Regulation of the initiation of DNA replication: Interplay between DNA topology and chromatin structure

by

Emmanouil Rampakakis

Department of Biochemistry
McGill University,
Montreal, Quebec, Canada

January 2009

A thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements of the degree of Doctor of Philosophy

TABLE OF CONTENTS

PREFACE

This thesis was written according to the guidelines provided by the Faculty of Graduate and Postdoctoral Studies, which state:

As an alternative to the traditional thesis format, the thesis can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following:

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)

2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceding and following each manuscript are mandatory.

3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: 1. a table of contents; 2. a brief abstract in both English and French; 3. an introduction which clearly states the rationale and objectives of the research; 4. a comprehensive review of the literature (in addition to that covered in the introduction to each paper); 5. a final conclusion and summary; 6. a thorough bibliography; 7. Appendix containing an ethics certificate in the case of research involving human or animal subjects, microorganisms, living cells, other biohazards and/or radioactive material.

4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis, the candidate must be the primary author (the author who has made the most substantial contribution) for all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

ABSTRACT

DNA replication initiates at replication origins and involves the ordered assembly of a pre-Replicative Complex (pre-RC), consisting of the ORC1-6, Cdc6, Cdt1 and MCM2-7 proteins. The Ku heterodimer (Ku70/Ku80) was previously implicated in the pre-RC assembly but its mechanism of action is yet unknown. Here, Ku is shown to form a complex with DNA topoisomerase IIβ (topo IIβ) both *in vitro* and *in vivo* and target it onto replication origins. The enzymatic activity of topo IIβ creates transient, origin-specific dsDNA breaks thus affecting the DNA topology during pre-RC assembly. Pharmacological inhibition of the topo II activity interferes with the pre-RC assembly at the level of ORC, Cdt1 and Cdc45 recruitment resulting in prolonged G_1 phase. Similarly, RNAi-mediated inhibition of the Ku80 expression leads to reduced chromatin loading of pre-RC proteins, decreased origin activation, and activation of a cell cycle checkpoint through a mechanism involving low Cdk2 activity. Overall, the data implicate Ku in the initiation of DNA replication through regulation of the DNA topology and suggest the interplay of the DNA replication and cell-cycle machineries.

Pre-RC assembly is followed by the recruitment of the replicative machinery which initiates DNA replication. Study of the chromatin structure of the human lamin B2 and hOrs8 and the monkey Ors8 origin revealed that dynamic changes within the levels of post-translationaly modified histone H3 occur during their activation, which differ between early- and late-firing origins. Late origins have a compact chromatin structure during S phase, which transiently opens during their activation, whereas early origins display histone modifications associated with open chromatin structure, which is only passively affected during their activation by passage of the replication fork.

RÉSUMÉ

La réplication de l'ADN commence aux origines de réplication et nécessite l'assemblage méthodique du complexe pré-réplicatif (pre-RC), qui consiste des protéines ORC1-6, Cdc6, Cdt1 et MCM2-7. L'hétérodimer Ku (Ku70/Ku80) a été précédemment impliqué dans l'assemblage du pre-RC, mais le mécanisme de cette action est inconnu jusqu'à présent. Ici, Ku est montré former un complexe avec la topoisomérase IIβ (topo IIβ), tant *in vitro* qu'*in vivo* et le conduire aux origines de la réplication. L'activité enzymatique de topo IIβ crèe de brisures transitoires et specifiques aux origines, lesquelles affectent la topologie de l'ADN pendant l'assemblage du pre-RC. L'inhibition pharmacologique de l'activité de topo IIβ tripote avec l'assemblage du pre-RC au niveau du récrutement des protéines ORC, Cdt1 et Cdc45, ayant comme résultat la prolongation de la phase G_1. De même, une inhibition de l'expéssion de Ku80 mène à un chargement réduit des proteins du pre-RC sur la chromatine, à une activation réduite des origines de réplication, ainsi qu' à l'activation d'un contrôle du cycle céllulaire par un mécanisme qui implique une activité réduite de Cdk2. En général, les donnés impliquent Ku dans l'initiation de la réplication de l'ADN par la régulation de la topologie de l'ADN et suggèrent une réciprocité entre les machinéries de la réplication de l'ADN et du cycle cellulaire.

L'assemblage du pre-RC est suivi du récrutement de la machinérie réplicative, qui initie la réplication de l'ADN. Une étude de la structure de la chromatine des origines de la réplication humaines lamin B2 et hOrs8, ainsi que celle du singe Ors8, a montré des changements dynamiques aux niveaux des histones H3 modifiés qui ont lieu pendant leur activation et lesquelles diffèrent entre les origines qui sont actives tôt et tard dans la

phase S. Les dernières possèdent une structure de chromatine qui est compacte pendant la phase S, laquelle s'ouvre d'une façon transitoire pendant l'activation de ces origines, tandis que les origines qui sont activées tôt montrent des modifications des histones associées avec une structure 'ouverte' de la chromatine, qui est passivement affectée pendant leur activation, seulement par le passage de la fourche de la réplication.

CLAIMS TO ORIGINALITY

The work presented in this thesis contains the following original scientific findings:

- The role of Ku in the initiation of DNA replication is mediated through regulation of the DNA topology.
- Topoisomerase IIβ is targeted onto replication origins by Ku.
- Ku, topoisomerase IIβ and PARP-1 bind specifically to the human lamin B2 and hOrs8 replication origins *in vivo*.
- The enzymatic activity of topoisomerase II generates two consecutive dsDNA breaks in proximity or within the origin area covered by the pre-RC complex, during early and mid-G_1 phase.
- Pharmacological inhibition of topoisomerase II results in the inhibition of pre-RC assembly and prolongation of G_1 phase.
- RNAi inhibition of Ku80 results in the activation of a cell cycle checkpoint which blocks cell-cycle progression in late G_1 phase with low Cdk2 activity due to increased p21 expression and decreased Cdc25A and Cdk2 levels.
- The late-firing hOrs8 origin has a compact chromatin structure (low levels of H3 Ac K9+K14 and H3 Me K4 but increased H3 Me K9 levels), whereas the early-firing lamin B2 origin has an open chromatin configuration (high levels of H3 Ac K9+K14 and H3 Me K4, but low levels of H3 Me K9) before activation.
- Dynamic changes occur in the chromatin structure of DNA replication origins during their activation.

CONTRIBUTION OF AUTHORS

Chapter 2: Rampakakis, E. and Zannis-Hadjopoulos, M. "Transient double-stranded DNA breaks during pre-Replication Complex assembly". Manuscript submitted to PNAS. I performed all experiments, analyzed the data and prepared the manuscript. Dr. Maria Zannis-Hadjopoulos supervised the work.

Chapter 3: Rampakakis, E., Di Paola, D. and Zannis-Hadjopoulos, M. "Ku is involved in cell growth, DNA replication and G1-S transition". J Cell Sci. 2008 Mar 1; 121(Pt 5):590-600. Domenic Di Paola carried out and analyzed the data of the nascent DNA strand assay (Figure 3B and 3C). I carried out all the other experiments, analyzed the data and prepared the manuscript. Dr. Maria Zannis-Hadjopoulos supervised the work.

Chapter 4: Rampakakis, E., Di Paola, D., Chan, M.K. and Zannis-Hadjopoulos, M. "Dynamic changes in the chromatin structure during replication origin activation. Journal of Molecular Biology". Manuscript submitted to the Journal of Molecular Biology. Domenic Di Paola carried out the chromatin immunoprecipitation assays (Figure 3) and Man Kid Chan the quantification of post-translationally modified histone H3 during S phase (Figure 4A and 4B). I conceived the study, designed the experiments, performed the remaining experiments, analyzed all the data and prepared the manuscript. Dr. Maria Zannis-Hadjopoulos supervised the work.

Other publications by the author can be found at the end of the thesis.

ABBREVIATIONS

ACS	ARS consensus sequence
APC	Anaphase-promoting complex
ARS	Autonomously replicating sequence
ATM`	Ataxia telangiecstasia-mutated
BrdU	5'-bromo-2'-deoxyuridine
BSA	Bovine serum albumine
CBP	Cruciform-binding protein
CDK	Cyclin-dependent kinase
DDK	Dbf4-dependent kinase
DSB	Double-strand break
DUE	DNA unwinding element
HDAC	Histone deacetylase
HR	Homologous recomgination
IR	Inverted repeat
MAR	Matrix attachment region
MEF	Mouse embryonic fibroblast
MCM	Minichromosome maintenance
NHEJ	Non-homologous end-joining
OBA	Origin-binding activity
ORC	Origin recognition complex
ORE	Origin recognition element
PARP-1	Poly-ADP ribose polymerase 1

Pre-IC	Pre-initiation complex
Pre-RC	Pre-replicative complex
PTM	Post-translational modification
RFC	Replication factor C
RNAi	RNA-interference
RPA	Replication protein A
Topo	Topoisomerase
TSA	Trichostatin A

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

First, I would like to thank my supervisor, Dr. Maria Zannis-Hadjopoulos, for giving me the opportunity to carry out my doctoral studies in her lab. I am also thankful for all her help and support, inside and outside of the laboratory, as well as for giving me the freedom to pursue my ideas.

I would like to thank my parents Vangelis and Mirsini and my brother Giannis for their constant support and for being behind me throughout my Ph.D.

I would like to thank several lab members for their support and entertainment, including Dr. Sahar Sibani for teaching me numerous techniques and for her invaluable scientific advice; Domenic Di Paola and Man Kid Chan for their support and humor; Annie Beaudry for laboratory maintenance and entertainment.

I would also like to thank Dr. Neil Osheroff for providing me with recombinant Topoisomerase IIβ to use in my research.

Finally, I would like to thank all of my friends for their psychological support and faith in me, providing me with strength and determination to get through my Ph.D.

Chapter 1:

General Introduction

Part of this chapter appears in the article "Metazoan Origins of DNA Replication: regulation through dynamic chromatin structure" (Emmanouil Rampakakis, Dina Arvanitis, Domenic Di Paola and Maria Zannis-Hadjopoulos), accepted for publication in the Journal of Cellular Biochemistry

1. DNA REPLICATION

The fundamental component required for the existence of a cell or a virus is their genome. Genomic maintenance and propagation is therefore of great importance for the maintenance of life. DNA replication in both prokaryotes and eukaryotes is tightly regulated at the level of initiation ensuring that the genome is accurately replicated once and only once per cell cycle and segregated equally to the daughter cells (reviewed in (Sclafani and Holzen 2007). Replication initiates at *cis*-acting elements, termed origins, which are recognized by *trans*-acting initiator proteins in order for DNA synthesis to occur. The mechanism of initiation is evolutionary conserved consisting of origin recognition, assembly of multiprotein pre-replication complexes, helicase activation and loading of the replicative machinery (replisome). Following recruitment, the replisome replicates the DNA bi-directionally in a semi-conservative manner. Due to the anti-parallel nature of the two strands of the double helix (i.e one strand runs in the 5'-3' direction, while the other has the opposite, 3'-5', polarity) and the enzymatic property of the DNA polymerases to synthesize DNA in only the 5'-3' direction, only one strand (leading strand) serves as a template for continuous DNA synthesis, while the other (lagging strand) is synthesized discontinuously involving multiple RNA-primed reinitiations and the generation of Okazaki fragments. These fragments are rapidly joined into a continuous strand by the enzymatic action of DNA ligase which catalyzes the formation of the phosphodiester bond that seals the nicks between the discontinuously synthesized strands.

2. ORIGINS OF DNA REPLICATION

Mammalian DNA replication is initiated at distinct chromosomal sites (origins) as evidenced by numerous different approaches, including DNA fiber autoradiography, electron microscopy, and ectopic replication potential (Cairns 1966; Huberman and Riggs 1968; Pearson, Shihab-El-Deen et al. 1994; Altman and Fanning 2001; Altman and Fanning 2004; Paixao, Colaluca et al. 2004). A replication origin is defined both functionally and genetically. The "*functional*" origin is the physical site where DNA replication begins, while the "*genetic*" origin (replicator) includes all the DNA sequences required for initiation of replication. In simple genomes, these two origins coincide (reviewed in (Todorovic, Falaschi et al. 1999; Zannis-Hadjopoulos and Price 1999; Cvetic and Walter 2005). While bacterial genomes are replicated by a single origin, eukaryotic chromosomes contain multiple replication origins. Fiber autoradiography studies suggest that *S.cerevisiae* has between 250 and 400 origins per genome while their number in mammals is on the order of 10^4-10^6, each spaced apart at approximately 50-250kb, depending on the stage of development, growth conditions, or cell transformation status (Edenberg and Huberman 1975; Hand 1978; Martin 1981; Anglana, Apiou et al. 2003). Replication origins and the DNA units replicating under their control (replicons) are grouped into clusters of 23-100 which are activated simultaneously. During S phase, the temporal order of cluster activation is tightly regulated resulting in a sequential replication of defined sets of chromosomal subregions (Edenberg and Huberman 1975; Hand 1978; Cimbora and Groudine 2001; Goren and Cedar 2003). The members of these clusters have been hypothesized to share a common DNA sequence, secondary structure or subnuclear localization (Stinchcomb, Stuhl et al. 1979; Struhl, Stinchcomb et al. 1979;

Small, Nelkin et al. 1982; Zannis-Hadjopoulos, Kaufmann et al. 1984; Zannis-Hadjopoulos, Frappier et al. 1988; DePamphilis 1993; Zink 2006).

2.1 Anatomy of Metazoan Origins

A typical mammalian origin of DNA replication consists of a number of core elements which are necessary for origin activation as well as auxiliary elements which function as modifiers of origin activity (Figure 1.1) (reviewed in (DePamphilis 1993; Zannis-Hadjopoulos and Price 1999). The core elements include the *o*rigin *r*ecognition *e*lement (ORE), the *D*NA-*u*nwinding *e*lement (DUE), the A/T-rich element, cruciforms, and *m*atrix *a*ttachment *r*egions (MARs), while adjacent transcription factor binding sites can function as enhancing or silencing auxiliary elements.

2.1.1 Origin Recognition Element (ORE)

The ORE is the genetic determinant of the origin whereby sequence-specific binding of replication initiator proteins occur. The best characterized eukaryotic ORE is the ACS (*A*utonomously replicating sequence *C*onsensus *S*ite), identified in *S.cerevisiae* (Tschumper and Carbon 1982; Broach, Li et al. 1983; Kearsey 1984; Van Houten and Newlon 1990), which is an AT-rich consensus sequence (A/TTTTAT/CA/GTTT A/T) able to confer autonomous replication to non-origin containing plasmids. A 36-base-pair (bp) degenerate nucleotide sequence was also identified as the human ORE (Price, Allarakhia et al. 2003). Versions of this consensus were shown to function in DNA replication in human, monkey, murine, bovine, chicken and fruit fly cells. Mutation analysis of the 36-bp consensus sequence suggested that an internal 20-bp human

consensus sequence is sufficient to act as a core origin element (5'-TMDAWKSGBYTSMAAWYWBC-3' where M=A or C; D=A, G or T; W=A or T; K=G or T; S=C or G; B=C, G or T; Y=C or T). The distribution of this 20mer consensus over 1Mb of human chromosomal DNA is similar, quantitatively and qualitatively to the distribution of the ACS on *S. cerevisiae* chromosomes. Since its identification the 20-bp consensus has been used to predict chromosomal regions that contain replication origins (Price, Allarakhia et al. 2003; Di Paola, Price et al. 2006) and currently is being used as bait for the identification of new replication initiator proteins (Di Paola et al., submitted)

2.2.2 DNA Unwinding Element (DUE)

The DUE is an easily unwound DNA region due to helical instability, associated with replication origins in bacteria, yeast and metazoa (Bramhill and Kornberg 1988; Huang and Kowalski 1993; Lin and Kowalski 1997; Liu, Malott et al. 2003; Casper, Kemp et al. 2005). This instability is determined by base-stacking interactions and, therefore, is reliant on nucleotide sequence rather than A/T content (Natale, Schubert et al. 1992). Deletion or mutation of the DUE region suppresses initiation of DNA replication (Kowalski, Natale et al. 1988; Umek and Kowalski 1988; Natale, Schubert et al. 1992; Liu, Malott et al. 2003) while heterologous DUEs restore origin activity suggesting that helical instability is essential for chromosomal origin activity (Lin and Kowalski 1997; Gilbert 2001). DUEs display hypersensitivity to single-strand-specific endonucleases, such as P1 endonuclease (Williams and Kowalski 1993), and have been linked to a variety of hereditary genome instability disorders such as the Werner

syndrome (Choudhary, Sommers et al. 2004), Bloom syndrome (Sun, Karow et al. 1998) and spinocerebellar ataxia (Matsuura, Fang et al. 2004; Liu, Bissler et al. 2007).

2.2.3 A/T Rich Element

A/T-rich sequences have been identified as important elements of DNA replication in a variety of eukaryotes (reviewed in (DePamphilis 1993)). Their role in the initiation of DNA replication is variable, acting either as OREs or origin enhancers; ARS (*A*utonomously *R*eplicating *S*equences) in *S.pombe* have an unusually high A/T content (Kelly and Brown 2000), which is recognized by the initiator protein SpORC (*O*rigin *R*ecognition *C*omplex) through the N-terminal binding domain of the ORC subunit 4 (ORC4) (Chuang and Kelly 1999). This domain contains nine copies of the HMG-I (Y)-related AT-hook motif which bind to the minor groove of A/T-rich DNA stretches sequence-non-specifically (Reeves and Beckerbauer 2001). A/T-rich elements have also been identified in *D.melanogaster* (Zhang and Tower 2004) and human replication origins (Keller, Ladenburger et al. 2002; Koina and Piper 2005). Although the exact role of the A/T-rich sequence in these organisms is not clear it has been shown that 4 to 7 consecutive adenines display intrinsic curvature, resulting in bent DNA (Barbic, Zimmer et al. 2003) which in turn has been identified in prokaryotic, SV40, yeast and hamster replication origins (DePamphilis 1996). Recently, the human as well as *D.melanogaster* ORC complex were found to display preferential binding to A/T-rich sequences (Vashee, Cvetic et al. 2003; Remus, Beall et al. 2004), suggesting ORC as the binding candidate for A/T rich elements.

2.2.4 Matrix Attachment Regions (MAR)

MARs are rich in AT and repetitive sequences, mapping to regions where the DNA is intrinsically curved or kinked, and has a propensity for base pairing (von Kries, Phi-Van et al. 1990; Bode, Kohwi et al. 1992; Liebich, Bode et al. 2002; Fiorini, Gouveia Fde et al. 2006). Sequences at or near the human lamin B2 and β-globin origins, the Chinese hamster dihydrofolate reductase (DHFR) genes, and the *Xenopus* and mouse c-myc origins function as dynamic MARs during the cell cycle (Razin, Vassetzky et al. 1991; Lagarkova, Svetlova et al. 1998; Phi-van, Sellke et al. 1998; Djeliova, Russev et al. 2001; Mesner, Li et al. 2003; Girard-Reydet, Gregoire et al. 2004). These regions are thought to anchor the replication origins to the nuclear matrix during G_1 phase, thus allowing the DNA replication machinery to assemble (Boulikas 1996). Once the origins are activated, they dissociate from the nuclear matrix until the next cell cycle (Brylawski, Cohen et al. 2000; Djeliova, Russev et al. 2001).

