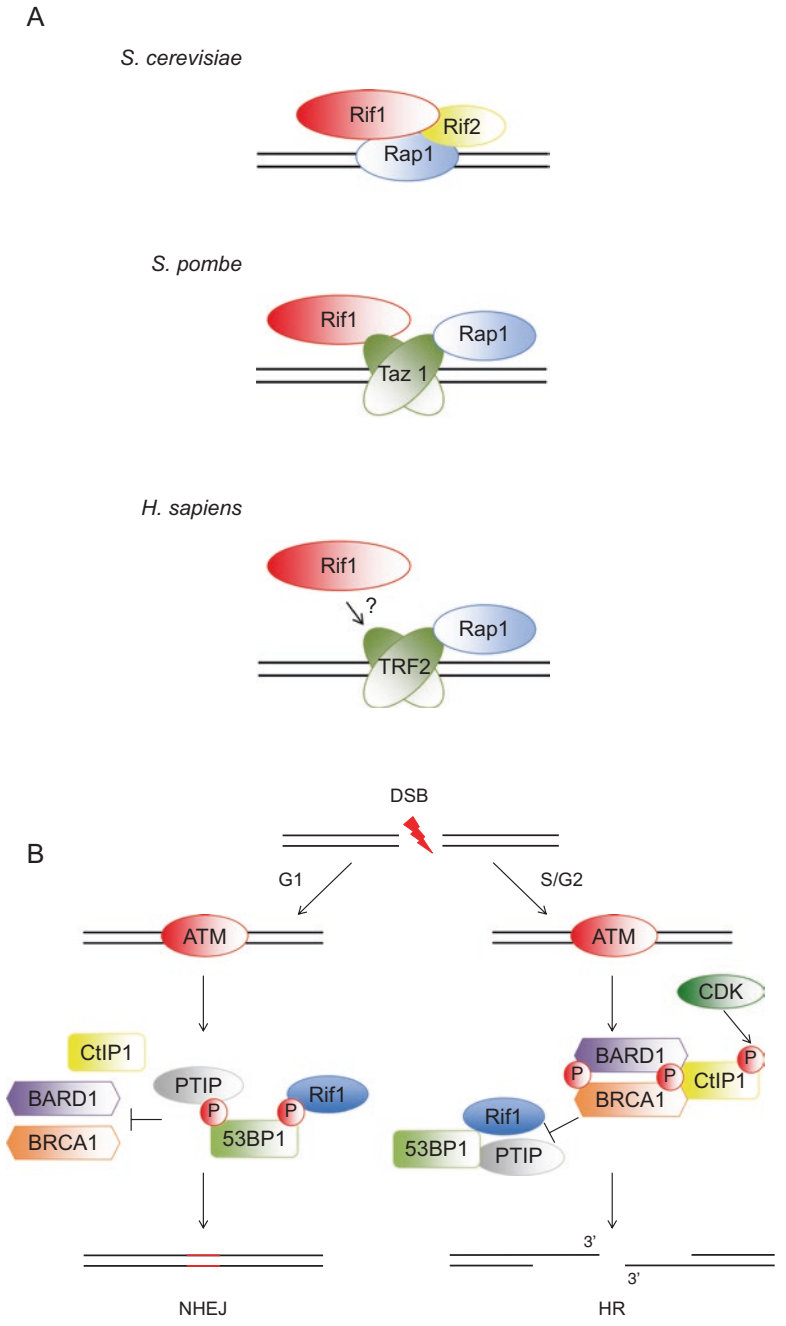


**Fig. 14.1** Roles of Rif1 protein in telomere, DSB repair, replication, and transcription. (**a**) Schematic representation of telomeric complexes involving Rif1 in budding yeast (*S. cerevisiae*), fission yeast (*S. pombe*), and human (*H. sapiens*). (**b**) A proposed role for Rif1 in regulation of repair of double-stranded DNA breaks (*DSBs*). In G1, 53BP1 is phosphorylated by ATM and localizes at the site of DSBs. Rif1 and PTIP are then recruited by the phosphorylated 53BP1 to the break

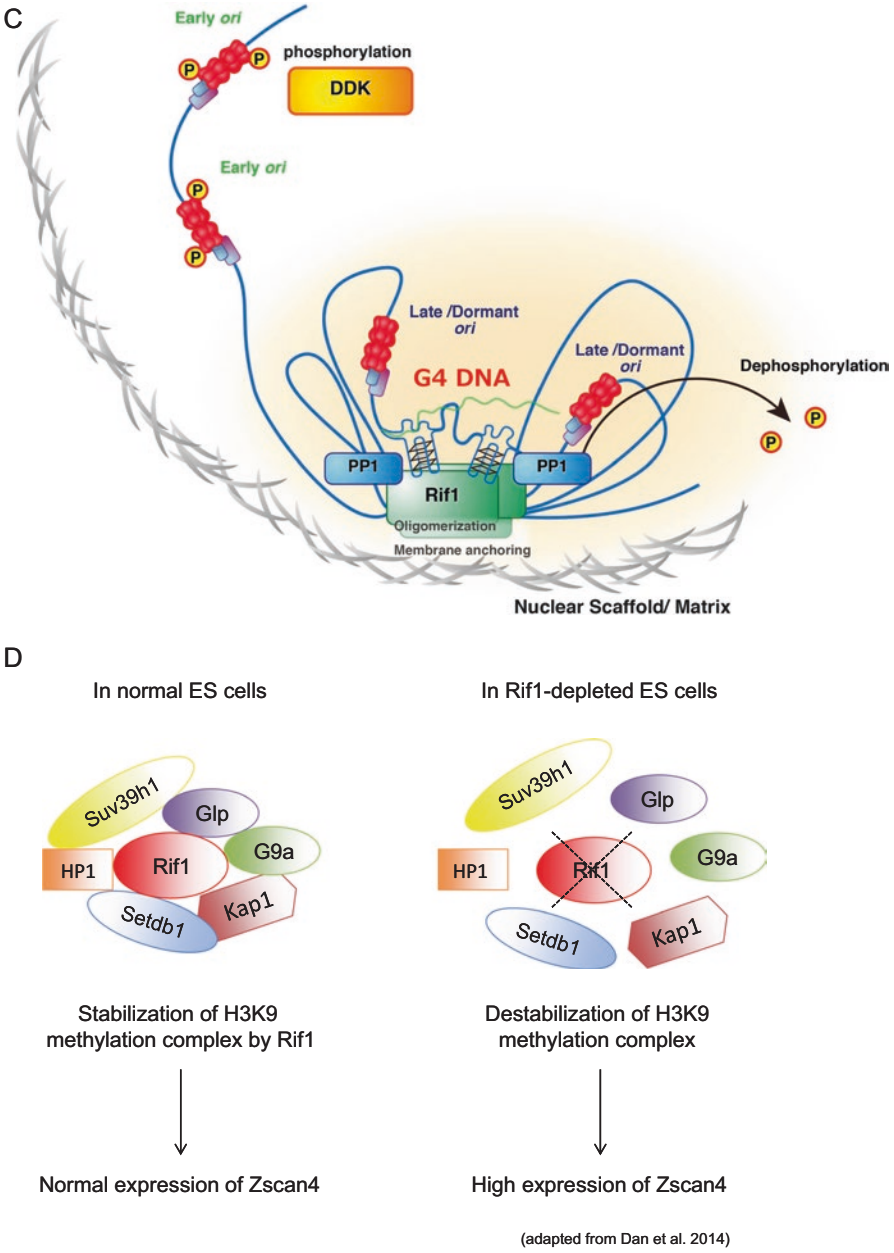


**Fig. 14.1** (continued) sites and inhibit the 5'–3' resection of the DNA ends. This process facilitates the repair of the DSBs by nonhomologous end joining (NHEJ). In S/G2, BRCA1 and BARD1 are phosphorylated by ATM, whereas CtIP is phosphorylated by CDK. The phosphorylated complex displaces 53BP1/Rif1/PTIP from the sites of DSBs and recruits the MRX complex to initiate 5'–3' resection and thus promote HR repair. (**c**) Rif1 binds to intergenic G4 structure to generate replication-suppressive chromatin domain near nuclear periphery by facilitating the chromatin loop formation through its G4 DNA-binding and oligomerization activities. (**d**) A model for Rif1-mediated transcriptional regulation of Zscan4 gene

homeostasis. SpRap1, but not SpRif1, is required to regulate 3′ overhang formation and prevents telomere fusions. Whereas *rap1*Δ exacerbates the cold sensitivity of *taz1*Δ cells, *rif1*Δ restores full viability of *taz1*Δ at 25 °C (Miller et al. 2005). In another yeast, *Candida glabrata*, Rif1 is required for correct telomere length regulation and also plays a positive role in subtelomeric silencing (Castaño et al. 2005; Rosas-Hernández et al. 2008).

The presence of Rif1 in higher eukaryotes suggests that the functions of this protein are conserved. Mouse ortholog of Rif1 (mRif1) is highly expressed in pluripotent stem cells, early embryonic cells, and germ cells. In an early report, mRif1 was also reported to be associated with telomeric DNA and to physically interact with a mouse telomere-associated protein, mTrf2 (Adams and McLaren 2004). However, hRif1 may not be involved in telomere regulation, as it can be found at non-telomeric locations and does not colocalize with human telomeric proteins Trf1, Trf2, or human Rap1 at normal telomeres, although overexpression of human Rif1 (hRif1) in *S. cerevisiae* interfered with telomere length control (Silverman et al. 2004; Xu and Blackburn 2004). Consistent with this prediction, Rif1 knockout mice or cells show no obvious telomere defects (Buonomo et al. 2009). On the other hand, Dan et al. (2014) reported that mRif1 controls telomere length through transcriptional silencing of Zscan4, a factor promoting recombination-mediated telomere elongation in ES cells. Thus, Rif1 appears to play an indirect role in telomere length homeostasis, possibly in a cell type-specific manner.

### *14.2.2 Rif1 in Regulation of Repair of Double-Stranded DNA Breaks*

The first indication of Rif1 involvement in DNA damage response came from the observation that human Rif1 only localized to damaged telomeres and accumulated at double-stranded DNA breaks (DSBs) in an ATM- and 53BP1-dependent manner (Silverman et al. 2004; Xu and Blackburn 2004). In vertebrates, Rif1 also participates in the intra-S-phase checkpoint to slow down DNA synthesis in response to DNA damage and also contributes to replication stress survival (Silverman et al. 2004; Buonomo et al. 2009; Xu et al. 2010). In this process, Rif1 accumulates at stalled replication forks in a manner dependent on ATR and 53BP1 (Buonomo et al. 2009). Rif1 interacts directly with BLM, a DNA helicase deficient in Bloom syndrome, and recruitment of Rif1 to stalled forks is delayed in BLM-deficient cells (Xu et al. 2010).

Subsequent studies have further uncovered the potential functions of vertebrate Rif1 in DNA damage responses. Cells respond to DSBs in two major pathways in order to repair the damages. One pathway is called nonhomologous end joining (NHEJ), which involves the religation of DNA ends. Another pathway is called homologous recombination (HR), which utilizes homologous sequence, usually the sister chromatids, to guide error-free repair. How cells select the repair pathways has remained unclear. Earlier studies showed that Rif1 participates in HR-mediated

repair of subsets of DSB (Buonomo et al. 2009; Wang et al. 2009). However, Xu et al. (2010) showed that Rif1 plays roles in the recovery of stalled forks generated by replication stress rather than in HR-dependent repair of DSB. More recent studies showed that Rif1 is a key player in DSB repair pathway choice and facilitates NHEJ (Fig. 14.1b). In G1 phase, when DSB occurs, Rif1 is recruited by the phosphorylated 53BP1 at the DSB sites, which in turn inhibits 5'–3' DNA end resection and thus promotes NHEJ. In S/G2 phase, CDK phosphorylation of CtIP facilitates its interaction with BRCA1. The CtIP-BRCA1 complexes displace Rif1 and 53BP1 at the DSB sites and recruit MRN complex to initiate resection that is required for HR repair (Chapman et al. 2013; Di Virgilio et al. 2013; Escribano-Díaz et al. 2013; Zimmermann et al. 2013). The recruitment of Rif1 upon DNA damage depends not only on 53BP1 but also on ATM in both human and mouse cells. Interactions among Rif1, ATM, and MRN complex are observed in *Xenopus* egg (Kumar et al. 2012).

It was previously reported that budding yeast ScRif1 does not significantly associate with DSB (Xue et al. 2011). However, more recent reports show that ScRif1 can also localize near DSB (Martina et al. 2014; Xue et al. 2016). Notably, ScRif1 does not inhibit end resection upon DSB. Instead, ScRif1 promotes DSB resection by cooperating with the MRX-Sae2 complex and thus is important for DSB repair by HR (Martina et al. 2014). Whether and how vertebrate Rif1 participates in HR repair is still under a debate and needs further studies.

### *14.2.3 Rif1 in Regulation of Temporal and Spatial Regulation of DNA Replication*

Besides its functions at telomere regulation and DNA damage response, Rif1 plays conserved and crucial roles in global regulation of replication timing program. DNA replication occurs at different timing and probably at different nuclear locations in different genome segments. This temporal and spatial program of DNA replication is determined at a discrete point during early G1 phase (known as “timing decision point”), where major chromatin repositioning and anchoring take place (Dimitrova and Gilbert 1999) to generate chromosome structures that dictate spatial and temporal organization of replication.

In fission yeast, *hsk1* (ortholog of budding yeast Cdc7) is not essential under certain physiological conditions or genetic backgrounds. In fact, *mrc1*Δ, in which late origins are fired in the presence of hydroxyurea, could suppress *hsk1*Δ and restore growth. Therefore, search for a novel suppressor of *hsk1*Δ cells was expected to lead to discovery of new factors that may regulate replication timing. This screening led to identification of *rif1*$^+$ (Hayano et al. 2012). In the absence of Rif1, subtelomeric regions as well as many late or dormant origins on the chromosome arms are vigorously fired in the early S phase. Similar results were later reported in budding yeast, although the replication timing effect seems to be more restricted to telomere-proximal regions (Lian et al. 2011; Peace et al. 2014). The striking effect of Rif1 in

global regulation of DNA replication was also observed in mouse and human cells (Cornacchia et al. 2012; Yamazaki et al. 2012; Masai et al. 2017). In Rif1-depleted cells, the mid-S-phase-specific pattern of replication foci was lost, and the replication timing program proceeded from early to mid/late S phase with early-replicating foci pattern, whereas the very late S-phase pattern with heterochromatin foci remained.

How does Rif1 influence replication timing on a genome-wide basis? It was initially discovered that phosphorylation of Mcm is significantly enhanced in Rif1-depleted human cells and that this hyper-phosphorylation depends on Cdc7 kinase (Yamazaki et al. 2012). One clue came from the findings that Rif1 possesses a conserved SILK/RVxF motif, which is a docking motif essential for the protein phosphatase 1 (PP1) interaction (Sreesankar et al. 2012; Davé et al. 2014; Hiraga et al. 2014; Mattarocci et al. 2014). In fact, yeast Rif1 interacts with PP1 through the SILK/RVxF motif, and Rif1/PP1 counteracts the DDK-dependent phosphorylation of Mcm complex, thus preventing DNA replication initiation (Fig. 14.1c; Davé et al. 2014; Hiraga et al. 2014; Mattarocci et al. 2014). Genetic and functional interactions between Rif1 and PP1 were also observed in *Drosophila* (Sreesankar et al. 2015). It was recently reported in human cells and *Xenopus* eggs that PP1 interacts with Rif1, and this interaction is required for suppression of Mcm phosphorylation (Hiraga et al. 2017; Alver et al. 2017). Considering the conserved Rif1-PP1 interaction, it is likely that the role of Rif1 in negatively regulating initiation through the recruitment of PP1 may be conserved across species.

### *14.2.4 Rif1 in Regulation of Transcription*

In budding yeast *Saccharomyces cerevisiae*, chromatin immediately adjacent to the telomeric repeats is transcriptionally silenced or heterochromatic (Gottschling et al. 1990). This epigenetic phenomenon, which is known as the telomere position effect (TPE), was proposed to link telomere structure to transcriptional regulation. TPE is mediated by the silent information regulator (Sir) proteins. Rap1 recruits the Sir proteins as well as the Ku70 complex to telomere to promote the spreading of heterochromatin in the subtelomeric region (Kueng et al. 2013). It was shown that *rif1* mutant cells are defective in silencing at *HMR* silencer, a phenotype that is similar to *rap1* mutant cells (Hardy et al. 1992), suggesting a role of Rif1 in transcriptional silencing. In fact, Rif1, together with the Sir proteins (Sir3 and Sir4), interacts with Rap1 to maintain the transcriptional silencing ability (Moretti et al. 1994). In fission yeast, SpRif1 also affects TPE in the subtelomeric region (Greenwood and Cooper 2012).

In HeLa cells, >600 genes are either upregulated or downregulated by depletion of Rif1, suggesting that Rif1 may affect transcription activity (Yamazaki et al. 2012). Notably, a megabase segment containing gene clusters are coordinately deregulated in the absence of Rif1 (Yamazaki et al. unpublished data). Zinc-finger and SCAN domain-containing protein 4 (Zscan4) is upregulated in ES cells depleted of Rif1 (Dan et al. 2014). Rif1 associates with a histone methylation complex in ES

cells (Fig. 14.1d), and Rif1 depletion leads to reduction of H3K9me3 in 33% of the genome including the pericentromeric segment and the subtelomeric region where the Zscan4 gene cluster is present (Dan et al. 2014). Coordinated regulation of transcription over several hundred kb by Rif1 may be consistent with the proposal that Rif1 regulates chromosome events through chromatin architecture. Recent report showed that Rif1 expression level in mouse ES cells is also tightly controlled by Smad3 and Oct4 (Li et al. 2015). Developmental control of Rif1 binding on chromatin and their relationship to long-range transcriptional regulation needs to be further studied.

## 14.3 Rif1 and G-Quadruplex

### *14.3.1 Interaction of Rif1 with Chromatin Through G-Rich Conserved Sequences*

Although an essential role of Rif1 in controlling global replication timing has become evident, how Rif1 is targeted to or selects its binding sites on chromosomes and how it affects origin firing still remain elusive. Recent study carried out in *Schizosaccharomyces pombe* provides evidence that Rif1 in fact can recognize and bind to G4-like structures at selected intergenic regions, thereby generating chromatin environment that can suppress local origin firing (Kanoh et al. 2015).

Using a chromatin immunoprecipitation-sequencing method, Kanoh et al. identified 35 high-affinity Rif1-binding sites (Rif1BSs) on the fission yeast chromosomes. Rif1BSs are associated with the following characteristics. (1) They are located in the intergenic regions. (2) They do not exactly overlap with the locations of pre-RC (Mcm binding sites) or promoter sequences. (3) The maximum chromatin binding is observed during G1, and the binding gradually decreases during S phase. (4) They are more enriched in the vicinity of the late-firing or dormant origins that are converted to early in *rif1*Δ cells.

Detailed analyses of Rif1BS sequences revealed the following features. (1) Rif1BSs contain a conserved motif, CNWWGTGGGGG (N, any nucleotide; W, A, or T), which is named the Rif1 consensus sequence (Rif1CS). (2) The strong Rif1BS almost always contain two or more Rif1CS, and they are arranged in head-to-tail orientation in over 75% of the strong Rif1BS. (Weak Rif1BS often contained only one Rif1CS.) (3) Mutations of the Rif1CSs resulted in the loss of chromatin binding of Rif1 protein specifically at the mutated site. (4) The mutations affected DNA replication initiation over ~100 kb segment encompassing the mutated Rif1BS.

Given the features of Rif1CS that it contains a stretch of five or six guanines and generally contains several other G-tracts, the authors examined a possibility that Rif1BSs adopt G4-like structures in vitro. Single-stranded DNA derived from Rif1BS as well as the double-stranded Rif1BS DNA that has been heat-denatured and reannealed can adopt G4-like structures in a manner dependent on the presence

of G-tracts in the Rif1CS as well as at other locations. Purified Rif1 protein binds to G4 DNA with affinity higher than other structured DNAs (see below).

These results strongly suggest that Rif1 binds to the G4-like structures that are generated in the intergenic region and contributes to the formation of the chromatin compartments that are related to the coordinated regulation of replication and possibly to that of transcription (Figs. 14.1c and 14.2a).

### *14.3.2 Structural and Functional Dissection of Rif1 Protein*

The N-terminal 340 amino acid segment of human Rif1 was initially predicted to fold into eight HEAT (huntingtin, elongation factor 3, a subunit of protein phosphatase 2A, and TOR) or armadillo-type helical repeats (Silverman et al. 2004), and this region is most conserved among Rif1 orthologs across the species. Bioinformatics analyses (Xu et al. 2010) predicted that the N-terminal domain of Rif1 consists of 14–21 tandem HEAT-like repeats, whose lengths depend on species (Fig. 14.2b). The HEAT unit consists of 35–45 amino acids bearing loosely conserved Asp and Arg at 19th and 25th residues, respectively, and forms a pair of antiparallel α-helices and a turn surrounding a virtual axis (Andrade et al. 2001). HEAT repeats are present in a variety of proteins and function as a scaffold that bridges different proteins in some cases. The HEAT repeat segment of condensin was reported to serve as a DNA-binding module (Piazza et al. 2014). We have recently found that the isolated HEAT repeat domain of mouse Rif1 not only binds to various G4 DNA but also forms multimers (K.M. and H.M. manuscript submitted). A nutrient-responsive kinase TOR also forms a dimer through its HEAT repeat domain-mediated self-association (Takahara et al. 2006; Baretić et al. 2016). Recently, it was reported that the Rif1 HEAT repeat domain is required for 53BP1-dependent foci formation of MAD2L2 (also known as MAD2B or REV7) following irradiation (Boersma et al. 2015). Thus, the Rif1 HEAT repeat may serve also as a scaffold to which 53BP1 and MAD2L2 are recruited.

Mishra and colleagues analyzed amino acid sequences of Rif1 homologues from 92 organisms (Sreesankar et al. 2012). They identified a highly conserved, Rif1-core region of 101–149 amino acids within the HEAT repeat domain. They also found a docking motif for type I protein phosphatase (PP1), consisting of SILK-RVxF sequences in multicellular organisms. The PP1-binding sequences can be found also in unicellular eukaryotes near the N-terminus in RVxF-SILK arrangement. The authors also pointed out that plant Rif1 homologues lack the PP1-interaction motifs.

In the context of Rif1-mediated regulation of replication timing, Rif1 recruits PP1 via this motif to dephosphorylate Mcm complex and Sld3, thereby repressing origin firing both in budding and fission yeasts (Hiraga et al. 2014; Davé et al. 2014; Mattarocci et al. 2014). Such repression is supposed to be reversed by phosphorylation of Rif1 near this motif probably via CDK/DDK, because this motif is neighbored by nine or more consensus Ser/Thr residues for CDK/DDK, and Asp

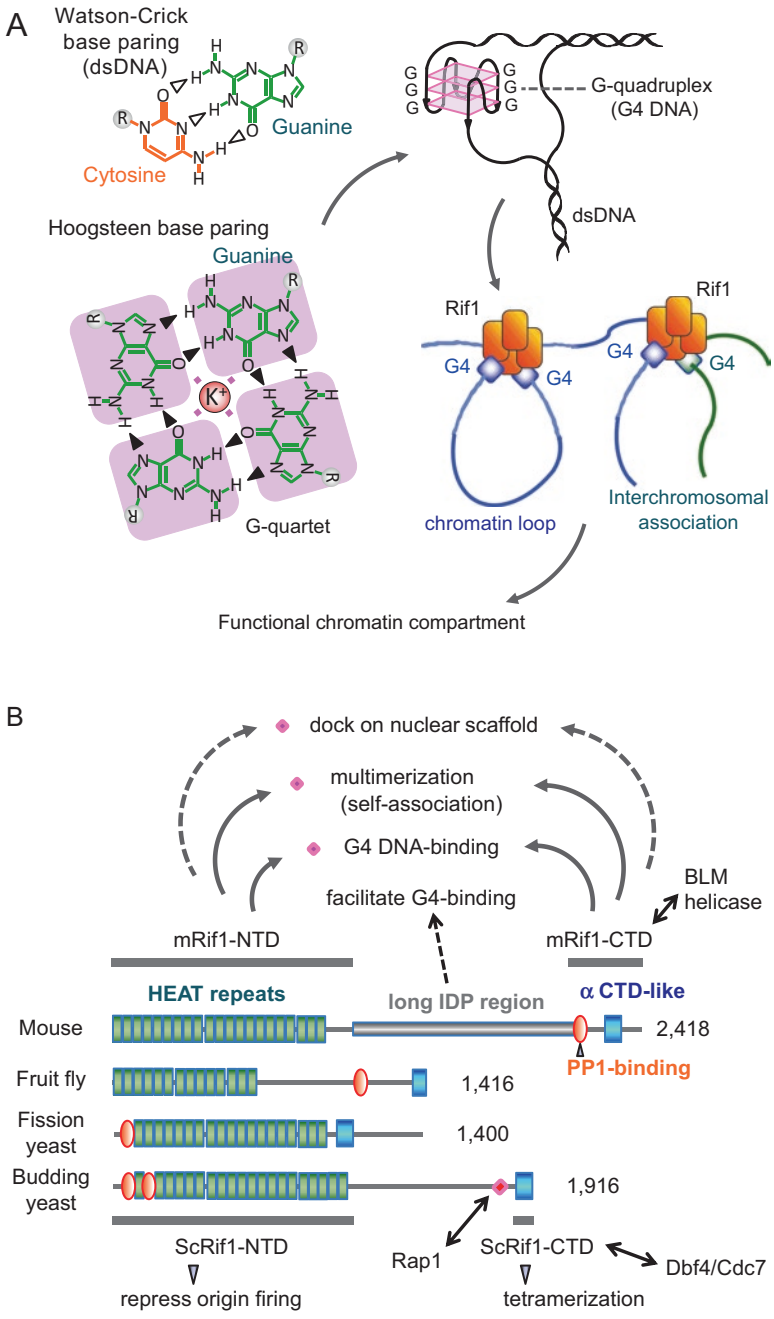


**Fig. 14.2** Rif1 protein and G4. (**a**) Schematic drawings of G-quadruplex DNA and its potential roles in Rif1-mediated formation of chromatin loops and association between distant chromosomes. Watson-Crick hydrogen bonds are indicated by open arrowheads, and Hoogsteen hydrogen

substitution of these residues abrogated PP1 binding (Hiraga et al. 2014; Davé et al. 2014; Mattarocci et al. 2014). Additionally, PP1 is involved in Rif1 function in suppression of telomere length in S phase as well as in resolution of non-telomeric chromosomal entanglement in M phase in fission yeast (Zaaijer et al. 2016).

The PP1 docking site exists in the C-terminal conserved domain of vertebrate Rif1 and is designated as CR-I (conserved region I). The CR-I of mouse Rif1 binds to PP1 more tightly than PP1-inhibitor I2 (Sukackaite et al. 2017). There are two additional conserved segments in the C-terminal domain (Rif1-CTD), designated as CR-II and CR-III (Xu et al. 2010). Xu et al. (2010) noticed sequence similarity between CR-II and αCTD domain of bacterial RNA polymerase and thus examined possible DNA binding by Rif1-CTD. They found that recombinant human Rif1-CTD bound branched DNAs, including cruciform and forked DNA, more tightly than dsDNA or ssDNA and that this binding was diminished by mutations in the CR-II region. They also identified Rif1-CTD as BLM-binding domain and suggested that Rif1 may contribute to the process of localizing stalled replication forks or broken DNAs through its ability to selectively recognize branched DNA. Similar DNA-binding properties of mouse Rif1-CTD were reported. It was reported that CR-II is sufficient to bind to cruciform and forked DNA (Sukackaite et al. 2014). An X-ray crystallographic study of the CR-II-related region of *S. cerevisiae* Rif1 (ScRif1) revealed that a short segment of the C-terminal 60 amino acids (residues 1857–1916) forms tetramer, composed of a pair of oppositely oriented dimers (Shi et al. 2013). They argued that the Rif1-CTD domain is conserved from yeast to humans based on their structure-based sequence alignment, and their notion is supported by the phylogenetic sequence analysis (Sreesankar et al. 2012). Another conserved sequence was noticed previously in Rif1-CTD (Silverman et al. 2004), although it appears to be distinct from the above tetramer-forming segment. Multiangle light scattering verified that ScRif1-CTD is also tetrameric in solution. L1905 (in CR-II-related region), highly conserved across the eukaryotic orthologs, is involved in intradimer packing. An arginine mutation of this residue not only led to loss of tetramer formation but also partially inhibited ScRif1 function at telomere (Shi et al. 2014). It was reported that the C-terminal 127 amino acids of ScRif1 associates with an N-terminal BRCT-like domain of Dbf4. However, deletion of C-terminal half of ScRif1 failed to suppress temperature-sensitive growth of *cdc7-1* strain, suggesting that CTD is dispensable for repression of premature origin firing by Rif1 in budding yeast (Hiraga et al. 2014; Mattarocci et al. 2014). We have recently found that isolated CTD of mouse Rif1 binds to various G4 DNAs more tightly than to branched DNAs and also exists as multimers. Thus, it appears to be a

**Fig. 14.2** (continued) bonds by closed arrowheads. Rif1 is present as an oligomer and is proposed to anchor multiple G4 DNAs to nuclear scaffold. (**b**) Domain organization of Rif1 orthologs along their polypeptides. The numbers indicate the length (amino acids) of each protein. PP1-binding motifs are indicated by ovals. Protein-protein interaction is shown by double-headed arrows. The repeat number of HEAT unit may not be accurate. αCTD-like segments are not well conserved among different species, and their assignments in species other than mouse may not be correct. See text for further functional dissections

conserved feature of Rif1 that the C-terminal segment is associated with DNA binding and oligomerization.

PP1 forms holoenzymes with more than 200 regulatory proteins, and most of them (≥70%) belong to the class of IDPs (intrinsically disordered proteins) (Choy et al. 2012). Computational disorder prediction indicates the presence of extremely long IDP region between HEAT repeat domain and CTD in vertebrate Rif1 (Sukackaite et al. 2014). As the term indicates, IDP region does not fold into stable tertiary structures under physiological conditions and is also called "naturally unstructured region" (reviewed in Oldfield and Dunker 2014; Shammas et al. 2016; Bah and Forman-Kay 2016). The isolated IDP region of mouse Rif1 does not show DNA-binding activity, but internal deletion of the IDP region resulted in substantial decrease of G4 binding in spite of the presence of DNA binding-proficient C- and N-terminal domains, suggesting that IDP may contribute to the DNA binding of Rif1 (K.M. and H.M. manuscript submitted). The functional and structural roles of the IDP in Rif1 functions will be an important issue to be addressed in the future.

### *14.3.3 Interaction of Rif1 with G-Quadruplex*

As described above, we have recently found that both SpRif1 and mRif1 bind to G4 DNA and presented evidence showing that this interaction is crucial for organization of replication timing domain in fission yeast (Kanoh et al. 2015; K.M. and H.M. manuscript submitted).

SpRif1 not only binds to short oligonucleotides adopting G4 conformation but also to longer dsDNA (derived from SpRif1BS) fragments containing Rif1BS that have been heat denatured/renatured (Kanoh et al. 2015). A classical consensus for G4-forming sequence is represented as $G_{\geq 3}N_{1-7}$ $G_{\geq 3}N_{1-7}G_{\geq 3}N_{1-7}G_{\geq 3}$ (Huppert and Balasubramanian 2005). However, fission yeast Rif1BS sequences deviate greatly from this formula and do not necessarily bear four or more G-tracts aligned on the same strand. In fact, 31 out of the 35 SpRif1BSs do not colocalize with the 446 putative G4 motifs predicted on the fission yeast genome (Sabouri et al. 2014). In spite of that, G4-like stretches in SpRif1BS sequences bind efficiently to a G4-specific antibody, and heat-denatured duplex Rif1BS generates specific higher-order structures, which are stabilized by a chemical compound 7OTD, a derivative of telomestatin, known to bind and stabilize G4 structures (Iida et al. 2013). These structures are not generated by DNA template in which guanine has been substituted by 7-deaza guanine. DNA polymerase stop assays and DMS footprinting analyses also indicated the generation of a specific higher-order structure at the G-tracts of Rif1BS that depends on Hoogsteen base paring. These results strongly point to the ability of Rif1BS to form G4-like structures.

The C-terminal domain of mammalian Rif1 has been reported to bind to branched DNAs including forked, flap, and cruciform DNAs (Xu et al. 2010; Sukackaite et al. 2014). We prepared various branched DNAs and used them in competition assays with the $dG_{24}$ probe, which is known to form a parallel-type G4 structure (Sengar

et al. 2014). In EMSA (electrophoretic mobility shift assay), even a tenfold excess of duplex, Y-fork, flap, forked, or cruciform DNA could not outcompete the binding of SpRif1 (Kanoh et al. 2015) or mRif1 (K.M. and H.M. submitted) to the G4 probe. Thus, Rif1 binds to G4 DNA with higher affinity than it does to other structured DNAs. Alternatively, it is also conceivable that G4 DNA and other branched DNAs bind to distinct sites on Rif1. As described in a previous section, both N-terminal and C-terminal domains of mRif1 bind independently to G4 DNA, and they exhibit similar structural preferences for parallel-type G4 DNA. It would be an interesting possibility that N-terminal and C-terminal domains may coordinate the high-affinity binding of Rif1 to G4 structures (Fig. 14.2b).

### *14.3.4 Potential Modes of Action of Rif1 Protein*

In mammals, Rif1 is localized at nuclease-insoluble structures, and Rif1 depletion causes an increase in chromatin loop sizes, suggesting a role in the regulation of higher-order chromatin structures (Cornacchia et al. 2012; Yamazaki et al. 2012). A more recent study in mouse embryonic stem cells (ESCs) further demonstrates that Rif1 is bound extensively with late-replicating regions which are associated with nuclear periphery and regulates chromatin architecture. The inter-replication domain interactions increase in the absence of Rif1 (Foti et al. 2016). Thus, Rif1 creates special nuclear architecture or chromatin domain that influences the regulation of replication timing. In budding yeast, Rif1 undergoes palmitoylation, a post-translational addition of fatty acid that anchors Rif1 to the nuclear periphery (Park et al. 2011).

Since fission yeast Rif1 can exert its inhibitory effect of origin firing as far as up to 50 kb away and biochemical characterization of Rif1 indicated that it forms oligomers (our unpublished data), we proposed that Rif1 potentially anchors to nuclear periphery through lipid modification and creates a specific chromatin architecture through recognition of the G4-like structures generated at specific intergenic regions (Kanoh et al. 2015). Rif1, in conjunction with its ability to recruit phosphatase, may tether multiple chromatin fibers through its ability to oligomerize and generate multiple chromatin loops and can negatively regulate the replication initiation over a long range (Fig. 14.1c). The ability of SpRif1 to interact with G4-DNA structures may provide an important clue to understand the link between nuclear architecture and replication timing regulation.

## 14.4 Emerging Biological Roles of G-Quadruplex

G-quadruplexes arise in G-rich sequences where four guanine bases make Hoogsteen base paring and thereby form planar G-quartets that stack with three looping strands connecting the G-quartet layers (Fig. 14.2a). G4 formation is kinetically fast, and

these structures are stable under physiological molecular crowding conditions, particularly in the presence of $K^+$ (Bochman et al. 2012). In double-stranded DNA, the opportunity for forming G4 structure may arise during DNA replication, transcription, and repair when DNA is rendered transiently single stranded. However, it was recently reported that Hoogsteen base pairs transiently form in canonical duplex DNA (Nikolova et al. 2011), suggesting that G4 formation may not necessarily require the prior unwinding or melting of DNA duplex. Since there have been a number of excellent reviews on G4 and its biological roles in the past several years (e.g, see Maizels and Gray 2013; Murat and Balasubramanian 2014; Maizels 2015; Rhodes and Lipps 2015; Hänsel-Hertsch et al. 2017), we will focus on very recent progresses on G4 biology that may serve to future study.

A single-chain antibody hf2 was used to map the locations of G4 structures on the human genome by sequencing the immunoprecipitated DNA fragments (Lam et al. 2013). Another recent high-resolution sequencing by using another single-polypeptide G4 antibody, BG4, identified 716,310 distinct G4 structures in the human genome (Chambers et al. 2015). A high G4 density was seen in functional regions, such as 5′ untranslated regions and splicing sites. The putative G4 sites were significantly associated with oncogenes, tumor suppressors, and somatic copy-number alterations related to cancer development. G4 motifs are highly enriched near promoters and transcription start sites. They are more frequently found on the non-template than on the template strand where they either enhance or inhibit transcription, respectively. A genome-wide analysis of the nearly 600,000 regulatory *cis* elements for all known genes in the human genome has shown that DNase hypersensitive (DHS) *cis*-regulatory elements are also enriched in guanines (Hegyi 2015). A possibility was proposed that these distal *cis* regions may form intermolecular G4 structure with the G-rich promoter regions, generating a G4-mediated looping between the non-template strands of the promoter and the enhancer, and this could lead to the generation of DHS segments (Fig. 14.3a).

It has been reported that 67–90% of mammalian replication origins have flanking G4 motifs (Besnard et al. 2012; Cayrou et al. 2012; Valton et al. 2014) and in higher densities near those origins that are used frequently (Besnard et al. 2012). As stated above, G4 motifs were shown to be necessary for origin function in two model origins in chicken DT40 cells, and G4 stability correlates with origin efficiency. In addition, G4 orientation determines the precise position of the replication start site (Cayrou et al. 2012; Valton et al. 2014). However, these studies used G4-sensitive lambda exonuclease (λ exo) to enrich short nascent strands (SNS) for origin mapping, and therefore, concerns about λ exo-associated biases have been pointed out frequently (Foulk et al. 2015; Urban et al. 2015; Prioleau and MacAlpine 2016). A bubble-seq study demonstrated that the majority (59%) of bubble-containing fragments did not bear G4 motifs (Mesner et al. 2013) and consistent with a more recent Okazaki fragment (OK)-seq analysis in HeLa and GM06990 cells (Petryk et al. 2016). Although a nascent strand capture and release (NSCR) approach indicated that, among 5% of the largest SNS peaks, 49.4% harbored associated G4 motifs within 2 kb, only <6% of SNS peaks exhibit a close and orientation-specific localization with G4 motifs in MEF cells (Kunnev et al. 2015). Another BrdU-NS-seq

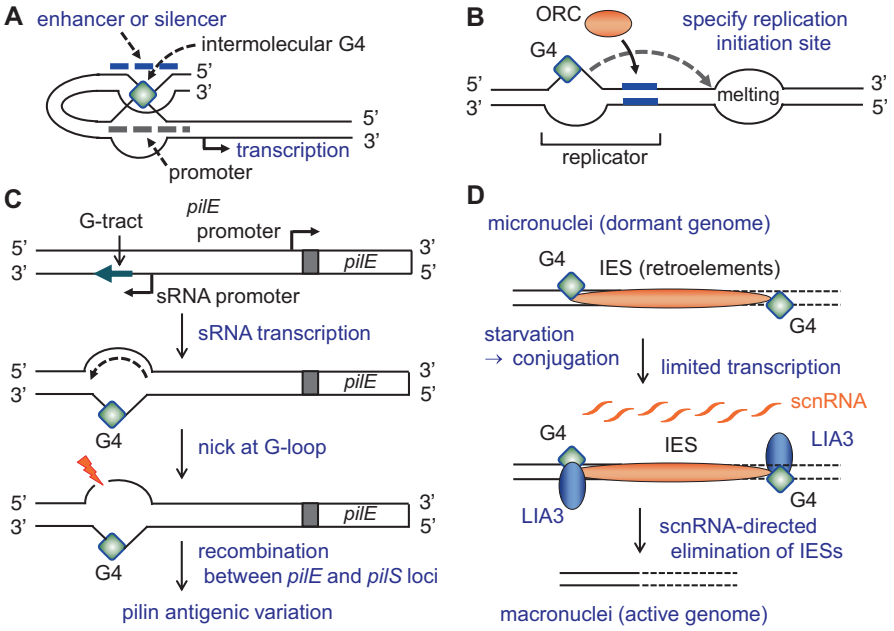


**Fig. 14.3** Various potential biological functions of G4 DNA. (**a**) A possible model for transcriptional regulation by G4-mediated DNA looping between gene promoter and its *cis*-regulatory element (Hegyi 2015). (**b**) A potential role of G4 DNA in specification of replication origin in vertebrate. (**c**) Recombination initiation pathway that generates pilin antigenic variation in *Neisseria*. (**d**) Programmed DNA elimination during development of macronucleus from micronucleus in *Tetrahymena*. See text for more details

study also revealed a limited overlap of G4 motifs (~37.5% of mapped origins; Mukhopadhyay et al. 2014). ChIP-seq analysis indicated that only 34.1% of Orc1-binding sites have flanking G4 motifs in HeLa cells (Dellino et al. 2013). A very recent Orc2-mapping study by Miotto et al. (2016) supports this result (31% overlap in K562 cells) and also demonstrated that ORC is enriched in open DNase I-accessible chromatin marked by active histone modifications such as H3K27ac, H3K4me2, and H3K4me3. The apparently discrepant locations between ORC sites and initiation sites (SNS peaks) may be in part explained by potential redistribution or sliding of Mcm2-7 complex after loading. However, it is noteworthy that most approaches found that a significant fraction (>30%) of identified origins (or initiation sites) overlapped with G4 motifs (Fig. 14.3b). The most recent mapping that directly analyzes the initiation site (Ini-seq) and does not involve the SNS methods showed 70 % colocalization of mapped TSS (transcription start site) origins with G4-forming sequences (44% of the total origins; Langley et al. 2016). Thus, it is likely that G4 is at least one of the determinants for origin specification.

Besides replication and transcription, G4 is involved in DNA recombination as well. Certain pathogens use DNA recombination to modify the proteins on their outer surfaces by rearranging their genes and avoid repeated detection by the

immune system. Cahoon and Seifert (2009) identified a *cis*-acting G4 motif near the variable pilin genes of the human pathogen *Neisseria gonorrhoeae* that controls recombination of the antigenic locus (Fig. 14.3c). Disruption of the G4 motif either by mutagenesis or with a G4 ligand, N-methyl mesoporphyrin IX (NMM), prevented nicks required for recombination from occurring within the G4 region and thereby inhibited pilin antigenic variation. Interestingly, folding into G4 structure of this site depends on transcription of sRNA, a small noncoding RNA (Cahoon and Seifert 2013). The nicks generated in the G4 DNA are subsequently processed by RecJ exonuclease and RecQ and Rep helicases. Most of the RecQ helicase family members encode one HRDC (helicase and RNase D C-terminal) domain, whereas the *N. gonorrhoeae* RecQ helicase bears three HRDC domains. Deletion of RecQ HRDC domains 2 and 3 causes a decrease in the frequency of pilin antigenic variation, consistent with a decrease in its G4-binding and unwinding activities (Cahoon et al. 2013).

BLM helicase, a Rif1-interacter, is one of the five human RecQ family helicases (Croteau et al. 2014) and is involved in class switch recombination (CSR) of immunoglobulin heavy-chain genes. CSR occurs within S regions, 1- to 8-kb segments containing repetitive G4 motifs, and joins a new constant region to the expressed variable region, thereby conferring new effector functions on the encoded antibody without affecting antigen specificity (Matthews et al. 2014). Recombination to a particular S region requires its prior transcription. The active transcription of S regions produces noncoding transcripts generating R loops that contain a stable RNA/DNA hybrid on the template strand and G-rich ssDNA on the non-template strand harboring repetitive G4 motifs (Zhang et al. 2014). The R-loop region is targeted by factors that promote CSR, including BLM helicase and MutSα (MSH2/MSH6 heterodimer). MutSα functions in telomere maintenance as well as in mismatch repair. Human MutSα binds to G4 in R loops formed by transcribed S regions and promotes synapsis between distinct S regions (Larson et al. 2005).

Another experimental evidence that G4 structure and its associated protein regulate chromosome reorganization emerged recently from the study of programmed DNA elimination events of *Tetrahymena thermophila* (Carle et al. 2016). This ciliate has a whole set of its genome in micronuclei (germline nuclei), but eliminates nearly one-third of its germline genome from each developing somatic nucleus (macronucleus). The excised chromosomal ends either form new telomeres by the action of telomerase or reconnected in a manner similar to DSB repair excluding the internal eliminated sequences (IESs) (Chalker and Yao 2011). Thus, in a sense, these programmed DNA elimination events seem reminiscent of formation of genomic domains or chromosomal boundaries in other eukaryotes (that would be contributed by Rif1; Zofall et al. 2016; Toteva et al. 2017), though these do not accompany genomic deletion. The IESs were targeted for removal by small RNA-directed heterochromatin formation. In cells lacking *LIA3 (ΔLIA3)*, the excision of IESs precisely at specific G-rich sequence (5′-AAAAAGGGGG-3′) was impaired and imprecise, whereas the removal of IESs without such controlling sequences was unaffected. The G-rich boundary controlling sequences form parallel G4 structures

in vitro that are specifically bound by Lia3 protein (Carle et al. 2016). Thus, G4 DNA and Lia3 crucially participate in global genomic rearrangement in a developmental stage-specific manner (Fig. 14.3d).

Most of IESs appeared to harbor retroelements in *Tetrahymena*. Other eukaryotes also carry huge amounts of retrotransposons which are often present on their genomes as clusters, but do not physically eliminate them during development. As ectopic transcription of retrotransposons is toxic to cells (Hatanaka et al. 2015), they must be silenced during the entire lifespan. Interestingly, knockdown of Rif1 leads to derepression of MuERV-L, a third class of LTR-type retrotransposons as well as other two-cell embryonic stage-specific genes in mouse ES cells (Dan et al. 2014; Yoshizawa et al. manuscript in preparation). LTRs of most LTR-type retrotransposons bear multiple G4 motifs (Lexa et al. 2014; Kejnovsky et al. 2015). G4 motifs are also present in non-LTR retrotransposons, such as LINE or SVA. G4 structure is proposed to affect not only transcription and translation but also reverse transcription and integration of retrotransposons (Lexa et al. 2014; Kejnovsky et al. 2015; Sahakyan et al. 2017). Although it is not known yet whether G4 structure is involved in silencing of retrotransposons or not, the fact that reduced expression of G4-binding proteins, such as Rif1 and chromatin remodeler ATRX (Sadic et al. 2015; Voon and Wong 2016), results in derepression of subsets of endoretroviruses implicates G4 structure as a critical *cis*-element for the gene silencing of retrotransposons. In support of this notion, recent study of immuno-electron microscopy showed exquisite specificity of a G4-specific antibody for heterochromatin (Hoffmann et al. 2016).

G4 structure is also involved in efficient infection and/or transcription of some pathogenic retroviruses including HIV as well as suppression of proviral expansion (Tosoni et al. 2015; Perrone et al. 2016; Métifiot et al. 2014; Harris and Merrick 2015). In addition, small molecule G4 stabilizers can reduce the copy number of episomal genomes of KSHV (Kaposi's sarcoma-associated herpesvirus) and EBV (Epstein-Barr virus) during their latent infection. Their latent-phase replication depends on human pre-RC and may proceeds in a manner similar to that of host DNA replication (Lieberman et al. 2007; Tempera and Lieberman 2010). TR (terminal repeat) of KSHV genome functions as a latent replication origin and harbors G4 motifs. Small molecule G4 stabilizers, such as PhenDC3, slow down fork speed due to fork stalling at the G4 structure and thereby trigger advanced firing of dormant replication origins (Madireddy et al. 2016). Slowing down the latent EBV replication with hydroxyurea also advances replication timing of *oriP*, a latent replication origin of EBV episome, and reduces its copy number (Zhou et al. 2009). BRACO-19, another small molecule G4-stabilizing agent, decreases the copy number of EBV latent genome by disrupting the interaction between EBNA1 and ORC and the ability of EBNA1 to tether to metaphase chromosomes (Norseen et al. 2009). Thus, in these γ-herpesviruses, late-to-early transition of replication timing seems to be intimately correlated with reduction of copy number of latent episomes, and affected by the interference or stabilization of G-quadruplex. These viral studies may provide important insight into spatiotemporal regulation of origin firing and of inter-chromatin associations in eukaryotic genomes.

## 14.5 Concluding Remarks

G4 was originally discovered from analyses of telomere-derived DNA. In spite of extensive analyses of chemical and physical nature of G4 structure, its biological roles have been rather elusive. However, recent development of new tools and genome-wide analyses led to the consensus that G4 is widespread on the genome. G4, due to their unusual structures, was considered to be inhibitory for transcription or replication. Indeed, numerous studies presented compelling evidence that it is the case. Thus, G4 has been regarded as a "hazardous" genome element. However, recent accumulating evidence points to crucial and unexpected roles of G4 in various aspects of chromosome regulation, including regulation of transcription, replication timing program, replication initiation, recombination, gene rearrangement, viral chromosome attachment, and immune evasion of pathogens.

In each case, G4 is recognized by a specific protein to execute the transaction. Rif1, a conserved nuclear factor, specifically recognizes intergenic G4 sequences to regulate DNA replication timing probably through modulation of chromatin architecture as well as by recruiting a phosphatase. Rif1 is involved also in regulation of telomere functions, DNA repair, and transcription. It would be interesting to examine whether G4 recognition is involved in these processes as well.

More than 370,000 G4-forming sequences are predicted to be present on the human genome, and recent genome-wide search has indeed confirmed this prediction, although it appears that the current algorithm does not precisely predict the in vivo G4 structures. In fact, in cells, Rif1, which shows strong preference for G4 structures in vitro, appears to bind to sequences that do not conform to the canonical consensus sequences. Although various probes or methods have been utilized to determine the genomic locations of G4 in the cells, it still appears to be difficult to obtain the consensus for the fully accurate profile of G4 structures.

Formation of G4 in the cells would be affected not only by sequence itself but also by physiological conditions, epigenetic features, and chromosome microenvironment. Thus, it would be also important to monitor dynamic local changes of DNA shapes in different environmental settings as well as in different cell types.

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# Chapter 15
# Chromatin Replication and Histone Dynamics

**Constance Alabert, Zuzana Jasencakova, and Anja Groth**

**Abstract** Inheritance of the DNA sequence and its proper organization into chromatin is fundamental for genome stability and function. Therefore, how specific chromatin structures are restored on newly synthesized DNA and transmitted through cell division remains a central question to understand cell fate choices and self-renewal. Propagation of genetic information and chromatin-based information in cycling cells entails genome-wide disruption and restoration of chromatin, coupled with faithful replication of DNA. In this chapter, we describe how cells duplicate the genome while maintaining its proper organization into chromatin. We reveal how specialized replication-coupled mechanisms rapidly assemble newly synthesized DNA into nucleosomes, while the complete restoration of chromatin organization including histone marks is a continuous process taking place throughout the cell cycle. Because failure to reassemble nucleosomes at replication forks blocks DNA replication progression in higher eukaryotes and leads to genomic instability, we further underline the importance of the mechanistic link between DNA replication and chromatin duplication.

**Keywords** DNA replication • Nucleosome assembly • Histone chaperone • Histone recycling • Chromatin • Epigenetics

## 15.1 Introduction

Genome function in eukaryotes is regulated by chromatin, a complex structure consisting of DNA, histones, RNA and a large number of structural and regulatory proteins. Chromatin compacts the genome, restricting access to the DNA template

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in a manner that is dependent on chromatin composition and chemical modifications on histones and DNA. Distinct chromatin states can be inherited through mitotic cell division, and this contributes to the correct execution of developmental programmes by establishment and maintenance of gene expression patterns. Duplication of chromatin structures in dividing cells thus impinges on the maintenance of epigenetic states and cell identity. However, while our knowledge about DNA replication is comprehensive, the process of chromatin duplication remains poorly understood. In this chapter we will discuss how chromatin is replicated with the emphasis on histone dynamics during DNA replication and maintenance of histone-based information.

The nucleosome consists of 147 base pairs of DNA wrapped around a histone octamer. Each octamer is composed of one centrally located H3-H4 tetramer, flanked by two H2A-H2B dimers. Histones come in different flavours, so-called variants, which along with the large array of modifications in the histone tails holds information important for the chromatin state. The current view is that the preservation of such histone-based information contributes to inheritance of chromatin states. Pioneering electron microscopy studies of replicating chromatin in *Drosophila* embryos revealed that nucleosomes are formed very rapidly after on the daughter DNA strands (McKnight and Miller 1977). Since then, we have learned that chromatin replication can be divided into five steps (Fig. 15.1): (1) disassembly

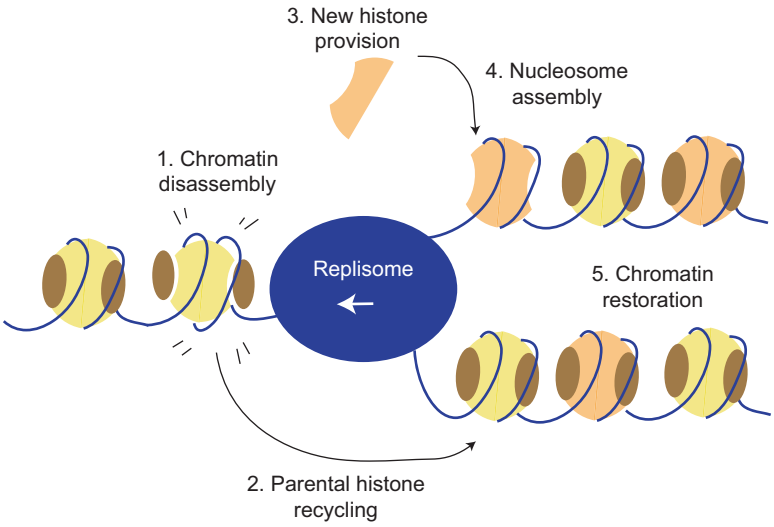


**Fig. 15.1** DNA replication in the context of chromatin. The process can be divided into five steps: (*1*) disassembly of chromatin ahead of the replisome, (*2*) recycling of parental histones in a presumably random fashion onto the two daughter strands, (*3*) supply of newly synthesized histones to sites of ongoing DNA replication, (*4*) nucleosome assembly on leading and lagging strands from 50% newly synthesized histones to 50% old recycled histones, (*5*) restoration of marks on DNA and histones and reassociation of other chromatin components including structural and regulatory proteins

of chromatin immediately ahead of the replication machinery, (2) recycling of evicted parental histones onto newly replicated DNA, (3) supply of newly synthesized histones to the sites of ongoing DNA replication, (4) nucleosome assembly from recycled parental and new histones to maintain nucleosome density on the two new daughter DNA strands and (5) restoration of marks on DNA and histones and association of additional chromatin components to restore the chromatin structure. Here we cover mainly steps 1–4 as step 5 is separated from the replication process. Further, we discuss current challenges to understand the dynamic and complex processes of chromatin replication and epigenome maintenance.

## 15.2 Replication Through Nucleosomes

The DNA molecule is wrapped in a left-handed manner around histone octamers to form nucleosomes (Fig. 15.2). Nucleosomes represent a barrier for DNA-based processes such as transcription, DNA replication and DNA repair. This section focuses

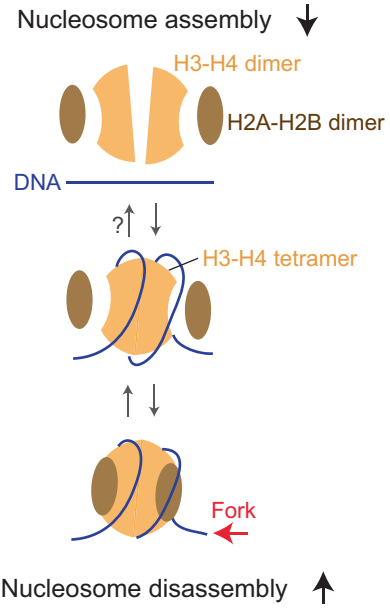


**Fig. 15.2** Nucleosome assembly and disassembly processes. In the nucleosome, a histone octamer is wrapped by 147 bp of DNA in a left-handed manner. Nucleosome disassembly (*bottom* to *top*) probably relies on unwinding of DNA by the helicase arriving at the proximal H2A-H2B dimer. This will first release a H2A-H2B dimer and then lead to full disruption of the octamer upon release of the H3-H4 tetramer. Parental histone H3-H4 do not mix with new histone H3-H4, suggesting that separate deposition pathways may exist (see main text). Newly synthesized histones H3-H4 are transported as dimer, which assemble into tetramers in the process of nucleosome assembly

on recent advances in understanding of how nucleosomes are transiently disassembled ahead of replication fork and how evicted histones are recycled on the newly replicated daughter strands.

### *15.2.1 Chromatin Disassembly Ahead of the Replisome*

To sustain an elongation rate of 1.5–2 kb per minute, one nucleosome has to be evicted every 7 second ahead of the replisome. The eviction mechanism involves local destabilization of one (maximally 2) nucleosomes ahead of each replication fork (Gasser et al. 1996; Sogo et al. 1986). Unzipping of the DNA duplex using optical tweezers is sufficient to provoke octamer eviction in vitro (Shundrovsky et al. 2006). However, in vivo studies and recent reconstituted replication systems suggest that a coordinated effort of histone chaperones and chromatin remodellers along with other events allow fork progression through chromatin.

#### 15.2.1.1 Histone Chaperones

Histone chaperones are defined proteins that handle non-nucleosomal histones in vivo and mediate the assembly of nucleosomes from histones in vitro. FACT, consisting of a heterodimer of Spt16 and SSRP1 in humans, is currently viewed as the key chaperone involved in chromatin disruption. FACT binds H2A-H2B dimers (Belotserkovskaya et al. 2003; Orphanides et al. 1999) and H3-H4 tetramer (Tsunaka et al. 2016) and serves a key role in transcription where it aids nucleosome disruption ahead of the RNA polymerase and restores the chromatin template behind (Hammond et al. 2017). Evidence from several organisms have linked FACT to DNA replication, and multiple interactions between FACT and replisome components have been reported (Hammond et al. 2017). However, the exact function of FACT in replication has been difficult to dissect as genetic analysis is hampered by its role in transcription. However, a recent study reconstituting DNA replication on a nucleosomal template from recombinant proteins revealed that FACT is required and sufficient to allow replisome progression in this system (Kurat et al. 2017). How FACT permits the progression of replisomes through nucleosome arrays remains to be determined. As FACT interacts with the replisome (Foltman et al. 2013), one possibility is that FACT promotes nucleosome disassembly as it collides with nucleosomes. As FACT is an extremely abundant protein (one per five nucleosomes in yeast McCullough et al. 2015), another possibility is that FACT destabilizes nucleosomes ahead of the replisome. Since FACT has also been proposed to deposit parental (Foltman et al. 2013) and new histones (Yang et al. 2016) behind the fork, it will be important to dissect these functions from disassembly. Indeed, both nucleosome disassembly ahead and reassembly behind replication fork may control elongation speed, as discussed in Sect. 15.3.

#### 15.2.1.2 ATP-Dependent Chromatin Remodelling Complexes

Chromatin remodellers are large multi-protein complexes that come in different flavours and allow the access to DNA by altering the structure, composition and/or position of nucleosomes. They were first found to play a key role in transcriptional regulation and are now recognized to be part of most chromatin-based processes including DNA replication (Narlikar et al. 2013). Several chromatin remodelling complexes have been suggested to destabilize or remove nucleosomes ahead of replisomes. Recent studies of reconstituted replication on a chromatin template did not find a requirement for ATP-dependent remodelling for fork progression, but the activity of INO80 and ISWIA enhanced the elongation rate (Kurat et al. 2017). Consistent with this, members of the INO80 and ISW2 complex promote efficient fork progression in *S. cerevisiae* (Iida and Araki 2004; Vincent et al. 2008). In mammals, members of the ISWI family (WICH and ACF) and of the INO80 complex have been shown to promote fork progression (Collins et al. 2002; Lee et al. 2014; Poot et al. 2004). However, chromatin remodellers also play an important role behind the fork in establishing nucleosome spacing and position, as discussed in Sect. 15.4. Thus, whether slow fork speed in vivo reflects a function of these remodellers ahead or behind the fork remains unclear.

#### 15.2.1.3 Other Mechanisms Promoting Nucleosome Disassembly

Progression of the replisome creates positive torsional stress of the DNA molecule ahead of the fork. In vitro studies show that nucleosome assembly stalls when DNA is under positive torsional stress (Gupta et al. 2009) and that H2A/H2B dimers could dissociate from the H3-H4 tetramer (Bancaud et al. 2006, 2007). Positive torsional stress could thus induce a structural change in nucleosome that aids disassembly and fork progression (reviewed in Teves and Henikoff 2014). Another mechanism that may aid chromatin disruption is phosphorylation of the linked histone H1. Phosphorylation of histone H1 by cyclin A-CDK2 can decompact chromatin fibres (Contreras et al. 2003) by increasing the dynamic exchange of histone H1. Given that Cdk2 may travel with the fork, it could trigger decompaction of replication domains to facilitate replisome progression (reviewed in Alabert and Groth 2012).

### *15.2.2 Histone Recycling at Replication Forks*

Nucleosomal histones carry modifications that are important for regulation of genome function; thus how they are handled during DNA replication impinges not only on fork progression but also on maintenance of epigenetic states. Histone octamers dissociate upon nucleosome disruption, releasing two H2A-H2B dimers and two H3-H4 dimers under physiological salt conditions (Hammond et al. 2017).

Most evidence suggest that the H3-H4 tetramer remains intact in the process of being recycled onto newly replicated DNA (Fig. 15.2, see below), in which case additional factors like histone chaperones must be involved to maintain the tetrameric state (Hammond et al. 2017). H2A-H2B dimers are also recycled (Alabert et al. 2015), but this process remains poorly understood.

#### 15.2.2.1 H3-H4 Transfer

Upon release from nucleosome, H3-H4 could be transferred as a tetramer or further split into dimers. This has been a major question in the field because of the implicates it has on inheritance of histone-based information; if new and old histone dimers mix after DNA replication, histone modification could be evenly distributed onto the two daughter stands and copied from old histones to new ones within one nucleosome. However, despite the appealing nature of this model, most evidence argue that there is no or only little mixing of new and old histones H3-H4 dimers in the process of DNA replication (Annunziato 2005; Xu et al. 2010). Two complementary technologies have been used to analyse the fate of H3-H4 tetramers upon recycling during DNA replication, differential metabolic labelling of pre-existing and newly synthesized histones (Jackson 1990; Yamasu and Senshu 1990) and differential tagging on newly synthesized and pre-existing histones (Katan-Khaykovich and Struhl 2011; Prior et al. 1980; Xu et al. 2010). Results obtained by both approaches suggest that parental histone H3-H4 do not mix with new histone H3-H4 dimer, which support the idea that the H3-H4 tetramer is transferred as an entity. However, it remains possible that the H3-H4 tetramers split into dimers transiently and reassociate as nucleosomes are assembled on newly replicated DNA. In either case, the absence of new and old H3-H4 dimer mixing in nucleosomes suggests that there are distinct pathways for replication-coupled deposition of new and old histones H3-H4. In relation to epigenetic cell memory, this argues that a potential copy-paste mechanism to propagate histones must operate between nucleosomes rather than internally between tails in the same nucleosome.

#### 15.2.2.2 Mechanisms of Histone Recycling

Pioneer studies of SV40 in vitro replication systems have suggested that histones remain in close proximity to the replisome during recycling (reviewed in Annunziato 2013); however it remains unclear how accurate the recycling process is. Will old histones reassociate with the same DNA sequence or will there be some displacement relative to their original position? A study in yeast used mathematical modelling to address this question, and they estimated that old histones would be recycled roughly within 400 bp of their original position (Radman-Livaja et al. 2011). Recent insights into histone-binding activities within the replisome provide some mechanistic insight into this process of histone segregation (Fig. 15.3).

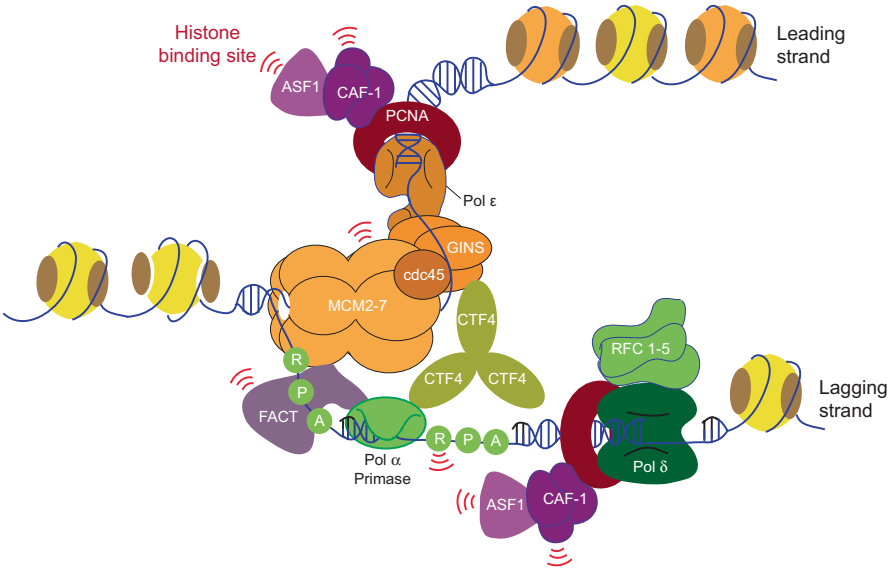


**Fig. 15.3** Contact between histones and the replisome. Contact points between histones and replication proteins are indicated in red

MCM2, part of the replicative helicase (MCM2-7), has histone chaperone activity (Ishimi et al. 2001) and binds histone H3-H4 in human (Groth et al. 2007) and yeast (Foltman et al. 2013). Recent crystal structures revealed that MCM2 can chaperone either a H3-H4 tetramer (Richet et al. 2015; Huang et al. 2015) or a H3-H4 dimer in complex with the ASF1 chaperone (Huang et al. 2015; Wang et al. 2015). The histone-binding domain of MCM2 binds H3-H4 tetramers by mimicking DNA in the nucleosome (Huang et al. 2015), which provide a very attractive binding mode for a chaperone involved in recycling. Indeed, MCM2 can handle all H3 variants including H3.3 and CENPA (Huang et al. 2015), and modification of the histone tails should not affect binding. Thus, MCM2 could act as an acceptor of evicted histone H3-H4 genome wide. Consistent with this, mutations of MCM2 histone-binding domain reduce silencing in yeast (Foltman et al. 2013) and slow down cell proliferation in cancer cell lines (Huang et al. 2015). Whether MCM2 operates alone or in conjunction with additional chaperones remains unclear. MCM2 form a co-chaperone complex together with ASF1 in which both chaperones make contact with a H3-H4 dimer (Groth et al. 2007; Huang et al. 2015; Wang et al. 2015). This co-chaperone interaction occurs at replication sites in mammalian cells (Huang et al. 2015), but it remains unclear whether it contributes to normal recycling of H3-H4, through a transient dimeric state, or mainly operates to catch evicted histone upon replication stress as this leads to accumulation of the complex with parental histones (Huang et al. 2015; Jasencakova et al. 2010).

MCM2 also forms a co-chaperone complex with FACT (Foltman et al. 2013). This chaperone travels with the replisome (Alabert et al. 2014; Foltman et al. 2013; Gambus et al. 2006) and can make contact to several replisome components including MCM4 (Gambus et al. 2006; Tan et al. 2006), Polα and RPA1 (VanDemark et al. 2006; Wittmeyer et al. 1999; Zhou and Wang 2004). FACT can bind H3-H4 tetramers (Stuwe et al. 2008; Tsunaka et al. 2016) as well as H2A-H2B dimers (Belotserkovskaya et al. 2003; Orphanides et al. 1999). Recently structural work showed that the binding of H2A-H2B and H3-H4 is not mutually exclusive, and therefore, in theory, FACT could transfer a H3-H4 tetramer together with at least one H2A-H2B dimer. This may happen through a co-chaperone interaction with MCM2, as the structural and biochemical data supports that they can bind H3-H4 simultaneously. FACT is required for replisome progression (Abe et al. 2011; Kurat et al. 2017; Tan et al. 2006); however, as mentioned in Sect. 15.1, genetic studies on FACT are complicated by its multiple functions in transcription and chromatin maintenance.