2.2.5 Cruciforms

Cruciforms are naturally occurring DNA secondary structures, which arise through intra-strand base pairing of palindromic (inverted repeat; IR) DNA sequences. Such structures are widely distributed in the DNA of both prokaryotes and eukaryotes (Wilson and Thomas 1974; Schmid, Manning et al. 1975; Panayotatos and Fontaine 1987) and may affect the supercoiling degree of DNA, nucleosome positioning, formation of other DNA secondary structures or directly interact with proteins (reviewed in (Pearson, Zorbas et al. 1996)). Numerous studies have shown that cruciforms serve as recognition signals at or near origins of DNA replication (Pearson, Zorbas et al. 1996;

Zannis-Hadjopoulos, Sibani et al. 2004). Using monoclonal antibodies raised against cruciform DNA structures it was shown that there is a dynamic formation of cruciforms in mammalian nuclei, reaching a maximum at the G_1/S boundary (Ward, McKenzie et al. 1990; Ward, Shihab-El-Deen et al. 1991). Addition of monoclonal anti-cruciform antibodies to permeabilized cells resulted in a 2- to 6-fold enhancement of DNA synthesis (Zannis-Hadjopoulos, Frappier et al. 1988) by a mechanism that is thought to involve cruciform stabilization and recognition by replication initiator proteins. In support of this, a cruciform-binding activity was purified which was identified as a member of the 14-3-3 protein family and participates in the initiation of DNA replication (Pearson, Ruiz et al. 1994; Todd, Cossons et al. 1998; Yahyaoui, Callejo et al. 2007) (reviewed in (Zannis-Hadjopoulos, Yahyaoui et al. 2008)).

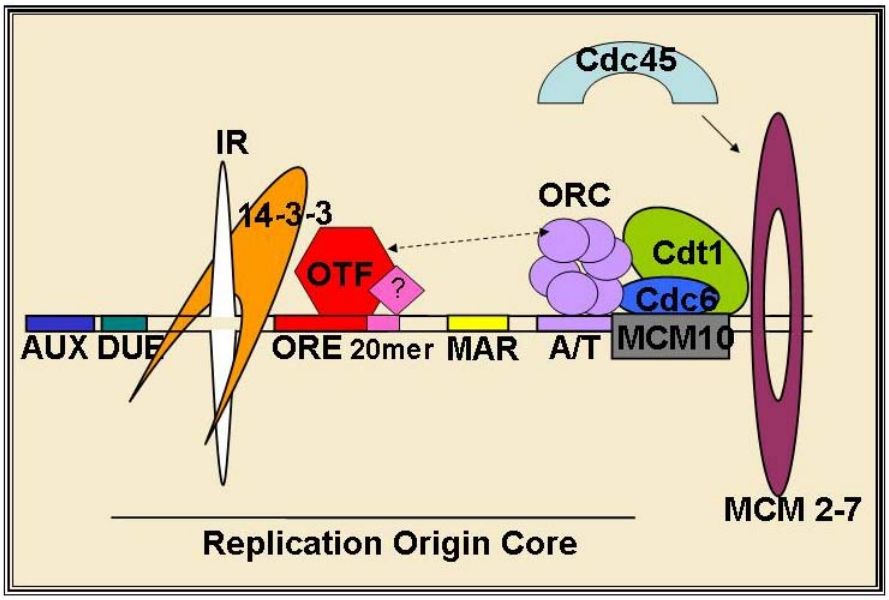


Figure 1.1: Model showing the anatomy of a metazoan replication origin as well as the various initiator proteins that bind onto it. The metazoan origin consists of several *cis*-acting elements that form the origin core, and auxiliary elements (AUX) that modify the activity of the origin. Core components include the origin recognition element (ORE) with the 20-bp consensus sequence (20mer), the DNA unwinding element (DUE), the A-T-rich sequence (A/T), the matrix attachment region (MAR), and inverted repeats (IR) that extrude into cruciforms. OTF: ORC targeting factor. The question mark indicates that other initiator proteins, unknown yet, may bind to the ORE/20mer (Rampakakis, Arvanitis et al. 2009).

3. INITIATION OF DNA REPLICATION

Initiation of DNA replication depends on the activation of replication origins (oris or replicators) distributed throughout the genome. These sites are marked by: a) the presence of the mammalian consensus sequence (Section 2.2.1) within a favorable chromatin context, and b) the highly organized binding of initiator proteins (IPs), which unwind the DNA (helicase activity) and recruit additional downstream proteins. The best characterized IP is the *o*rigin *r*ecognition *c*omplex (ORC; reviewed in (Sasaki and Gilbert 2007)), which acts as a cell-cycle-regulated landing dock for the downstream initiator proteins Cdc6 and Cdt1 (Figure 1.2). Binding of the latter two proteins during G_1-phase permits the subsequent loading of the putative DNA helicase, the minichromosome maintenance protein complex (MCM2-7) (Cook, Chasse et al. 2004), forming the pre-replication complex (pre-RC), and thus licensing the replication origins [(DePamphilis, Blow et al. 2006) and refs. therein]. The MCM2-7 complex has very low helicase activity *in vitro* by itself (Ishimi 1997; Lee and Hurwitz 2000), but is activated when in complex with Cdc45 and GINS (Moyer, Lewis et al. 2006). At the G_1/S transition, the activity of two kinases, the Dbf4-dependent kinase (DDK) (Cdc7-Dbf4) and the cell-cycle dependent kinase 2 (Cdk2-Cyclins E/A), results in the formation of a pre-initiation complex, containing the cdc45 and GINS proteins as well as the activation of the putative DNA helicase and the recruitment of the replicative DNA polymerases (Mimura and Takisawa 1998; Tanaka and Nasmyth 1998; Sheu and Stillman 2006; Yabuuchi, Yamada et al. 2006; Krasinska, Besnard et al. 2008). Upon entry into S phase, multiple mechanisms, which will be further discussed later, ensure that the replication initiation

machinery is inactivated so as to avoid re-replication of chromosomal regions and genome instability (Blow and Dutta 2005; Hook, Lin et al. 2007).

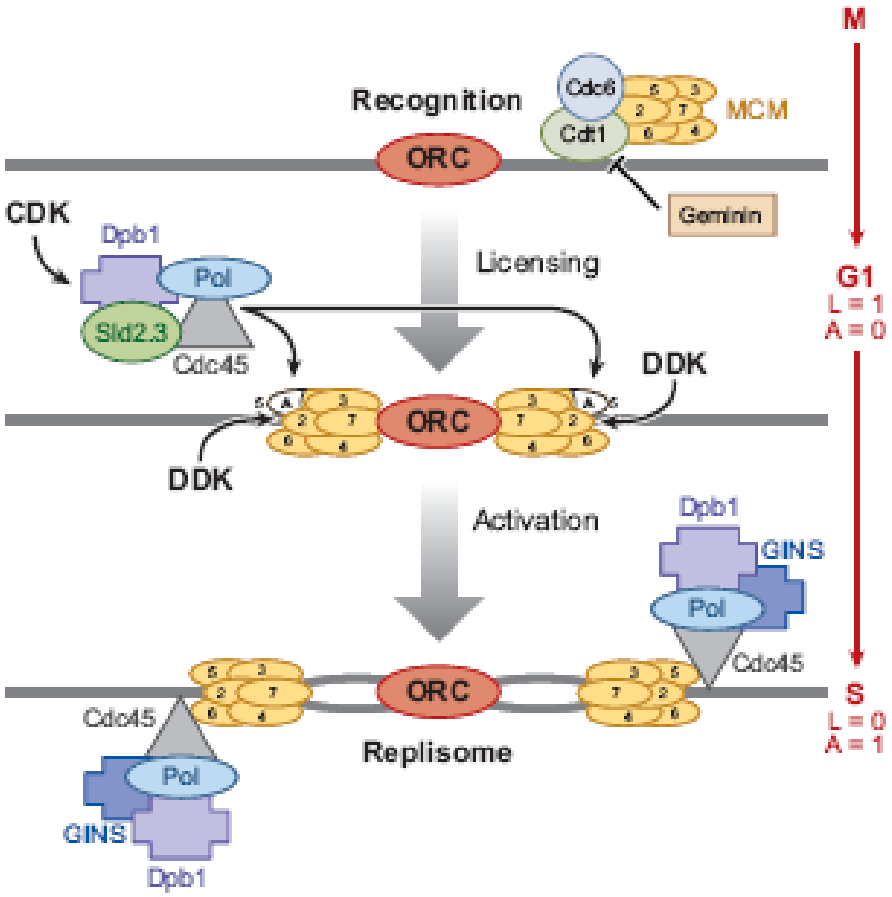


Figure 1.2: Overview of the assembly of initiator proteins at a eukaryotic origin. The origin is initially recognized by ORC, followed by Cdc6 and Cdt1, which load the hexameric MCM helicase to form the pre-RC in G_1 phase. Geminin inhibits Cdt1 and pre-RC formation. Cdk and DDK become active in late G_1, activating the MCM helicase and loading the replisome that contains the replicative DNA polymerases. See section 3.1 for details. (Sclafani and Holzen, 2007)

3.1 Pre-Replicative Complex (pre-RC) Assembly

3.1.1 Origin Recognition Complex (ORC)

The ORC is a hetero-hexameric complex containing ORC1-6 subunits in equal stoichiometry (reviewed in (Chesnokov 2007; Sasaki and Gilbert 2007), originally identified as an ACS (*A*RS *c*onsensus *s*equence) binding protein in *Saccharomyces cerevisiae* (Sc) (Bell and Stillman 1992). Ever since, homologues of all six subunits have been identified in all eukaryotic species, including *S.pombe* (Sp) (Gavin, Hidaka et al. 1995), *D.melanogaster* (Dm) (Gossen, Pak et al. 1995), *Xenopus laevis* (Xl) (Rowles, Chong et al. 1996) and *Homo sapiens* (Hs) (Gavin, Hidaka et al. 1995; Takahara, Bong et al. 1996; Ishiai, Dean et al. 1997; Quintana, Hou et al. 1997; Quintana, Thome et al. 1998; Tugal, Zou-Yang et al. 1998; Pinto, Quintana et al. 1999; Dhar and Dutta 2000).

All ORC subunits, except for ORC6, belong to the superfamily of AAA^+ ATPases (*A*TPases *A*ssociated with various cellular *A*ctivities) with conserved Walker A, B, C and D motifs (Koonin 1993; Bell and Dutta 2002). ATP binding by the ORC1 subunit is required for DNA binding by the complex (Bell and Stillman 1992; Chesnokov, Remus et al. 2001; Klemm and Bell 2001), while using a frog *in vitro* system the ATP-binding activities of HsORC4 and HsORC5 were also found to be necessary for DNA replication (Giordano-Coltart, Ying et al. 2005). Once bound to origin DNA, the ORC ATPase activity is inhibited until the recruitment of downstream initiator proteins (Bell and Dutta 2002).

3.1.1.1 ORC targeting to DNA

Although the ORC proteins are conserved among eukaryotes, their DNA-binding properties have diverged over the course of evolution. With the exception of ScORC (Bell, Kobayashi et al. 1993), all other ORC complexes have lost their sequence dependence (Vashee, Cvetic et al. 2003; Remus, Beall et al. 2004; Schaarschmidt, Baltin et al. 2004), suggesting the existence of different mechanisms for origin specification in these species. Thus, it has been proposed that targeting of metazoan ORC to specific replication origins, relies on DNA topology (Falaschi, Abdurashidova et al. 2007), accessory targeting factors that exhibit sequence-specificity (Gerbi, Strezoska et al. 2002; Kearsey and Cotterill 2003; Cvetic and Walter 2005) and/or the presence of permissive local chromatin structure (Aladjem 2007).

Human, frog and *S.pombe* ORCs exhibit a clear preference for A-T rich rather than consensus sequences (Chuang and Kelly 1999; Vashee, Cvetic et al. 2003). Interestingly enough though, DmORC displays significant differences in affinity for sequences with similar A/T content (Austin, Orr-Weaver et al. 1999). Recently, it was proposed that this slight preference for high A/T content by DmORC may be due to the topological conformation adopted from these sequences. Indeed, the affinity of DmORC for negatively supercoiled DNA was found to be 30-fold higher than for either relaxed or linear DNA (Remus, Beall et al. 2004). Moreover, DNA topoisomerases, the regulatory enzymes of DNA topology, functionally interact with a human replication origin during origin activation, further supporting a role for DNA topology in the origin-recognition by ORC (Abdurashidova, Radulescu et al. 2007; Falaschi, Abdurashidova et al. 2007).

Another proposed mechanism for metazoan origin specification involves the function of accessory proteins that target ORC to replication origins. The Epstein-Barr virus (EBV) replicates its genome from the latent origin of replication, oriP, using the ORC of the host. Initiation of DNA replication from oriP is affected by the viral transactivator protein EBNA-1 (Schepers, Ritzi et al. 2001), the telomere repeat factor 2 (TRF2) (Atanasiu, Deng et al. 2006) and the high mobility group protein A1a (HMGA1a) (Thomae, Pich et al. 2008), which have been proposed to recruit HsORC to the replicator by binding to specific DNA sequences (EBNA-1, TRF2) or the minor groove of AT-tracks (HMGA1a) at chromosomal origins. In mice, the transcriptional repressor protein A1F-C was shown to be able to recruit Orc1 to the rat aldolase B origin and thus affecting replicator activity from this origin (Minami, Takahashi et al. 2006). Finally, a role for ORC (HsORC) positioning was also described in humans; human cells hypomorphic for the Ku DNA repair protein displayed decreased origin usage and prolonged G1 phase due to defective ORC assembly (Sibani, Price et al. 2005; Sibani, Price et al. 2005). Recently, a direct, transcription-independent role for c-Myc in replication initiation involving replication origin binding and pre-RC interaction was also proposed (Dominguez-Sola, Ying et al. 2007).

Finally, a role for chromatin structure and nucleosome positioning in origin specification has also been proposed (Wyrick, Aparicio et al. 2001). In *D.melanogaster,* ORC targeting to, and amplification of the chorion locus requires histone acetylation (Aggarwal and Calvi 2004; Hartl, Boswell et al. 2007). HBO1, a histone acetyltransferase, interacts with ORC and is required for loading of MCM onto chromatin in human cells and *X.laevis* extracts (Iizuka and Stillman 1999; Burke, Cook et al. 2001; Iizuka, Matsui et al. 2006). Finally, insertion of mammalian replicators to ectopic sites

with histone modifications characteristic of heterochromatin resulted in histone acetylation patterns characteristic of euchromatin (Fu, Wang et al. 2006). It remains to be determined whether chromatin solely represents a limiting factor to ORC's function or ORC also actively modifies chromatin during its association with DNA.

3.1.2 Cdc6

Similarly to ORC, Cdc6 is also an AAA^+-ATPase participating in the initiation step of DNA replication, downstream of ORC, through loading of the MCM helicase during the G_1 phase. ATP binding of Cdc6 leads to a conformational change that promotes its association with chromatin (Tatsumi, Tsurimoto et al. 2000). Binding of Cdc6 to DNA-bound ORC leads to the activation of the ORC1 ATPase activity (Bell and Dutta 2002), as well as, to a conformational change which increases the stability and specificity of the ORC-Cdc6-DNA complex (Mizushima, Takahashi et al. 2000; Speck, Chen et al. 2005; Speck and Stillman 2007).

Cdc6 exhibits significant sequence similarity to *R*eplication *F*actor *C* (RFC), the eukaryotic clamp (PCNA) loader, and has therefore been proposed to act as the MCM clamp-loader (Carpenter, Mueller et al. 1996; Pflumm and Botchan 2001). According to the "clamp loading" model, the clamp loader loads a ring-shaped molecule onto DNA by inducing the opening of the ring (Davey, Jeruzalmi et al. 2002). Different subunits of the clamp loader function as "wrench", "motor" and "stator" to unlock the ring, with associated energy consumption. By analogy, the ORC/Cdc6 complex is considered as the clamp-loader of the ring-shaped MCM complex participating in its chromatin loading (Perkins and Diffley 1998). Sequential cooperating ATP hydrolysis by ORC and Cdc6

was shown to regulate the correct temporal and spatial targeting of MCM onto origins (Randell, Bowers et al. 2006). However, there is no genetic evidence for a direct interaction of Cdc6 with MCM. Instead, it is thought that a Cdt1-MCM complex is loaded onto the ORC-Cdc6-DNA complex during initiation (Nishitani, Lygerou et al. 2000; Tanaka and Diffley 2002; Yanagi, Mizuno et al. 2002; Cook, Chasse et al. 2004; Randell, Bowers et al. 2006).

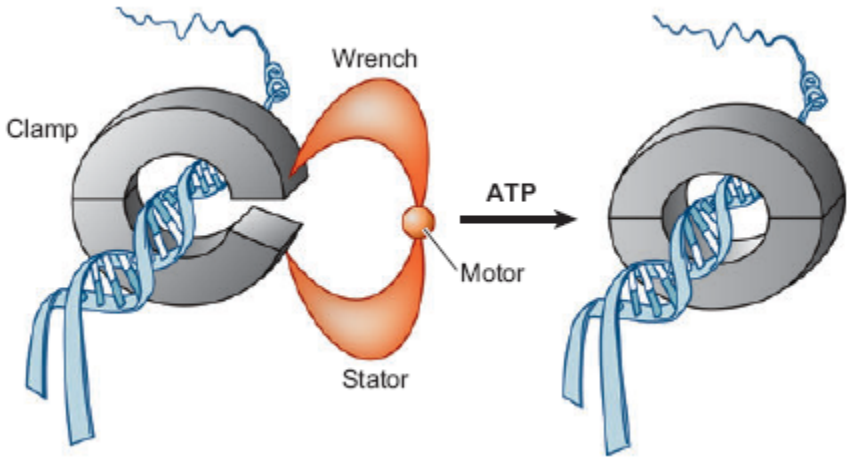


Figure 1.3: The clamp loading model. The clamp loader opens clamp using energy from ATP hydrolysis. The clamp loader consists of "stator", "wrench" and "motor" functions. The "stator" fixes clamp onto the DNA while the "wrench" and "motor" transiently open the clamp to bind the DNA. MCM corresponds to the clamp, whereas different subunits of the ORC/Cdc6 complex function as the clamp loader. (Davey, Jeruzalmi et al. 2002)

3.1.3 Cdt1

Cdt1 is an evolutionary conserved replication initiator protein first identified in fission yeast (Nishitani, Lygerou et al. 2000) and afterwards in all eukaryotes (Bell and Dutta 2002). The *Cdt1* gene is under the transcriptional control of the Cdc10/Sct1 transcription factor, therefore its protein levels are cell cycle regulated, peaking at G_1 and decreasing in S and G_2 phases (Hofmann and Beach 1994). Cdt1 physically interacts with components of the MCM hexamer participating in their nuclear translocation and chromatin loading (Nishitani, Lygerou et al. 2000; Tanaka and Diffley 2002; Yanagi, Mizuno et al. 2002; Cook, Chasse et al. 2004), as well as, with Cdc6 in both *S.cerevisiae* and human cells (Nishitani, Lygerou et al. 2000; Dhar, Yoshida et al. 2001). Cdc6 must already be bound to chromatin before Cdt1 joins the ORC-Cdc6 complex, consistent with a role for Cdt1 in bridging MCM and the clamp-loader. This ordered assembly of ORC-Cdc6-DNA, Cdt1 and MCM is very stringently regulated, as reconstruction in a *Xenopus in vitro* system showed that binding of Cdt1 onto DNA prior to Cdc6 leads to insufficient MCM loading and origin licensing (Tsuyama, Tada et al. 2005). Following MCM loading onto ORC-Cdc6, Cdc6 and Cdt1 dissociate from origins and finally ATP hydrolysis by ORC completes the MCM helicase loading reaction (Speck, Chen et al. 2005; Randell, Bowers et al. 2006; Speck and Stillman 2007).