## 15.3 New Histone Provision

During replication, pre-existing parental histones are recycled as described above, and in addition newly synthesized histones are delivered and deposited onto the newly synthesized daughter DNA strands to maintain nucleosome density. In human cells about 33 million new nucleosomes have to be assembled in each S phase. To match this high demand for nucleosome assembly, the biosynthesis of new histones and their transfer to replicating DNA are regulated in multiple ways. The replication-dependent canonical histones (H3.1, H3.2, H4, H2A, H2B and H1) are multicopy genes, which are induced at the onset of DNA replication and tightly regulated at both the transcription and post-transcription levels (reviewed in Marzluff et al. 2008). The shortage or excess of histones can block DNA replication and trigger genomic instability in yeast and mammals (Groth et al. 2007; Gunjan and Verreault 2003; Han et al. 1987; Kim et al. 1988; Meeks-Wagner and Hartwell 1986; Mejlvang et al. 2014; Nelson et al. 2002). The massive production of canonical histones also represents a logistic challenge: newly synthesized histones need to be efficiently guided to sites of ongoing replication, a process that is carried out by histone chaperones (reviewed in Hammond et al. 2017). Histone chaperones are a broad class of proteins that handle non-nucleosomal histones in vivo and in vitro can mediate the assembly of nucleosomes when mixed with histones and DNA.

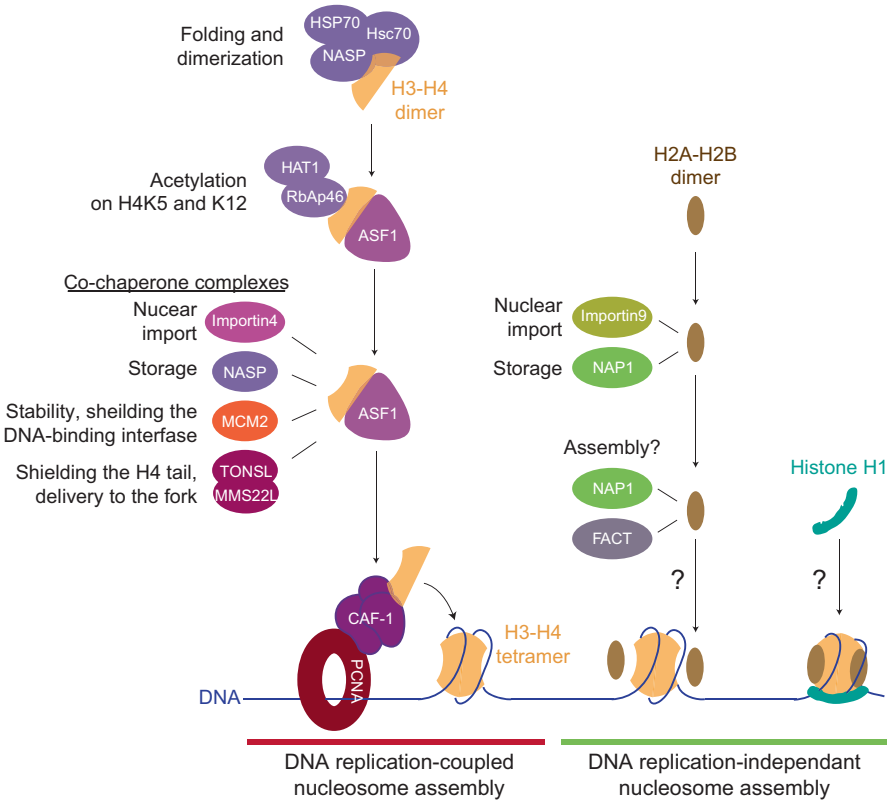


**Fig. 15.4** Network of histone chaperones, handling histones from their site of synthesis to their deposition onto newly replicated DNA (See text for details)

### *15.3.1 Delivery of Newly Synthesized H3.1/H3.2-H4*

Soon after their synthesis, histones H3.1/H3.2 and H4 engage with histone chaperones and are transported to sites of DNA replication as dimers (Benson et al. 2006; Tagami et al. 2004). The histone supply chain involves a network of specialized chaperones that are connected via histone-dependent and histone-independent interactions that allow 'on-the-go' modification of the histone, handover of histones between different chaperone complexes and culminates with CAF-1 that assembles H3.1-H4 dimers and newly replicated DNA into tetrasomes, which are completed into nucleosomes upon the addition of H2A-H2B (reviewed in Hammond et al. 2017) (Fig. 15.4).

#### 15.3.1.1 Histone H3.1-H4 Chaperone Network

Biochemical and proteomic characterization of protein complexes interacting with soluble histones has uncovered a large number of histone chaperones, and the list is still growing. While it remains unclear exactly how all these chaperones are organized with respect to each other in the supply chain, the position and role of several key players have been identified based on both biochemical and functional studies. Initially, rapidly after histone synthesis in the cytoplasm, histone H3.1/H3.2/H3.3-H4 can interact with HSC70, HSP90 and NASP that promote the folding and formation of histone dimers (Alvarez et al. 2011; Bowman et al. 2017; Campos and Reinberg 2010). NASP is found in co-chaperone complexes with ASF1 and RBAP46-HAT1 (catalyses H4 acetylation, discussed below) and thus presumably works upstream of these in the network (Bowman et al. 2017; Haigney et al. 2015; Jasencakova et al. 2010).

ASF1 is a central histone H3-H4 chaperone that then engages with several additional histone chaperones, histone modifiers and other accessory partners to form dynamic multi-protein complexes that carry out specialized tasks in histone supply. These tasks include nuclear import (IMPORTIN-4), acetylation of histone tails (RBAP46-HAT1) and regulating the storage pool of soluble histones (NASP or MCM2) (reviewed in Hammond et al. 2017) (Fig. 15.4). These interactions represent co-chaperone relationships where two or more chaperones bind histones concomitantly. ASF1 interacts with the H3 alpha2-alpha3 helices that constitute the H3-H4 tetramerization interface (English et al. 2006; Natsume et al. 2007). Thus, a major consequence of ASF1 binding is to maintain H3-H4 dimeric, while leaving other histone interfaces available for other chaperones like MCM2 (Groth et al. 2007; Huang et al. 2015), RBAP46 (Haigney et al., 2015; Jasencakova et al. 2010), VPS75 (Hammond et al. 2016), NASP (Bowman et al. 2017; Jasencakova et al. 2010) and TONSL (Saredi et al. 2016). It remains to be understood how this dynamic network of multi-chaperone interactions is regulated. However, ASF1 is subject to phosphorylation by the S phase active kinases TLK1 and TLK2 (Sillje and Nigg 2001; Sillje et al. 1999), which are targets of the checkpoint kinase Chk1 (Groth et al. 2003; Krause et al. 2003). TLKs specifically target histone-free ASF1, and phosphorylation in turn promotes histone binding and interaction with downstream chaperones like CAF-1 (Klimovskaia et al. 2014). In contrast, Codanin-1 can act as a negative regulator of histone supply by sequestering ASF1 in the cytoplasm (Ask et al. 2012). Once ASF1 translocate with H3-H4 dimers into the nucleus, it makes direct contact with the two downstream chaperones CAF-1 and HIRA (De Koning et al. 2007; Mello et al. 2002; Tang et al. 2006; Tyler et al. 2001) and, somehow, hand over histone H3-H4 dimers to these chromatin-bound complexes. Whereas CAF-1 deposits canonical histones H3.1/H3.2-H4 in a replication-coupled manner (Sect. 15.3.3) (Smith and Stillman 1989; Tagami et al. 2004), HIRA deposit H3.3-H4 in a replication-independent manner (Ray-Gallet et al. 2002; Tagami et al. 2004) and may serve a gap-filling role under conditions where replication-coupled nucleosome assembly is impaired (Ray-Gallet et al. 2011).

#### 15.3.1.2 Post-translational Modifications of Newly Synthesized H3.1-H4

Newly synthesized histones H4 are acetylated on lysine 5 and 12 by the histone acetyl transferase HAT1 in complex with RBAP46 (Kleff et al. 1995; Parthun et al. 1996; Sobel et al. 1995). This histone H4 di-acetylation is a highly conserved hallmark of new histones, yet its exact function remains unclear. It may contribute to a wide range of events including import of histones into the nucleus (Blackwell et al. 2007; Glowczewski et al. 2004), repair of replication forks (Barman et al. 2006) and CAF-1-dependent chromatin assembly in vivo (Ejlassi-Lassallette et al. 2011; Zhang et al. 2013). Acetylation of new histones generates an open and accessible chromatin organization behind the replication fork (Annunziato and Seale 1983), in part by delaying binding of the linker histone H1 until the acetylations are removed by histone deacetylases 10–20 min after fork passage (Alabert et al. 2014; Perry and Annunziato 1989; Sirbu et al. 2011). Histone H3 is also acetylated, but the sites and abundance differ between species. In yeast, ASF1-bound histone H3 is subject to acetylation on lysine 56 by Rtt109 (Driscoll et al. 2007; Han et al. 2007; Tsubota et al. 2007), and this is a very abundant modification which has major implications for genome stability (Masumoto et al. 2005). H3 K56ac promotes binding to the downstream chaperones CAF-1 and RTT106 and thus facilitates nucleosome assembly (Burgess et al. 2010; Li et al. 2008). The function of H3K56ac does not appear to be conserved in mammals, as H3K56ac is not very abundant (Jasencakova et al. 2010; Ray-Gallet et al. 2011; Xu et al. 2011). In human, soluble H3 is acetylated on lysine 14 and 18 (Jasencakova et al. 2010; Loyola et al. 2006), but their functions remain unclear. Soluble histone H3.1/H3.2 can also be monomethylated on lysine 9 by SETDB1 (Loyola et al. 2006) both early on in the process of translation (Rivera et al. 2015) and late in the assembly pathway as part of a heterochromatin-associated CAF-1 complex containing HP1a and SetDB1 (Loyola et al. 2009). Pre-deposition mono-methylation of H3K9 can prime for the establishment of H3K9me3, which is associated with repressive chromatin (Loyola et al. 2009; Pinheiro et al. 2012); however H3K9me1 may also serve additional roles in the assembly process itself.

### *15.3.2 Delivery of Newly Synthesized H2A-H2B*

The supply chain that delivers histone H2A-H2B to newly replicated DNA appears to be less complex (reviewed in Hammond et al. 2017), although this may reflect that fewer studies have focused on H2A-H2B chaperones. NAP1, the main chaperone for soluble H2A-H2B, stabilizes the H2A-H2B dimer (D'Arcy et al. 2013) and together with Importin-9 facilitates nuclear import (Straube et al. 2010). The binding of NAP1 prevents unscheduled accumulation of H2A-H2B on DNA (Andrews et al. 2010; D'Arcy et al. 2013), and NAP1 is a prime candidate to deliver H2A-H2B to newly formed tetrasomes to complete nucleosome assembly. However, given that FACT is also a histone H2A-H2B (Belotserkovskaya and Reinberg 2004; Hondele

et al. 2013) and it is present at replication forks, it might also contribute to H2A-H2B deposition (reviewed in Hammond et al. 2017).

### *15.3.3 De Novo Deposition*

Based on the average replication rate of 1.5–2 kb/min, about one nucleosome assembles on leading and lagging strands every 7 second. Electron microscopy data of replication intermediates suggest that the first nucleosome is assembled about 225 and 285 bp behind the fork for leading and lagging strands, respectively (Gasser et al. 1996). The nucleosome assembly is a stepwise process: the H3-H4 tetramer assembles first followed by two H2A-H2B dimers (Hammond et al. 2017; Smith and Stillman 1991) (Fig. 15.4). While new and old H3-H4 dimers are segregated into separate nucleosomes, new and old H2A-H2B dimers can associate with both new and old H3.1-H4 tetramers (Jackson 1987; Xu et al. 2010). Deposition of histone H1 occurs later as part of the chromatin maturation process, probably depending on deacetylation of the new histones and nucleosome remodelling to establish order nucleosome arrays (Alabert et al. 2014; Perry and Annunziato 1989).

#### 15.3.3.1 Mechanisms of Nucleosome Assembly

Under physiological salt concentrations, histones bind nonspecifically to naked DNA and do not form nucleosomes, and efficient nucleosome assembly thus relies on the concerted action of histone chaperones (reviewed in Hammond et al. 2017). The key chaperone responsible for histone H3.1-H4 deposition onto newly replicated DNA is CAF-1 (Smith and Stillman 1989), which receives histone dimers from ASF1 (Tyler et al. 1999). CAF-1 is composed of three subunits: p150, p60 and RBAP48 (reviewed in Hammond et al. 2017). CAF-1 p150 binds directly to PCNA via a dual PIP-box motif (Moggs et al. 2000; Shibahara and Stillman 1999), elegantly coupling CAF-1 activity to DNA synthesis. ASF1 binds directly to the B domain of the CAF-1 p60 subunit (Mello et al. 2002; Tang et al. 2006; Tyler et al. 2001) through a surface that is an opposite site to the H3-H4-binding site on ASF1 (English et al. 2006; Natsume et al. 2007) (Fig. 15.4). While CAF-1 potentially could bind H3-H4 through RBAP48 (Verreault et al. 1996), recent data argue that it is mainly the p150 subunit that interacts with histones (Kim et al. 2016; Liu et al. 2016). CAF-1 has been found to mainly bind H3.1-H4 dimers (Tagami et al. 2004), but biophysical studies of CAF-1 function show that two CAF-1 complexes may work together to assembly a tetramer during assembly (Mattiroli et al. 2017); Sauer et al. 2017).

In addition to CAF-1, additional pathways for replication-coupled nucleosome assembly are emerging. However, their relative importance and integration with each other remain unclear. RTT106, a yeast chaperone with no clear mammalian homolog, works together with CAF-1 in replication-coupled nucleosome assembly

(Clemente-Ruiz et al. 2011; Li et al. 2008), but it is not clear how it is recruited to sites of DNA replication. Yeast RPA, a critical constituent of DNA replication machinery that binds ssDNA, was recently found to bind histone H3-H4 and facilitate replication-coupled deposition of new histones H3-H4 (Liu et al. 2017). In yeast, FACT has also been implicated in new histone H3-H4 deposition (Yang et al. 2016), and there is evidence that yeast ASF1 interacts with RFC3, part of the clamp loader (Franco et al. 2005). In addition, MCM2 is able to bind both new and old histones at the replication fork and could thus play a role in new histone deposition together with ASF1 (Huang et al. 2015; Jasencakova et al. 2010), although interaction with MCM2 is not a prerequisite for H3.1-H4 incorporation in mammalian cells (Huang et al. 2015). A critical question for future research is whether any of these deposition pathways are specific to either new or old histone H3-H4 and how their activities are coordinated to ensure balanced deposition of new and old H3-H4 on both leading and lagging strands as predicted in the current models.

## 15.4 Coordination of Nucleosome Assembly and Fork Progression

In yeast, cells can complete one round of DNA replication in the absence of newly synthesized histones (Kim et al. 1988; Prado and Aguilera 2005). In mammals, inhibition of histone production rapidly blocks replisome progression (Mejlvang et al. 2014; Seale and Simpson 1975; Weintraub 1972), reflecting a requirement to coordinate DNA replication and nucleosome assembly. The pool of newly synthesized histones represents only about 1% of the total number of histones in the cells, which explain the need for high rates of histone biosynthesis throughout S phase. The prediction is that replication rates will be sensitive to small changes in new histone availability, as it is the case for deoxyribonucleotides (Earp et al. 2015). However, whereas lack of dNTPs rapidly induces a checkpoint response in part due to excessive DNA unwinding at replication forks (Zeman and Cimprich 2014), lack of histones and impaired nucleosome assembly slows down replication forks without exposing ssDNA and triggering a strong checkpoint response (Groth et al. 2007; Mejlvang et al. 2014). Nevertheless, forks arrested due to lack of nucleosome assembly do become unstable over time giving rise to DNA damage and genomic instability (Hoek and Stillman 2003; Mejlvang et al. 2014; Ye et al. 2003). While replication-coupled nucleosome assembly is not required for fork progression in yeast, it is necessary to maintain integrity of advancing forks. Indeed, mutants defective in replication-coupled chromatin assembly show large chromosomal rearrangements, elevated sister chromatid exchange and loss of replication fork integrity (Clemente-Ruiz et al. 2011; Clemente-Ruiz and Prado 2009; Myung et al. 2003; Prado et al. 2004).

One mechanism proposed to directly link nucleosome assembly and replisome progression involves nucleosome assembly on the lagging strand. Here, histones

can be in principle be deposited onto the growing Okazaki fragment once sufficient DNA has been synthesized. Consistent with this idea, the two enzymes important for Okazaki fragment processing, FEN1 and DNA ligase I, can operate efficiently on a nucleosomal substrate (Chafin et al. 2000; Huggins et al. 2002). Intriguingly, Okazaki fragment size is close to the size of a nucleosome repeat (about 200 bp in human cells), suggesting a link between the two processes. This link has now been supported by in vitro and in vivo work in yeast (Devbhandari et al. 2017; Kurat et al. 2017; Smith and Whitehouse 2012). In vivo mapping of Okazaki fragments in yeast revealed that the ligation sites between Okazaki fragments are found in close proximity to the nucleosome dyads and that Okazaki fragment length is increased in CAF-1 mutants deficient in nucleosome assembly (Smith and Whitehouse 2012). More recently, elegant systems for reconstituting DNA replication in vitro demonstrated that nucleosome assembly determines Okazaki fragment length by restricting Pol delta (Polδ) progression (Devbhandari et al. 2017). The evidence thus support a model in which newly assembled nucleosomes will block Polδ progression as the polymerases reaches the nucleosome, which in turn is the signal to release the polymerase and ligate the fragment to the lagging strand. Consistent with this, PCNA unloading, which requires ligation of Okazaki fragments (Kubota et al. 2015), is also impaired when nucleosome assembly is impaired (Mejlvang et al. 2014). Nucleosome assembly on the lagging strand would therefore prime for Polδ release, Okazaki fragment maturation and PCNA unloading, potentially tethering replisome progression to chromatin assembly. How the interplay and potential interdependency between DNA synthesis by Pol epsilon and PCNA-dependent nucleosome assembly operate on the leading strand remains unclear.

## 15.5 Epigenome Maintenance

DNA replication leads to a genome-wide disruption of chromatin along with two-fold dilution of histone modification as new histones are deposited to double the nucleosome content (Alabert et al. 2015; Alabert and Groth 2012). How chromatin-based information is restored on the two new daughter DNA strands remains a major question. The mechanisms governing restoration of DNA methylation have been extensively studied, and here the maintenance DNA methyltransferase DNMT1 is recruited to hemi-methylated sites in new DNA shortly after fork passage through a mechanism that involves the UHFR1/2 cofactors directly recognizing hemi-methylated CpG sites (Almouzni and Cedar 2016). This section focuses on the restoration of nucleosome positioning and histone modifications. Reincorporation of histone variants and restoration of high-order structures are reviewed elsewhere (Dileep et al. 2015; Talbert and Henikoff 2017).

### 15.5.1 Nucleosome Positioning

The position of nucleosomes contributes to gene regulation by controlling access to DNA. Accordingly, active promoters and enhancers consist of well-defined nucleosome-free regions (NFR). At these loci, newly assembled nucleosomes thus have to be repositioned to match the pre-replicative state. Nucleosome positioning behind replication forks has recently been analysed genome-wide in yeast and *Drosophila*. In yeast, nucleosomes realign according to their initial profile within minutes (Fennessy and Owen-Hughes 2016; Vasseur et al. 2016). The positioning occurs more rapidly in gene bodies of highly transcribed genes, suggesting a role for transcription elongation (Vasseur et al. 2016). However, transcription elongation-independent mechanisms are also involved in nucleosome positioning, and transcription factors have been suggested to work as so-called rulers that define upstream and downstream nucleosome occupancy patterns (Fennessy and Owen-Hughes 2016; Vasseur et al. 2016). In *Drosophila*, nucleosome positioning is blurred after DNA replication with nucleosome gain at NFR and weakening of strong promoter nucleosome positioning (Ramachandran and Henikoff 2016). One hour later, nucleosomes largely regain patterns, an occupancy pattern resembling the pre-replicative state. These observations suggest that in *Drosophila*, but not in yeast, nucleosomes may outcompete transcription factor during chromatin assembly, potentially creating a window for switching expression states by forming a post-replicative chromatin state characterized by a more uniform nucleosome occupancy pattern.

### 15.5.2 Histone Post-translational Modifications

A 1:1 mix of newly synthesized and recycled histones is assembled into nucleosomes on the daughter DNA strands (Alabert et al. 2015) (Fig. 15.1), leading to a twofold dilution of histone modifications as the new histones carry mainly pre-deposition-specific acetylation marks. How the pre-replication level of histone modifications is restored after DNA replication is a central question in epigenetics, given that histone modifications sustain transcriptional programmes and must be stably maintained across cellular division (Pengelly et al. 2013). Mass spectrometry data in human cells and genetic analysis in *C. elegans* and fission yeast support that histones retain their modifications during recycling (Alabert et al. 2015; Audergon et al. 2015; Gaydos et al. 2014; Ragunathan et al. 2015; Scharf et al. 2009; Xu et al. 2011). Once incorporated, newly synthesized histones must acquire modification similar to the old ones in the locus where they are incorporated. Several studies have followed this process (Alabert et al. 2015; Pesavento et al. 2008; Scharf et al. 2009; Xu et al. 2011; Zee et al. 2012) and conclude that restoration of the pre-replicative

histone modification level is a slow process continuing even in G1 after cell division. This argues that the level of histone modification at a given site oscillates during the cell cycle. It remains unclear how these fluctuations affect chromatin-based processes such as transcription. However, new histones were recently shown to provide a signature of post-replicative chromatin that is recognized by a protein complex involved in homologous recombination (Saredi et al. 2016). This signature relies on the unmethylated state of histone H4 K20, which remains unmodified on new histones until late G2 (Alabert et al. 2015; Pesavento et al. 2008), at which time almost all new histones acquire mono- and di-methylation at this site in a stepwise fashion. In this way, unmethylated H4K20 marks regions of chromatin that have been replicated as good substrates for homologous recombination (Saredi et al. 2016).

How newly assembled histones acquire modifications to restore the pre-replicative state remains unclear. Several histone modifiers bind to PCNA (reviewed in Alabert and Groth 2012) which could serve as a landing platform and provide a direct link between DNA replication and histone modification. However, the slow rates with which marks are established on the new histones question the idea of a tight coupling to the replication process. One possibility is that the replication machinery and parental histones recruit activities that prime histones towards a certain modification state (e.g. H3K9me1 and H3K27me1 are imposed very rapidly, Alabert et al. 2015) and that general chromatin maintenance mechanisms known from studies of transcriptional regulation ensure that the final modification state is restored prior to the next round of cell division. These processes are under intense studies since they are instrumental for maintenance of epigenetic states in cycling cells and likely to play an important role in both organismal development and disease.

## 15.6 Concluding Remarks

Our understanding of how histones are handled at replication forks is continuing to expand. In the future it will also be important to understand how non-nucleosomal proteins, such as regulatory factors, structural proteins and transcription factors, reassociate on daughter strands and whether their occupancy pattern is affected by DNA replication. Another exciting area of research is to understand how replication stress impinges on the ability of cells to maintain chromatin states (reviewed in Dabin et al. 2016; Svikovic and Sale 2016). As shown in this chapter, nucleosome dynamics are tightly linked to replisome progression, and it remains unclear how fork arrest, processing and collapse will affect the process of chromatin duplication.

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# Chapter 16
# The Temporal Regulation of S Phase Proteins During $G_1$

**Gavin D. Grant and Jeanette G. Cook**

**Abstract** Successful DNA replication requires intimate coordination with cell-cycle progression. Prior to DNA replication initiation in S phase, a series of essential preparatory events in $G_1$ phase ensures timely, complete, and precise genome duplication. Among the essential molecular processes are regulated transcriptional upregulation of genes that encode replication proteins, appropriate post-transcriptional control of replication factor abundance and activity, and assembly of DNA-loaded protein complexes to license replication origins. In this chapter we describe these critical $G_1$ events necessary for DNA replication and their regulation in the context of both cell-cycle entry and cell-cycle progression.

**Keywords** Cell cycle • Origin licensing • RB • E2F • Cyclin • CDK • APC/C • Quiescence • DREAM complex

## 16.1 Introduction

Proper progression from the $G_1$ cell-cycle phase into S phase and the accurate duplication of chromosomal DNA are key for successful completion of the cell division cycle. In this chapter we describe the molecular events of $G_1$ that are critical for successful initiation and completion of DNA replication and, thus, S phase. We primarily focus on events that have been studied in human cell lines, but when appropriate, we expand our focus to other model systems. While the ultimate goal of $G_1$ is to prepare for S phase, there are many sequential $G_1$ processes required for the proper transition into S phase including appropriate control of gene expression,

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protein accumulation, and protein-DNA complex assembly. We have divided our discussion of $G_1$ into three temporal segments: $G_0$, early $G_1$, and late $G_1$.

Because error-free DNA replication is so critical, many different cell-cycle checkpoints and levels of regulation ensure that this process is precise. Many of these checkpoints happen in $G_1$ before DNA replication begins. Given that DNA replication is essentially irreversible, it makes sense that multiple inputs are integrated into the decision to begin replication. If cells receive appropriate inputs in $G_1$, and the external signals and environment are compatible with another round of the cell cycle, then an intracellular signaling cascade ultimately results in irrevocable commitment to S phase entry; this commitment is known as passage through the "restriction point" (Pardee 1974). The restriction point, as canonically known, is the point at which cells transition from requiring mitogens (extracellular growth factors) for S phase entry to no longer requiring sustained mitogenic signaling for progressing into S phase and through the rest of the cell cycle (Blagosklonny and Pardee 2002). If mitogenic stimuli are removed before cells pass the restriction point, they may enter $G_0$, or quiescence, a temporary withdrawal from the cell cycle. Quiescence is a normal part of organismal development and homeostasis and is important for regenerative processes and the long-term maintenance of stem cell populations (Rumman et al. 2015).

When reduced to its simplest form, progression through $G_1$ is relatively straightforward: there is a period of low cyclin-dependent kinase (CDK) activity after mitosis or during $G_0$. Then mitogenic signals trigger intracellular signal transduction networks that ultimately activate the heterodimeric protein kinase cyclin D/CDK4 or cyclin D/CDK6; CDK4 is the catalytic subunit responsible for phosphotransfer but is inactive in the absence of cyclin binding (Choi and Anders 2014). (Current evidence indicates that cyclin-dependent kinases 4 and 6 are functionally redundant in $G_1$; thus, to simplify, we will only designate CDK4.) Cyclin D/CDK4, in turn, upregulates cyclin E/CDK2, which both drives expression of genes required for DNA replication and triggers the initiation of DNA synthesis (Fig. 16.1). These two major cyclin/CDK pairs drive cells through early then late $G_1$ and into S phase. There are three different human genes for cyclin D (1, 2, and 3) and two different genes that encode cyclin E (1 and 2); we refer to the class of genes (e.g., cyclin D) instead of specifying each gene product.

Regulatory events or genetic perturbations can prevent, delay, speed up, or enhance any of the essential $G_1$ processes. Both positive and negative regulation during $G_1$ influences the rate of cell-cycle progression. For instance, cyclins fluctuate with peak abundance at different cell-cycle phases, and these peaks are driven by accumulation (largely from gene induction) and induced protein degradation. The catalytic subunits of CDKs are present at relatively stable levels throughout the cell cycle but are not active unless bound to a cyclin. Once bound by cyclin, CDK undergoes a series of modifications (protein domain remodeling, phosphorylations, dephosphorylations, etc.) that either activate or inhibit kinase activity. The importance of controlling these events in the cell cycle is illustrated by the fact that most of the proteins involved are misregulated in some way during tumorigenesis.

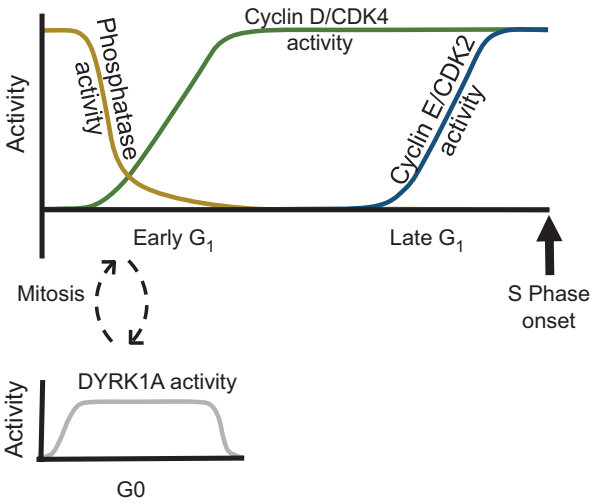


**Fig. 16.1** Activities of cell cycle-dependent kinases and phosphatases during $G_1$ and $G_0$. Dashed lines indicate entry and exit from $G_0$

Complete and precise genome duplication in organisms with large genomes divided among many discrete chromosomes requires a complex network of pathways and molecules. Decades of research in both the cell cycle and the DNA replication fields has identified a great many proteins that act at key points in $G_1$ entry and progression. To aid readers, we provide a list of a subset of principle $G_1$ proteins and their general functions relevant to our discussion in Table 16.1.

## 16.2 Quiescence ($G_0$)

Before passing through the restriction point, cells can enter a nonproliferative phase, called $G_0$ or quiescence, which is considered to be outside of the cell cycle. This noncycling state is fundamentally different from other cell-cycle exits such as senescence or terminal differentiation, which are irreversible; cells can re-enter the cell cycle from $G_0$ by transitioning into and progressing through $G_1$. $G_0$ is critically important to organismal health and maintenance because, when needed, populations of stem cells in $G_0$ can re-enter the cell cycle to replenish the overall cell population (Rumman et al. 2015). The availability of $G_0$ cells is especially important in wound healing, the immune response, the routine regeneration of the intestinal epithelium, and in bone marrow (where hematopoietic stem cells produce blood cells). Similarly, populations of stem cells in $G_0$ provide protection against acute toxic events such as radiation or chemotherapy. Many types of chemotherapy are designed to induce toxic damage in cycling cells, often by forming DNA adducts that prevent cells from successfully replicating their DNA. It has been proposed recently, however, that some populations of cancer cells are able to escape genotoxic drugs and thus the effects of chemotherapy by entering a $G_0$ state. After treatment has ceased, these

**Table 16.1** A subset of important $G_1$ proteins and their functions

| Factor | Function |
|---|---|
| *Kinases* | |
| Cyclin D/CDK4 | Rb phosphorylation |
| Cyclin E/CDK2 | Rb phosphorylation, initiating DNA synthesis at origins |
| DYRK1A | DREAM complex assembly, Lin52 phosphorylation |
| GSK3β | p130 stabilization and cyclin D destabilization in $G_0$ |
| *Protein kinase inhibitors* | |
| p21 | CDK2 inhibition (cyclin D/CDK4 assembly) |
| p27 | CDK2 inhibition |
| *Phosphatases* | |
| PP1 | Rb dephosphorylation |
| PP2A | Rb dephosphorylation |
| *Transcription factors* | |
| E2F1,2,3 | Cell-cycle gene activation |
| E2F4,5,6,7,8 | Cell-cycle gene repression |
| DP1 | Obligate binding partner of activating E2F1,2,3 |
| *Transcriptional corepressors* | |
| Rb | Activator E2F repression in $G_1$ |
| p107 | Activator E2F repression in S phase |
| p130 | Cell-cycle gene repression in G0 (DREAM complex) |
| HDAC | Histone deacetylase |
| DREAM complex | Repression in $G_0$ (E2F4/DP1/p130 and Lin52-containing MuvB complex) |
| *Ubiquitylation* | |
| APC/C | E3 ubiquitin ligase complex in mitosis, $G_1$, and $G_0$ |
| Cdc20 | Substrate-recruiting subunit of APC/C in mitosis |
| Cdh1 | Substrate-recruiting subunit of APC/C in mitosis, $G_1$, and $G_0$ |
| Emi1 | Pseudosubstrate inhibitor of $APC/C^{Cdh1}$ in $G_1$ |
| SCF | E3 ubiquitin ligase complex throughout the cell cycle |
| Skp2 | Substrate-recruiting subunit of SCF |
| 26S proteasome | Degradation of ubiquitylated proteins |
| *Origin licensing* | |
| MCM | DNA helicase (Mcm2-Mcm7) |
| ORC | DNA-binding MCM loading enzyme (Orc1-Orc6) |
| Cdc6 | MCM loading enzyme |
| Cdt1 | MCM recruitment and loading |
| Geminin | Cdt1 inhibitor |
| Set8 (PR-Set7) | Histone H4 K20 monomethylase |

cells can re-enter the cell cycle and form a new tumor, causing a cancer relapse (O'Connor et al. 2014). Thus, quiescence provides a reservoir of proliferative capacity that maintains adult tissues but also presents a challenge for cancer treatment.

### *16.2.1 Global Transcriptional Control in $G_0$*

One characteristic of quiescent cells is the active repression of transcriptional activity, especially the repression of genes involved in cell-cycle progression. The molecular mechanisms that occur during the transition from the cell cycle into $G_0$ are largely unknown, but investigators have identified some key molecular characteristics of cells that are already in $G_0$ (Litovchick et al. 2007, 2011). Whole-genome transcriptomics studies revealed unique quiescence-associated transcriptional programs (Coller et al. 2006). This microarray based approached catalogued groups of up- or downregulated genes in primary human fibroblasts driven into $G_0$ by three different methods (serum starvation, contact inhibition, or "loss of adhesion"). While the investigators found one set of genes that were similarly regulated in each of the three methods, indicating there is a "core" transcriptional program in quiescent cells, the regulation of some genes were synchronization method specific. The differences among these three datasets suggest that there are different transcriptional programs active in quiescence depending on the mechanism of cell-cycle exit (Coller et al. 2006). Many of the genes that were downregulated in at least one condition are central to DNA replication and cell-cycle control such as the mitotic cyclin B1, enzymes required to create DNA synthetic precursors, and subunits of the major E3 ubiquitin ligases that act in S phase or mitosis. Among the commonly upregulated genes were transcriptional repressors such as *E2F4* and *E2F5*, which cooperate with the so-called pocket proteins to suppress the expression of DNA replication and cell-cycle genes during $G_0$.

### *16.2.2 E2F and Pocket Proteins*

The majority of genes that encode proteins needed for cell-cycle progression, DNA synthesis, and mitosis are regulated by a class of transcription factors known as E2Fs. The activating subclass of E2F transcription factors (E2F1, E2F2, and E2F3) is one of the main drivers for the cell-cycle transcriptional program. We will refer to the class of activating transcription factors and their binding partners, DP1 or DP2, as E2F, instead of the individual gene products. A second subclass of E2F transcription factors, known as the repressor E2Fs (E2F4, E2F5, E2F6, E2F7, and E2F8), generally inhibit expression of target genes (Frolov and Dyson 2004). Each E2F protein has a DNA-binding domain, an RB-binding domain, and a DP1-binding domain that consists of a leucine-repeat sequence and the marked-box domain (Fig. 16.2a). E2F is primarily functional during late $G_1$ and early S phase as cells accumulate proteins

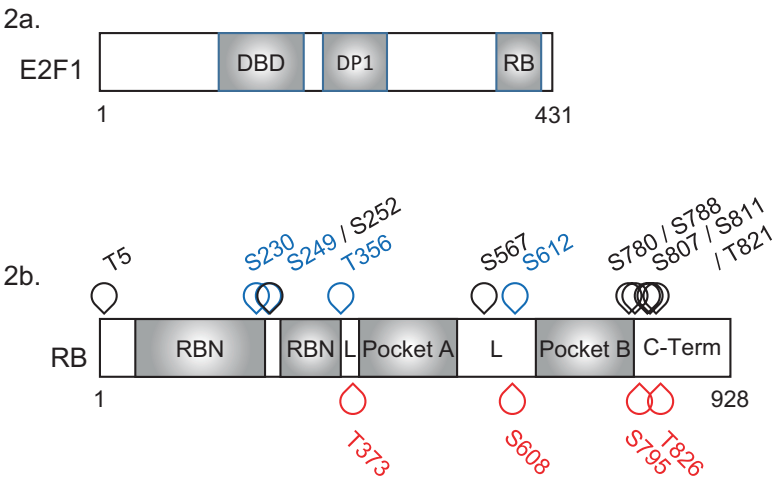


**Fig. 16.2** E2F and RB proteins. (**a**) Domain structure of the transcription factor E2F1 as an representative activator E2F. *DBD* DNA-binding domain, *DP1* DP-binding domain, *RB* RB-binding domain. (**b**) RB phosphorylation sites. Blue sites have been implicated in increased binding affinity to E2F transcription factors; red sites decrease affinity for E2F, and black indicates no change in E2F affinity. *RBN* N-terminal domains of RB, *L* linker domains

in preparation for DNA replication. E2F transcriptionally activates the expression of genes encoding proteins involved in positive feedback loops, such as E2F itself, and cyclin E that drive the cell into S phase. During mid-S phase through mitosis and early $G_1$, E2F is bound to and repressed by the pocket protein RB which is encoded by the human retinoblastoma gene, the first tumor suppressor identified (Knudson 1971; Benedict et al. 1983; Murphree and Benedict 1984). E2F-regulated genes are actively repressed during $G_0$ by the action of E2F-associated corepressors and that repression is mediated by E2F interaction with the p130 and RB-pocket proteins.

Each member of the activating E2F subclass can largely compensate for the loss of the other two members; however, loss of all three E2F proteins results in severely impaired cell proliferation (Wu et al. 2001). *E2f1* null mice are viable and reproduce normally, indicating that the other two activating E2F transcription factors are able to compensate for the loss of E2F1 (Field et al. 1996). Likewise, $E2f1^{-/-}$ $E2f2^{-/-}$ mice also survive to adulthood. Interestingly, $E2f3^{-/-}$ mice die early in development, indicating E2F3 plays a critical role in mouse development, but $E2f3^{-/-}$ cells proliferate nonetheless (Wu et al. 2001). Using conditional knockout mice, Wu et al. generated mouse fibroblast lines with combinations of two or three null alleles in the three activating *E2f* genes. The cells deficient for two of the three E2F transcription factors proliferated slowly, whereas cells lacking all three had no measurable proliferation (Wu et al. 2001).

There are three E2F-binding "pocket proteins" encoded by mammalian genomes: the retinoblastoma protein (RB), p107 (RBL1), and p130 (or RBL2). They are named for a structural domain containing a protein-binding cleft. Though they do

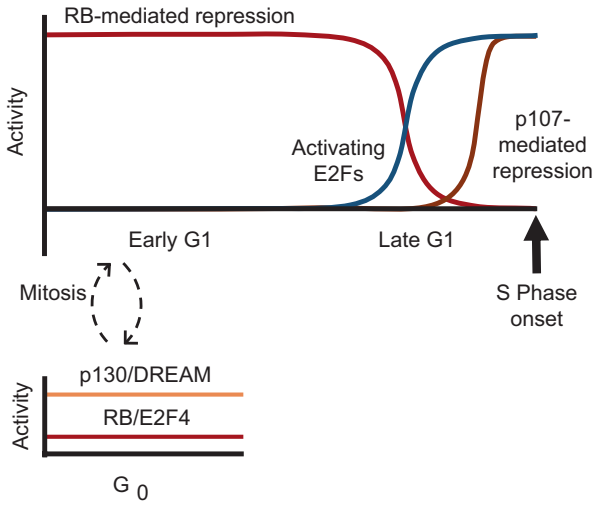


**Fig. 16.3** Activities of the pocket proteins RB, p107, and p130 and activating E2F transcription factors during $G_1$ and $G_0$. Dashed lines indicate entry and exit from $G_0$

not bind DNA directly, these proteins mediate transcriptional repression of many cell-cycle genes and are key regulators of cell-cycle progression. Each pocket protein consists of five major domains: the N- and C- terminal domains, the A- and B-pocket domains, and a linker region between the two pocket domains (Fig. 16.2b).

The three pocket proteins have both overlapping and distinct roles, patterns of expression, regulation, and binding partners during the cell cycle (Classon and Harlow 2002). RB is primarily active in $G_1$ by binding to and inactivating the activating family of E2F transcription factors (E2F1, E2F2, and E2F3). p130 is highly expressed in $G_0$, whereas p107 expression increases after mitogenic stimulation to peak in S phase (Fig. 16.3) (Litovchick et al. 2007; Devoto et al. 1992; Cobrinik et al. 1993; Burkhart et al. 2010). If one pocket protein is lost through mutation, the others can functionally compensate in some but not all settings.

### *16.2.3 E2F and Pocket Proteins in $G_0$*

During $G_0$, p130 is abundant and bound to DNA through E2F4 as a member of a multisubunit complex, known as the DREAM complex (Litovchick et al. 2007; Guiley et al. 2015). In contrast, RB is much less abundant, and p107 is almost undetectable in quiescent cells (Hurford et al. 1997; Moberg et al. 1996). The p130-containing DREAM complex only assembles during $G_0$ and is named for its components: DP, RB-like (e.g., p130), E2F, and MuvB subcomplex, which itself consists of subunits LIN9, LIN37, LIN52, LIN54, and RBAP48. The DREAM complex represses both E2F target genes and genes that are expressed in late S phase and $G_2$. This repression prevents aberrant expression that could lead to improper cell-cycle reentry (Litovchick et al. 2007; Muller et al. 2012, 2016).

DREAM complex assembly requires phosphorylation of the LIN52 subunit of MuvB at serine 28. In $G_0$, Lin52 Ser28 is phosphorylated by DYRK1A kinase, a protein kinase with multiple roles in cell proliferation and neuronal development (Fig. 16.1) (Chen et al. 2013; Soppa et al. 2014). Phosphorylation of p130 also contributes to DREAM assembly in $G_0$ by increasing p130 stability. Glycogen synthase kinase 3β (GSK3β), a protein kinase that is highly active during $G_0$, phosphorylates three sites in the loop region in the B-pocket domain of p130. These three phosphorylations stabilize p130 to promote DREAM assembly but are removed during cell-cycle reentry to facilitate DREAM disassembly (Litovchick et al. 2004). Cyclin D/CDK4 phosphorylates p130 to disrupt its incorporation into the DREAM complex; cyclin D/CKD4 activity is low in $G_0$, allowing the DREAM complex to fully assemble (Sandoval et al. 2009). These p130 phosphorylations occur in the p130 interdomain linker region and disrupt the association between p130 and LIN52 to derepress DREAM-regulated genes (Guiley et al. 2015).

The MuvB subcomplex associates with DP, RB-like, and E2F proteins to form the DREAM complex in $G_0$, but it also binds to the transcription factor BMYB during S phase to form the MMB complex. The MMB complex is important for transactivation of genes involved in $G_2$ and M phase progression in tandem with the transcription factors BMYB and FOXM1 (Sadasivam et al. 2012). Somewhat paradoxically, the MuvB subcomplex is critical for the maintenance of quiescence as part of the transcriptionally repressive DREAM complex but is also critical for the proper transcriptional expression of $G_2$/M genes.

Consistent with the idea that cells are sometimes considered to be "resting" during quiescence, overall mRNA abundance is reduced, as are global levels of translation (Williams and Penman 1975; Levine et al. 1965; Degen et al. 1983). These reductions in both mRNA and protein may be due to the ability of RB and p130 to downregulate rRNA gene expression by inhibiting RNA polymerase I (Hannan et al. 2000), or the unique ability of RB to reduce tRNA levels by inhibition of RNA polymerase complex TFIIIB (Scott et al. 2001; White et al. 1996). The resulting reduction in ribosomal RNAs and tRNAs slows protein translation globally.

## 16.3 Early $G_1$

Despite its temporal distance from S phase, many events that occur in early $G_1$ influence S phase progression. These early $G_1$ events may ultimately impact when a segment of DNA is replicated in S phase, help determine the length of $G_1$, or ensure that proteins required for S phase do not accumulate prematurely. As described briefly in the introduction, $G_1$ progression requires the function of two heterodimeric kinases, cyclin D/CDK4 and cyclin E/CDK2. Cyclin E/CDK2 is also essential to initiate DNA synthesis in S phase. All forms of cell cycle-driving CDKs are inactivated at mitosis or kept inactive during $G_0$ through multiple mechanisms targeting both the cyclin and CDK subunits individually and the complexes themselves. These inactivating mechanisms are reversed in early $G_1$.

### *16.3.1 Cyclin D/CDK4 in Early $G_1$*

During $G_0$ and also between anaphase in mitosis and mid-$G_1$ in actively proliferating cells, CDK activity is minimal due to low transcriptional activation and targeted destruction of cyclins. Immediately after cell division, cyclin D protein levels are very low due to a combination of inactive *cyclin d* gene expression and active cyclin D protein degradation. Unlike cyclins E, A, or B, the protein levels of cyclin D do not rise and fall in a cell cycle-dependent fashion but instead are regulated by the presence of growth factors in the environment (Reviewed in (Choi and Anders 2014)). There are three primary mechanisms of cyclin D accumulation. The first is transcriptional activation by transcription factors controlled by mitogenic signaling pathways (e.g. c-Jun, c-Fos, or MYC) activated by cytokines (e.g., NF-κB) or other signaling factors (e.g., Notch or CREB). The second method of cyclin D accumulation is through the PI3K-AKT-mTOR-S6 K1 signaling pathway, which downregulates glycogen synthase kinase β and upregulates cyclin D translation (Diehl et al. 1998) (Koziczak and Hynes 2004; Fornoni et al. 2008; Katoh and Katoh 2006). The third method is stabilization and nuclear localization of cyclin D, which is regulated by phosphorylation of cyclin D itself. Cyclin D phosphorylation by GSK3β creates a phospho-dependent binding site for an E3 ubiquitin ligase complex, SCF (Alt et al. 2000; Barbash et al. 2009; Diehl et al. 1998). SCF family enzymes are multisubunit ubiquitylases that use a variety of substrate-targeting subunits and mechanisms. Substrate-binding stimulates polyubiquitination which typically targets proteins to a degradative protein complex called the 26S proteasome. Since GSK3β is inhibited in $G_1$, cyclin D1 is not phosphorylated and therefore not targeted for ubiquitylation and destruction; hence cyclin D1 accumulates in $G_1$.

Many different cofactors associate with cyclin D/CDK4 complexes to regulate kinase activity either positively or negatively depending on context. These cofactors include the Cip/Kip family of proteins (p21, p27, and p57). Although Cip/Kip proteins are strictly inhibitors of cyclin E/CDK2 and mitotic CDKs, they can act as positive regulators of cyclin D/CDK4 (Bao et al. 2006; Cheng et al. 1999; LaBaer et al. 1997; Chen et al. 1995; Fotedar et al. 1996; Luo et al. 1995). Specifically, phosphorylated forms of these proteins play integral roles in cyclin D/CDK4 assembly, activation, and nuclear localization. The Cip/Kip family of proteins is required for cyclin D/CDK4 complex assembly, which is a precursor for activation. After the cyclin D-CDK4-Cip/Kip tertiary complex is formed, a substrate-blocking loop in CDK4 is exposed for an essential activating phosphorylation by the CDK7/cyclin H/MAT1 complex, known as CDK-activating kinase (CAK) (Schachter et al. 2013). This phosphorylation event physically relocates the T-loop from the active site of the CDK subunit, allowing substrates access to the active kinase site. Finally, cyclin D/CDK4 complexes do not have nuclear localization sequences themselves, whereas Cip/Kip proteins have bipartite nuclear localization sequences. Thus, the association between cyclin D/CDK four complexes and Cip/Kip proteins promotes the assembly and nuclear localization of the cyclin D/CDK4 complex, and therefore access to nuclear substrates, most notably RB.

### 16.3.2 E2F and Pocket Proteins in Early $G_1$

Perhaps the most consequential event leading to the transition from $G_1$ into S phase is passing through the restriction point, a cell-cycle checkpoint between early $G_1$ and S (Pardee 1974; Blagosklonny and Pardee 2002). Cells that have not reached the restriction point can either progress through the cell cycle or can transition into quiescence, $G_0$. Cells that have progressed past the restriction point are destined to initiate DNA replication and complete the cell cycle. Progression past the restriction point is generally correlated with the phosphorylation state of RB. RB phosphorylation is dependent on cyclin-dependent kinases, in particular cyclin D/CDK4 in early $G_1$ and cyclin E/CDK2 in late $G_1$. Prior to the restriction point, RB is not fully phosphorylated and binds activating E2F transcription factors (E2F1–3) to prevent activated E2F target gene expression. Once RB is fully phosphorylated, it releases E2F, allowing activated transcription of most of the genes that encode DNA replication proteins.

RB activity effectively represses the transcription of E2F target genes, particularly those necessary for DNA replication, during early $G_1$, and prevents cells from prematurely passing the restriction point and irreversibly committing to S phase entry. The mechanism of repression is primarily through local chromatin changes at E2F-dependent promoters. RB recruits HDAC1 or HDAC2, two histone deacetylases that repress gene transcription by maintaining a closed chromatin state at the promoters of E2F target genes (Brehm et al. 1998; Takaki et al. 2004). RB is also linked to the mSin·HDAC complex (containing RBAP46, RBAP48, SAP18, and SAP45) via the RBP1 and SAP30 linker proteins (Fig. 16.4) (Suryadinata et al. 2011). The ultimate effect of these interactions is not only the inability of E2F to stimulate transcription but also active gene repression.

RB is phosphorylated by the $G_1$ cyclin/CDK complexes, and these phosphorylation events induce structural changes in RB that, in turn, weaken (but do not fully disrupt) the interaction of RB with E2F. The human retinoblastoma protein has 16 potential CDK phosphorylation sites (S/T-P sites) (Fig. 16.2b). Twelve of the 16 RB phosphorylation sites are optimal CDK consensus sites (S/T-P-X-K/R motifs) and can be readily phosphorylated by cyclin/CDK complexes (Ubersax and Ferrell 2007). Each of the 16 phosphorylation sites is also detectably phosphorylated in vivo (Brown et al. 1999; Lents et al. 2006; Zarkowska and Mittnacht 1997; Lees et al. 1991; Harbour et al. 1999; Huttlin et al. 2010; Dephoure et al. 2008; Connell-Crowley et al. 1997). Using a combination of in vitro kinase assays and two-dimensional tryptic phosphopeptide maps, Zarkowska and Mittnacht found that RB is preferentially phosphorylated by different cyclin/CDK pairs (Zarkowska and Mittnacht 1997; Zarkowska et al. 1997). Cyclin D/CDK4 preferentially phosphorylates T5, S249, T252, T356, T373, S608, S788, S795, S807, S811, and T826. Cyclin E/CDK2 phosphorylates T5, T373, S612, S795, and T821. Finally, cyclin A/CDK2 best phosphorylates RB positions T5, S608, S612, S795, and T821.While some positions are only phosphorylated by a single cyclin/CDK pair (for instance, S811 by cyclin D/CDK4), many can be phosphorylated by two different pairs or even, like T5, all three pairs (Zarkowska and Mittnacht 1997).

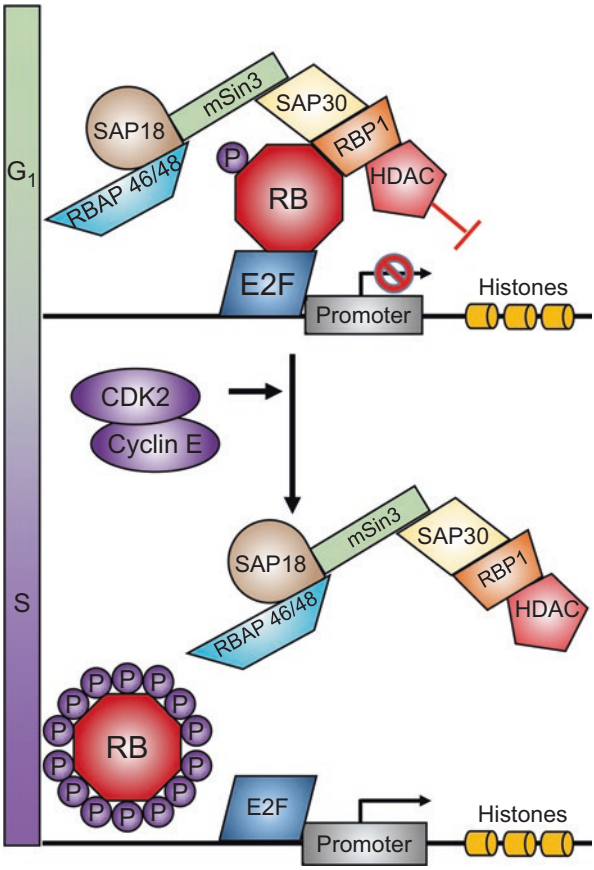


**Fig. 16.4** Inactivation of E2F target genes by the RB-associated SAP30-mSin3-HDAC complex in $G_1$. Monophosphorylated RB binds activating E2F transcription factors at promoters of E2F target genes. The SAP30-mSin3-HDAC complex binds to RB, and the associated HDACs deacetylate the E2F target gene, repressing transcription. Hyperphosphorylated RB releases E2F and dissociates from the SAP30-mSin3-HDAC complex, relieving the transcriptional repression of E2F target genes

These cyclin/CDK RB phosphorylation site preferences, combined with the temporal regulation of cyclin abundance (Fig. 16.1), suggest a temporal order for when individual sites are likely phosphorylated in vivo: In early $G_1$, RB is phosphorylated by cyclin D/CDK4 at eight sites (S249, T252, T356, S608, S788, S807, S811, and S826) (Zarkowska and Mittnacht 1997). After these initial eight sites are phosphorylated, a next round of phosphorylation occurs via either cyclin D/CDK4 complexes or cyclin E/CDK2 complexes at threonine 373. Finally, cyclin E/CDK2 phosphorylates serine 612 (Zarkowska and Mittnacht 1997). However, to date there has been no biochemical proof that these phosphorylations occur in the temporal order suggested, nor are there biochemical data precisely defining what functionally constitutes hypophosphorylated vs. hyperphosphorylated RB (Narasimha et al. 2014). Using two-dimensional isoelectric focusing (2D IEF), Narasimha et al. showed that during early $G_1$, RB is monophosphorylated by cyclin D/CDK4 at any one of 14 different sites, but there was no evidence for individual RB molecules with more than one phosphate until very late in $G_1$. (The T5 and S567 sites were not analyzed in that study.) Later in $G_1$, upon upregulation of cyclin E expression and activation

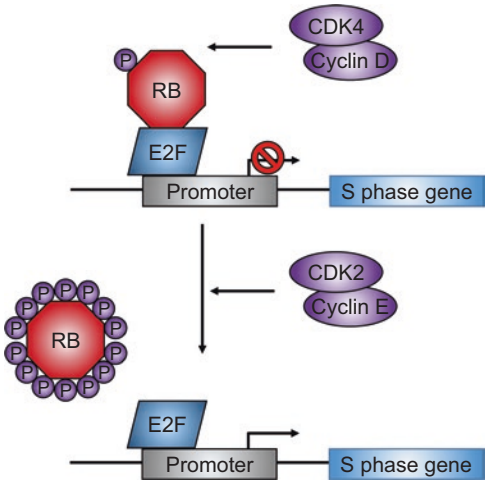


**Fig. 16.5** Inactivation of E2F by monophosphorylation of RB. In early $G_1$, E2F is bound and inactivated by RB, which is monophosphorylated by cyclin D/CDK4. Later in $G_1$, cyclin E/CDK2 becomes active and hyperphosphorylates RB, releasing E2F which then activates transcription of target genes

of cyclin E/CDK2, the vast majority of RB molecules were hyperphosphorylated on at least 14 sites and no longer bound to E2F (Fig. 16.5). Reducing RB hyperphosphorylation with different concentrations of the CDK2 inhibitor roscovitine, a compound that has little effect on cyclin D/CDK4, suggested that cyclin E/CDK2 is the principle processive RB kinase that rapidly converts monophosphorylated RB molecules to hyperphosphorylated RB molecules in a single binding step.

While phosphorylation of either S788 or S795 somewhat reduces RB affinity for E2F, simultaneous phosphorylation of both residues is additive. Characterizing the effects of multiple phosphorylation events on RB, Burke et al. showed that phosphorylation of S788/S795 destabilizes the interaction of RB and the E2F1-DP1 complex as well as inducing a conformational shift in RB, allowing a disordered linker, RB amino acids 787–816, to bind to the pocket domain. This phosphorylated linker region competes with E2F to bind the pocket domain of RB (Burke et al. 2014). These phosphorylation events weaken the overall interactions between RB and the E2F-DP1 complex as cells progress to late $G_1$, thus stimulating the production of gene products necessary for DNA replication.

Why is RB phosphorylation so complex (Fig. 16.2b)? For example, monophosphorylation at four sites (S230, S249, T356, or S612) may in fact increase the affinity of RB for E2F1, while phosphorylation at three other sites (T373, S608, or S795) decrease RB-E2F1 affinity (Burke et al. 2010, 2012, 2014; Narasimha et al. 2014). Currently, the biological significance of the various individual phosphorylation sites on RB interactions affinity is unclear. These different phosphorylation sites may modulate binding affinities for the different members of the activating E2F transcription factor family. Another possibility is that the differences in RB affinity for E2F1 allow for activation of some subset of E2F1 target genes, but not other subsets. Furthermore, CDK-dependent phosphorylation affects not only RB-E2F binding but also induces conformational changes in RB that disrupt interactions with histone deacetylases and associated proteins (Takaki et al. 2004). The abun-

dance of phospho-sites on RB may also facilitate integration of different signaling pathways and prevent aberrant E2F release and activation.

During early $G_1$ the other two pocket proteins, p107 and p130, are complexed with E2F4 at promoters contributing to gene repression, though they are less abundant than RB. In situations where RB has been lost, such as in an *Rb* null mouse, transcriptional repression and $G_1$ restraint is partially covered by the action of p130 and p107 (Cobrinik et al. 1996). Because RB phosphorylation and the subsequent E2F release stimulate progression from $G_1$ into S phase, this process is susceptible to oncogenic perturbation. Interestingly, there are only a few cancer types, such as retinoblastoma, osteosarcoma, and small cell lung cancer, which are associated with inactivating *RB* mutations, possibly reflecting the partial redundancy with p107 and p130 (Wikenheiser-Brokamp 2006; Kaye and Harbour 2004). In contrast, many tumors have mutations in upstream regulators of pocket proteins that render them constitutively inactive in cell-cycle control (Wikenheiser-Brokamp 2006; Paternot et al. 2010).

### *16.3.3 $APC^{CDH1}$-Mediated Protein Degradation in Early $G_1$*

In addition to the complex network of transcriptional control described above, post-transcriptional regulation ensures that early $G_1$ is free from both mitotic and S phase activities, to provide essential protected time to properly prepare for S phase. Prior to the cascade of RB phosphorylation events, both during cell-cycle reentry from $G_0$ and in early $G_1$ after mitosis, somatic cells experience a period of low cyclin-dependent kinase activity and high phosphatase activity (Fig. 16.1). This period removes mitosis-associated phosphorylations and introduces a cell-cycle pause to be receptive to inputs from the environment, such as growth factors. Activities that ensure this low CDK period, which ultimately determines the length of $G_1$, are the APC/C and protein phosphatases.

The anaphase-promoting complex/cyclosome, APC/C, is a multisubunit E3 ubiquitin ligase named for its essential role in mitotic progression, but APC/C also has critical functions throughout $G_1$. The substrate-targeting subunit of APC/C varies such that substrates in anaphase are recognized by the CDC20-targeting subunit, whereas substrates in $G_1$ are recognized by the CDH1 subunit (Manchado et al. 2010). As cells exit mitosis, $APC/C^{CDC20}$ targets the mitotic cyclins for degradation via the 26S proteasome, reducing the cellular kinase activity and allowing the activation of mitotic exit phosphatases, such as PP1 or PP2A (Wu et al. 2009; van Leuken et al. 2008). The reduced mitotic kinase activity promotes the assembly of $APC/C^{CDH1}$. Like $APC/C^{CDC20}$, the newly formed $APC/C^{CDH1}$ targets mitotic cyclins and kinases for degradation. $APC/C^{CDH1}$ also targets CDC20 for destruction, allowing for the complete switch from $APC/C^{CDC20}$ to $APC/C^{CDH1}$. Once cells have progressed through telophase and have completed the division into two daughter cells, $APC/C^{CDH1}$ continues to target mitotic proteins for destruction throughout $G_1$. One of the mitotic cyclins is cyclin A, which is active from mid-S phase until early mitosis (Fig. 16.1), and since cyclin A/CDK2 can trigger DNA synthesis, its removal

before and during $G_1$ helps prevent premature DNA replication (Coverley et al. 2002; Erlandsson et al. 2000).

Alongside the elimination of cyclin/CDK activity, phosphatases reverse mitotic (or $G_0$) phosphorylations. During mitosis, protein phosphatase 1 (PP1) is inactivated by CDK-mediated phosphorylation at threonine 320. In anaphase, PP1 removes the inhibitory phosphorylation on threonine 320 by auto-dephosphorylation as CDK activity drops. PP1 also inactivates its own inhibitor, inhibitor-1, a protein uniquely expressed in $G_2$/M (Wu et al. 2009). Activated PP1 then removes the phosphates from hyperphosphorylated RB until it is in the hypophosphorylated state which reestablishes RB-mediated repression. This dephosphorylation activity lasts from anaphase to mid-$G_1$ when the kinase activity toward substrates outpaces the phosphatase. Another phosphatase, the PP2A holoenzyme, also dephosphorylates the pocket proteins. The PP2A holoenzyme can also dephosphorylate the three pocket proteins after a variety of signals that, if ignored, can result in genome instability. These signals include oxidative stress, UV radiation, and DNA damage (Avni et al. 2003; Cicchillitti et al. 2003; Magenta et al. 2008; Voorhoeve et al. 1999). The dephosphorylation of RB delays activating E2F activity, allowing time to recover from the genomic insult.

In early $G_1$, APC/$C^{CDH1}$ also targets the SKP2 substrate receptor of the SCF (SKP1-Cullin-F-box) E3 ubiquitin ligase (Bassermann et al. 2014; Bashir et al. 2004; Wei et al. 2004). The resulting loss of SCF activity leads to accumulation of SCF substrates that include the cyclin-dependent kinase inhibitors $p21^{Cip1}$ and $p27^{Kip1}$. High levels of p21 and p27 help to maintain low CDK activity in early $G_1$. In human cells and mouse models lacking the CDH1 substrate-targeting subunit for APC/C, the exit from mitosis occurs normally, but cells begin $G_1$ with aberrantly high CDK activity. High CDK activity shortens $G_1$, i.e., causes premature entry into S phase. Early entry into S phase, in turn, can result in increased endogenous DNA damage, presumably from inadequate $G_1$ preparation or an uncoordinated $G_1$/S transition.

APC/$C^{CDH1}$ substrates include other proteins involved in controlling the length of $G_1$. ETS2, one such target, induces cyclin D expression. APC/$C^{CDH1}$ directed destruction of ETS2 delays *cyclin D* gene expression and prolongs $G_1$. Two of the repressor E2F transcription factors, E2F7 and E2F8, are also targets of APC/$C^{CDH1}$ and repress transcription of the CDH1 inhibitor, the EMI1 pseudosubstrate. The APC/$C^{CDH1}$ directed destruction of these two E2F repressors and subsequent de-repression of EMI1 contribute to CDH1 inactivation in late $G_1$. Throughout $G_1$ APC/$C^{CDH1}$ targets cyclin F, a substrate receptor of SCF; the targeting of cyclin F prevents the $SCF^{cyclin\ F}$ from reciprocally degrading its target,CDH1, and shortening G1 phase (Choudhury et al. 2016). APC/$C^{CDH1}$ also targets CDC6 and ORC1, two proteins involved in origin licensing for DNA replication which itself contributes to CDK2 activation in late $G_1$ (described below). Altogether, APC/C targets a cohort of proteins in early $G_1$ including both cyclins and CDK regulators, to ultimately set the timing of the onset of DNA replication.

### *16.3.4 Nuclear and Chromatin Architecture Changes in Early $G_1$*

Transcriptional and post-transcriptional control are not the sole determinants of DNA replication parameters that are established during $G_1$. One special aspect of DNA replication in eukaryotes is the phenomenon of replication timing. Genomic DNA replication does not initiate simultaneously at all sites at the beginning of S phase, but rather some loci are replicated early in S phase and others much later in S phase. Much of this replication timing pattern is, in fact, established during early $G_1$. Local chromatin structure is an important contributor to replication timing, and progress toward understanding this phenomenon is described in chapters by Zhao, Rivera-Mulia and Gilbert and by Alabert, Jasencakova, and Groth in this book.

As a test of when replication timing is determined, Lu et al. (2010) introduced nuclei isolated from cells in specific cell-cycle phases (e.g., early or late $G_1$) into a cell-free DNA replication initiation system that triggers initiation immediately. They found that nuclei from cells in mid-to-late $G_1$ displayed a pattern of temporal regulation of DNA in replication domains similar to their pattern in intact cells, but nuclei from cells in early $G_1$ or $G_2$ lacked the temporal replication (Lu et al. 2010). They proposed that DNA replication timing is determined in a ~1 h window during early $G_1$, a model strengthened by more recent live cell imaging analysis by Wilson et al. (2016).

Wilson et al. (2016) used live cell imaging to show that as cells differentiate, the replication domains coalesce into larger regions of dense chromatin that are replicated in a coordinated fashion (Wilson et al. 2016; Dixon et al. 2015). It is well known that the nucleus has a three-dimensional architecture and that this architecture is modified as animal cells progress through development (Hiratani et al. 2010). The interior of the nucleus generally contains highly transcribed genes that are replicated early in S phase, while portions of chromosomes that are closer to the nuclear periphery are likely to be transcriptionally silent and replicated later in S phase. For example, in mammalian females, the inactivated X chromosome is compacted and localized to the nuclear periphery and is replicated much later in S phase than the active X chromosome (Lyon 1961; Morishima et al. 1962; Barr and Bertram 1949). This architectural compaction, DNA replication delay, and localization to the periphery also apply to pluripotency genes when they are silenced during differentiation (Meshorer et al. 2006; Hiratani et al. 2010). Thus, both chromatin and nuclear architecture that are established in early $G_1$ influence subsequent S phase events.

## 16.4 Late $G_1$

The transition from "early $G_1$" to "late $G_1$" is not readily defined by any discrete molecular markers; nonetheless, a collection of molecular events are typically confined to a period of $G_1$ just before the onset of S phase. Many of these late $G_1$ events

depend on successful completion of early $G_1$ steps, such as initial RB phosphorylation to begin the accumulation of essential DNA replication proteins. In particular, a burst of cyclin E/CDK2 activity coincides with both induced E2F-dependent gene expression and with the completion of DNA replication origin licensing in late $G_1$. A sequence of events in late $G_1$ reflects passage through the restriction point and leads inexorably to the initiation of DNA synthesis in S phase. Disruptions to this sequence, particularly after cells are already committed to S phase initiation, increase the likelihood of replication failure or genome instability. Recent advances in live cell imaging have begun to elucidate stereotypical orders of molecular events in a window of 1–2 h (in somatic human cells) at the $G_1$/S transition; these tools will ultimately support investigations of how that order promotes normal S phase completion (Cappell et al. 2016; Coleman et al. 2015).

### *16.4.1 Transcriptional Activation via E2F*

As cells pass the restriction point, commit to S phase, and therefore commit to progression through the cell cycle, they must accumulate the proteins required for DNA replication. Full release of E2F does not occur until late $G_1$ upon full RB hyperphosphorylation and is one indicator that the requirements of the restriction point have been fulfilled. Once fully released by RB hyperphosphorylation, activating E2F family members stimulate transcription of key genes involved in DNA replication and S phase onset (Grant et al. 2013; Muller et al. 2016; Helin 1998; Johnson et al. 1994; Slansky and Farnham 1996). Additionally, the dissociation of RB from the activating family of E2F transcription factors allows for the association of other transcriptional coactivators with E2F, such as p300 or CBP (Ait-Si-Ali et al. 2000; Ferreira et al. 1998).

E2F1 specifically is part of two positive feedforward loops that help to drive the cell into S phase with little possibility of turning back to a $G_1$-like state. The first of these loops involves *cyclin E* and was described in the previous section: cyclin E/CDK2-mediated RB hyperphosphorylation stimulates E2F1-mediated transcription of the *cyclin E* gene to further increase cyclin E/CDK2. The second feedforward loop is autoregulatory: E2F1 binds to and activates its own promoter, driving expression of more E2F1. Additionally, E2F1 is part of a negative feedback loop by activating transcription of the gene encoding the E3 ubiquitin ligase substrate receptor, SKP2. SKP2 is active from late $G_1$ through $G_2$ (Marti et al. 1999) and is responsible not only for the eventual ubiquitin-mediated degradation of E2F1 in S phase but also for ubiquitylating the Cip/Kip family of CDK inhibitors. p27 destruction leads to higher cyclin E/CDK2 activity and yet more robust RB hyperphosphorylation creating another feedforward loop (Sheaff et al. 1997; Yung et al. 2007). The result of these reinforcing relationships is maximal cyclin E/CDK2 activity and E2F protein levels at the $G_1$-to-S phase transition.

The E2F-mediated induction of genes involved in the positive feedback loops occurs before the transcriptional activation of genes involved in the negative feed-

back loops (Grant et al. 2013; Whitfield et al. 2002; Eser et al. 2011). This temporal arrangement provides time for cells to accumulate sufficient DNA replication proteins while still limiting that time, so replication protein levels do not increase to unregulatable levels. The offset in expression timing also prevents $G_1$-S phase genes from being expressed in late S phase or during $G_2$ when they are not needed or could be disruptive.

Nearly every protein required for DNA replication preparation in $G_1$ or DNA synthesis in S phase is the product of an E2F-regulated gene. Using a combination of microarray expression analysis of cell cycle-regulated transcription in synchronous cells and genome-wide chromatin precipitation followed by high-throughput sequencing (ChIP-seq), E2F1 has been detected at a majority of genes involved in DNA replication in cancer cells and at the promoters of over 2,500 genes across the genome (Grant et al. 2013). Since E2F transcription factors regulate the expression of many genes and are active during late $G_1$ and S phase, it is not surprising that they regulate a large number of genes involved in all stages of DNA replication. E2F-dependent genes encoding proteins critical for DNA replication include DNA polymerase delta 3 (*POLD3*), replication factor C subunit 4 (*RFC4*), proliferating cell nuclear antigen (*PCNA*), and histone mRNA stem-loop binding protein (*SLBP*). RFC4 and PCNA are accessory proteins for leading strand DNA replication by polymerase delta. SLBP binds to stem loops at the 3′ end of histone RNAs; this binding stabilizes the RNAs and promotes histone protein production (Townley-Tilson et al. 2006; Wang et al. 1996; Whitfield et al. 2000). Many genes whose products are involved in nucleotide metabolism are E2F targets including thymidine kinase 1 (*TK1*), thymidylate synthetase (*TYMS*), dihydrofolate reductase (*DHFR*), and ribonucleotide reductase subunit M2 (*RRM2*) (DeGregori et al. 1995, 1997). Genes whose products are essential for origin licensing that are regulated by E2F transcription factors include *CDC6*, *CDT1*, *ORC1*, and *MCM2–7* (Yoshida and Inoue 2004b; Ohtani et al. 1996, 1998, 1999; Tsuruga et al. 1997; Leone et al. 1998; Yan et al. 1998b).

### *16.4.2 Origin Licensing*

Creating a single exact copy of the genome is, of course, an S phase event and is a critical process for somatic cell viability and genome stability. To replicate the vast quantity of DNA in each eukaryotic cell in a timely fashion, cells initiate DNA replication at thousands of DNA replication origins located throughout the genome. Eukaryotic origins are not strictly defined by specific DNA sequences and are strongly influenced by local chromatin structure. For example, histone H4 lysine 20 monomethylation shows a strong positive correlation with origin activity (Tardat et al. 2010; Jorgensen et al. 2013). Discussions of the molecular features associated with eukaryotic origins, and ongoing efforts to identify molecular determinants of mammalian origins, can be found in other chapters in this book, and therefore are not addressed here.

Prior to replication initiation DNA replication origins must have been pre-loaded in G1 phase with mini-chromosome maintenance (MCM) complexes. MCM complexes are the core component of the replicative DNA helicase (Bell and Kaguni 2013; Remus and Diffley 2009), and the process of loading MCM complexes onto DNA is known as "origin licensing." MCM complexes are stable heterohexameric ring-shaped complexes of subunits MCM2-MCM7, and their sequences are conserved not only throughout eukarya but also in archaeal species (Bell 2012). The majority of knowledge to date about the mechanism of MCM loading comes from pioneering studies with purified *Saccharomyces cerevisiae* or *Xenopus laevis* proteins to reconstitute the MCM loading reaction (Remus and Diffley 2009; Gillespie et al. 2001), but the strong evolutionary conservation among licensing proteins gives confidence that insights into the functions of licensing components are readily extrapolatable to mammalian licensing systems. Despite this strong functional conservation, the regulation of origin-licensing proteins themselves varies among different eukaryotic species. In addition, some mammalian licensing proteins have non-replication functions including roles in transcriptional control, centrosome duplication, CDK regulation, chromosome segregation, and cell division (Kawasaki et al. 2006; Varma et al. 2012; Prasanth et al. 2002, 2004, 2010; Hemerly et al. 2009; Tachibana and Nigg 2006; Hossain and Stillman 2012).

During $G_1$, MCM complexes are loaded such that double-stranded DNA passes through their central channels (Fig. 16.6) (Bell and Botchan 2013; Gambus et al. 2011; Remus and Diffley 2009; Evrin et al. 2009); a detailed discussion of MCM structure can be found in the chapter by Zhai and Tye (Chapter 9). Functionally licensed origins have at least two MCM complexes loaded in anticipation of their lead roles at bidirectional replication forks in S phase (Sun et al. 2014; Li et al. 2015). MCM complexes are loaded to create these double hexamers by the concerted action of three essential loading factors: the heterohexameric origin recognition complex (ORC), the CDC6 protein, and the CDT1 protein (Siddiqui et al. 2013; Nishitani and Lygerou 2004). ORC and CDC6 are each members of the AAA+ family of ATPases (Duncker et al. 2009; Lee and Bell 2000; Speck et al. 2005; Clarey et al. 2006) and bear some sequence similarity to replication factor C (RFC) which is responsible for the DNA loading of another ring-shaped complex, the PCNA sliding clamp (Schepers and Diffley 2001). By analogy to RFC function, ORC and CDC6 are thought to bind and open MCM rings to allow double-stranded DNA to pass into their central channels (Samel et al. 2014; Bochman and Schwacha 2008). Non-productive MCM hexamer loading attempts are removed through the ATPase activity of CDC6 (Cocker et al. 1996; Frigola et al. 2013). The CDT1 protein binds MCM and is required for MCM loading, but CDT1 has no known enzymatic activity (Xouri et al. 2007; Zhang et al. 2010; Jee et al. 2010; Khayrutdinov et al. 2009). At least one role for CDT1 in origin licensing is to recruit MCM complexes to ORC and CDC6, which are resident at origins in $G_1$ (Xouri et al. 2007).

Based on the reconstituted yeast MCM loading reaction, once a first MCM hexamer is properly loaded onto DNA, CDT1 and CDC6 are released and can presumably recruit additional MCM complexes (Fig. 16.6) (Randell et al. 2006; Chen et al. 2007; Duzdevich et al. 2015; Ticau et al. 2015). This dynamic behavior of CDT1

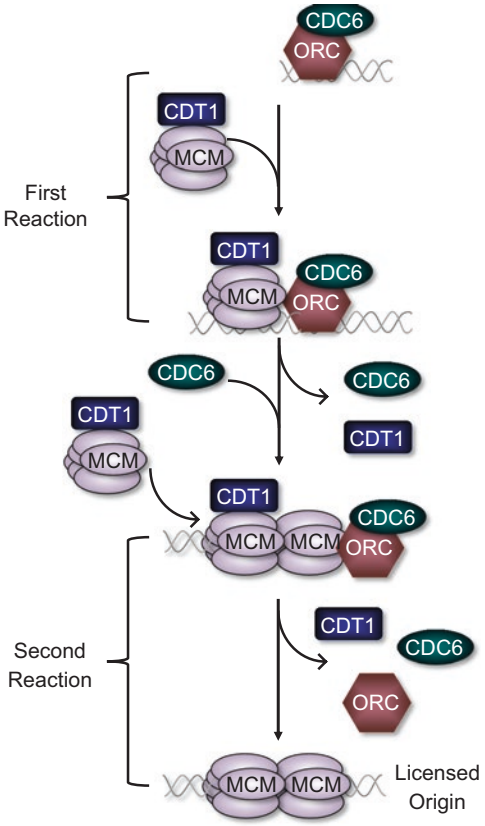


**Fig. 16.6** Origin licensing. DNA replication origins are licensed by loading MCM complexes. The first CDT1-associated MCM complex is loaded onto DNA through the action of the DNA bound origin recognition complex (ORC) and CDC6. Once the first MCM complex is successfully loaded by ORC, CDC6, and CDT1, CDC6 and CDT1 dissociate from the DNA bound MCM and ORC complexes. A second CDC6 molecule then associates with the DNA-bound complexes. Finally, a second CDT1 associated MCM complex is loaded onto the DNA in a head to head fashion, and CDC6 and CDT1 are released

relative to ORC implies that CDT1 and CDC6 can readily participate in MCM loading at many origins over the course of $G_1$ (Xouri et al. 2007). Perhaps for this reason, human CDT1 in particular is the most highly regulated of the origin-licensing proteins, and this regulation is critical to restrict licensing to only $G_1$. CDT1 is degraded during S phase, bound by the geminin inhibitor protein during S phase and $G_2$, and inhibited by phosphorylation during $G_2$ and M phase (Wohlschlegel et al. 2000; Xouri et al. 2007; Chandrasekaran et al. 2011; Coulombe et al. 2013; Nishitani et al. 2006). A second molecule of CDC6 assists with loading a second MCM hexamer. During this process, the second molecules of CDC6 and CDT1 are removed from the complex, completing origin licensing (Ticau et al. 2015; Duzdevich et al. 2015).

An important feature of origin licensing is that once MCM complexes are loaded, none of the loading factors are required for MCM to maintain stable DNA associations or replication competence (Rowles et al. 1999; Yeeles et al. 2015; Tsakraklides and Bell 2010; Ticau et al. 2015; Bowers et al. 2004). Each replication origin that does fire should fire at most once per S phase; only a subset of licensed origins fire in a typical S phase (Woodward et al. 2006; Ge et al. 2007). The complex mechanisms and regulations for converting a licensed origin to an active replication fork

are described in several chapters of this book, including Parts IV, V, VI, and XVIII. If an origin fires twice during a single-cell cycle, it results in DNA re-replication, a form of endogenous DNA damage that increases genome instability (Green and Li 2005; Vaziri et al. 2003; Davidson et al. 2006; Arentson et al. 2002; Liontos et al. 2007). Origin licensing can only occur during a period beginning in late mitosis (telophase) throughout all of $G_1$ until the onset of S phase; telophase and $G_1$ are the only times when all licensing proteins are abundant and active. At all other times from the beginning of S phase until the end of mitosis, origin licensing is prevented by an extensive series of overlapping molecular mechanisms that inactivate or destroy MCM loading proteins. More extensive descriptions of the mechanisms preventing re-replication after $G_1$ may be found in several other chapters, such as those contributed Li, by Abbas and Dutta, and by Teixeira and Reed.

### *16.4.3 Origin-Licensing Regulation During $G_1$*

Origin licensing is blocked during $G_2$ and mitosis by several mechanisms; one of these involves tight binding of the licensing inhibitor, geminin, to CDT1 (Wohlschlegel et al. 2000). Geminin accumulates throughout S phase and reaches peak levels in late $G_2$. Geminin binding to CDT1 interferes with the CDT1-MCM interaction, and since that interaction is essential for origin licensing, high levels of geminin block origin licensing during $G_2$ and mitosis (Cook et al. 2004; Yanagi et al. 2002). Geminin is subject to ubiquitin-mediated proteolysis during anaphase, so its reduction by the beginning of $G_1$ releases CDT1 to once again bind MCM (McGarry and Kirschner 1998). CDT1 is also hyperphosphorylated during $G_2$ and M phase, and at least some of these phosphorylations interfere with CDT1 function, though the mechanism of that interference is not yet known. CDT1 is dephosphorylated in early $G_1$, which presumably increases CDT1 licensing activity (Chandrasekaran et al. 2011; Coulombe et al. 2013).

Each protein in the licensing system is the product of an E2F-regulated gene. As a consequence, each of the genes for licensing proteins is induced during cell-cycle reentry from quiescence, and at least some of them are also subject to cell cycle-dependent fluctuations during active proliferation (Ohtani et al. 1996; DeGregori et al. 1995; Yoshida and Inoue 2004a; Yan et al. 1998a). For example, five of the six subunits of the origin recognition complex (*ORC2–6*) are constitutively expressed throughout the cell cycle, while *ORC1* is cell cycle regulated with peak expression at $G_1$/S (Whitfield et al. 2002; Grant et al. 2013).

In addition to the genes encoding proteins that act at origins, E2F-dependent *cyclin E* expression impacts origin licensing during late $G_1$. Like geminin, the CDC6 protein is targeted for degradation during mitosis. Both geminin and CDC6 are substrates of the APC/$C^{Cdh1}$ E3 ubiquitin ligase, which is activated in anaphase and remains active until the onset of S phase (McGarry and Kirschner 1998; Petersen et al. 2000). CDC6 escapes APC/C $^{Cdh1}$-mediated degradation in late $G_1$ because cyclin E/CDK2 phosphorylates CDC6 near the APC/$C^{Cdh1}$-binding site, and this

phosphorylation blocks CDC6 ubiquitylation (Mailand and Diffley 2005). (CDK-mediated CDC6 phosphorylation at a second site also stimulates cytoplasmic translocation later in S phase (Yim et al. 2013).) As a result, cyclin E accumulation and cyclin E/CDK2 activation in late $G_1$ stabilize CDC6; the subsequent increase in CDC6 stimulates MCM loading. Cyclin E has also been ascribed non-kinase roles in origin licensing, though the mechanism of these roles remain to be determined (Geng et al. 2007). The relationship between CDK activity and licensing is complex, however, because very high levels of cyclin E/CDK2 activity can block origin licensing through multiple routes that are independent of CDC6 stabilization. These routes include premature S phase onset, phosphorylation-stimulated CDT1 and ORC1 degradation, and phosphorylation-mediated inhibition of interactions among licensing proteins or between licensing proteins and chromatin (Wheeler et al. 2008; Ekholm-Reed et al. 2004; Mendez and Stillman 2000; Takeda et al. 2005). Thus, the licensing inhibitory CDK function helps block origin relicensing and the consequent genome instability.

### *16.4.4 The Origin-Licensing Checkpoint*

Due to both the enormity of the task and the potential dire consequences of even small errors, there are checks and balances built into replication control, particularly at the transition from $G_1$ to S phase. Mechanisms that prevent origin relicensing during S phase set up a separate challenge when considering the need for complete genome duplication. Entering S phase with too few licensed origins to fully duplicate each chromosome results in under-replication which, like re-replication, is also a form of endogenous DNA damage. Reducing the number of licensed origins in $G_1$ can lead to sections of unreplicated DNA segregating during mitosis which then require DNA repair in the subsequent $G_1$ (Moreno et al. 2016). In addition, a destabilizing mutation in an MCM subunit causes reduced origin licensing, chromosomal instability, and development of cancer in mice (Shima et al. 2007; Pruitt et al. 2007). Moreover, replication forks can stall if they encounter bulky lesions or interstrand crosslinks, but as long as a fork converges from the other side of the lesion, repair and replication can be completed (Raschle et al. 2008; Moreno et al. 2016). In response to fork stalling, nearby licensed origins are activated to generate such converging forks, but those origins must have been licensed in the previous $G_1$ (Woodward et al. 2006; Ge et al. 2007; Ge and Blow 2010; Ibarra et al. 2008).

The tight regulation of licensing means that there is no opportunity to license additional origins after $G_1$, but cells typically license many more origins than are strictly required to accommodate replication fork stalling and to ensure complete replication. Moreover, individual origins can be loaded with more than one MCM double hexamer. At least in vitro, loaded MCM complexes can slide away from their initial loading site, freeing origins to receive additional MCM complexes (Remus and Diffley 2009; Gros et al. 2015; Evrin et al. 2009). As long as the MCM complexes remain DNA-loaded as double hexamers, they can be activated in S phase

with no need for ORC, CDC6, or CDT1. The additional licensed origins may never be utilized during S phase, but these dormant origins can be activated if needed.

Though it is imperative that enough origins are licensed to support complete replication before S phase begins, it is not clear how cells couple the completion of origin licensing to the timing of S phase onset. In the early years of origin-licensing investigations, the consequences of licensing failure were evident, but the existence of an origin-licensing checkpoint that can prevent S phase onset before some critical threshold of licensing was reached and sensed was questioned. Budding and fission yeasts with null alleles in essential licensing components do not execute a cell-cycle arrest in $G_1$ but rather proceed through cell division without replication (Piatti et al. 1995; Kelly et al. 1993; Hofmann and Beach 1994). Depleting origin-licensing proteins by RNAi in the most commonly used cancer-derived human cell lines results in the predicted DNA synthesis defects, but no $G_1$ arrest (Nevis et al. 2009; Shreeram et al. 2002). These early results argued against any origin-licensing sensing mechanism.

Subsequently, however, several groups found that depleting origin-licensing proteins in non-transformed human cells did indeed cause delayed S phase onset. The delay could only be detected in untransformed cell lines, suggesting that growth control pathways disrupted in cancer-derived cell lines are normally required to link origin-licensing status in $G_1$ to S phase initiation (Nevis et al. 2009; Shreeram et al. 2002; Teer et al. 2006). In each of these studies, reducing origin licensing caused defects in the activation of cyclin-dependent kinases, especially cyclin E/CDK2.

Cyclin E/CDK2 is a major driver in the transition from $G_1$ into S phase; constitutive or ectopic expression of cyclin E leads to a shortened $G_1$ (Resnitzky and Reed 1995) (reviewed in (Clurman et al. 1996). CDK activity is required for origin firing, and early S phase origin firing is driven by CDK2 complexes with cyclin E or cyclin A (Fig. 16.1); chapters on replication initiation describe the molecular mechanisms of replication initiation. Delayed CDK2 activation from impaired origin-licensing consequently delays origin firing and, by definition, S phase entry. No single molecular mechanism to link origin licensing to CDK activity has yet emerged. In different studies, elevated CDK inhibitor proteins, CDK localization or phosphorylation, or expression of $G_1$ cyclins were each implicated in the S phase delay caused by insufficient origin licensing (Nevis et al. 2009; Shreeram et al. 2002; Liu et al. 2009; Teer et al. 2006; Lunn et al. 2010). It may be that each of these mechanisms can operate in all non-transformed cells, but different cell types are more or less dependent on specific ones.

### *16.4.5 APC/C$^{CDH1}$ Inactivation and S Phase Entry*

The CDH1 substrate-targeting subunit of APC/C must be degraded for proper entry into S phase to allow full CDK activation and cyclin A accumulation. There are three main mechanisms for inactivating APC/C$^{CDH1}$. The first mechanism is the E2F-dependent accumulation of the APC/C$^{CDH1}$ pseudosubstrate early mitotic inhibitor-1 (EMI1). EMI1 binds to CDH1 complexed with APC/C but is not

degraded, thus acting as a competitive inhibitor. Once EMI1 binds APC/$C^{CDH1}$, an irreversible cascade toward APC/C inactivation is triggered (Cappell et al. 2016). The EMI1-driven partial reduction in APC/$C^{CDH1}$ activity stabilizes cyclin A. Cyclin A/CDK2 phosphorylates CDH1, preventing it from binding APC/C. Cyclin A/CDK2 phosphorylates CDH1 at four different amino acids (S40, S151, S163, and T121) (Lukas et al. 1999). The phosphorylation of serine 40 and threonine 121 create two binding domains for the protein kinase, PLK1. PLK1 can then bind and phosphorylate CDH1 at serines 138 and 146 (Fukushima et al. 2013). Phosphorylated CDH1 molecules are then targeted for degradation by the $SCF^{cyclin\ F}$ complex (Fig. 16.7) (Fukushima et al. 2013; Choudhury et al. 2016).

Cells that lack CDH1 enter S phase early but also experience increased rates of DNA damage. This increased damage could be from insufficient origin licensing if origin firing begins before chromosomes are fully licensed (Ayuda-Duran et al. 2014). In addition CDH1-deficient cells show enhanced mutagenesis. This increased mutation rate could be due to the accumulation of a pair of proteins involved in dTTP formation: thymidine kinase 1 (TK1) and thymidylate kinase (TMPK) (Ke et al. 2005, 2007). The over-accumulation of these two proteins leads to an imbalance in the dNTP pool. This imbalance then leads to an increased rate of dNTP misincorporation, reducing the fidelity of DNA replication and increasing the mutation rate (Ke et al. 2005). Conversely, human osteosarcoma cells that overexpress CDH1 using inducible expression have a delayed S phase entry as well as a slower rate of DNA replication (Sorensen et al. 2000). Thus, the proper regulation of CDH1 is important for proper cell-cycle progression and S phase entry.

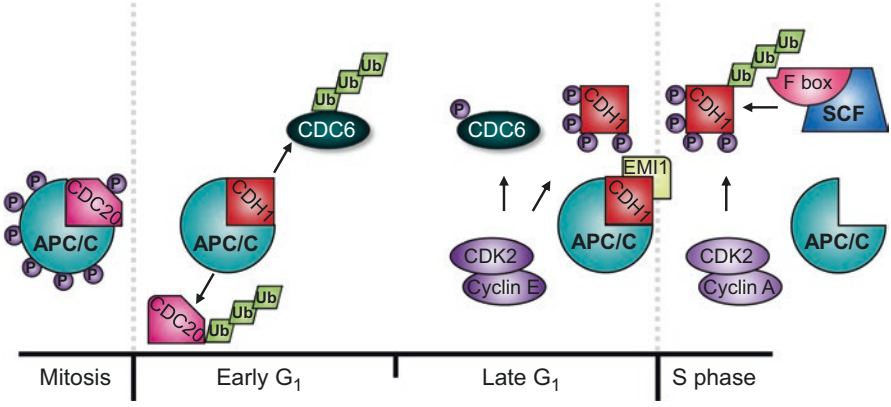


**Fig. 16.7** Modifications and activation of APC/$C^{CDH1}$ from mitosis to the onset of S phase. In late mitosis, CDH1 replaces CDC20 as the substrate receptor of the APC/C E3 ligase and is active during early $G_1$. Two APC/$C^{CDH1}$ substrates in early G1 are CDC20 and the origin-licensing protein, CDC6. In late $G_1$ cyclin E/CDK2 phosphorylates and protects CDC6. Also in late G1, the pseudosubstrate, EMI1, binds to CDH1, inactivating the complex. In late $G_1$ and early S phase, CDH1 is phosphorylated by cyclin E/CDK2 and cyclin A/CDK2, respectively; this phosphorylation dissociates CDH1 from APC/C, thus inactivating the complex (From S phase through mitosis, the E3 ligase CDH1 is polyubiquitylated by the $SCF^{cyclin\ F}$ complex and degraded by the 26S proteasome)

## 16.5 Conclusion

Successful completion of S phase relies on proper progression through $G_1$. In this chapter we have discussed some of the many different $G_1$ events and checkpoints that contribute to the proper initiation and completion of DNA replication including quiescence, the restriction point, origin licensing, and cyclin-dependent kinase regulation. Many of the topics covered in this chapter are regulated to occur normally in a stereotypical order and were initially studied using assays of bulk cell populations such as immunoblots, microarray analysis, or ChIP-seq. As technology progresses, researchers will be able to analyze cell-cycle kinetics of individual cells using single-cell assays such as single-cell RNA-seq or high-content live cell imaging with cell-cycle biosensors (Kaida et al. 2011; Pauklin and Vallier 2013; Sugiyama et al. 2009; Wilson et al. 2016; Cappell et al. 2016; Spencer et al. 2013; Coleman et al. 2015; Purvis et al. 2012). These next-generation technologies to study cell-cycle progression with timescales of mere minutes have begun to reveal new timelines of $G_1$ progression (Cappell et al. 2016; Spencer et al. 2013; Coleman et al. 2015; Matson and Cook 2017).

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**Conflicts of Interest** The authors have no conflicts of interest.

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# Chapter 17
# Roles of SUMO in Replication Initiation, Progression, and Termination

**Lei Wei and Xiaolan Zhao**

**Abstract** Accurate genome duplication during cell division is essential for life. This process is accomplished by the close collaboration between replication factors and many additional proteins that provide assistant roles. Replication factors establish the replication machineries capable of copying billions of nucleotides, while regulatory proteins help to achieve accuracy and efficiency of replication. Among regulatory proteins, protein modification enzymes can bestow fast and reversible changes to many targets, leading to coordinated effects on replication. Recent studies have begun to elucidate how one type of protein modification, sumoylation, can modify replication proteins and regulate genome duplication through multiple mechanisms. This chapter summarizes these new findings, and how they can integrate with the known regulatory circuitries of replication. As this area of research is still at its infancy, many outstanding questions remain to be explored, and we discuss these issues in light of the new advances.

**Keywords** DNA replication initiation • Replication progression • Posttranslational modifications • Sumoylation • Ubiquitination • Phosphorylation

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## 17.1 Overview of Eukaryotic DNA Replication

### *17.1.1 Replication Initiation*

DNA replication occurs in three stages, namely, initiation, progression, and termination. Each of these stages entails multi-step DNA transactions carried out by dozens of proteins. Most of the replication steps and proteins are highly conserved from simple model organisms, such as yeasts, to humans. Replication initiation begins with the licensing of genomic sites called origins. Origin licensing takes place in late M to G1 phase when origins become bound by the MCM complex, a ring-shaped complex composed of MCM2-7 subunits (Fig. 17.1). This process is achieved by interaction between MCM and MCM-loading factors. In budding yeast, wherein origin licensing is best understood, the MCM-loading factors include the origin recognition complex (ORC), Cdc6, and the MCM binding partner Cdt1 (Fig. 17.1) [reviewed in (Diffley et al. 1994; Kelly and Brown 2000; Bell and Dutta 2002; Sclafani and Holzen 2007; Remus and Diffley 2009; Li and Araki 2013; Bell and Labib 2016)]. ORCs demarcate origins by interacting with specific DNA sequences and chromatin components [reviewed in (Hoggard and Fox 2016; Gutiérrez and MacAlpine 2016)]. Cdc6 recruits the MCM-Cdt1 complex to ORC through interactions with both factors (Santocanale and Diffley 1996; Donovan et al. 1997; Speck et al. 2005; Randell et al. 2006; Sun et al. 2013). Subsequently, ATP hydrolysis by ORC, Cdc6, and MCM enables a pair of MCM rings to enclose DNA at origins (Fig. 17.1) (Bowers et al. 2004; Randell et al. 2006; Remus et al. 2009; Fernández-Cid et al. 2013; Frigola et al. 2013; Coster et al. 2014). This multi-step process produces an MCM double hexamer with its central channel enclosing DNA [reviewed in (Diffley et al. 1994; Kelly and Brown 2000; Bell and Dutta 2002; Sclafani and Holzen 2007; Remus and Diffley 2009; Wei and Zhao 2016b; Li and Araki 2013; Bell and Labib 2016)].

Once loaded, the MCM double hexamer must be kept inactive until the onset of S phase when origin firing takes place. One critical event during origin firing is the conversion of MCM into the replicative helicase, composed of MCM and its cofac-

**Fig. 17.1** (continued) and the ORC complex. During the origin firing step in S phase, DDK and CDK kinases activate the loaded MCM. DDK-mediated phosphorylation of loaded MCM recruits Sld3 and its binding partners Cdc45 and Sld7. CDK-mediated phosphorylation of Sld2 and Sld3 promotes the recruitment of Pol ε, Dpb11, and the GINS complex. Cdc45, MCM, and GINS form the active replicative helicase CMG. Subsequent recruitment of additional protein factors results in the formation of the replisome. SUMO can counteract DDK-mediated MCM phosphorylation. A fraction of loaded Mcm2-7 subunits are sumoylated to prevent premature replication initiation. This is partly achieved as SUMO aids the recruitment of the Rif1-PP1 phosphase complex that can antagonize DDK action in G1. As S phase starts, rising DDK levels are associated with increased MCM phosphorylation and decreased MCM sumoylation levels. Such a switch of MCM modification states is also seen in other organisms. Note that recently findings suggest that MCM also switches from enclosing dsDNA to enclosing mostly leading strand ssDNA template when DNA synthesis begins and that the N-terminal tier of the MCM ring faces the moving replication fork

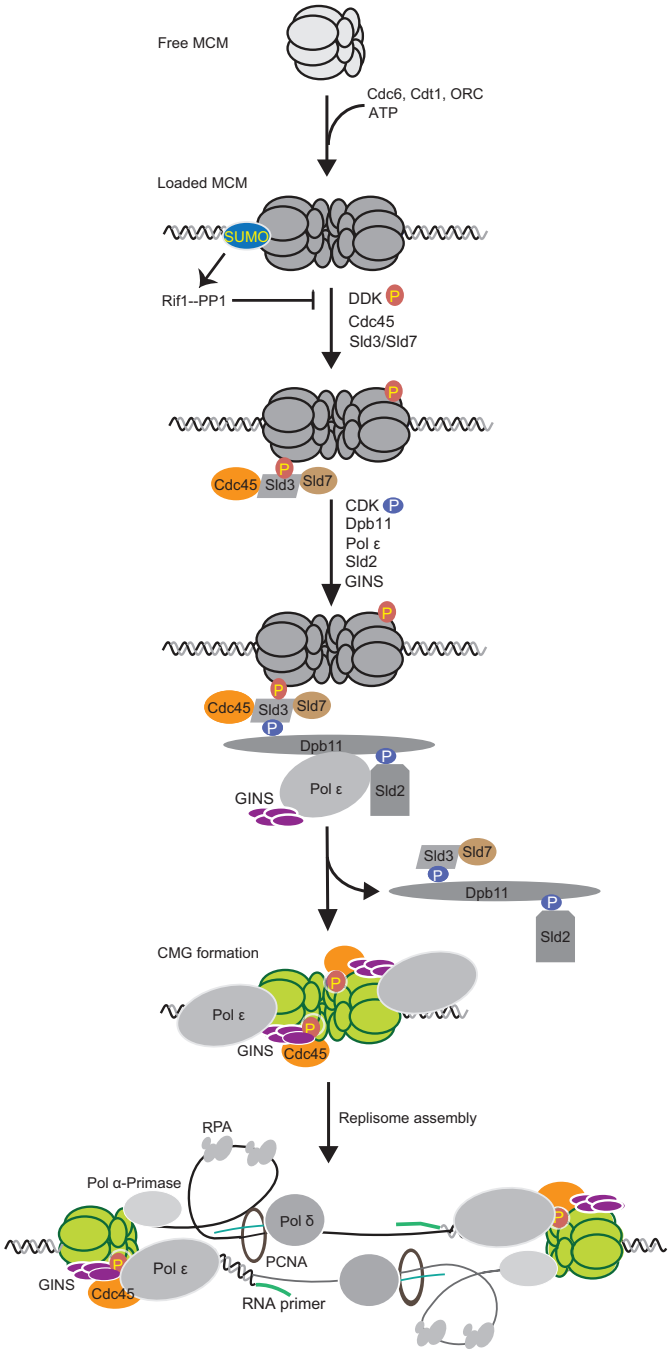


**Fig. 17.1** Summary of main steps of DNA replication initiation in budding yeast. The origin licensing step during late M to G1 phases entails free MCM being loaded onto replication origins as a double hexamer. This process requires several MCM-loading factors, including Cdc6, Cdt1,

tors Cdc45 and the four-subunit GINS complex. This conversion requires two protein kinases, DNA polymerase ε, and several scaffolding proteins, including Sld2, Sld3–Sld7, and Dpb11 in budding yeast and their homologs in other organisms (Gambus et al. 2006; Moyer et al. 2006; Pacek et al. 2006; Ilves et al. 2010; Muramatsu et al. 2010; Kang et al. 2012). The first kinase, the Dbf4-dependent kinase (DDK), phosphorylates MCM subunits (Fig. 17.1). Mcm4 phosphorylation is particularly important as it is recognized by Sld3 in partnership with Sld7 and Cdc45 (Fig. 17.1) (Sheu and Stillman 2006; Sheu and Stillman 2010; Deegan et al. 2016). Subsequent to this step, the second kinase, cyclin-dependent kinase (CDK), phosphorylates Sld2 and Sld3, enabling their interaction with Dpb11 in cooperation with Pol ε and GINS (Fig. 17.1) (Tanaka et al. 2007; Zegerman and Diffley 2007; Muramatsu et al. 2010). As a result of this cascade of protein interactions, Cdc45 and GINS are delivered to MCM, resulting in the formation of the replicative helicase CMG (Cdc45-MCM-GINS) (Fig. 17.1) (Gambus et al. 2006; Moyer et al. 2006; Pacek et al. 2006; Ilves et al. 2010; Kang et al. 2012).

Subsequent to CMG formation, DNA polymerase α, the chromatin remodeling complex FACT, and several scaffolding proteins are recruited to CMG and Pol ε to form the replisome (Fig. 17.1) (Gambus et al. 2006, 2009; Morohashi et al. 2009). In the meantime, the origin firing scaffolds, such as Sld2, Sld3, and Dpb11, leave the CMG and are recycled to additional origins that fire later in S phase (Fig. 17.1) (Mantiero et al. 2011). The temporal order of the origin firing program is determined by multiple factors, such as the affinity between ORCs and origin sequences and local chromatin environment [reviewed in (Masai et al. 2010; Fragkos et al. 2015)]. At both early- and late-fired origins, the formation of a pair of replisomes establishes divergent replication forks that travel in opposite directions.

### *17.1.2 Replication Progression*

As DNA synthesis begins, the DNA primase-Pol α complex generates primer sequences (Fig. 17.1). These primers can be extended by Pol ε for continuous leading strand synthesis and by Pol δ to produce many Okazaki fragments during discontinuous lagging strand synthesis (Fig. 17.1) [reviewed in (Kelly and Brown 2000; Bell and Dutta 2002; Sclafani and Holzen 2007; Bell and Labib 2016)]. The maturation of Okazaki fragments requires several additional conserved enzymes. In budding yeast, these include the flap endonuclease Rad27, the DNA helicase-nuclease Dna2, the exonuclease Exo1, the DNA helicase Pif1, and the ligase Cdc9 [reviewed in (Waga and Stillman 1998; Bell and Labib 2016)]. Collaboration among these factors enables ligation of Okazaki fragments.

During replication progression, a major challenge is coping with many types of template blockages. These can include (i) the topological stress generated by DNA unwinding, (ii) tightly bound nonhistone proteins, (iii) difficult-to-replicate genomic loci, (iv) collision with transcription machinery, and (v) DNA lesions generated from both intrinsic and extrinsic sources. Topological stress is largely relieved by

replisome-associated topoisomerases. Several scaffold proteins within the replisome play pivotal roles in dealing with other replication impediments. Depending on the types of blocks, different proteins and strategies are used, and in many cases, additional DNA metabolism proteins are recruited to overcome template blocks. For example, tightly bound nonhistone proteins can be removed by DNA helicases, such as Rrm3 in budding yeast, allowing the resumption of DNA synthesis (Ivessa et al. 2000; Calzada et al. 2005; Azvolinsky et al. 2006). In the case of template damage, such as UV-induced thymidine dimers, translesion polymerases can mediate synthesis bypass of these sites [reviewed in (Waters et al. 2009)].

Besides template blockage, other issues that must be managed during replication progression include the removal of template nucleosomes ahead of replication forks and reestablishment or recycling of nucleosomes with correct positioning behind replication forks. In addition, replication progression is coupled with the establishment of sister chromatid cohesion and inheritable epigenetic markers. These topics have been recently summarized, and we refer the readers to several reviews for details [reviewed in (Jeppsson et al. 2014; Almouzni and Cedar 2016; Bell and Labib 2016)].

### *17.1.3 Replication Termination*

As two opposing replication forks from adjacent origins converge, replication terminates. In general, replication termination sites are determined by the meeting point of two forks, but in some instances, termination occurs at replication pausing sites where one fork has more retention time (Labib and Hodgson 2007; Fachinetti et al. 2010). Three major events are required for replication termination, including the completion of local DNA synthesis, decatenation of the two daughter strands by topoisomerases, and disassembly of replisome. Compared with replication initiation and progression, replication termination is less well understood. More recently, new findings have implicated MCM ubiquitination in the disassembly of replisomes during termination (Maric et al. 2014; Priego Moreno et al. 2014; Dewar et al. 2015).

## 17.2 The SUMO Modification Cycle

Protein modifications underpin many regulatory mechanisms during the three stages of replication. Phosphorylation and ubiquitination have been found to be critical in all stages of replication [reviewed in (Wei and Zhao 2016b; Kelly and Brown 2000; Diffley 2004; Vodermaier 2004; Blow and Dutta 2005; Arias and Walter 2007; Sclafani and Holzen 2007; Moreno and Gambus 2015; Sivakumar and Gorbsky 2015; Garcia-Rodriguez et al. 2016)]. More recently, sumoylation has also been found to influence replication and is important for genome integrity. In this chapter,

we summarize the findings that begin to unravel the mechanisms of SUMO-based replication regulation after a brief introduction of protein sumoylation.

### *17.2.1 Principles of the Sumoylation Process*

SUMO (small ubiquitin-like modifier) is a highly conserved member of the ubiquitin family of protein modifiers. With approximately 100 amino acids, SUMO assumes an ubiquitin fold but with a distinct surface charge distribution. The SUMO-specific E1 (or activating enzyme), E2 (or conjugating enzyme), and E3 (or ligase) enzymes conjugate SUMO to the ε-amino group lysine residue of a substrate (Johnson 2004) (Fig. 17.2). Most organisms contain one SUMO E1 and E2 but multiple SUMO E3s. SUMO E2 can directly bind to the so-called sumoylation consensus or reverse consensus sequences, [ΨKX(D/E)] or [(D/E)XKΨ] (Ψ, a hydrophobic residue; X, any residue). With the help of E3s, SUMO is then transferred from E2 to the lysine within such sequences (Gareau and Lima 2010; Lamoliatte et al. 2014). However, proteomic studies found that many of these sites are not sumoylated, suggesting that additional factors must also influence the sumoylation process (Gareau and Lima 2010; Lamoliatte et al. 2014). For example, it has been noted that sumoylation sites are often in loop regions (Gareau and Lima 2010), likely because of the ability of such regions to adopt local conformational changes that favor productive contact between the E2~SUMO thioester bond and the acceptor lysine. This principle could also explain findings that while sumoylation is conserved among many protein homologs, the sites of modification often vary (Golebiowski et al. 2009; Dou et al. 2010; Elrouby and Coupland 2010; Cremona et al. 2012; Psakhye and Jentsch 2012; Hendriks et al. 2014; Ma et al. 2014; Tammsalu et al. 2014).

SUMO E3s play important roles partly by bridging E2 and substrates. For instance, budding yeast has the homologous Siz1 and Siz2 SUMO E3s and the Mms21 E3, all of which possess an SP-RING domain that can associate with Ubc9 (Johnson and Gupta 2001; Takahashi et al. 2001; Zhao and Blobel 2005; Gareau and Lima 2010). In addition, each E3 can associate with specific substrates. For example,

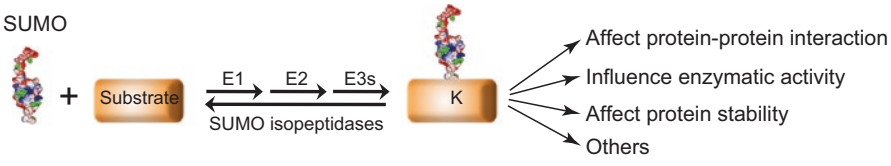


**Fig. 17.2** The SUMO conjugation cycle and SUMO's effects on substrate proteins. SUMO E1, E2, and E3 enzymes can conjugate a SUMO molecule to a lysine residue of a substrate. Sumoylation can also occur on multiple lysines of a substrate or in the form of SUMO chain (not shown). Sumoylation can have several biological effects on the substrates, and three frequently observed molecular consequences are indicated. Sumoylation can be reversed by SUMO isopeptidases

Siz2 interacts with the ssDNA-binding protein RPA, promoting RPA sumoylation and Siz2 localization to DNA breaks (Chung and Zhao 2015). Mms21 is a part of the Smc5/Smc6 complex that localizes to several genomic loci to influence sumoylation events in these places (Murray and Carr 2008; De Piccoli et al. 2009; Hang et al. 2015; Bonner et al. 2016; Bermudez-Lopez et al. 2016). These interactions and the ability of E3s to enable productive alignment between E2 and substrates for SUMO transfer make SUMO E3s indispensible for sumoylation in vivo [reviewed in (Gareau and Lima 2010)]. Based on studies from several organisms, SUMO E3s often exhibit substrate redundancy, likely reflecting their similar SUMO transfer mechanisms (Hang et al. 2014; Sarangi et al. 2014).

Mammalian cells have larger numbers of SUMO E3s than yeast. At least ten E3s have been described in human cells thus far (Gareau and Lima 2010; Cappadocia et al. 2015; Eisenhardt et al. 2015). These can be divided into several groups, including (1) PIAS proteins that are homologs of the yeast Siz1 and Siz2, (2) the Mms21 homolog NSMCE2, (3) the ZNF451 type of SUMO E3s that utilize tandem SUMO-interacting motifs (SIMs) to enable sumoylation (Cappadocia et al. 2015; Eisenhardt et al. 2015), and (4) more specialized SUMO E3s that target specific processes, such as the nuclear pore protein Ran binding protein 2 (RanBP2), the polycomb group protein Pc2, and the promyelocytic leukemia (PML) protein. The increased numbers of SUMO E3s in human cells are associated with the presence of multiple SUMO isoforms (Gareau and Lima 2010; Liang et al. 2016). SUMO2 and SUMO3 have 97% sequence identity and can form SUMO chains, while the more divergent SUMO1 is less frequently found in SUMO chains [reviewed in (Gareau and Lima 2010)]. The acquirement of different SUMO isoforms and the many types of SUMO chains that they can form, in conjunction with the multiple types of SUMO E3s in human cells, can meet the needs of more complex genomes and increased demands for regulation.

### *17.2.2 Principles of the Desumoylation Process*

Sumoylation can be reversed by multiple SUMO-specific cysteine proteases, known as desumoylation enzymes (Mukhopadhyay and Dasso 2007; Hickey et al. 2012) (Fig. 17.2). The substrate selectivity of desumoylation enzymes is partly achieved by their distinct localizations. Using budding yeast as an example, one of its desumoylation enzymes, Ulp1, primarily associates with nuclear pore complexes, whereas the other enzyme, Ulp2, can be seen concentrated in the nucleolus (Li and Hochstrasser 1999, 2000; Panse et al. 2003; Kroetz et al. 2009; Srikumar et al. 2013). Consistent with these localization patterns, Ulp1 and Ulp2 have different substrates (Makhnevych et al. 2009; de Albuquerque et al. 2016; Wei and Zhao 2016a). In addition, Ulp1 enables SUMO maturation by removing the tail of the precursor SUMO molecule (Li and Hochstrasser 1999), while Ulp2 has a major role in removing SUMO chains (Li and Hochstrasser 2000). Both Ulp1 and Ulp2 are required for cell fitness and resistance to a broad range of genotoxins (Li and

Hochstrasser 1999, 2000; Schwartz et al. 2007). As Ulp2 mutant defects are suppressed to a large degree by mutating the lysine residues on SUMO, which prevents SUMO chain formation, accumulation of SUMO chains is deleterious (Bylebyl et al. 2003). Human cells contain at least six desumoylation enzymes, called sentrin-/SUMO-specific proteases (SENPs) (Hannoun et al. 2010; Hickey et al. 2012). SENP1, 2, 3, and 5 are more related to Ulp1, whereas SENP6 and 7 are similar to Ulp2 [reviewed in (Mukhopadhyay and Dasso 2007)]. As is the case for Ulp1 and Ulp2 in yeast, SENPs have distinct activities and cellular localization patterns, and their mutants cause a wide range of defects [reviewed in (Mukhopadhyay and Dasso 2007; Hickey et al. 2012)].

### *17.2.3 Biochemical Effects of Sumoylation*

Protein sumoylation affects a myriad of biological processes, such as transcription, nuclear transport, DNA metabolism, and protein quality control [reviewed in (Sarangi and Zhao 2015)]. The conjugation and removal of SUMO from proteins can alter protein-protein interactions, partly because SUMO modules can interact with SIMs on other proteins or the substrate itself. As such, SUMO-SIM interactions can promote the assembly of protein complexes and the formation of a membrane-free nuclear compartment [reviewed in (Shen et al. 2006)]. On the other hand, SUMO sometimes disrupts existing protein-protein interactions or protein aggregation, possibly due to steric hindrance posed by the SUMO moiety. Additionally, sumoylation can alter a protein's interaction with DNA or chromatin, its enzymatic activities, or its protein levels. Each of these effects has been observed in DNA metabolism processes, particularly in DNA repair. For example, sumoylation enhances inter-subunit association among the DNA helicase-topoisomerase Sgs1-Top3-Rmi1 complex and promotes its function in resolving Holliday junctions (Bermudez-Lopez et al. 2016; Bonner et al. 2016). On the other hand, sumoylation of the DNA nuclease cofactor Sae2 helps convert the protein from insoluble aggregates to a soluble form, which is required for DNA-end resection (Sarangi et al. 2015). In the case of the recombination mediator protein Rad52, sumoylation leads to association with the segregase Cdc48/p97 that removes proteins from DNA using its ATPase activity (Bergink et al. 2013). These examples illustrate some of the mechanisms by which sumoylation regulates genome maintenance. Recent studies have begun to reveal the roles of sumoylation during DNA replication, and the following sections summarize findings in this area.

## 17.3 SUMO-Based Regulation of Replication Initiation

Proteomic studies in multiple organisms have shown that protein factors that help replisomes cope with template obstacles are enriched among SUMO substrates (Golebiowski et al. 2009; Elrouby and Coupland 2010; Cremona et al. 2012; Hendriks et al. 2014; Ma et al. 2014; Tammsalu et al. 2014). Genetic and biochemical studies of individual SUMO substrates and SUMO enzymes have revealed some mechanisms of SUMO-based regulation of these proteins and how they affect DNA replication.

### *17.3.1 MCM Sumoylation Inhibits Replication Initiation in Budding Yeast*

In budding yeast, all six subunits of MCM are sumoylated (Cremona et al. 2012; de Albuquerque et al. 2016; Wei and Zhao 2016a). MCM sumoylation has a distinct spatial and temporal pattern relative to the cycle of DNA replication (Wei and Zhao 2016a). Spatially, MCM is only sumoylated when loaded onto origins (Wei and Zhao 2016a). Temporally, Mcm2-6 sumoylation levels peak during G1 phase prior to DDK-mediated Mcm4 phosphorylation, then decline as cells enter S phase, and again increase during G2/M phase, coincident with the next MCM-loading cycle (Wei and Zhao 2016a). The opposing patterns of Mcm2-6 sumoylation and MCM phosphorylation during the cell cycle indicate a negative role of MCM sumoylation during replication initiation. Indeed, increased MCM sumoylation causes a reduction in the levels of Mcm4 phosphorylation, CMG, and origin firing (Wei and Zhao 2016a). These defects are partly because hyper-sumoylated MCM has increased association with the PP1 phosphatase, which reverses Mcm4 phosphorylation (Wei and Zhao 2016a; Davé et al. 2014; Hiraga et al. 2014; Mattarocci et al. 2014). MCM sumoylation levels subside at the start of S phase, partly through the action of Ulp2 (Wei and Zhao 2016a; de Albuquerque et al. 2016). These findings suggest that MCM sumoylation serves as a safeguard to prevent premature helicase function before S phase and that initiation of DNA synthesis requires removing this modification (Wei and Zhao 2016a). Future work is needed to examine whether the observed effects are due to a particular MCM subunit or contributions from multiple subunits and whether sumoylation alters other MCM features in addition to PP1 regulation.

### 17.3.2 MCM Sumoylation in Higher Eukaryotes

MCM sumoylation has also been detected in higher eukaryotes (Golebiowski et al. 2009; Elrouby and Coupland 2010; Hendriks et al. 2014; Ma et al. 2014; Schimmel et al. 2014; Tammsalu et al. 2014). For example, human MCM2, 3, 4, and 7 proteins are sumoylated. Importantly, Mcm4 sumoylation levels exhibit a similar pattern to that of yeast Mcm2-6 during the cell cycle, peaking in G1, declining in S phase, and increasing again during the subsequent G1 phase (Schimmel et al. 2014). It is reasonable to envision that human MCM sumoylation also provides a regulatory mechanism to restrain origin firing. A negative role for sumoylation in replication initiation can also be inferred from findings in *Xenopus*, wherein increased origin firing occurs after reducing sumoylation, either by expression of a dominant-negative SUMO E2 or addition of SUMO-specific proteases (Bonne-Andrea et al. 2013). Given *Xenopus* MCM subunits are sumoylated (Ma et al. 2014), it is worthy of consideration whether this modification underlies the negative effect of sumoylation in replication initiation in this system. Considering that PP1- and DDK-mediated MCM regulation is conserved across species (Wotton and Shore 1997; Lee et al. 2003; Cho et al. 2006; Masai et al. 2006; Montagnoli et al. 2006; Tsuji et al. 2006; Cornacchia et al. 2012; Hayano et al. 2012; Yamazaki et al. 2012), the targeting of this pathway by MCM sumoylation to prevent premature initiation, as seen in yeast, may be conserved. Direct tests of these ideas will clarify the roles of MCM sumoylation in higher eukaryotes.

### 17.3.3 ORC2 Sumoylation Prevents Re-replication at Centromeric Regions

ORC, composed of ORC1–6 subunits, binds to replication origins and is critical for MCM loading during origin licensing (Diffley et al. 1994; Kelly and Brown 2000; Bell and Dutta 2002; Sclafani and Holzen 2007; Remus and Diffley 2009; Li and Araki 2013). In addition, ORC2 can dissociate from replication origins and localize to centromeric regions during G2/M phase (Craig et al. 2003; Prasanth et al. 2004; Lee et al. 2012), coincident with the appearance of SUMO2-modified ORC2 in human cells (Huang et al. 2016). Elimination of ORC2 sumoylation by mutating its two sumoylation sites leads to re-replication, polyploidy, and genome damage (Huang et al. 2016). Mechanistically, ORC2 sumoylation promotes the recruitment of KDM5A (Huang et al. 2016), a histone H3 lysine 4 (H3K4) demethylase (Defeo-Jones et al. 1991; Christensen et al. 2007; Klose et al. 2007). Loss of ORC2 sumoylation results in elevated levels of tri-methylated H3K4 (H3K4me3) in centromeric chromatin, reduced transcription of α-satellites at centromeres, and decondensation of pericentric heterochromatin, which correlates with re-replication of the pericentric region (Huang et al. 2016). It remains to be determined how the change in chromatin environment caused by the perturbation of the ORC2-KMD5A axis

leads to re-replication, despite the presence of multiple mechanisms that prevent re-replication. In budding yeast, multiple ORC subunits are sumoylated (Cremona et al. 2012), but the functions of this modification have yet to be determined.

### *17.3.4 Other Potential SUMO Substrates Affecting Replication Initiation*

Several other proteins involved in replication initiation are SUMO substrates, such as the ssDNA-binding protein RPA and CDK (Dou et al. 2010; Cremona et al. 2012; Bonne-Andrea et al. 2013). Sumoylation of both human and yeast RPA has been reported to promote homologous recombination during DNA repair, though whether it also has a role in replication initiation has not been tested (Dou et al. 2010; Psakhye and Jentsch 2012). Cyclin E has been shown to be a SUMO2/SUMO3 substrate in *Xenopus* (Bonne-Andrea et al. 2013). Its sumoylation is detectable during replication and is independent of its kinase activity (Bonne-Andrea et al. 2013). A direct effect of cyclin E sumoylation in replication initiation remains to be determined. Because multiple proteins involved in origin licensing and firing are SUMO substrates, we anticipate the presence of multiple mechanisms through which SUMO regulates timing and efficiency of origin firing and prevents harmful re-replication events.

## 17.4 SUMO-Based Regulation of Replication Progression

Genetic studies using SUMO E2 and E3 mutants have shown that reducing sumoylation retards replication progression, particularly under replicative stress (Cremona et al. 2012; Schimmel et al. 2014; Hang et al. 2015). For example, mutating SUMO E3s in budding yeast impairs replication when cells are treated with the DNA-alkylating agent methyl methanesulfonate (MMS) (Cremona et al. 2012). In human cell lines, reducing UBC9 function leads to a prolonged S phase (Schimmel et al. 2014). The effects of sumoylation in replication progression may be broad, as many proteins central for this process are subject to sumoylation. Aside from MCM, several other replisome components are sumoylated, including Pol ε, Pol δ, the processivity factor PCNA, topoisomerases, DNA primase, and the nucleosome remodeling factor FACT (Golebiowski et al. 2009; Elrouby and Coupland 2010; Cremona et al. 2012; Hendriks et al. 2014; Ma et al. 2014; Tammsalu et al. 2014). In addition, several proteins that collaborate with replisome for DNA synthesis are sumoylated (Golebiowski et al. 2009; Elrouby and Coupland 2010; Cremona et al. 2012; Hendriks et al. 2014; Ma et al. 2014; Tammsalu et al. 2014). Some examples include subunits of the SMC complexes (cohesin, condensin, and Smc5/Smc6), the SMC-like Mre11 complex, and the clamp loader RFC complex (Golebiowski et al.

2009; Elrouby and Coupland 2010; Cremona et al. 2012; Hendriks et al. 2014; Ma et al. 2014; Tammsalu et al. 2014). Among these proteins, the sumoylation of PCNA has been well examined and shown to recruit the anti-recombinase Srs2 to impaired replication forks in order to prevent toxic recombination events (Papouli et al. 2005; Armstrong et al. 2012). The molecular function of this modification, in conjunction with other types of PCNA modifications, has been extensively reviewed elsewhere [reviewed in (Mailand et al. 2013; Ulrich and Takahashi 2013)]. Below we focus on recent findings regarding additional effects SUMO has on replication progression, mostly derived from studying the combined effects of loss of sumoylation of many substrates, but with a few mechanistic studies as well.

### *17.4.1 SUMO-Based Regulation of Replisome Components*

Following up earlier observations that Mms21 SUMO ligase mutations impair replication, our group showed that under MMS conditions, Mms21 and the associated Smc5/Smc6 complex promote sumoylation of Mcm6 and Pol2, the catalytic subunit of Pol ε (Hang et al. 2015). As physical interactions are detected between the Smc5/Smc6 complex and these substrates, the effects seen are likely to be direct (Hang et al. 2015). In addition, as Smc5/Smc6 deficiency impairs replication at regions far from fired origins, the Smc5/Smc6 complex may use sumoylation to facilitate later stages of replication. Future tests can help us understand whether MCM sumoylation has a distinct role during replication stress and whether Pol2 sumoylation influences DNA polymerization.

Several replisome members have been found to be sumoylated in human cells (Golebiowski et al. 2009; Hendriks et al. 2014; Schimmel et al. 2014; Tammsalu et al. 2014). Interestingly, under ATR inhibition conditions, sumoylation of replisome components can lead to fork collapse (Ragland et al. 2013). This largely depends on the SUMO-targeted ubiquitin ligase RNF4 and endonuclease scaffold protein SLX4 (Ragland et al. 2013). It is thought that under such conditions, RNF4 can target sumoylated replisomes for degradation, rendering replication forks accessible for SLX4-mediated cleavage (Ragland et al. 2013). In another study, sumoylation of two Fanconi anemia (FA) proteins, namely, FANCD2 and FANCI, was shown to trigger RNF4-mediated ubiquitination of these proteins and subsequent removal from DNA damage sites (Gibbs-Seymour et al. 2015). Lack of this regulation reduces the ability of cells to cope with replication stress, likely due to blockage of FANCD2 and FANCI from recycling among different damage sites (Gibbs-Seymour et al. 2015). Both of these studies highlight the complex interplay between sumoylation and ubiquitination for replication fork management.

The above notion is further extended by another study utilizing iPOND (isolation of proteins on nascent DNA) coupled with mass spectrometry-based protein analyses in human cells. It has been reported that SUMO is enriched on nascent chromatin, while ubiquitin molecules are enriched on mature chromatin (Lopez-Contreras et al. 2013). A follow-up study showed that the SUMO deubiquitinase, USP7, contributes

to the establishment of this SUMO-high and ubiquitin-low nascent chromatin environment (Lecona et al. 2016). USP7 can remove ubiquitin molecules that are conjugated to SUMO2 *in vitro* and *in vivo* and is associated with nascent chromatin and MCM4 (Lecona et al. 2016). The authors suggest that through this mechanism, USP7 reduces ubiquitin levels and allows enrichment of SUMO2 on replisome components. This role likely contributes to USP7's function in maintaining normal rates of fork speed and origin firing (Lecona et al. 2016). These studies suggest that enrichment of SUMO and reduction of ubiquitin at or near replisomes can be advantageous for replication progression. Further investigation is needed to provide mechanistic insights into the relevant sumoylation events critical for DNA synthesis and the full spectrum of the effects of USP7 and RNF4 in keeping a balance between sumoylation and ubiquitination of replisome components under different conditions.

### *17.4.2 SUMO-Based Regulation of Lagging Strand Synthesis*

Lagging strand Okazaki fragment processing involves sequential reactions of gap filling by polymerase δ, flap cleavage by flap endonuclease 1 (FEN1), and nick ligation by DNA ligase 1 (LIG1) (Waga and Stillman 1998). In human cells, modification of FEN1 by SUMO3 at lysine 168 occurs in S phase and peaks in G2/M phase (Guo et al. 2012). The sumoylation of FEN1 is dependent on its phosphorylation, which occurs during G1 phase, peaking in late S phase. Sumoylation of FEN1 appears to occur after its phosphorylation, as the non-phosphorylatable FEN1 mutant (S187A) is not sumoylated (Guo et al. 2012). Furthermore, sumoylation of FEN1 triggers its ubiquitination and subsequent proteasome-mediated degradation, presumably by recruiting the SIM-containing ubiquitin E3 ligase PRP19 (Guo et al. 2012). The timely degradation of FEN1 via this cascade of modifications is critical for maintaining genome stability, as its deregulation leads to cell cycle delay and polyploidy (Guo et al. 2012). Other than FEN1, polymerase δ is also reported to be SUMO substrate in organisms from yeast to humans (Cremona et al. 2012; Hendriks et al. 2014; Tammsalu et al. 2014). It remains to be determined if polymerase δ sumoylation has a role in DNA lagging strand synthesis.

### *17.4.3 SUMO-Based Regulation of Sister Chromatid Cohesion*

As replication progresses, the two sister chromatids stay close together partly through the function of cohesin [reviewed in (Blow and Tanaka 2005; Sherwood et al. 2010)]. The ring-shaped cohesin complex is loaded onto chromatin before S phase and encloses sister chromatids to keep them connected during S phase [reviewed in (Blow and Tanaka 2005; Sherwood et al. 2010)]. The resulting sister chromatid cohesion is important for supplying faithful templates for DNA repair and for ensuring each daughter cell receives one set of chromosomes during mitosis

[reviewed in (Blow and Tanaka 2005; Sherwood et al. 2010)]. Four subunits of cohesin, including the rod-shaped Smc1 and Smc3 proteins and the associated Scc1 and Scc3 proteins, are sumoylated in yeast and human cells, partly in an Mms21-dependent manner (Denison et al. 2005; Potts et al. 2006; Almedawar et al. 2012; Golebiowski et al. 2009; Hendriks et al. 2014; Tammsalu et al. 2014). In budding yeast, sumoylation of Smc1 and Scc1 occurs after cohesin loading, and Scc1 sumoylation is independent of another important modification for cohesion establishment, namely, Smc3 acetylation (Almedawar et al. 2012). Decreasing cohesin sumoylation impairs cohesion, without affecting the integrity of the cohesin complex (Almedawar et al. 2012). Based on these observations, it was proposed that cohesin sumoylation is required for the establishment of cohesion.

Another study further highlights the importance of SUMO-based regulation of cohesion. When a cohesion establishment factor, Pds5, is mutated in budding yeast, Scc1 becomes hyper-sumoylated and is degraded (D'Ambrosio and Lavoie 2014). This correlates with precocious separation of sister chromatids (D'Ambrosio and Lavoie 2014). Such a defect can be suppressed through removal of the SUMO E3 ligase Siz2 or the SUMO-targeted ubiquitin ligase Slx5/Slx8, supporting the hypothesis that toxic sumoylation events underlie *pds5-1* defects (D'Ambrosio and Lavoie 2014). The authors suggest that Pds5 plays a role in preventing hyper-sumoylation of cohesin and maintaining cohesin levels until mitosis. The full picture of how SUMO regulates cohesion is likely more complex. For example, Pds5 itself is sumoylated by Siz2 from S to G2/M phase, though the biochemical effect of this modification is not known (Stead et al. 2003). The timing of sumoylation and ubiquitination of cohesin is critical, and it will be of interest to determine how their temporal order is established, perhaps through the regulation of sumoylation and desumoylation enzymes or through other cohesion regulators.

### *17.4.4 SUMO-Based Regulation of Topoisomerase Function*

Topoisomerases are essential for releasing topological stress and promoting replication fork progression (Champoux 2001; Wang 2002; Vos et al. 2011). They also contribute to the removal of transcription-generated DNA-RNA hybrids, known as R-loops, which pose a barrier for replication (Gan et al. 2011; Aguilera and Garcia-Muse 2012). A recent study showed that sumoylation of human TOP1 provides a means to reduce R-loop-mediated replication fork stalling via two distinct mechanisms (Li et al. 2015). Upon sumoylation by PIAS1, TOP1 showed improved interactions with the active form of RNA polymerase II (RNAPIIo), leading to recruitment of splicing factors to avoid R-loop formation (Li et al. 2015). In addition, sumoylation of TOP1 reduces its enzymatic activity, potentially leading to reduced TOP1-induced DNA nicking at transcriptionally active regions (Li et al. 2015). Both effects of TOP1 sumoylation could contribute to lessening barriers for replication forks. It remains to be tested if these effects of TOP1 sumoylation are conserved in other organisms.

## 17.5 Potential SUMO Substrates Affecting Replication Termination

Although a full understanding of replication termination is still elusive, several new discoveries have shed light into this process. In budding yeast, *C. elegans*, and *Xenopus*, ubiquitination of Mcm7 has been shown to be a key event for disassembly of the replisome. In budding yeast, the Dia2 ubiquitin ligase that is part of the replisome can ubiquitinate Mcm7 when replication forks converge. This modification is then recognized by the segregase Cdc48/p97, leading to removal of MCM from chromatin and replisome disassembly (Maric et al. 2014; Priego Moreno et al. 2014). In *C. elegans* and *Xenopus,* replisome-associated E3 ligase *CUL-2*$^{LRR-1}$ and the segregase remove CMG during termination (Sonneville et al. 2017; Dewar et al. 2017). Interestingly, in budding yeast, Mcm7 sumoylation appears to be regulated distinctly from that of Mcm2-6, with its levels only declining when the bulk of DNA replication has been completed (Wei and Zhao 2016a). It will be interesting to investigate whether Mcm7 sumoylation could trigger its ubiqutination or contribute to replication termination.

During replication termination, decatenation of sister chromatids requires Top2. Top2 sumoylation has been found in human, mouse, *Xenopus*, and yeast [reviewed in (Lee and Bachant 2009)]. Sumoylation of Top2 in vertebrates promotes the recruitment of Top2 or the chromosomal passenger complex to kinetochores during mitosis to facilitate chromosome segregation [reviewed in (Lee and Bachant 2009)]. Whether Top2 sumoylation plays a role in decatenation during replication termination is not known. With more molecular details of replication termination becoming available in the future, the examination of SUMO substrates involved in replication termination will reveal more details of this process.

## 17.6 Concluding Remarks

Each stage of DNA replication is intricately regulated to ensure precise genome duplication. Posttranslational modifications provide a dynamic regulatory means at multiple stages of the replication process. Phosphorylation- and ubiquitination-based modes of regulation are essential for replication, and the role of sumoylation in replication is emerging from several recent studies. During the replication initiation stage, sumoylation of MCM (yeast) and ORC2 (human) can influence origin firing. In addition, sumoylation promotes replication progression through multiple mechanisms, such as lagging strand synthesis, reducing R-loops, replication fork metabolism, and sister chromatid cohesion. However, only a small number of SUMO substrates have been studied thus far as summarized in Table 17.1, and our understanding of how sumoylation regulates replication is still at an early stage. With more advanced methods to map sumoylation sites and tools to alter the sumoylation status of substrates, detailed molecular mechanisms of how sumoylation

**Table 17.1** Summary of sumoylated substrates involved in DNA replication

| Replication | Substrate (function) | Molecular effect(s) of sumoylation |
|---|---|---|
| Initiation | *sc*MCM (replicative helicase) | Inhibit replication initiation via phosphatase recruitment |
| | *hs*ORC2 (replication origin recognition) | Prevent centromeric region re-replication by recruitment of the histone demethylase KDM5A |
| Progression | *hs*FANCD2 and *hs*FANCI (Fanconi anemia proteins) | Promote replication stress survival by triggering self-ubiquitination and removal from DNA damage sites |
| | *hs*FEN1 (flap endonuclease) | Promote genome stability, prerequisite for its ubiquitination and degradation |
| | *sc*Cohesin subunits (chromatid cohesion) | Promote sister chromatid cohesion |
| | *hs*TOP1 (topoisomerase) | Prevent R-loop formation by promoting interaction with splicing factors; reduce DNA nicking |

Note that only the major function is indicated for each substrate
sc *Saccharomyces cerevisiae*, hs *Homo sapiens*

regulates each substrate will be elucidated. Future work will also help to establish a clear picture of how sumoylation is coordinated with other types of protein modifications during DNA replication. In addition, examination of how sumoylation and desumoylation enzymes are themselves regulated can also reveal how SUMO modification cycles facilitate DNA replication. As SUMO enzyme deficiencies, such as SUMO E1 and E2 depletion or SUMO E3 mutations, have been implicated in cancer and inherited human syndromes (Eifler and Vertegaal 2015; He et al. 2015; Yu et al. 2015), understanding their roles in genome duplication will provide new avenues for disease detection and treatment strategies.

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# Chapter 18
# The Multiple Roles of Ubiquitylation in Regulating Challenged DNA Replication

**Sara Villa-Hernández, Avelino Bueno, and Rodrigo Bermejo**

**Abstract** DNA replication is essential for the propagation of life and the development of complex organisms. However, replication is a risky process as it can lead to mutations and chromosomal alterations. Conditions challenging DNA synthesis by replicative polymerases or DNA helix unwinding, generally termed as replication stress, can halt replication fork progression. Stalled replication forks are unstable, and mechanisms exist to protect their integrity, which promote an efficient restart of DNA synthesis and counteract fork collapse characterized by the accumulation of DNA lesions and mutagenic events. DNA replication is a highly regulated process, and several mechanisms control replication timing and integrity both during unperturbed cell cycles and in response to replication stress. Work over the last two decades has revealed that key steps of DNA replication are controlled by conjugation of the small peptide ubiquitin. While ubiquitylation was traditionally linked to protein degradation, the complexity and flexibility of the ubiquitin system in regulating protein function have recently emerged. Here we review the multiple roles exerted by ubiquitin-conjugating enzymes and ubiquitin-specific proteases, as well as readers of ubiquitin chains, in the control of eukaryotic DNA replication and replication-coupled DNA damage tolerance and repair.

**Keywords** DNA replication • Replication forks • Replication stress • DNA damage response • DNA repair • DNA damage tolerance • Ubiquitin • Ubiquitin ligases • DUBs • Cdc48/p97

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## 18.1 The Ubiquitin System

Ubiquitylation is a highly regulated three-enzyme process consisting in the covalent attachment of ubiquitin moieties to a determined substrate. First, an E1 ubiquitin-activating enzyme forms a high-energy thioester bond with the carboxyl group of the terminal glycine residue of ubiquitin (Fig. 18.1a). This activated ubiquitin is then transferred to an E2 ubiquitin-conjugating enzyme by transesterification. An E3 ubiquitin ligase then catalyzes the formation of an isopeptide bond between a lysine in the substrate and the activated carboxyl group of ubiquitin (Komander and Rape 2012). Multiple rounds of this process, using lysines on ubiquitin as a substrate, lead to the formation of different types of polyubiquitin chains (Fig. 18.1b). Any of the seven lysines present on ubiquitin (K6, K11, K27, K29, K33, K48, and K63), as well as the amino-terminal methionine (Met1) of the ubiquitin monomer, can serve as isopeptide bond acceptors (Komander and Rape 2012; Kulathu and Komander 2012). Moreover, not only homotypic ubiquitin chains can be formed but also atypical chains, such as mixed chains (in which different lysines are successively used to link ubiquitin moeties) or branched chains (in which different lysines are ubiquitylated within a given ubiquitin molecule).