3.1.4 Geminin

Geminin was first identified as an *a*naphase-*p*romoting *c*omplex (APC) substrate with an inhibitory activity on pre-RC assembly and has since emerged as a key regulator of metazoan DNA replication (McGarry and Kirschner 1998). It is conserved from

Caenorhabditis elegans (*C.elegans*) to humans but no homologs have been identified in yeast. Geminin is localized in the nucleus during S, G_2, and M phases of the cell cycle but is absent in G_1 phase. The presence of its mRNA throughout the cell cycle indicated that its protein expression is regulated by post-transcriptional mechanisms (Kulartz, Kreitz et al. 2003). Indeed, it has been shown that geminin is destabilized during metaphase-anaphase transition through ubiquitination by APC and subsequent proteasome degradation. This degradation requires a "destruction box" motif located at its NH_2-terminus (Quinn, Herr et al. 2001) and allows pre-RC formation during G_1 phase.

The inhibitory effect of geminin on pre-RC assembly is exerted through binding to Cdt1 and inhibiting MCM loading (Saxena and Dutta 2005). Geminin inhibits Cdt1 binding to DNA, Cdc6 and MCM (Yanagi, Mizuno et al. 2002; Cook, Chasse et al. 2004) by dimerizing and forming a negative coiled cylinder interacting with Cdt1 (Saxena, Yuan et al. 2004). The surface of the coiled-coil domain in the central region of geminin has an array of glutamic acid residues, which provides negative charges for ionic interaction with three positively charged residues in the central region of Cdt1. Cocrystals of Cdt1 and geminin also revealed that the ionic bonds are reinforced by van der Waals contacts at the binding surface (Lee, Hong et al. 2004; Saxena, Yuan et al. 2004). An unstructured region adjacent to the coiled-coil domain of geminin provides a second interface of interaction with Cdt1. Mutagenesis in either interface abolishes geminin's ability to inhibit replication suggesting that both interactions are crucial for inhibition of pre-RC assembly.

3.1.5 MCM 2-7

The *MCM* genes were first identified in a genetic screen for mutants that were defective for the *m*aintenance of *m*ini-*c*hromosomes (MCM) in *S.cerevisiae* and are conserved in all eukaryotes (reviewed in (Tye 1999; Forsburg 2004; Lei 2005)). All six members of the gene family are essential from yeast to humans, suggesting an important and highly conserved function. The MCM2-7 proteins belong to the AAA^+ ATPase superfamily with similarity to DNA helicases and represent the putative replicative DNA helicase. All MCM subunits possess the MCM box, a 200 aminoacid region harboring Walker A and B motifs as well as an arginine finger motif (SRFD) (Koonin 1993; Kearsey and Labib 1998; Neuwald, Aravind et al. 1999; Hingorani and O'Donnell 2000; Tye and Sawyer 2000).

The MCM subunits associate *in vivo* in a 1:1:1:1:1:1 stoichiometry through interaction of the arginine finger motif of one subunit with the P-loop of the Walker motif of the other (Davey, Indiani et al. 2003). Purification of *in vivo* MCM complexes in human cells led to the identification of a MCM4/6/7 subcomplex with ATPase, ssDNA-binding, dsDNA-binding and helicase activity. This subcomplex is believed to be the catalytic core of the MCM hexamer, while MCM2/3/5 represent the regulatory subunits (Ishimi, Ichinose et al. 1996; Ishimi 1997). This model was further confirmed by *in vitro* reconstitution experiments using recombinant MCM subunits from yeast, frog and mouse cells (Schwacha and Bell 2001; You, Ishimi et al. 2002; Ying and Gautier 2005). However, the helicase activity of the MCM complex was shown to be very weak and not processive as one would expect from the replicative helicase (Patel and Picha 2000). This was later explained by the fact that the MCM helicase activity is greatly enhanced by the

Cdc45 and GINS co-factors in both *X.laevis* (Masuda, Mimura et al. 2003; Pacek and Walter 2004) and *D.melanogaster* (Moyer, Lewis et al. 2006).

The structure of the MCM helicase has been deduced by using the atomic structure of the N-terminus of the MCM protein from *Methanobacterium thermoautotrophicum* (Mth-MCM) (Figure 1.4A) and the SV40 T antigen (Figure 1.4B) as models (Fletcher, Bishop et al. 2003; Li, Zhao et al. 2003; Pape, Meka et al. 2003; Sclafani, Fletcher et al. 2004; Gomez-Llorente, Fletcher et al. 2005). Mth-MCM is a true homologue of eukaryotic MCM, while the SV40 T antigen is an analogue resulting from convergent evolution. Using this reconstructive method the MCM helicase is believed to be a planar, double hexamer in head-to-head conformation. The N-terminal domain is believed to be responsible for oligomerization and DNA binding while the C-terminal contains the catalytic ATPase and helicase domains. In agreement with this model, using electron microscopy (EM), the eukaryotic MCM complex was shown to have a central large opening between the two hexamers (34 Å) which is thought to accommodate dsDNA participating in its unwinding (Figure 1.4C) (Yabuta, Kajimura et al. 2003). According to the current model, the eukaryotic MCM is believed to reside in fixed positions on the chromosome, at a distance from the replication machinery, acting as a rotary pump, accommodating the dsDNA, rotating it and generating unwound DNA at the replication origin or fork (Figure 1.4D). As the MCMs rotate the DNA, they also push it towards the replication machinery, thus allowing replication of the genomic DNA (reviewed in (Laskey and Madine 2003)).

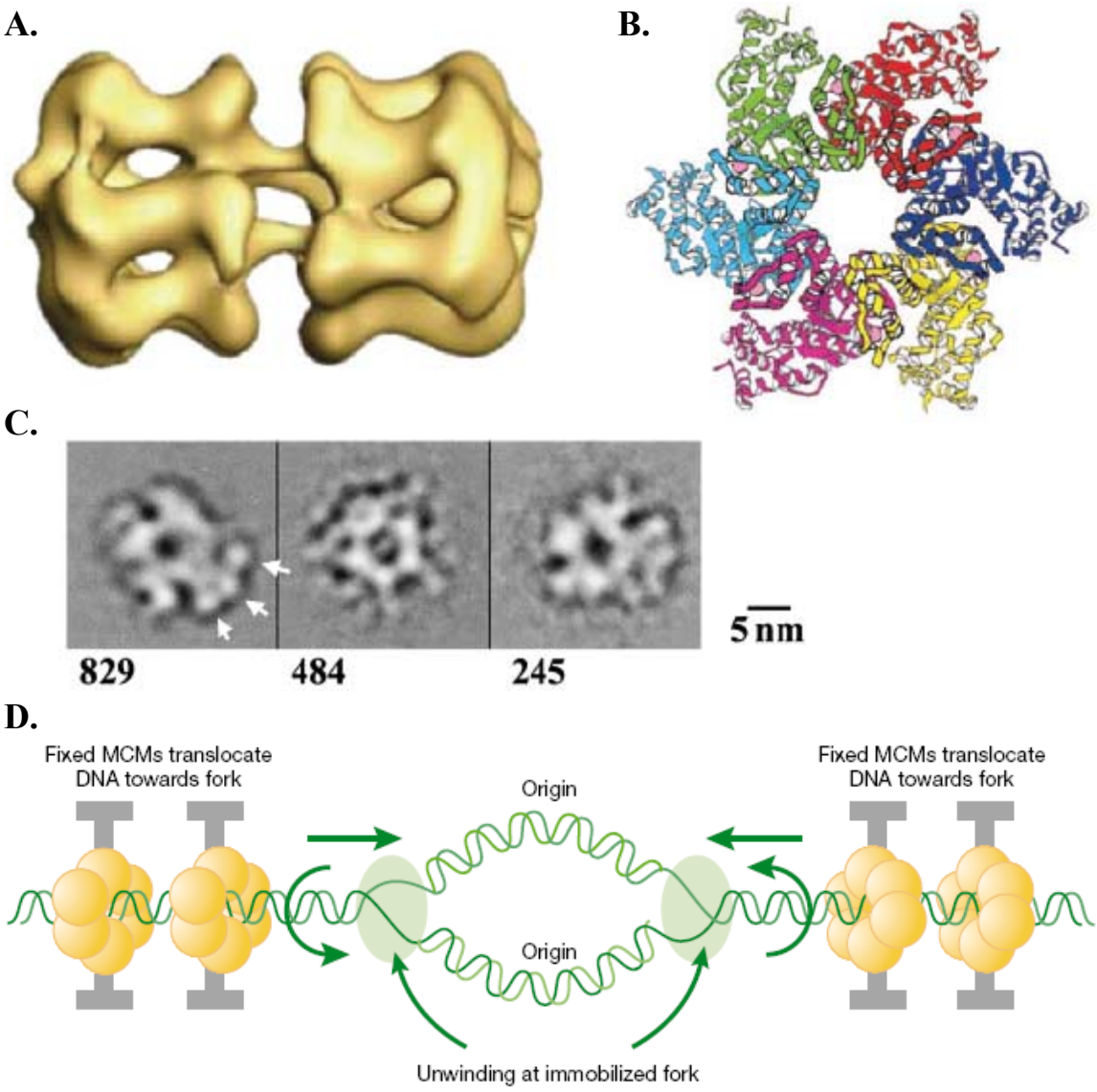


Figure 1.4: The MCM complex. A. Electron microscopy reconstruction of the full-length archaeal double hexameric Mth-MCM complex (Gomez-Llorente, Fletcher et al. 2005). **B.** Ribbon diagram of the atomic structure of the SV40 T-antigen (Li, Zhao et al. 2003). **C.** Electron micrograph of negatively stained human MCM complexes. Numbers below represent the frequency of the images used to obtain the averaged image (Yabuta, Kajimura et al. 2003). **D.** The rotary pump model of MCM function (Laskey and Madine 2003).

3.1.6 Cdc45 and GINS complex

Cdc45 binds onto origins after MCM recruitment, but prior to DNA unwinding and polymerase recruitment (Mimura, Masuda et al. 2000; Walter and Newport 2000) as well as travels with the replication fork (Aparicio, Stout et al. 1999), thus being important for both initiation and fork elongation (Tercero, Labib et al. 2000; Zou and Stillman 2000).

GINS (*G*o, *I*chi, *N*ii, *S*an which stand for the numbers 5, 1, 2, 3 in Japanese) is a recently identified complex, required for DNA replication, composed of the Sld5, Psf1, Psf2 and Psf3 proteins. GINS has a ring-like structure in the electron microscope and functions interdependently with Cdc45 in the loading of the replisome including the DNA polymerases and RPA, the eukaryotic *s*ingle-*s*tranded DNA *b*inding protein (SSB) (Aparicio, Stout et al. 1999; Kubota, Takase et al. 2003; Takayama, Kamimura et al. 2003). Cdc45/GINS also act as cofactors of the MCM complex, enhancing its helicase activity (Moyer, Lewis et al. 2006), thereby coupling helicase activation and replisome loading (Botchan 2007).

3.2 Pre-RC inactivation

Upon entry into S phase, multiple mechanisms ensure that the replication initiation machinery is inactivated so as to avoid re-replication of chromosomal regions and genome instability (Blow JJ and Dutta A., 2005; Hook SS et al., 2007). In yeasts, a number of S-phase CDK-dependent events prevent re-licensing of replicated DNA which include: i) Cdc6 phosphorylation at the G_1/S transition which targets it for degradation (Elsasser S et al., 1999; Drury LS et al., 2000); ii) decrease of the S.pombe Cdt1 (SpCdt1) levels during S-phase, possibly by a CDK-mediated degradation mechanism

(Nishitani H et al., 2000); iii) inactivation of the ORC complex by CDK phosphorylation (Vas A et al., 2001) and; iv) nuclear export of Cdt1 and MCM during S phase, G_2 and mitosis preventing them from gaining access to chromosomal DNA (Labib, Diffley et al. 1999; Nguyen, Co et al. 2000). An alternative mechanism of pre-RC inactivation after origin firing was also described in yeast involving direct interaction of CDKs with ORC6 and Cdc6, which maintains them in an inactive state during S phase, G_2 and mitosis (Mimura S et al., 2004; Wilmes GM et al., 2004).

In metazoans, the CDK levels are also important for regulating the licensing system but their role is less clear (Nishitani and Lygerou 2004). The ORC1 subunit, the ATPase subunit of ORC, is targeted for degradation in S-phase by a CDK- and SCF (Skp2)-dependent ubiquitination reaction (Mendez J et al., 2002). In some cell lines ORC1 remains stable in S phase, but is mono-ubiquitinated and/or phosphorylated as S phase progresses, which is believed to contribute in regulation of the licensing system (Li and DePamphilis 2002; Li, Vassilev et al. 2004). Furthermore, human Cdc6 is exported from the nucleus following CDK activity, which may play a role in preventing re-replication (Saha P et al., 1998; Petersen BO et al., 1999). The dominant pathway that is functioning in preventing metazoan origin re-licensing though involves blockage of the Cdt1 activity; geminin has emerged as a key regulator of metazoan replication and Cdt1 activity. Geminin is destabilized during G_1 phase and accumulates during S, G_2 and M phases of the cell cycle binding directly to Cdt1 and preventing the loading of the MCM2-7 complex to it (Yanagi K et al., 2002; Cook G et al., 2004). Furthermore, phosphorylation of Cdt1 by CDK at the G_1/S transition triggers its degradation by the proteasome, through Skp2-dependent and –independent pathways (Liu E et al., 2004; Nishitani H et al., 2004;

Arias EE and Walter JC., 2005; Thomer M et al., 2004). In *C.elegans*, a CUL-4 ubiquitin ligase is required to downregulate Cdt1 levels at the end of G_1, deletion of which leads to Cdt1-dependent re-licensing and re-replication (Zhong, Feng et al. 2003).

Finally, a new evolutionary conserved back-up mechanism was recently described in the case of re-replication-induced DNA damage; geminin depletion as well as overexpression of Cdt1 or Cdc6 causes re-replication which could ultimately lead to tumorigenesis. However, re-replication induced by such manipulations is incomplete suggesting the existence of backup events which restrain re-replication once it begins. Studies in both Drosophila and human cells suggest that Cdt1 and Cdc6 ubiquitination and degradation are coupled with an associated geminin overexpression, which provides a salvage pathway, minimizing the extent of re-replication and genomic instability (Mihaylov IS et al., MCB 2002; Ballabeni A et al., EMBO 2004; Hall JR et al., JBC 2008).

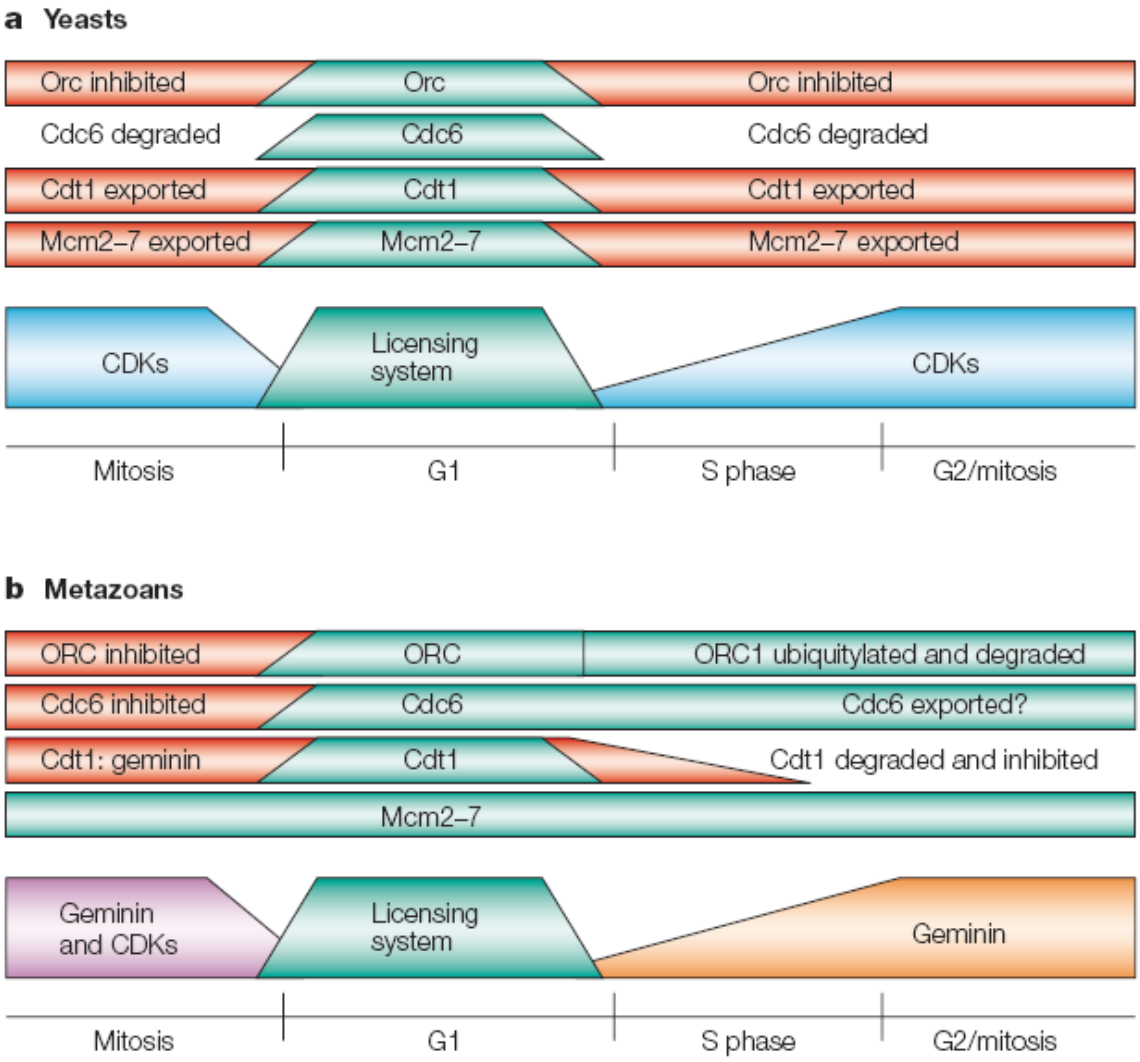


Figure 1.5: Inactivation of the pre-RC complex during cell division. The activity of components of the licensing system during the cell cycle in yeast **(a)** and metazoans **(b)** is shown. In the lower part of each figure, the activity of the licensing system is shown. This is active (green) only in G_1, while inactive in S, G_2 and M phases. (Blow and Dutta 2005)

3.3 Replication timing

DNA is replicated during S phase according to a defined and reproducible temporal program (reviewed in (Donaldson 2005; Zink 2006)). Distinct genomic loci are organized in replication timing domains which are synchronously activated either early or towards the end of S phase. Early evidence in mammalian cells showed that the active X chromosome replicated early while the homologous inactive X chromosome in females replicated late suggesting a correlation between early replication and active transcription (Brown 1966). In support of this, imprinted genes that show allele-specific patterns of gene expression, replicate asynchronously (Simon, Tenzen et al. 1999; Gribnau, Hochedlinger et al. 2003). More recent studies using microarray analyses have further supported this idea in *Drosophila* (Schubeler, Scalzo et al. 2002; MacAlpine, Rodriguez et al. 2004) and human cells (White, Emanuelsson et al. 2004; Woodfine, Fiegler et al. 2004; Jeon, Bekiranov et al. 2005; Woodfine, Beare et al. 2005) revealing a clear association between replication timing and features correlated with gene expression such as GC content and gene density. Using a mouse cell line containing an inserted transgene, Lin *et al.* further showed that replication timing is most probably related to the transcriptional permissivity of chromatin rather than transcription *per se* (Lin, Fu et al. 2003); when the transgene was inserted into the chromosome in a transcription-permissive orientation, it replicated early, whereas in the silenced orientation it replicated late. Interestingly, the replication timing switch did not require active transcription from the locus since a promoter-dead version of the transgene showed a similar dependence on insertion orientation.

On the other hand, whole-genome studies in *S.cerevisiae* identified no correlation between transcription and origin usage (Raghuraman, Winzeler et al. 2001), suggesting that the mechanisms that determine the replication time differ in yeast and higher eukaryotes. However, there is a relation between origin and transcriptional terminators (Nieduszynski, Knox et al. 2006). Short *cis*-acting sequences at varying distances from replication origins can specify late origin firing (Friedman, Diller et al. 1996), most likely by modifying the chromatin conformation. Two studies examined the effect of histone acetylation on yeast replication timing. Deletion of the budding yeast histone deacetylase Rpd3 increases histone acetylation levels at many replication origins, causing them to fire earlier in S phase (Aparicio, Viggiani et al. 2004). Similarly, targeting the histone acetylase Gcn5 to a late origin, induced an "open chromatin" status and accelerated its activation timing (Vogelauer, Rubbi et al. 2002). Taken together, these data emphasize the importance of histone acetylation in determining replication timing in budding yeast.

In human cells, treatment with the histone deacetylase (HDAC) inhibitor trichostatin A (TSA), led to a more dispersive pattern of initiation site selection as well as an earlier activation of some late-firing origins (Bickmore and Carothers 1995; Kemp, Ghosh et al. 2005), again highlighting histone acetylation as a clear regulator of origin activation. However, histone acetylation is neither the sole nor a necessary determinant of origin firing in metazoa (Prioleau, Gendron et al. 2003). The methylation status of histone H3 also seems to be an important molecular event during the initiation of DNA replication (Stedman, Deng et al. 2004; Zhou, Chau et al. 2005), but it alone is not sufficient to dictate the spatial and temporal regulation of chromosomal domains (Wu, Terry et al. 2005). Altogether, there seems to be a clear relationship between "open"

chromatin structure and early replication but the molecular features regulating replication timing in metazoans are not well understood yet.

4. OTHER INITIATORS OF DNA REPLICATION

4.1 OBA/Ku

Using a 186bp fragment of the ors8 replication origin as a bait, an *o*rigin *b*inding *a*ctivity was purified from HeLa cell extracts, which was able to support *in vitro* replication of an ors8 plasmid (Ruiz, Pearson et al. 1995). Peptide sequence analysis identified its DNA binding subunit as the 86 kDa subunit of Ku antigen (Ruiz, Matheos et al. 1999). Affinity-purified OBA, also contains DNA-PKcs, the catalytic subunit of the DNA-dependent protein kinase, as well as, topoisomerase II. Subsequent studies further implicated Ku in DNA replication, registering it as an emerging regulator of replication initiation. A more detailed description of Ku's role in DNA replication can be found in section 5.3.5.

4.2 DNA topoisomerases

One of the basic features of DNA is the intertwining of the DNA strands, which results in a global underwinding of DNA (negatively supercoiled) in all species (Schvartzman and Stasiak 2004). This underwinding facilitates sister DNA strand separation and affects accessibility of trans-acting factors to DNA (Hiasa and Marians 1994). Thus, the topological state of the DNA drastically affects biological processes such as DNA replication, transcription and chromatin remodeling.

The topological state of the DNA in the cell is being managed by the DNA topoisomerases (topos) (Champoux 2001), which accomplish this by creating either a single-strand (type I subfamily) or double-strand (type II subfamily) break in DNA, thus permitting the rotation of one or two DNA strands respectively around the DNA axis and relaxation of the torsional stress. The role of topoisomerases in transcription (Ju, Lunyak et al. 2006), chromosome condensation (Cuvier and Hirano 2003), recombination (Grainge, Bregu et al. 2007), and replication fork progression (Sakaguchi and Kikuchi 2004) has been well established. A role for topoisomerases in the initiation step of DNA replication has also been proposed, as DNA topoisomerases are required for the activation of the origins of the SV40 (Tsurimoto, Melendy et al. 1990; Halmer, Vestner et al. 1998), bovine papillomavirus (BPV) (Hu, Clower et al. 2006), Epstein-Barr virus (EBV) (Kawanishi 1993). Furthermore, both mammalian topoisomerases I and II were recently found to associate with the human replication origin Lamin B2 in a cell cycle- and sequence-specific manner and participate in the formation of the pre-RC (Abdurashidova, Radulescu et al. 2007). Finally, the use of specific topoisomerase II inhibitors resulted in the abolition of origin firing (Lallev, Anachkova et al. 1993; Narayana, Khodarev et al. 1998; Abdurashidova, Radulescu et al. 2007), suggesting that DNA topology plays an essential role in origin activation. Mammalian cells possess two isoforms of topo II, α and β, with high sequence homology (68% identity and 86% similarity) (Jenkins, Ayton et al. 1992; Austin, Sng et al. 1993) and almost identical enzymatic properties *in vitro* (with the exception of a preferential relaxation of positive supercoils by topo II α) (McClendon, Rodriguez et al. 2005), which complicates the study of their differential functions. Recently, it was reported that it is the topo IIβ

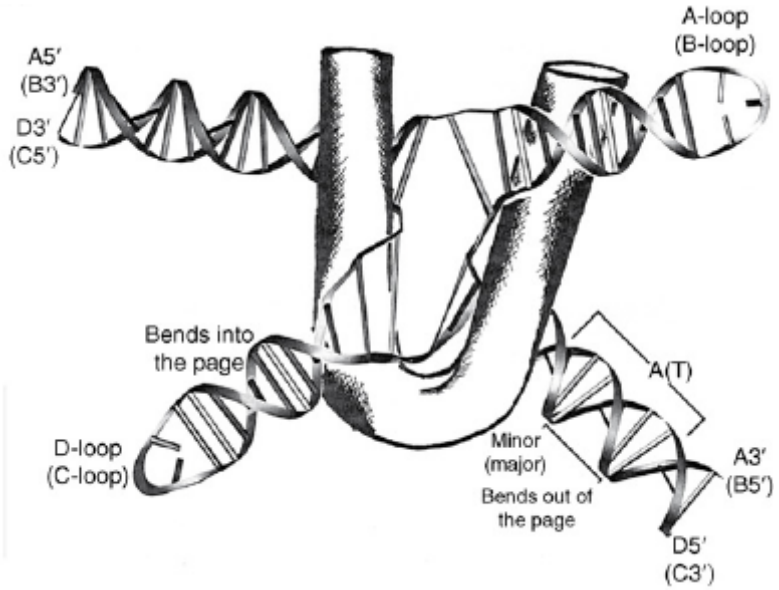
isoform that is implicated in the initiation of DNA replication of Kaposis's sarcoma-associated herpes virus (KSHV), which utilizes the host molecular machinery in order to proliferate, suggesting that topo IIβ is the DNA replication-specific isoform (Wang, Li et al. 2008).

4.3 CBP/14-3-3

The *c*ruciform *b*inding *p*rotein (CBP) was initially purified via its ability to specifically interact with cruciform DNA regardless of its sequence (Pearson, Ruiz et al. 1994). The interaction of CBP with DNA was localized to the four-way junction at the base of the cruciforms, binding onto the elbows of the junctions in an asymmetric fashion (Pearson, Zannis-Hadjopoulos et al. 1995). By microsequencing, CBP was subsequently identified as a member of the 14-3-3 protein family (Todd, Cossons et al. 1998). The CBP complex contains the 14-3-3 isoforms β, γ, ε, ζ and σ (Alvarez, Novac et al. 2002) which form specific heterodimers as shown by the ability of 14-3-3 ζ to dimerize only with β, ε, and ζ but not with γ and σ (Alvarez, Callejo et al. 2003). This limitation in heterodimer formation is believed to confer specificity on 14-3-3 function (Aitken, Baxter et al. 2002). The crystal structure of the 14-3-3 dimers revealed a U-shaped molecule, the center of which binds onto the cruciform (Liu, Bienkowska et al. 1995). CBP/14-3-3 binds *in vivo* to replication origins in mammalian and *S.cerevisiae* cells in a cell cycle-dependent manner, peaking at late G_1 and decreasing thereafter (Callejo, Alvarez et al. 2002; Novac, Alvarez et al. 2002; Yahyaoui, Callejo et al. 2007). With regard to its mechanism of action, CBP/14-3-3 is believed to act as sensor of transient cruciform extrusion during the G_1 phase (see section 2.2.5), serving as an attachment site

for initiator proteins (reviewed in (Zannis-Hadjopoulos, Sibani et al. 2004; Zannis-Hadjopoulos, Yahyaoui et al. 2008)). In support of this model, a number of proteomic studies have shown the association of 14-3-3 with initiator proteins and members of the pre-RC complex, among which, the MCM helicase, Ku, Replication Factor C and DNMT1 (Meek, Lane et al. 2004; Pozuelo Rubio, Geraghty et al. 2004; Satoh, Nanri et al. 2006).

A.



B.

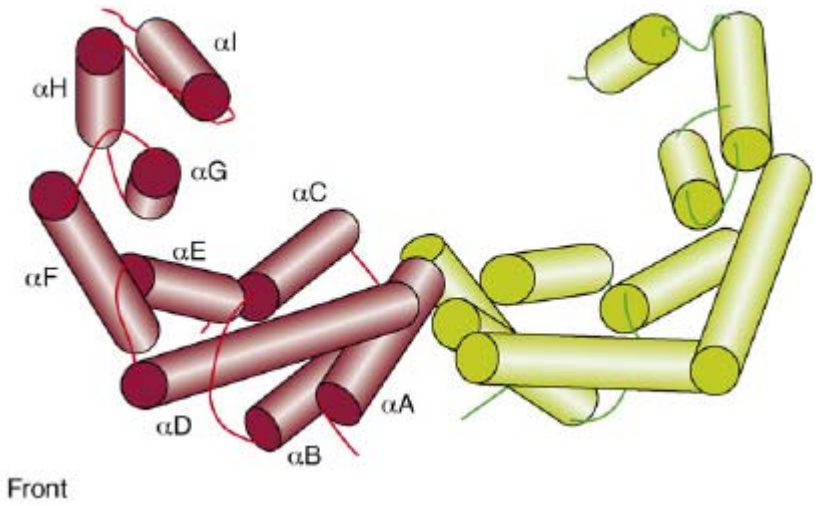


Figure 1.6: The CBP protein. A. Model of the structure of the CBP-cruciform complex. **B.** 3D structure of 14-3-3. (Zannis-Hadjopoulos, Yahyaoui et al. 2008)

5. KU ANTIGEN

The heterodimeric Ku protein (reviewed in (Downs and Jackson 2004; Zannis-Hadjopoulos, Sibani et al. 2004; Gullo, Au et al. 2006)) consists of 70 kDa and 80 (or 86) kDa subunits and is present in all prokaryotic and eukaryotic organisms, suggesting a conserved function. It is predominantly a nuclear protein that was originally identified as an autoantigen in sera of patients with autoimmune diseases. As the DNA-binding subunit of DNA-PK, Ku allosterically activates DNA-PKcs. This trimeric complex (DNA-PK-Ku70/Ku80) is implicated in the non-homologous end joining (NHEJ) of DNA double strand break (DSB) repair, site-specific V(D)J recombination, transcription, telomeric maintenance and DNA replication. Ku is also involved in the maintenance of genomic integrity, suppression of chromosomal aberrations and apoptosis inhibition.

5.1 Expression and subcellular localization

Studies on the subcellular localization of Ku have yielded differing and sometimes conflicting results which may be due to differences in the use of detection methods, type of cells, species, phase of the cell cycle, phase of development or phase of differentiation. However, Ku's predominant location is the nucleus where it oscilates between the nucleoplasm and the nucleolus (reviewed in (Koike 2002)). During G_1 and S phases Ku80 resides to the nucleoplasm and starts accumulating in the nucleolus during S phase, reaching a maximum at late S and G_2 phase (Li and Yeh 1992). This change was not accompanied by alterations in its protein expression but was rather due to migration of the nucleoplasmic fraction into the nucleolus (Li and Yeh 1992; Novac, Matheos et al. 2001). Using confocal microscopy Koike *et al.* showed that Ku70 and Ku80 are confined

to the nucleus of interphase cells in several cancer and normal human cell lines, while during metaphase they are mainly diffusely distributed in the cytoplasm with a small fraction residing at the periphery of condensing chromosomes (Koike, Awaji et al. 1999a). This is in agreement with previous studies showing that Ku dissociates from chromatin and localizes to the periphery of condensed chromosomes during early prophase and metaphase (Reeves 1987; Higashiura, Shimizu et al. 1992; Li and Yeh 1992).

Nuclear translocation of Ku is mediated through association of their intrinsic nuclear localization signals (NLS) with NLS receptors. Ku70 contains a bipartite NLS consisting of two basic motifs (KVTKRKHDNEGSGSKRPK) at aminoacids 539-556 separated by a nonbasic intervening region, while Ku80 contains a simple 7 aminoacid basic signal (PTAKKLK) at aminoacids 561-569 (Koike, Ikuta et al. 1999b; Koike, Ikuta et al. 1999c; Bertinato, Schild-Poulter et al. 2001). Ku70 and Ku80 can translocate to the nucleus both as monomers and heterodimers, the latter being more efficient. Interestingly, the nuclear transport of Ku70 during M/G_1 transition preceded that of Ku80 which occurred after nuclear membrane formation. These data suggest that the nuclear transport of Ku is both selective and cell cycle-dependent.

5.2 Activities of Ku

5.2.1 DNA-end binding

The best characterized DNA binding activity of Ku is its DNA-end binding activity. Initial experiments indicated that Ku can bind to digested plasmid DNA in a manner proportionate to the number of cleavages that were introduced, suggesting a sequence-

independent DNA end binding by Ku (Mimori and Hardin 1986). Indeed, it was later proven that Ku can bind to short double-stranded oligonucleotides (14-18bp) containing either blunt ends, 3' or 5' overhangs as well as IR-induced breaks with high affinity (K_d between 5 x 10^{-10} and 25 x 10^{-10} M^{-1}) (Mimori and Hardin 1986a; Paillard and Strauss 1991; Blier, Griffith et al. 1993; Falzon, Fewell et al. 1993; Ono, Tucker et al. 1994; Pang, Yoo et al. 1997; Yaneva, Kowalewski et al. 1997). Electrostatic forces were identified as the major contributors of Ku-DNA interaction, with 12 ions (9 cations and 3 anions) released upon formation of the Ku-DNA complex (Arosio, Costantini et al. 2004). *In vitro* studies revealed that multiple Ku molecules can bind DNA with free ends, each molecule being displaced inwards by 25bp without the requirement of ATP, resulting in a "beads threaded on a string" arrangement (Arosio, Cui et al. 2002). If the DNA ends are ligated, the resulting Ku-DNA complex is extremely stable (Paillard and Strauss 1991). *In vivo*, however, only one molecule binds each free end as evidenced by a seven-fold preference for DNA ends compared to internal sequences (Taghva, Ma et al. 2002). Biochemical studies indicated that both Ku subunits make contact with double-stranded DNA (ds-DNA), with Ku70 located closer to the end (Ono, Tucker et al. 1994; Wu and Lieber 1996; Ochem, Skopac et al. 1997). Neither subunit alone could effectively bind DNA ends. Using *in vitro* translated proteins, the C-terminal 40kDa of Ku70 and C-terminal 45kDa of Ku80 were found to be responsible for DNA end binding (Wu and Lieber 1996), a result later confirmed by the crystal structure of Ku on DNA (described in detail in section 5.4).