Ubiquitin chain variants can occur and determine different outcomes of the modified substrates. While K11 and K48 chains more frequently signal proteins for degradation, mono-ubiquitylation and K63 chains usually modulate protein-protein interactions. However, these regulatory functions are not strict, and proteolytic outcomes of K63 chains and non-proteolytic ones for K48 have been described (Shibata et al. 2012; Maric et al. 2014). K6, K27, K29, and K33 ubiquitin chains have been reported only for a small number of substrates, and their function is still poorly understood (Kulathu and Komander 2012; Pinder et al. 2013; Yau and Rape 2016). As in the case of other posttranslational modifications, ubiquitylation can be reversed, and this is achieved by the action of ubiquitin-specific proteases or deubiquitinases (DUBs). DUBs are cysteine proteases (with the exception of budding yeast Rpn11 that is a zinc metalloprotease), which catalyze the hydrolysis of the isopeptide bonds connecting ubiquitin with its substrate (Nijman et al. 2005b; Sahtoe and Sixma 2015). Ubiquitylation modulates a great variety of cellular processes and is regulated in a more sophisticated way than initially anticipated by factors that promote either substrate ubiquitylation or deubiquitylation. Noteworthy, pairs of coordinated E3 ligases and DUBs have been identified, in which the two enzymes act on the same substrates to fine-tune ubiquitylation levels (Kee et al. 2005; Sowa et al. 2009). Another important layer of regulation comes from ubiquitin chain editing, which requires the concerted action of additional ubiquitin ligases and/or DUBs that change the topology of the ubiquitin chains and thus alter substrate fate (Newton et al. 2008).

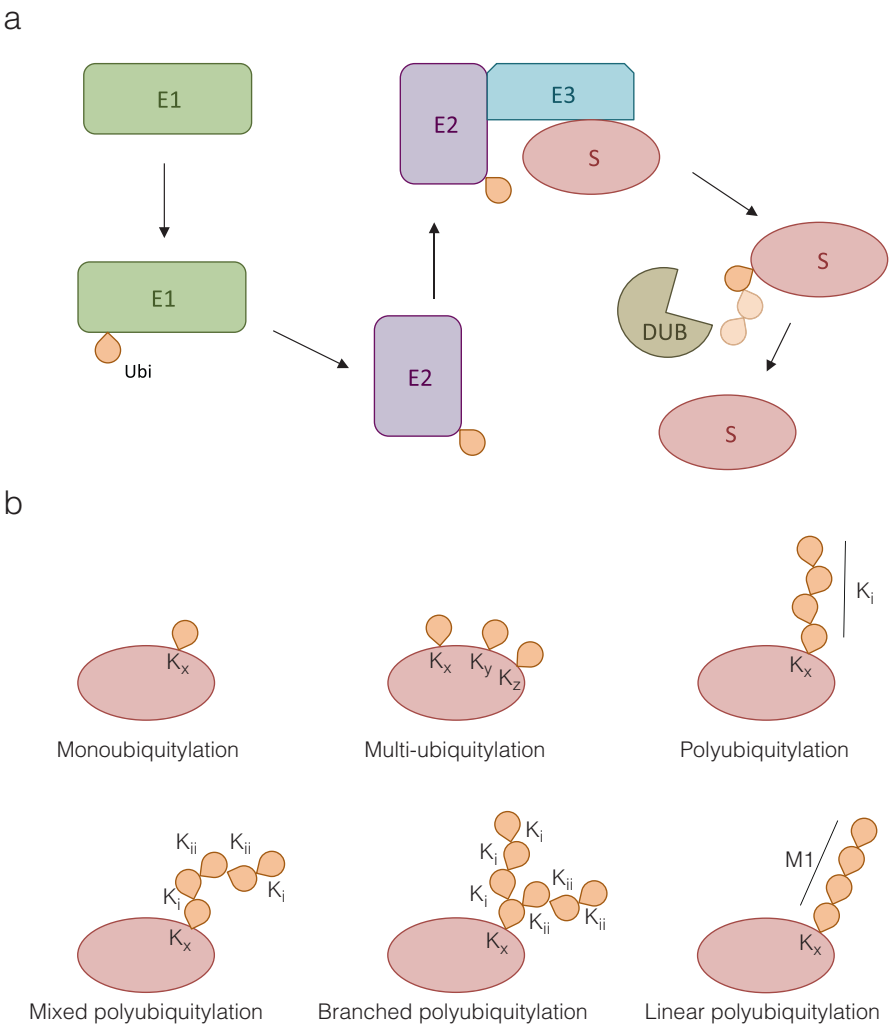


**Fig. 18.1** The ubiquitin system. (**a**) Mechanism of ubiquitin conjugation and deconjugation. Ubiquitin conjugation requires the activities of three factors: an E1 activating enzyme, an E2 conjugating enzyme, and an E3 ubiquitin ligase (see text for details). Ubiquitylation can be reversed by ubiquitin proteases, also known as deubiquitinases (DUBs), which hydrolyze lysine-ubiquitin bonds, and can remove single ubiquitin moieties or entire chains. (**b**) Different types of ubiquitylation have been reported. Attachment of a single ubiquitin moiety in a given lysine (Kx) is referred to as mono-ubiquitylation, while conjugation of ubiquitin moieties to different lysines (Kx-z) results in multi-ubiquitylation. Polyubiquitylation occurs when multiple rounds of ubiquitin conjugation are preformed on a substrate-conjugated ubiquitin. The lysine used to extend ubiquitin chains can be the same (Ki) along the chain or vary (Ki-Kii) giving rise to mixed polyubiquitylation. In addition, more than one lysine on an ubiquitin moiety can be used to extend chains resulting in branched polyubiquitin chains. Linear polyubiquitin chains arise if the residue for chain elongation is the initial methionine on the ubiquitin moiety

## 18.2 Control of Unperturbed DNA Replication by Ubiquitylation

DNA replication is initiated at sites of the genome known as origins of replication, to which the origin recognition complex (ORC) binds (Bell and Stillman 1992). ORC is required for the recruitment of the MCM2–7 complex to origin DNA, by the action of Cdc6 and Cdt1 proteins in a process termed origin licensing (Diffley et al. 1994). These sequentially loaded proteins conform the pre-replicative complex (pre-RC), which marks sites that can potentially initiate replication (Fig. 18.2). Upregulation of CDK (cyclin-dependent kinase) and DDK (Dbf4-dependent kinase) activities after the transition from G1 to S phase leads to the phosphorylation and recruitment of additional factors and the formation of a pre-initiation complex (pre-IC) (Francis et al. 2009; Randell et al. 2010). Among these factors are Cdc45 and the GINS (Go-Ichi-Ni-San) complex, which together with MCM2–7 conform the functional replicative helicase (Ilves et al. 2010), as well as Mcm10, Dpb11, and DNA polymerase ε (Muramatsu et al. 2010). Once MCM2–7 complex is phosphorylated and activated, the double hexamer divides into two hexamers that start

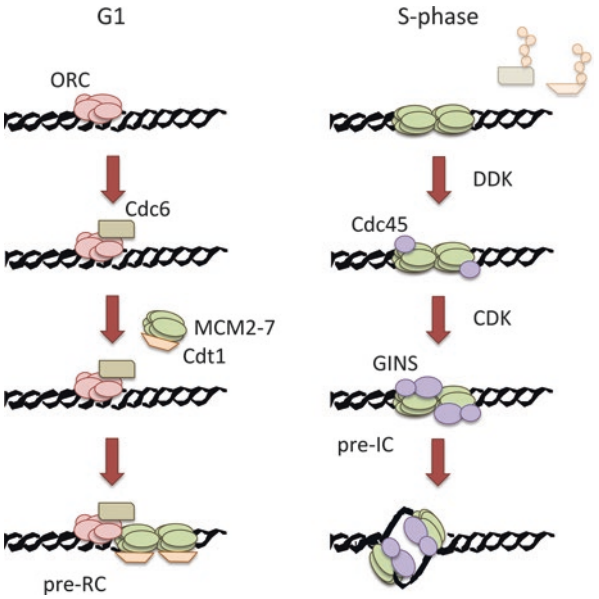


**Fig. 18.2** Ubiquitylation in the control of replication origin licensing. In G1 ORC binds origin DNA. Cdc6 and Cdt1 mediate the loading of the MCM2-7 helicase complex, in a process called origin licensing, and lead to the formation of pre-replication complexes (pre-RC) determining potential replication initiation sites. During the G1-S transition, Cdc6 and Cdt1 are ubiquitylated and degraded in order to avoid loading of new MCM2-7 complexes to already replicated DNA and prevent re-replication. Origin firing involves the formation of a pre-initiation complex (pre-IC) that depends on the phosphorylation of several replication factors by DDK and CDK. DDK and CDK activities are also required to activate the replicative helicase and promote initial DNA unwinding

unwinding DNA (Quan et al. 2015). Helicase activation generates replication forks to which additional factors are recruited to build replisomes capable of efficient DNA synthesis (Bell and Labib 2016).

Origin licensing and firing are restricted to G1 and S phase, respectively, in order to ensure that replication occurs once and only once per cell cycle (Siddiqui et al. 2013). This is achieved by coupling origin function to the oscillation in the activity of cyclin-dependent kinases. Origin licensing occurs when CDK levels are low due to degradation of mitotic cyclins and the CDK-activating phosphatase Cdc25, mediated by the anaphase-promoting complex (APC) ubiquitin ligase and its adaptor Cdh1 (Donzelli et al. 2002). Additionally, helicase loading to origin DNA is prevented outside of G1 by the degradation of the licensing factors Cdc6 and Cdt1 (Fig. 18.2). Cdc6 is marked for ubiquitin-mediated proteolysis by phosphorylation by CDK upon S-phase onset. In budding yeast, phosphorylated Cdc6 is ubiquitylated by the Skp1-Cullin-F-box $SCF^{Skp2}$ E3 ligase complex (Sánchez et al. 1999), leading to its degradation and thus prevention of MCM2–7 recruitment. In human cells instead, CDC6 phosphorylation leads to its export to the cytoplasm (Yim et al. 2013), where it cannot exert its MCM chromatin loading function. Nonetheless, it has been recently proposed that human CDC6 undergoes ubiquitylation-mediated degradation in a mechanism reminiscent of the one described in yeast (Walter et al. 2016).

Similarly to Cdc6, the Cdt1 origin-licensing factor is degraded by ubiquitin-mediated proteolysis. In human cells, CDT1 can be ubiquitylated by both the E3 Cullin-RING ubiquitin ligase complex $CRL4^{Cdt2}$ and the $SCF^{Skp2}$ E3 ligase (Li et al. 2003; Nishitani et al. 2006). CDT1 degradation is induced by its phosphorylation by CDK, which promotes the interaction between CDT1 and Skp2 (Sugimoto et al. 2004). CDT1 degradation is also promoted by ATR checkpoint-dependent phosphorylation of the Cdt2 adaptor in response to DNA damage (Sakaguchi et al. 2012), as part of a mechanism that may contribute to limit origin licensing and firing upon checkpoint activation. In addition, it has been recently reported that the ATM (ataxia-telangiectasia mutated) checkpoint kinase promotes the degradation of CDT1 during unperturbed replication (Iwahori et al. 2014). However, the identity of the signals determining ATM activation and CDT1 degradation in the absence of exogenous perturbations remains unclear. CDT1 is also regulated by deubiquitylation. Cells ablated for the USP37 ubiquitin protease exhibit hyper-ubiquitylated CDT1, and USP37 has been shown to deubiquitylate CDT1 in vitro (Hernández-Pérez et al. 2016). Ablation of USP37 leads to defects in S-phase progression, but whether this reflects alterations in origin licensing or a yet unidentified function for CDT1 is promoting ongoing replication remains to be elucidated. In metazoans, CDT1 function is subject to an additional layer of regulation through inhibition by the small nuclear protein geminin (Wohlschlegel et al. 2000). Geminin is expressed upon transition from G1 to S phase and is degraded at the end of mitosis through a destruction box targeted by APC (McGarry and Kirschner 1998). Thus, multiple redundant mechanisms control licensing factors' function and stability to restrict helicase loading to the G1 phase of the cell cycle.

Ubiquitylation is also important during termination of DNA replication (Fig. 18.3). Both in budding yeast and *Xenopus*, K48 ubiquitin chains are linked to

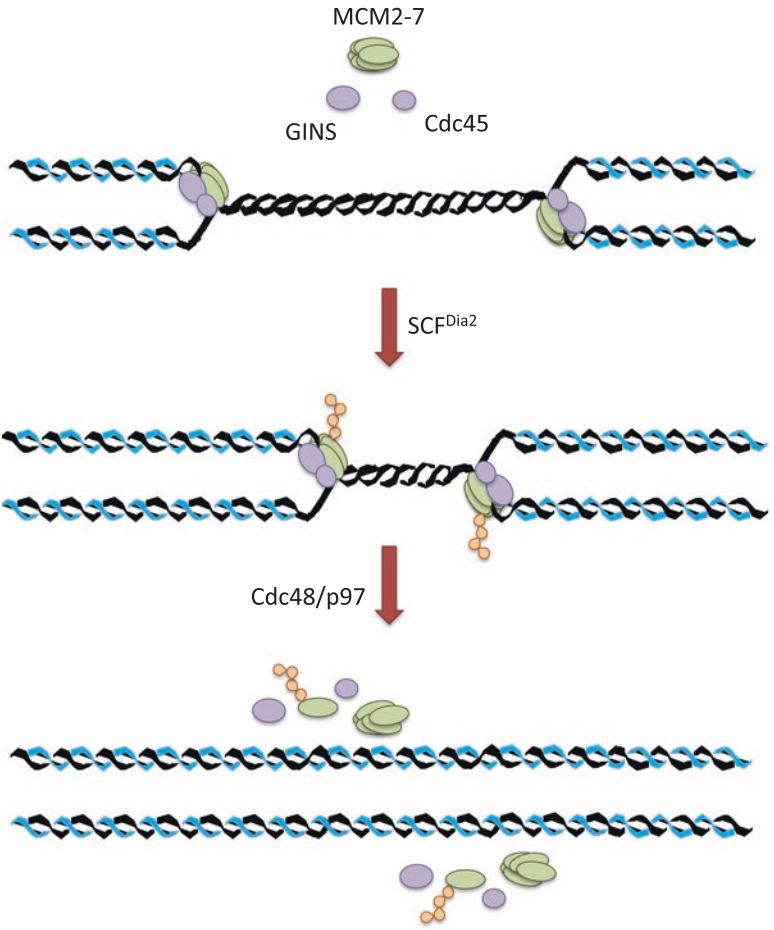


**Fig. 18.3** CMG ubiquitylation and extraction from DNA during replication termination. Upon replication fork convergence during replication termination, the $SCF^{Dia2}$ ubiquitin ligase polyubiquitylates the MCM7 subunit of the CMG replicative helicase. MCM7 ubiquitylation determines the disassembly and extraction of CMG helicase components, mediated by the Cdc48/p97 ubiquitin-selective segregase, presumably to facilitate the last steps of DNA synthesis

Mcm7 during the last steps of replication. Mcm7 ubiquitylation by yeast $SCF^{Dia2}$ or *Xenopus* $CRL2^{Lrr1}$ ubiquitin ligases leads to the disassembly of the CMG helicase from chromatin by action of the Cdc48/p97 ubiquitin-selective segregase (Priego Moreno et al. 2014; Maric et al. 2014; Maculins et al. 2015; Dewar et al. 2017). In this process, ubiquitylation by $SCF^{Dia2}$ does not trigger Mcm7 degradation and appears to be restricted to converging forks through mechanisms that are not yet understood. It had been previously reported that CDC48/p97 is required for the extraction from chromatin and degradation of CDT1 in *Caenorhabditis* embryos and *Xenopus* extracts (Franz et al. 2011). In this study, accumulation of CDC45/

GINS on chromatin was also observed upon ablation of CDC48, and it was proposed that CDT1 degradation might be linked to GINS release from chromatin. Cdc48/p97 has also been involved in the extraction of proteins associated to chromatin during replication elongation (Lecona et al. 2016). It was reported that in human cells proteins bound to nascent DNA have higher levels of modification by the SUMO (small ubiquitin-like modifier) protein, while ubiquitylation is more abundantly detected in mature chromatin. The replisome-associated USP7 ubiquitin protease was found to counteract the accumulation of ubiquitylated proteins around nascent strands and thus limit their extraction by Cdc48/p97. Impairment of USP7 function results in fork progression defects, suggesting that limiting the levels of ubiquitylated proteins is important to sustain processive chromatin replication.

## 18.3 Ubiquitin in the Modulation of DNA Replication in Conditions of Stress

DNA replication can be challenged in conditions that impede replication fork progression, broadly termed as replication stress (Gaillard et al. 2015). Replication stress can result from inhibition of DNA synthesis, as in the case of dNTP pool depletion or polymerase-blocking DNA lesions, or be due to blockage of DNA unwinding by replicative helicases, as in the case of interstrand cross-links (ICLs) or DNA-protein cross-links (DPCs). These conditions are generally thought to cause an excessive accumulation of ssDNA, either due to the uncoupling between the replicative DNA polymerases and helicases or owing to nucleolytic resection of replication/repair intermediates (Jossen and Bermejo 2013). Stalled replication forks are unstable and prone to accumulate DNA breaks priming genomic instability (Branzei and Foiani 2010). Several mechanisms have been described that contribute stabilized replication forks and prevent their collapse. A prominent role in preserving genome stability is played by the replication checkpoint, which is mediated by highly conserved kinases Mec1/ATR and Rad53/CHK1 that monitor problems in fork progression. Upon fork stalling, extended ssDNA filaments coated by replication protein A (RPA) complex recruit Mec1/ATR through its partner protein Ddc2/ATRIP (Zou and Elledge 2003). At replication forks, Mec1 phosphorylates several targets, which include the Mrc1/CLASPIN protein (Alcasabas et al. 2001; Tanaka and Russell 2001). Mrc1 is a replisome component that acts as a mediator likely facilitating Rad53 *in trans* autophosphorylation leading to its full kinase activation required for the stabilization of replication forks (Alcasabas et al. 2001; Pellicioli and Foiani 2005). Checkpoint kinases interplay with factors dedicated to preserve replisome architecture and functionality, as well as with DNA repair and DNA damage tolerance pathways (Branzei and Foiani 2010; Ulrich and Walden 2010). Homologous recombination (HR) proteins are also required for replication fork stabilization, though their role in this process, likely related to ssDNA protection, is not yet fully understood (Carr and Lambert 2013). DNA lesions blocking the

progression of replicative DNA polymerases can be bypassed through DNA damage tolerance (DDT) pathways (Chang and Cimprich 2009), which are driven by error-prone translesion synthesis (TLS) and the error-free template switching (TS) mechanisms. These are mediated by ubiquitylation of PCNA (proliferating cell nuclear antigen), and their impairment leads to increased mutagenesis and loss of viability upon treatment with genotoxic agents.

### *18.3.1 Ubiquitylation of Single-Stranded DNA Binding Proteins at Stalled Replication Forks*

Replication protein A (RPA) complex is composed of three subunits (Rfa1/RPA70, Rfa2/RP32, and Rfa3/RPA14) and is phosphorylated by checkpoint kinases (Vassin et al. 2009; Ghospurkar et al. 2015). It has been recently described that all RPA subunits are ubiquitylated in response to replication fork stalling induced by agents causing dNTP depletion or DNA damage (Elia et al. 2015). RPA ubiquitylation depends on the RFWD3 E3 ligase and does not induce degradation by the proteasome. RFWD3-depleted cells show defects in stalled fork restart and increased sister chromatid exchange events in conditions inducing fork stalling. Based on these observations, it was proposed that RFWD3-dependent ubiquitylation of RPA promotes homologous recombination-mediated restart of stalled forks (Fig. 18.4). It was suggested that ubiquitylation of RPA favors HR by promoting displacement of

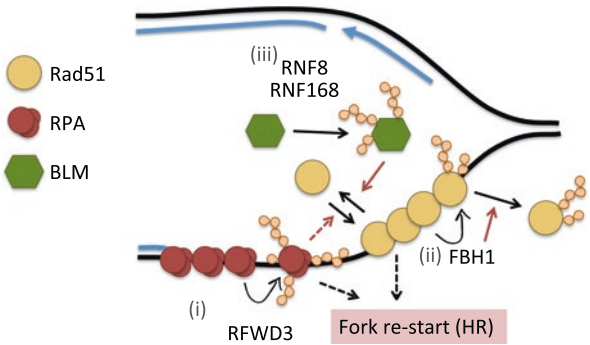


**Fig. 18.4** Modulation of RPA/Rad51 function and homologous recombination at stalled replication forks. (i) RPA ubiquitylation by RFWD3 upon replication stress induction promotes fork restart. Ubiquitylation likely promotes exchange of RPA by Rad51, thus promoting fork restart perhaps through recombination-mediated mechanisms. (ii) Besides its ability to disassemble Rad51 filaments, the FBH1 helicase/E3 ligase ubiquitylates Rad51. Ubiquitylation of Rad51 likely promotes its eviction from DNA, thus limiting toxic recombination impairing stalled fork functionality. (iii) RNF8 and RNF168 E3 ligases polyubiquitylate BLM in three different lysines. This modification is required for BLM recruitment to sites of stalled replication, where it is thought to suppress unscheduled recombination events by promoting the disruption of Rad51 nucleofilaments

RPA from ssDNA to facilitate RAD51 filament formation. Alternatively, ubiquitylated RPA may provide a signal for attracting RAD51 to stalled replication forks. Previously, RPA subunit ubiquitylation by a different E3 ligase, PRP19, in response to DNA damage had been reported (Maréchal et al. 2013). It was shown that depletion of PRP19 reduced DNA damage-induced ubiquitylation of RPA and a role for this modification in promoting ATR-ATRIP signaling and fork progression was proposed. This conclusion was challenged by the finding that the system used to interfere PRP19 expression resulted in a collateral increase of the overall levels of exogenously expressed ubiquitin (Elia et al. 2015). Hence, further work will be required to clarify the relative contribution of RFWD3 and PRP19 to RPA ubiquitylation and stalled fork protection.

A role for the ubiquitylation of the homologous recombination (HR) mediator RAD51 in response to replication stress conditions has also been proposed. Rad51 is ubiquitylated in vitro by the FBH1 helicase/F-box protein (Chu et al. 2015). FBH1 helicase function had been previously implicated in modulating HR-mediated DNA repair as it can displace RAD51 nucleofilaments (Simandlova et al. 2013). Conversely, FBH1 ubiquitin ligase activity has been proposed to limit recombination-driven genomic instability by modulating RAD51 association to stalled forks (Chu et al. 2015). Expression of a RAD51 K58/64R variant, which shows impaired ubiquitylation in vitro, results in increased recombination and promotes viability and fork stability in response to replication stress-inducing agents. It was hence proposed that ubiquitylation may counteract RAD51 accumulation at stalled forks, thus limiting unscheduled recombination events (Fig. 18.4). The RecQ-family BLM (Bloom) helicase also plays an important role in the regulation of HR-mediated DNA repair. It has been proposed that BLM exerts both pro- and anti-recombinogenic functions at replication forks, by limiting HR events upon fork stalling or, conversely, promoting Rad51 recruitment to collapsed forks to promote repair (Böhm and Bernstein 2014). BLM function at replication forks is regulated by posttranslational modifications. Upon HU treatment, recruitment of BLM to sites of stalled replication requires its polyubiquitylation on three different lysines (K105/K225/K259) by the RNF8 and RNF168 E3 ligases (Tikoo et al. 2013). Expression of non-ubiquitylatable forms of BLM results in increased HR levels. This and the fact that BLM can disrupt RAD51 nucleofilaments in vitro (Bugreev et al. 2007) lead to the notion that ubiquitylation enhances BLM anti-recombinogenic function at stalled forks (Fig. 18.4). Conversely, BLM SUMOylation mediates the pro-recombinogenic role of BLM upon fork collapse, though the underlying mechanism is less characterized (Böhm and Bernstein 2014). Collectively, this evidence hints at ubiquitylation as a mechanism to modulate the balance between RPA and Rad51 association to ssDNA and fine-tune HR events at stalled replication forks.

### *18.3.2 Cross Talk Between DNA Damage Checkpoint Signaling and Ubiquitin Systems*

Several lines of evidence point at a regulation of replication stress signaling by the checkpoint response through ubiquitylation. In *S. cerevisiae*, the Mrc1/CLASPIN adaptor is ubiquitylated by the $SCF^{Dia2}$ E3 ligase in vitro. In line with this observation, Mrc1 is degraded upon induction of DNA damage in S phase in a manner dependent on Dia2 (Mimura et al. 2009). In *Schizosaccharomyces pombe*, deletion of the replication fork protection complex factor Swi1 (homolog of Tof1/TIMELESS) induces degradation of Mrc1, dependent on the $SCF^{Dia2}$ homolog $SCF^{Pof3}$ (Roseaulin et al. 2013). This evidence suggests that Mrc1 is degraded through ubiquitylation via Dia2, which is a fork-associated factor. Cells ablated for Dia2 are sensitive to DNA damage induced in S phase and show defects in recovering from checkpoint activation, both suppressed by checkpoint-adaptor-deficient alleles of *MRC1* (Fong et al. 2013). It was thus proposed that Mrc1 degradation through Dia2-mediated ubiquitylation is important to achieve checkpoint inactivation. Of note, Dia2 itself is ubiquitiylated and degraded via the proteasome in a mechanism counteracted by checkpoint kinases (Kile and Koepp 2010), thus hinting at the existence of a feedback loop promoting checkpoint inactivation by coupling stabilization of Dia2 and degradation of Mrc1. Mrc1 has also been described to interplay with the $Rtt101^{Mms22}$ ligase in response to replication stress in a pathway unrelated to Dia2 (Buser et al. 2016). Mutants impairing $Rtt101^{Mms22}$ function show sensitivity to the alkylating agent methyl-methanesulfonate (MMS), which is suppressed by *MRC1* deletion, but not by alleles abrogating its checkpoint mediator function. $Rtt101^{Mms22}$ is recruited to replication forks via the Ctf4 replisome component and does not appear to affect Mrc1 stability. These data suggest that in response to MMS-induced damage, Mrc1 imposes a requirement for a yet unknown $Rtt101^{Mms22}$ function, perhaps in preserving replication fork integrity. Alternatively, $Rtt101^{Mms22}$ may counteract a yet not characterized function of Mrc1 in promoting DNA replication that may result deleterious in the presence of a damaged template. In human cells, CLASPIN is ubiquitylated by the $APC/C^{Cdh1}$ ubiquitin ligase complex, which promotes CLASPIN degradation via the proteasome both in the G1 phase of the cell cycle and in G2/M during recovery from treatment with DNA damaging agents (Bassermann et al. 2008). CLASPIN ubiquitylation by $APC/C^{Cdh1}$ is counteracted by the USP28 ubiquitin protease. The $SCF^{\beta TrCP}$ ubiquitin ligase complex has also been involved in CLASPIN ubiquitylation upon entry in mitosis following recovery from genotoxic stress (Mailand et al. 2006). This modification has been proposed to contribute to checkpoint inactivation by limiting CLASPIN function as an adaptor and is counteracted by the ubiquitin protease USP7. Additionally, a recent work identified USP29 as an ubiquitin protease counteracting CLASPIN ubiquitylation and degradation (Martín et al. 2014). Downregulation of USP29 function results in S-phase progression defects and impaired activation of the CHK1 kinase, though the ligase introducing the ubiquitin chains removed by USP29 remains to be elucidated. Lastly, a role in modulating CHK1 function through

CLASPIN deubiquitylation has also been reported for the USP20 ubiquitin protease (Zhu et al. 2014). Collectively, this evidence points at a high control of Mrc1/CLASPIN function through ubiquitylation and degradation in the modulation of checkpoint signaling following replication stress.

It was recently shown that the ATR checkpoint response can negatively regulate ubiquitylation machineries. In particular, silencing of CHK1 destabilizes APC/$C^{Cdh1}$ targets in human cells experiencing replication blocks, in a fashion dependent on expression of the ligase (Yamada et al. 2013). It was thus suggested that the checkpoint kinase inhibits the ubiquitin ligase complex in a process required to stabilize the Dbf4/ASK component of the DDK complex and promote DNA damage bypass mechanisms. In consistence with this idea, chromatin loading of both the Rad18 ubiquitin ligase and Pol η translesion polymerase is impaired by depletion of CHK1 upon induction of interstrand cross-links. Thus bidirectional cross talk likely occurs between checkpoint and ubiquitylation machineries in the response to DNA lesions generating replication stress.

### *18.3.3 Ubiquitylation in the Regulation of DNA Polymerases' Association to DNA and Chromatin Dynamics*

Ubiquitylation of DNA polymerases also appears to be important in the response to replication stress. In eukaryotes DNA polymerase ε primarily carries out leading strand synthesis, while DNA polymerase δ extends Okazaki fragments generated at lagging strands (Pursell et al. 2007). Ubiquitylation of the catalytic subunits of either replicative polymerase has been described in yeast cells (Roseaulin et al. 2013; Daraba et al. 2014). Inactivation of the replication fork protection complex by ablation of Swi1/Tof1 in *S. pombe* cells, which presumably enhances replication stalling and collapse at natural fork pausing sites, leads to ubiquitylation by $SCF^{Pof3}$ and degradation of the Pol2 catalytic subunit of DNA polymerase ε (Roseaulin et al. 2013). Ultraviolet (UV) light irradiation of *S. cerevisiae* cells induces DNA polymerase δ catalytic subunit Pol3 ubiquitylation and proteasomal degradation in a fashion dependent on Def1, a factor also involved in RNA polymerase II degradation (Daraba et al. 2014). Sensitivity of *def1* mutants to MMS treatment is epistatic to that conferred by genes required for the error-free pathway of DNA damage tolerance, which raised the possibility that degradation might facilitate exchange of Pol3 by translesion synthesis polymerases. In agreement with this hypothesis, DNA polymerase δ auxiliary subunits Pol31 and Pol32 are not degraded in these conditions and form a complex with DNA polymerase ζ-associated Rev1 protein, while Def1 ablation reduces UV-induced mutation rates.

Ubiquitylation has been shown to mediate the extraction of translesion polymerases from DNA by the action of the Cdc48/p97 cofactor DVC1/SPARTAN (Mosbech et al. 2012). DVC1 is recruited to sites of stalled replication in fashion dependent of its UBZ (ubiquitin-binding zinc finger) domain, which leads to the hypothesis that it

might interact with ubiquitylated replisome components. Interference of DVC1 or expression of an ATPase-deficient version of p97 results in increased Pol η interaction with PCNA in UV-irradiated cells. DVC1 also enhances p97 association to UV-damaged chromatin, which suggests that polyubiquitylation of Pol η may promote engagement by p97 to mediate its extraction during the last steps of translesion synthesis. In addition to this mechanism, it has been described that Pol η is mono-ubiquitylated in unperturbed cells and that this modification decreases upon treatment with genotoxic agents (Bienko et al. 2010). Mono-ubiquitylation inhibits Pol η interaction with PCNA and in this way may limit the basal frequency of TLS events.

DVC1/SPARTAN, as its budding yeast paralog Wss1, bears a metalloprotease activity, which is dependent on its association to DNA and is involved in replication-dependent repair of DNA-protein cross-links (DPC) (Stingele and Jentsch 2015; Stingele et al. 2016; Vaz et al. 2016). DVC1 is enriched at nascent DNA and interacts with replisome factors such as PCNA and the MCM complex, suggesting that it acts on DPCs as they are encountered by replication forks (Stingele et al. 2016; Vaz et al. 2016). Consistently, ablation of DVC1 in human or worm cells leads to lethality and fork progression defects upon treatment with agents inducing protein cross-linking to DNA. DVC1 is ubiquitylated, and its deubiquitylation promotes its access to chromatin, perhaps by disengagement of *in cis* interactions of its UBZ domain. Whether DVC1 function in replication-coupled DPC repair involves its role as an adaptor for Cdc48/p97 in extraction of ubiquitylated proteins from chromatin remains to be elucidated.

Ubiquitylation also plays a critical role in regulating replication in the chromatin context. Polycomb repressive complexes (PRCs) mediate the establishment of repressive chromatin through ubiquitylation of H2A (Di Croce and Helin 2013). In addition, H2A ubiquitylation by PCRs has recently emerged as a regulator of DNA replication. PRCs associate to nascent DNA in mouse cells, and interference of their function impairs replication fork progression in the absence of external perturbations (Piunti et al. 2014). These observations suggest that PCRs may modulate chromatin dynamics in particular chromosome territories to facilitate fork progression. Consistently, inactivation of RING ubiquitin ligase activities associated to PCRs leads to S-phase defects and increased asymmetry in replication fork progression (Bravo et al. 2015). Replication impairment in these conditions is specific to pericentromeric heterochromatic (PCH) domains and can be suppressed by targeted restoration of H2A ubiquitylation at PCH. The exact function of H2A ubiquitylation during replication remains to be understood but might reflect a necessity to modulate chromatin structure at hard-to-replicate domains or limit fork conflicts with derepressed transcription.

H2B is mono-ubiquitylated by Bre1/RNF20 E3 ubiquitin ligase, and this modification plays a well-characterized role in transcriptional regulation (Meas and Mao 2015). In budding yeast, Bre1 and mono-ubiquitylated H2B are enriched around active replication origins, suggesting that Bre1 associates to replication forks to promote H2B ubiquitylation at nascent chromatin DNA (Trujillo and Osley 2012; Lin et al. 2014). Non-ubiquitylatable lysine-123 H2B mutants are sensitive to HU treatment and show altered stalled fork progression as well as defects in recovery

from replication stress, likely independent of transcriptional regulation of dNTP pools. It was thus suggested that H2B ubiquitylation is important in coupling chromatin assembly and replication fork progression.

### *18.3.4 Modulation of the Ubiquitylation of the Sliding Clamp PCNA as a Central Regulator of DNA Damage Tolerance Mechanisms*

At the core of replicating chromatin, PCNA works as an essential processivity factor for DNA polymerases (Moldovan et al. 2007; Ulrich 2009; Mailand et al. 2013). PCNA interacts with a number of proteins involved in replication itself as well as with factors involved in the maintenance of genome integrity (Mailand et al. 2013). The posttranslational modification of PCNA licenses a layer of control in PCNA-protein interactions that facilitates fork progression under normal or perturbed replication (Andersen et al. 2008; Ulrich 2009; Bergink and Jentsch 2009; Mailand et al. 2013). During S phase, cells face damaged DNA or lesions that pose significant barriers to the progression of replication forks (Curtin 2012). In living organisms, tolerance mechanisms ensure that DNA can be replicated when it is damaged (Friedberg 2003). These mechanisms prevent irreversible fork collapse when the replisome encounters bulky lesions at damaged sites that block replicative DNA polymerases (Friedberg et al. 2005). Cells have evolved tolerance to DNA lesions to ensure progression of replication forks past unrepaired damage (Lawrence 1994; Chang and Cimprich 2009).

Tolerance to DNA damage is based either on translesion synthesis (TLS) that is carried out by specialized low-fidelity, potentially error-prone, TLS DNA polymerases or on template switching, an error-free mechanism that involves sister-strand pairing within or nearby the replication fork. In the face of a DNA lesion, the ubiquitylation of the sliding clamp controls the choice of translesion synthesis in eukaryotes. While mono-ubiquitylation of PCNA at $Lys^{164}$ enhances the affinity of error-prone TLS DNA polymerases (Hoege et al. 2002; Stelter and Ulrich 2003; Watanabe et al. 2004), further $Lys^{63}$-linked polyubiquitylation of mono-ubiquitylated $Lys^{164}$-PCNA promotes template switching (Zhang and Lawrence 2005; Vanoli et al. 2010). This control mechanism is well characterized and widely conserved in eukaryotic organisms (Moldovan et al. 2007; Ulrich 2009; Mailand et al. 2013). On the contrary, the significance of the deubiquitylation of PCNA remains insufficiently understood.

The ubiquitylation of PCNA is catalyzed by proteins in the Rad6 epistasis group (Hoege et al. 2002; Stelter and Ulrich 2003) (Fig. 18.5). The Rad6 group includes genes required for post-replication repair in the budding yeast *S. cerevisiae* and consists of *RAD6* and *RAD18* ($Lys^{164}$-PCNA-ubiquitin ligase) or *RAD5*, *MMS2*, and *UBC13* ($Lys^{63}$-linked $Lys^{164}$-PCNA-ubiquitin ligase) (Lawrence 1994; Zhang and Lawrence 2005). The Rad6 epistasis group is a central player in DNA damage bypass

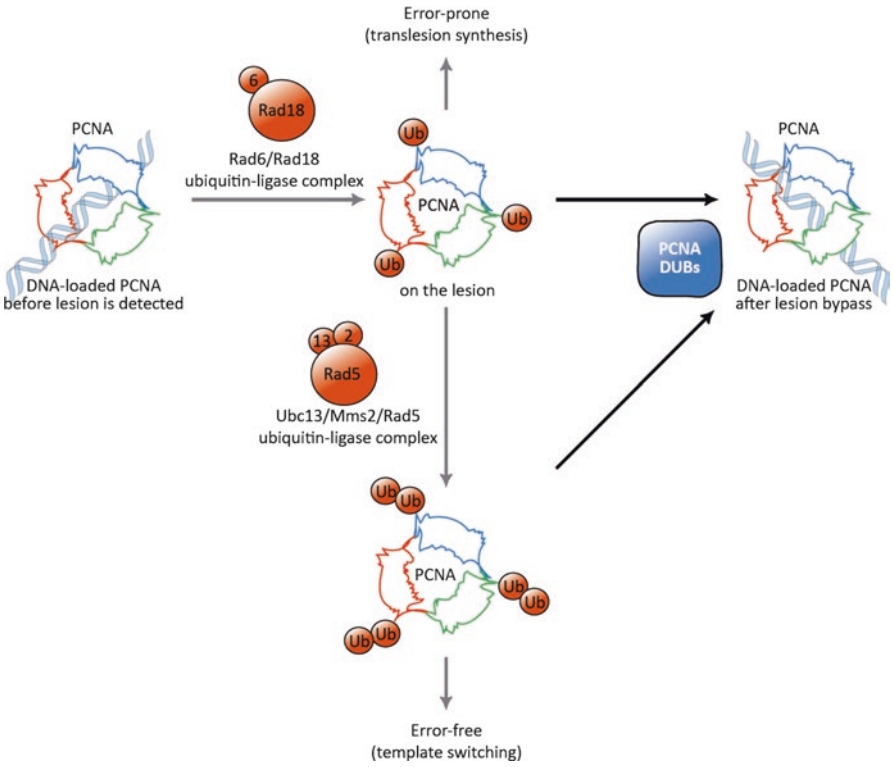


**Fig. 18.5** Model for PCNA ubiquitylation/deubiquitylation in the modulation of tolerance of DNA damage encountered by replication forks. PCNA progresses in association with the replisome on leading strands and associates to Pol δ on lagging strands. Bulky lesions block the progression of replicative polymerases and induce Rad6-/Rad18-mediated PCNA mono-ubiquitylation. Ubiquitylated PCNA enhances the interaction with translesion synthesis DNA polymerases. Further Lys63-linked ubiquitylation of PCNA Lys164-conjugated ubiquitin by a second ubiquitin ligase complex (Ubc13/Mms2/Rad5) promotes template switching events. After lesion bypass, PCNA-DUBs deubiquitylate PCNA to switching back to replicative DNA polymerases and resume processive DNA replication. Dynamic ubiquitylation and deubiquitylation of PCNA may regulate DNA damage tolerance at replication forks and/or post-replicatively

and tolerance pathways (historically known as post-replication repair) as these enzyme complexes are involved in protein ubiquitylation of PCNA and translesion synthesis DNA polymerases. PCNA mono-ubiquitylation is regarded as a central step in translesion synthesis during normal or perturbed DNA replication. However, DT40 avian cell line evidence indicates that Pol η (a TLS-DNA polymerase) and PCNA interact in the absence of PCNA ubiquitylation (Edmunds et al. 2008). Furthermore, there is general consensus that PCNA ubiquitylation is important but not essential for translesion DNA synthesis in mammalian cells (Edmunds et al. 2008; Hendel et al. 2011). Nevertheless, all eukaryotic models tested to date mono-ubiquitylate $Lys^{164}$-PCNA to detectable levels in response to replication blocks or

DNA damage to direct TLS polymerases to damaged sites (Hoege et al. 2002; Kannouche et al. 2004; Watanabe et al. 2004; Arakawa et al. 2006; Frampton et al. 2006). Of particular interest is the fact that PCNA is mono-ubiquitylated in *Xenopus* and *S. pombe* and di-ubiquitylated in *S. pombe*, during unperturbed S phase in a Rad6-/Rad18-dependent manner (Leach and Michael 2005; Frampton et al. 2006).

Rad18 is the predominant PCNA E3 ubiquitin ligase in eukaryotes. However, evidence from mammalian cells indicates that other ubiquitin ligases, including RNF8, HLTF, and $CRL4^{CDT2}$, may have a role in this process in the absence of Rad18 or during unperturbed DNA replication (Zhang et al. 2008; Terai et al. 2010; Lin et al. 2011). The choice between the two branches of damage tolerance, error-prone or error-free, relies respectively on the mono-ubiquitylation and polyubiquitylation of the sliding clamp PCNA. However, the timing of PCNA ubiquitylation, and therefore the timing of lesion bypass, relative to DNA replication remains under discussion. It has been shown that tolerance to DNA damage mediated by ubiquitin-dependent modification of PCNA can be uncoupled from bulk DNA replication, at least in *S. cerevisiae* (Karras and Jentsch 2010; Daigaku et al. 2010), suggesting the hypothesis that DNA damage bypass may work exclusively as a post-replicative mechanism (Karras and Jentsch 2010). It is worth to mention that PCNA-ubiquitin ligase Rad18 associates with replisomes at sites of newly synthesized DNA in human cells (Dungrawala et al. 2015; Despras et al. 2016), indicating that the machinery for ubiquitylation of PCNA travels with the replisome at replication forks.

Along the last decade, mammalian USP1, human Usp10, and budding yeast Ubp10 have been identified as major deubiquitylating enzymes (DUBs) for PCNA (Huang et al. 2006; Gallego-Sánchez et al. 2012; Mailand et al. 2013; Park et al. 2014). USP1, the first PCNA-DUB identified, is a ubiquitin protease that deubiquitylates mono-ubPCNA and mono-ubFANCD2 in human cells (Huang et al. 2006). USP1 undergoes inactivation by auto-proteolysis when cells are irradiated with UV light. Therefore, upon UV irradiation, USP1 is inactivated and PCNA becomes ubiquitylated; this observation suggests that USP1 continuously deubiquitylates PCNA in the absence of DNA damage. Consistent with a role as a PCNA-DUB, the depletion of chicken USP1 in DT40 cells or in murine USP1−/− MEFs increases PCNA, and also FANCD2, mono-ubiquitylation (Oestergaard et al. 2007; Kim et al. 2009). Recently, it has been shown that upon UV-mediated DNA damage, HeLa cells rely on USP10 to deubiquitylate ISGylated PCNA (Park et al. 2014). Two unicellular model systems have been examined in detail, budding and fission yeast. In the budding yeast *S. cerevisiae*, the ubiquitin protease Ubp10 deubiquitylates $Lys^{164}$ mono- and di-ubiquitylated PCNA during S phase (Gallego-Sánchez et al. 2012, 2013). More recently, it has been shown that Ubp2, Ubp15, Ubp16, and Ubp12 ubiquitin proteases revert PCNA ubiquitylation in the fission yeast *S. pombe* during S phase (Álvarez et al. 2016). To date, all the identified PCNA-DUBs belong to the USP/UBP subfamily of ubiquitin-specific proteases.

All the published evidence provides robust evidence that PCNA-DUBs revert the $Lys^{164}$ mono- and di-ubiquitylation of PCNA in vitro and in vivo. Therefore, PCNA-DUB activities may counterbalance the ubiquitin ligase activity of the Rad6/Rad18 complex responsible for PCNA mono-ubiquitylation at sites of newly synthesized

DNA. Thus, these ubiquitin-specific proteases have the potential to be part of a safeguard mechanism limiting the residence time of TLS DNA polymerases on replicating chromatin (Fig. 18.5).

### *18.3.5 Ubiquitylation-Mediated Control of the Fanconi Anemia DNA Repair Pathway*

Fanconi anemia (FA) is a rare disorder characterized by sensitivity to DNA interstrand cross-linking agents, bone marrow failure, and susceptibility to both acute myeloid leukemia and solid tumors. These features can also be accompanied by congenital abnormalities and infertility (Risitano et al. 2016). The Fanconi anemia pathway (named after the syndrome) responds to DNA interstrand cross-links and replication fork-stalling lesions that hamper not only the DNA synthesis by polymerases but also strand separation by replicative helicases. The FA pathway promotes the repair of DNA ICLs orchestrating several processes that include recognition of the ICL (Fig. 18.6), incision of the lesion by structure-specific endonucleases, bypass by TLS polymerases, and HR-mediated repair of the incised strand. The pathway is initiated by the recruitment of the core FA complex, which contains the E3 ubiquitin ligase FANCL, to ICLs upon engagement by replication forks (Longerich et al. 2009). Unloading of the CMG helicase complex from chromatin, a step required for the last steps of DNA synthesis and subsequent nucleolytic incision, follows ICL recognition. CMG extraction requires polyubiquitylation of MCM7 that is mediated by BRCA1 E3 ligase, in contrast with replication termination which is mediated by $SCF^{Dia2}$, which promotes engagement by the Cdc48/p97segregase (Long et al. 2014; Fullbright et al. 2016). Upon recruitment to stalled forks, the catalytic subunit FANCL mono-ubiquitylates the FANCD2-FANCI dimer. This ubiquitylation activates downstream events leading to lesion processing and repair. How these events are coordinated to achieve ICL removal starts to be elucidated, as some key mechanistic details have been recently described. Ubiquitylation of FANCD2 is essential for its interaction with a UBZ4 domain in the SLX4 scaffold protein. SLX4 in turn promotes recruitment and activation of several nucleases, such as ERCC4-ERCC1, MUS81-EME1, and SLX1, which mediate the incisions of a DNA strand flanking the ICL. Ubiquitylation of FANCD2 also mediates its interaction with the UBZ domain of FAN1 (Fanconi anemia nuclease 1), yet another nuclease involved in the FA-mediated repair (Liu et al. 2010; Yoshikiyo et al. 2010). FAN1 is dispensable for the incision of ICLs in vitro, and FAN1 mutations are not associated to typical FA phenotypes (Klein Douwel et al. 2014), suggesting that it may mediate FA pathway functions unrelated to ICL repair. Accordingly, FANCD2 recruits FAN1 to replication forks stalled by HU treatment where it is required for efficient restart (Lachaud et al. 2016). Of note, FANCD2 patient-derived cells exhibit increased replication fork stalling at common fragile sites (CFS) (Madireddy

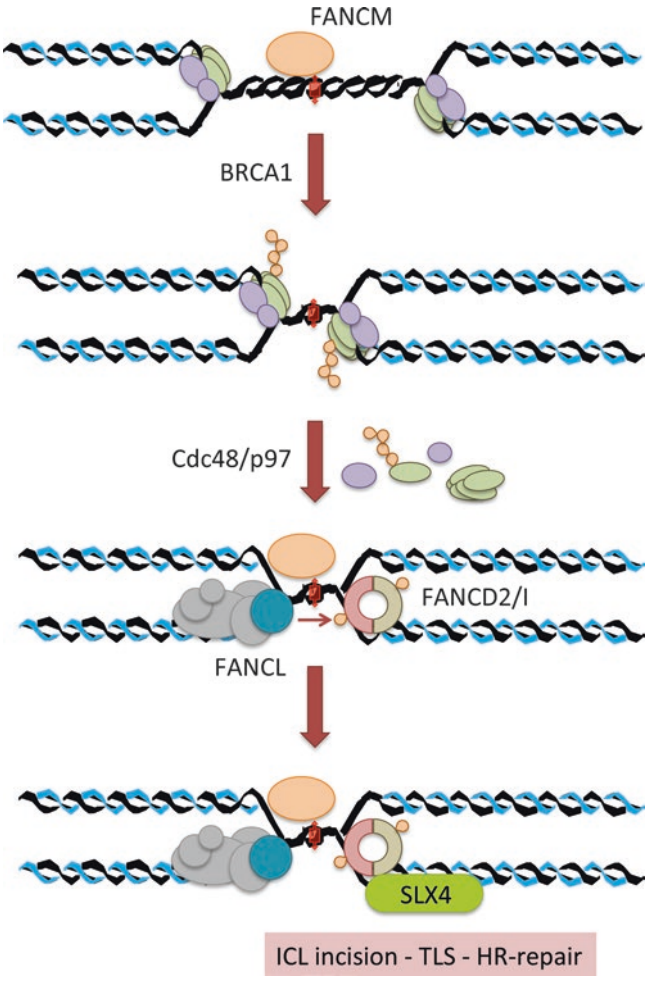


**Fig. 18.6** Ubiquitylation is central in the modulation of the Fanconi anemia DNA repair pathway. Upon collision with replication forks, the FANCM translocase recognizes interstrand cross-links (ICL) and initiates the FA repair pathway. CMG ubiquitylation by BRCA1 E3 ligase drives its eviction through the action of the Cdc48/p97 segregase, which enables synthesis of nucleotides nearby the lesion. Then the Fanconi anemia core complex is recruited to the lesion, where the FANCL ubiquitin ligase promotes the mono-ubiquitylation of the FANCD2/I heterodimer. This ubiquitylation is the central event that mediates the recruitment of the SLX4 protein, which serves a scaffold for nucleases that promote incisions on the DNA backbone around the lesion. DNA incision triggers downstream events in the FA pathway such as base flipping, translesion synthesis, and HR-mediated repair of the incised strand

et al. 2016), suggesting that FANCD2 ubiquitylation may serve as a signal mediating distinct fork protection mechanisms besides the repair of ICLs.

There is evidence of cross talk between the FA pathway and key regulators of the DNA damage tolerance mechanisms. As mentioned above, Rad18 mediates PCNA mono-ubiquitylation and is also required for the ubiquitylation and activation of FANCD2/I (Geng et al. 2010; Williams et al. 2011). However, how Rad18 contributes to activation of the FA pathway is a matter of debate. On the one hand, it was proposed that FANCL interacts with ubiquitylated PCNA and that this interaction is necessary for recruitment of FANCL to chromatin and FANCD2 mono-ubiquitylation (Geng et al. 2010). On the other hand, it has been reported that Rad18 promotes FACND2/I ubiquitylation in a fashion independent on PCNA-K164 (Williams et al. 2011), in apparent contradiction with the previous report. In addition, it has been shown that FANCD2 interacts with PCNA through a PIP box and that FANCD2 PIP-box mutants show impaired ubiquitylation (Williams et al. 2011), though the involvement of Rad18 or PCNA mono-ubiquitylation in this mechanism was not explored. Another link between FA and DDT pathways occurs through the USP1 ubiquitin protease that, as mentioned, reverts the mono-ubiquitylation of both PCNA and the FANCD2-I complex (Nijman et al. 2005a). Silencing of USP1 expression increases mutation frequencies in UV-treated cells (Nijman et al. 2005a), suggesting that a fine regulation of these two processes is important to promote replication fidelity. Inactivation of the FA pathway involves SUMOylation of FANCD2 and FANCI proteins by PIAS1 and PIAS4 SUMO E3 ligases (Gibbs-Seymour et al. 2015). SUMOylated FANCD2-I is engaged by the RNF4 SUMO-targeted ubiquitin ligase, which in turn polyubiquitylates FANCD2-I and promotes eviction of the dimer from chromatin. FANCD2-I is extracted by the p97 segregase with the aid of its adaptor DVC1, thus locally interrupting FA pathway signaling.

## 18.4 Concluding Remarks

In recent years our knowledge on the flexibility and complexity of the ubiquitylation code has rapidly expanded. This is reflected in the current understanding of how ubiquitylation dynamically regulates DNA replication. Ubiquitin-mediated degradation controls the stability of factors spatiotemporally regulating replication initiation, as well as proteins mediating replication stress signaling. Ubiquitylation of replication factors also modulates important DNA repair and DNA damage tolerance pathway choices in response to replication stress. Lastly, the importance of ubiquitylation as a signal to control the extraction of key proteins from replicating chromatin by ubiquitin-selective segregase complexes during termination and upon fork stalling is emerging. It is thus expected that research in years to come may elucidate key mechanistic details of how ubiquitin regulates DNA replication, replication fork protection, and the coordination of replication with other chromosome metabolic processes.

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# Chapter 19
# Regulation of Mammalian DNA Replication via the Ubiquitin-Proteasome System

**Tarek Abbas and Anindya Dutta**

**Abstract** Proper regulation of DNA replication ensures the faithful transmission of genetic material essential for optimal cellular and organismal physiology. Central to this regulation is the activity of a set of enzymes that induce or reverse posttranslational modifications of various proteins critical for the initiation, progression, and termination of DNA replication. This is particularly important when DNA replication proceeds in cancer cells with elevated rates of genomic instability and increased proliferative capacities. Here, we describe how DNA replication in mammalian cells is regulated via the activity of the ubiquitin-proteasome system as well as the consequence of derailed ubiquitylation signaling involved in this important cellular activity.

**Keywords** DNA replication • Ubiquitin • Ubiquitylation E3 ligases • DNA rereplication • Cancer

## 19.1 Introduction

Eukaryotic DNA replication is a highly regulated process that ensures the faithful transmission of genetic material to daughter cells (Machida et al. 2005). The process is coupled both with cell cycle progression and with the ability of cells to cope with various intrinsic and extrinsic insults that constantly threaten the integrity of the genome. Various repair mechanisms have evolved to cope with these insults,

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permitting the repair of various lesions prior to the completion of DNA replication (Hustedt and Durocher 2016; Ganai and Johansson 2016; Berti and Vindigni 2016). Critical to this regulation is the ability of cells to sense environmental perturbations, to enforce appropriate checkpoints, and to activate a number of cellular processes conductive of DNA repair prior to the initiation or resumption of DNA replication. Posttranslational modifications of certain proteins play fundamental role in the timely execution of most, if not all, of these cellular activities.

Similar to many physiological processes in the cell, proper regulation of DNA replication is governed through the balanced production and termination of key cellular proteins controlling the various steps involved. DNA replication depends on key enzymatic activities that are controlled through the action of cellular proteins and cofactors that are actively synthesized, modified, or destroyed to achieve optimal activity. ATP-dependent protein polyubiquitylation plays an important role in almost all physiological processes and in many diseases including cancer owing to its role in the global regulation of protein turnover (Schwartz and Ciechanover 2009; Hershko 2005; Amir et al. 2001; Glickman and Ciechanover 2002; Kornitzer and Ciechanover 2000; Ciechanover and Schwartz 2002). The highly coordinated and reversible process ensures timely downregulation of proteins via the activity of the 26S proteasome, where the polyubiquitylated proteins, roughly 80% of all intracellular proteins, are digested into small peptides, and ubiquitin molecules are recycled (Skaar et al. 2014).

Proteasomal degradation through the ubiquitin-proteasome system (UPS) is triggered following the covalent attachment of multiple ubiquitin molecules linked together through lysine 48 (Lys-48) to substrate proteins (Teixeira and Reed 2013; Groll and Huber 2003). Other forms of polyubiquitylation (e.g., linkage through Lys-63 of ubiquitin) do not result in proteasomal degradation but regulate other functions, such as protein trafficking, protein-protein interaction, and kinase activation (Yang et al. 2010; Behrends and Harper 2011). Polyubiquitylation is comprised of three distinct and consecutive enzymatic steps (Fig. 19.1): ubiquitin activation by an E1 ubiquitin-activating enzyme, the transfer of the activated ubiquitin to an E2 ubiquitin-conjugating enzyme (UBC), and the transfer of ubiquitin to the substrate through the activity of an E3 ubiquitin ligase (Kornitzer and Ciechanover 2000; Groll and Huber 2003; Glickman and Ciechanover 2002). This latter activity by E3 ubiquitin ligases is particularly important as it confers specificity for the substrate to be targeted for ubiquitylation (Fig. 19.1).

While polyubiquitylation is an efficient mechanism by which cellular proteins are eliminated or modified, the process is reversible, and the removal of ubiquitin chains from substrate proteins is carried by a class of highly specific cysteine proteases, collectively termed “deubiquitinases” or “DUBs” (Fig. 19.1). DUBs hydrolyze the isopeptide bonds between the ε-amino group of lysine side chains of the target substrate and the C-terminal group of ubiquitin or the peptide bond between the α-amino group of the target protein and the C-terminal of ubiquitin (Wilkinson 1997). DUBs play a pivotal role as regulators of the turnover rate, activation, recycling, and localization of many proteins and thus are essential for regulating several signaling pathways and for cellular homeostasis (Komander et al. 2009; Reyes-Turcu et al. 2009). The role of the UPS in controlling DNA replication following replication stress and in response to DNA damage is described in excellent recent

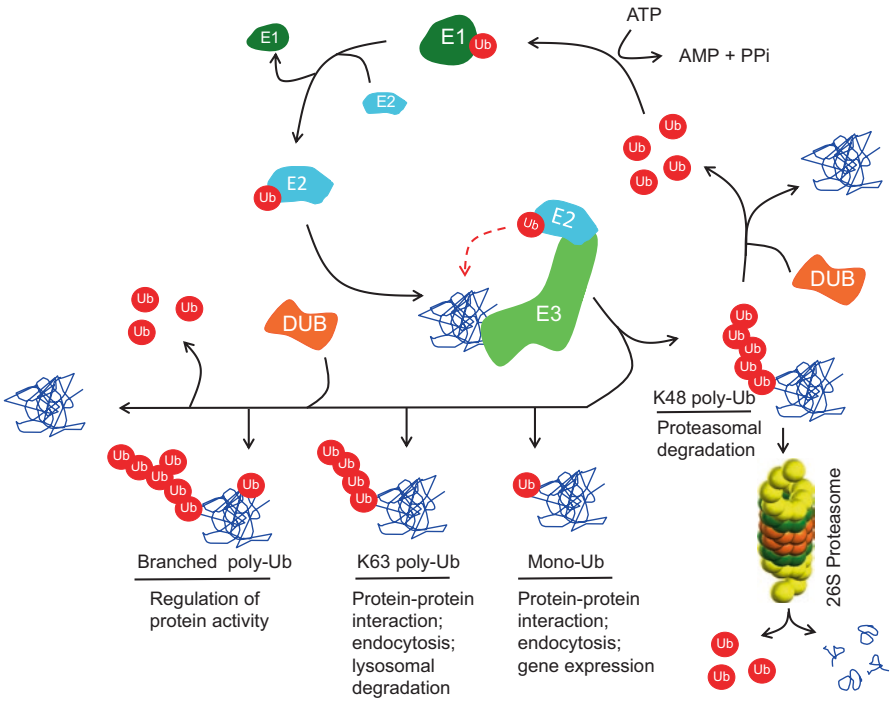


**Fig. 19.1** Schematic of the various steps involved in the ubiquitylation of protein substrates via the UPS. Ubiquitin (Ub) is conjugated to various ubiquitylation substrates through various linkages to form monoubiquitylated (mono-Ub), lys-48-linked polyubiquitylated (K48 poly-Ub), lys-63-linked polyubiquitylated, and branched polyubiquitylated (branched poly-ub) species. Other forms of ubiquitin linkages are not shown. The process begins with ubiquitin first activated and bound through a thioester bond by the ubiquitin-activating enzyme (E1). Activated ubiquitin is subsequently transferred to one of several ubiquitin-conjugating enzymes (E2) through another thioester bond. An E3 ubiquitin ligase (E3) then promotes the transfer of ubiquitin to the target substrate through interaction with the E2-charged ubiquitin, whereby a covalent isopeptide bond is formed between the C-terminus of ubiquitin and a specific lysine residue on the substrate. Elongation of the ubiquitin chain is effected when the C-terminus of another ubiquitin moiety is linked to one of seven lysine residues (e.g., K48) or the fist methionine residue (M1) on the first ubiquitin. Polyubiquitylation through K48 or the less common K29 linkages targets the ubiquitylated substrate for proteasomal degradation via the 26S proteasome. Other polyubiquitylation linkages (e.g., K63) serve non-proteolytic functions. Ubiquitin and polyubiquitin chains can be removed from the substrates through the activity of one of many highly specific cysteine proteases, called deubiquitylating enzymes (DUBs), which can cleave both the isopeptide bond between the ubiquitin and ε-amino group of the lysine on the substrate or on ubiquitin (in a polyubiquitin chain) or the peptide bond between ubiquitin and the N-terminal methionine of ubiquitin

reviews (Garcia-Rodriguez et al. 2016; Renaudin et al. 2016; Sommers et al. 2015). In this chapter, we focus on protein ubiquitylation leading to proteasomal degradation or modification of function of key proteins to control DNA replication, with an emphasis on key E3 ubiquitin ligases and DUBs.

## 19.2 Structure and Function of Cullin-RING Ubiquitin Ligases Controlling DNA Replication

The **C**ullin-**R**ING (*R*eally *I*nteresting *N*ew *G*ene) E3 ubiquitin **L**igases (CRLs) represent the largest family of E3 ligases and are responsible for the ubiquitylation of approximately 20% of total cellular proteins degraded through the proteasome (Soucy et al. 2009). The other major E3 ligases belong to the HECT (*H*omologous to the *E*6-AP *C*arboxyl *T*erminus) domain containing E3 ubiquitin ligases (Li et al. 2008; Deshaies and Joazeiro 2009; Skaar et al. 2014). CRLs play significant roles in various processes including cell cycle regulation, cell proliferation, and tumorigenesis (Petroski and Deshaies 2005; Hotton and Callis 2008; Bosu and Kipreos 2008). Family members include eight cullin proteins (cullins 1, 2, 3, 4A, 4B, 5, 7, and 9 (also known as PARC or p53-associated parkin-like cytoplasmic protein)) and a cullin-like protein ANAPC2 or APC2. The general description of the structure and function of CRLs has been described in several excellent reviews (Chen et al. 2015; Lydeard et al. 2013; Sarikas et al. 2011; Duda et al. 2011; Hua and Vierstra 2011; Lipkowitz and Weissman 2011; Deshaies and Joazeiro 2009; Hotton and Callis 2008). The SCF (**S**KP1-**C**ullin1-**F**-Box protein; also known as CRL1, Fig. 19.2) is one of the well-characterized members of CRL ligases best known for its role in the regulation of cell cycle progression, cellular proliferation, apoptosis, and differentiation (Nakayama and Nakayama 2005; Welcker and Clurman 2008; Maser et al. 2007; Huang et al. 2010; Lee and Diehl 2014; Duan et al. 2012). SCF recognizes and promotes the ubiquitylation and degradation of its substrates through association with one of many substrate receptors (69 in mammalian cells), collectively known as F-box proteins, constituting a large family of distinct SCF ligases with varying specificity (Heo et al. 2016; Kipreos and Pagano 2000; Cardozo and Pagano 2004; Skaar et al. 2013; Wang et al. 2014). These substrate receptors utilize their F-box motifs to associate with the SKP1 (S-phase kinase-associated protein 1) bridge subunit (Wang et al. 2014). The SKP1 subunit of the SCF ligase bridges the F-box proteins (along with their cognate substrates) to the N-terminal domain of the cullin 1 subunit. The C-terminal domain of cullin 1 interacts with a small RING domain protein (RBX1 or RBX2), which is essential for the recruitment of E2 UBCs necessary for the polyubiquitylation of substrates distend for proteolytic degradation (Fig. 19.2).

Three subfamilies (FBXW, FBXL, and FBXO) characterize the F-box proteins, depending on whether they contain WD40 repeats (FBXW or FBW series), leucine-rich repeats (FBXL or FBL series), or variable other or "catch-all" domains (FBXO or F-box only series) (Skaar et al. 2014). Through their ability to assemble distinct

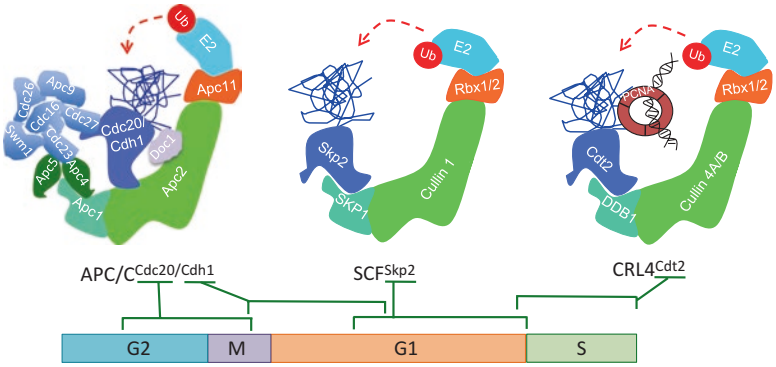


**Fig. 19.2** Molecular architecture of the three multi-subunit E3 ubiquitin ligases controlling DNA rereplication and their activity during the cell cycle. The schematic illustrates the general architecture of the APC/C (CDC20 or CDH1), cullin 1 (SCF), and cullin 4 (CRL4) E3 ubiquitin ligases. Ubiquitin molecules (red circles) are transferred to the substrate (blue ribbons) through the activity of one of the three E3 ubiquitin ligase complexes (made of a cullin or cullin-like subunit (light green), a bridge protein(s) (cyan), substrate adaptors (royal blue), and small RING protein (orange)) and E2 conjugating enzymes (baby blue). Multiple proteins bridge the substrate receptors CDC20 or CDH1 to the APC2 scaffold subunit of the APC/C ligase. SKP1 and DDB1 (damage-specific DNA-binding protein 1) bridge the substrate receptors SKP2 and CDT2 to the cullin 1 or cullin 4 (A or B) of the $SCF^{SKP2}$ or $CRL4^{CDT2}$ E3 ligases, respectively. $CRL4^{CDT2}$ recognizes its ubiquitylation substrates when bound to chromatin-bound trimeric proliferating cell nuclear antigen (PCNA), encircling DNA (black helix). *G1* first gap phase of the cell cycle, *S* DNA synthesis phase, *G2* second gap phase of the cell cycle, *M* mitosis

SCF ligases, many of these F-box proteins are involved in the regulation of DNA replication and cell cycle progression, and their expression or activity is altered in human malignancies (Heo et al. 2016). For example, the $SCF^{SKP2}$ ubiquitin ligase (Fig. 19.2) composed of the core SCF ligase associating with the substrate receptor and SKP2 (S-phase kinase-associated protein 2; also known as FBL1) is an essential driver of cell cycle progression, primarily through impacting DNA replication either directly or indirectly. $SCF^{SKP2}$ directly controls DNA replication through its ability to promote the timely ubiquitin-dependent degradation of several components of the pre-replication complexes (pre-RCs) from mid-G1-phase (Fig. 19.3). This ensures that licensing of replication origins occurs only from late M till mid-G1-phase of the cell cycle and is prevented from occurring again until cells complete the genome duplication and following the segregation of daughter chromosomes in late M (Fig. 19.4; see below). $SCF^{SKP2}$ also activates DNA replication through activating cyclin-dependent kinases (CDKs) primarily through the ubiquitylation and degradation of negative CDK regulators (e.g., the CDK inhibitors $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$). Later in the cell cycle, $SCF^{SKP2}$ is also responsible for the targeted proteolysis of positive regulators of CDKs (e.g., cyclin D1 and cyclin A) to promote the handover of CDK activity from one CDK to the next (Fig. 19.4). The latter activity ensures the availability of CDK molecules for assembling distinct cyclin-CDK complexes for catalyzing various specific activities necessary for the irreversible pro-

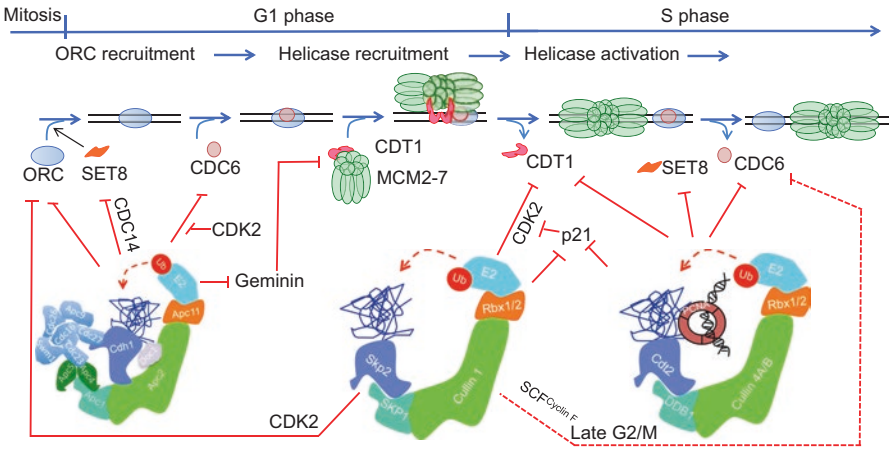


**Fig. 19.3** Control of MCM2-7 loading via the UPS. The APC/$C^{Cdh1}$ ligase promotes MCM2-7 loading in late M and in G1 by degrading Geminin. However, APC/$C^{Cdh1}$ also appears to limit the abundance of certain licensing factors like *Drosophila* ORC1 and mammalian CDC6 in G1, narrowing to late M-phase the window in the cell cycle when there is enough ORC, CDC6, and active CDT1 available to load MCM2-7 on origins. The E3 ubiquitin ligases, $SCF^{SKP2}$ and $CRL4^{CDT2}$ limit the abundance of key proteins involved in MCM2-7 loading (pre-RC assembly) in the S-, G2-, and early M-phases of the cell cycle. The SCF ubiquitin ligase also utilizes the substrate recognition subunit cyclin F ($SCF^{Cyclin\ F}$; *red dashed lines*) to suppress origin relicensing by promoting the ubiquitylation and degradation of CDC6 in late G2- and early M-phase of the cell cycle

gression of the cell cycle. For example, the ubiquitin-dependent proteolysis of cyclin A via the $SCF^{SKP2}$ ligase ensures the availability of CDK1 molecules to assemble cyclin B-CDK1 complexes essential for G2 progression. Likewise, the degradation of cyclin E, mediated through the activity of the SCF ligase with the substrate receptor FBXW7 ($SCF^{FBXW7}$) following cyclin E phosphorylation by CDK2, ensures the availability of CDK2 molecules to assemble cyclin A-CDK2 complexes necessary for S-phase progression (Clurman et al. 1996; Koepp et al. 2001).