5.2.2 Sequence-specific DNA binding

Several studies have shown that Ku also exhibits sequence-specific DNA binding, binding to regulatory DNA elements and affecting gene transcription (reviewed in (Dynan and Yoo 1998)). The heterodimer associates with the negative regulatory element 1 (NRE1) present in the long terminal repeat of the murine mammary tumor virus (MMTV) (Giffin, Torrance et al. 1996), the U5 region of the human T-cell leukemia virus type I (HTLV-I) long terminal repeat (LTR) (Okumura, Takagi et al. 1994), the Heat Shock Element (HSE) of the hsp70 promoter (Kim, Ouyang et al. 1995), the Proximal Sequence Element (PSE) of the U1 gene (Knuth, Gunderson et al. 1990), the GATA sequence of the Glycophorin B promoter (Camara-Clayette, Thomas et al. 1999), the enhancer core sequence from the IAP (Intracisternal A-particle) LTR (Long Terminal Repeat) of the mouse c-mos (Falzon, Fewell et al. 1993) and the antigen receptor response element (ARRE) in the IL-2 promoter of Jurkat T-cells (Shi, Qiu et al. 2007). Furthermore, Ku binds to the A3/4 and ors8 replication origins in a sequence-specific manner (Ruiz, Matheos et al. 1999; Schild-Poulter, Matheos et al. 2003), affecting their activity (Matheos, Novac et al. 2003; Sibani, Price et al. 2005). Although all these transcription and replication elements do not appear to have a common consensus recognition sequence, they are all polypurine/polypyrimidine rich, a sequence binding preference that Ku appears to exhibit (Jeanson and Mouscadet 2001). Study of the binding of Ku to its two best characterized sequences, A3/4 and NRE-1, and two other internal sequences, using bandshift analyses and UV-crosslinking experiments, indicated that Ku reaches to its different binding sequences through multiple mechanisms classified into eight categories (Schild-Poulter, Matheos et al. 2003). Ku has greater affinity for

specific DNA sequences than DNA ends (Giffin, Torrance et al. 1996; Giffin, Gong et al. 1999; Schild-Poulter, Matheos et al. 2003). With regard to NRE-1, Ku70 initially recognizes it, and upon addition of 10mM $MgCl_2$ and 4mM ATP, a structural transition upstream of NRE-1 occurs which leads to the recruitment of Ku80 (Torrance, Giffin et al. 1998; Giffin, Gong et al. 1999). On the other hand, both Ku70 and Ku80 interact with both DNA strands of A3/4 independently of ATP and $MgCl_2$ (Schild-Poulter, Matheos et al. 2003). Using Southwestern analysis, only Ku80 was found to bind to the double-stranded A3/4 sequence (Ruiz, Matheos et al. 1999).

5.2.3 RNA binding

Immunostaining studies of Ku's subcellular localization indicated a cell cycle-dependent oscillation between the nucleoplasm and the nucleolus which was dependent on RNA and DNA (Reeves 1985; Li and Yeh 1992). Binding of Ku to total HeLa RNA was found to be weak and nonexistent in the case of tRNA (Mimori and Hardin 1986a; Kaczmarski and Khan 1993). However, Ku bound TAR (trans-activation response element), a stable hairpin structure present in all HIV-1 LTR RNAs, with high specificity forming a protein-RNA complex. This RNA-binding activity of Ku was 5-fold higher compared to DNA ends and was hypothesized to affect HIV-1 gene expression (Rosen, Sodroski et al. 1985; Kaczmarski and Khan 1993).

Recently, Ku was also found to associate with nuclear DNA helicase II (NDH II or RNA helicase A) in an RNA-dependent manner participating in hnRNP complexes (Zhang, Schlott et al. 2004). Zhang *et al.* showed that Ku80 binds to RNA and recruits

DNA-PK which then acts as RNA-dependent protein kinase, phosphorylating NDH II and affecting its subnuclear distribution along with the localization of hnRNP C.

Finally, the RNA template for telomerase, the telomere-specific reverse transcriptase, has also been recognized as an RNA substrate for Ku both in *S.cerevisiae* and human cells. In yeast, Ku binds to a 48 nucleotide (nt) stem-loop structure within the 1.3kb TLC1 sequence (the RNA component of yeast telomerase) during telomere replication and promotes the recruitment of Estp2, the catalytic protein subunit of telomerase (Fisher, Taggart et al. 2004). In the absence of Ku Estp2 was not telomere-associated during G_1 phase, while its association and telomere addition kinetics during S phase were compromised (Stellwagen, Haimberger et al. 2003; Fisher, Taggart et al. 2004). Similarly, in humans Ku interacts with a 47 nt region of the 3' end of the RNA component of human telomerase (hTR), which resembles the stem-loop region of the yeast Ku-binding domain on TLC1, independently of the human telomerase reverse transcriptase protein (hTERT) (Ting, Yu et al. 2005). These data suggest that the interaction of Ku with telomerase RNA is conserved among various eukaryotic species indicating its implication in telomere metabolism.

5.2.4 ATPase and helicase activity

Ku was purified to homogeneity from HeLa cells as a DNA-dependent ATPase activity (Vishwanatha and Baril 1990). The ATPase activity of Ku resides in both subunits as well as the heterodimer and is single-stranded DNA-dependent (Tuteja, Rahman et al. 1994; Vishwanatha, Tauer et al. 1995; Ochem, Skopac et al. 1997). Ku's ATPase activity is upregulated upon phosphorylation by DNA-PKcs (Cao, Pitt et al.

1994) and stimulates DNA synthesis by the DNA polymerase α/primase in HeLa cells (Vishwanatha and Baril 1990).

Furthermore, Ku was identified as the ATP-dependent human DNA helicase II (HDH II) purified from HeLa cells (Tuteja, Rahman et al. 1994). HDH II/Ku unwinds exclusively DNA duplexes, prefering partially unwound and fork-like substrates, but not DNA-RNA and RNA-RNA substrates. Its direction of translocation is exclusively in the 3' to 5' direction on the bound strand with greater helicase efficiency in the presence of short length duplexed DNA with a 5' overhang. Ku's helicase activity resides uniquely in the Ku70 subunit, while its DNA-binding activity resides in the heterodimeric form (Ochem, Skopac et al. 1997).

5.2.5 DNA-PKcs allosteric activation

DNA-PKcs is the catalytic subunit of DNA-PK which, along with the Ku heterodimer, forms the DNA-PK holoenzyme (Gottlieb and Jackson 1993; Collis, DeWeese et al. 2005). It is a 470kDa protein, coded for by 4127 aminoacids, which is only found in vertebrates, since homologues of DNA-PKcs in lower eukaryotes such as *Drosophila melanogaster*, *Arabidopsis thaliana* and *Saccharomyces cerevisiae* do not exist (Downs and Jackson 2004). DNA-PK has been implicated in the regulation of gene transcription, DNA replication and recombination, as well as, DNA repair through non-homologous end-joining (NHEJ), the latter being the best characterized (see section 5.3.1). DNA-PKcs can bind both double-stranded and single-stranded DNA, albeit with low affinity and its kinase activity is stimulated by association with DNA ends (Hammarsten, DeFazio et al. 2000; Martensson and Hammarsten 2002). However, DNA-

bound Ku increases the affinity of DNA-PKcs for DNA fragments longer than 26bp by 100-fold and stimulates its kinase activity (Yaneva, Kowalewski et al. 1997; West, Yaneva et al. 1998). Ku and DNA-PKcs are not constitutively associated when they are not bound to DNA since they do not co-purify under high or low salt condition in the absence of DNA (Dvir, Peterson et al. 1992; Gottlieb and Jackson 1993). The current model for the recruitment of DNA-PKcs to DNA breaks is that Ku binds to DNA, creating a dual binding dock (protein and DNA), which allows for a stronger interaction with either partner. Additional changes in the conformation of Ku which lead to the exposure of the last 12 aminoacids of Ku80, known to interact with DNA-PKcs (Gell and Jackson 1999), or the generation of new interaction sites may also contribute to DNA-PKcs binding and activation (Lehman, Hoelz et al. 2008).

5.3 Functions of Ku

5.3.1 Non-homologous end-joining (NHEJ)

DNA double strand breaks (DSBs) are considered to be among the most detrimental forms of DNA damage and can arise both from exogenous stimuli (i.e., DNA damaging agents, ionizing radiation) and endogenous processes, such as base oxidation due to reactive oxygen species (ROS), DNA depurination due to hydrolysis and replication fork collapse (Branzei and Foiani 2008), and enzymatic DNA cleavage during gene activation (Lis and Kraus 2006) and lymphocyte maturation (Soulas-Sprauel, Rivera-Munoz et al. 2007). It is estimated that a mammalian cell suffers at least ten spontaneous DSBs per day (Haber 2000). If unrepaired, DSBs may either be lethal to the cell or cause its transformation into a cancer cell. Chromosome rearrangements including large deletions,

duplications, inversions or translocations, resulting from DSBs, are frequent in a variety of haematological cancers and solid tumors (Lengauer, Kinzler et al. 1998; Mitelman, Johansson et al. 2007). In response to this threat, two highly efficient DSB repair pathways have evolved in eukaryotes: (i) homologous recombination (HR) which acts upon sister chromosomes and therefore is active during the S and G_2 phases of the cell cycle (West 2003) and; (ii) non-homologous end joining (NHEJ), which is not reliant on a homologous template and therefore is not restricted to a specific cell cycle phase (Weterings and Chen 2007) (Figure 1.7).

NHEJ is initiated by the association of Ku with the two DNA ends, which forms a ring cradling two full turns of DNA and protects them from nucleolytic degradation (Walker, Corpina et al. 2001). The DNA-Ku complex then functions as a scaffold for the binding of DNA-PKcs, the 460 kDa serine/threonine kinase subunit of DNA-PK (see section 5.2.5). This leads to the formation of a continuous channel enclosing and protecting the DNA ends at the site of the lesion (Weterings, Verkaik et al. 2003) and the generation of a synaptic complex consisting of two Ku and two DNA-PKcs molecules juxtaposing the two DNA ends (Spagnolo, Rivera-Calzada et al. 2006). Analysis of the 3D structure of this synaptic complex, using single-particle electron microscopy (EM), revealed that upon binding to Ku, DNA-PKcs is subject to substantial changes in its conformation, which alter the relative position of conserved C-terminal regions directly attached to the two lobes of the catalytic domain (Rivera-Calzada, Maman et al. 2005). These conformational changes are believed to result in the activation of the DNA-PKcs which phosphorylates a number of targets, including XRCC4, Ku, Artemis, p53 and DNA-PKcs itself (Burma and Chen 2004). Before the religation step takes place, it is

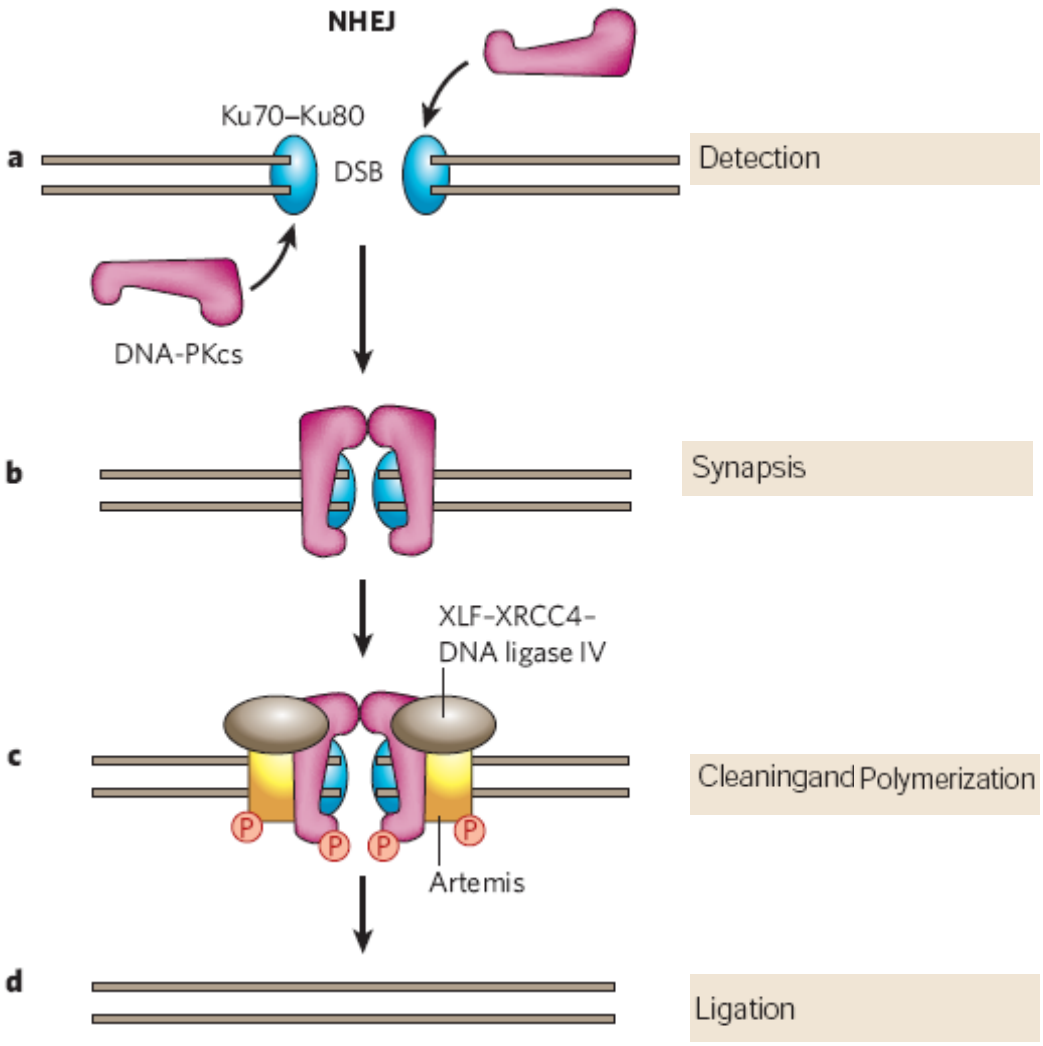


Figure 1.7: Non-homologous end-joining. A DSB is recognized by the Ku heterodimer, which recruits the DNA-PKcs, and the two DNA ends are synapsed by the DNA-PK holoenzyme. Artemis is phosphorylated by DNA-PKcs and "cleans up" ends of the DNA to generate 3' hydroxyl groups and 5' phosphate groups. Polymerase μ fills in any gaps present, and ligase IV then ligates the two DNA fragments. (Downs, Nussenzweig et al. 2007)

important that non-complementary ends are processed. Single-stranded overhangs are removed by the endonucleolytic activity of the DNA-PK-activated Artemis (Ma, Pannicke et al. 2002), while in the case of partially cohesive DNA ends Polymerase μ is responsible for filling the missing nucleotides (Mahajan, Nick McElhinny et al. 2002). Phosphorylation of DNA-PKcs and Ku promotes the alignment of the two DNA ends, while phosphorylation of XRCC4 results in recruitment of the XRCC4/ligase IV complex, the ligase responsible for ligation of the DNA termini. A recently discovered protein, XLF (XRCC4-like factor), is also thought to participate in this step, acting as a co-factor of ligase IV (Ahnesorg, Smith et al. 2006). Autophosphorylation of DNA-PKcs is also thought to destabilize its interaction with DNA ends, leading to its release from DSBs upon recruitment of ligase IV (Uematsu, Weterings et al. 2007).

5.3.2 Telomere biology

Telomeres are nucleoprotein structures at the ends of eukaryotic chromosomes consisting of G-rich DNA repeats bound by an array of specialized proteins (Verdun and Karlseder 2007). Telomere maintenance involves three challenges emanating from their structure, which have resulted in the evolution of unique regulatory mechanisms. First, the chromosome ends need to be protected from nucleolytic attack so as to avoid loss of genetic formation; second, chromosome ends must be concealed from detection and inappropriate repair by the DSB-repair machineries and; third, the discontinuous replication of the lagging strand and removal of the 5' RNA-primer results in shortened chromosomes, which is referred to as the end-replication problem (Chakhparonian and Wellinger 2003). The actual terminus of the chromosome is not blunt-ended but

protrudes forming a 3' single-stranded DNA overhang on the G-rich leading strand, known as G tail. This overhang is conserved in humans, yeast, trypanosomes and plants suggesting an essential role in telomere biology (Verdun and Karlseder 2007). Studies in mouse and human cells have shown that the G tail can invade homologous double-stranded telomeric repeats, resulting in a lasso-like structure termed telomeric loop (t-loop) (Griffith, Comeau et al. 1999), which is thought to protect chromosome ends (Figure 1.8A). Terminal loops are associated with the shelterin complex, which consists of telomeric-repeat-binding factor 1 (TRF1), TRF2, TRF1-interacting protein 2 (TIN2), the transcriptional repressor/activator protein RAP1, protection of telomeres 1 (POT1) and the POT1- and TIN2-organizing protein TPP1 (de Lange 2005). This complex is important for chromosome end protection as well as telomere length control (van Steensel, Smogorzewska et al. 1998).

The end-replication problem at telomeres is dealt with in embryonic and immortalized cells by expressing telomerase, a reverse transcriptase (TERT) that elongates the 3' end of the chromosome using an RNA primer (TERC) (Feng, Funk et al. 1995; Cong, Wright et al. 2002). Telomerase introduces telomeric repeats in the leading strand which are copied onto the lagging strand by DNA polymerase α/primase, thus providing a buffer to counteract replication-associated shortening.

The role of Ku in telomere maintenance has been extremely difficult to decipher due to the different species-specific phenotypes observed. In particular, yeast defective for Ku proteins show loss of telomeric repeats and deregulation of the G tail (Cervantes and Lundblad 2002), whereas plants deficient for Ku proteins show massive telomere elongation, as well as elongation of the G tail (Riha, Watson et al. 2002; Riha and

Shippen 2003). Mouse embryonic fibroblasts from Ku80-deficient mice show increased frequencies of telomere fusions suggesting a role for Ku80 in telomere capping (Bailey, Meyne et al. 1999; Hsu, Gilley et al. 1999; Hsu, Gilley et al. 2000; d'Adda di Fagagna, Hande et al. 2001), while the impact on telomere length was controversial (Samper, Goytisolo et al. 2000; d'Adda di Fagagna, Hande et al. 2001; Espejel, Franco et al. 2002). Finally, in humans, Ku80 deficiency causes telomere shortening to a new stable length and increased chromatid-type fusions (Jaco, Munoz et al. 2004; Myung, Ghosh et al. 2004).

The role of Ku in telomere-length regulation may be mediated by its ability to interact with TLC1 and hTR, the RNA components of the yeast and human telomerase respectively (Stellwagen, Haimberger et al. 2003; Ting, Yu et al. 2005). This interaction was indeed found to promote telomere addition by enabling telomerase to act both at broken and normal chromosome ends (Stellwagen, Haimberger et al. 2003). Alternatively, Ku may participate in the control of telomere length through its ability to inhibit homologous recombination between sister telomeres (telomere sister-chromatid exchange, T-SCE). This process can shorten and elongate individual telomeres when the exchanged segments are not equal (Bailey, Brenneman et al. 2004; Bechter, Zou et al. 2004). Sister telomere exchanges occurred at approximately 15% of the chromosome ends in mouse $Ku70^{-/-}$ cells, indicating the importance of Ku in telomere protection (Celli, Denchi et al. 2006).