The other major E3 ubiquitin ligase regulating DNA replication is the APC/C (anaphase-promoting complex/cyclosome) ubiquitin ligase (Fig. 19.2). This ubiquitin ligase utilizes the APC2 cullin-like scaffold to assemble the largest multi-subunit CRL ligase in mammalian cells (van Leuken et al. 2008). APC/C associates with one of two substrate adaptor proteins, CDH1 and CDC20, for recognizing and promoting the polyubiquitylation (both K48- and K11-linked ubiquitin conjugation) of a large number of key drivers of cell cycle progression (Visintin et al. 1997; Zachariae and Nasmyth 1999; Pines 2006). Unlike the SCF-FBX ligases, which recognize the vast majority of target substrates through phosphorylation-dependent sequence motifs or "phospho-degrons" within these substrates (Skaar et al. 2013), APC/C ligases recognize proteins containing D-boxes and KEN-boxes, such as cyclin A and cyclin B (Pfleger and Kirschner 2000; Pfleger et al. 2001). The assembly of APC/$C^{CDC20}$ is driven by CDK-mediated phosphorylation of CDC20 and is active primarily in mitosis (Rahal and Amon 2008); this is critical for the initial

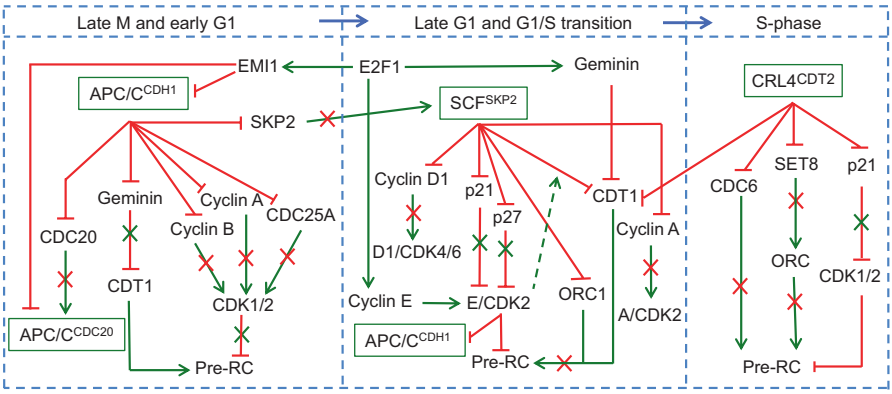


**Fig. 19.4** UPS control of DNA replication via direct and indirect mechanisms. MCM2-7 is loaded (Pre-RCs established) in late M (and perhaps early G1)-phase of the cell cycle and is prevented from being loaded again from the $G_1$/S transition until the latest part of the next mitosis. This regulation is dependent primarily on the activity of CDK, which is maintained at low levels through late mitosis and $G_1$ through the activity of APC/$C^{CDH1}$ ubiquitin ligase. CDK activity peaks again in late G1 and at the G1/S transition through the activity of the $SCF^{SKP2}$ ligase, which promotes the proteolysis of negative regulators of CDK2. CDK2 activity remains high in S-phase through the targeted proteolysis of p21 via $CRL4^{CDT2}$. Such elevated levels of CDK suppress pre-RC in late G1 and in S and prevent aberrant origin relicensing and DNA rereplication. All three E3 ligases also directly control the abundance of pre-RC proteins such that replication occurs only once in the cell cycle. The circuit receives transcriptional input from the E2F1 transcription factor, which in addition to promoting S-phase entry into the cycle through upregulation of cyclin E and the consequent elevation of cyclin E/CDK2 activity provides a negative feedback control to shut down the activity of APC/C ligase (via EMI1 upregulation) and inactivation of CDT1 (via Geminin upregulation). This ensures that cells proceed in S-phase without aberrant licensing. Green arrows, positive regulation. Red lines, negative regulation

targeted proteolysis of mitotic cyclins and for exit from mitosis. Assembly of APC/$C^{CDH1}$ on the other hand is stimulated through dephosphorylation of the CDH1 subunit by the CDC14A phosphatase (Cdc14 in budding yeast) and is active in late mitosis and though G1-phase of the cell cycle (Jaspersen et al. 1999; Robbins and Cross 2010; Sullivan and Morgan 2007). The oscillating activities of APC/C ligases during the cell cycle, as described in more detail below, are critical for not only controlling the timing of DNA replication but also for driving cell cycle progression and for guarding against genomic instability and cancer development (van Leuken et al. 2008; Nakayama and Nakayama 2006).

## 19.3 Regulation of Origin Licensing via the UPS

The control of eukaryotic DNA replication begins at the end of mitosis and through the G1-phase of the cell cycle, whereby the sequential binding of origin recognition complex proteins (ORCs) followed by CDC6 and CDT1 loads the replicative

helicase MCM2-7 onto replication origins: the establishment of pre-RCs (Fig. 19.3). Origin licensing is inhibited by CDK activity, which suppresses pre-RC formation either by promoting the phosphorylation-dependent nuclear exclusion of certain replication licensing factors (e.g., mammalian CDC6) or through the targeted proteolysis of a number pre-RC components though the UPS (Zhu et al. 2005). The degradation of SKP2 by the APC/$C^{CDH1}$ ligase is particularly important for the stabilization of key replication factors in late M- and G1-phases of eukaryotic cell cycles (Fig. 19.4). For example, human ORC1, the largest subunit of ORC, is stable in G1 because SKP2 is degraded by APC/ $C^{CDH1}$ in G1 but is specifically degraded in S-phase cells via the activity of the $SCF^{SKP2}$ ubiquitin ligase (Mendez et al. 2002; Tatsumi et al. 2003). *Drosophila* ORC1 is paradoxically ubiquitylated and degraded via the APC/$^{CFZr/CDH1}$ E3 ligase as cells exit mitosis and during G1 phase of the cell cycle, and this requires the non-conserved N-terminal domain of ORC1 (Araki et al. 2003, 2005; Narbonne-Reveau et al. 2008). Thus, ORC would have had to finish its licensing activity (MCM2-7 loading) before cells exit mitosis. The *Drosophila* ORC1 protein reappears in late G1 following induction of ORC1 RNA by E2F1 after the latter is activated by cyclin-CDK.

CDC6, another protein important for MCM2-7 loading, is also regulated extensively by the UPS. Yeast Cdc6 is degraded via an $SCF^{Cdc4}$-dependent proteolytic pathway, while the phosphorylation of mammalian CDC6 by increasing CDK activity in late G1 and in S-phase triggers its relocalization to the cytoplasm, thereby preventing origin licensing from occurring until CDK activity drops again in late mitosis (Aparicio et al. 1997; Tanaka et al. 1997; Fujita et al. 1999; Jiang et al. 1999; Petersen et al. 1999; Saha et al. 1998; Alexandrow and Hamlin 2004). Ubiquitin-mediated proteolysis of mammalian CDC6 in G1 also controls its abundance in G1 and in quiescent non-cycling cells. This is carried out by the APC/$C^{CDH1}$ ligase and is mediated through interaction between the destruction box and KEN box motifs of CDC6 and CDH1 (Petersen et al. 2000). This too suggests that as with fly ORC1 above, the degradation of mammalian CDC6 in G1 implies that the licensing activity must be completed before cells exit mitosis. The degradation of CDC6 by APC in G1 poses a special problem for cells entering the cell cycle from G0. It turns out that CDC6 is phosphorylated by cyclin E/CDK2 in cells entering the cell cycle from quiescence, and this inhibits APC/$C^{CDH1}$-mediated proteolysis of CDC6 (Mailand and Diffley 2005). This protection from ubiquitylation ensures that sufficient origins of replications are licensed before cells enter S-phase (Fig. 19.3). Once cells enter S-phase, CDC6 is targeted for ubiquitylation and degradation again, but this is mediated via the $CRL4^{CDT2}$ ligase (Clijsters and Wolthuis 2014). Late in the cell cycle (in G2 and early M), CDC6 is targeted for proteolysis via the $SCF^{Cyclin\ F}$ ligase (Walter et al. 2016).

The third protein important for loading MCM2-7 is CDT1. CDK kinase activity promotes the ubiquitylation and degradation of CDT1 via the $SCF^{SKP2}$ ligase at the G1/S transition of the cell cycle, and this, along with the targeted proteolysis of CDT1 in S-phase via the $CRL4^{CDT2}$ ligase (see below), ensures that CDT1 is not available for relicensing origins of replication in late G1 or in S-phase. Metazoans employ a second mechanism by which CDT1 is inactivated in S-phase through interaction with and inhibition by Geminin (Wohlschlegel et al. 2000; Tada et al.

2001). Geminin, however, is degraded in G1 through the activity of the APC/C E3 ligase (McGarry and Kirschner 1998), which is associated with low CDK activity. Origin licensing in cycling cells therefore can only proceed with low CDK activity (Figs. 19.3 and 19.4).

The mechanisms that maintain low CDK activity during late mitosis and in G1 are themselves under the control of the UPS. For example, the APC/C ligase is responsible for the decrease of mitotic CDK activity by promoting the ubiquitylation and degradation of the mitotic cyclins A and B (den Elzen and Pines 2001). This is first mediated through APC/$C^{CDC20}$, which promotes the degradation of cyclin A and cyclin B in prometaphase and metaphase, respectively. As cells proceed in metaphase, the degradation of cyclin B is mediated by APC/$C^{CDH1}$. APC/$C^{CDH1}$ additionally suppresses CDK2 activity in late M and in early G1 by promoting the degradation of the dual-specificity CDC25A phosphatase, which catalyzes the removal of inhibitory phosphorylations on CDK2 (Donzelli et al. 2002). Low CDK activity in late M and early G1 is also aided through the accumulation of elevated levels of the CDK inhibitors (CKI) p21 and p27, which can bind to and inhibit CDK2 in early G1 (Abbas and Dutta 2009). The protein levels of these two CDK inhibitors in G1 are under the control of the $SCF^{SKP2}$ ligase, which is active only in late G1.

## 19.4 Feedback Control of Origin Licensing Through the UPS

The ubiquitylation reactions involved in regulating origin licensing function in "self-regulating" networks with multiple feedbacks, whereby elevated CDK activity in mitosis turns on specific ubiquitylation reactions that feedback to decrease CDK activity during late mitosis and early G1 (Fig. 19.4). In both yeast and man, elevated G2 and mitotic CDK activity renders the APC/$C^{CDH1}$ inactive due to phosphorylation-dependent conformational changes in CDH1 precluding assembly of the active ligase. However, exit from mitosis requires inactivation of mitotic CDK, and this requires (a) targeted proteolysis of mitotic cyclins via the APC/$C^{CDH1}$ (or its homologue in yeast APC/$C^{Hct1}$) and (b) the stabilization of the CDK inhibitor p21 (or its homologue in yeast, Sic1) through degradation of CDC20. APC/$C^{CDC20}$ targets p21 for proteasomal activity during mitosis (Shirayama et al. 1999; Amador et al. 2007). APC/$C^{CDC20}$ ligase also promotes (through an unknown mechanism) the release of the yeast Cdc14 phosphatase from the nucleolus or the human CDC14A from centrosomes (Mocciaro et al. 2010; Kaiser et al. 2002; Chen et al. 2016; Shirayama et al. 1999; Bembenek and Yu 2001). In yeast, Cdc14 dephosphorylates and activates the Cdh1/Hct1 subunit, which competes with and targets Cdc20 for ubiquitylation and proteolysis, thereby assembling active APC/$C^{Cdh1/Hct1}$ and inactivating the APC/$C^{Cdc20}$ ligase (Jaspersen et al. 1999; Robbins and Cross 2010; Sullivan and Morgan 2007). Thus, the simultaneous activation of APC/$C^{CDH1}$ and inactivation of APC/$C^{CDC20}$ trigger CDK inactivation, culminating in exit from mitosis. As cells progress through early G1-phase of the cell cycle with low CDK activity, APC/$C^{CDH1}$ promotes the degradation of SKP2 (Bashir et al. 2004; Wei et al. 2004), and this prevents the premature formation of the

$SCF^{SKP2}$ complex and consequent destabilization of its ubiquitylation substrates p21 and p27, thus maintaining low CDK activity (Fig. 19.4).

At the G1/S transition, cyclin E-CDK2 phosphorylates CDH1 leading to $APC/C^{CDH1}$ inactivation (Cappell et al. 2016). In addition CDH1 binds to and is inhibited by the F-box protein and early mitotic inhibitor 1 (EMI1), marking a "point of no return" driving cells entry into S-phase (Cappell et al. 2016). As cells proceed through S-phase, the activity of both $APC/C^{CDH1}$ and $APC/C^{CDC20}$ is kept low through interaction between EMI1 with CDH1 or CDC20 (Cappell et al. 2016; Reimann et al. 2001). Inhibition of $APC/C^{CDC20}$ by EMI1 is essential for the stabilization of mitotic cyclins A and B, thereby ensuring the completion of DNA synthesis, prevention of DNA rereplication (see below), and progression through G2-phase (Di Fiore and Pines 2007).

As CDK1 activity builds up, the $APC/C^{CDC20}$ activity is increased in prometaphase through the coordinated sequential phosphorylation of the APC3 and APC1 subunits of the APC/C ligase by CDK1 and the docking of CDC20 onto the APC/C ligase (Fujimitsu et al. 2016). Yeast $APC/C^{Cdc20}$ is similarly activated by mitotic Clb-CDK activity, and this is critical for metaphase-anaphase transition (Rahal and Amon 2008). Elevated CDK1 activity in G2 and in early mitosis is enhanced by the targeted ubiquitylation and degradation of the CDK1 tyrosine kinase and inhibitor Wee1 via the activity of an SCF ligase, which utilizes the β-transducin repeat-containing protein 1 (βTRCP1) as substrate recognition subunits ($SCF^{\beta TRCP1}$) (Watanabe et al. 2004). Interestingly, this same E3 ligase also promotes the ubiquitylation and degradation of EMI1 (Guardavaccaro et al. 2003) and thus contributing to the rising activity of $APC/C^{CDC20}$ necessary for mitotic progression. This, along with the targeted ubiquitylation and degradation of EMI1 via the $SCF^{\beta TRCP}$ ligase, ensures optimal activity of $APC/C^{CDC20}$ ligase activity to drive mitotic progression. Thus, fluctuating CDK activity throughout the cell cycle, itself regulated through ubiquitin-mediated proteolysis, ensures that the ubiquitylation machinery required for the irreversible progression through the cell cycle is temporally coordinated with successive stabilization and destabilization of key drivers of cell cycle progression.

## 19.5 Inhibition of Origin Relicensing and DNA Rereplication Through the UPS

An extensive body of literature demonstrates a critical role for the UPS in restricting origin licensing to late M- and early G1-phase of the cell cycle and thus preventing aberrant relicensing and refiring of replication origins or rereplication (Fig. 19.3). As CDK activity builds up in G1 cells, the phosphorylation of the tumor suppressor protein pRb, first by cyclin D1/CDK4/6 and subsequently by cyclin E/CDK2, results in its dissociation from the E2F1 transcription factor, which transactivates Geminin and dozen other genes essential for S-phase progression (Wong et al. 2011). Increased Geminin transcription by E2F1 and increased Geminin protein stability through cyclin E/CDK2-dependent suppression of $APC/C^{CDH1}$ prevent origin

relicensing by directly binding CDT1, which sterically hinders its ability to recruit MCM2-7 complexes to origins of replication (Fig. 19.4). Second, and as cells proceed through S-phase, E2F1-dependent transcription of cyclin A coupled with enhanced protein stability of cyclin A due to inhibition of APC/$C^{CDC20}$ by EMI1 causes cyclin A-CDK2 activity to increase dramatically. Cyclin A/CDK2 phosphorylates CDT1 at a conserved N-terminal threonine residue (Thr-29) creating a phospho-degron that is specifically recognized by SKP2, which is itself stabilized due to inhibition of the APC/$C^{CDH1}$ activity in S-phase. The newly assembled $SCF^{SKP2}$ promotes phospho-CDT1 ubiquitylation and degradation (Li et al. 2003, 2004; Takeda et al. 2005). Thus, increased EMI1 levels at the G1/S transition and throughout S-phase with the consequent inhibition of APC/C ubiquitylation activity are critical for suppressing origin relicensing in mammalian cells, both by inhibiting CDT1 activity by Geminin and by promoting its proteolysis by the CDK-dependent and $SCF^{SKP2}$-mediated activity. Interestingly, alleviating Geminin-mediated suppression of CDT1, or inhibiting cyclin A-dependent proteolytic degradation of CDT1 via the $SCF^{SKP2}$ ligase alone, is sufficient to promote origin relicensing and rereplication in certain cancer cell types. This, however, is insufficient to induce rereplication in some other cancer cell types or in non-cancer cells (Zhu and Depamphilis 2009; Benamar et al. 2016; Machida and Dutta 2007). Inhibition of EMI1 (e.g., by short-interfering RNAs (siRNA)) on the other hand is sufficient to trigger robust rereplication both in cancer and non-cancer cells (Machida and Dutta 2007; Benamar et al. 2016). Thus, APC/C activity in S-phase inhibits origin licensing through the timely inhibition and degradation of CDT1 (Sivaprasad et al. 2007).

Whereas the $SCF^{SKP2}$ ligase promotes the degradation of cyclin A/CDK2-phosphorylated soluble CDT1 in S-phase, the ubiquitylation and degradation of chromatin-bound CDT1 in S-phase occur through a phosphorylation-independent mechanism that requires the activity of the $CRL4^{CDT2}$ ubiquitin ligase (Nishitani et al. 2006; Arias and Walter 2006; Senga et al. 2006; Jin et al. 2006). The overall composition and architecture of CRL4 ligases are very similar to CRL1 ligases (Fig. 19.2) (Angers et al. 2006; Higa and Zhang 2007). The core complex is composed of one of two E3 ubiquitin ligases (cullin 4A or cullin 4B), DDB1 (DNA damage-specific protein-1), which is a bridge protein analogous to the SKP1 subunit in CRL1, and functions to bridge one of many substrate receptors (also known as DCAFs; DDB1 and cullin 4 associated factors) to the cullin subunit, and through that to RBX1 or RBX2, required for the recruitment of E2 UBCs. DCAFs include at least 49 family members of WD motif-rich proteins that function to recruit substrates to the CRL4 ligase similar to the function of the F-box proteins in the CRL1 ligases (Angers et al. 2006; He et al. 2006; Higa et al. 2006; Jin et al. 2006). CRL4 is emerging as a master regulator of genome stability, and recent findings suggest that it orchestrates a variety of physiological processes, particularly those related to chromatin regulation and genomic stability (Jackson and Xiong 2009). The substrate adaptor CDT2 assembles with CRL4 to form a rather unique E3 ubiquitin ligase that does not recognize CDT1 (or several other ubiquitylation substrates) directly but specifically recognizes the substrate when it interacts with proliferating cell nuclear antigen (PCNA) (Arias and Walter 2006; Senga et al. 2006). PCNA,

the processivity factor for DNA polymerase δ, serves this role as an accessory factor for recognition by CDT2 only when PCNA is bound to chromatin, a condition that is established only in S-phase and following certain types of DNA damage (Havens and Walter 2011; Abbas and Dutta 2011; Abbas et al. 2013). Thus DNA damage is another stimulus that uses this pathway to degrade CDT1 and other replication factors (Higa et al. 2003).

The interaction between CDT1 and PCNA occurs through a specialized PCNA-interacting protein (PIP) box motif. This PIP box, commonly referred to as the "PIP degron," is a modified version of the PIP box motif utilized by many PCNA-interacting proteins; in that it contains, in addition to the canonical sequence (Q-X-X-(I/L/M)-X-X-(F/Y)-(F/Y)), conserved threonine and aspartic acid residues at positions 5 and 6, respectively, as well as a basic amino acid residue C-terminal of the PIP box (at position +4) and a second basic amino acid residue at position +3 or +5 (or both) (Abbas et al. 2010; Havens and Walter 2011; Michishita et al. 2011; Havens and Walter 2009). The importance of $CRL4^{CDT2}$-mediated ubiquitylation and degradation of CDT1 is manifested by the fact that cells from various organisms that are deficient in cullin 4, DDB1, or CDT2 exhibit rereplication and genomic instability reminiscent to that seen in cells overexpressing CDT1 (Jin et al. 2006; Lovejoy et al. 2006; Vaziri et al. 2003; Tatsumi et al. 2006; Zhong et al. 2003; Kim et al. 2008; Sansam et al. 2006), and this is associated with double-strand DNA breaks, rereplication, and activation of the ATM and ATR-dependent checkpoints (Zhu et al. 2004; Zhu and Dutta 2006), which can be partially suppressed by co-depletion of CDT1 (Lovejoy et al. 2006). The ubiquitylation-dependent proteolysis of CDT1 via $CRL4^{CDT2}$ is a feature of all eukaryotes except for budding yeast, where the CDT1-MCM complexes are exported to the cytoplasm (Tanaka and Diffley 2002; Devault et al. 2002). This is likely due to the fact that although budding yeast contains orthologs for cullin 4 and DDB1, they lack an identifiable ortholog for CDT2 (Zaidi et al. 2008).

Synchronization experiments in human cancer cells have shown that the levels of CDT1 protein begin to degrease as cells enter S-phase but re-accumulate late in S-phase and reach significantly higher levels in G2 (Abbas et al. 2010). In late S and in G2 cells, CDT1 is protected from $CRL4^{CDT2}$-mediated ubiquitylation and degradation through two phosphorylation-dependent and distinct mechanisms. The first one employs the phosphorylation of CDT1 by the stress-activated mitogen-activated protein kinases (MAPK) p38 and JNK precluding recognition by $CRL4^{CDT2}$ (Chandrasekaran et al. 2011). The second mechanism is dependent on CDK1-dependent phosphorylation of CDT1 preventing its recruitment to chromatin (Rizzardi et al. 2015). Just as the ubiquitylation and degradation of CDT1 in S-phase are critical for preventing rereplication and cell cycle progression, its re-accumulation in late S and in G2 is equally important for cell cycle progression, although its role in G2 is not fully understood (Rizzardi et al. 2015). Although the steady state level and protein half-life of CDT1 are clearly increased in late S and in G2 cells, a recent report suggests that its abundance particularly in G2-phase is also under the control of another E3 ubiquitin ligase, the $SCF^{FBXO31}$ ligase (Johansson et al. 2014). In this

study, the authors demonstrated that depletion of cancer cells of the substrate adaptor and putative tumor suppressor FBXO31 protein by siRNA induces low levels of DNA rereplication (7.6% vs. 4.6% in control cells), which is insufficient to inhibit growth (Johansson et al. 2014). It remains unclear however, how the stabilized CDT1 in these G2 cells gain access to chromatin to trigger rereplication in the presence of elevated CDK1 activity. An interesting possibility is that the loss of $SCF^{FBXO31}$ activity in tumors lacking FBXO31 or with FBXO31 inactivating mutations may result in a minute amount of rereplication that do not interfere with proliferation but are sufficient to induce gene amplification and/or genome instability exacerbating the tumorigenic phenotype (Green et al. 2010).

In addition to CDT1, the $CRL4^{CDT2}$ ligase promotes the ubiquitylation of several other proteins whose proteolysis in S-phase is critical for preventing origin relicensing and DNA rereplication. These include the CDK inhibitor p21, the histone H4 methyltransferase SET8 and CDC6 (Abbas et al. 2008, 2010; Nishitani et al. 2008; Kim et al. 2008; Tardat et al. 2010; Centore et al. 2010; Oda et al. 2010; Jorgensen et al. 2011; Clijsters and Wolthuis 2014). Ubiquitin-dependent degradation of p21 in S-phase via the $CRL4^{CDT2}$ ligase is critical for ensuring elevated CDK activity necessary for S-phase progression and for promoting DNA replication by freeing PCNA from inhibitory p21 (Abbas and Dutta 2009). Recent evidence also demonstrates that increased p21 stability downstream of $CRL4^{CDT2}$ inhibition stimulates rereplication in cancer cells, presumably due to suppression of CDK activity, a condition compatible with origin licensing (Kim et al. 2008; Benamar et al. 2016). Because these studies utilized the overexpression of a mutant p21 protein, which fails to interact with PCNA – a PIP degron mutant of p21 – these rereplicating cells are able to replicate DNA free from p21-mediated suppression of PCNA. Although ectopic expression of PIP degron mutant p21 induces only minor rereplication, the p21 protein is required for rereplication induced by CDT2 depletion (Benamar et al. 2016). Unlike the case for p21, the expression of PIP degron mutant of SET8, but not wild type SET8, induces robust rereplication, and this required the catalytic activity of this enigmatic methyltransferase (Abbas et al. 2010; Tardat et al. 2010). SET8 is also required for DNA rereplication in cells depleted of CDT2 (Benamar et al. 2016). SET8, also known as PR-SET7, is an enzyme that deposits a single methyl group on lysine 20 of nucleosomal histone 4 (H4K20me1) (Nishioka et al. 2002; Xiao et al. 2005). H4K20 can also be di- and tri-methylated (H4K20me2/3), but this is carried out by the SUV4-20H1 and SUV4-20H2 histone methyltransferases, which utilize H4K20me1 as substrate (Schotta et al. 2008). How SET8 promotes rereplication following $CRL4^{CDT2}$ inactivation is not entirely clear, but likely dependent on its ability to mono-methylate H4K20 at replication origins (Tardat et al. 2010). In fact, tethering SET8 to a specific genomic locus permitted loading of pre-RC proteins on chromatin and induced rereplication from that site. Interestingly, the rereplication phenotype associated with ectopic expression of PIP degron mutant SET8 was correlated with increased di- and tri-methylation of H4K20 with a concurrent reduction of H4K20me1 and required SUV4-20H1 and SUV4-20H2 (Abbas et al. 2010; Beck et al. 2012). Consistent with this, it was shown that the ability of SET8 to nucleate origins of replication is mediated through SUV4-20H1- and SUV4-20H2-dependent

recruitment of ORC1 and the ORC-associated protein (ORCA) proteins to chromatin and that both of these proteins are capable of binding H4K20me2/3 in vitro (Beck et al. 2012). Intriguingly, $CRL4^{CDT2}$-mediated proteolytic degradation of SET8 is critical for S→G2 cell cycle progression and for proper chromatin condensation, and inactivation of this pathway in cancer cells results not only in the induction of rereplication, but also in the repression of histone gene transcription and the consequent chromatin decondensation as well as repression of E2F1-driven gene expression (Abbas et al. 2010). These latter toxicities are likely mediated through enrichment of the repressive chromatin marks (H4K20me2/3) at the promoters of these genes (Abbas et al. 2010). Thus, cell viability is critically dependent on $CRL4^{CDT2}$-dependent downregulation of SET8 in S-phase for the maintenance of a stable epigenetic state. In M-phase SET8 protein is phosphorylated at Ser-29 by cyclin B/CDK1 from prometaphase to early anaphase, preceding the accumulation of H4K20me1 (Wu et al. 2010). This phosphorylation is reversed in late M-phase by the CDC14 phosphatase, which is activated by $APC/C^{CDC20}$. SET8 dephosphorylation renders the protein subject to proteolytic degradation via $APC/C^{CDH1}$, which facilitates mitotic progression (Wu et al. 2010).

As discussed above, CDC6 is another pre-RC component, which is targeted for ubiquitylation and degradation in S-phase via the $CRL4^{CDT2}$ ligase, and this is dependent on a conserved PIP degron contained near its N-terminus (Clijsters and Wolthuis 2014). However, although CDC6 depletion prevented rereplication induced by CDT2 depletion, it is not clear whether failure to degrade CDC6 via this ligase is sufficient to induce rereplication. As mentioned above, CDC6 is also ubiquitylated and degraded via the $SCF^{Cyclin\ F}$ ligase in G2- and in early M-phase, and this is also critical for preventing DNA rereplication as the depletion of cyclin F or the expression of a stable mutant form of CDC6 promotes rereplication and genome stability in cells lacking Geminin (Walter et al. 2016). Finally, in *Drosophila melanogaster*, $CRL4^{CDT2}$ also promotes the ubiquitylation and degradation of the transcription factor E2f1, and this is dependent on E2f1 interaction with PCNA through a PIP degron, which is not conserved in human E2F1 protein (Shibutani et al. 2008). From these studies, it is becoming abundantly clear that the $CRL4^{CDT2}$ ligase is a major inhibitor of origin licensing in S- and G2-phase of the cell cycle and does so through the degradation of key positive regulators of origin activity via their specialized interaction with chromatin-bound PCNA.

## 19.6 Targeting the Ubiquitylation Machinery Controlling DNA Replication Licensing for Therapeutic Gain

Deregulated origin licensing can have serious consequences. On one hand, failure of the ordered assembly of pre-RCs on sufficient origins of replications in late M and in G1 inhibits cell proliferation. This is demonstrated by the fact that targeted knockout or knockdown of many of the pre-RC components in various eukaryotes interferes

with viability, and thus some of these components, e.g., the MCM2-7 helicases, are considered promising chemotherapeutic targets in cancer (Lei 2005; Simon and Schwacha 2014). On the other hand, excessive origin licensing can result in DNA rereplication, which is deleterious to cells owing to the accumulation of replication intermediates and collapsed replication forks (Abbas et al. 2013). Substantial evidence shows that induction of rereplication in cancer cells through genetic manipulation (e.g., by depletion of Geminin, EMI1, or CDT2) results in growth inhibition through the accumulation of DNA damage, cell cycle checkpoint activation, and the induction of growth arrest and/or cell death (Zhu et al. 2005, 2004; Abbas and Dutta 2011; Abbas et al. 2013;Zhu and Dutta 2006). The first indication showing that pharmacological induction of rereplication is associated with inhibitory antiproliferation activity came from the accidental discovery that a small molecule, called MLN4924, designed to inhibit protein neddylation, inhibits the proliferation of human cancer cell lines and is associated with DNA rereplication (Soucy et al. 2009; Lin et al. 2010; Wei et al. 2012; Jazaeri et al. 2013). Neddylation of cullins is a posttranslational modification necessary for the activity of cullin-RING ligases. A small protein called NEDD8 is covalently attached to target protein substrate by an enzyme cascade system similar to ubiquitylation (Merlet et al. 2009). MLN4924 (also known as pevonedistat), currently in multiple clinical trials for hematologic (NCT00722488, NCT00911066) and solid (NCT01011530) malignancies, inhibits the NEDD8-activating enzyme (NAE), which catalyzes the first step in this enzymatic cascade, and its ability to suppress cullin neddylation and induction of rereplication suggested that the anticancer activity of this investigational drug is mediated through the suppression of $CRL4^{CDT2}$ and the stabilization of its ubiquitylation substrate CDT1 (Soucy et al. 2009). Subsequent studies, however, demonstrated that MLN4924, in addition to inhibiting all CRLs, inhibits a number of signal transduction pathways critical for cell proliferation, including the NFκB, AKT, and mTOR signal transduction pathways (Soucy et al. 2009; Lin et al. 2010; Milhollen et al. 2010, 2011; Gu et al. 2014; Godbersen et al. 2014;Li et al. 2014a, b). We have recently shown that the stabilization of the $CRL4^{CDT2}$ substrates SET8 and p21 is critical for the induction of DNA rereplication and senescence in melanoma cells treated with MLN4924 (Benamar et al. 2016). In particular, melanoma cells with hypomorphic expression of either of these two proteins are resistant to MLN4924-induced rereplication and senescence in culture, and, more importantly, tumor xenografts of these melanoma lines are refractory to inhibition by MLN4924 in nude mice. Interestingly, MLN4924 induces rereplication only in cancer cells but not in nonmalignant primary cells, suggesting that normal cells and tissues may be protected from the toxicity induced by this agent (Benamar et al. 2016). Thus, MLN4924 represents the first compound with anticancer activity, which selectively inhibits cancer cell proliferation through the induction of rereplication (Nawrocki et al. 2012; Zhao and Sun 2013; Tanaka et al. 2013; Zhao et al. 2014; Jiang and Jia 2015; Benamar et al. 2016).

Although specific inhibitors for $CRL4^{CDT2}$ ligases are yet to be developed, specific inhibitors of the other major E3 ligase controlling DNA replication, $SCF^{SKP2}$, already exist and exhibit potent anticancer activity in nude mice (Chan et al. 2013). However, because SKP2 promotes the degradation of many other proteins in

addition to the ones that impact DNA replication, it is unclear whether such antitumor activity is dependent on dysregulated DNA replication. An image-based high-throughput screening for compounds with the ability to induce rereplication selectively in cancer cells has identified other small molecules that selectively inhibit cancer cell proliferation (Zhu et al. 2011). However, whether these compounds exhibit antitumor activity or induce rereplication via targeting components or the UPS remains to be determined.

## 19.7 Regulation of DNA Synthesis via the UPS

Whereas the role of the UPS for controlling origin licensing and entry of cells into S-phase is well established, its role in regulating ongoing DNA replication and in coordinating the associated chromatin dynamics in unperturbed cells is just beginning to be appreciated. The latter is accomplished through the activity of histone proteins, histone chaperones, nucleosome-remodeling complexes, histone and DNA methylation-binding proteins, and chromatin-modifying enzymes. Together, these factors facilitate nucleosomal disassembly ahead of incoming replication forks and promote their reassembly following their passage. Many of these proteins and protein complexes are regulated through the UPS, and several excellent recent reviews describe these activities in details (Garcia-Rodriguez et al. 2016; Talbert and Henikoff 2017; Henikoff 2016; Almouzni and Cedar 2016). In this section, we focus on the role of the UPS in regulating replication initiation, progression, and termination.

A high-resolution proteome-wide mass spectrometry-based identification of ubiquitylated peptides in unperturbed cells identified many components of the replication machinery as substrates for ubiquitin modification, although in some cases this is not associated with proteasomal degradation (Wagner et al. 2011). These include components of various complexes regulating DNA replication, such as the MCM2-7 helicase, GINS, and replication factor C (RFC) clamp loader complexes. In addition, almost all DNA polymerases and their associated factors had detectable ubiquitylated peptides identified in this screen. Although the individual components coordinating these ubiquitylation events and their functional significance remain to be defined, this study suggests that ubiquitylation plays an important role in regulating DNA replication and its fidelity. Not all of these ubiquitylation events are associated with proteolysis of the DNA replication factors. For example, the two subunits of the mammalian DNA polymerase δ, p66 and p12, undergo non-proteolytic ubiquitylation during S-phase that is thought to regulate protein-protein interactions within the polymerase complex or with other replication factors (Liu and Warbrick 2006). Following DNA damage, the p12 subunit is degraded through a PCNA-dependent ubiquitylation via the $CRL4^{CDT2}$ ligase, and this is critical for inhibiting fork progression (Terai et al. 2013). Similarly, the *S. pombe* Pol2, the catalytic subunit of polymerase ε, which is responsible for leading strand synthesis, undergoes ubiquitin-dependent proteolysis, and this is mediated via the $SCF^{Pof3}$ ligase (Roseaulin et al. 2013). Because the catalytic subunit of polymerase δ

(responsible for lagging strand synthesis) remains stable, despite undergoing ubiquitylation, the results suggest that DNA synthesis of the leading strand requires a "fresh" supply of DNA polymerase, whereas the synthesis of the discontinuous lagging strand does not (Roseaulin et al. 2013). This example highlights the complexity in the mechanisms regulating the abundance and activity of replication factors through the UPS in response to various stimuli. Minichromosome maintenance protein 10 (MCM10) is another important DNA replication protein, which undergoes non-proteolytic ubiquitylation (Das-Bradoo et al. 2006). MCM10 is dispensable for the assembly of the MCM2-7 replicative helicase but facilitates strand separation during the early stages of replication initiation (Kanke et al. 2012; van Deursen et al. 2012; Thu and Bielinsky 2013). In fission yeast, Mcm10/Cdc23 was shown to function after pre-RC assembly and facilitates Cdc45 chromatin binding (Gregan et al. 2003). The budding yeast Mcm10 protein was also suggested to play a role in replication elongation through interacting with and stabilization of the catalytic subunit of DNA polymerase α (POL1) (Ricke and Bielinsky 2004). Similarly, mammalian MCM10 interacts with and stabilizes the catalytic subunit of DNA polymerase α (p180) and is essential for efficient DNA synthesis (Chattopadhyay and Bielinsky 2007; Zhu et al. 2007). In G1- and in S-phase, MCM10 is monoubiquitylated at two residues, and this promotes its interaction with PCNA, which may be important for the release of polymerase α and the recruitment of polymerase δ necessary for the extension of Okazaki fragments (Das-Bradoo et al. 2006; Thu and Bielinsky 2013). The identity of the E3 ligase responsible for MCM10 monoubiquitylation is currently unknown. MCM10 is also subject to ubiquitylation-dependent proteolysis via the $CRL4^{VBRBP}$ E3 ligase, both in unperturbed cells and following exposure to UV, although the biological significance of this regulation remains elusive (Kaur et al. 2012; Romani et al. 2015).

Perhaps the most prominent example where the regulation of DNA replication involves coordination between proteolytic and non-proteolytic ubiquitylation machineries concerns the enigmatic PCNA protein (Fig. 19.5). PCNA is a homotrimeric ring-shaped protein complex that functions as a platform for coordinating the activity of many proteins involved in DNA replication, repair, and chromatin-related transactions (Ulrich and Takahashi 2013; Choe and Moldovan 2017). PCNA has long been shown to undergo DNA damage-induced monoubiquitylation at a conserved lysine residue (Lys-164), and this is critical to recruit translesion Y-family DNA polymerases for bypassing replication-stalling DNA lesions by translesion DNA synthesis (TLS) (Yang et al. 2013). This recruitment is dependent on the enhanced interaction of TLS polymerases with the modified PCNA through their ubiquitin-binding domains (Bienko et al. 2005; Plosky et al. 2006). PCNA monoubiquitylation at Lys-164 is catalyzed by the Rad18 E3 ligase and mediated by the E2 Rad6. The monoubiquitin moiety can be converted to Lys-63-linked polyubiquitylation by a heterodimeric E2, Ubc13-Mms2, to initiate an error-free pathway of repair, template switching, which uses the newly replicated sister chromatid as a template for replication (Hedglin and Benkovic 2015; Branzei 2011). Rad18, however, is not the only E3 ligase that can monoubiquitylate PCNA at Lys-64 as this modification can still be detected in the absence of functional Rad18 (Simpson et al.

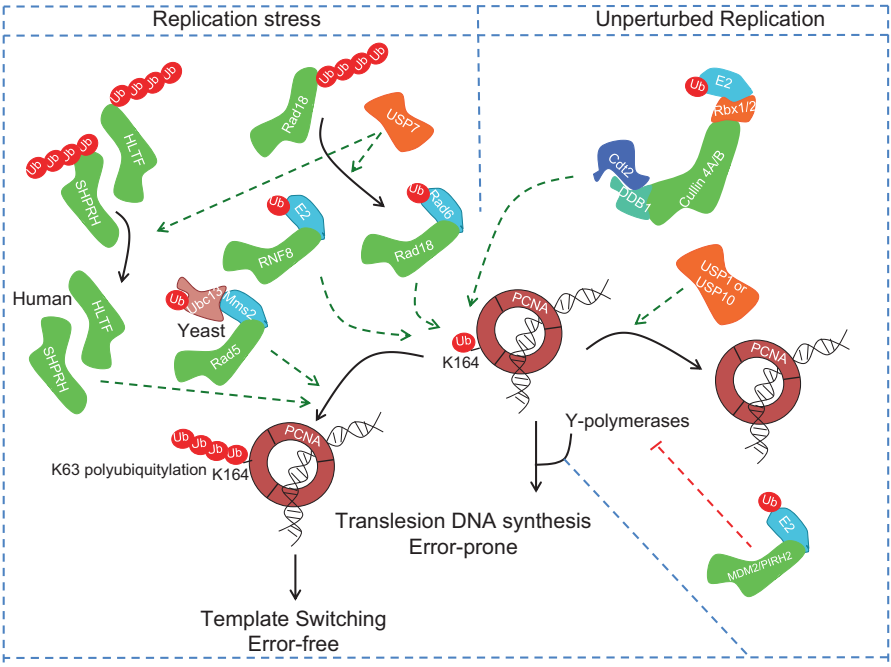


**Fig. 19.5** Control of PCNA ubiquitylation and its role in normal DNA replication and following replication stress. The trimeric PCNA is subject to monoubiquitylation via the activity of multiple E3 ligases both in unperturbed cells (via CRL4-CDT2 E3 ligase) and following replication stresses via Rad18/Rad5 E2/E3 or RNF8 E3 ligase. PCNA monoubiquitylation is essential for the recruitment of Y-family DNA polymerases, such as DNA polymerase eta, and this is critical for translesion DNA synthesis (TLS). This is reversed by the action of the deubiquitylating enzymes USP1 and USP7 (Orange). The monoubiquitin on PCNA at Lys-164 (K164) can be extended by the Rad5 E3 ligase and heterodimeric Ubc13/Mms2 in yeast or through the activity of the HLTF and SHPRH E3 ligases in mammals to form K63-linked polyubiquitin chain, and this promotes template switching and minimizes mutagenesis due to excessive activity of TLS polymerases. Other E3 ligases, MDM2 and PRH2, limit the activity of TLS polymerases to limit error-prone TLS either via the targeted proteolysis of TLS polymerase eta (MDM2) or ubiquitin-dependent suppression of its activity (PRH2)

2006). Consistent with this, two other E3 ligases, RNF8 and $CRL4^{CDT2}$, were shown to promote PCNA monoubiquitylation (Zhang et al. 2008; Terai et al. 2010). Intriguingly, PCNA undergoes monoubiquitylation in normal replicating cells and without exposure to external stresses (Frampton et al. 2006; Leach and Michael 2005; Terai et al. 2010). Although the role of this ubiquitylation in unperturbed DNA replication is not clear, PCNA monoubiquitylation appears to be important for efficient DNA replication and for promoting TLS that may be necessary to cope with replication-associated stress (Leach and Michael 2005; Terai et al. 2010).

In mammalian cells, two E3 ubiquitin ligases, SNF2 histone linker plant homeodomain RING helicase (SHPRH) and helicase-like transcription factor (HLTF), promote the Lys-63-linked polyubiquitylation at Lys-164 to suppress

PCNA-dependent TLS and mutagenesis (Unk et al. 2008; Motegi et al. 2008). This latter activity is aided by the activity of the DUB USP7, which deubiquitylates and stabilizes HLTF and Rad18, promoting template switching through interaction between the unubiquitylated Rad18 and HLTF (Qing et al. 2011; Zeman et al. 2014). In response to replication stress, USP7 also deubiquitylates and stabilizes both Rad18 (so that it can monoubiquitylate PCNA) and the TLS polymerase, polymerase eta (POL-η), and this facilitates the bypass of lesions through the error-prone TLS pathway (Qian et al. 2015; Zlatanou et al. 2016). Another E3 ubiquitin ligase, the TNF receptor-associated factor (TRAF)-interacting protein (TRIP), and its homologue in *Drosophila*, "no pole" or NOPO, promotes the Lys-63-polyubiquitylation of POL-η, and this stimulates TLS by promoting the localization of POL-η to nuclear foci (Wallace et al. 2014). Intriguingly, Rad18 itself undergoes monoubiquitylation, and this prevents its interaction with SHPRH or HLTF and at the same time suppresses its ability to promote PCNA monoubiquitylation and TLS (Lin et al. 2011; Moldovan and D'Andrea 2011; Zeman et al. 2014). Kashiwaba et al. have additionally shown that USP7 can deubiquitylate PCNA, but this is not coupled to DNA replication and likely plays a role in DNA repair (Kashiwaba et al. 2015).

Just as it is important to initiate TLS in the face of replication-associated fork stalling, it is critical that the TLS activity is restricted to prevent increased mutations caused by low-fidelity polymerases. Two potential mechanisms ensure that TLS activity is restrained in cells (Fig. 19.5). The first involves the ubiquitin-dependent inhibition of TLS polymerases via both proteolytic and non-proteolytic ubiquitylation of TLS polymerases. For example, POL-η undergoes ubiquitylation-dependent proteolysis via the E3 ligase activity of MDM2 (Jung et al. 2012). On the other hand, the E3 ligase PIRH2 monoubiquitylates POL-η to suppress its interaction with monoubiquitylated PCNA (Jung et al. 2010, 2011). In both cases, this results in suppression of POL-η-dependent TLS.

Homologues of the TLS polymerases in yeast also undergo ubiquitin-dependent proteolysis. For example, both Rad30 (the S. *cerevisiae* homologue of POL-η) and Rev1 undergo cell cycle-dependent proteolysis to limit mutagenic activity, and in the case of Rad30, this is mediated via the SCF ubiquitin ligase with the F-box protein Ufo1 (Waters and Walker 2006; Skoneczna et al. 2007; Plachta et al. 2015). The second, and perhaps more important, mechanism restraining TLS activity is mediated through deubiquitylation of monoubiquitylated PCNA via the USP1 isopeptidase (Huang et al. 2006). An interesting study has recently shown that the deubiquitylation of mammalian PCNA, at least in the context of DNA damage induced by ultraviolet irradiation (UV), is aided by the deubiquitylating activity of USP10. In response to UV irradiation, USP10 is recruited to PCNA following its ISGylation by the ubiquitin-like modifier ISG15 (Park et al. 2014). Following deubiquitylation by USP10 and the release of POL-η, PCNA is de-ISGylated by UBP43, which renders PCNA available for reloading the replicative DNA polymerases and the resumption of DNA replication. Surprisingly, inactivation of Ubp10, the deubiquitinase for PCNA monoubiquitylation in yeast, does not appear to cause increased mutagenesis, arguing that other mechanisms contribute to inhibiting TLS-induced mutagenesis in yeast (Gallego-Sanchez et al. 2012).

In addition to PCNA monoubiquitylation at Lys-164, large-scale studies have identified additional lysine residues on PCNA that are ubiquitylated, but the E2/E3 enzymes carrying these ubiquitylation events and their biological functions are yet to be determined (McIntyre and Woodgate 2015). PCNA is also subject to extensive regulation through the ubiquitin-related SUMOylation pathway, and this is critical to suppress spontaneous and DNA damage-induced homologous recombination and help unload PCNA during DNA replication (Zilio et al. 2017; Garcia-Rodriguez et al. 2016). In this case, ubiquitylation and SUMOylation of the same protein and at the same residue appear to be tightly coordinating for ensuring optimal activity for this important multifunctional protein.

Termination of DNA replication is also regulated via the UPS (Fig. 19.6). Replication termination is initiated upon the convergence of replications forks, and this is triggered by the disassembly of the replicative helicase complex CMG (CDC45-MCM-GINS) DNA. The mechanism underlying replisome disassembly is

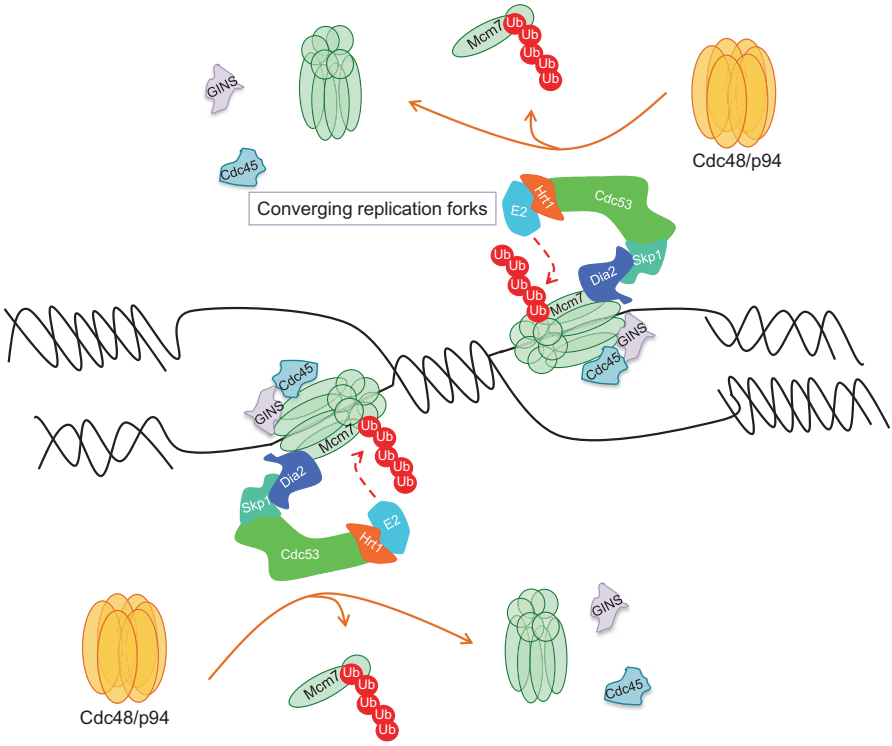


**Fig. 19.6** Control of replication termination *in S. cerevisiae* via the activity of the $SCF^{Dia2}$ E3 ubiquitin ligase. A model depicting the K-48-polyubiquitylation of Mcm7 by the $SCF^{Dia2}$ E3 ubiquitin ligase in *S. cerevisiae* ($CRL2^{Lrr1}$ E3 ubiquitin ligase in metazoans) at the sites of converging replication forks, followed by its extraction from the MCM2-7 helicase by the activity of the p97 chaperon, leading to the replisome disassembly and replication termination. GINS and CDC25, components of the CMG helicase complex, are also shown

not entirely clear, but two recent studies in yeast and in *Xenopus laevis* egg extracts provided the first insights into how replication termination is achieved (Maric et al. 2014; Moreno et al. 2014). Both studies have shown that the disassembly of the CMG complex is dependent on Lys-48-linked polyubiquitylation of the MCM7 subunit of the MCM2-7 complex, and this triggers its recognition by the ubiquitin-dependent segregase Cdc48 (also known as p97), a AAA+ adenosine triphosphatase (ATPase) that forms a hexameric ring, which undergoes conformational changes upon ATP hydrolysis to drive protein unfolding (Maric et al. 2014; Moreno et al. 2014; Barthelme et al. 2014). This results in the translocation of the MCM7 subunit through the p97 hexameric ring and the opening of the MCM2-7 ring allowing DNA exit and replication termination (Bell 2014; Lengronne and Pasero 2014). Mcm7 polyubiquitylation in *S. cerevisiae* is catalyzed by the activity of the SCF E3 ligase and the F-box protein Dia2, which has been previously shown to localize to the replisome (Maric et al. 2014; Moreno et al. 2014; Morohashi et al. 2009). Although $SCF^{Dia2}$ inactivation prevents CMG disassembly and results in its retention on chromatin causing replication defects consistent with inhibition of replication termination, blocking Mcm7 proteolysis does not do so, arguing that Mcm7 polyubiquitylation is most important for its extraction from the Mcm2-7 complex and unloading of the Mcm2-7 ring (Maric et al. 2014; Moreno et al. 2014). The role of MCM7 polyubiquitylation in replisome disassembly and replication termination is conserved in higher eukaryotes, but this was recently shown to be mediated through the activity of the $CRL2^{Lrr1}$ ubiquitin ligase (Dewar et al. 2017).

## 19.8 Summary and Concluding Remarks

The last few decades have witnessed an extensive understanding of the role of the UPS in regulating almost all aspect of cell physiology, and in many cases, deregulation of key regulators of this ubiquitous regulatory system contributes to disease development. This regulation is even more important for cellular processes that are dependent on the efficient temporal and spatial interplay between many factors and is subject to endogenous and external perturbations, as is the case for the regulation of eukaryotic DNA replication. While many factors influencing DNA replication undergo extensive regulation through the UPS leading to proteasomal degradation, non-proteolytic ubiquitin signaling coordinating protein-protein interactions is being subjected to increasing scrutiny. Various components of the UPS operate with extensive crosstalk and feedback mechanisms as well as interactions with other posttranslational modifications, and together, they provide a complex network of protein-protein communications to control the specificity and robustness of DNA replication and cell cycle control. When faced with stresses that impact the replication machinery and threaten the fidelity of DNA replication, for example, when replication forks encounter replication-stalling lesions, cells must be able to quickly respond and adopt the appropriate measures to cope with these stresses. The dynamic and reversible activities associated with the UPS allow the cell to adjust to

these insults. Even in the absence of these stresses, the non-proteolytic as well as proteolytic mechanisms ensure that DNA replication proceed unperturbed with exquisite accuracy. The diversity in the specificity of the various E2-E3 pairs in conjugating various polymers of ubiquitin chains on target substrates adds another layer of complexity that will require more effort to fully understand and appreciate. Key regulators, such as the APC/C, $CRL4^{CDT2}$, and SCF ubiquitin ligases, and the USP7 and USP1 deubiquitinases, dominate the scene and regulate the steady state levels or activities of many proteins controlling DNA replication and cell cycle progression. They are also important for regulating appropriate cellular responses to perturbations by activating cellular checkpoints. It is therefore understandable that significant research is targeting some of these key regulators for therapeutic gain, but this will require a greater understanding of the molecular and biochemical details and structural information of these regulators. Proteome-wide studies suggest that we are yet to understand the functional significance of new ubiquitin modifications of many replication-associated factors. The development of novel techniques for genome editing and for gain- or loss-of-function screens as well as the development of new protocols for enriching for and identifying various ubiquitin modifications will no doubt be great assets for making these discoveries.

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# Chapter 20
# Coordinating Replication with Transcription

**Yathish Jagadheesh Achar and Marco Foiani**

**Abstract** DNA topological transitions occur when replication forks encounter other DNA transactions such as transcription. Failure in resolving such conflicts leads to generation of aberrant replication and transcription intermediates that might have adverse effects on genome stability. Cells have evolved numerous surveillance mechanisms to avoid, tolerate, and resolve such replication-transcription conflicts. Defects or non-coordination in such cellular mechanisms might have catastrophic effect on cell viability. In this chapter, we review consequences of replication encounters with transcription and its associated events, topological challenges, and how these inevitable conflicts alter the genome structure and functions.

**Keywords** DNA replication • Transcription • Topology • Topoisomerase • Chromatin structure • Genome instability • RNA:DNA hybrids

## 20.1 Introduction

Every cell, whether it is a prokaryotic or eukaryotic cell, has to duplicate its genome prior to its division. To ensure a faithful completion of genome duplication, cells have to protect the replication apparatus from numerous intrinsic and extrinsic factors that might hinder its progression. Along with extrinsic sources, which might alter chromatin integrity, several natural obstacles like DNA lesions, DNA-protein complexes, and alternative DNA structures also hinder the replication process. One such major natural hindrance for replication progression is transcription.

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S-phase in eukaryotic cells is the most vulnerable period during the cell cycle as replication and transcription coexist in space and in time. Collision between the two processes is inevitable as both compete for the same template to synthesize nucleic acid chains. In principle, replication and transcription are two independent processes, which are expected to perform their respective tasks that are to synthesize new DNA or RNA molecules using the complimentary DNA strand. Both processes are highly processive, strictly follow 5–3′ polarity, and are carried out by brigades of respective polymerases along with a plethora protein partners and other cofactors. These two process need to be coordinated as both must be completed with high fidelity to preserve genetic information and genome stability.

It has been known for many years that transcription could interfere with genome integrity. The very first indication that transcription could passively challenge genome stability came from bacterial studies where a reporter gene showed higher mutational rates when highly transcribed (Herman and Dworkin 1971; Savic and Kanazir 1972). Later, direct pieces of evidence for the effect of transcription on genome integrity were reported in several organisms including bacteria (Dul and Drexler 1988; Vilette et al. 1992), yeast (Keil and Roeder 1984; Brill et al. 1987; Thomas and Rothstein 1989), and mammalian cells (Nickoloff 1992). Along with causing spontaneous mutagenesis (Datta and Jinks-Robertson 1995), transcription-associated recombination (TAR) was also discovered in yeast (Keil and Roeder 1984; Voelkel-Meiman et al. 1987). Interestingly, TAR is known to depend on replication as proven in yeast (Prado and Aguilera 2005) and mammalian cells (Gottipati et al. 2008). These events eventually lead to one of the important questions in molecular biology "how do replication forks contend transcription complexes?" Research in the last 25 years has provided evidence that conflicts between transcription and replication constitutes major natural source of threat on genome stability. Current knowledge of both replication and transcription suggests that the mechanisms and factors involved in resolving conflict are more complex than previously foreseen.

How does a process involved in gene expression create problems for the gene duplication process? The answer lies in the speed at which these two processes are carried out. DNA replication complexes move faster than transcription complexes; the two complexes must inevitably collide considering that they occupy the same template. Cells could solve this problem by replisome-induced dissociation of transcription complex from the template. However in physiological conditions, it is not an efficient solution, as the transcription process is complex and associates with several factors and events. A replication fork has to uncouple these factors before dissociating the RNA polymerases (RNAPs) from the template. Along with hindering replication movement by occupying the DNA template, transcription is accompanied by co-transcriptional processes, such as RNA maturation and export. During transcription elongation, RNAPs can pause at regulatory sequences and DNA damage sites (Selby et al. 1997; Landick 2006). A halted RNAP complex can give rise to consequent arrays of stalled RNAP complexes, as stalled transcription units can

also be barriers for the following RNAPs, which are transcribing the same template (Artsimovitch and Landick 2000). In highly transcribing genes, repeated loading of the RNAP at promoters lead to the piling of RNAP, creating a potentially formidable obstacle to replication and also for damage repair. To rescue such a scenario, the RNAP complex translocates backward along the template (Park et al. 2002). Such type of reverse translocation is known to occur spontaneously at the pausing sites displacing the 3′ end of transcripts from active sites. This results in an extremely stable, inactive complex, which can form potential replicative barrier and give rise to a tandem array of barriers.

Along with occupying templates, transcription also induces additional chromatin structural changes, which could also challenge replication fork progression. Eukaryotic cells must lessen the secondary effects enforced by each other through topological perturbations. By unwinding the DNA duplex, replication and transcription generate topological alterations that are solved by specialized enzymes, the DNA topoisomerases. Replication can accumulate hemicatenane-like structures behind the fork in response to DNA damage (Lopes et al. 2001; Liberi et al. 2005), whereas negative supercoiling is induced due to transcription-mediated formation of Z-DNA (Ha et al. 2005). These alternative DNA transitions can be hazardous structures if they occur out of their natural context by having a negative impact on transcription elongation and also can be endangering for replication fork integrity (Aguilera and Garcia-Muse 2012).

Interrelation between replication and transcription became evident when a new set of genes, known to be involved in transcription and RNA processing, was discovered, and their subsequent mutation was shown to cause an increase in DNA damage, higher mutation rates, and hyper-recombination (Wahba et al. 2011). Recent observations show that mutations in DNA topoisomerases (El Hage et al. 2010), RNA-elongating proteins (Senataxin 1) (Mischo et al. 2011), RNA export complexes (THO/TREX complex) (Gomez-Gonzalez et al. 2009), and splicing factors (ASF/SF2) (Li and Manley 2005) have been linked to genome instability. Slight alterations in the functionality of these transcription-associated events might challenge the integrity of the genome via a common mechanism that would induce the formation of persistent, transcription-associated RNA:DNA hybrids (Aguilera 2002). Formation of RNA:DNA hybrids out of their natural context can lead to distinctive DNA structures including R-Loops (nascent RNA bound to one of the DNA strands by displacing the other strand) and triple helix DNA termed as H-DNA (RNA bound to duplex DNA via Hoogsteen base pairing with separating strands).

Cells have evolved to counteract the conflict between replication and transcription. Checkpoint genes, which are often mutated in cancer cells, might play a role in sensing the physiological stress caused by topological intermediates. On the other hand, oncogenes that alter the transcriptional programs are known to cause replication stress and checkpoint activation (Bermejo et al. 2012). Possible consequences, topological challenges, and chromatin structure alterations arising due to the replication-transcription conflicts are outlined in the following sections.

## 20.2 Replication and Transcription Collision

Initial attempts to characterize the collision between transcription and replication were examined in vitro, using T4 bacteriophage replication apparatus and purified *E.coli* RNAP (Bedinger et al. 1983). Several subsequent efforts were made to understand the consequences of replication-transcription collision using in vitro assays in bacteria, yeast, and mammalian cells. The existence of collisions between the major molecular processes was raised mainly in bacteria due to the differences of the speed of replication fork progression and transcription elongation. The speed of replication fork progression in *Escherichia coli* is ~700 bp per second (Hirose et al. 1983; Mok and Marians 1987; Breier et al. 2005), while the elongation rate of RNAP is ~50 nt per second (Gotta et al. 1991; Epshtein et al. 2003; Proshkin et al. 2010). Since replication is 12-fold faster than transcription, collision between the two processes is unavoidable in bacteria where both processes continue throughout the cell cycle. First in vivo evidence of replication-transcription collision came from *E. coli* (French 1992) by placing the rrnB ribosomal operon on either side of the inducible replication origin. Upon inducing replication, EM visualization revealed that replication fork that is head-on direction with the rrnB operon was slower compared to the codirectional fork. Since then, several studies in bacteria and other prokaryotes have shown transcription complexes as natural impediments to replication (Merrikh et al. 2012).

Since prokaryotes with circular genome cannot completely avoid the replication-transcription collision, a simple solution would be the replisome-induced dissociation of RNAPs from the template. However, pieces of evidence linking genome stability to transcription-induced replication blockage suggest it is not an efficient solution in vivo (Brewer 1988; Rudolph et al. 2007). To avoid such scenarios, bacterial genomes have evolved a simple strategy to reduce the effect of transcription-replication collision by placing highly transcribed and essential genes on codirectional orientation relative to replication fork movement. This kind of gene arrangement is conserved in most bacteria studied (Guy and Roten 2004). These codirectional arrangements are known to reduce the effect of conflict in lower organisms as reversing the orientation has shown to impact the progression of replication and genome stability (Srivatsan et al. 2010).

Unlike prokaryotes, eukaryotic cells have the luxury of restricting genome duplication to one phase of the cell cycle, S-phase, leaving the other phases free of replication so that transcription and other chromatin-associated events can occur in an uninterruptible way. However, since transcription in a eukaryotic cell cannot be completely turned off in S-phase, it is believed that cells can spatially separate replication through chromatin domain architecture (Wansink et al. 1994; Wei et al. 1998). Eukaryotic cells also initiate replication from hundreds of origins, which will initiate at different time points, some early and some very late during S-phases (Wyrick et al. 2001). This will allow cells to temporally separate transcription and replication events through inverse correlation. Early replicating genes are more strongly transcribed late in S-phase, and late replicating genes are preferably transcribed in early S-phase (Meryet-Figuiere et al. 2014). Moreover, interference between replication and transcription in eukaryotes debated for long due to compa-

rable speeds at which both processes operate. Unlike in prokaryotes, in eukaryotes replication and transcription machineries operate at similar speeds of 17–33 nt $S^{-1}$ and 17–72 nt $S^{-1}$, respectively (Raghuraman et al. 2001; Hiratani et al. 2008; Spiesser et al. 2010). First evidence for the occurrence of collision in eukaryotes came from the discovery of replication fork barrier (RFB) at the 3′ end of the *S. cerevisiae* 35S rRNA genes and tRNA transcription units (Brewer and Fangman 1988; Linskens and Huberman 1988). Later, transcription-dependent pausing sites were also mapped in plants (Lopez-Estrano et al. 1999), *Xenopus laevis* (Maric et al. 1999), *Tetrahymena thermophila* (Zhang et al. 1997), and mammals (Lopez-estrano et al. 1998). Replication barriers, which are observed in cases of highly expressed and specialized units such as rDNA and tRNA, are not normally observed at the RNA pol-II genes. In such protein coding regions of the genome, transcription and replication seem to be separated temporally, by inversely coordinating the transcription with replication timing (Gilbert 2002; Hiratani et al. 2009; Rivera-Mulia and Gilbert 2016).

Temporal and spatial segregation of transcription with replication in S-phase of eukaryotic cells still cannot rule out the possibility of interference between the two processes. Recent studies show that eukaryotic replication and transcription units do arise in certain situations and at specific genomic regions. Moreover, recent genome-wide transcription data reveal that the majority of the genome is transcribed well beyond boundaries of annotated gene bodies. This phenomenon was observed in several eukaryotic cells like *S. cerevisiae* (David et al. 2006), mammalian (Carninci et al. 2005), *S. pombe* (Dutrow et al. 2008), rice (Li et al. 2006), and *D. melanogaster* (Stolc et al. 2004). This only shows that transcription and replication collisions are more pervasive than previously anticipated.

Other than arrangement of transcription units, collision in prokaryotes and eukaryotes also differs in the way it occurs. In bacteria such as *E. coli*, the replicative helicase responsible for separation of the two strands is DnaB that acts in a 5–3′ direction. During replication fork progression, DnaB is associated with the lagging arm of the replication fork (Fig. 20.1a). In eukaryotes, the CMG helicase takes care

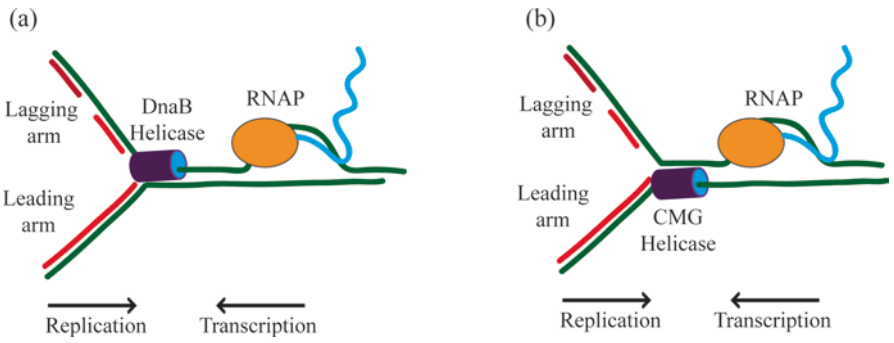


**Fig. 20.1** Difference in polarity of prokaryotic and eukaryotic DNA replicative helicases. (**a**) Prokaryotic helicase like DnaB has 5–3′ polarity hence travels on lagging arm. (**b**) Eukaryotic helicase like CMG has 3–5′ polarity hence travels on leading arm of replication fork

of strand separation and is associated with the leading arm of the replication fork as it has 3–5′ helicase activity. Since RNAP translocates in a 3–5′ direction on the transcribed strand, a head on collision might interfere directly with the replicative helicase in prokaryotes. However, in eukaryotes, same head on collision will not interfere with replicative helicase as RNAP and helicase translocate on different strands (Fig. 20.1b).