With regard to the role of Ku in the protection of chromosome ends, it is believed to be mediated through its ability to promote heterochromatinization of telomeric DNA (Figure 1.8A). In support of this, Ku promotes transcriptional silencing of genes in close

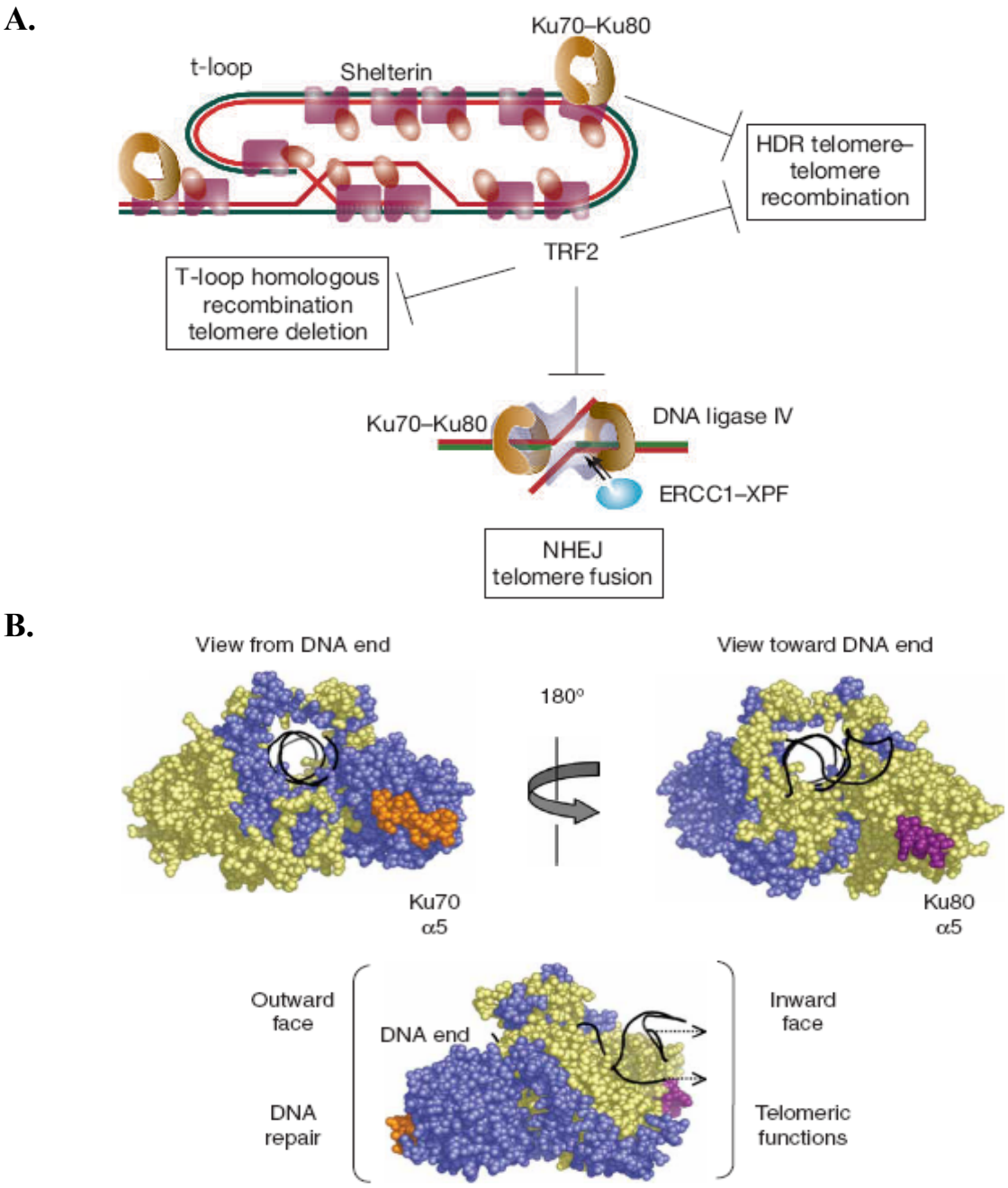


Figure 1.8: Schematic representation of the role of Ku at telomeres. A. Ku inhibits telomere-telomere recombination and end-to-end fusions by promoting hetero-chromatinization of the t-loop (Downs, Nussenzweig et al. 2007). **B.** The two-face model. Residues constituting the outward face, such as the Ku70 NHEJ-specific α-helix are involved in DSB repair, whereas surface residues constituting the inward face, such as the Ku80 telomeric silencing α-helix, are involved in telomeric functions (Ribes-Zamora, Mihalek et al. 2007).

proximity to telomeres (Boulton and Jackson 1998; Laroche, Martin et al. 1998; Nugent, Bosco et al. 1998), as well as induces late replication timing of replication origins located close to telomeres (Cosgrove, Nieduszynski et al. 2002). This silencing mechanism seems to involve the recruitment of the silent information regulator (Sir) protein 4 onto telomeric regions *in vivo* (Roy, Meier et al. 2004). Roy *et al.* isolated a separation-of-function *S.cerevisiae* Ku mutant strain, which was defective in telomeric silencing and could no longer interact efficiently with Sir4, indicating that the Yku80-Sir4p interaction plays a vital role in the assembly of telomeric heterochromatin.

Interestingly, distinct faces of the Ku heterodimer were found to mediate DNA repair and telomeric heterochromatin formation (Ribes-Zamora, Mihalek et al. 2007). Using real-valued evolutionary trace (rvET), Ribes-Zamora *et al.* identified two ancestrally related α-helices in yeast Ku, one on the Ku70 surface that is required in for NHEJ, and a second on the Ku80 surface that is required for telomeric heterochromatinization. When bound to a DNA end, the Ku heterodimer is oriented with the Ku70 helix toward the DNA terminus, whereas when bound to a telomere, the Ku80 helix faces toward telomeric chromatin suggesting a "two-face" model for Ku's dual role in NHEJ and telomere maintenance (Figure 1.8B).

5.3.3 V(D)J recombination

Vertebrates generate diversity in B-cell-immunoglobulin and T-cell-receptor genes by three main mechanisms, variable (V)-diversity (D)-joining (J) gene-segment (V(D)J) recombination, class-switch recombination and somatic hypermutation. V(D)J involves the generation of sequence-specific DSBs by the RAG1/RAG2 protein complex at the

ends of the two coding segments that are to be joined. This process generates four DNA ends: two blunt 5' phosphorylated signal ends and two coding ends terminating in DNA hairpin structures (McBlane, Van Gent et al. 1995; Van Gent, McBlane et al. 1995). Accordingly, there are two DNA-end joining events, one including the two coding ends (coding-joint formation), and a second including the linear fragments (signal-joint formation) (Figure 1.9).

Ku has been implicated in both coding- and signal-joint formation by a mechanism probably analogous to its role in the NHEJ of DNA DSBs (Bassing, Swat et al. 2002; Ma, Pannicke et al. 2002). Furthermore, Ku was found to recruit the terminal deoxynucleotidyl transferase (TdT) at recombination junctions, a process that is thought to promote variability by the addition of random nucleotides (N regions) to the DNA ends (Purugganan, Shah et al. 2001), as well as to directly interact with RAG1/RAG2 providing a biochemical link between the two phases of V(D)J recombination (Raval, Kriatchko et al. 2008). Interestingly, the latter interaction was also found to modulate DNA cleavage by RAG, increasing its specificity, suggesting an additional role for Ku in the DSB generation during V(D)J recombination (Sawchuk, Mansilla-Soto et al. 2004).

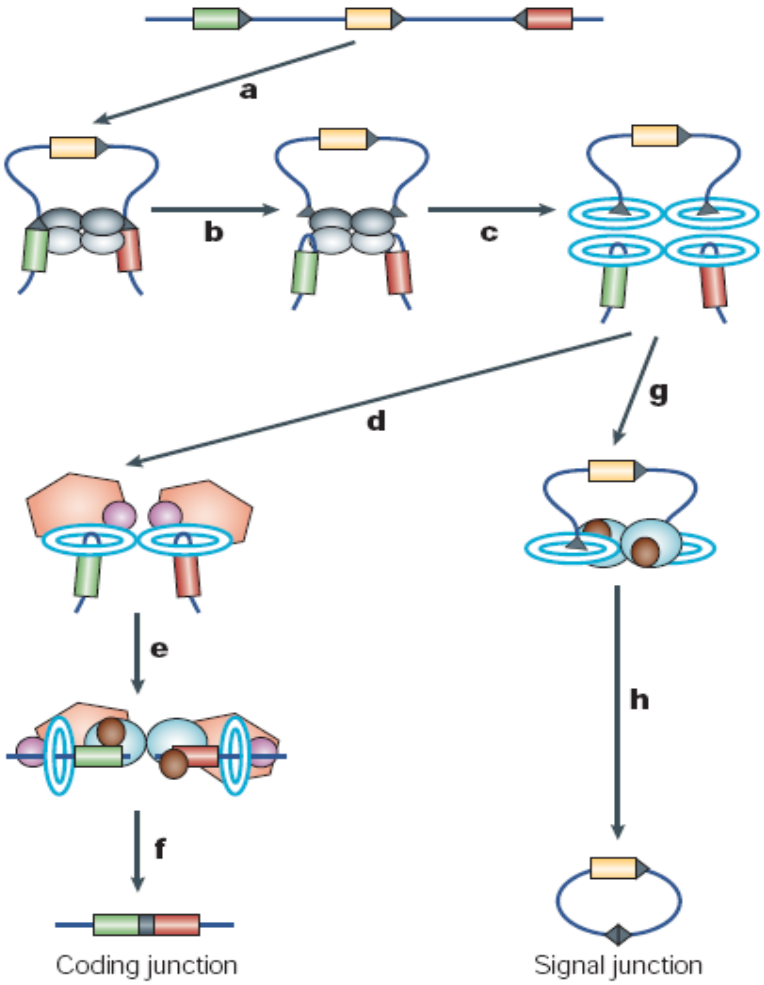


Figure 1.9: V(D)J recombination. Mechanism of genomic rearrangements at the variable (V), diversity (D) and joining (J) gene-segments of the immunoglobulins and T-cell receptors. The RAG proteins bind to the recombination signal sequences of the gene segments to be joined (a) and introduce a nick in the DNA followed by a *trans*-esterification reaction which result in the formation of a hairpin structure (b). It is thought that Ku may have a role in this stage by affecting the sequence-specificity of the RAG enzymes. The recruitment of the NHEJ machinery (DNA-Pkcs, Artemis, ligase IV, XRCC4) by Ku (c) is thought to facilitate opening of the hairpin through the enzymatic activity of Artemis (d), and repair of the resulting DNA ends (e). (Downs and Jackson 2004)

5.3.4 Transcription

A number of reports in the literature indicate that Ku is implicated in transcription regulation by binding in a sequence-specific manner to promoter elements (Giffin, Torrance et al. 1996). Furthermore, Ku has been found associated with RNA polymerase II (pol II) elongation sites (Mo and Dynan 2002), as well as to inhibit Pol-I-mediated transcription (Kuhn, Gottlieb et al. 1995). However, while in some cases Ku represses transcription, in others it functions as transcription activator. Among others, Ku has been shown to bind onto and repress transcription from the negative regulatory element 1 (NRE1) in the long terminal repeat of MMTV (Giffin, Torrance et al. 1996), the U5 region of the human T-cell leukemia virus type I (HTLV-I) long terminal repeat (LTR) (Okumura, Takagi et al. 1994), the Heat Shock Element (HSE) of the hsp70 promoter (Kim, Ouyang et al. 1995), the GATA sequence of the Glycophorin B promoter (Camara-Clayette, Thomas et al. 1999), the enhancer core sequence from the IAP (Intracisternal A-particle) LTR (Long Terminal Repeat) of the mouse c-mos (Falzon and Kuff 1989) and the negative regulatory element of the HIV-1 long terminal repeat (Jeanson and Mouscadet 2002). The mechanism by which Ku inhibits transcription is believed to include phosphorylation and associated inactivation of transcription factors (Giffin, Kwast-Welfeld et al. 1997), chromatin remodeling (Laroche, Martin et al. 1998; Roy, Meier et al. 2004) and/or displacement of transcription factors from their binding sites within the promoter of the target gene (Wang, Fang et al. 2004; Lienard, De Mees et al. 2006).

On the other hand, Ku has also been reported to bind and participate in the transcriptional activation of a number of genes including the Proximal Sequence Element

(PSE) of the U1 gene (Knuth, Gunderson et al. 1990), the IL-2 proximal promoter of activated Jurkat T-cells (Shi, Qiu et al. 2007) and the promoter of the estrogen-responsive pS2 gene (Lis and Kraus 2006). Recently, Ju *et al.* added a new and interesting dimension with regard to the mechanism of Ku-mediated gene activation. The authors showed that estrogen-dependent transcription of pS2 requires a promoter intermediate containing a double-strand break (DSB) (Ju, Lunyak et al. 2006), which is generated by a protein complex containing three enzymes, topoisomerase II (TopoIIβ), poly(ADP-ribose) polymerase (PARP-1), and Ku/DNA-PK. The action of this complex during estradiol-dependent transcriptional response alters the structure and the molecular composition of the pS2 promoter leading to the replacement of a corepressor complex – containing nucleolin, nucleoplasmin, and Hsp70 (Ju, Solum et al. 2004)- by an activating complex containing the acetyltransferase CBP and Pol II .

5.3.5 DNA replication

There is increasing evidence implicating Ku in DNA replication ((Zannis-Hadjopoulos, Sibani et al. 2004) and refs. therein). Ku is identical to the DNA-dependent ATPase purified from HeLa cells (Cao, Pitt et al. 1994), which cofractionated with a 21S multiprotein complex that is able to support SV40 *in vitro* DNA replication (Malkas, Hickey et al. 1990; Vishwanatha and Baril 1990) and a human protein initiation complex, important for the replication of Kaposi's sarcoma associated HSV (KHSV) (Wang, Li et al. 2008). Furthermore, Ku was purified as the Origin Binding Activity (OBA) present in HeLa cells, providing additional evidence for its role in DNA replication. OBA was first semi-purified from HeLa cells through its ability to interact specifically with the minimal

186bp core origin of the monkey replication origin ors8 and support ors8 replication *in vitro* (Ruiz, Pearson et al. 1995). OBA was subsequently further purified by affinity chromatography through its ability to bind to A3/4 (Ruiz, Matheos et al. 1999), a version of the mammalian origin consensus sequence(Price, Allarakhia et al. 2003). Southwestern analysis revealed that Ku80 was the origin binding protein (Ruiz, Matheos et al. 1999), while *in vitro* DNA footprinting revealed that the heterodimer bound to A3/4 and p186 in a sequence-specific manner (Schild-Poulter, Matheos et al. 2003). The interaction of Ku with the origin was independent of Mg^{2+} or ATP and was demonstrated to be stronger than the interaction of Ku with DNA ends.

Thereafter, Ku has been implicated in the initiation step of DNA replication, where the majority of the regulatory events occur (reviewed in (Zannis-Hadjopoulos, Sibani et al. 2004; Sclafani and Holzen 2007)). Using a ChIP assay Ku was shown to associate *in vivo* with the Ors8 and ors12 origins of replication in a cell-cycle dependent manner, peaking at the G1/S border and decreasing thereafter (Novac, Matheos et al. 2001). Co-immunoprecipitation studies showed that Ku forms a complex with DNA replication proteins Orc2, DNA pol α,δ and ε, Topoisomerase II, RFC and RPA (Matheos, Ruiz et al. 2002), while more recently a tandem affinity purification (TAP) procedure showed that Ku80 associates *in vivo* with the replicative helicase Mcm2-7 (Burckstummer, Bennett et al. 2006).

Ku binds to several replication origins, including the adenovirus type 2 origin (de Vries, van Driel et al. 1989), the lamin B2 human origin (Toth, Marusic et al. 1993), the mammalian replication origin consensus sequence, A3/4 (Ruiz, Matheos et al. 1999), the Chinese hamster DHFR replication origin, oriβ (Ruiz, Matheos et al. 1999), and the

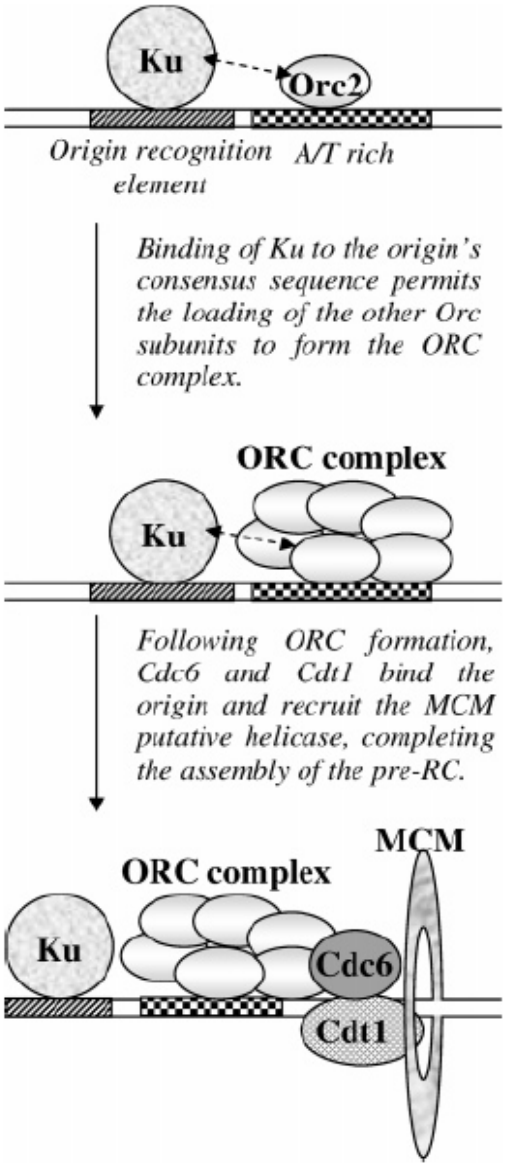


Figure 1.10: Model of the role of Ku in the initiation of DNA replication. HsORC and Ku bind to the A/T-rich region and an origin recognition element, respectively, independently of each other. Ku interacts with the HsORC2 subunit and possibly modifies the DNA environment so as to allow binding of the other HsORC subunits to HsORC2 and assembly of the HsORC complex. This is followed by the binding of the Cdc6, Cdt1 and the MCM complex, completing the assembly of the pre-RC (Sibani, Price et al. 2005).

human origins lamin B2, β-globin, c-myc and *dnmt*1 (DNA-methyltransferase) (Araujo, Knox et al. 1999; Sibani, Price et al. 2005).

A recent study, using a Ku80 hypomorphic HCT-116 cell line showed that those cells displayed a prolonged G1 phase and decreased usage of the lamin B2, c-myc and globin replication origins, as measured by nascent strand abundance (Sibani, Price et al. 2005). Interestingly the binding of Ku to those origins was decreased accordingly, suggesting a direct correlation between Ku and initiation of DNA replication. In a subsequent study, using ChIP assays it was shown that Ku80 binds to these replication origins prior to, and affects the assembly of the ORC complex (Figure 1.10) (Sibani, Price et al. 2005).

A number of possible roles for Ku in DNA replication are postulated, including a protective role in the event of DNA damage at the origins (Park, Ciccone et al. 2004; Shimura, Martin et al. 2007). Alternatively, Ku may be required to repress DNA transcription and prevent head-on collision of the transcription and replication machineries, as supported by its association with TTF1 at the mouse rDNA origin (Wallisch, Kunkel et al. 2002) or to unwind DNA through its helicase activity and allow other DNA replication proteins to bind (Tuteja, Rahman et al. 1994). Finally, Ku may act to stabilize pre-RC formation; that role has been attributed to its yeast homologue, OBF2, which was found necessary for the stable assembly of an ORC-like complex at the ARS121 origin *in vitro* (Shakibai, Kumar et al. 1996).

5.4 Structure of Ku

5.4.1 Domain structure

The Ku family of proteins is relatively poorly conserved at the primary sequence level, but recognition of homologues is facilitated by the identification of primary homology regions (PHRs) within their sequence (Gell and Jackson 1999). Each Ku subunit consists of an amino-terminal von Willebrand A domain (vWA), a central "core" consisting of a β-barrel domain and a helical C-terminal arm, and a divergent carboxy-terminal region (Figure 1.11A, D). vWA domains are protein-protein-interacting modules and, in Ku, they are involved in heterodimerization. The PHRs map to the vWA and core regions of the Ku subunits.

The carboxy-terminal region of Ku70 contains a SAP domain, which is a version of helix-extension-helix fold and is thought to be a DNA-binding domain (Aravind and Koonin 2000; Aravind and Koonin 2001). This domain has been proposed to be responsible for the pausing of the Ku heterodimer at specific DNA sequences during translocation on linear molecules (Walker, Corpina et al. 2001). The carboxy-terminal domain of Ku80 is longer and in higher eukaryotes contains an extension that binds the DNA-PKcs.

5.4.2 Three-dimensional structure

The crystal structure of the Ku heterodimer bound to a DNA end revealed that the two subunits form an asymmetric ring with an expansive base that cradles DNA, and a very narrow bridge that encircles approximately two turns of DNA (Figure 1.11B) (Walker, Corpina et al. 2001).

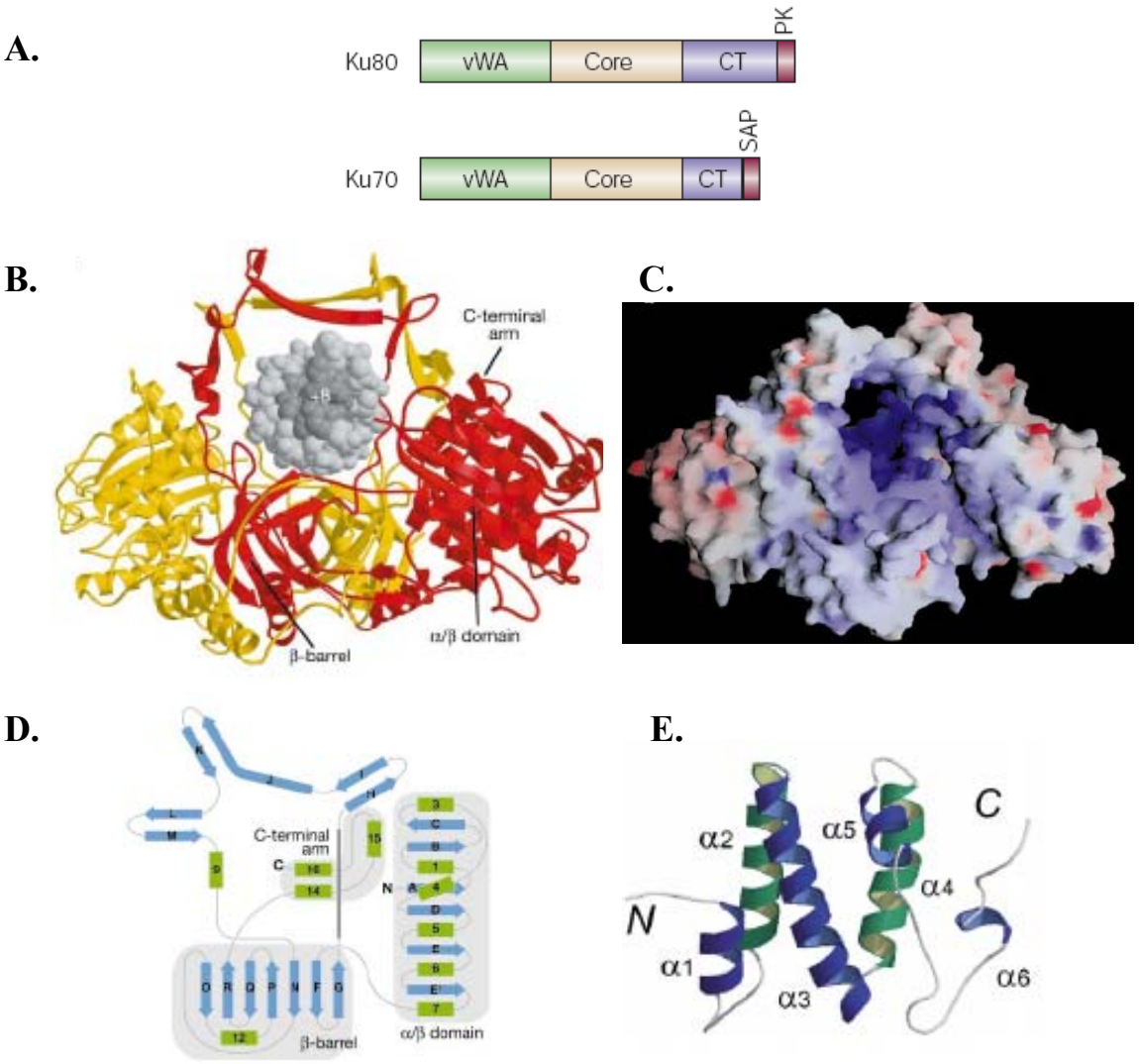


Figure 1.11: Structure of the Ku heterodimer. A. Domain organization of the Ku proteins which comprise of an amino-terminal von Willebrand A domain (vWA), a central "core" consisting of a β-barrel domain and a helical C-terminal arm, and a divergent carboxy-terminal region. **B.** View down the DNA helix. Ku70 is coloured in red and Ku80 orange. The DNA sugar-phosphate backbone is coloured light grey and the bases dark grey. **C.** Molecular surface representation of Ku according to electrostatic potential, showing the positively charged residues (blue) lining the DNA binding ring. **D.** Topology diagram showing the fold of Ku70 and Ku80. **E.** Structure of the Ku80 CTR showing its six helical organization. (Walker, Corpina et al. 2001; Harris, Esposito et al. 2004)

The folds of Ku70 and Ku80 are closely related and the two form a quasi-symmetrical molecule (Walker, Corpina et al. 2001), indicating that they diverged from an ancestral homodimer, as predicted from their sequence homology (Dynan and Yoo 1998; Gell and Jackson 1999). The two subunits are intertwined in the ring structure which is consistent with the high stability of the heterodimer even in the absence of DNA. There is a polarization of positive electrostatic charge focused on the inner surface of the ring and along the DNA-binding cradle, which allows Ku to bind DNA through a limited set of interactions with the sugar phosphate backbone (Figure 1.11C). The absence of any interaction with a DNA base explains the lack of sequence specificity in end binding during NHEJ.

The structure of the extreme carboxy-terminus of Ku80, responsible for DNA-PKcs recruitment, which was absent in the original crystal structure of the Ku heterodimer, was also solved recently by two individual groups (Harris, Esposito et al. 2004; Zhang, Hu et al. 2004). Zhang *et al.* and Harris *et al.* independently showed that a linker region between the DNA-binding domain and the carboxy-terminal domain is unstructured, whereas the extreme region folds into a highly helical structure, consisting of six helices organized in helical hairpins that pack together to form a right-handed superhelix overall (Figure 1.11E). A second 28bp DNA region (spanning residues 705-732), containing residues implicated in DNA-PKcs binding, was also found to be unstructured. This region has a highly-helical propensity and may fold upon binding its protein partner. This was indeed proven to be the case in a study from Rivera-Calvada *et al.* who prerformed 3D reconstruction by electron microscopy to show that the C-terminal domain of Ku80 undergoes a substantial displacement when DNA-PKcs is recruited, detaching from the

core of the Ku protein to interact with DNA-PKcs (Rivera-Calzada, Spagnolo et al. 2007).

5.5 Ku80 mutants

5.5.1 *Saccharomyces cerevisiae: hdf2* mutants

The yeast Ku80 homologue, *HDF2*, was targeted by homologous recombination in *S.cerevisiae* (Feldmann, Driller et al. 1996). These cells exhibited a temperature-sensitive phenotype for growth, being able to grow at 30^{o}C but not at 37^{o}C. This phenotype was suppressed by inactivation of the Rad53 checkpoint kinase (Teo and Jackson 2001), which is normally activated in response to DNA damage or replication stress and arrests cells at G_1/S. The *hdf2* mutants were sensitive to bleomycin and methyl methane sulfonate (MMS), two radiomimetic drugs known to introduce double-strand breaks in DNA molecules (Feldmann, Driller et al. 1996). Furthermore, they displayed defective telomeric maintenance phenotypes, including illegitimate recombination between telomeric ends and deregulation of C-strand resection, resulting in end-to-end fusions and elongation of the G tail respectively (Boulton and Jackson 1996). Finally, Ku was also found to regulate telomere origin activation in yeast, with earlier firing observed in knockout cells relative to their wildtype counterparts (Donaldson and Blow 1999).

5.5.2 Mice: Ku80$^{-/-}$

Ku80 was inactivated by deleting 3.4 kilobases of the mouse Ku80 locus, including 100bp of the promoter and the first two exons, using Cre-loxP technology (Nussenzweig, Chen et al. 1996). Within three days of birth Ku80$^{-/-}$ mice have an 80% survival rate and

are significantly smaller than their wild-type littermates. Adult Ku80$^{-/-}$ mice are fertile but their average size is 50% smaller than Ku80$^{+/+}$ mice, although they do not appear to have a smaller cell size, as examined in kidney, muscle and liver sections. Furthermore, they are immuno-compromised, lacking T and B lymphocytes in the spleen, lymph nodes and thymus. Examination of the bone marrow and thymus showed that both T- and B-cell development was blocked at early precursor stages due to a 2-3 fold decrease in D-J joint formation.

When mouse embryonic fibroblasts (MEFs), derived from Ku80$^{-/-}$ embryos, were monitored for their growth *in vitro*, they grew about twice as slowly as Ku80$^{+/+}$ fibroblasts. Only 75% of Ku80$^{-/-}$ MEFs were actively cycling, compared to 94% for the controls. Of the non-cycling Ku80$^{-/-}$ cells, 81% were in G_1, 19% were in G_2/M, whereas 99% of the non-cycling controls were arrested in G_1 (Nussenzweig, Chen et al. 1996). In addition, Ku80$^{-/-}$ primary MEFs failed to divide after 4-6 doublings, arresting as polyploid cells. Inactivation of p53 alleviated this growth arrest, leading to a cellular proliferation state similar to that of p53$^{-/-}$ MEFs (Difilippantonio, Zhu et al. 2000). Interestingly, however, loss of p53 did not rescue the size of Ku80$^{-/-}$ mice, indicating that the dwarfism is unrelated to DNA damage.

Studies examining the effect of Ku80 inactivation in mice yielded contradictory data with some studies reporting a telomerase-dependent telomere elongation in Ku80$^{-/-}$ MEFs (Hande, Slijepcevic et al. 1999; Espejel, Franco et al. 2002), while others described a shortening of the telomeres (d'Adda di Fagagna, Hande et al. 2001). However, both studies demonstrated end-to-end chromosomal fusions, indicating a role for Ku80 in telomeres capping.

5.5.3 Chinese Hamster Ovary (CHO) cells: *Xrs5*

The CHO Xrs5 cells were initially characterized as being sensitive to ionizing radiation due to an impaired ability to rejoin radiation-induced DNA double-strand breaks (Jeggo, Hafezparast et al. 1992), a phenotype that was rescued by the exogenous expression of Ku80 (Taccioli, Gottlieb et al. 1994). When tested for their ability to replicate a mammalian origin-containing plasmid *in vivo* and *in vitro*, the Xrs5 cells were deficient compared to the CHO wild-type cells, implicating the Ku antigen in DNA replication (Matheos, Novac et al. 2003).

5.5.4 Human: *HCT-116 Ku80*$^{+/-}$

Deletion of the promoter and first exon of both alleles of the Ku80-encoding *XRCC5* gene in human colon cancer cells produced null cells that are not viable, suggesting that Ku80 is an essential gene in human somatic cells (Li, Nelsen et al. 2002). HCT-116 Ku80$^{-/-}$ cells died of apoptosis after 8-10 doublings. Their heterozygous counterparts were viable, however they display haploinsufficiency. HCT-116 Ku80$^{+/-}$ cells express 20-50% of the Ku80 and Ku70 levels of the wildtype cells, and their cycling time is increased from 17.7 hours to 20.5 hours, while they have elevated levels of p53 protein. Examination of their telomeres revealed that they were shortened from an average of 5.6kb in HCT116 cells to 3.1kb in Ku80$^{+/-}$ and they displayed telomeric fusions and chromosomal instability in a frequency of 6 out of 23 metaphases (Myung, Ghosh et al. 2004). This phenotype was partially rescued after 30 generations following re-introduction of a Ku80 cDNA.

Finally, cell cycle analysis of asynchronous and late G_1 synchronized Ku80$^{+/-}$ cells revealed prolonged G_1 phase by comparison to wild-type cells. Furthermore, these cells display a 4-fold decrease in nascent strand DNA abundance at the *lamin B2*, *β-globin* and *c-myc* replication origins (Sibani, Price et al. 2005). These effects were found to be due to decreased chromatin binding of Ku80 and the ORC subunits 3, 4 and 6, suggesting a role for Ku in the initiation of DNA replication by affecting the loading of members of the pre-Replicative Complex (Sibani, Price et al. 2005).

RESEARCH OBJECTIVES

Initiation of DNA replication is a tighly regulated process ensuring proper genome duplication, which involves the stepwise recruitment of replication initiator proteins onto the origins. The initiation process can be divided into three phases: 1) formation of the pre-replication complex (pre-RC) during the G_1 phase of the cell cycle; 2) activation of the pre-RC complex during the G_1/S transition and; 3) recruitment of the replicative machinery onto the replication origin at various timepoints during S phase, and DNA synthesis.

Previously, the Ku protein (Ku70/Ku80 heterodimer) was identified as an initiator protein associating with replication origins in a sequence- and cell cycle-dependent manner. Ku deficiency was shown to result in impaired origin activation both *in vitro* and *in vivo* by affecting the pre-RC assembly. However, the molecular mechanism of action of Ku in the initiation of DNA replication has not been determined to date.

Furthermore, the recruitment of the replicative machinery onto origins is biphasic occurring either early or late in S phase. This process has been proposed to be epigenetically regulated, although the exact regulatory events governing this process are unknown. The goal of my research was to elucidate some of the mechanisms regulating the initiation of DNA replication. The research objectives of this thesis were to:

- Elucidate the molecular mechanism of action of the Ku protein in the pre-RC assembly (addressed in Chapter 2).
- Determine the effect of RNAi inhibition of Ku expression on DNA replication and cell cycle progression (Chapter 3).

- Comparatively analyze the molecular events taking place during the recruitment of the replicative machinery at early- and late-firing origins of DNA replication (Chapter 4).

Chapter 2:

Transient dsDNA breaks during pre-Replication Complex assembly

Emmanouil Rampakakis and Maria Zannis-Hadjopoulos

This chapter has been submitted for consideration for publication in the Proceedings of the National Academy of Sciences (PNAS).

ABSTRACT

Initiation of DNA replication involves the ordered assembly of the multi-protein pre-replicative complex (pre-RC) during the G_1 phase of the cell cycle. Previously, DNA topoisomerase II (topo II) was shown to associate with the DNA replication origin located in the lamin B2 gene in a cell cycle-modulated manner. Here we report that activation of both the early-firing lamin B2 and the late-firing hOrs8 human replication origins requires DNA topo II-dependent, transient, site-specific dsDNA break formation. Topo IIβ in complex with the DNA repair protein Ku associates *in vivo* and *in vitro* with the pre-RC region, introducing dsDNA breaks in a biphasic manner, during early and mid-G_1 phase. Inhibition of topo II activity interferes with the pre-RC assembly resulting in prolonged G_1 phase. Our data mechanistically link DNA topo IIβ-dependent dsDNA breaks and the components of the DNA repair machinery with the initiation of DNA replication and suggest an important role for DNA topology in origin activation.

INTRODUCTION

Eukaryotic DNA replication is a stringently regulated and remarkably precise multi-step process ensuring duplication of all chromosomes once and only once per cell cycle (Sclafani and Holzen 2007). Replication is initiated at multiple origins scattered along each chromosome which are marked by the binding of the evolutionarily conserved origin recognition complex (ORC1-6). ORC acts as a cell-cycle-regulated landing dock for the assembly of the pre-RC, which consists of ORC, Cdc6, Cdt1 and the MCM2-7 complex, and its targeting onto chromatin is sufficient to create a functional artificial mammalian replication origin (Takeda, Shibata et al. 2005). However, unlike *Saccharomyces cerevisiae* ORC (*Sc*ORC) which recognizes the 11-bp ARS consensus sequence, metazoan ORC exhibits low or no preference for specific DNA sequences (Gilbert 2004; Cvetic and Walter 2005; Rampakakis, Arvanitis et al. 2009).

One of the basic features of DNA is the intertwining of the DNA strands, which results in a global underwinding of DNA (negatively supercoiled) in all species (Schvartzman and Stasiak 2004). This underwinding facilitates sister DNA strand separation and affects accessibility of trans-acting factors to DNA (Hiasa and Marians 1994). The topological state of the DNA in the cell is being managed by the DNA topoisomerases (Champoux 2001), which accomplish this by creating either a single-strand (type I subfamily) or double-strand (type II subfamily) break in DNA thus permitting the rotation of one or two DNA strands respectively around the DNA axis and relaxation of the torsional stress. A role for topoisomerases in the initiation step of DNA replication has been proposed, as they are required for the activation of the simian virus 40 (SV40) (Tsurimoto, Melendy et al. 1990; Halmer, Vestner et al. 1998; Trowbridge,

Roy et al. 1999; Simmons, Gai et al. 2004), bovine papilloma virus (BPV) (Hu, Clower et al. 2006), and Epstein-Barr virus (EBV) replication origins (Kawanishi 1993). Furthermore, it was shown that *Drosophila melanogaster* ORC (*Dm*ORC) exhibits mild sequence specificity, but strong preference for negatively supercoiled DNA, suggesting that the topological state of DNA is a critical factor for origin specification in *D.melanogaster* (Remus, Beall et al. 2004). Recently, topos I and II were also found to interact specifically with the human lamin B2 replication origin in a cell cycle-regulated manner, indicating that the role of DNA topology during pre-RC assembly may be applicable in mammalian genomes as well (Abdurashidova, Radulescu et al. 2007; Falaschi, Abdurashidova et al. 2007).