Replication speed depends on the orientation of the gene with respect to the fork movement when replicating across a gene. However, no particular bias was observed with respect to the dislodgement of RNAPs during collision (French 1992). In contrast, in vitro studies showed that RNAP resumes transcription after replication passes in either orientation (Liu et al. 1993; Liu and Alberts 1995; Elias-Arnanz and Salas 1997, 1999). Based on the influence of directionality and impact, replication-transcription collision is grouped into head-on and codirectional collisions. Differences between these two collisions and possible consequences are discussed in the below section.

## 20.3 Head-On Versus Codirectional Collision

Since both replication and transcription processes have the same polarity for synthesis of new nucleic acid chains, the template strand used for transcription determines the orientation of a gene with respect to replication. A head-on collision will take place when transcription from RNAP II uses the lagging strand as a template (Fig. 20.2b), wherein codirectional uses the leading strand (Fig. 20.2a). Comparison between the two types of collisions rose due to the difference in consequences of collision. Regardless of whether collision occurs in a codirectional or a head-on orientation, replication fork cannot progress past an elongating RNAP, so their encounter causes replication fork pausing (Azvolinsky et al. 2009). Earlier work,

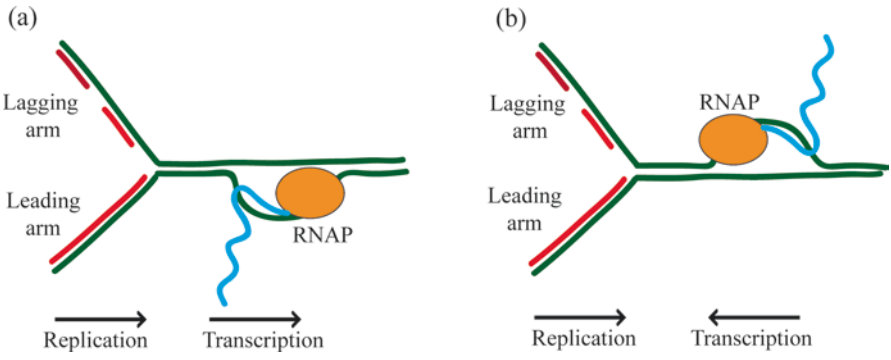


**Fig. 20.2** Comparison of head-on and codirectional collisions between replication fork and transcription unit. (**a**) In codirectional collision, RNAP uses leading arm as a template. (**b**) In head-on collision, RNAP uses lagging arm as a template

particularly in bacteria, suggested that head-on collisions were more deleterious causing replication to stall, but codirectional collisions were generally not detected in vivo and hence thought to be less harmful (French 1992; Mirkin and Mirkin 2005; Wang et al. 2007; Srivatsan et al. 2010). However, recent findings contradicted this notion and indicated that both types of collisions can disrupt replication progression in vivo (Dutta et al. 2011; Merrikh et al. 2011).

Fork stalling, a major outcome of head-on collision, can lead to accumulation of alternative DNA structures such as DNA knots (Olavarrieta et al. 2002). In vitro experiments have shown that fork restart can only take place after the displacement of RNAP machinery from the DNA template during head-on conflicts (Pomerantz and O'Donnell 2008). Consistent with this idea, yeast genetic experiments also suggest that replication forks are blocked more predominantly in head-on collisions. When head-on encounters were promoted in yeast using an artificial system, replication pause sites along with hyper-recombination events were observed. On the contrary, codirectional collisions did not lead to replication pausing or high levels of recombination (Prado and Aguilera 2005). Moreover, a codirectional collision barely affects fork progression in vitro (Liu and Alberts 1995; Pomerantz and O'Donnell 2008), unless the elongating RNAP is stalled (Elias-Arnanz and Salas 1997; Dutta et al. 2011). Occasionally, the replication machinery can use mRNAs to reprime DNA synthesis after codirectional collision with RNAP (Kogoma 1997; Pomerantz and O'Donnell 2008), probably supporting the notion that codirectional collisions are less harmful than the head-on collisions. The key event that determines the effect of collision is RNAP displacement from the template. Although it is believed largely that RNAP units are more easily and quickly displaced in a codirectional manner. However, mechanistic details supporting this notion remain to be unknown.

One of the major influential pieces of evidence for codirectional conflicts to be less harmful came from observing the arrangements of transcription units in bacteria. In almost all bacteria, highly transcribed genes are co-oriented with replication. In *B. subtilis*, around 75% of all genes are co-oriented relative to replication fork direction (Kunst et al. 1997). In *E.coli*, fewer genes, only 55%, show such an orientation (Blattner et al. 1997). However, if only essential and highly transcribed genes like rRNA and tRNA are considered, a number of genes in co-orientation increase to 70% and 90% in *E. coli* and *B. subtilis*, respectively. Since such bias is not obvious in eukaryotic cells, they seemed to have evolved other alternative strategies to reduce head-on collision. In *S. cerevisiae*, replication fork barriers (RFBs) block fork progression at the highly transcribed rDNA locus to avoid the head-on encounter (Brewer and Fangman 1988; Linskens and Huberman 1988). Such type of programmed replication blockage by RFBs at rDNA locus is conserved in other yeast species (Sanchez-Gorostiaga et al. 2004; Krings and Bastia 2005). Other than rDNA loci, replication barriers are known to exist in other part of the genome; for example, replication termination sequence 1 (RTS1) in fission yeast is responsible for unidirectional replication at the mating-type locus, avoiding the head-on collision (Dalgaard and Klar 2001). Some other barriers such as polar barriers that form at tRNA genes (Deshpande and Newlon 1996) and bidirectional barriers present at

centromere (Greenfeder and Newlon 1992) are also known to stall fork progression in yeast. Higher eukaryotes like mammalian cells also contain RFBs at rDNA loci (Gerber et al. 1997), and their genes are potentially organized collinearly with replication (Huvet et al. 2007). Along with replication barriers, higher eukaryotic cells also use genome organization as a secondary line of defense against collisions. One such example is demonstrated in mammalian cells, where transcription of rDNA genes is spatially separated at nucleoli (Smirnov et al. 2014).

Codirectional encounters can also be disruptive for replication, if encounter occurs with a highly transcribed gene. Codirectional conflicts were detected at rRNA genes only under fast-growing conditions, where there is a high level of transcription of rRNA genes. However, the effect of conflict was not detected under slow-growing conditions, where there is a decrease of transcription levels (Merrikh et al. 2011). This probably indicates that codirectional conflicts can be disruptive to replication in vivo, most likely when the replication apparatus encounters more than one RNAP or an array of RNAPs.

Consequences of challenging the bias by inverting a gene from codirectional to head-on orientation can have a severe effect. Inverting the direction of highly transcribed gene like rRNA not only stalls replication fork but also disrupts the integrity of the replication fork (Boubakri et al. 2010; Srivatsan et al. 2010). However, inverting a genomic region containing different classes of genes showed only a mild reduction in replication fork progression (Wang et al. 2007). Disruption of a replication fork is more deleterious than fork slowdown or pausing, as disruption can trigger cell cycle checkpoints and eventually delays cell division. Indeed, failure to repair replication disruption due to head-on collision is associated with cell growth arrest and leads to cell death (Boubakri et al. 2010; Srivatsan et al. 2010). This might explain why codirectional arrangements for highly transcribed genes like tRNA and rRNA are evolutionally conserved in most of prokaryotes (Guy and Roten 2004). However, how essential genes also follow codirectional orientation conservation across species is not clearly known, as comparative genomic studies indicate that essential genes are selected on the basis of essentiality rather than level of its expression (Rocha and Danchin 2003).

It is clear that head-on collisions negatively impact DNA replication more than codirectional collisions. However, the nature of aspects of head-on collision that makes it more deleterious is not clearly known. One previously thought hypothesis is that the replicative helicase might encounter RNAPs before the other replication components on the lagging strand (McHenry 2011), which might make them nonfunctional, leading to replication fork stalling. However, this hypothesis only explains the scenario in prokaryotes but not in eukaryotic cells, where replicative helicase travels on the leading arm unlike in prokaryotes. Moreover, recent findings question if directionality matters at all in eukaryotes. In yeast, the relative directions of replication and transcription do not matter on a genome-wide scale, as the naturally occurring sites of replication-fork pausing at transcribed regions appear to be independent of polarity (Azvolinsky et al. 2009). DNA damage studies carried out in yeast mutants defective in RNA-processing factors also show damage accumulation at the ORFs regardless of directionality (Stirling et al. 2012). Recent belief, at

least in eukaryotes, is that whenever a replication fork encounters an elongating transcription complex in either direction has the potential to result in DNA breakage. This situation holds true particularly if transcription is hampered (Garcia-Muse and Aguilera 2016). If directionality does not affect the collision consequences, what determines lethality? A possible contributing factor could be the difference in the supercoiling state at the site of encounter, which will be discussed in details in the following section.

## 20.4 Topological Consequences of Collision

Along with the obvious physical collision, what replication and transcription machineries have to resolve is the topological stress imposed on each other. Topological status of DNA depends on several cellular and molecular aspects like nucleosome organization, loop-like structures, stable protein-DNA interactions, and its association with immobile elements like nuclear membrane. All chromosomal processes that require opening or folding of the DNA helix are affected by DNA topology status. On the other hand, these processes also introduce changes in DNA topology. Topological status of DNA can be negatively or positively supercoiled when the double helix under-winds or overwinds, respectively (Wang 2002). The term topological stress is used when the unwinding of DNA challenges the topological status of DNA without removing intertwines between two strands. On a linear DNA fragment, this stress can diffuse away and off the end of DNA by the axial rotation of the double-stranded DNA. However, within cells, topological or torsional stress created during the biological process simply cannot diffuse by turning the chromosome ends. Chromosomes are very large and complex structures, and often DNA is anchored to the rigid structures like the nuclear membrane or the chromosome scaffolds that act as topological barriers.

Based on "twin topological domain" model (Liu and Wang 1987), during transcription, DNA in front of the elongating RNAP becomes overwound and coils around itself forming positive supercoils. Behind the transcription machinery, the DNA helix also coils around itself owing to under-winding (Fig. 20.3a). On the other hand, advancing replication fork generates positive supercoiling ahead of the forks (Fig. 20.3b) (Peter et al. 1998). Behind the replication fork, sister chromatid intertwinings arise because the fork occasionally rotates with the turn of the DNA helix. Both polymerases can rotate around the double axis as they move along the DNA to counteract the topological changes (Harada et al. 2001; Schalbetter et al. 2015). In theory, rotation of both polymerases should facilitate the movement as it will avoid accumulation of supercoiling (Fig. 20.3c, d).

One potential explanation of why head-on collisions inhibit the fork movement to a greater extent than codirectional collisions is attributed to the accumulation of higher positive supercoiling from forward movement of both machineries. It is indeed hypothesized that positive supercoiling generated ahead of the transcription bubble might act as a topological barrier rather than a direct physical block to fork

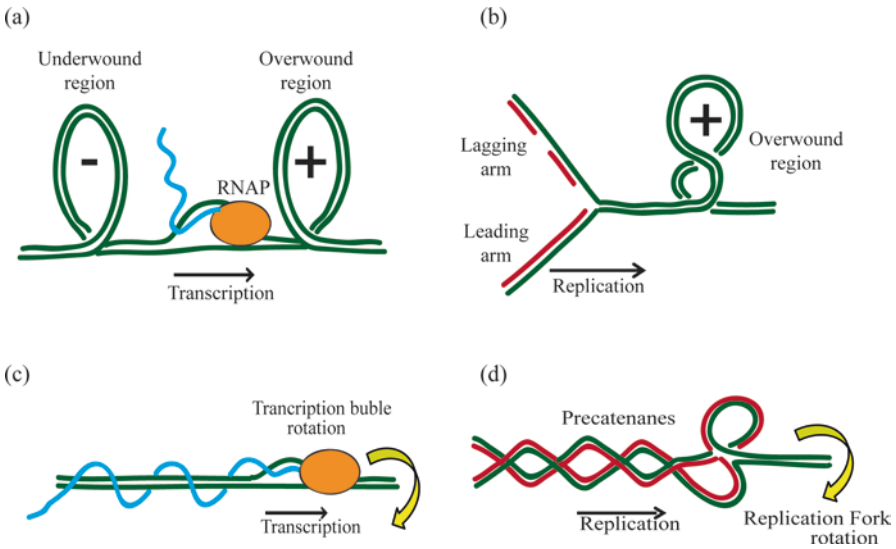


**Fig. 20.3** Topological alterations arising during DNA replication and transcription. (**a**) Transcription induces formation of both under- and overwound DNA in the back and front of elongating RNAP, respectively. (**b**) Elongating replication fork induces overwound DNA ahead of the fork. (**c**) Rotation of RNAP resolves topological alterations. (**d**) Replication fork rotation induces precatenanes formation behind the fork

movement (Wu et al. 1988; Olavarrieta et al. 2002). However, studies from head-on collisions in bacteria suggest that blockage of fork movement occurs by direct contact between replisomes and RNAPs rather than higher accumulation of positive supercoiling (Mirkin and Mirkin 2005). Experiments carried out on topologically unconstrained DNA also support this idea as *E.coli* replisome could displace a stalled RNAP rapidly in vitro when collision occurs codirectionally but not when it is head on. This explains how easily RNAP could be displaced when codirectional, allowing a smoother movement of replication fork. However, it is still unclear why head-on collisions show higher inhibitory effect on fork movement. One possible hypothesis could be that in codirectional collision, the replicative helicase could reach a very close proximity to RNAP, which will initiate the disassociation of RNAP complex. Due to the higher positive supercoiled topological barrier in head-on collision, replicative helicase and RNAP might not reach such a close proximity until the topoisomerase resolves positive supercoiling. This hypothesis might be more suitable for eukaryotes as topology is more complex due to tethering of DNA to the nuclear envelope (Bermejo et al. 2011).

It is not clear whether the RNA and DNA polymerases ever actually make contact. It is possible that before the physical contact, topological changes might induce transcription- and replication-mediated chromatin and DNA structural modifications. These alterations might attenuate the progression of the polymerases. Unequal chromatin environment throughout the genome makes it difficult to identify and unravel the importance of chromatin alterations formed through the accumulation of topological stress. During transcription, topological stress arising depends on its

gene position, expression level, and chromatin environment. One way to keep these alterations at a desired level is by diffusing the topological tension across the other domains. Studies on global genome architecture have shown that chromosome fibers are organized into distinct higher topological domains (Bermudez et al. 2010; Kouzine et al. 2013; Naughton et al. 2013). These domains are separated by insulator binding protein CTCF, housekeeping genes, tRNAs, and short interspersed element (SINE) retro transposons (Dixon et al. 2012), which most likely act as a barrier to the diffusion of topological stress. Apart from global chromosomal architecture, other factors such as density of nucleosomes packaging also affect topological stress diffusion (Salceda et al. 2006).

In theory, in a codirectional collision between a fork and a transcribed unit, the negative supercoiling generated behind the RNAP might absorb the positive supercoiling generated by the incoming fork (Fig. 20.4), thus avoiding consequences of

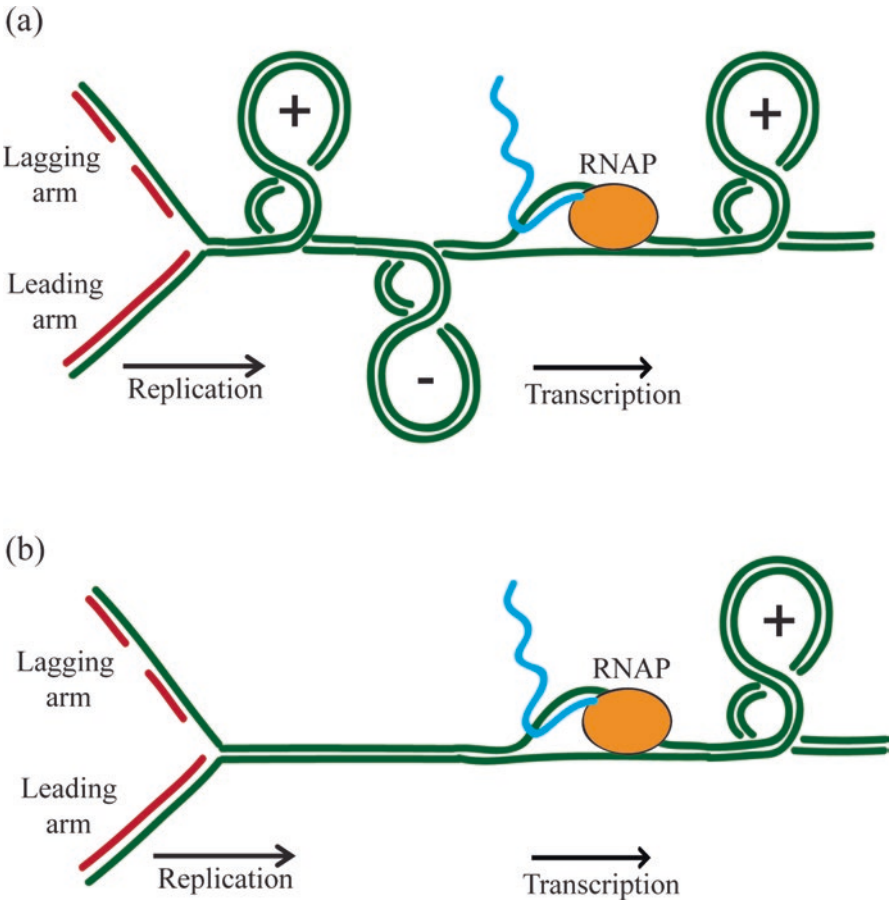


**Fig. 20.4** Possible topological resolutions during codirectional collision. (**a**) Negative supercoiling generated behind elongating RNAP might absorb positive supercoiling at the front of replication fork. (**b**) Possible outcome of codirectional collision is the complete negotiation of topological alteration between RNAP and replication fork

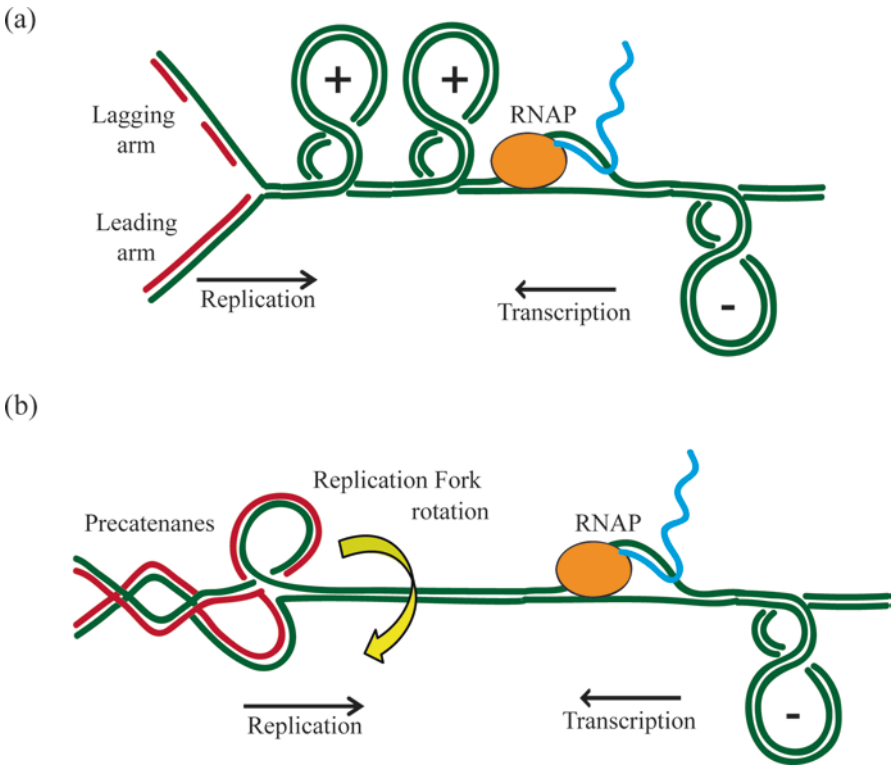


**Fig. 20.5** Possible topological resolutions during head-on collision. (**a**) Positive supercoiling accumulated at the site inhibits elongation of both transcription and replication. (**b**) Fork rotation might resolve the hyper-positive supercoiling at the site creating precatenanes structures

topological stress (Wang 2002; Bermejo et al. 2012). However, in a head-on orientation, hyper-positive accumulation generated by both mechanisms need to be resolved by specialized mechanisms. One such way to prevent topological stress building up ahead of the fork is to swivel around the helix to make a rotation (Fig. 20.5) (Champoux and Been 1980). If the replication fork is free to rotate, the positive supercoiling stress in front of the fork can diffuse to create an intertwining of the daughter duplexes behind the fork, forming "precatenanes" (Postow et al. 2001). Following the completion of replication, precatenanes mature to full-DNA catenanes that are resolved by the action of topoisomerases prior to chromosome separation (Fachinetti et al. 2010). Recent evidence of frequent fork rotation at the site of transcription might hint toward precatenanes role in diminishing positive supercoil during head-on collisions (Jeppsson et al. 2014). Although fork rotation could be one possible way to resolve excessive positive supercoiling during the head-on conflict, mechanistic details explaining the process is yet to be discovered.

Considering the processivity of the replicative helicase, a replication fork had to rotate at a considerable rate and more often to counteract the positive supercoiling. This may work at certain restricted chromatin regions but may not be considered as a general phenomenon. Identification of anti-fork-rotating complexes such as Timeless/Topf1 and Tipin/Csm3 proves this point, as these are known to inhibit excessive fork rotation and precatenation formation (Schalbetter et al. 2015). Another way a cell can reduce the positive supercoiling during replication is through reserving some portions of the chromatin to be "negatively supercoiled" regions. Such kind of reservoir of under-wound DNA absorbs the positive supercoiling from the incoming replication fork. This hypothesis makes sense considering the fact that DNA in most of the organisms is known to be slightly under-wound (Champoux 2001), however, lacks experimental support.

Alternatively, cells can use DNA topoisomerases to keep supercoiling at a controlled level. DNA topoisomerases are conserved proteins from bacteria to humans and known to participate in both replication and transcription process. Possible role of DNA topoisomerases in resolving the transcription-replication conflict is discussed in the following section.

## 20.5 Topoisomerases at the Center of Conflict

Both prokaryotes and eukaryotes utilize topoisomerase enzymes that can relax topological stress by introducing temporary strand breakage into the DNA. Type I topoisomerase nicks one strand, whereas type II topoisomerase breaks both strands while passing another DNA strand through the break (Wang 2002). Topoisomerases, along with relaxing negative or positive supercoiling, can also introduce either negative (bacterial DNA gyrase) or positive supercoiling (reverse gyrase). Besides altering supercoiling, strand-passing activities of topoisomerases can promote the catenation and decatenation of both circular and chromosome DNAs. In eukaryotes, both type IB topoisomerase (Top1) and type II topoisomerase (Top2) regulate the torsional state of the DNA. Although Top1 and Top2 are implicated in supporting replication and transcription, their resolving functions are redundant in many instances.

Positive supercoiling generated ahead of the replication fork can be resolved by both Top1 and Top2 topoisomerases. If the replication machinery could readily rotate, Top2 could function behind the fork as well to remove precatenanes. In yeast, both Top1 and Top2 travel with the replication fork (Bermejo et al. 2007); however, either one of the topoisomerases is sufficient for replication fork progression, confirming the functional redundancy in resolving positive supercoils. Simultaneous inactivation of Top1 and Top2 prevents DNA replication synthesis (Brill et al. 1987). Although one of the topoisomerases is sufficient for replication fork progression, studies in bacteria and yeast indicate that type II (Top2) topoisomerases are indis-

pensable for replication termination and chromosome segregation (Wang 2002; Fachinetti et al. 2010).

Like the elongation step of replication, transcription could also generate positive supercoiling if free rotation of the transcription machinery is inhibited. Along with the sheer size and composition of the transcriptional machinery, its association with the nuclear membrane (Cook 1999; Bermejo et al. 2011) and co-coupling with the translation process (Iborra et al. 2001) inhibits its rotation. Like replication, association and coordination with the topoisomerase are crucial for an efficient transcription process (King et al. 2013). Top1 protein is associated with the elongating transcription unit, and its activity is regulated by RNAP II itself, to favor efficient transcription (Baranello et al. 2016). In yeast, loss of both Top1 and Top2 activity causes a rapid decrease in rRNA transcription but only modest changes at shorter tRNA genes and lower expressed RNA pol II genes (Brill et al. 1987). However, it is recently shown that transcription of longer mRNAs is sensitive to the inactivation of the Top2 protein (Joshi et al. 2012). A genome-wide study has also shown that Top2 binds at the beginning and the end of transcribed genes, specifically during S-phase (Bermejo et al. 2009). This indicates that topoisomerase activity is required to relax topological stress preferably at highly expressed and longer genes where topological stress might build up during replication.

Since DNA topoisomerases are associated with both replication and transcription, hints at their role in resolving the conflict have been proposed. The head-on collision is likely to cause both sterical and topological problems due to hyper-positive supercoiling accumulation. This topological scenario mimics the one during the replication termination zones, where opposing forks converge creating an excess of positive supercoiling ahead of them (Wang 2002). In both scenarios, non-replicated DNA becomes very short, making it difficult for DNA topoisomerases to bind and resolve overwounded DNA. Cells resolve this issue, at least in replication termination regions by generating precatenanes possibly through fork rotation, which later can be resolved by Top2. Indeed in *E. coli*, as well as *S. cerevisiae* and *S. pombe*, Top2 topoisomerase is required for the timely replication termination and to prevent chromosomal breaks during chromosome segregation (Champoux 2001; Wang 2002; Fachinetti et al. 2010). Similarly, fork rotation-induced precatenane resolution by Top2 could be hypothesized at least in prokaryotes, as head-on conflicts are less frequent. However, such scenario may not be possible in eukaryotic cells due to the frequent occurrence of head-on clashes and topological barriers inhibiting the fork rotation. Interestingly, both replication termination and head-on collision zones are known to be replication-dependent fragile sites (Song et al. 2014). Although it is still not clear if there is a direct connection between positive supercoiling accumulation and fragile site formation, a possibility cannot be ruled out.

Replication- and transcription-mediated transient supercoils are necessary, as several processes depend on them. Proteins, like chromatin remodelers often depend on such transient supercoil modifications to activate a variety of cellular responses (Kim and Deppert 2003). Slight alterations in DNA supercoiling can have a strong impact on gene expression in both prokaryotes and eukaryotes. In bacteria, it was

shown that loss of chromosomal supercoiling significantly and rapidly affected the expression of 7% of genes (Peter et al. 2004). Several complexes that involve extensive DNA-protein interactions, including replication and transcription initiation, can only occur during particular supercoiling conditions (Bates and Maxwell 2005).

## 20.6 Transcription and Alternative DNA Structures

Along with being a natural obstacle for replication fork progression, transcription could also interfere through its secondary effects. To increase efficiency and robustness of RNA production, the transcriptional machinery generates several artifacts. These unnatural features could facilitate RNA synthesis and export mechanisms but could challenge replication progression. These features include chromatin structural changes like gene loops, RNA:DNA hybrids, secondary DNA structures like hairpins, triplex-like structures and hinged (H)-DNA), non B-DNA structures such as G-quadruplex, and Z-DNA.

### *20.6.1 Gene Gating and Gene Loops*

In eukaryotes, RNAP II-transcribed genes are organized in loops (Ansari and Hampsey 2005). In *S. cerevisiae*, Top2 along with high-mobility group protein Hmo1 mediate formation of DNA loops. In S-phase, integrity as well as topological complexity of DNA loops depends on the concerted action of both Top2 and Hmo1 (Bermejo et al. 2009). DNA looping assists transcription by facilitating RNAP recycling for concomitant rounds of transcription, a key process in highly transcribed genes (Ansari and Hampsey 2005). These structures also influence the capability of mRNA genes to memorize the previous transcriptional status through a process known as transcriptional memory (Tan-Wong et al. 2009). These specialized architectures of chromatin function as a barrier for incoming forks, topologically insulating the transcriptional apparatus (Azvolinsky et al. 2009). Since DNA loops act as topological insulators, they keep the supercoiling generated by transcription within the loop, maintaining the neighboring chromatin topologically unaffected. This, at least in theory, explains how head-on collisions can be less harmful if loops are formed as DNA topoisomerases have to resolve only positive supercoils generated by the replication fork. It is still unclear how replication forks can dismantle loop structures and what factors might assist them during the process. Loops can also couple mRNA transcription with their export by bringing chromatin into contact with the nuclear pore complexes (NPCs), a process termed as gene gating (Strambio-De-Castillia et al. 2010; Burns and Wente 2014). Evidence in yeast suggests that every RNAPII-transcribed gene associates with gating factors (Casolari et al. 2004; Gomez-Gonzalez et al. 2011). Recent observations in yeast suggest that transcribed genes are unleashed from the nuclear envelope to

allow fork progression (Bermejo et al. 2011). This process is mediated by the Mec1-Rad53 checkpoint that phosphorylates Mlp1, a key protein located in the inner basket of the nuclear pore. Fork passage through transcribed genes not only counteracts gene gating but also allows the dismantling of DNA loop. The key question here is how does a loop anchored to the nuclear membrane sense the incoming fork? A recent finding in mammalian cells demonstrates that nuclear membrane could sense topological tension accumulated at the chromatin. This leads to accumulation of the major checkpoint protein, ATR, at the nuclear envelop, which initiates a signal cascade to relax loop-like structures (Kumar et al. 2014). This assembly and disassembly of gene loops during replication shows that transcription domains are cell cycle dependent. Indeed, gene gating is regulated by cyclin-dependent kinases (CDKs) through phosphorylation of Nup1 nucleoporin. Moreover, transcriptional memory has been linked to the chromosome architecture of transcribed genes (Tan-Wong et al. 2009; Light et al. 2010). It is expected that replication across RNA genes would influence the capability of genes to memorize their transcriptional status, as it is crucial to reestablish the transcription process after fork passage.

In addition to mRNA-generating genes, rDNA loci where replication fork barriers are essential to avoid replication conflicts are also anchored to the nuclear membrane through a different mechanism. The function of RFB relies on the Fob1 protein, which mediates the perinuclear attachments of rDNA repeats through its interactions with cohibin/CLIP complex (Kobayashi and Horiuchi 1996; Huang et al. 2006; Mekhail et al. 2008). Anchoring of rDNA loci to the nuclear envelope might impose a series of topological constraints leading to fork pausing. On the other hand, releasing rDNA repeats from the nuclear envelope destabilizes the repeats. Due to hyperactive transcription at rDNA loci, DNA topoisomerase is absolutely necessary to maintain supercoiling. Yeast devoid of both Top1 and Top2 not only shows drastic reduction in the rRNA levels but also its rDNA repeats are excised as extrachromosomal circles (Kim and Wang 1989).

### *20.6.2 R-Loops and RNA-DNA Hybrids*

Both replication and transcription are required to form short RNA-DNA hybrids, which are used for priming polymerases. These short stretches of hybrids are more stable than double-stranded DNA as they adopt a heteromeric confirmation, which is a mix of the “B” form of dsDNA and the “A” form of double-stranded RNA (Roberts and Crothers 1992). Often, longer forms of RNA:DNA hybrids occur in most of the genome. On the basis of “thread-back model,” hybrid structures can be generated by the hybridization of nascent RNA molecules with the template DNA strand (Westover et al. 2004). This longer hybrid and the resultant displaced single-stranded DNA are collectively known as R-loops. Formation and stabilization of R-loops in vivo depend on several structural features such as negative supercoiling, GC-content, and tendency to form alternative structures in the displaced ssDNA

such as G-quartets (Duquette et al. 2004; Roy and Lieber 2009; Aguilera and Garcia-Muse 2012).

R-loops are found in all living organisms and generally considered to have deleterious effects except in unusual situations such as in immunoglobulin (Ig) class-switch recombination (CSR). It has been shown in yeast and human cells that deficiency in RNA and DNA metabolic factors leads to R-loop-mediated DNA breaks, recombination and chromosome rearrangements, and losses (Santos-Pereira and Aguilera 2015). It is still unclear what forces R-loops to generate DNA breaks. A largely accepted hypothesis is that R-loop-induced replication fork stalling might generate DNA breaks. First, evidence of RNA:DNA hybrids causing genome instability came from bacteria where loss of RNaseH1 provoked the SOS response (Kogoma et al. 1993). *E. coli* cells defective in DNA damage response (DDR) show lethality when an rDNA operon was inverted to have a head-on collision with a replication fork. However, this lethality was rescued by overexpression of RNaseH, showing the connection between R-loops, replication stalling, and DDR (Boubakri et al. 2010). Experiments in both *E. coli* and mammalian cells revealed that R-loop-mediated chromosomal rearrangements and recombination could be rescued by impairing DNA replication (Gan et al. 2011). Genome-wide replication analysis in several yeast mutants such as THO (*tho*) (Gomez-Gonzalez et al. 2011), Sen1 (*sen1-1*) (Alzu et al. 2012), and RBP Npl3 *(npl3)* (Santos-Pereira et al. 2013) revealed R-loop-mediated replication-transcription conflicts. Replication analysis on *TOP1*-depleted mammalian cells shows transcription interference with replication in an R-loop-dependent manner (Tuduri et al. 2009). Moreover, common fragile sites associated with replication and transcription collisions in human cells are known to accumulate R-loop structures (Helmrich et al. 2011). It is now evident that R-loop structures do have the potential to impair replication progression by inducing DNA breaks. However, it is to be noted that not all RNA-DNA hybrid-forming regions may give rise to R-loop-like structures, suggesting that only a fraction of such regions participate in replication stalling. Possibility of an arrested RNAP II at the site of R-loop-forming region cannot be ruled out, as it will further hinder replication progression.

In addition to causing DNA breaks, R-loops may also be involved in promoting mutagenic DNA replication. In *E. coli*, RNA-DNA hybrids can be used as primers to initiate noncanonical replication (Kogoma 1997). In yeast cells, rDNA loci also show origin-independent replication but only in the absence of Top1 and RNaseH proteins, confirming the role of RNA-DNA hybrids in replication initiation. Although R-loop-mediated replication initiation has been reported in several instances, fidelity and consequences are little known. R-loop-mediated replication is largely considered to be highly mutagenic, as cells may use such origin-independent replication only in extreme conditions such as DNA breaks (Deem et al. 2011).

A recent finding shows the involvement of tumor suppressor and DNA repair genes such as *BRCA2* and *BRCA1* in R-loop-mediated homeostasis, revealing a direct link between R-loop accumulation and mutagenesis (Bhatia et al. 2014). Studies revealing the role of RNA-DNA helicases such as Sen1, Rrm3, and Pif1 in

assisting replication fork progression across pausing sites open new perspectives into our understanding of R-loop-mediated replication stress and DNA breaks (Alzu et al. 2012; Rossi et al. 2015).

### *20.6.3 Hinged DNA (H-DNA)*

H-DNA can be formed with three DNA strands and uses Hoogsteen hydrogen bonds for its stability and specificity (Frank-Kamenetskii and Mirkin 1995). One possible role of H-DNA in vivo was revealed by identifying a segment in the promoter of the human cMYC gene capable of adopting H-DNA (Kinniburgh 1989). Interestingly, this region overlaps with one of the major breakage hotspots found in c-MYC-induced lymphomas and leukemias (Joos et al. 1992; Wilda et al. 2004). Later, certain fragile spots or hotspot regions of the genome are mapped in or near the genes carrying polypurine-polypyrimidine sequences known to form H-DNA confirmation (Bacolla et al. 2006). It is well known that naturally occurring H-DNA structures are intrinsically mutagenic in mammalian cells (Wang and Vasquez 2004). The existence of H-DNA structures may effectively impede both transcription and replication processes (Jain et al. 2008); at least in vitro, it is known to impose a very strong barrier for polymerases (Hoyne and Maher 2002). Although the exact mechanism is yet to be known, naturally occurring intermolecular triple-strand DNA like H-DNA are inherently mutagenic and recombinogenic and might play a role in replication and transcription collisions.

### *20.6.4 Z-DNA*

Till now, definitive biological functions for Z-DNA have not been found. Z-DNA is believed to provide torsional stress relaxation, particularly from negative supercoiling at the transcription site (Ha et al. 2005). Indeed, formation of Z-DNA is directly proportional to accumulation of negative supercoiling (Azorin et al. 1983), and topoisomerases can convert Z-DNA back to B-DNA (Rich and Zhang 2003). In yeast, Z-DNA structures can be induced and stabilized by Z-DNA-binding proteins, which might have a role to play in gene regulation and chromatin remodeling (Wang et al. 2007). Till now, the effect Z-DNA on DNA replication was only reported in trypanosomas, where unwinding of DNA due to intercalation leads to Z-DNA formation and subsequent inhibition of replication (Roy Chowdhury et al. 2010). However, the impact of Z-DNA on replication in other organisms cannot be ruled out since negative supercoiling generated by transcription directly influences its formation and Top1 function is required to counteract its formation. Particularly, the occurrence of Z-DNA-forming sequences at chromosomal breakpoints in human

tumors suggests its role in double-strand breaks. In fact, occurrence of Z-DNA forming sequences at chromosomal breakpoints in human tumors suggests its possible role in generation of double-strand breaks (Wang et al. 2006).

### *20.6.5 G-Quadruplex (G4) DNA*

A guanine-rich genomic region has the ability to form a quadruplex (G4), stabilized by Hoogsteen hydrogen bonds at physiological concentrations (Sen and Gilbert 1988). The formation of G4 structures is favored by DNA transition processes, which involve unwinding of the double helix such as transcription and replication (Maizels and Gray 2013). It is estimated that in human genome, over 350,000 sequences have the potential to form G4 structures that can act as potential replication barriers (Huppert and Balasubramanian 2005; Todd et al. 2005). Recent studies using either G4-recognizing molecules or antibodies show that G4 sequences are present in cells and they increase during S-phase (Rodriguez et al. 2012; Biffi et al. 2013; Henderson et al. 2014). G4 structures have been involved in several biological processes including telomere maintenance, gene expression, replication initiation, and the immune response (Maizels and Gray 2013).

Transcription, particularly at G-rich regions, induces the formation of stable G4 structures (Duquette et al. 2004). Recently, it is revealed that G4-DNA is enriched in the promoters and 5′UTRs of highly transcribed genes, particularly in genes related to carcinogenesis (Hansel-Hertsch et al. 2016). During DNA replication, the inappropriate intermolecular folding like in G4 must be resolved in order to allow replication fork movement. It is well known that G4-DNA, if not unwound, could inhibit fork stalling, possibly causing fork collapse and DNA breaks (Woodford et al. 1994; Usdin and Woodford 1995). Cells have developed several mechanisms to counteract the G4-DNA formed due to transcription during replication. Recent evidence implicating specialized proteins in the unwinding of G4-DNA is now accumulating. Proteins such as human ATRX (Law et al. 2010), XPB, and XPD (Gray et al. 2014) and yeast Pif1 (Paeschke et al. 2011) and Rif1 (Kanoh et al. 2015) have been shown to accumulate near G-rich genomic regions predicted to form G4-structures, indicating their possible role in assisting replication fork progression. Other than helicases, cells also use specialized DNA polymerases known as translesional synthesis (TLS) polymerases to replicate across G4-DNA structures. TLS polymerases such as pol kappa, pol eta, and Rev1 are required for efficient replication across G4 motifs (Betous et al. 2009; Sarkies et al. 2010). It is now well established that specific helicases and specialized polymerases are involved in replication of G4-DNA; loss of these factors leads to severe genetic disorders leading to premature aging and higher cancer risk (Rhodes and Lipps 2015).

## 20.7 Negotiating Replication and Transcription Conflict

Cells have evolved several mechanisms to reactivate, destabilize, or even remove transcription roadblocks to minimize the effects of transcription-replication collisions. Conceptually, clearing the pathway ahead of a replication fork is the simplest mechanism of resolving conflicts. Alternatively, a codirectional conflict may be resolved by slowing down the replication fork behind the RNAP until transcription termination. However, it remains unanswered whether a replication fork has the luxury of waiting until the transcription is completed. Preventing the formation of backtracked or stalled RNAPs will also be beneficial for replication. Cells achieve this by ensuring an appropriate RNAP elongation rate. In prokaryotes, transcription elongation factors GreA and GreB clear the pathway by reactivating backtracked and stalled RNAPs (Opalka et al. 2003). Another bacterial transcription factor, DksA, promotes elongation by reducing nucleotide disincorporation leading to backtracking and pausing of RNAPs (Tehranchi et al. 2010; Roghanian et al. 2015). Another mechanism by which prokaryotes keep backtracking at minimal levels is by coupling transcription with translation. Ribosomes are loaded simultaneously onto nascent RNA as they emerge from the RNAP complex (Proshkin et al. 2010; Dutta et al. 2011) leading to increased transcription efficiency. Same phenomenon can be attributed in yeast as ongoing transcripts simultaneously anchor to the nuclear membrane, likely ensuring the elongation rate (Bermejo et al. 2011). Higher eukaryotes have additional protein factors including RECQL5, which reduces stalling and backtracking by controlling transcription elongation rate (Saponaro et al. 2014).

RNAP arrested by encountering damaged bases can only proceed when the lesion is repaired by repair mechanisms, such as nucleotide excision repair (NER). Prokaryotes use two different NER factors Mfd and UvrD, to dislodge arrested RNAP at the lesion (Ganesan et al. 2012; Epshtein et al. 2014). In eukaryotes, how NER dislodges RNAP is not clear. However, if NER fails, RNAP is polyubiquitinated and degraded by proteasomes (Wilson et al. 2013). Interestingly, mammalian cells lacking NER exhibit apoptosis during S-phase, suggesting lethality associated with irreversibly arrested RNAP (McKay et al. 2002). In eukaryotes, TFIIS, a Pol II transcription elongation factor, can also stimulate transcript cleavage to restart arrested RNAP (Cheung and Cramer 2011).

For RNAPs to be cleared on their own will pose detrimental time delays and cells inevitably need to swiftly clear the path for replication forks. Recent in vivo and in vitro studies support the long-lasting hypothesis that auxiliary helicases promote replication through roadblocks including transcription complexes. The best-known examples are REP and UvrD in *E. coli* and their homolog PcrA in *B. subtilis*, all of which are known to associate with replication forks to promote replication though transcribed genes (Guy et al. 2009; Gwynn et al. 2013; Merrikh et al. 2015). In eukaryotes, Rrm3, Pif1, and Sen1 are also associated with replisomes and facilitate progression through highly transcribed genes (Azvolinsky et al. 2009; Alzu et al. 2012; Rossi et al. 2015). Additionally, RNaseH overexpression significantly reduces

the association of Rrm3 with these genes, indicating RNA:DNA hybrid accumulation as an intermediate step in these genes (Gomez-Gonzalez et al. 2011). Since most eukaryotes encode at least one of these helicases, the function of these proteins to promote replication fork progression across highly expressed genes may be conserved.

## 20.8 Replication-Transcription Collision and Genome Instability

Until now, transcription and its associated events represent major endogenous sources causing replication stress. Certain regions in the genome are prone to form gaps, breaks, and rearrangements in response to replication stress and are generally referred to as chromosomal fragile sites (Glover et al. 1984; Yunis and Soreng 1984; Beroukhim et al. 2010; Bignell et al. 2010; Wilson et al. 2015; Wei et al. 2016). These common fragile sites (CFSs) tend to coincide with transcribed genes, and transcription itself is believed to be the source of genomic instability at these regions (Huertas and Aguilera 2003; Helmrich et al. 2011; Barlow et al. 2013; Saponaro et al. 2014; Wilson et al. 2015; Kantidakis et al. 2016). It has been known for many years that transcription can lead to higher recombination (Keil and Roeder 1984; Voelkel-Meiman et al. 1987; Thomas and Rothstein 1989; Nickoloff 1992; Prado et al. 1997). Moreover, transcription-dependent recombination strictly depends on replication, and it is the collision of replication with transcription that causes recombination as proven in yeast (Prado and Aguilera 2005) and mammalian cells (Gottipati et al. 2008). Recent sequencing of cancer genomes has identified specific mutation signatures arising due to replication-transcription collisions (Hatchi et al. 2015). Even in bacteria, mutations arising due to replication and transcription conflicts show a distinct mutation pattern (Sankar et al. 2016), indicating pathological consequences of such collisions. However, linking collision-related mutation accumulation directly to genome stability will be a daunting task, as gene expression patterns differ significantly between different cell and tissue types. It is tentative to speculate that this difference might be the reason behind cell- or tissue-specific mutation variations in cancer cells.

There is a vast amount of data connecting transcription-induced replication pausing, chromosome breaks, and genome rearrangements. It is yet to become clear what percentage of collisions will lead to fork collapse, leading to DNA breaks. Since cells cannot completely avoid collisions, they acquire several layers of protection to avoid the lethal consequences of collisions. Particularly, in response to environment stimuli, whole arrays of proteins remodel genome architectures by activating or suppressing genes. Cells also need to repair lesions and base modifications, which arise due to various external and internal stimuli. Amidst, faithful replication has to negotiate and resolve conflicts of genome trafficking and is essential for genome integrity.

## 20.9 Conclusions and Future Perspectives

A cell can either avoid collisions between replisomes and RNAPs or resolve them through specific mechanisms. A precise dissection of molecular mechanisms, which could explain the impact of collisions, is still very unclear. Extensive overlap between processes that ensure faithful completion of both DNA replication and RNA production makes it difficult to unravel the details of such mechanisms. Research from the last 30 years has indicated the importance of replication-transcription conflicts, as they emerged as important natural sources of genome instability. Particularly, identification of mutations in genes that are involved in either prevention or resolution of conflicts is connected to several cancer-prone syndromes and neurodegenerative diseases. In the future, it will be important to distinguish deleterious collisions that cause mutations from the nonhazardous counterparts. It is well known that collisions lead to genome instability; however, it is still unknown if damage occurs at the site of collision. If damage occurs at the site, do DDR or S-phase checkpoints play any roles in resolving the conflicts? It is also important to understand how an encounter affects the stability of both replication forks and transcription units. Among all the factors studied that have implications on collision-mediated consequences, DNA topology is least understood. Since DNA topoisomerases have an impact on both processes, it is important to understand how their action might help to coordinate these two processes. In higher eukaryotes, since replication origins and termination regions are not fixed, it is difficult to reliably predict the orientation of collisions. This makes it not trivial to associate collision hotspots with DNA sequences and chromatin modifications. Identifying mutational patterns arising from replication-transcription conflicts and associating them with known cancer biomarkers might have biomedical and therapeutic relevance.

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# Chapter 21
# Fragility Extraordinaire: Unsolved Mysteries of Chromosome Fragile Sites

**Wenyi Feng and Arijita Chakraborty**

**Abstract** Chromosome fragile sites are a fascinating cytogenetic phenomenon now widely implicated in a slew of human diseases ranging from neurological disorders to cancer. Yet, the paths leading to these revelations were far from direct, and the number of fragile sites that have been molecularly cloned with known disease-associated genes remains modest. Moreover, as more fragile sites were being discovered, research interests in some of the earliest discovered fragile sites ebbed away, leaving a number of unsolved mysteries in chromosome biology. In this review we attempt to recount some of the early discoveries of fragile sites and highlight those phenomena that have eluded intense scrutiny but remain extremely relevant in our understanding of the mechanisms of chromosome fragility. We then survey the literature for disease association for a comprehensive list of fragile sites. We also review recent studies addressing the underlying cause of chromosome fragility while highlighting some ongoing debates. We report an observed enrichment for R-loop forming sequences in fragile site-associated genes than genomic average. Finally, we will leave the reader with some lingering questions to provoke discussion and inspire further scientific inquiries.

**Keywords** Chromosome fragility • Common and rare fragile sites • DNA double-strand breaks • DNA replication stress • R-loops • Aphidicolin • Folate stress • Neurological disorders • Cancer

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## 21.1 Introduction

Chromosome fragile sites are defined as gaps, constrictions, or breaks on metaphase chromosomes that are induced by various DNA replication inhibitors. The Human Genome Database currently documents 120 chromosome fragile sites (30 of the rare type and 90 of the common type, Tables 21.1 and 21.2). According to the HUGO Gene Nomenclature Committee, each fragile site is reported as "FRA" followed by the chromosome number and a letter denoting the order of nomenclature, such as FRA1A. In the last decade, fragile sites have been the subject of many comprehensive reviews with respect to the molecular basis, mechanisms of expression, and relevance to human diseases (Debacker and Kooy 2007; Durkin and Glover 2007; Fungtammasan et al. 2012; Lukusa and Fryns 2008; Mirkin 2006; Thys et al. 2015). Here we approach this subject from an alternative angle. By retracing some of the early developments in fragile site discoveries, we hope to identify the gaps in our knowledge and bring awareness to some "cold cases," so to speak, in chromosome fragile sites (we apologize to countless researchers whose work may have been omitted from this review due to space limitation). We review the classification of common vs. rare fragile sites and provide some insights into the DNA structural determinants of fragility. We also provide updated information regarding disease associations of known fragile sites in the database as well as summarize recent genome-wide studies of fragile sites. Finally, we highlight three "unsolved mysteries" in chromosome fragile sites and provide speculations.

## 21.2 Early History of Chromosome Fragile Site Discoveries Revisited

### *21.2.1 Discovery of the First Fragile Site*

Early history of chromosome fragile site discoveries makes a captivating read. The term "fragile site" was coined in 1969 by Frederick Hecht to describe hitherto reported spontaneous breaks at a specific site in a human metaphase chromosome. However, the first known fragile site was observed 4 years before the genesis of its terminology (Dekaban 1965). Notably, the definition of fragile site later expanded to include constrictions and gaps in the chromosome as well. In this broader definition, the first chromosomal "secondary constriction," to be distinguished from the primary constriction or the centromere, was discovered still earlier (Ferguson-Smith et al. 1962).

Nevertheless, the first fragile site in the pedantic sense was observed in a woman who had received multiple X-ray irradiations for eczematous dermatitis and later had borne a malformed child (Dekaban 1965). It was unclear if the fragile site observed on the presumed Chromosome 9 from the woman was linked to the birth of this child. Limited survey of the woman's family showed normal chromosomes

**Table 21.1** Updated summary of *RFSs*

| Fragile site | Type | Repeat | Cyto-band | Population freq. (%)[a] | Size (kb) | No. of RLFSs | No. of RLFSs/kb | No. of genes with > = 1 RLFS/kb | Freq. (%) in folate stress[a] | Freq. (%) in APH[b] | Assoc. genes | Disease |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| FRA1M | Folate | | 1p21.3 | | 4,900 | 48 | 0.010 | 0 | | 3.333 | | ID (D'Angelo et al. 2015) |
| FRA2A | Folate | CGG | 2q11.2 | 0.0014 | 6,400 | 266 | 0.042 | 1 | 27.00 | 0.089 | *AFF3* | Schizophrenia (Chen et al. 1998); ID (Metsu et al. 2014a) |
| FRA2B | Folate | | 2q13 | | 5,200 | 177 | 0.034 | 0 | | 0.067 | | |
| FRA2K | Folate | | 2q22.3 | | 3,700 | 20 | 0.005 | 0 | | 0.626 | | ID (Mulatinho et al. 2012) |
| FRA5G | Folate | | 5q35 | | 13,458 | 898 | 0.067 | 6 | | 0.120 | | Found in a FX patient at 50% expression and a normal brother at 36% (Howell et al. 1990) |
| FRA6A | Folate | | 6p23 | | 2,000 | 99 | 0.050 | 1 | | 0.009[c] | | Schizophrenia (Olavesen et al. 1995) |
| FRA7A | Folate | CGG | 7p11.2 | | 3,500 | 89 | 0.025 | 1 | | 0.058 | *ZNF713* | ASD (Metsu et al. 2014b) |
| FRA8A | Folate | | 8q22.3 | | 4,500 | 164 | 0.036 | 2 | | 0.018 | | |
| FRA9A | Folate | | 9p21 | 0.0041 | 12,900 | 50 | 0.004 | 0 | 21.00 | 0.102 | | |
| FRA9B | Folate | | 9q32 | | 2,800 | 136 | 0.049 | 0 | | 1.513 | | |
| FRA10A | Folate | CGG | 10q23.3 | 0.0028 | 16,707 | 168 | 0.010 | 0 | 35.67 | 0.124 | *FRA10AC1* | Single case of turner syndrome (Li et al. 2015; Maltby and Higgins 1987); Alzheimer's (Li et al. 2015) |

(continued)

**Table 21.1** (continued)

| Fragile site | Type | Repeat | Cyto-band | Population freq. (%)[a] | Size (kb) | No. of RLFSs | No. of RLFSs/kb | No. of genes with > = 1 RLFS/kb | Freq. (%) in folate stress[a] | Freq. (%) in APH[b] | Assoc. genes | Disease |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| FRA11A | Folate | CGG | 11q13.3 | | 1,500 | 165 | 0.110 | 2 | | 0.075 | *C11orf8c/ MPPED2* | |
| FRA11B | Folate | CGG | 11q23.3 | | 5,300 | 459 | 0.087 | 1 | | 0.098 | *CBL2* | Jacobsen syndrome (Jones et al. 1994, 1995; Michaelis et al. 1998) |
| FRA12A | Folate | CGG | 12q13.1 | 0.0007 | 4,678 | 382 | 0.082 | 8 | 17.67 | 0.075 | *DIP2B* | ASD and MR (Winnepenninckx et al. 2007) |
| FRA12D | Folate | | 12q24.13 | | 2,000 | 93 | 0.047 | 0 | | Not induced | | Segregating in FX families (Amarose et al. 1987; Barletta et al. 1991; Sutherland and Baker 1993) |
| FRA16A | Folate | CGG | 16p13.11 | 0.0007 | 2,000 | 116 | 0.058 | 1 | 12.00 | 0.053 | N.D. | |
| FRA19B | Folate | | 19p13 | | 19,800 | 4,650 | 0.235 | 88 | | 0.036 | | |
| FRA20A | Folate | | 20p11.23 | | 3,400 | 70 | 0.021 | 0 | | 0.027[c] | | |
| FRA22A | Folate | | 22q13 | 0.0014 | 13,791 | 2,445 | 0.177 | 17 | 5.67 | 0.328 | | MR (Webb and Thake 1984) |
| FRAXA | Folate | CGG | Xq27.3 | | 5,000 | 17 | 0.003 | 0 | | Not induced | *FMR1, ASFMR1/ FMR4* | FXS, MR, ID, FXTAS, FXPOI (Galloway and Nelson 2009; Santoro et al. 2012; Usdin et al. 2014) |
| FRAXE | Folate | CGG | Xq28 | | 8,014 | 615 | 0.077 | 13 | | 0.018[c] | *AFF2/ FMR2* | MR (Bensaid et al. 2009) |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| FRAXF | Folate | CGG | Xq28 | | 8,014 | 615 | 0.077 | 13 | | 0.018[c] | *FAM11A* | MR; ASD; psychosis, pyramidal signs, macroordism (Holden et al. 1996; Lindsay et al. 1996; Vianna-Morgante et al. 1996) |
| FRA8E | DA | | 8q24.1 | | 28,526 | 1,394 | 0.049 | 24 | | 0.799 | | Azoospermia (Seki et al. 1992); hereditary multiple exostoses 1 gene and two overlapping Langer-Giedion syndrome deletion endpoints (Hill et al. 1997) |
| FRA11I | DA | | 11p15.1 | | 5,609 | 175 | 0.031 | 1 | | 0.510 | | Azoospermia (Seki et al. 1992) |
| FRA16B | DA | AT-rich MS | 16q22.1 | | 4,200 | 491 | 0.117 | 9 | | 0.488 | N.D. | Difficulty achieving pregnancy (Martorell et al. 2014); dominant inheritance of cleft palate, microstomia, and micrognathia (McKenzie et al. 2002) |
| FRA16E | DA | | 16p12.1 | | 5,900 | 178 | 0.030 | 1 | | 0.027 | | Batten disease (common form of neuronal ceroid lipofuscinoses [NCLs]) (Dooley et al. 1994) |
| FRA17A | DA | | 17p12 | 0.0049 | 4,700 | 91 | 0.019 | 2 | 20.00 | 0.049 | | Azoospermia (Shabtai et al. 1982) |

(continued)

**Table 21.1** (continued)

| Fragile site | Type | Repeat | Cyto-band | Population freq. (%)[a] | Size (kb) | No. of RLFSs | No. of RLFSs/kb | No. of genes with > = 1 RLFS/kb | Freq. (%) in folate stress[a] | Freq. (%) in APH[b] | Assoc. genes | Disease |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| FRA10B | BrdU | AT-rich MS | 10q25.2 | | 3,100 | 45 | 0.015 | 0 | | 0.191 | N.D. | |
| FRA12C | BrdU | | 12q24 | | 24,850 | 1,907 | 0.077 | 6 | | 0.288 | | Segregating in FX families (Amarose et al. 1987; Barletta et al. 1991; Sutherland and Baker 1993) |
| FRA8F | UC | | 8q13 | | 7,900 | 104 | 0.013 | 0 | | 0.004[c] | | |

*Folate* folate stress, *DA* distamycin A, *BrdU* bromodeoxyuridine, *UC* unclassified, *MS* minisatellite, *RLFS* R-loop forming sequence, *ID* intellectual disability, *ASD* autism spectrum disorder, *MR* mental retardation, *FXTAS* fragile X-associated tremor/ataxia syndrome, *FXPOI* fragile X-associated premature ovarian insufficiency, *Freq.* frequency of fragile site expression

[a]Data derived from Kähkönen et al. (1989)

[b]Data derived from Mrasek et al. (2010)

[c]Fragile site detected in fewer than three individuals when induced by aphidicolin

**Table 21.2** Summary of *CFS*-associated genes and their implications in human diseases

| Fragile site | Induction method | Cyto- band | Freq. in APH (%)[a] | Assoc. genes | Disease |
|---|---|---|---|---|---|
| FRA1A | APH | 1p36 | 0.488 | | |
| FRA1B* | APH | 1p32 | 1.491 | *DAB1* | Brain and endometrial cancer (McAvoy et al. 2008) |
| FRA1C | APH | 1p31.2 | | *IL23R-C1orf141* | Vogt-Koyanagi-Harada syndrome; LOH/MSI associated with advanced tumor stage in prostate cancer (Brys et al. 2013; Hou et al. 2014) |
| FRA1D | APH | 1p22 | 0.009 | | |
| FRA1E* | APH | 1p21.2 | 0.067 | *DPYD* | Colorectal, breast, and ovarian cancer (Hormozian et al. 2007) |
| FRA1F | APH | 1q21 | 0.098 | | |
| FRA1G | APH | 1q25 | 0.799 | | |
| FRA1I | APH | 1q44 | 2.299 | | Nasopharyngeal carcinoma (Xia et al. 1988) |
| FRA1K | APH | 1q31 | 0.138 | | |
| FRA1L | APH | 1p31 | 0.479 | | |
| FRA2C* | APH | 2p24.2 2q24.3 | 1.513 | *MYCN* | Two FSs, FRA2Ccen and FRA2Ctel, flank the *MYCN* amplicon; locus-specific and genomic rearrangements in neuroblastoma and multiple cancers (Blumrich et al. 2011; Lipska et al. 2013) |
| FRA2D | APH | 2p16.2 | 2.325 | | |
| FRA2E | APH | 2p13 | 0.235 | | Posttransplant diffuse large B-cell lymphoma (Rinaldi et al. 2010) |
| FRA2F | APH | 2q21.3 | 0.994 | *LRP1B* | Alzheimer's; Kawasaki disease (Lin et al. 2014; Shang et al. 2015) |
| FRA2G* | APH | 2q31 | 0.306 | *GAD1* | Schizophrenia (Bharadwaj et al. 2013) |
| FRA2H* | APH | 2q32.1 | 3.905 | *DIRC1, PMS1, MIRN589, MIRN1245* | Breakpoints characterized in K562 cells (Pelliccia et al. 2010) |
| FRA2I | APH | 2q33 | 1.722 | | |
| FRA2J | APH | 2q37.3 | 0.657 | | |
| FRA3A | APH | 3p24.2 | 1.327 | | |

(continued)

**Table 21.2** (continued)

| Fragile site | Induction method | Cyto- band | Freq. in APH (%)[a] | Assoc. genes | Disease |
|---|---|---|---|---|---|
| FRA3B* | APH | 3p14.2 | 14.153 | *FHIT* | Loss or change of *FHIT* expression associated with common cancers including bladder, esophageal, lung, breast, and prostate cancers (Saldivar et al. 2010) |
| FRA3C | APH | 3q27 | 1.061 | | |
| FRA3D | APH | 3q25 | 0.546 | | FS expression linked to schizophrenia (Demirhan et al. 2006) |
| FRA4A | APH | 4p16.1 | 0.612 | | |
| FRA4C | APH | 4q31.1 | 2.175 | | |
| FRA4D | APH | 4p15 | 0.111 | | |
| FRA4F* | APH | 4q22 | 0.683 | *GRID2* | Autosomal recessive cerebellar ataxia associated with retinal dystrophy; ataxia and tonic upgaze (Hills et al. 2013; Van Schil et al. 2015) |
| FRA5C | APH | 5q31.1 | 0.373 | *SMAD5* | Copy number gain and increased expression in human hepatocellular carcinoma cells (Zimonjic et al. 2003) |
| FRA5D | APH | 5q15 | 0.834 | | Concomitant t(5;21)(q15;q22) and del(5)(q13q33) events in myelodysplastic syndrome (Kasi Loknath Kumar et al. 2016) |
| FRA5E | APH | 5p14 | 0.293 | | Copy number loss observed at 52.4% in early Barrett's esophagus (Lai et al. 2010) |
| FRA5F | APH | 5q21 | 0.515 | | FS expression significantly higher in patients with rectum cancer and their first-degree relatives (Tunca et al. 2000) |
| FRA6B | APH | 6p25.1 | 0.985 | | Copy number change associated with osteoporotic fractures, gyral pattern anomaly, and speech and language disorder in two dizygotic twins (Bozza et al. 2013; Oei et al. 2014) |
| FRA6C | APH | 6p22.2 | 0.062 | BAC CITB.564_C_7 | High LOH in cervical tumors; HPV integration site in cervical tumor CC171 (Rader et al. 1996; Thorland et al. 2000) |

(continued)

**Table 21.2** (continued)

| Fragile site | Induction method | Cyto- band | Freq. in APH (%)[a] | Assoc. genes | Disease |
|---|---|---|---|---|---|
| FRA6E* | APH | 6q26 | 2.823 | *PARK2* | Parkinson's; poor outcome in breast cancer (Ambroziak et al. 2015; Letessier et al. 2007) |
| FRA6F* | APH | 6q21 | 0.608 | *REV3L, DIF13, FKHRL*, etc. | Cancer; schizophrenia (Karayianni et al. 1999; Morelli et al. 2002) |
| FRA6G | APH | 6q15 | 0.115 | | Chromosome breakpoints in metastatic melanoma (Limon et al. 1988) |
| FRA7B* | APH | 7p22 | 0.799 | *THSD7A, SDK1, MAD1L1, MIRN589, MIRN339* | Recurrent breakpoint in multiple cancers (Bosco et al. 2010) |
| FRA7C | APH | 7p14.2 | 0.541 | | |
| FRA7D | APH | 7p13 | 0.55 | | Highly expressed in a female presenting severe immunodeficiency (Conley et al. 1986) |
| FRA7E* | APH | 7q21.2 | 0.501 | | |
| FRA7F | APH | 7q22 | 0.107 | | Fragile site expression linked to bipolar disorder and schizophrenia (Demirhan et al. 2006, 2009) |
| FRA7G* | APH | 7q31.2 | 0.111 | Caveolin-1, caveolin-2, *TESTIN* | Reduced *TESTIN* expression in 22% of cancer cell lines and 44% of the cell lines derived from hematological malignancies; caveolin-1 gene proposed to be a candidate tumor suppressor (Engelman et al. 1998; Tatarelli et al. 2000) |
| FRA7H* | APH | 7q32.3 | 2.374 | | Translocation involving 7q32 found in the RC-K8 cell line derived from a patient with terminal diffuse large B-cell lymphoma (Mishmar et al. 1998; Schneider et al. 2008) |
| FRA7I* | APH | 7q36 | 0.084 | *CNTNAP2* | Implicated in ASD (Rodenas-Cuadrado et al. 2014) |
| FRA7J | APH | 7q11 | 0.83 | *LIMK1, EIF4H(WBSCR1)* | Breakpoint found between the *LIMK1* and *EIF4H* (*WBSCR1*) genes in patients with Williams-Beuren syndrome and ASD (Plaja et al. 2015) |

(continued)

**Table 21.2** (continued)

| Fragile site | Induction method | Cyto- band | Freq. in APH (%)[a] | Assoc. genes | Disease |
|---|---|---|---|---|---|
| FRA8B | APH | 8q22.1 | 0.923 | | |
| FRA8C* | APH | 8q24.1 | 0.799 | *MYC* | Cluster of HPV18 integrations at 8q24 in primary cervical carcinoma; CFS expression frequent in bladder cancer (Ferber et al. 2004; Moriarty and Webster 2003) |
| FRA8D | APH | 8q24.3 | 0.182 | | |
| FRA9D | APH | 9q22.1 | 0.231 | | FS expression frequent in bladder cancer (Moriarty and Webster 2003) |
| FRA9E* | APH | 9q32 | | *PAPPA* | LOH, particularly loss of *PAPPA*, is linked to ovarian cancer (Callahan et al. 2003) |
| FRA10D | APH | 10q22.1 | 0.306 | *CTNNA3* | Decreased gene expression of *CTNNA3* in oropharyngeal squamous cell carcinomas (Gao et al. 2014) |
| FRA10E | APH | 10q25.2 | 0.191 | | |
| FRA10F* | APH | 10q26.1 | 0.408 | *FATS* | |
| FRA10G* | APH | 10q11.2 | 0.142 | *RET, NCOA4* | FS generates oncogenic *RET/PTC* rearrangements in human thyroid cells (Gandhi et al. 2010) |
| FRA11C | APH | 11p15.1 | 0.51 | | FS expression frequent in bladder cancer (Moriarty and Webster 2003) |
| FRA11D | APH | 11p14.2 | 0.928 | | |
| FRA11E | APH | 11p13 | 0.621 | | Translocation breakpoints at 11p13 found in CML patients. |
| FRA11F | APH | 11q14.2 | 1.771 | | Gene amplification linked to FRA11F expression in oral cancer (Reshmi et al. 2007) |
| FRA11G* | APH | 11q23.3 | 0.098 | | Interstitial deletions at 11q23.3 associated with abnormal ultrasound findings during prenatal diagnosis (Fechter et al. 2007; Liu et al. 2014) |
| FRA11H* | APH | 11q13 | 0.075 | | |
| FRA12B | APH | 12q21.3 | 0.364 | | |
| FRA12E | APH | 12q24 | 0.288 | | |
| FRA13A* | APH | 13q13.2 | 1.061 | *NBEA* | Neuropsychiatric disorders (Savelyeva et al. 2006a, b) |

(continued)

**Table 21.2** (continued)

| Fragile site | Induction method | Cyto- band | Freq. in APH (%)[a] | Assoc. genes | Disease |
|---|---|---|---|---|---|
| FRA13C | APH | 13q21.2 | 0.124 | | HPV16 integration site in the cervical tumor cell line SiHa (Thorland et al. 2000) |
| FRA13D | APH | 13q32 | 0.178 | | Fragile site expression linked to bipolar disorder and schizophrenia (Demirhan et al. 2006, 2009) |
| FRA14B | APH | 14q23 | 1.429 | | Found as a constant FS in bloom syndrome cell lines (Barbi et al. 1984; Shiraishi and Li 1993) |
| FRA14C | APH | 14q24.1 | 0.382 | | Found as frequent HPV integration site in HPV-related cancers (Bodelon et al. 2016) |
| FRA15A | APH | 15q22 | 0.08 | *RORA* | *RORA* gene expression is low in breast, prostate, and ovarian cancers (Zhu et al. 2006) |
| FRA16C | APH | 16q22.1 | 0.488 | | High expression at FRA16B/C found in peripheral blood lymphocytes in the male of a couple having trouble conceiving. Chr 16 instability detected in the sperm of the male and in embryos (Martorell et al. 2014) |
| FRA16D* | APH | 16q23.2 | 7.576 | *WWOX/WOX1/FOR* | *WWOX* gene dysregulation associated with multiple cancers, including pancreatic adenocarcinoma, renal cell carcinoma, and endocrine and exocrine carcinomas (Li et al. 2014) |
| FRA17B | APH | 17q23.1 | 0.071 | | HPV integration site in cervical tumor CC226 (Thorland et al. 2000) |
| FRA18A | APH | 18q12.2 | 0.475 | | High frequency of LOH on chr 18 in esophageal squamous cell carcinoma (Karkera et al. 2000) |
| FRA18B | APH | 18q21.3 | 0.182 | *DCC* | Found as FSs with frequent interstitial deletions in HIV lymphomas (Capello et al. 2010) |

(continued)

**Table 21.2** (continued)

| Fragile site | Induction method | Cyto- band | Freq. in APH (%)[a] | Assoc. genes | Disease |
|---|---|---|---|---|---|
| FRA18C* | APH | 18q22.1–18q22.2 | 0.067 | *DOK6* | FS expression in the father of a patient with Beckwith-Wiedemann syndrome and a chromosome truncation 18q22-qter (Debacker et al. 2007) |
| FRA20B | APH | 20p12.2 | | | |
| FRA22B | APH | 22q12.2 | 0.275 | | Elevated FS expression in bone marrow and peripheral blood of young cigarette smokers (Kao-Shan et al. 1987) |
| FRAXB* | APH | Xp22.31 | 5.494 | *STS, GS1 HDHD1 MIR4767* | Deletions within FRAXB seen in 15% of primary tumors and cell lines examined (Arlt et al. 2002) |
| FRAXC | APH | Xq22.1 | 2.121 | *DMD, IL1RAPL1* | |
| FRAXD | APH | Xq27.2 | 0.209 | | |
| FRA1H* | 5-AZ | 1q41–q42.1 | 0.129 | *USH2A, ESRRG, MIRN194–1, MIRN215* | |
| FRA1J | 5-AZ | 1q12 | 0.12 | | |
| FRA9F | 5-AZ | 9q12 | 0.453 | | |
| FRA19A | 5-AZ | 19q13 | 0.053 | | |
| FRA4B | BrdU | 4q12 | 0.142 | | |
| FRA5A | BrdU | 5p13 | 0.191 | | |
| FRA5B | BrdU | 5q15 | | | |
| FRA6D | BrdU | 6q13 | 0.031 | | |
| FRA9C | BrdU | 9p21 | | | |
| FRA10C | BrdU | 10q21 | 0.089 | | |
| FRA13B | BrdU | 13q21 | | | |
| FRA4E | UC | 4q27 | 0.382 | | |

Molecularly mapped fragile sites are indicated by an asterisk
5-*AZ*, 5-azacytidine, *APH* aphidicolin, *BrdU* bromodeoxyuridine, *UC* unclassified, *FS* fragile site, *LOH* loss of heterozygosity, *MSI* microsatellite instability, *ASD* autism spectrum disorder
[a]Data derived from Mrasek et al. (2010)

in her abnormal child, her husband, and her father (Dekaban 1965). However, this study raised a question that is still relevant in the research field today. There were two types of chromosome abnormality in the woman's blood cultures. A relatively more frequent type consisted of a break near the third telomere-proximal portion of the long arm on the presumed Chromosome 9 (24.8%) and a less frequent one involved aberrations such as dicentrics, rings, and deletions on random chromosomes

(7.1%). In contrast, her skin culture contained a much lower frequency of breakage on Chromosome 9 (6%), despite a comparable level of other chromosome aberrations (8.8%). Moreover, the break seen in the skin culture occurred at a different location (telomere-distal) on Chromosome 9 from that seen in the blood cultures (telomere-proximal). One theory put forth suggested that the X-ray irradiations had induced a clone of abnormal cells with a telomere-proximal fragile site on Chromosome 9 in the woman's blood-forming tissues but not in skin tissues. Alternatively, though it could not be ascertained thoroughly due to family members in absentia, there existed tissue-specific formation of chromosome fragile sites. The latter possibility was subsequently more convincingly demonstrated (Kuwano et al. 1990; Murano et al. 1989a). However, the mechanism of tissue-specific fragile site formation still remains a major challenge in the field.

### *21.2.2 First Report of a Heritable Fragile Site*

The first heritable fragile site was demonstrated in a mother-and-daughter case, which involved a fragile site on the long arm of Chromosome 2 near the centromere (Lejeune et al. 1968). The heritability of this fragile site was later confirmed independently (Ferguson-Smith 1973). Curiously, this fragile site gave rise to duplication of the centromere-distal two-thirds of 2q and formation of a three-armed chromosome, which was referred to as qh(2). It was speculated that this endo-duplication event was the result of interruption of a centromere-originated DNA replication signal by the fragile site. Alternatively, it was thought that qh(2) formation was reminiscent of interstitial telomere element-induced endo-duplications elsewhere (Hsu 1963). It was shown that 2q indeed contains interstitial telomere elements, but this fusion site is distinct from the fragile site band 2q11.2 (Ijdo et al. 1991). Finally, it was also surmised that qh(2) formation was due to chromatid breakage followed by mitotic nondisjunction (Ferguson-Smith 1973). To this date, the mechanism for the duplicated q-arms remains a mystery.

Phenotypically the mother and daughter were both of short stature and had intellectual deficiencies, which may or may not be related to the expression of the fragile site. However, subsequent studies demonstrated that the fragility at 2q11.2, which resides in the presumed cytoband responsible for the formation of qh(2), was observed at a significantly higher frequency in "mentally subnormal" school children (Kahkonen et al. 1986) as well as in patients with schizophrenia (Chen et al. 1998). It was not until recent that this fragile site was molecularly characterized as a site with CGG repeat expansion impacting the *AFF3* gene and was detected in families with a broad range of neurodevelopmental disorders (Metsu et al. 2014a).

### 21.2.3 First Demonstration of Mendelian Transmission of a Fragile Site

A fragile site on 16q22 was examined in a large family of a boy who had recurrent cold urticaria and immunoglobulin A deficiency (Magenis et al. 1970). Transmission of the fragile site followed a Mendelian pattern. However, later it was demonstrated that this fragile site is distal from the haptoglobin gene whose deficiency is linked to IgA deficiency, suggesting that the IgA condition in the propositus was likely a coincidence (Simmers et al. 1986). Fragility at 16q22 has since been implicated in a wide spectrum of neurological disorders (Demirhan et al. 2006; Kerbeshian et al. 2000) as well as in other conditions such as neutropenia (Glasser et al. 2006; Tassano et al. 2010) and cleft palate (Bettex et al. 1998; Dunner et al. 1983; Janiszewska-Olszowska et al. 2013; McKenzie et al. 2002). A recent study also reported elevated fragile site formation at 16q22.1 in an embryo from a couple who had difficulty achieving pregnancy and in the sperm from the father (Martorell et al. 2014). However, cytogenetic breakage and potential disease-associated gene(s) at this locus are yet to be molecularly characterized.

### 21.2.4 First Fragile Site Linked to a Clinical Phenotype

The vast majority of fragile sites during the early discoveries appeared innocuous and not associated with any phenotypic abnormalities, even when present in homozygous conditions such as those fragile sites at 10q25.2 and 12q24 (Sutherland 1981; Voiculescu et al. 1991). The only fragile site definitively associated with a clinical phenotype was that which resides on Xq27.3 (FRAXA) and was associated with the Martin-Bell or the fragile X syndrome, an X-linked and most common familial form of mental retardation (Giraud et al. 1976; Harvey et al. 1977; Lubs 1969). Subsequent studies demonstrated that the FRAXA fragility is the result of a CGG repeat expansion and is correlated with hypermethylation, gene silencing, as well as delayed replication at the fragile X mental retardation 1 (*FMR1*) gene locus (Bell et al. 1991; Dietrich et al. 1991; Hansen et al. 1992, 1993; Heitz et al. 1991; Pieretti et al. 1991). A full mutation (>200 CGG repeats) at the *FMR1* locus causes gene silencing and the loss of the protein product, FMRP, which causes the loss of synaptic plasticity and the fragile X pathology. Fragile X biology has been the subject of numerous and up-to-date reviews to which we direct the reader for a comprehensive understanding of the disease etiology, mechanism, and intervention (Ligsay and Hagerman 2016; Lozano et al. 2016; Santoro et al. 2012; Wang et al. 2012; Zhao and Usdin 2016).

## 21.3 Classification of "Common" vs. "Rare" Fragile Sites

### *21.3.1 Population Frequencies*

The Edinburgh survey estimated that from 3% to 5% of the population contained identifiable chromosomal variants (Court Brown 1966). Subsequent population studies defined two types of fragile sites: those that were present in <1% of the general population and those that were present at a theoretical frequency between 1% and 99% or polymorphic (Hecht 1988). Later, this fine distinction between the two groups was relinquished, and collectively they were referred to as "rare fragile sites" with an observed maximal frequency of 5% (Schmid et al. 1986). This classification was also necessitated by the discovery of what appeared to be common or ubiquitous fragile sites, which were present in all 12 subjects regardless of sex or clinical phenotypes (Glover et al. 1984). Thus, presently, fragile sites are classified as "rare" and "common" fragile sites (RFSs and CFSs, respectively) based on the definitions above.

Both RFSs and CFSs can be further characterized by another important metric—the frequency at which fragile sites are observed/expressed in the cells from a given individual, which is akin to a measurement of penetrance. As current literature makes broad reference to frequencies of fragile sites—i.e., at a population level vs. at a cellular level within an individual—it is important to note the difference between these two metrics. For instance, the frequency of FRAXA expression in fragile X individuals can be as high as 50% (Glover et al. 1984). In contrast, the frequency of aphidicolin-induced CFS expression in individuals can be as low as 0.01% based on a large-scale study (Mrasek et al. 2010). Therefore, arguably a CFS with extremely low penetrance may be better characterized as a RFS because it is conceivable that certain individuals, yet to be identified, might exhibit abnormally high penetrance.

### *21.3.2 Methods of Induction*

It was serendipity that led to the discovery that many RFSs discovered by routine diagnostic cytogenetic screen in early studies were unstable in medium 199, which is deficient for folic acid (Sutherland 1977). This finding thus defined the first group of RFSs, which are sensitive to folate stress induced by folic acid or thymidine deprivation, inhibitors of folate metabolism, inhibitors of thymidylate synthetase, and excess thymidine (Sutherland 1991). Systematic analyses revealed that other culture conditions, such as pH, also impact the frequency of fragile site expression (Sutherland 1979). RFSs can also be induced by a group of chemicals of the non-folate stress type such as a nucleotide analog, bromodeoxyuridine, and a base intercalator, distamycin A. In contrast, CFSs are primarily induced by a DNA polymerase inhibitor, aphidicolin, and to a lesser extent by other chemicals including bromodeoxyuridine and 5′-azacytidine.

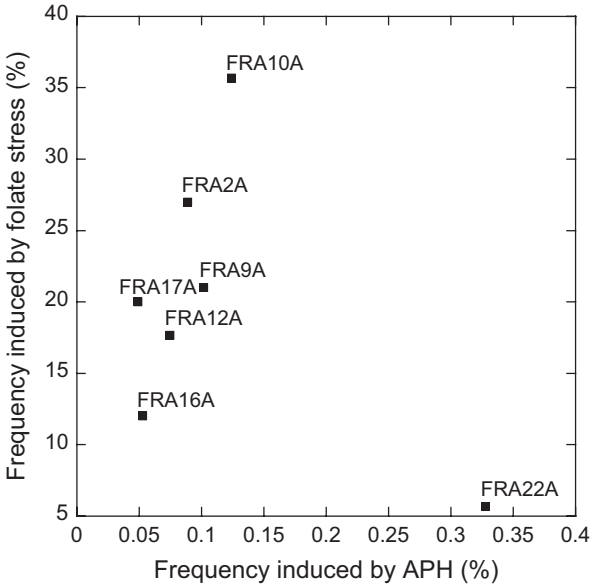


**Fig. 21.1** Comparison between *RFS* expression frequencies induced by folate stress and by aphidicolin (APH). Experimental values obtained from Kähkönen et al. (1989) and Mrasek et al. (2010), respectively

It is unclear why RFSs and CFSs show drug-specific induction. Genomic loci may be differentially susceptible to drug-induced replication perturbation. This apparent drug specificity of fragile site expression is likely also due to limitations of the cytological screening methods. Consequently, a given fragile site has been traditionally associated with a specific drug though other drugs could also induce its expression, albeit not as potently. Consistent with this notion, the most frequent aphidicolin-induced CFSs have been shown also inducible by thymidylate stress (Kähkönen et al. 1989). Thus, thymidylate stress can induce not only RFSs but also CFSs, demonstrating effectively "cross-induction" of these two classes of fragile sites. The cross-induction of RFSs by aphidicolin was also confirmed by a genomic scale survey (Mrasek et al. 2010). We further asked if there is a correlation between the expression frequencies (percentage of cells showing fragile sites) of seven RFSs when induced by folate stress (Kähkönen et al. 1989) vs. by aphidicolin (Mrasek et al. 2010). For the study by Käukönen et al., we calculated the expression frequencies only from phenotypically normal individuals for comparison with the study by Mrasek et al. With the exception of a single outlier, FRA22A, which appeared to be highly inducible by aphidicolin, the remaining six RFSs showed a positive correlation ($R = 0.8$) between the induction frequencies by the two conditions (Fig. 21.1). Nevertheless, the differential mechanisms of drug induction of fragile site expression await further exploration.

### *21.3.3 DNA Sequence and Structural Features*

Molecular cloning permitted structural distinction between RFSs and CFSs beyond population frequencies and induction methods. RFSs are characterized by repetitive DNA sequences. Of the 30 RFSs, 12 have so far been identified with a repeat motif with 10 containing a CGG trinucleotide repeat tract and 2 containing an AT-rich minisatellite repeat (Table 21.1). Repetitive DNA can generate non-B DNA structures or alternative conformations with unusual secondary structures (Choi and Majima. 2011). For instance, folate-sensitive RFSs are enriched in CGG repeats that can form stable hairpins, slipped strand structures, G-quadruplexes, and i-tetraplex structures (Fry and Loeb 1994; Usdin and Woodford 1995; Kang et al. 1995). CGG repeats are capable of pausing or stalling replication forks both in vitro and in vivo (Samadashwily et al. 1997). These repeats are polymorphic in normal individuals but undergo dynamic expansion/contraction, resulting in the cytogenic expression of fragile sites under folate stress (Kremer et al. 1991; Verkerk et al. 1991). Non-folate-sensitive RFSs on the other hand consist of expandable AT-rich minisatellite repeats and are specifically induced by AT-dinucleotide binding chemicals such as distamycin A and berenil. For instance, the most frequently observed RFS, FRA16B, contains a polymorphic 33 bp AT-rich minisatellite repeat, which can expand to 2000 copies in certain individuals (Yu et al. 1997). Fourteen copies of this 33 bp AT-rich minisatellite repeat were shown to form DNA secondary structures and cause replication fork stalling, fork regression, and polymerase skipping in vitro (Burrow et al. 2010). Similarly, the BrdU-induced non-folate-sensitive RFS, FRA10B, shares homology with the 33 bp minisatellite in FRA16B and contains an 11 bp inverted repeat that can form hairpins (Handt et al. 2000; Yu et al. 1997).

In contrast, CFSs are not characterized by expandable di- or trinucleotide repetitive sequence. However, some of the cloned CFSs are enriched for short interrupted AT-rich islands with high torsional flexibility and high propensity to form stable secondary structures, similar to the RFSs (Dillon et al. 2013; Zlotorynski et al. 2003). Moreover, a study using a yeast-based genetic assay identified a flexibility peak region (Flex1) of FRA16D with a perfect AT/TA repeat element capable of stalling replication forks (Zhang and Freudenreich 2007). Interestingly, these repeats resemble the AT-rich minisatellites found in non-folate RFSs. The extent of stalling depends on the length of the repeat. Therefore, there appears to be a shared mechanism of repeat-based secondary structure formation and perturbation of the replication fork between RFSs and CFSs, the difference being that RFSs have greater expandability than CFSs (Schwartz et al. 2006). Are there any other cis elements, in addition to DNA secondary structures, that define fragile sites? An RNA/DNA hybrid molecule known as R-loop appears to qualify. R-loops play a wide array of functions in normal cells. For instance, R-loops occur in replication origins in mitochondria as primers for DNA replication (Lee and Clayton 1996). R-loops at CpG islands may also facilitate replication origin specification by generating single-stranded DNA in the nuclear genome (Lombrana et al. 2015). In stimulated B-lymphocytes, R-loop formation at the immunoglobulin heavy chain locus

facilitates class switch recombination (Yu et al. 2003). Finally, it was shown that R-loop formation in the guanine-rich transcription pause sites downstream of the poly-A signals is required for transcription termination (Skourti-Stathaki et al. 2011). The genome-wide association of GC skew (asymmetry in guanine distribution between the two strands of DNA), which is conducive to R-loop formation, with transcription termination sites was subsequently validated (Ginno et al. 2013). On the flip side, R-loops are increasingly associated with genome instability and human diseases (Groh and Gromak 2014; Santos-Pereira and Aguilera 2015). In yeast, R-loop formation can sensitize DNA to damaging agents, causing double-strand breaks (Huertas and Aguilera 2003; Li and Manley 2005; Sordet et al. 2009). R-loops at transcription termination sites also require proper resolution by factors such as senataxin, and unresolved R-loops can cause genome instability (Hatchi et al. 2015; Skourti-Stathaki et al. 2011). R-loops can also trigger epigenetic changes in the DNA and bring about the formation of repressive chromatin, which in turn could impede DNA replication (Groh et al. 2014). Evidence for blockage of replication fork by R-loop formation was demonstrated for FRA16D (Madireddy et al. 2016). Finally, R-loop formation can endanger genome stability by incurring replication-transcription conflicts, as exemplified by their association with CFS formation, particularly in large genes such as *FHIT* (Helmrich et al. 2011). The question becomes what roles do R-loops play in replication stress-induced fragile site formation, the corollary being, is there a genome-wide correlation between R-loop content/density and the probability of fragile site formation?

Recent advance in experimental mapping of R-loops capitalized on the utility of the S9.6 antibody, which has a sequence-independent affinity toward the A/B helical RNA/DNA duplex, and coupled the immunoprecipitation of the DNA/RNA hybrid with high-throughput sequencing. Variations of this methodology include DRIPc-seq (DNA/RNA immunoprecipitation followed by cDNA conversion coupled with high-throughput sequencing, an improved version of the previous DRIP-seq method) (Sanz et al. 2016) and RDIP (RNA/DNA immunoprecipitation, implementing key technical modifications in RNase I pretreatment and DNA fragmentation by sonication) (Nadel et al. 2015). DRIPc-seq detected ~70,000 R-loop peaks in the human embryonic carcinoma Ntera2 cells, which is comparable to the number of R-loops detected by RDIP-seq (~64,000 and 39,000 in IMR-90 and HEK 293 T cells, respectively). Consistent with R-loops being co-transcriptional structures, DRIPc-seq peaks were found predominantly in RNA polymerase II-transcribed genes, with two- to three-fold enrichment at the promoter and terminator regions (Sanz et al. 2016). Interestingly, this co-transcriptional model of R-loop formation was contended by the RDIP-seq study, at least at the ribosomal DNA loci (Nadel et al. 2015). Moreover, using a more sensitive readout (than RNA-seq) for transcription, the global run-on sequencing (GRO-seq) method, it was found that only 47.7% of the RNA/DNA hybrids are associated with active transcription (Nadel et al. 2015). Finally, the RDIP-seq study reported a moderate depletion of R-loops at the terminators, in contrast to a twofold enrichment at the promoters (included in a 1.5 kb window downstream from the transcription start site) (Nadel et al. 2015). These studies highlighted the dynamic nature of R-loops and their potential cell

**Table 21.3** Distribution of *RLFS*s in the genes located in 1973 *RFS*-associated and 13 cloned *CFS*-associated genes vs. all annotated genes in the genome

| No. of RLFSs per gene | RFSs | | CFSs | | Whole genome | |
|---|---|---|---|---|---|---|
| | No. of genes | % | No. of genes | % | No. of genes | % |
| 1 | 416 | 21.08 | 0 | | 16,362 | 41.19 |
| 2 | 279 | 14.14 | 1 | 7.69 | 7,910 | 19.91 |
| 3 | 219 | 11.10 | 2 | 15.38 | 4,563 | 11.49 |
| 4 | 164 | 8.31 | 1 | 7.69 | 2,880 | 7.25 |
| 5 | 137 | 6.94 | 0 | | 1,992 | 5.02 |
| 6–10 | 366 | 18.55 | 2 | 15.38 | 3,811 | 9.59 |
| 11–50 | 362 | 18.35 | 7 | 53.85 | 2,132 | 5.37 |
| 51–100 | 24 | 1.22 | 0 | | 59 | 0.15 |
| >100 | 6 | 0.31 | 0 | | 11 | 0.03 |
| Total | 1973 | 100 | 13 | 100 | 39,720 | 100 |

Statistics from the whole genome were previously reported (Wongsurawat et al. 2012)

type-specific formation. They also necessitate an unbiased approach to identify R-loops in the human genome.

Sequences with high CG content are more conducive to R-loop formation due to high thermostability of the rG·C base pair (Roy and Lieber 2009; Sugimoto et al. 1995; Tracy et al. 2000). Training on previously discovered R-loops, a quantitative model of R-loop forming sequences (RLFSs) has been developed and used to predict RLFSs in the known transcribed regions of the human genome (Wongsurawat et al. 2012). Using the R-loop database (http://rloop.bii.a-star.edu.sg/), we tallied the number of R-loops in each gene of the RFSs as well as calculated their density (number of R-loops per kb in a given gene), as reported in Table 21.1. Our analysis shows that genes residing in RFSs have relatively higher concentration of R-loop forming sequences compared to all genes (Table 21.3). We also queried which RFSs and genes have the highest R-loop content (top 1%), either based on total number or density (Fig. 21.2). The analysis reveals that FRA22A is enriched for genes with the highest number of R-loops (more than 70 R-loops per gene) and that FRA19B is enriched for genes with the highest R-loop density (more than 2.5 R-loops per kb sequence). High R-loop content, together with the fact that many RFSs contain CGG repeat, is intriguing. Because the computational model for R-loop prediction defines an R-loop core sequence as clusters of 3–4 contiguous Gs interspersed by a single nucleotide, CGG trinucleotide repeats per se are not R-loop forming sequences. It would be interesting to test if these two entities have coevolved and that the higher than average R-loop content in the RFSs predisposes CGG repeats to break.

We performed similar analysis with a subset ($n$ = 11) of CFSs, which have been cloned, and the associated genes identified (13 total, with FRAXC and FRA10G each housing two genes): FRA1B(*DAB1*), FRA2F(*LRP1B*), FRA3B(*FHIT*), FRA4F(*GRID2*), FRA6E(*PARK2*), FRA10D(*CTNNA3*), FRA10G(*RET*, *NCOA4*), FRA15A(*RORA*), FRA16D(*WWOX*), and FRAXC(*DMD*, *IL1RAPL1*). Seven of

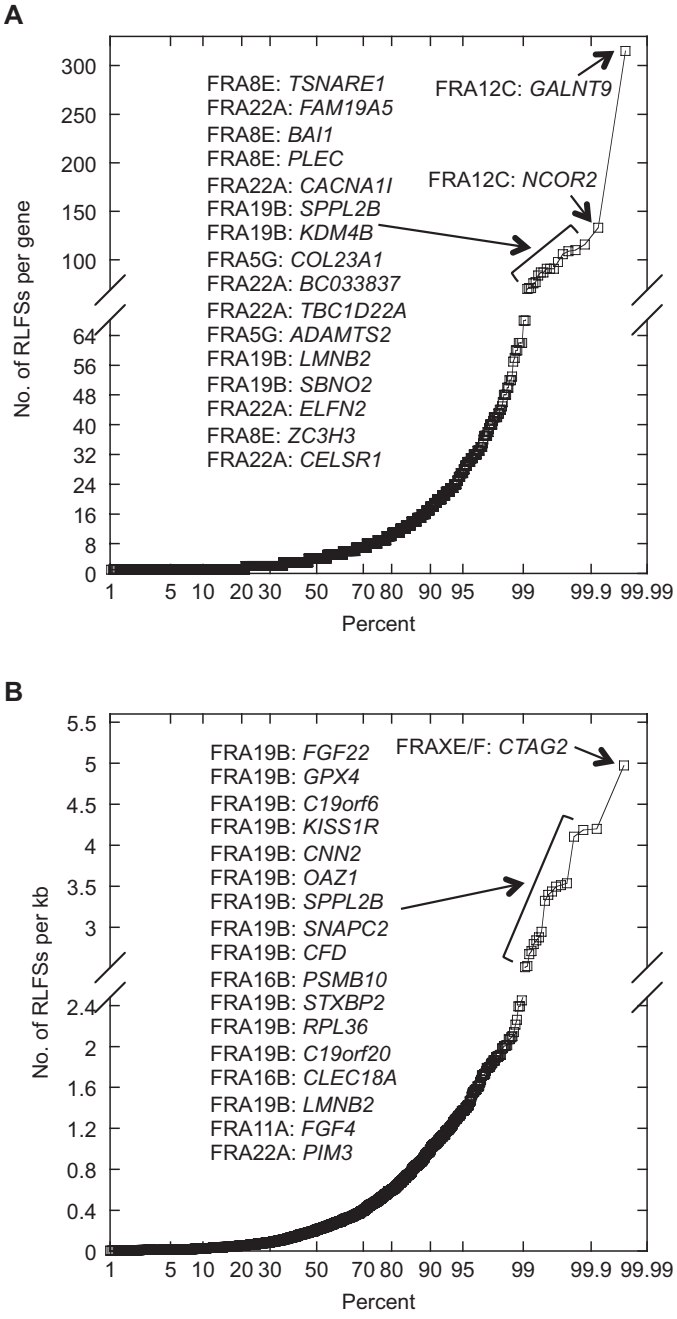


**Fig. 21.2** Probability plots showing the distribution of 1973 genes within *RFSs* by the number of RLFSs per gene (**A**) or by the number of *RLFSs* per kb for a given gene (**B**). The top 1% of genes in each plot are as shown

these 13 genes (54%) contain 11–50 R-loops, which appeared significantly higher than the reported genomic average (5%) with the same level of R-loop content (Table 21.3). Thus, R-loop formation might also be associated with CFSs, consistent with previous findings that breakage at CFSs harboring large genes can be due to R-loop formation deterring transcription complexes (Helmrich et al. 2011; Wilson et al. 2015).

### *21.3.4 Mechanisms of Fragile Site Formation*

Early studies observed that the fragile X chromosome showed delayed or incomplete replication due to the slowing down or stalling of replication forks (Hansen et al. 1993). This study presented an attractive model for fragile site development as approximately 1% and 20% of the genome in transformed and non-transformed human cells, respectively, undergoes replication even during mitosis (Widrow et al. 1998). Subsequent studies also confirmed late replication timing of a handful of CFSs (Handt et al. 2000; Hellman et al. 2000; Le Beau et al. 1998; Palakodeti et al. 2004; Pelliccia et al. 2008; Wang et al. 1999). However, not all fragile sites appear to replicate late—some are located in the interface of early- and late-replicating regions (El Achkar et al. 2005; Handt et al. 2000), and others are in fact associated with early replication timing (Barlow et al. 2013). Besides replication timing anomalies, CFSs can also be sites of collisions between the replication and the transcription machineries (Helmrich et al. 2011). Additionally, defective processing of CFSs in a Rad52-dependent DNA repair pathway has also been proposed to contribute to fragile site instability (Bhowmick et al. 2016; Sotiriou et al. 2016). However, the requirement for DNA repair is likely linked to a potential underlying defect in DNA replication. Therefore, there appears to be two major factors that underlie fragile site instability: defective DNA initiation/progression and replication-transcription conflicts (Le Tallec et al. 2014; Ozeri-Galai et al. 2014; Sarni and Kerem 2016). Here, we highlight two major areas with competing models, one regarding the replication defects at fragile sites and the other pertaining to the impact of transcriptional status of large genes on chromosome fragility.

While a strong correlation exists between DNA secondary structure formation and fork stalling in both micro- and minisatellites of RFSs, evidences for such direct implication for instability in CFSs are contradictory. Is chromosome fragility the consequence of replication fork stalling at AT-rich sequences in CFSs? Previous studies have shown that replication across CFSs is delayed compared to other regions of the genome, particularly in the presence of aphidicolin (Hellman et al. 2000; Le Beau et al. 1998; Palakodeti et al. 2004; Wang et al. 1999). However, recent studies employing DNA combing have countered that replication fork speed is not different between fragile sites (FRA3B and FRA6E) and non-fragile regions, with or without aphidicolin treatment (Letessier et al. 2011; Palumbo et al. 2010). It was further shown that fragility at FRA3B is the consequence of deficient origin activation within the fragile site when replication fork is slowed down by aphidicolin

(Letessier et al. 2011). In contrast, using a similar DNA combing approach, it was shown that replication forks indeed stall at AT-rich sequences in FRA16C (Ozeri-Galai et al. 2011). These contradictory results can be partly explained by significant differences in experimental conditions for replication fork rate measurement, such as the duration of pulse labeling by nucleoside analogs (in DNA combing) and aphidicolin dosage. Considering that fragile site expression level varies significantly with aphidicolin concentrations (Glover et al. 1984), direct comparison between fork rates measured under such different conditions is tenuous. The fact that FRA16C coincides with a RFS, FRA16B, further precludes direct comparison between these studies. Thus, future studies will undoubtedly benefit from standardized experimental conditions. However, both studies agree that paucity of origins in the fragile site region is at least partially responsible for the delayed replication completion of fragile sites (Letessier et al. 2011; Ozeri-Galai et al. 2011; Palumbo et al. 2010).

Another contentious topic is whether the transcriptional status of a large gene impacts fragile site formation. Helmrich et al. suggested that collision between replication and transcription at large genes causes chromosome fragility and, further, breakage frequency is correlated with gene expression level (Helmrich et al. 2012). The authors further posited that R-loop formation as a consequence of transcription along the fragile locus could pose a serious obstacle to the replication fork. However, Le Tallec et al. argued that the expression level of large genes does not correlate with chromosome fragility (Le Tallec et al. 2013). Again, this apparent discrepancy between the two studies is at least attributable to multiple differences in experimental conditions including aphidicolin concentration, definition of large genes, and calculation of break frequency. It further underscores the importance of applying standardized or comparable experimental conditions for effective comparisons between studies.

Finally, an altered epigenetic environment in the fragile loci could be another cause for fragile site instability. The inability to undergo condensation at the time of mitosis could result in DNA strand breakage. Using an in vitro nucleosome reconstitution assay, it was shown that CGG repeats with greater than 50 copies exclude nucleosome and that this exclusion is dependent on the length of the repeat (Wang and Griffith 1996; Wang et al. 1996). It was also shown that the expanded AT-rich minisatellites in FRA16B can exclude nucleosome assembly albeit only in the presence of distamycin A (Hsu and Wang 2002). Finally, it has been shown that hypoacetylation occurs in CFSs compared to genomic average, indicating a compact chromatin around CFSs (Koch et al. 2007; Savelyeva and Brueckner 2014).

### *21.3.5 Disease Associations*

Fragile sites have been associated with both genetic and epigenetic instability (Smith et al. 2010). Currently there are 11 RFSs that have been molecularly mapped, i.e., the gene(s) that are impacted by fragile site formation have been identified.

Many of them are associated with a definitive human disease, predominantly a neurological disorder (Table 21.1). Interestingly, folate-sensitive RFSs are specifically associated with neuropsychiatric disorders including schizophrenia, autism spectrum disorders (ASDs), and mental retardation, while non-folate-sensitive RFSs are associated with a more diverse set of disorders such as infertility and Langer-Giedion syndrome. Schizophrenia, ASDs, and bipolar disorders are all complex neurological disorders that have intricate association with genetics and the environment (Kerner 2014; Miles 2011; Smith et al. 2010). No single gene can account for all the symptoms that characterize these diseases. Therefore, it stands to reason that the genes impacted by chromosome fragility at the folate-sensitive RFSs have a global impact on gene expression and/or protein production in the brain. Many of the CGG repeat-containing genes indeed have high expression in the brain (*AFF3*, *ZNF713*, *FAM10AC1*, *FMR1*, *FMR2*) (Uhlen et al. 2010). As the biochemical functions of these RFS-impacted genes become clear, it will help us understand the disease etiology in each of the associated diseases. For instance, the protein product of *FMR1*, FMRP, is an RNA-binding protein and is estimated to bind 4% of the mRNAs in the brain and regulate their translation (Santoro et al. 2012). Therefore, it remains a challenge to fully understand the genomic impact of FMRP deficiency in the fragile X syndrome.

Moreover, differential expression levels of the disease-associated genes can cause different diseases. For instance, in the case of the *FMR1* gene at FRAXA, a full mutation (>200 CGG repeats) induces *FMR1* gene silencing and results in the fragile X syndrome, while premutation alleles (55–200 CGG repeats) cause fragile X-associated ataxia/tremor syndrome and fragile X-associated primary ovarian insufficiency [note: premutation alleles do not cause chromosome fragility] (Galloway and Nelson 2009; Garcia-Arocena and Hagerman 2010; Santoro et al. 2012; Usdin et al. 2014). These observations suggest that impaired function of the RFS-associated genes can also impact organs other than the brain. The aforementioned brain-expressed genes associated with RFSs also show high expression in the reproductive organs (Uhlen et al. 2010). In addition, *FAM11A* gene expression is high in bone marrow, nervous system, and endocrine glands (Unger et al. 2013). Finally, there seems to be a dominance of neurological disorders, as opposed to cancer, associated with RFSs for unknown reasons. *CBL2* is the only CGG repeat-associated gene that is both seen in neurological disorders and cancer—while CGG repeat expansion and fragility at *CBL2* are linked to Jacobsen syndrome (Jones et al. 1995), *CBL2* has also been reported as a proto-oncogene associated with cancer breakpoints in several forms of leukemia and lymphomas (Fu et al. 2003).

In contrast, CFSs are clearly associated with cancer breakpoints and their expression linked to carcinogenesis (Arlt et al. 2006; Ma et al. 2012). Interestingly, some CFSs are also implicated in neurological disorders (Parkinson's, schizophrenia, and intellectual disability) and a variety of diseases associated with immunodeficiency, bone disorders, and infertility (Table 21.2). Twenty-eight of the 90 CFSs have genes associated with schizophrenia (Smith et al. 2010). Two CFSs, FRA7H and FRA7F, also showed high expression in cells from bipolar disorder patients compared to normal individuals when induced with folate-deficient media (Demirhan et al.

2009). These studies indicate that CFSs may trigger neurological symptoms more frequently than previously thought. The latter study also highlights the importance of understanding the cross-induction of fragile sites by chemicals and growth conditions.

Molecular cloning has enabled the identification of disease-associated gene(s) within a broadly defined fragile site. As shown above, R-loops are enriched in both RFSs and CFSs compared to genomic average. R-loops have been associated with neurological disorders such as the dominant juvenile form of amyotrophic lateral sclerosis type 4 (*ALS4*) and a recessive form of ataxia oculomotor apraxia type 2 (*AOA2*) (Chen et al. 2004; Moreira et al. 2004). A firm link between R-loop and oncogenesis was also established when it was shown that *BRCA2*, mutated in breast and ovarian cancer, is required to prevent R-loop accumulation and genome instability (Bhatia et al. 2014). We speculate that RLFSs can serve as a marker for disease-associated genes. Among the genes residing in RFSs, *GALNT9* contains the highest number (315) of R-loops (Fig. 21.2). It has been shown that *GALNT9* is frequently methylated and silenced in breast to brain metastasis (Pangeni et al. 2015) and its gene expression is a prognostic marker in neuroblastoma patients (Berois et al. 2013). Whether the high R-loop content plays a role in epigenetic regulation of *GALNT9* is an interesting question that warrants further investigation. Similarly, *CTAG2* shows the highest R-loop density (5 per kb sequence) among RFS-associated genes (Fig. 21.2). In CFSs, the *RET* gene at FRA10G has the highest number of R-loops and the highest R-loop density (1 every 1.6 kb, Fig. 21.2). *RET* rearrangements have been observed in several cancerous cell lines (Dillon et al. 2010). Undoubtedly, systematic analysis of R-loop distribution in all known CFSs would be vitally important.

## 21.4 Genome-Wide Mapping and Analysis of Fragile Sites

Many of the questions regarding population frequency and penetrance of fragile sites can be more effectively addressed by studies with increased scale and resolution. For instance, are there multiple breakage hot spots in a fragile site? How do the breakage spectra vary between cell types and inducing drugs? Mrasek et al. systematically identified aphidicolin-induced CFSs by screening 25,000 metaphase chromosome spreads isolated from lymphocytes of three normal and unrelated individuals (Mrasek et al. 2010). As alluded to above, this study demonstrated that the classically defined RFSs are less dependent on folate stress for induction than previously considered—all but 3 (FRA6A, FRA12D, and FRAXA) of the 30 RFSs were induced by aphidicolin, and only 4 were present in fewer than 3 individuals (total frequency among 3 individuals for each site listed in Table 21.1). However, based on our analysis of the data reported by Mrasek et al., the penetrance of RFS when induced by aphidicolin is significantly lower than that of CFSs, with the median levels being 0.075% and 0.464%, respectively, confirming the biased potency of aphidicolin toward CFSs.

Cytogenetic screens, while discernible and powerful, are not amenable for genome-wide and high-resolution identification of fragile sites. The advent of deep sequencing technology now permits fragile site mapping at a global scale and a faster pace. Currently the following methods have been applied to map DNA double-strand breaks (DSBs) in the human genome: BLESS (direct in situ break labeling, enrichment on streptavidin, and next-generation sequencing) (Crosetto et al. 2013), RAFT (rapid amplification of forum termini involving direct ligation of biotinylated oligonucleotides to DNA DSBs) (Tchurikov et al. 2015), and DSB-seq (using terminal deoxyribonucleotidyl transferase labeling of DSBs with biotinylated nucleotides followed by streptavidin pull-down and library construction) (Baranello et al. 2014). However, only one study applied conditions to induce CFSs, and it identified over 2429 aphidicolin-induced DSBs after correction for copy number variation in HeLa cells (Crosetto et al. 2013). The authors stated "many CFSs were scored as sensitive to aphidicolin following our approach." Based on our analysis, 190 (8%) and 574 (24%) of these 2429 DSBs overlap with the known rare and common fragile site regions (defined as cytoband coordinates from the UCSC Human Genome Database), respectively. The apparent lack of correlation for these aphidicolin-induced DSBs with RFSs is not unexpected. But the moderate level of overlap with CFSs begs discussion, and we attribute it to the following reasons. First, the moderate level of concordance is most likely the result of cell type (HeLa)-specific fragile site expression with fragile sites primarily defined in lymphocytes. Second, it also highlights the fundamental difference between methodologies. On one hand, cytologically defined chromosome breakage might include also single-stranded DNA breakage which would evade detection by BLESS. On the other hand, computationally predicted micro-fragile chromosomal regions might be missed by cytological screening (Thys et al. 2015). Finally, the usage of growth medium and concentrations of inducing drugs, e.g., aphidicolin, are not standardized across different studies and impinge on fragile site formation. For these reasons, it is at once a necessity and a challenge for future genomic mapping of fragile sites to compare different cell types with standardized inducing conditions.

## 21.5 Unsolved Mysteries of Chromosome Fragile Sites

So far, we have discussed some of the contentious topics in chromosome fragility in sections above. In this section we highlight three phenomena that still confound researchers and remain not understood. We also note that additional mysteries previously articulated in "Forgotten fragile sites and related phenomena" still remain unsolved (Sutherland and Baker 2003).

### 21.5.1 What Is the Underlying Cause for Tissue-Specific Fragile Site Formation?

The very first reported case of fragile site already noted cell type-dependent fragile site expression as discussed earlier in this document. Mounting evidence further confirmed this observation (Hosseini et al. 2013; Kuwano et al. 1990; Le Tallec et al. 2013; Letessier et al. 2011; Murano et al. 1989a, b). Since chromosome fragility is enhanced by replication fork instability, the varying locations and activation patterns of origins of replication across tissue types would directly impact fragile site expression. In addition, tissue-specific gene expression would also influence sites of replication-transcription conflict-induced chromosome fragility. Related to this latter point, we have recently postulated that the inducing agent for fragile site expression plays a dual role in simultaneously generating replication stress and untimely gene expression while replication is still incomplete, resulting in replication-transcription conflicts at distinct loci in different cells (Hoffman et al. 2015). This hypothesis was derived from the model organism *Saccharomyces cerevisiae*, and we are currently testing it in human cell lines. Future studies mapping fragile sites in different cell lines with simultaneous measurements of origin activities and gene expression levels will be ideal for understanding tissue-specific fragility.

### 21.5.2 Why Are RFSs Preferentially Induced by Folate Stress?

Perhaps one of the foremost interesting questions is how do different classes of drugs define fragile sites. For instance, folic acid deprivation, thymidylate synthase inhibition (e.g., fluorodeoxyuridine), and thymidine deprivation or excess can all induce a class of folate-sensitive RFSs. One of the outstanding features of the folate-sensitive fragile sites is that nearly half of them (10 out of 22) have been found to contain CGG repeats thus far. Does folate deficiency preferentially lead to DNA breaks at CGG repeats? Folic acid is crucial for methyl metabolism, which in turn impacts DNA replication and repair. Deprivation of folic acid blocks the methylation of dUMP to TMP and triggers an increase in dUTP level. Similarly, intracellular fluorodeoxyuridine is converted to fluorodeoxyuridine monophosphate, which in turn inhibits thymidylate synthase and also causes an increase of dUTP. Consequently, there is an increase in the incorporation of uracil into DNA. Uracil in DNA is removed by the uracil DNA glycosylase (UDG), which, through a facilitated diffusion mechanism, locates the damaged/modified DNA bases (Schonhoft et al. 2013). It has been proposed that folate-sensitive chromatid breakage is the result of a catastrophic DNA repair cycle where excision of uracil is followed by reincorporation of uracil due to continuous blockage of the dTTP pool (Reidy 1987).

The question then becomes is there a higher level of uracil incorporation occurring at CGG repeat regions than other chromosomal regions, or are CGG repeats more conducive to uracil excision? There seemed to be evidence supporting both arguments. A genome-wide study in *S. cerevisiae* has demonstrated that the uracil content is relatively lower in early-replicating regions than late-replicating ones (Bryan et al. 2014). RFSs such as FRAXA tend to be late-replicating (Hansen et al. 1993; Subramanian et al. 1996; Webb 1992) and therefore might incorporate uracil at a higher rate. Alternatively but not mutually exclusively, cytosine deamination to uracil at the CGG repeats might be an underlying cause for increased uracil content. On the other hand, incorporated uracil might be easier to recognize by the UDG enzyme in the context of secondary structures due to CGG repeats. Supporting this hypothesis is the observation that a related DNA glycosylase, the human alkyladenine DNA glycosylase, can capture the site of DNA damage more efficiently on a flexible DNA template containing kinks, bubbles, or gaps than on a continuous B-form DNA duplex (Hedglin et al. 2015). Conceivably, this characteristic can also extend to UDG acting on a DNA template enriched for CGG repeats which readily form hairpin structures.

### *21.5.3 What Is the Underlying Cause for Sex-Biased Transmission of Autosomal Fragile Sites?*

Sherman and Sutherland reported in a population study that folate-sensitive and BrdU-sensitive autosomal fragile site expression was higher when the carrier parent was the mother than if it was the father (Sherman and Sutherland 1986). Similarly, Kähkönen et al. also observed that there was a maternal bias of the transmission of autosomal RFSs (16 out of 19 families were maternal carriers and 1 was a paternal carrier) (Kähkönen et al. 1989). This phenomenon was once again reported much later in a study demonstrating predominantly maternal transmission of ring chromosome 15 (Nikitina et al. 2003). What is the underlying cause for this sex-biased fragile site inheritance? Is the fragile site expression dependent on genomic imprinting of the maternal genes? Or is the maternal transmission of fragile sites a consequence of nonrandom chromosome arrangement in germ lines? Finally, is this phenomenon unique to folate stress? Large-scale population studies are required to confirm these findings. Further genetic and epigenetic characterizations of the autosomal RFSs will also shed new light on this genetic puzzle.

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# Chapter 22
# Cyclin E Deregulation and Genomic Instability

**Leonardo K. Teixeira and Steven I. Reed**

**Abstract** Precise replication of genetic material and its equal distribution to daughter cells are essential to maintain genome stability. In eukaryotes, chromosome replication and segregation are temporally uncoupled, occurring in distinct intervals of the cell cycle, S and M phases, respectively. Cyclin E accumulates at the G1/S transition, where it promotes S phase entry and progression by binding to and activating CDK2. Several lines of evidence from different models indicate that cyclin E/CDK2 deregulation causes replication stress in S phase and chromosome segregation errors in M phase, leading to genomic instability and cancer. In this chapter, we will discuss the main findings that link cyclin E/CDK2 deregulation to genomic instability and the molecular mechanisms by which cyclin E/CDK2 induces replication stress and chromosome aberrations during carcinogenesis.

**Keywords** Cell cycle • Cyclin E • CDK2 • FBW7 • Replication stress • Chromosome aberration • Genomic instability • Fragile sites • Cancer

## 22.1 Introduction

Progression through the cell cycle is regulated by association of cyclin-dependent kinases (CDKs) with specific regulatory subunits known as cyclins. Oscillations in cyclin levels primarily dictate oscillations in CDK activity, ensuring the order and timing of cell cycle phases (Hochegger et al. 2008; Malumbres and Barbacid

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2009). The E-type cyclin family is composed of two proteins, cyclin E1 and cyclin E2, which exhibit high sequence similarity and are functionally redundant (Lew et al. 1991; Koff et al. 1991; Gudas et al. 1999; Lauper et al. 1998; Zariwala et al. 1998). Cyclin E levels are tightly regulated during normal cell cycles, accumulating at the G1/S transition and being completely degraded by the end of S phase (Koff et al. 1992; Dulic et al. 1992). Consistent with its expression pattern, cyclin E binds to and activates CDK2 to control S phase entry and progression (Koff et al. 1992; Dulic et al. 1992; Ohtsubo and Roberts 1993; Resnitzky et al. 1994). Cyclin E mRNA levels are mostly induced by E2F transcription factors (Ohtani et al. 1995; Geng et al. 1996), whereas cyclin E protein is degraded by the $SCF^{Fbw7}$ ubiquitin ligase complex in a phosphorylation-dependent manner (Won and Reed 1996; Clurman et al. 1996; Strohmaier et al. 2001; Moberg et al. 2001; Koepp et al. 2001). Cyclin E/CDK2 activity is also controlled by the CDK inhibitors $p21^{Cip1}$ and $p27^{Kip}$, and potentially other CDK-inhibitory proteins, which are able to bind to and inactivate the cyclin E/CDK2 complex (Harper et al. 1993; Gu et al. 1993; Xiong et al. 1993; Polyak et al. 1994; Toyoshima and Hunter 1994; Reynaud et al. 1999).

Once activated, the cyclin E/CDK2 complex promotes the G1/S transition largely through phosphorylation and inactivation of the RB protein and the subsequent release of E2F transcription factors (Chellappan et al. 1991; Hinds et al. 1992; Dyson 1998; Harbour and Dean 2000). E2F proteins then promote S phase entry by regulating the expression of numerous genes required for DNA replication, such as the pre-replication complex components ORC1, CDC6, CDT1, and MCMs (Ohtani et al. 1996, 1998, 1999; Yan et al. 1998; Yoshida and Inoue 2004); the enzymes required for nucleotide and DNA synthesis, such as dihydrofolate reductase (DHFR), thymidine kinase (TK), and DNA polymerase α (Blake and Azizkhan 1989; Dou et al. 1994; Pearson et al. 1991); and the histone H2A (Oswald et al. 1996). Besides the RB protein, cyclin E/CDK2 directly phosphorylates and regulates other substrates required for S phase entry and progression, such as the DNA replication factors CDT1 and CDC6 (Liu et al. 2004; Mailand and Diffley 2005); the replication initiator Treslin (Kumagai et al. 2011); the activator of histone expression NPAT (Zhao et al. 2000; Ma et al. 2000); the transcription factors CBP/p300, E2F5, SMAD3, and MYC (Ait-Si-Ali et al. 1998; Morris et al. 2000; Matsuura et al. 2004; Hydbring et al. 2010); the centrosome proteins NPM, MPS1, and CP110 (Okuda et al. 2000; Tokuyama et al. 2001; Fisk and Winey 2001; Chen et al. 2002); and the DNA repair protein BRCA1 (Ruffner et al. 1999).

Regulation of E-type cyclins and the function of cyclin E/CDK2 in normal and aberrant cell cycles have been extensively reviewed elsewhere (Hwang and Clurman 2005; Caldon and Musgrove 2010; Siu et al. 2012). Here, we will focus on how deregulation of cyclin E/CDK2 causes replication stress and chromosome aberrations that may lead to genomic instability in cancer.

## 22.2 Cyclin E-Mediated Chromosome Instability

Several lines of evidence support the notion that cyclin E/CDK2 deregulation causes genomic instability. The initial finding that linked cyclin E to chromosome instability was the observation that constitutive cyclin E overexpression induced chromosome gains and losses in non-transformed rodent fibroblasts and human mammary epithelial cells, leading to aneuploidy (Spruck et al. 1999). Importantly, constitutive overexpression of cyclin D1 or cyclin A2 had no effect on the number of chromosomes in these cells. Later, it was shown that deletion of *FBXW7*, the gene encoding the F-box protein FBW7 involved in cyclin E recognition and degradation by the $SCF^{Fbw7}$ E3 ubiquitin ligase complex, resulted in increased frequency of micronucleus formation, multipolar spindles, and eventually chromosome instability in colorectal cancer cells (Rajagopalan et al. 2004). Even though FBW7 is involved in the degradation of other oncoproteins, such as c-MYC, c-JUN, and NOTCH (Davis et al. 2014), downregulation of cyclin E was sufficient to revert micronucleus formation in FBW7-depleted cells (Rajagopalan et al. 2004). More recently, generation of a hyperactive *CDK2* knockin allele in a human colorectal cancer cell line that expresses high cyclin E-associated kinase activity also showed increased rates of micronucleus formation when compared to *CDK2* wild-type cells (Hughes et al. 2013). Together, this evidence supports a causal role for cyclin E in chromosome instability during carcinogenesis.

One of the proposed mechanisms to explain chromosome instability in cancers is centrosome amplification, which leads to the formation of merotelic attachments and eventually chromosome segregation errors (Fig. 22.1) (Godinho and Pellman 2014). Normal cyclin E/CDK2 activity is required to ensure initiation of centrosome duplication in *Xenopus* egg extracts (Hinchcliffe et al. 1999; Lacey et al. 1999). In mammalian cells, it is also clear that CDK2 activity is necessary for

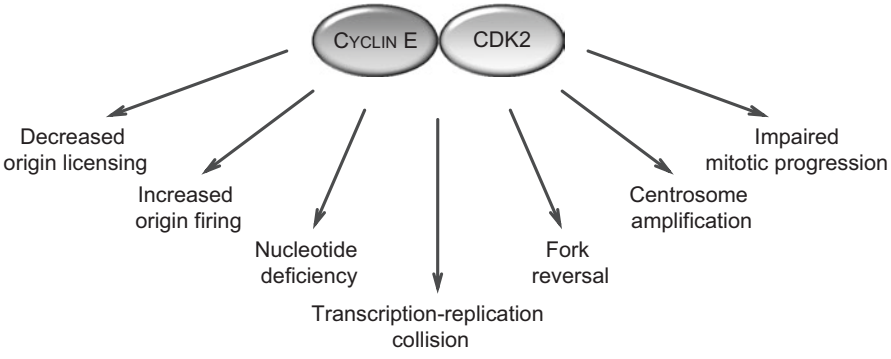


**Fig. 22.1** Mechanisms of cyclin E-induced genomic instability. Cyclin E/CDK2 deregulation may cause impaired assembly of pre-replication complex, increased origin initiation, deficiency of nucleotide biosynthesis pathway, collisions between replication and transcription machineries, formation of aberrant replication intermediates, such as fork reversal, centrosome amplification, and impairment of mitotic progression and checkpoint function

centrosome duplication; however, it is still uncertain whether cyclin E or cyclin A plays a major role in CDK2-dependent centrosome duplication (Matsumoto et al. 1999; Meraldi et al. 1999; Hanashiro et al. 2008). Cyclin E overexpression alone does not efficiently induce centrosome amplification in mouse embryonic fibroblasts (MEFs), normal human fibroblasts, and epithelial cells (Spruck et al. 1999; Mussman et al. 2000; Kawamura et al. 2004). However, high levels of cyclin E synergize with loss of p53 function to induce centrosome amplification and chromosome instability in human cell lines and tumors (Mussman et al. 2000; Kawamura et al. 2004). Furthermore, MEFs from a hyperactive *CDK2* knockin mouse model, which show elevated cyclin E- and cyclin A-associated kinase activities, had an elevated number of centrosomes when compared to wild-type MEFs (Zhao et al. 2012).

Cyclin E/CDK2 localizes to centrosomes (Matsumoto and Maller 2004), where it phosphorylates and dissociates NPM protein, initiating separation of paired centrioles and duplication of centrosomes (Okuda et al. 2000; Tokuyama et al. 2001). It is therefore possible that deregulation of cyclin E/CDK2 kinase activity, in combination with other insults, impairs NPM release from centrosomes, leading to centrosome amplification and chromosome instability. Indeed, deletion of NPM causes aberrant mitotic figures with multiple centrosomes and aneuploidy in MEFs (Grisendi et al. 2005), and alterations in *NPM* are frequently observed in human cancers (Grisendi et al. 2006). As discussed above, the centrosome proteins MPS1 and CP110 are also directly phosphorylated by cyclin E/CDK2 (Fisk and Winey 2001; Chen et al. 2002) and therefore may represent potential targets for cyclin E-induced chromosome instability as well.

Another mechanism that drives chromosome instability in tumorigenesis is impairment of mitotic checkpoint function and progression through mitosis, which may cause chromosome missegregation and aneuploidy (Fig. 22.1) (Varetti et al. 2014). It has been shown that cyclin E overexpression delays progression through early stages of mitosis, leading to accumulation of cells in prometaphase and unaligned metaphase (Keck et al. 2007). Impairment of mitotic progression was caused by cyclin E/CDK2-mediated phosphorylation and inactivation of the APC/C adaptor protein CDH1 and subsequent accumulation of the APC/$C^{Cdh1}$ ubiquitin ligase substrates cyclin B1 and securin, resulting in mitotic failure and polyploidy. In agreement, *FBXW7*-deficient cells, which have increased cyclin E-associated CDK2 activity, also exhibit increased levels of the APC/C substrates cyclin B1 and securin and accumulation of cells in prometaphase (Bailey et al. 2015). Interestingly, a genome-wide RNAi screen in these cells identified synthetic lethality with BUBR1, a spindle assembly checkpoint (SAC) protein, and high sensitivity to depletion of two other SAC components BUB1 and MPS1. These results suggest that cells with increased levels of cyclin E may depend on intact mitotic checkpoints for survival. Moreover, it has also been shown that cyclin E/CDK2 phosphorylates and prematurely activates the protein phosphatase CDC25C, leading to increased activity of the mitotic kinases cyclin B1/CDK1 and PLK1 and delayed mitotic progression (Bagheri-Yarmand et al. 2010).

## 22.3 Cyclin E-Mediated Replication Stress

Replication stress is characterized by the slowing or stalling of DNA replication forks, which may lead to fork collapse, DNA damage, and ultimately genomic instability (Zeman and Cimprich 2014). Activated oncoproteins and mutated tumor suppressors that drive sustained cellular proliferation cause replication stress and genomic instability, two events that are frequently observed in human cancers (Hills and Diffley 2014; Macheret and Halazonetis 2015). Indeed, this is the case for the oncoprotein cyclin E. Overexpression of cyclin E has been shown to cause replication stress, typified by slowed progression and premature termination of replication forks, DNA damage, and loss of heterozygosity at fragile sites (Bartkova et al. 2005, 2006; Bester et al. 2011). Cyclin E-mediated replication stress most likely is linked to elevated CDK2 kinase activity, as a *CDK2* hyperactive knockin allele was sufficient to delay replication fork progression, induce DNA damage, and increase micronucleus formation without cyclin E overexpression (Hughes et al. 2013). In a seminal series of articles, it has been proposed that oncogene-induced replication stress, including the oncoprotein cyclin E, activates the DNA damage response (DDR) pathway and leads to cell cycle arrest, cell death, and senescence, acting as an inducible barrier to tumor progression (Bartkova et al. 2005, 2006; Gorgoulis et al. 2005; Di Micco et al. 2006). Disruption of the DDR pathway facilitates cell proliferation and increases replication stress, leading to genomic instability in preneoplastic lesions.

The primary mechanism underlying cyclin E/CDK2-induced replication stress and genomic instability is interference of the nucleotide biosynthesis pathway (Fig. 22.1). Nucleotides are structural components of nucleic acids and therefore essential for a wide variety of biological processes, such as cell growth, DNA replication, and transcription (Lane and Fan 2015). Cyclin E overexpression, through disruption of the RB/E2F pathway, enforces cell proliferation of human fibroblasts with insufficient nucleotide levels (Bester et al. 2011). Nucleotide deficiency induced by cyclin E overexpression slowed replication fork progression and caused double-strand DNA breaks. Importantly, either exogenous supplementation of nucleosides or upregulation of nucleotide metabolism genes attenuated cyclin E-mediated replication stress and DNA damage. Consistent with this, replication stress in the form of impaired fork progression has been shown to generate structural as well as numerical chromosome instability during mitosis (Burrell et al. 2013).

Collisions between DNA replication and transcription machineries are another important source of replication stress (Fig. 22.1). Transcription complexes represent natural obstacles to the progression of replication forks, especially at fragile sites that contain extremely long genes (>800 kb), where replication forks have a high probability of encountering transcription complexes during the period of one cell cycle (Helmrich et al. 2011). Transcription-replication collisions may generate increased DNA topological tension and formation of R-loops (RNA-DNA hybrid structures), inducing replication fork stalling, DNA damage, and fragile site instability (Bermejo et al. 2012; Helmrich et al. 2013). Oncogenic events that interfere with the timing and location of DNA replication and transcription may increase

the probability of transcription-replication collisions. In cells overexpressing cyclin E, it has been shown that inhibition of transcription elongation attenuates replication stress and DNA damage (Jones et al. 2013). This study also showed that inhibition of replication initiation restores normal levels of fork progression in cyclin E-overexpressing cells, suggesting that increased replication initiation and transcription-replication collisions contribute to the replication stress upon high levels of cyclin E (Fig. 22.1). One potential consequence of transcription-replication collisions is the formation of aberrant replication intermediates, such as reversed replication forks (Neelsen and Lopes 2015). Consistently, it has been shown that cyclin E overexpression induces accumulation of reversed forks and chromosome breakage in human cells, suggesting that DNA topological stress also underlie cyclin E-mediated replication stress and genomic instability (Fig. 22.1) (Neelsen et al. 2013). Furthermore, it has been shown that cyclin E-induced collapsed forks may be processed and repaired by break-induced replication (BIR) repair, which generates copy number alterations, such as segmental genomic duplications (Costantino et al. 2014).

## 22.4 Genomic Instability in Cyclin E Mouse Models

Cyclin E is frequently overexpressed in human tumors, and its deregulation has been associated with poor prognosis and decreased survival of cancer patients (Scuderi et al. 1996; Porter et al. 1997; Iida et al. 1997; Erlanson et al. 1998; Fukuse et al. 2000; Muller-Tidow et al. 2001; Keyomarsi et al. 2002; Schraml et al. 2003). Overexpression of cyclin E in mouse models has been shown to induce mammary and lung carcinomas as well as hematopoietic malignancies, further supporting a causative role for cyclin E in carcinogenesis (Bortner and Rosenberg 1997; Karsunky et al. 1999; Geisen et al. 2003; Loeb et al. 2005; Smith et al. 2006; Ma et al. 2007; Minella et al. 2008; Siu et al. 2014).

Several tissue-specific transgenic and knockin mouse models have provided significant information on the role of cyclin E deregulation in genomic instability. A knockin mouse expressing a nondegradable form of cyclin E in MEFs showed increased chromosome breaks, translocations, and aneuploidy in a $p21^{-/-}$ background (Loeb et al. 2005). In this model, cyclin E overexpression also cooperated with p53 deficiency and RAS activation to cause cellular transformation, induce whole chromosome gains and losses, and accelerate lung carcinogenesis. Consistent with this, transgenic mice expressing either wild-type or degradation-resistant cyclin E in the lungs incurred multiple pulmonary adenocarcinomas with specific gains of chromosomes 4 and 6 (Ma et al. 2007). In mammary gland transgenic mouse models, cyclin E overexpression has been shown to induce p53 loss of heterozygosity and drastically increase tumor formation in a $p53^{+/-}$ background (Smith et al. 2006; Akli et al. 2007). Lastly, a knockin mouse with expression of nondegradable cyclin E in the hematopoietic stem cell compartment exhibited abnormal hematopoiesis, chromosome instability illustrated by chromosome gains

and losses, and decreased latency of T-cell malignancies in a $p53^{-/-}$ background (Minella et al. 2008; Siu et al. 2014). Again, p53 and p21 deficiencies were synergistic with cyclin E deregulation in promoting chromosome instability. Indeed, it has been shown that cyclin E-associated genomic instability is restrained by the p53/p21 pathway (Bartkova et al. 2005; Minella et al. 2002, 2007). Disruption of the inducible barrier established by the p53/p21 pathway may allow cyclin E overexpression to trigger genomic instability through some of the mechanisms discussed above, such as centrosome amplification and replication stress. Therefore, current mouse models support the notion that cyclin E deregulation contributes to tumorigenesis by promoting genomic instability in vivo.

## 22.5 Cyclin E Deregulation Promotes Replication Failure at Targeted Sites

We have discussed above the relationship between cyclin E and replication stress. Since cyclin E overexpression promotes replication stress and therefore slows replication fork progression (Bester et al. 2011; Jones et al. 2013; Liberal et al. 2012), we hypothesized that cells experiencing cyclin E deregulation might enter mitosis with incompletely replicated chromosomes. This in turn would lead to abnormal segregation and chromosomal damage during anaphase. Consistent with this, we observed that cyclin E-overexpressing non-transformed cells exhibited high levels of anaphase chromosomal anomalies such as bridged chromosomes and nonattached chromosomal fragments up to the size of the entire chromosome arms (Teixeira et al. 2015). If this observed chromosomal damage is a result of incompletely replicated chromosomal segments impairing segregation of sister chromatids, there are two obvious models that could account for the under-replication. Cyclin E-mediated replication stress could promote under-replication without any regional or feature specificity, or under-replication could occur at specific sites or regions possessing features that might sensitize them. To distinguish between these alternatives, we harvested cells blocked in mitosis immediately following cyclin E overexpression and analyzed their DNA by comparative genomic hybridization (CGH) array analysis. Indeed, a relatively small number of specific regions varying in size from approximately 200 to 100,000 base pairs had frequently failed to complete replication prior to entry into mitosis (Teixeira et al. 2015). Presumably, these under-replicated regions were responsible for the anaphase segregation anomalies we had observed in real time after cyclin E overexpression. Based on these observations, one would predict that these under-replicated regions would be included in deleted chromosomal segments subsequent to anaphase. We interrogated both mixed populations and single cells after cyclin E overexpression and found that deletion of these specific loci did indeed occur at high frequency (Teixeira et al. 2015). However, it appears that most cells carrying such deletions were incapable of clonal expansion, suggesting that checkpoint barriers eliminate cells with severely

damaged genomes, should surveillance mechanisms be intact. This presumably reduces the impact of cyclin E-mediated damage at the population level and is therefore protective from potentially oncogenic events (Bartkova et al. 2005). Indeed, the link between cyclin E deregulation and the p53 surveillance system has been discussed above.

## 22.6 Genomic Features Associated with Replication Failure

### *22.6.1 Late-Replicating Genomic Regions*

The majority of under-replicated regions detected in our study have been annotated as large late-replicating domains (Teixeira et al. 2015; Weddington et al. 2008). These are, for the most part, heterochromatic regions with a paucity of replication origins. The fact that origins in these domains fire late during the replication cycle combined with the low density of origins provides a likely explanation for failure to complete replication under conditions of replication stress (Le Tallec et al. 2014; Ozeri-Galai et al. 2014). However, these properties alone cannot explain the specific locations and boundaries of the under-replicated regions, as they were relatively small compared to the larger domains and highly targeted to specific sites. Therefore, other features of these sites must be relevant.

### *22.6.2 Recombinational Hotspots/Translocation Breakpoints*

A number of the sites have been annotated as recombinational hotspots or translocation breakpoints. A subset of these has been classified as fragile sites, as well. Both recombination and translocation are processes that are initiated by double-strand DNA breaks. Repair involving homologous sequences versus heterologous sequences containing microhomology determines the outcome (Berti and Vindigni 2016). Significantly, within this context, one characteristic of fragile sites is a tendency to experience double-strand breaks at abnormally high frequencies, presumably due to replication barriers within these sites (Le Tallec et al. 2014; Ozeri-Galai et al. 2014; Thys et al. 2015). These observations suggest that features of fragile sites might impede DNA replication under conditions of cyclin E-mediated replication stress leading to local replication failure. One feature of fragile sites suggested as causative for replication impairment and double-strand breaks is unusual DNA structures, such as palindromic sequences leading to formation of hairpins and loops (Thys et al. 2015; Ozeri-Galai et al. 2011). Such structured nonlinear DNA could easily explain why stressed replication forks might stall or collapse. However, this cannot completely explain the specificity of under-replicated sites under conditions of cyclin E overexpression, since only a small subset of fragile sites,

recombinational hotspots, and translocation breakpoints is affected. In addition, two recent studies showing that cyclin E overexpression causes instability at genomic regions with fragile site characteristics have given somewhat different results (Teixeira et al. 2015; Miron et al. 2015). Interestingly, among the susceptible genomic regions identified on each study (16 and 26, respectively), only one chromosome band was coincident for both (3q26). Since one study was carried out in mammary epithelial cells and the other in fibroblasts, a possible interpretation of the data is that cyclin E-mediated fragility may also be cell type specific, as has been shown previously in cells from different tissues (Le Tallec et al. 2011, 2013; Hosseini et al. 2013). In addition, not every under-replicated site was associated with fragile site features, suggesting factors unique to cyclin E-mediated replication stress must come into play (see below).

### *22.6.3 Low Origin Density and Licensing*

Interrogating a number of databases of origin distribution in human cells compiled using diverse methodologies, we found that most of the under-replicated sites were located in chromosomal regions characterized by extremely low origin density (Teixeira et al. 2015). Under conditions of replication stress leading to fork collapse, the probable lack of nearby functional forks is likely to eliminate the most common mechanism for rescue of localized replication failure: processing of unreplicated DNA by an adjacent replicon (Letessier et al. 2011; Kawabata et al. 2011). However, it is likely that replication stress caused specifically by cyclin E overexpression compounds the logistical problems of completing the replicative cycle. This is because, in addition to replication stress, cyclin E overexpression impairs assembly of the pre-replication complex (Ekholm-Reed et al. 2004). Specifically, high cyclin E/CDK2 activity at the M/G1 boundary inhibits chromatin loading of MCM proteins, which constitute the primary replicative helicase. Based on investigation of the impact of direct MCM protein depletion, it is unlikely that this effect of cyclin E overexpression would alter DNA replication during an unperturbed replicative cycle. Very high percentages of individual MCM proteins can be depleted via RNAi-mediated silencing with no apparent effect on unperturbed replication (Ge et al. 2007; Ibarra et al. 2008). However, MCM-depleted cells are extremely sensitive to replication stress, as they fail to assemble backup origins. These origins are competent but normally remain dormant except under conditions of replication stress, when they are mobilized to rescue collapsed and/or poorly functioning replication forks (McIntosh and Blow 2012). Since it is probable that cyclin E overexpression via impaired MCM loading leads to a deficiency of backup origins, but also simultaneously causes replication stress, the problem of rescuing collapsed forks in origin-sparse regions is likely exacerbated, leading to replication failure.

### 22.6.4 Transcription-Replication Collisions

As stated above, one possible source of cyclin E-mediated replication stress is collision between the replication and transcriptional machineries. These encounters would be predicted to occur most frequently at fragile sites containing very long genes (>300 kb). Consistent with this, cyclin E overexpression produced copy number losses at two very long genes, *EPHA6* and *NCAM2*, in human mammary epithelial cells (approximate size of 935 kb and 545 kb, respectively) (Teixeira et al. 2015). In addition, transcriptional changes were observed at two other long genes, *DAB1* and *NRXN3*, in human fibroblasts (approximate size of 430 kb and 1.7 Mb, respectively) experiencing deregulated levels of cyclin E (Miron et al. 2015).

### 22.6.5 Sensitive DNA Structures

As alluded to the above, a number of the sites under-replicated after cyclin E overexpression correspond to fragile sites, a characteristic of which is the presence of nonlinear DNA structures expected to pose barriers to replication fork progression (Thys et al. 2015). However, most fragile sites were not represented as under-replicated sites in our analysis. One fragile site, nevertheless, is likely to be informative: FRA11B/G (Fechter et al. 2007; Burrow et al. 2009). This site on chromosome 11 is particularly interesting because it is the breakpoint for rearrangements in mixed-lineage leukemia (MLL); hence, the locus is referred to as MLL (Muntean and Hess 2012). It is also noteworthy that this site is also frequently deleted in breast cancer (see below). The under-replicated segment detected in our study was 4,331 bp, which contains part of the breakpoint cluster region (BCR) for MLL translocations in leukemia (Muntean and Hess 2012). We therefore scanned this region for DNA sequences predictive of the ability to form energetically favorable hairpin loop structures. Interestingly, two such structures were detected near the center of the segment separated by approximately 500 base pairs (Teixeira et al. 2015). To determine whether these structured DNA elements posed a barrier to replication under conditions of cyclin E-mediated replication stress, we cloned the segment containing them, with and without the palindromic sequences, into an episomal vector that replicates autonomously in mammalian cells. While both the control and palindrome-containing plasmids were well maintained in the absence of cyclin E overexpression, only the control plasmid was maintained when cyclin E was overexpressed. These data are consistent with the hypothesis that palindromic structures pose a barrier to replication, specifically under conditions of cyclin E-mediated replication stress (Teixeira et al. 2015). Therefore, two structural barriers to replication in close proximity may represent a feature that promotes sensitivity to cyclin E-mediated replication stress, although such structures are likely to be sensitive to other sources of replication stress as well (see below). However, it is worth noting that cyclin E overexpression has been determined to be associated with MLL

translocations in the context of B-cell precursor acute lymphoblastic leukemia (BCP-ALL) (Accordi et al. 2010).

## 22.7 Cyclin E and Genomic Instability in Breast Cancer

What does the observation that cyclin E overexpression promotes replication failure at a small subset of specific loci implicate for oncogenesis? The first question one might ask is are deletions in the chromosomal regions surrounding the under-replicated sites found in actual cancer? To address this, we interrogated a database of approximately 2000 breast tumors subjected to CGH array analysis (Teixeira et al. 2015; Curtis et al. 2012). As a surrogate for cyclin E overexpression, we employed copy number increases of the *CCNE1* locus, presumably due to gene amplification. This undoubtedly represents an underestimation of tumors overexpressing cyclin E and likely introduces experimental noise, as several posttranslational mechanisms have been shown to elevate cyclin E levels. Nonetheless, when *CCNE1* copy number increase was compared with copy number decrease at each of the under-replicated sites, a highly significant correlation was observed for many of them. This suggests that cyclin E overexpression is a driver of deletion at these sites. It should be noted that overall these specific sites experience deletions at relatively low frequency, and their detailed analysis is likely to yield clues concerning what characteristics constitute sensitivity specifically to cyclin E-mediated replication stress. On the other hand, some of the sites that were more frequently deleted in the data set did not show a significant correlation with cyclin E copy number increase. The MLL locus was one of these. Presumably, features of the MLL locus, specifically two likely hairpin loops in close proximity, render this site sensitive to multiple forms of replication stress, including but not exclusive to cyclin E. The second relevant question is whether these specific deletions have a direct role in oncogenesis. Unfortunately, at this point, we do not know the magnitudes or boundaries of deletions that occur surrounding these sites when they have not completed replication but are forced through anaphase. However, one might speculate that large deletions could drive oncogenesis by promoting loss of heterozygosity at tumor suppressor loci and deletions over fragile sites (Bignell et al. 2010). It is interesting to note that tumor suppressor genes have been identified on chromosomes 3q (Guo et al. 2002; Schwaenen et al. 2009; Thean et al. 2010) and 21q (Lee et al. 2003; Silva et al. 2003; Yamada et al. 2008), the arms where cyclin E-driven deletions occur in breast cancer.

In our study using immortalized non-transformed human mammary epithelial cells, it was clear that few cells that had sustained deletions after exposure to cyclin E overexpression were capable of expanding robustly and forming colonies. Although reassuring from a human health perspective, this observation raises the question of how cyclin E-driven deletions might get fixed in an expanding premalignant population. The answer probably lies in the fact that individual breast cancers when they present commonly possess more than 100 genetic modifications

(Nik-Zainal et al. 2016). At least some of these are likely to have been selected to override checkpoint barriers, thereby allowing clonal expansion of chromosomally damaged cells.

## 22.8 Relevance to Other Sources of Replication Stress

The discussion above has focused on mechanisms of genomic instability associated with cyclin E overexpression/deregulation (Fig. 22.1). However, other oncogenic events have been associated with replication stress, e.g., overexpression of c-MYC (Dominguez-Sola et al. 2007; Srinivasan et al. 2013; Rohban and Campaner 2015) or mutation of RAS (Di Micco et al. 2006; Miron et al. 2015; Maya-Mendoza et al. 2015). Although the mechanisms whereby these overexpressed or mutant proteins cause replication stress are likely to differ, one can infer that in severe instances, incompletely replicated genomes will enter mitosis with the end result being genomic instability.

A number of model system experiments support this idea. A hypomorphic allele of the mouse MCM helicase component MCM4, designated Chaos3, which promotes instability of the pre-replication complex (Kawabata et al. 2011), causes anaphase aberrations similar to what we observed for cyclin E overexpression. Although $MCM4^{Chaos3}$ does not appear to affect the number of origins fired, the number of dormant backup origins is reduced, and the number of stalled replication forks is increased, as it has been proposed for cyclin E overexpression. Presumably, intrinsic levels of replication stress during the normal replicative cycle require such backup origins in order to avoid under-replicated regions and the resulting aberrant anaphases. Interestingly, the $MCM4^{Chaos3}$ mouse is cancer prone (Shima et al. 2007), indicating that this type of chromosomal damage is directly linked to oncogenesis.

In yeast, the absence of the cohesin-like complex Smc5-Smc6 causes replication impairment at loci that contain replication barriers, such as the rDNA cluster (Torres-Rosell et al. 2007). Yet, cells progress through mitosis resulting in elevated rates of chromosomal nondisjunction, even though all checkpoints are intact. These results confirm that no robust checkpoint exists that can detect and respond to small quantities of unreplicated DNA in eukaryotes ranging from yeast to human (Mohebi et al. 2015; Koundrioukoff et al. 2013).

## 22.9 Conclusions

Elevated cyclin E has been shown to be associated with aggressive disease and poor outcome in at least some human malignancies. The link between cyclin E overexpression and genomic instability suggests a mechanism whereby cyclin E might promote oncogenesis. Our recent work showing that cyclin E overexpression promotes replication failure at a small number of specific loci and, as a consequence,

chromosomal damage following anaphase leaves some important unanswered questions relevant to oncogenesis. First and foremost is a detailed description of the genomic damage that occurs in individual cells. Such information will allow the assessment of whether the classic two-hit tumor suppressor model is applicable or whether more complex mechanisms apply such as amplifications and translocations. The second important issue to be resolved is whether cyclin E can serve as a prototype for other oncoproteins that cause replication stress and promote genomic instability. On the one hand, as outlined above, some of the modalities of cyclin E function in the context of the replication stress are likely to be unique. On the other, there are certain to be mechanistic commonalities. Only investigation of the pathways leading to replication stress and from replication stress to genomic alterations for other oncoproteins will resolve this question.

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# Chapter 23
# Replication Through Repetitive DNA Elements and Their Role in Human Diseases

**Advaitha Madireddy and Jeannine Gerhardt**

**Abstract** Human cells contain various repetitive DNA sequences, which can be a challenge for the DNA replication machinery to travel through and replicate correctly. Repetitive DNA sequence can adopt non-B DNA structures, which could block the DNA replication. Prolonged stalling of the replication fork at the endogenous repeats in human cells can have severe consequences such as genome instability that includes repeat expansions, contractions, and chromosome fragility. Several neurological and muscular diseases are caused by a repeat expansion. Furthermore genome instability is the major cause of cancer. This chapter describes some of the important classes of repetitive DNA sequences in the mammalian genome, their ability to form secondary DNA structures, their contribution to replication fork stalling, and models for repeat expansion as well as chromosomal fragility. Included in this chapter are also some of the strategies currently employed to detect changes in DNA replication and proteins that could prevent the repeat-mediated disruption of DNA replication in human cells. Additionally summarized are the consequences of repeat-associated perturbation of the DNA replication, which could lead to specific human diseases.

**Keywords** DNA replication • Repeat sequences • Human diseases • Replication fork stalling • DNA helicases • Secondary DNA structures • Non-B DNA • Repeat expansion • Genome instability

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## 23.1 Introduction

A considerable fraction of the genome in nearly all organisms consists of repetitive DNA sequences. It has been suggested that these repetitive sequences might play a role in several cellular processes, such as recombination, nuclear architecture, and transcription. In human cells two-thirds of the genome sequence is compromised by repetitive DNA sequences (de Koning et al. 2011), which can be broadly classified into two major groups: interspersed repeats and tandem repeats. Interspersed repeats are repeated sequences that are scattered throughout the genome. Tandem repeats are repetitions of one or more nucleotides directly adjacent to each other. Depending on the size of the repeat unit, tandem repeats can be further categorized into satellite, minisatellite, and microsatellite DNA.

During DNA replication, each cell copies 3 billion base pairs within a period of 6–8 h, with minimal errors. Repetitive DNA sequences can be challenging for the replication machinery to replicate accurately because repeats are able to adopt alternative secondary DNA structures, which consist of non-B double helix DNA. During DNA replication, these secondary repeat sequences could cause replication fork stalling, which can lead to replication fork reversal or in extreme cases replication fork collapse. The consequences of replication fork stalling could be DNA polymerase slippage or incomplete DNA duplication, leading to genome instability such as repeat expansions (reviewed in Kim and Mirkin 2013; Lopez Castel et al. 2010) and chromosome fragility (reviewed in Durkin and Glover 2007).

It is normal to find variation in the length of repetitive DNA sequences in humans. This variation in the repeat size can be used as a tool to distinguish a person from another during forensics analysis. However, in some individuals the repetitive sequences are expanded beyond the normal threshold causing several human diseases. Large repetitive sequences in the genome can disturb or modify gene transcription, cause alternate mRNA transcription and mutant protein expression, or silence a gene entirely. In turn, this can lead to human diseases such as hereditary neurological and muscular repeat expansion diseases (Lopez Castel et al. 2010). Besides the effect on gene transcription, repeats can also alter the epigenetic landscape and cause genome fragility in human cells (Durkin and Glover 2007). Stalling of the replication machinery at repetitive DNA sequences could delay the DNA synthesis and prompt fork collapse leading to double-strand DNA breaks. Various forms of cancers are suspected to be the result of incomplete DNA replication and genome instability at specific repetitive sequences called fragile sites.

Fragile sites are broadly classified into rare fragile sites (RFS), common fragile sites (CFS), early replicating fragile sites (ERFs), and regions that resemble fragile sites such as telomeres. Treatment with low doses of aphidicolin induces CFS expression (Glover et al. 1984; Hecht and Glover 1984), while incubation with hydroxyurea exacerbates breaks at ERFs in human cells. However, treatment with chemotherapeutic drugs could destabilize both classes of fragile sites (Dillon et al. 2010). CFS and ERFs consist of non-expanding repeats and are an inherent part of the cellular genome, whereas RFS are only present in a few individuals where the

repeats are abnormally expanded. Interestingly, CFS and ERFs have numerous chromosomal locations and exist in all humans, whereas RFS are only expressed in rare individuals containing the disorder and are located at a specific chromosomal location; these are perhaps the most important distinctions between RFS and the other classes of fragile sites.

Considering the deleterious effects these repetitive DNA sequences in the human genome can have, it is very surprising that fragile sites are evolutionarily conserved in mammals across different species (Smeets and van de Klundert 1990). Their evolutionary conservation has led to the speculation that they have a protective role in the cell. One possible explanation for their conservation is that they were engineered to act as sensors and first responders of endogenous toxicity, to alert the DNA damage response pathway (O'Keefe and Richards 2006). However, not all repetitive DNA sequences are fragile sites in the genome. For example, chromosomal fragility is observed only in a few repeat expansion diseases like at the expanded CGG repeats (FRAXA site) in cells of fragile X syndrome (FXS) patients. In this section we will discuss unusual DNA structures and effects of non-fragile as well as fragile repetitive DNA sequences, which as a consequence of their repetitive nature can lead to human disorders.

The first repeat expansion diseases were discovered in 1991 when several groups found that the root of the FXS, an X-linked inherited disease causing intellectual disabilities, is a CGG repeat expansion (Heitz et al. 1991; Pieretti et al. 1991; Verkerk et al. 1991). In the same year, another inherited X-linked disease, spinal and bulbar muscular atrophy (also called Kennedy's disease), was discovered, which is caused by a CAG repeat expansion in the androgen receptor gene (La Spada et al. 1991). These diseases belong to the trinucleotide repeat expansion (TNR) diseases, which form the largest category of repeat expansion diseases currently known (see Table 23.1). Though most of these disorders are a consequence of repeat expansions, multiple skeletal dysplasias are an exception where the symptoms can appear from the addition or deletion of a repeat sequence. While there are several diseases caused by a repeat expansion, not all of them contain a RFS. FXS is an example of a disease that is caused by CGG repeat expansions that has been also characterized as a RFS. In contrast, for example, the expanded GAA repeats in Friedreich's ataxia cells do not show fragility in vivo and are hence not classified as RFS. Repeat expansion diseases can also be triggered by larger repeat sequences, such as tetra-, penta-, and hexanucleotides, and even dodecanucleotide repeats. For instance, myotonic dystrophy type 2 (DM2) is caused by a CCTG expansion and spinocerebellar ataxia type 10 (SCA10) by an AATCT repeat expansion (reviewed in Lopez Castel et al. 2010; Mirkin 2007). Additionally more and more diseases caused by repeat instability are being discovered, for example, recently it was determined that in a fraction of patients with amyotrophic lateral sclerosis (ALS, also known as Lou Gehrig's disease), the disease symptoms are triggered by a GGGGCC repeat expansion (DeJesus-Hernandez et al. 2011). However, currently there is no cure for these repeat expansion diseases, and the mechanism leading to repeat instability is still unclear.

**Table 23.1** Examples of diseases caused by a repeat expansion: summarized are repeat expansion diseases, the potential secondary DNA structures formed by the repeats, and effects the repeat expansion has at the endogenous locus and on the molecular processes in the cell

| Disease | Repeat | Structure | Gene | Effect |
|---|---|---|---|---|
| *Promoter* | | | | |
| Epilepsy progressive myoclonic (EPM1) | $C_4GC_4GCG$ | G-quadruplex, hairpins, i-motif | CSTB | Reduced gene transcription |
| Spinocerebellar ataxia 12 | CAG | Hairpins | PPP2R2B | Altered expression of splice variants |
| *5′UTR* | | | | |
| Fragile X syndrome (FXS) | CGG | G-quadruplex, hairpins | FMR1 | Epigenetic modification, FMR1 gene silencing |
| FXS-associated tremor/ ataxia syndrome (FXTAS) | CGG | G-quadruplex, hairpins | FMR1 | Increased FMR1 transcription, FMRpolyG inclusions |
| FXS primary ovarian insufficiency (FXPOI) | CGG | G-quadruplex, hairpins | FMR1 | Increased FMR1 transcription, FMRpolyG inclusions |
| Fragile X syndrome E (FRAXE) | CCG | Hairpins | FMR2 | FMR2 gene silencing |
| *Exons* | | | | |
| Huntington's disease (HD) | CAG | Hairpins | HTT | Mutant protein expression (huntingtin) |
| Spinocerebellar ataxia 1 | CAG | Hairpins | ATXN1 | Polyglutamine protein |
| Spinocerebellar ataxia 2 | CAG | Hairpins | ATXN2 | Polyglutamine protein |
| Spinocerebellar ataxia 3 | CAG | Hairpins | ATXN3 | Polyglutamine protein |
| Spinocerebellar ataxia 6 | CAG | Hairpins | CACNA1A | Polyglutamine protein |
| Spinocerebellar ataxia 7 | CAG | Hairpins | ATXN7 | Polyglutamine protein |
| Spinocerebellar ataxia 17 | CAG | Hairpins | TBP | Polyglutamine protein |
| Kennedy's disease; spinal and bulbar muscular atrophy (SBMA) | CAG | Hairpins | AR | Mutant protein expression (mutant androgen receptor) |
| Dentatorubral-pallidoluysian atrophy (DRPLA) | CAG | Hairpins | ATN1 | Polyglutamine protein |
| *Introns* | | | | |
| Friedreich's ataxia (FRDA) | GAA | Triplexes | FXN | Epigenetic modifications, reduced gene transcription |
| Spinocerebellar ataxia 10 | ATTCT | Unpaired | ATXN10 | Loss of function |
| Spinocerebellar ataxia 31 | TGGAA | Purine-rich duplexes | TK2/BEAN | Form RNA aggregates |
| Myotonic dystrophy type 2 (DM2) | CCTG | Hairpins | CNPB (ZNF9) | Form RNA aggregates |
| Frontotemporal dementia/ amyotrophic lateral sclerosis | GGGGCC | G-quadruplex | C9orf72 | Form RNA aggregates, reduced gene transcription |

(continued)

**Table 23.1** (continued)

| Disease | Repeat | Structure | Gene | Effect |
|---|---|---|---|---|
| *3'UTR* | | | | |
| Myotonic dystrophy type 1 (DM1) | CTG | Hairpins | DMPK | Form RNA aggregates |
| Spinocerebellar ataxia 8 (SCA8) | CTG | Hairpins | ATXN8OS/ ATXN8 | Form RNA aggregates polyglutamine protein |
| Huntington's disease-like 2 (HDL2) | CAG | Hairpins | JPH3 | Form RNA aggregates |

Several models that could lead to repeat instability were suggested including abnormalities in the DNA replication, DNA repair, transcription, and recombination process (reviewed by McMurray 2010; Mirkin 2007; Usdin et al. 2015). This section summarizes errors during DNA replication, which could trigger repeat expansion and chromosomal fragility in human cells. Furthermore disorders caused by repetitive DNA sequences are also discussed in this chapter.

## 23.2 Secondary Repeat Structures

Repeat sequences are able to form unusual secondary non-B DNA structures by engaging in hydrogen bonds that differ from the canonical Watson-Crick bonding structures. When the DNA is unwound, single-stranded repeat sequences are able to adapt secondary DNA structures, such as hairpins, triplexes, G-quadruplexes, i-motifs, DNA:RNA hybrids (R-loops), and slipped DNA structures (Gacy and McMurray 1998; Mirkin 2007; Pataskar et al. 2001; Wells 2008), which are shown to form in vitro plus some of these secondary repeat structures were also shown to form in vivo.

### *23.2.1 Secondary Structures Formed by Expanding Repeats*

In vitro, it was found that CTG, CGG, CAG, and CCG form imperfect hairpins whose stability depends on the repeat sequences (Gacy et al. 1995; Paiva and Sheardy 2004). In addition, CGG repeats were implicated in the formation of tetrahelical structures called G-quadruplexes (Fry and Loeb 1994). GAA repeats are able to form intermolecular (H-DNA) or intramolecular ("sticky DNA") triplexes (Wells 2008). Although such structures are shown to form in vitro, it is not clear yet whether they form in vivo at the endogenous repeats in the human genome. Several evidences suggest that such structures are indeed formed at the endogenous disease locus. A good example of this is the observation that AGG interruptions in the CGG repeats in FXS patients are linked to fewer repeat expansion events (Nolin et al. 2015). The interruptions in the repeat sequence could prevent the formation of

specific secondary DNA structures at the genomic locus in patient cells. Similarly, other TNR expansion diseases such as Friedreich's ataxia (FRDA), myotonic dystrophy type 1 (DM1), and spinocerebellar ataxia diseases (SCA) contain interruptions in the repeat sequences which stabilize the repeats (Lopez Castel et al. 2010; Pearson et al. 1998). In addition, it was discovered that Msh2, which is shown to bind to hairpins formed by CTG repeats (Pearson et al. 1997) and CAG repeats (Owen et al. 2005) in vitro, can be found at the expanded GAA repeat tract at the endogenous *Frataxin* (*FXN*) gene locus in FRDA cells in vivo (Du et al. 2012). This indicates that Msh2 might bind to secondary repeat structures formed by the endogenous repeats at the disease locus.

Recently some of these secondary structures such as G-quadruplexes and R-loops were detected in vivo in human cells (Lam et al. 2013; Phillips et al. 2013). R-loops are structures that are formed during transcription when nascent RNA hybridizes to the DNA template behind the elongating RNA polymerase. A newly developed antibody was used to detect R-loops at the endogenous repeats in FRDA and FXS cells (Groh et al. 2014; Lam et al. 2013; Loomis et al. 2014). Because the *fragile X mental retardation (FMR1)* gene is silenced in FXS cells, the *FMR1* gene transcription was reactivated before analysis of the R-loops by treatment with the DNA methylation inhibitor 5-aza-2′-deoxycytidine (Groh et al. 2014). Using antibodies it was also reported that triplexes are formed in human cells in vivo (Agazie et al. 1994; Burkholder et al. 1988; Lee et al. 1987). However it is still not clear whether secondary structures such as hairpins, G-quadruplexes, and triplexes are formed at the endogenous repeats in disease cells. Small molecules, which prevent triplex formation in vitro, were used to release the stalled replication forks at the GAA repeats indicating that GAA triplexes may indeed form at the expanded GAA repeats in FRDA cells (Gerhardt et al. 2016).

Nevertheless, despite the disturbances secondary repeat structures could cause, certain noncanonical repeat structures appear to be involved in normal cell function. The high concentration of G-quadruplex motifs near promoter regions indicates potential function for G-quadruplex structures in gene regulation. G-quadruplexes were also suggested to have a role in replication initiation, recombination, or meiosis (Bochman et al. 2012). R-loops were suggested to function as regulators of chromatin structure and transcription (Al-Hadid and Yang 2016). In addition, triplexes were implicated in transcriptional regulation, epigenetic modification, and organization of the chromatin structure (Buske et al. 2011). Triplexes may also be involved in posttranscriptional processing of RNAs (Buske et al. 2011). However, these non-B DNA repeat structures could also perhaps hinder RNA polymerases. Repeat-mediated inhibition of the transcription by formation of secondary repeat structures in vitro has been observed in model systems (Pandey et al. 2015). Thus, transcription inhibition and DNA polymerase slippage at the repeats could lead to reduced gene expression and repeat expansion. Furthermore increasing repeat number worsens the symptoms of the disorders and causes an earlier start of the disease in patients. The process where these repeat expansions (also called dynamic mutations) increase in size through generations, resulting in an earlier onset of the disease and increased severity of symptoms, is called genetic anticipations.

### *23.2.2 Secondary Structures Formed by Non-expanding Repeats*

Non-expanding repeats exist at numerous sites in the human genome and can be composed of either A/T-rich repeats (e.g., at CFS loci) or G-rich repeats (e.g., at ERFs loci, telomeres). Both classes of repetitive sequences have been shown to have the potential to form a variety of secondary structures similar to expanding repeats. Instability at CFS loci has been widely attributed to the replication stalling at secondary structure-prone repetitive DNA elements (Schwartz et al. 2006; Walsh et al. 2013). Polymorphic A/T-rich repeat sequences at CFS-FRA16D can potentially form hairpins as well as cruciform DNA structures that could stall replication machinery in a manner that is dependent on the length of the repeat sequence (H. Zhang and Freudenreich 2007). In addition to $[TA]_n$ dinucleotide repeat sequences, mononucleotide $[A]_{28}$ repeats located at the FRA16D locus have also been shown to stall replication forks in vitro (Walsh et al. 2013).

Another class of repetitive sequences that are strongly implicated in genome instability are quasi-palindromes. At CFS, biochemical studies indicate that quasi-palindrome sequences can form stem structures that can stall replication forks (Walsh et al. 2013). Computational analysis of GC-rich sequences at ERFs revealed that they have the potential to form G-quadruplex structures (Madireddy et al. unpublished). While the G-rich DNA at ERFs has the potential to form secondary structures such as R-loops and G-quadruplexes, their contribution to ERFs instability is yet to be determined. However, the formation of most of these secondary structures has been observed so far by biochemical studies carried out in vitro due to the lack of suitable reagents (e.g., antibodies) that can facilitate their detection in vivo.

In addition to repeat-associated secondary structures, current literature suggests that transcription-associated obstacles are a major cause for instability at both ERFs that contain actively transcribed genes and CFS that contain very long genes (Helmrich et al. 2011, 2013; Mortusewicz et al. 2013). Collisions between the transcriptional machinery and replication machinery and/or R-loops can lead to genomic instability by obstructing the progression of replication machinery or by making the cell more susceptible to genotoxic stress (Aguilera and Garcia-Muse 2012; Sollier and Cimprich 2015).

## 23.3 Replication Fork Stalling at the Repetitive DNA

Blockage of replication forks at the repeats was observed first in vitro using various DNA polymerases and model substrates. Primer extension assays have been used to demonstrate polymerase pausing at CTG, CGG, and GAA repeats (Gacy et al. 1998; Kang et al. 1995; Usdin and Woodford 1995). Later, the first in vivo replication assay confirmed that replication fork stalling occurs at the CGG, CTG (Pelletier et al. 2003; Samadashwily et al. 1997; Voineagu et al. 2009), and GAA repeats

(Krasilnikova and Mirkin 2004). An increase in replication fork stalling arises when the repeats expand to certain repeat lengths similar to the repeat lengths observed in patients. However, the question remains whether secondary repeat structures indeed form and stall DNA polymerases at endogenous genomic loci in human cells. For example, early studies showed that the fragile X locus is replicated later in S phase in FXS cells, in contrast to unaffected cells (Hansen et al. 1993; Subramanian et al. 1996). This suggested that DNA replication of the *FMR1* locus in FXS cells is slowed down probably by encountering an obstruction. Indeed recently a short replication fork pause was detected at the repeats at the endogenous *FMR1* locus in FXS human embryonic stem cells (hESCs) (Gerhardt et al. 2014) (Fig. 23.1). Surprisingly, pausing occurred at the short CGG repeats in unaffected cells to a similar degree as at the expanded CGG repeats in FXS patient cells. However, the presence of numerous human helicases that are capable of unwinding secondary structures such as G-quadruplexes quickly could explain the relatively short pause of the replication fork at the endogenous CGG repeats.

How about other secondary structures such as triplexes, DNA cruciform, and slipped-stranded structures where both DNA strands are involved in the formation of the secondary DNA structures? GAA repeats, which are able to form triplexes, when inserted into plasmids were shown to transiently pause (Krasilnikova and Mirkin 2004) and reverse the replication forks in human cells (Follonier et al. 2013). Recently, a major replication fork stall was observed in FRDA-induced pluripotent stem cells (iPSCs) at the endogenous *Frataxin* locus (Gerhardt et al. 2016) (Fig. 23.1). The intensity of the fork stall at the GAA repeats was much higher than in FXS cells indicating that the expanded GAA repeats pose a bigger challenge than the expanded CGG repeats for the DNA polymerases and perhaps even the DNA helicases. During replication fork stalling, the DNA polymerase slippage could add additional repeats, which would explain the observed GAA repeat expansion in FRDA cells. Interestingly, when triplex formation was inhibited by treatment of FRDA stem cells with small molecules, the replication fork stall was reversed (Gerhardt et al. 2016), and the GAA repeats become stable (Du et al. 2012). This indicates that the release of the stalled forks and stabilization of the repeats could have a therapeutic effect for patients where the repeats continue to expand during their lifetime. In this manner, stabilizing the *Frataxin* gene transcription by preventing GAA repeat expansion could be a therapeutic strategy to improve the lives of FRDA patients.

In addition, in vitro and in vivo studies suggest that the $[AT]_n$ or $[AT/TA]_n$ non-expanding flexible sequences found at CFS (Glover 2006; Lukusa and Fryns 2008; Zhang and Freudenreich 2007) lead to polymerase pausing (Walsh et al. 2013). Replication stalling has been shown to occur either in the presence of replicative stress-inducing agents such as aphidicolin (e.g., FRA16C) (Ozeri-Galai et al. 2011, 2013) or in the absence of essential proteins that facilitate replication fork movement at CFS (e.g., pausing at FRA16D in the absence of FANCD2) (Fig. 23.1 (Madireddy et al. 2016a). Whether the replication machinery also stalls at other CFS and expanded repeats, such as CAG and CTG, at the endogenous genomic locus in patient cells has to be still determined.

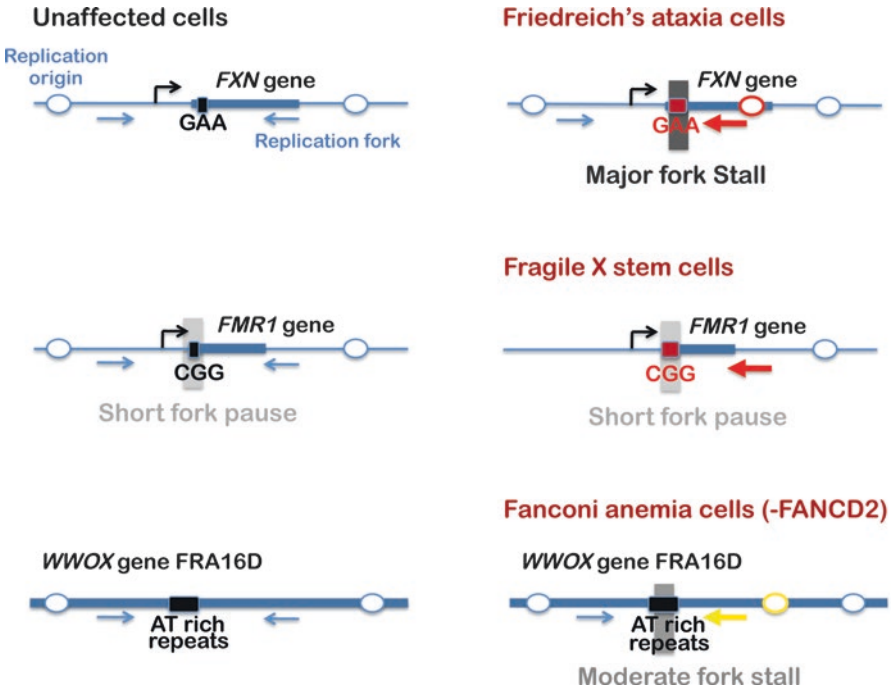


**Fig. 23.1** Alterations in the DNA replication program in disease cells: severe changes in the DNA replication program are observed in Friedreich's ataxia (FRDA), fragile X syndrome (FXS), and Fanconi anemia complementation group D2 (FANCD2) cells. Fork stalling was detected at the endogenous repeats probably caused by secondary structures, such as triplexes, G-quadruplexes, and R-loops, in human patient cells (gray box, different shades represent the severity of the replication fork block). In addition the absence or activation of replication origins (red circles) alters the replication fork direction through the repeat in FRDA and FXS stem cells (red arrow). Replication fork stalling together with the altered replication fork direction possibly triggers repeat expansion in FRDA and FXS patient stem cells. At non-expanding repeats in FANCD2 patient cells, replication fork stalling potentially activates dormant origins (yellow circle) to rescue the stalled forks and complete DNA replication. Nonetheless spontaneous fragile site breaks occur in these patients probably caused by the inability to recruit proteins (e.g., helicases) in the absence of FANCD2 that eliminate secondary DNA structures

## 23.4 Models for Repeat Instability

Depending on the cell type, repeat sequence, repeat size, and location of the repeat in the genome, different mechanisms could trigger repeat instability. Several models suggest that errors during DNA replication, DNA repair, and DNA recombination process could cause instability of repetitive DNA sequences (Durkin and Glover 2007; McMurray 2010; Mirkin 2007; Usdin et al. 2015). In addition the repeat size plays a major role in the repeat instability mechanism since an increase in the repeat length could hinder cellular processes more severely and so worsen the instability of the repeat in human cells.

### 23.4.1 Models Leading to Repeat Expansions and Contractions

Diverse models of repeat expansion were proposed for different repeat expansion diseases in different patient tissues (Lopez Castel et al. 2010). For example, inaccuracies during the DNA replication process could lead to repeat expansions or contractions in proliferating cells. One model suggests that to prevent uncoupling of the DNA helicase from a stalled DNA polymerase at the repeat, the template DNA containing the repeat sequence is pushed out and bypassed by the DNA polymerase, causing repeat contractions (Delagoutte et al. 2008). Small changes in the repeat length could be a result of DNA polymerase slippage during DNA synthesis at the remaining 5′ flap or by misalignment of template and daughter DNA strands (Kim and Mirkin 2013). Large repeat expansions were proposed to occur during replication fork stalling after replication fork reversal (Follonier et al. 2013; Fouche et al. 2006; Kerrest et al. 2009) and generation of a four-way junction (chicken foot) structures. This could result in the formation of a secondary repeat structure and synthesis of additional repeats in the nascent DNA (Mirkin 2006). Another model suggests that repeat expansion could happen when the leading strand DNA polymerase switches templates using the nascent lagging strand as template and then switches back to the leading strand template leaving additional repeats, the size of an Okazaki fragment in the nascent DNA (Shishkin et al. 2009). The template-switch model was supported by experimental evidence showing that deletion of genes impeding the switch of the DNA templates increased the repeat expansion rate (Shishkin et al. 2009).

Cumulative findings indicate that a combination of several inaccuracies in different cellular processes including mutations in *cis*-elements and deregulation of *trans*-factors could facilitate repeat expansion or contraction in patient cells. Faulty regulation of several DNA repair proteins, such as Msh2 (MutS protein homolog 2), was suggested to promote repeat expansion in mammalian cells and mouse models (McMurray 2010; Usdin et al. 2015). *Trans*-factor, such as Msh2, could bind and stabilize these non-B DNA structures. Indeed human Msh2 is upregulated in stem cells making the cells more susceptible to repeat instability (Du et al. 2012; Gerhardt 2015). Replication fork stalling and polymerase slippage at the expanded repeats, together with upregulated Msh2 level, would explain the progressive repeat instability in disease cells already containing expanded repeat sequences. Although these *trans*-factors significantly facilitate repeat instability, repeat expansion occurs only at one specific genomic locus in trinucleotide repeat expansion disorders, suggesting that *cis*-elements close to the expanding repeats probably trigger repeat instability.

Only a few *cis*-elements have been identified in close proximity to the repeats in FXS, DM1, and SCA7 cells (Cleary et al. 2010; Gerhardt et al. 2014a, b; Libby et al. 2008, 2003). These *cis*-elements were detected through studies of mutations in the binding sites of the CCCTC-binding factor (CTCF) and the DNA replication program at or in close proximity to the repeats. In addition, both the length and the

purity of the repeat tract influence repeat instability (Holloway et al. 2011). Interruptions in the repeat sequence probably reduce the formation of secondary DNA structures and decrease repeat expansion events in patients. Expanded uninterrupted repeats could trigger more frequent expansion events. However, the initial events triggering repeat expansion of the normal repeat length allele are unknown.

One possibility is that repeat instability is initiated in human cells by alterations in *cis*-elements near the repeats such as the position of replication origins, which could lead to altered replication fork progression and lead to a higher propensity of forming secondary repeat structures. In plasmid-based model systems, it was shown that the placement of a replication origin at a certain distance and orientation from the CGG, GAA, or CTG repeats can cause repeat expansion or contractions (Cleary et al. 2002; Freudenreich et al. 1997; Rindler et al. 2006; Samadashwily et al. 1997). Subsequently, in the origin switch model, it was suggested that a switch in the direction of the replication fork, for example, due to the absence of replication initiation sites could cause repeat expansions (Mirkin 2007). In another model, the origin shift model, repeat expansion was triggered by changes in the distance of the closest replication origins to the repeats causing alteration in the position of the repetitive sequence within Okazaki initiation zone (OIZ). A third model, which does not require altered replication origin activation or relocation, suggests that repeat expansion is triggered by *cis*-elements and *trans*-factors influencing the mode of the replication fork progression, thereby altering the location of the repeats in the single-stranded lagging strand template (Cleary and Pearson 2005; Mirkin 2007). In line with these models, in mouse tissues containing the human *DM1* locus, differential replication origin usage was observed (Cleary et al. 2010). Furthermore, we found that the replication fork direction is altered in FXS and FRDA stem cells due to altered location of replication origin(s) (Gerhardt et al. 2016; Gerhardt et al. 2014) (Fig. 23.1). We observed an increase in replication forks in the direction of the TTC repeats in the lagging strand template, which is the direction shown in experiments utilizing plasmid-based model systems to increase repeat instability (Rindler et al. 2006). The altered replication fork direction together with DNA polymerase stalling would explain the repeat instability in these proliferating cells.

However, an altered replication fork direction could also be the primary cause for repeat expansion and transcriptional inhibition in somatic patient tissues, including terminally differentiated nonproliferating cells. For example, human cardiomyocytes and some neuronal cells are polyploid (Adler and Friedburg 1986; Brodsky and Uryvaeva 1977), and genome multiplication (endoreplication or rereplication) has been speculated to promote cell survival and tissue regeneration under stressful conditions (Anatskaya and Vinogradov 2007). More recently rereplication was proposed as the cause for repeat instability in proliferating and differentiated cells under conditions of induced stress (Chatterjee et al. 2015). It was suggested that environmental stress could activate DNA rereplication in human cells (Chatterjee et al. 2015). Replication fork stalling during rereplication could then promote repeat expansion and further impede gene transcription in differentiated polyploid cells, such as the FRDA cardiomyocytes, thus contributing to the progression of the disease.

### 23.4.2 *Models Leading to Repeat Instability of Non-expanding Repeats*

Non-expanding repeats such as those found in CFS loci are stable under normal replicative conditions; however, exposure to replicative stress renders them highly susceptible to chromosomal breakage, deletions, translocations, and sister chromatid exchanges (Glover and Stein 1987, 1988; Wang et al. 1997). While it has been shown that replicative polymerases find it difficult to replicate through the repetitive sequences at fragile sites (Walsh et al. 2013), the primary cause for the polymerase stalling and the mechanisms that promote replication restart and completion have remained unclear. However, repeat instability at CFS has been attributed to the following classic features that define these genomic regions:

*A/T-Rich Sequences* CFS are A/T-rich, highly flexible genomic sequences (Arlt et al. 2002; Mishmar et al. 1998; Ried et al. 2000). Computational modeling has shown that CFS-derived repeat sequences composed of $[AT]_n$ or $[AT/TA]_n$ repeats have the potential to form stable secondary structures (Fungtammasan et al. 2012; Zhang and Freudenreich 2007). Accordingly, some in vitro and in vivo studies from different model systems have demonstrated that these structure-prone sequences at CFS can impede replicative polymerases such as DNA polymerase delta and lead to replication fork pausing (Burrow et al. 2010; Ozeri-Galai et al. 2013; Shah et al. 2010; Walsh et al. 2013) (Fig. 23.2a). Transfection of these repetitive sequences to other non-fragile regions renders the new regions unstable (Ragland et al. 2008); however, the partial removal of some of these A/T-rich sequences from CFS reduces but does not completely eliminate the CFS instability (Corbin et al. 2002). This shows that the presence of A/T-rich sequences while contributing to instability is not the only factor leading to CFS fragility.

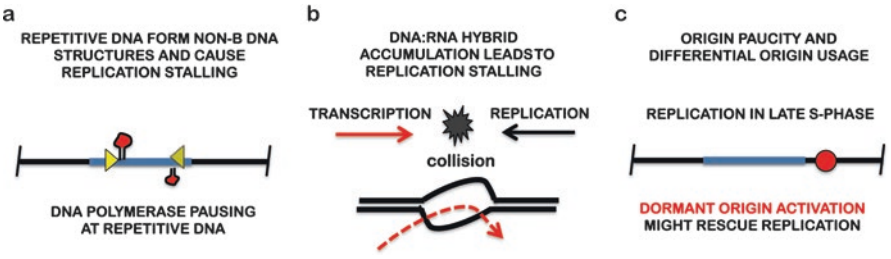


**Fig. 23.2** Models of repeat instability: (**a**) Replication of repetitive DNA sequences can be delayed due to replication fork stalling at potential non-B DNA structures and so lead to repeat instability. (**b**) Collisions between transcription and replication machinery or stalling at transcription-associated DNA:RNA hybrids could cause repeat instability. (**c**) The activation of dormant origins, to complete replication, is one of the mechanisms by which replication of repetitive DNA sequences can be rescued. However, alterations in the location and scarcity of replication origins at late replicated regions in S phase lead to a risk of incomplete or altered DNA replication, which could result in repeat instability

*Located in Long Genes* The most frequently unstable CFS loci harbor long underlying genes. These genes have largely been classified as tumor suppressor genes or proto-oncogenes at some of the most commonly expressed CFS loci, such as FRA3B, FRA16D, and FRA7I, and instability at these sites have been closely correlated with multiple types of cancer (Bednarek et al. 2000; Ciullo et al. 2002; Hellman et al. 2002; Siprashvili et al. 1997). Since the transcription of long genes takes longer than one cell cycle, there is a high likelihood for both replication and transcription to take place at the same time. This could result in collision between the replication and transcriptional machinery and can also lead to the formation of DNA:RNA hybrids (R-loops) (Helmrich et al. 2011) (Fig. 23.2b). Obstruction of transcription and/or replication can lead to genomic instability and dysregulation of the tumor suppressor genes located at CFS loci. In addition CFS are located in regions of the genome that are replicated late during S phase (Hansen et al. 1997; Hellman et al. 2000; Le Beau et al. 1998; Palakodeti et al. 2004; Wang et al. 1999). Any further delay in replication due to physiological stress or replication stalling at secondary structures can lead to incompletely replicated DNA upon reaching G2/M. This manifests as ultrafine DNA bridges that have to be resolved between cell division (Chan et al. 2009).

*Paucity of Replication Origins* CFS have a scarcity of replication initiation events at their loci (Letessier et al. 2011). Due to this, origins that fire outside the locus have to travel long distances to complete CFS replication before cell division begins (Fig. 23.2c). Interestingly, the absence of origins at CFS loci is a cell type-specific phenomenon. The striking differences in replication initiation events at CFS loci in different cell types (e.g., lymphocytes have no origins whereas fibroblasts have an abundance of origins at CFS) closely correlate with CFS fragility. Variations in the epigenetic regulation of the locus are thought to be a possible mechanism behind this cell type specificity. Collectively, these reports show that instability of long non-expanding repeats is multifactorial, and each unique characteristic contributes to its fragility.

## 23.5 Effect of Repetitive DNA Sequences and Human Diseases

Position of the repeats in the genome determines the effect that repetitive sequences and expanded microsatellite repeats have on various cellular processes.

### *23.5.1 Effect of Expanded Repeats and Human Diseases*

Repeat expansion disorders are categorized as loss or gain of function diseases depending on whether the gene is transcribed or a mutant protein or mutant RNA is produced (Table 23.1). Located in the exons of specific genes, repeats can modify

the gene transcription. For example, the expansion of CAG repeats causes expression of a mutant protein in Huntington's disease (HD) patients, spinocerebellar ataxia (SCA) patients, and Kennedy's disease patients (Orr and Zoghbi 2007). In addition to mutant protein expression produced by conventional RNA translation, proteins expressed by repeat-associated non-ATG (RAN) translation can accumulate in disease cells (Banez-Coronel et al. 2015; Cleary and Ranum 2013). Repeat expansion may also result in the production of a toxic RNA, such as in the case of DM1 (Ranum and Day 2004) and ALS (Donnelly et al. 2013). These mutant RNAs contain the expanded repeat sequence and form intra-nucleoplasmic hairpin loops. The presence of these toxic RNAs in the cells leads to the sequestration of proteins such as the splicing regulator MBNL1 resulting in distinctive foci in DM1 cells (Cho and Tapscott 2007; Jiang et al. 2016; Zhang et al. 2016).

Examples for loss of function disorders are FXS and FRDA. Inhibition of RNA polymerases by expanded GAA repeats leads to reduced *Frataxin* gene transcription in FRDA patient cells (Koeppen 2011). In fragile X patients, epigenetic modifications lead to complete silencing of the *FMR1* gene (Santoro et al. 2011; Sutcliffe et al. 1992). Enrichment of heterochromatin marks is observed close to the repeats in several repeat expansion diseases, such as FRDA, FXS, and DM1 (Cho et al. 2005; Kumari and Usdin 2012; Sutcliffe et al. 1992) suggesting that repeat sequences could alter chromatin composition and trigger epigenetic modifications, which could result in the inhibition of gene transcription or lead to gene silencing. Besides epigenetic alterations, large repetitive sequences and the associated non-B DNA secondary structures formed could probably also impede the RNA polymerases. In line with this, it has been shown that repeats inhibit gene transcription in vitro (Krasilnikova et al. 2007; Pandey et al. 2015) and in vivo (Li et al. 2015).

The stalling of the replication machinery could also have an effect on the transcription machinery. For example, the altered direction of the replication fork and the replication stall at the endogenous GAA repeats could lead to head-on collisions of the RNA polymerase with the DNA polymerase in FRDA cells (Gerhardt et al. 2016). After collision, the replication machinery might be able to dislodge the transcriptional machinery as shown by electron microscopy imaging following insertion of an inducible replication origin either upstream or downstream of an *E. coli* rRNA gene (French 1992). In vitro studies with bacterial enzymes show a slow displacement of transcriptional elongation complexes and replisome pausing during head-on collisions whereas no delay during the codirectional progress of replication and transcription (Pomerantz and O'Donnell 2008, 2010). Thus, head-on collision of RNAPII with replication machinery in the 3'–5' direction may not only lead to the GAA expansions but also affect *FXN* transcription progression in FRDA cells. In addition to the mechanistic impact, replication-transcription collisions have been demonstrated in yeast to facilitate establishment and maintenance of heterochromatin, a phenomenon not yet demonstrated in higher eukaryotes (Nikolov and Taddei 2015). This could explain the enrichment of heterochromatic marks surrounding the repeat tracts. Deciphering the interplay between transcription and replication could be critical from the perspective of therapeutic approaches for repeat expansion diseases.

It was recently shown that R-loops are formed at the endogenous repeat sequences (Groh et al. 2014; Lam et al. 2013; Loomis et al. 2014). R-loops could potentially stall the replication and transcription machinery in these cells, and/or secondary repeat structures could form in the single-stranded DNA strand of the R-loop. In this case R-loops could trigger repeat instability. However, R-loops could likewise be a result of the paused transcription machinery, which is held up by a stalled replication fork. Conversely, R-loops are also formed in unaffected cells, but the function(s) of these R-loops are not clear. It has been suggested that R-loops could function as regulators of chromatin dynamics and are implicated in transcription initiation and termination by modulating the chromatin architectures (Al-Hadid and Yang 2016). When, why, and how R-loops form at the endogenous repeats and whether they positively or negatively impact repeat stability is currently unknown. Also, it has yet to be determined whether R-loops form at the expanded repeats or whether they are a consequence of replication fork stalling at the endogenous locus in patient cells.

### *23.5.2 Effect of Large Repetitive Sequences and Human Diseases*

Non-expanding repetitive sequences like CFS are largely implicated in the development and progression of cancer (Arlt et al. 2003; Glover 2006). DNA breaks have been observed at precancerous lesions, indicating that the instability at CFS loci is an important initial event in cancer (Gorgoulis et al. 2005). In addition, these regions are thought to be hotspots of chromosomal abnormalities such as deletions, duplications, and translocations in a number of cancer cell lines (Chesi et al. 1998; Finnis et al. 2005; O'Keefe and Richards 2006). Furthermore, more than 80% of copy number variations, in preneoplastic cell lines, have been found at repetitive DNA sequences (Tsantoulis et al. 2008). Maintenance of CFS integrity is critical because most of the commonly expressed CFS contain tumor suppressor genes and proto-oncogenes which when altered are associated with a large spectrum of cancers (Ciullo et al. 2002; Hellman et al. 2002; Siprashvili et al. 1997). Breaks at two of the most commonly expressed CFS, FRA3B and FRA16D, result in the destabilization of tumor suppressor genes *FHIT* and *WWOX*, respectively (Bednarek et al. 2000; Ohta et al. 1996; Virgilio et al. 1996). Mutations and downregulation of the *PARK2* gene located at the third most commonly expressed fragile site FRA6E are associated with ovarian cancer, colorectal cancer, and glioblastoma (Denison et al. 2003; Poulogiannis et al. 2010; Veeriah et al. 2010). Furthermore, CFS are also the preferred sites for viral integration (Popescu and DiPaolo 1989; Wilke et al. 1996) and are highly susceptible to oncogenic stress (Bartek et al. 2007). Other repetitive DNA sequences such as ERFs sites are also known to be hotspots of rearrangements in B cell lymphomas (Barlow et al. 2013).

Instability of CFS is a cell type-specific phenomenon. Most of the aphidicolin-induced breaks in lymphocytes are confined to CFS loci (Hecht and Glover 1984).

Accordingly, lymphocyte-specific fragile sites (e.g., FRA3B and FRA16D) break less frequently in fibroblasts, and likewise, fibroblast-specific fragile sites (e.g., 3q13.3 and 1p31.1) are less unstable in lymphocytes (Le Tallec et al. 2011; Letessier et al. 2011). This is possibly one of the factors contributing to the strong association between CFS instability and hematological malignancies (Gumus et al. 2002). However, CFS instability is implicated in cancers originating from different cell lineages as well. This could be because of the fact that although there is a strong cell type-specific correlation between replication initiation and fragility, the repetitive sequences at these loci can still pose a threat to the replication machinery in any cell type. This is further supported by the fact that the absence of proteins implicated in resolving potential structures at CFS repetitive sequences renders even the initiation proficient cell types unstable at these loci (Casper et al. 2002; Durkin et al. 2006; Focarelli et al. 2009; Madireddy et al. 2016a; Pirzio et al. 2008).

Deficiency of some of the most important proteins implicated in maintaining CFS stability is associated with debilitating diseases characterized by premature aging, genomic instability, and cancer. Mutations in the ATR gene, one of the primary regulators of fragile site stability (Casper et al. 2004), results in a condition called Seckel syndrome (O'Driscoll et al. 2003). Werner syndrome and Bloom syndrome are a result of absences of the respective RecQ helicases implicated in CFS instability (Pirzio et al. 2008; Sidorova et al. 2013). The former is associated with premature aging, while the latter is characterized by genomic instability and an increased predisposition to cancer (Ellis and German 1996; Yu et al. 1996). The absence of polymerase eta that induces spontaneous breaks at CFS (Rey et al. 2009) and the absence of nucleases XPF-ERCC1 that are recruited to ultrafine DNA bridges at CFS loci (Naim et al. 2013) are associated with a skin cancer predisposition syndrome called xeroderma pigmentosum (Cleaver 1972). Considering the strong association between fragile sites and malignancies, it is conceivable that instability at these sites contributes to the etiology of each of these diseases.

## 23.6 Techniques to Determine Replication Errors

Relevant techniques are essential to understand the disease mechanisms and to develop therapeutic approaches. Non-human models for repeat instability (e.g., mouse) often do not recapitulate the disease phenotype exactly (Perdomini et al. 2013), limiting the tools which could be used to study mechanism for genome instability. To detect DNA replication initiation sites, the direction of the replication fork, replication fork stalling, and DNA replication termination sites in human cells, methods such as nascent strand analysis, 2-D gel electrophoresis, DNA combing, and single-molecule analysis of replicated DNA (SMARD) are used.

*Nascent Strand Abundance Analysis* Mapping of replication initiation sites in the mammalian genome is achieved through measurement of the relative abundance of nascent DNA throughout a specific region of the chromosome. Therefore, newly

synthesized leading strand DNA is first isolated. Then the relative abundance of the nascent DNA is measured at specific loci using quantitative PCR. It is anticipated that the abundance of nascent DNA within the origin region is greatest at the site where DNA replication begins as compared to a genomic region without a replication initiation site. With this technique replication start sites of the laminB2 origin within an ~500-bp segment (Giacca et al. 1994), the replication initiation sites within the hamster dihydrofolate reductase gene locus (Kobayashi et al. 1998) and the *FMR1* promoter in FXS cells (Gray et al. 2007) were mapped.

*2-D Gel Electrophoresis* The neutral/neutral two-dimensional (2-D) agarose gel technique is a useful tool for understanding the mechanisms leading to the complete duplication of eukaryotic chromosomes. This technique has been used to localize and characterize origins of replication as well as fork progression in a variety of experimental settings. To distinguish different DNA shapes produced by the traveling replication forks, 2-D gels were first used by Brewer and Fangman in 1987. In the first dimension, the DNA molecules are separated by size. Then a gel slice containing the continuum of replicating DNA is cut and subjected to a second round of electrophoresis in a second dimension to resolve replication intermediates of varying topology. In the second dimension, the molecules are separated mainly on the basis of their shape. Nonlinear DNA molecules travel anomalously on agarose gels when compared to linear DNA. To examine replication at a specific DNA segment, the 2-D gel is then blotted and hybridized with specific DNA probes. Two-dimensional gels allow detection of replication fork stalling, which led to the visualization of replication fork pausing at the CGG and GAA repeats (Krasilnikova and Mirkin 2004). Electron microscopy methods are used in combination with 2-D gel electrophoresis to reveal the structure of in vivo DNA replication intermediates. For example, using replication intermediates excised form 2-D gels, it was demonstrated that replication fork reversal occurs after replication fork stalling in human cells (Follonier et al. 2013). These experiments, although advantageous, were limited in their ability to recapitulate the effects of repeats on the DNA replication process at the genomic loci in patient cells. The recent development of single-molecule assays has shed new light on the dynamics of DNA replication at the level of individual chromosomes, such as DNA combing and SMARD.

*DNA Molecular Combing* analyzes single DNA molecules encompassing large genomic regions. Therefore the DNA is stretched on silanized glass cover slips, and the DNA region of interest is observed by hybridization with specific FISH probes. Since DNA analyses using this technique are single molecule, genomes from different cells can be compared to find anomalies, with implications for diagnosis of cancer and other genetic alterations. In 1994, Bensimon and colleagues (1994) used this technique for the first time to extend bacteriophage lambda DNA molecules. In addition, DNA molecular combing is used for the detection of the DNA replication program in large genomic regions. With molecular combing it is possible to detect replication initiation events and calculate replication fork speed and fork stalling on chromatin fiber. DNA replication studies using combed molecules are based on the fluorescent detection of modified nucleotides, such as bromodeoxyuridine (BrdU),

incorporated into newly synthesized DNA at a high resolution. However, the technique is limited due to partial labeling of the DNA fibers.

*Single-Molecule Analysis of Replicated DNA* SMARD is an approach which was adapted from the molecular combing technique and is used to reveal DNA replication initiation events, fork progression, pausing, and termination sites in large genomic regions (Norio and Schildkraut 2001). To observe the DNA replication by SMARD, cells are pulse labeled with two differentially halogenated nucleoside analogs of thymidine. SMARD in contrast to other single-molecule approaches uses longer halogenated nucleoside incorporation times. The labeling period is longer than the time required to fully replicate the genomic region of interest, but short enough to prevent the occurrence of multiple replication cycles. After labeling, the replicated DNA molecules are stretched on silanized glass slides, and the incorporated nucleotides during replication are visualized by immunostaining. FISH is used to detect the genomic loci of interest. To achieve a higher yield of the region of interest, the DNA is enriched by restriction enzyme digestion followed by size separation of the DNA by pulsed-field gel electrophoresis (PFGE). At the end, the mixture of single DNA molecules, representing all stages of the DNA replication process in cells, gives a composed picture of the DNA replication events in a particular cell line. This approach is sensitive enough to study even short replication fork pauses as well as the direction of the replication forks at specific genomic sites. Examination of the sites and severity of replication fork stalling provide more information about the location of secondary repeat structures. This technique can be used to detect variations in replication origin usage and changes in the DNA replication program during cellular development as well as in disease cells.

## 23.7 Strategies to Prevent Genome Instability at Repetitive Sequences

The lack of repeat-associated instability under unperturbed "normal" conditions implies that cells have well-defined mechanisms that facilitate the replication of these loci to prevent genome instability. To rescue replication fork stalling, recruitment of proteins that unravel, nucleolytically process, and/or bypass replication impediments is perhaps essential to complete the DNA replication of repetitive DNA sequences. Following replicative stress, stability of both non-expanding repeats, CFS and ERFs, is associated with the activation of ATR checkpoint-signaling pathway (Barlow et al. 2013; Koundrioukoff et al. 2013; Ozeri-Galai et al. 2008). In the absence of ATR, spontaneous breaks have been reported at repetitive DNA sequences, indicating that ATR is an important regulator of CFS stability (Casper et al. 2002). Accordingly, Chk1, a crucial component of the ATR signaling pathway, has also been implicated in maintaining CFS stability (Durkin et al. 2006). In addition, it was found that deficiency of ATR and ATM facilitates expansions of CGG repeats in mice models (Entezam and Usdin 2009). However, while the

inactivation of the ATM kinase does not directly result in CFS instability, the combined loss of both ATM and ATR leads to increased breaks at CFS (Ozeri-Galai et al. 2008). As alternative the DNA replication after replication fork stalling at repetitive sequences could be rescued and completed by activation of dormant replication origins to avoid genome instability and breaks.

### *23.7.1 Helicases Capable of Unwinding Secondary Repeat Structures*

DNA helicases are an important group of proteins involved in DNA replication, DNA repair, and gene transcription to ensure that these processes continue unobstructed. During replication, helicases unwind DNA so that the DNA can be copied by the DNA polymerases. In addition, helicases remove obstacles, such as secondary DNA structures, which could hinder the DNA replication machinery. Secondary repeat structures could be generated during lagging strand synthesis in the Okazaki initiation zone, which is a stretch of single-stranded DNA. Several helicases have been shown to unwind these noncanonical DNA structures in vitro.

Helicases from the RecQ family were one of the first helicases shown to unwind G-quadruplex structures. *E. coli* RecQ helicases, the *S. cerevisiae* homolog Sgs1 and their human homologs Bloom (BLM) and Werner (WRN), are all capable of efficiently unwinding G-quadruplexes in DNA, including tetrahelical structures formed by the CGG repeats in vitro (Fry and Loeb 1999). Furthermore, it was shown that purified BLM and WRN helicases could unwind a DNA triple helix structure (Brosh et al. 2000). Sgs1 and Srs2 are two yeast helicases, which act as inhibitors of trinucleotide repeat expansion (Anand et al. 2011; Bhattacharyya and Lahue 2004; Dhar and Lahue 2008; Kerrest et al. 2009). Deletion of these helicases causes repeat instability (Anand et al. 2011). Using a single-molecule fluorescence assay, it was found that these helicases resolve trinucleotide hairpins in a complementary manner (Qiu et al. 2015).

As described previously, many members of the RecQ helicases play an important role in stabilization of CFS. The WRN protein plays an essential role in rescuing stalled replication forks by removing DNA secondary repeat structures that impede replication fork movement (Brosh and Bohr 2002; Shen and Loeb 2000). WRN, a helicase and an exonuclease, has been shown to functionally interact with Pol delta and enhance the processivity of Pol delta across CFS repetitive sequences (Kamath-Loeb et al. 2000; Shah et al. 2010). In addition, WRN patient cells display increased spontaneous breaks at CFS, indicating that WRN is a key regulator of CFS stability. The five helicases of the RecQ family have unique substrate specificities; however, multiple reports suggest mild functional redundancy (Mendonca et al. 1993; Wang et al. 2003). In support of this, the WRN and BLM proteins have been shown to work additively to help in normal replication fork progression (Sidorova et al. 2013).

In addition to the RecQ helicases, other helicases are also important in protecting against genomic instability arising at repetitive sequences. PIF1 helicase is a 5‘–3’ DNA helicase important for genome stability and is capable of unwinding G-quadruplexes in vitro. The yeast Pif1 helicase unwinds G-quadruplexes with higher efficiency than other substrates such as Y-structures, and unwinding of G-quadruplexes by Pif1 occurs at equimolar concentrations of the helicase and its substrate (Paeschke et al. 2013). These in vitro experiments describe the Pif1 helicase as one of the most effective helicases that unwind G-quadruplexes. Deletion of the PIF1 helicase affects repeat stability only when the G-rich strand is the leading strand template during replication, which relies on the presence of intact G-quadruplex motifs (Lopes et al. 2011). In vivo, the absence of the PIF1 helicase leads to instability of G-quadruplex forming human minisatellites introduced into the yeast genome (Ribeyre et al. 2009). This effect is further enhanced by the action of G-quadruplex-specific compounds, PhenDC3 and PhenDC6, which stabilize G-quadruplexes (Piazza et al. 2010) and enhance replication fork stalling (Madireddy et al. 2016b). Higher eukaryotes possess only one PIF1 helicase, which is shown to specifically recognize and unwind DNA structures resembling putative stalled replication forks (George et al. 2009).

Another 5‘–3’ helicase known to unwind secondary repeat structures is the Fanconi anemia (FA) complementation group J (FANCJ) RAD3-family DNA helicase. FANCJ belongs to a group of 21 genes that together constitute the FA pathway. Mutations in any 1 of 21 genes cause FA, a rare genetic disorder. The disease is characterized by genomic instability, bone marrow failure, developmental abnormalities, and highly increased predisposition to cancer. Several lines of evidence indicate that FANCJ acts directly during DNA replication to unwind G-quadruplex structures. Furthermore, FANCJ interacts with the replication-associated single-strand binding protein RPA (Gupta et al. 2007; Wu et al. 2008). RPA stimulated FANCJ G-quadruplex unwinding is inhibited by the mismatch repair complex MSH2/MSH6. FANCJ may possibly be loaded 5′ to the G-quadruplex structure on the single-stranded DNA fragment generated prior to Okazaki fragment synthesis.

### *23.7.2 Proteins That Prevent Replication Fork Collapse at Repetitive Sequences*

Besides FANCJ other proteins of the FA tumor suppressor pathway have also been implicated in ensuring stability of difficult to replicate DNA such as repetitive sequences. The FA proteins, although prominently characterized for their essential role in removing inter-strand cross-links (ICL) from DNA, have in the last decade been implicated in preserving genome stability even in response to replicative stress. This has been demonstrated by the observation that the FA pathway is strongly activated in response to replisome stalling under condition of replicative stress (Petermann and Helleday 2010; Petermann et al. 2010; Taniguchi et al. 2002). Involvement of the FA proteins at repetitive sequences is attributed to their role in

cooperatively resolving ultrafine DNA bridges that result from under-replicated DNA at CFS (Chan and Hickson 2009; Naim et al. 2013). In addition, the absence of key FA proteins, such as FANCA, FANCD2, and BRCA2/FANCD1, has been associated with severe genomic instability at stalled replication forks (Chaudhury et al. 2014; Karanja et al. 2014; Schlacher et al. 2012), further supporting its role in replication. In the absence of FA proteins, replication forks spontaneously pause at the A/T-rich fragility core of CFS loci (Madireddy et al. 2016a). Moreover, spontaneous CFS breaks are observed in FANCD2 patient-derived lymphocytes, and this is further exacerbated in the presence of aphidicolin-induced replicative stress (Howlett et al. 2005; Madireddy et al. 2016a). Given the requirement for FA proteins in protecting stalled replication forks, it is not surprising that the FA pathway is one of the prominent cellular mechanisms that ensures efficient and timely replication of repetitive DNA sequences.

In addition to the FA pathway and helicases, a number of other proteins are involved in restoring DNA synthesis. When replication forks stall at repetitive DNA sequences, they have a tendency to regress spontaneously to form four-way DNA junctions referred to as chicken foot structures (Fouche et al. 2006; Neelsen and Lopes 2015; Sogo et al. 2002). Nucleases, such as DNA2, are a class of proteins that prevent the collapse of stalled replication forks. This is possibly one of the reasons why DNA nucleases have been implicated in maintaining fragile site stability. Under stress, the absence of nucleases such as XPF-ERCC1 and Mus81-EME1 has been associated with increased breaks at CFS, further supporting their role in preventing CFS instability (Naim et al. 2013; Ying et al. 2013). However it is important to note that the stabilization of stalled forks by the FA proteins, BRCA1 and RAD51, is crucial to prevent fork regression and collapse. The absence of fork stabilizing proteins leads to nucleolytic degradation of nascent DNA and genomic instability (Schlacher et al. 2012). Another DNA nuclease that is recruited to paused replication forks to prevent genomic instability is the Fanconi anemia-associated nuclease 1 (FAN1) which is recruited by the FANCD2 protein (Lachaud et al. 2016). So in addition to fork stabilization, FANCD2's role at CFS could be attributed to its role in recruiting facilitator proteins to sites of replication fork stalling. This role of FANCD2 is further supported by the fact that FANCD2 recruits CtIP to sites of stalled replication forks (Yeo et al. 2014).

Translesion DNA polymerases are another class of proteins that facilitate the efficient synthesis of repetitive sequences, thereby ensuring replication fork stability. In support of this, Pol eta and Pol kappa have been shown to be more efficient at in vitro synthesis of repetitive sequences (Bergoglio et al. 2013; Walsh et al. 2013). Moreover, the depletion of another translesion polymerase, Rev3 (the catalytic subunit of Pol zeta), was shown to induce ultrafine DNA bridges and CFS fragility (Bhat et al. 2013). Although biochemical studies have demonstrated the involvement of translesion polymerases at repetitive sequences, the mechanism behind both their recruitment and their actual function at these sequences is unclear. Given the previously described role of FANCD2 in recruiting Pol eta to the sites of damage (Fu et al. 2013), one possibility could be that FANCD2 recruits Pol eta to fragile site loci by a similar mechanism. Overall, the processes of unperturbed CFS replication

and maintenance appear to require the collaborative action of a number of proteins. This is further substantiated by reports that show that the WRN helicase physically interacts with and co-localizes with translesion polymerase eta to alleviate replication fork stalling (Kamath-Loeb et al. 2007).

### *23.7.3 Dormant Origin Activation to Prevent Genome Instability at Repetitive Sequences*

In addition to the recruitment of proteins to sites of replication stalling, dormant origin activation could be an additional and important mechanism by which cells complete replication in regions surrounding the stalled forks. In 1977, J. Herbert Taylor first described that cells license more origins than are actually utilized during the DNA replication process (Taylor 1977). The 3–20 fold excess replication origins that are licensed but not used during each S phase are termed as dormant origins (Blow and Ge 2009; Blow et al. 2011; Wong et al. 2011). Slowing or stalling of the replication fork results in the activation of dormant origins (Alver et al. 2014; Blow and Ge 2009). Dormant origin activation was observed in response to fork stalling at the A/T-rich fragility core of the endogenous CFS-FRA16D locus in cells deficient for the FANCD2 protein (Madireddy et al. 2016a). However, not all CFS loci activate dormant replication origins in response to stress. For example, the FRA16C CFS locus fails to activate dormant replication origins after replicative stress, and this has been implicated in genome instability (Ozeri-Galai et al. 2011). Due to the paucity of replication origins at certain repetitive DNA sequences, completion of the genomic DNA replication is in jeopardy in some human cells.

## 23.8 Conclusion

The faithful and timely completion of the DNA synthesis is essential to maintain normal cellular functions. Therefore the cell has developed several strategies to ensure the accurate duplication of the genome to prevent chromosomal instability. As described above, complex and well-coordinated networks of proteins work together to ensure the successful completion of the DNA replication process. The inactivation of any one of these proteins can lead to genomic instability. Repetitive sequences because of their unique characteristic features are particularly vulnerable genomic regions, which have the tendency to impede DNA replication leading to replication fork blockage, DNA breaks, and genomic instability. Inaccuracies during DNA replication and the deficiency of proteins, which could stabilize and/or restart replication fork, can lead to repeat expansions, chromosomal instability, and several severe human diseases. Despite decades of research, there is still no cure for these diseases that result from repeat expansions and chromosomal fragility. We believe the reason is the inherent complexity of the mechanisms triggering repeat

instability in human cells. While it is known that defects during DNA replication contribute to the overall etiology of repeat-associated diseases, there is a need to elucidate the exact molecular mechanisms that trigger the replication inaccuracies, causing these diseases.

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