In this study, we describe the functional synergy of the DNA topology and DNA repair machineries during pre-RC assembly. We demonstrate that Ku and topo IIβ directly interact with replication origins *in vivo* and *in vitro,* and that their targeting onto chromatin during G_1 phase leads to topologic changes in the chromosomal regions corresponding to replication origins. The generation of two consecutive topo II-dependent dsDNA breaks in proximity to or within the area covered by the pre-RC complex, during early and mid-G_1 phase, is concurrent with the pre-RC formation. Pharmacological inhibition of topo II results in the inhibition of pre-RC and the downstream pre-Initiation Complex (pre-IC) assembly. Our data suggest that dynamic changes in the DNA structure occur during pre-RC assembly and indicate that DNA topology is a critical factor affecting the initiation of DNA replication.

MATERIALS AND METHODS

Cell Culture, Cell-Cycle Analysis and Drug Treatments.

HeLa cells were cultured in αMEM (minimum essential medium) (Invitrogen, Carlsad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), penicillin (100 μg/ml), streptomycin (100 μg/ml) and L-glutamine (1 mmol/L) at 37^0C and 5% CO_2. For M phase synchronization, cells at 70-80% confluency were incubated with 100 ng/ml Nocodazole at 37ºC for 24 hours. Upon Nocodazole treatment, approximately 98% of cells were synchronized at M phase obtaining the characteristic round shape. Mitotic cells were removed into suspension by gently shaking the culture flasks, washed with warm PBS and harvested in order to be used for downstream applications. For G_1 phase synchronization, cells were re-seeded and released from M-phase synchronization into the cell cycle by the addition of pre-warmed fresh complete medium. Treatments with the topoisomerase II inhibitor merbarone (100μM) (Calbiochem) and the DNA-PK inhibitor NU-7026 (10μM) (Calbiochem) were performed for 1 hour at 37^oC. Cell-cycle progression was monitored by FACS analysis; cells were washed twice in ice-cold phosphate buffered saline (PBS), resuspended in Vindelov's solution (3.4 mM Tris, 75μM Propidium Iodide, 0.1% NP-40, 700 U/L RNase A (Roche), 0.01 M NaCl) (Vindelov 1977) overnight at 4^oC and then analysed using a Beckman flow cytometer and the WinMDI program.

Extract Preparation, Immunoblot Analysis and Quantification

Nuclear cell extracts (NEs) were prepared as previously described (Dignam, Lebovitz et al. 1983) and the protein concentration of each extract preparation was determined using the Bradford Protein Assay (BioRad, Hercules, CA). Western blot analysis was carried out according to standard protocols (Sambrook, Fritsch et al. 1989). Briefly, 20µg of NEs were resuspended in SDS loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), boiled for 10 minutes and loaded on an 8% SDS-PAGE gel. Following electrophoresis, the proteins were transferred onto a PVDF membrane and the membrane was immunoblotted with the indicated primary and corresponding HRP-conjugated secondary antibodies. The following antibodies were used: anti-Cyclin E (M-20), anti-Cyclin A (H-432), anti-Ku80 (H-300), anti-PARP-1 (F-2), anti-ORC2 (H-300), anti-ORC4 (H-300), anti-Cdc45 (H-300) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Cyclin B (BD Biosciences), anti-Cdt1 (generous gift from Dr. Zoi Lygerou, University of Patras, Greece), anti-TopoIIβ (2010-3; Topogen).

Electrophoretic mobility-shift assay (EMSA)

Nuclear extracts (10µg) or 100ng of recombinant Ku (TREVIGEN), TopoIIβ (generous gift from Dr. Neil Osheroff, Vanderbilt University Medical Center) and 14-3-3 proteins, were incubated with 0.4fmoles of ^{32}P-labeled PCR products corresponding to the 186bp core element of the hOrs8 origin of DNA replication, for one hour on ice. Binding to the oligonucleotide was performed in the presence of 2µg poly dI-dC (Amersham-Pharmacia), used as nonspecific competitor, and in a final volume of 20µl

including binding buffer (10mM Tris-HCl, pH7.5, 80mM NaCl, 1mM EDTA, 10mM β-Mercaptoethanol, 0.1% Triton X-100, 4% glycerol). The mixtures were subjected to electrophoresis on a native 5% PAGE at 160 Volts in 0.5x TBE and the gels were then dried and subjected to autoradiography. For electrophoretic mobility-shift competition assays, increasing molar excess amounts of cold probe were included in the reactions as indicated in the figures. For the interference experiments the nuclear extracts were pre-incubated with 1μg of anti-Ku80 (M-20), anti-TopoIIβ (C-19), anti-ORC2 (H300) (Santa Cruz Biotechnology, Santa Cruz, CA) or normal rabbit serum in binding buffer for 1h at 4^{o}C followed by the standard binding reaction.

Chromatin immunoprecipitation assay (ChIP)

For the ChIPs, sheared chromatin lysates from 2x10^{7} cells at the indicated timepoints in G_1 phase were pre-cleared by incubation with 50 μl of Protein G or Protein A agarose (Roche Molecular Biochemicals) to reduce background DNA precipitation caused by nonspecific binding to the beads, as previously described (Sibani, Price et al. 2005). Pre-cleared lysates were incubated overnight with 20 μg of anti-Ku80 (H-300), anti-TopoIIβ (C-19), anti-PARP-1 (F-2), anti-ORC2 (H-300), anti-ORC4 (H-300), anti-Cdt1 (H-300), anti-Cdc45 (H-300) or pre-immune serum (Santa Cruz Biotechnology, Santa Cruz, CA) with constant shaking. Protein G or A was added and incubated at 4^{o}C for one hour. The pelleted beads were washed successively with 1 ml Lysis Buffer for 15 minutes at 4^{o}C, followed by 1 ml of WB1 (50mM Tris-HCl pH 7.5, 500mM NaCl, 0.1% NP40, 0.05% sodium deoxycholate, complete protease inhibitors tablet (Roche Molecular Biochemicals)), 1ml of WB2 (50mM Tris-HCl pH 7.5, 0.1% NP40, 0.05% sodium

deoxycholate, complete protease inhibitors tablet) and 1 ml sterile TE lacking any protease inhibitors. The beads were subsequently resuspended in 200 µl TE/1% SDS, incubated at room temperature for 15 minutes and centrifuged at 1000g for 1 minute. Half of the supernatant was then incubated overnight at 65^{o}C to reverse the crosslinks, followed by digestion with 100 µg of Proteinase K at 55^{o}C for two hours. The DNA was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA), and eluted in 100µl 10 mM Tris-HCl (pH 8.0). The remaining half of the supernatant was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

DNA-break labeling and ChIP

Detection of topoII-mediated transient DNA strand break formation, was done as previously described, with slight modifications (Ju, Lunyak et al. 2006). Briefly, merbarone-, NU-7026-treated or untreated cells were fixed with Streck Tissue Fixative (STF, Streck Laboratories), which does not cause any DNA damage (Kodym and Horth 1995) for 10 minutes at 37^{o}C. The cells were harvested, washed twice with ice-cold PBS, once with Buffer A (0.25% Triton X-100, 10mM EDTA, 10mM HEPES [pH6.5] and once with Buffer B (200mM NaCl, 1mM EDTA, 10mM HEPES [pH6.5]), and permeabilized with Buffer C (100mM Tris-HCl [pH7.4], 50mM EDTA, 1% Triton X-100) for one hour at 4^{0}C. Subsequently, the nuclei were sequentially washed with ice-cold PBS, deionized water and 1x terminal deoxynucleotide transferase (TdT; Promega) reaction Buffer. The DNA breaks were labeled with biotin-16-dUTP using 50U of TdT for 1 hour at 37^{o}C and residual biotin-dUTP was washed away with Buffer D (100mM

Tris-Cl [pH7.4], 150mM NaCl). Nuclei were refixed and chromatin immunoprecipitation was performed using an anti-biotin antibody (A1559; Sigma), as described above.

Real-Time PCR quantification of DNA

Chromatin immunoprecipitated DNA was quantified using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. PCR reactions were carried out in a total volume of 20 µl, as previously described (Sibani, Price et al. 2005). The sequences and amplification conditions for all primer sets are listed in Table 1. Non-replicating genomic DNA from serum starved HeLa cells was included in each run to create a standard curve necessary for the quantification of the PCR products. A negative control without template DNA was also included within each set of reactions. The PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide and visualized with an Eagle Eye apparatus (Speed Light/BT Sciencetech-LT1000). None of the primers sets produced non-specific PCR products. The amount of DNA within each sample was expressed in molecules using the formula: $\frac{\text{(DNA amount)(Avogadro's number)(dilution factor)}}{\text{(molecular weight of amplicon)}}$.

Sequence Analyses

DNA sequences of characterized replication origins were scanned for the presence of the 12bp mammalian topo II consensus sequence (Fosse, Rene et al. 1991), using the EMBOSS alignment tool (http://www.ebi.ac.uk/Tools/emboss/align/index.html) of the European Bioinformatics Institute (EBI), allowing no gaps.

Table 1. Sequences and amplification conditions of primers used for the real-time PCR quantification of DNA

Primer name	Sequence (5'-3')	Amplicon size (bp)	$T_{Annealing}(C^{o)}$
LB2-F	GGCTGGCATGGACTTTCATTTCAG	232	66
LB2-R	GTGGAGGGATCTTTCTTAGACATC		
LB2C-F	GTTACCAGTCAGGCGCATGGGCC	240	66
LB2C-R	CCATCAGGGTCACCTCTGGTTCC		
LB2 Set 1-F	AATCCGATCATGCACCTGTCCTCA	215	68
LB2 Set 1-R	CTGAAATGAAAGTCCATGCCAGCC		
LB2 Set 2-F	TCTGCCCTAATGAAGCGGATGTCT	282	68
LB2 Set 2-R	TCACTTTGTACTCACGCTCTGCCT		
Ors8-F	TTGCACTTCACAGAGCAGTCAT	320	66
Ors8-R	GACCCACAAAGGCAAAAGTACC		
Ors8+2kb F	CCCTGAGGCAGGAGTGTTTGCC	520	65
Ors8+2kb R	GTATGCTCAATCTGCCCAACGG		
Ors8 Set 1-F	AGCAGAATCAGGATGACCTAGTCAGG	238	68
Ors8 Set 1-R	TTTGCCTTTGTGGGTCCCTTCCTT		
Ors8 Set 2-F	CCCAAATCCCACCATCTCAGTTAG	247	68
Ors8 Set 2-R	AGCACTGTGATGAACATCCTTG		

Names and sequences of primers used for real-time quantification of DNA with the LightCycler 480 (Roche Diagnostics). 'F' and 'R' designate the forward and reverse primers respectively. The size of the PCR products in base pairs (bp) and the annealing temperature ($T_{Annealing}$) used in the PRC cycling conditions in ^{0}C is also indicated.

RESULTS

Ku and topo IIß bind onto replication origins

Topo II was previously shown to bind onto the lamin B2 replication origin in a cell cycle-modulated manner (Abdurashidova, Radulescu et al. 2007). However, although purified topo II bound lamin B2 in a sequence-specific manner *in vivo*, it had no sequence preference *in vitro*, suggesting that it is targeted to specific sites by another protein rather than by direct enzyme/sequence recognition. A human *o*rigin *b*inding *a*ctivity, containing the Ku protein and topo II, was previously purified through its ability to interact specifically with the 186bp minimal monkey replication origin of Ors8 and support its replication (Ruiz, Pearson et al. 1995). We therefore examined whether Ku targets topo II onto replication origins. For this, we performed electrophoretic mobility shift assays using antisense oligonucleotides corresponding to the 186bp core of the Ors8 origin which acts as a *bona fide* metazoan replication origin (Todd, Landry et al. 1995; Callejo, Sibani et al. 2006). In the presence of large excess of poly-dI·dC which blocks non-specific DNA binding, a predominant protein-DNA complex was detected onto the replication origin (Fig.1A, lane 2; indicated with an arrow). Oligonucleotide competition experiments demonstrated greater inhibition of complex formation in the presence of increasing amounts of cold-specific (origin) oligonucleotide (Fig.1A, lanes 3-5) compared to a non-specific oligo of the same size and GC-content (Fig.1A, lanes 6-8). The kinetics of the competition reaction suggests that the affinity of the complex for the origin region is six-fold higher as compared to the non-specific probe. We next examined whether Ku and topo IIβ are members of the protein-DNA complex formed onto the origin, using antibodies against the 80 kDa subunit of Ku (Ku80) and topo IIβ. As can be

Fig.1

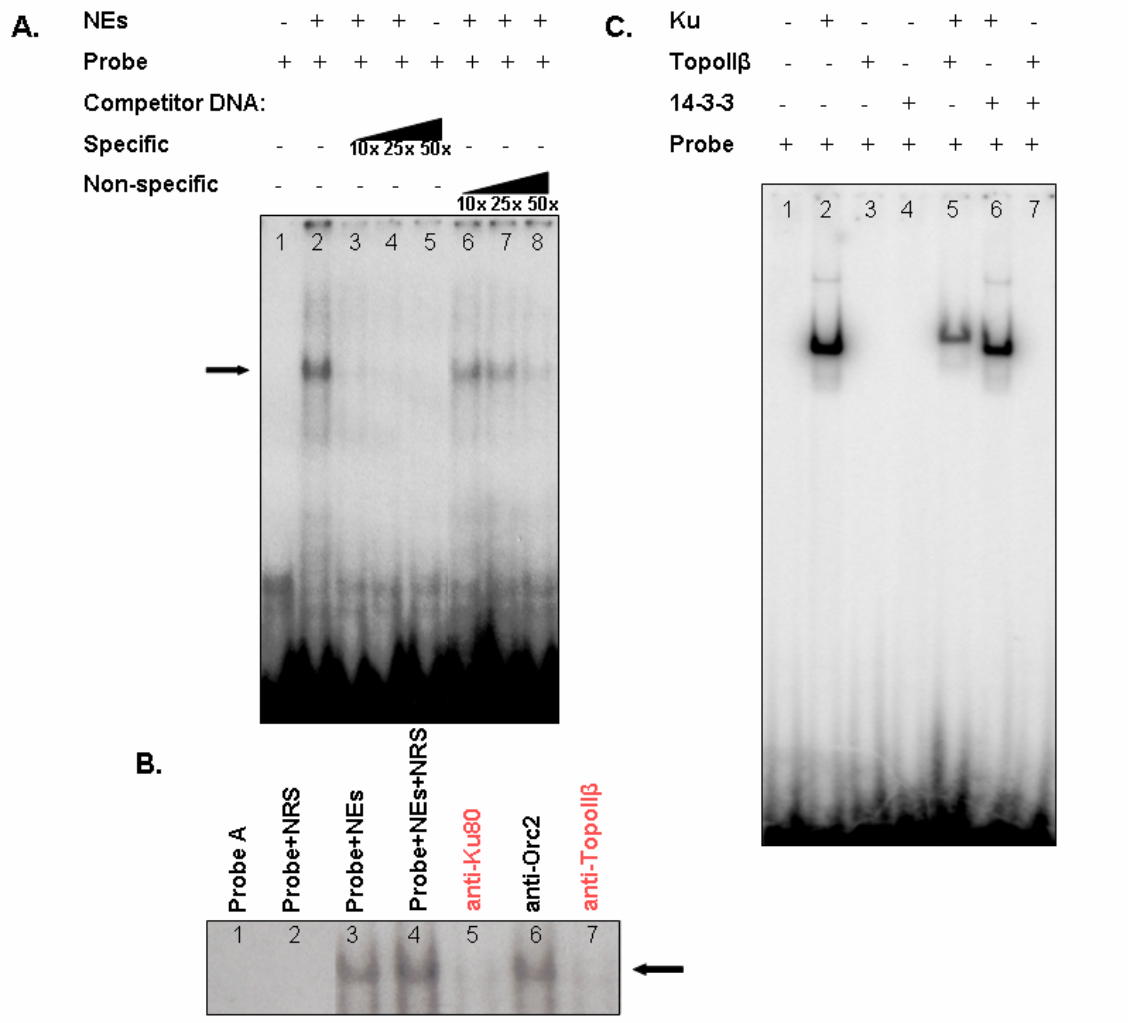
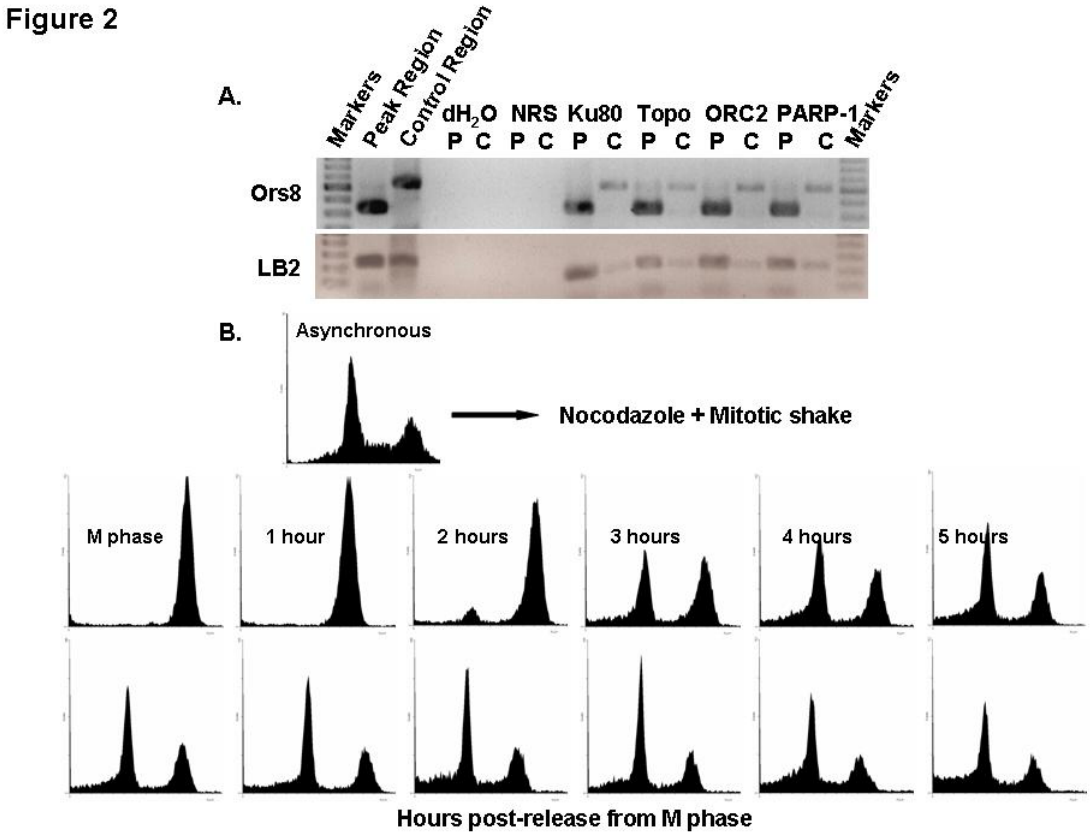
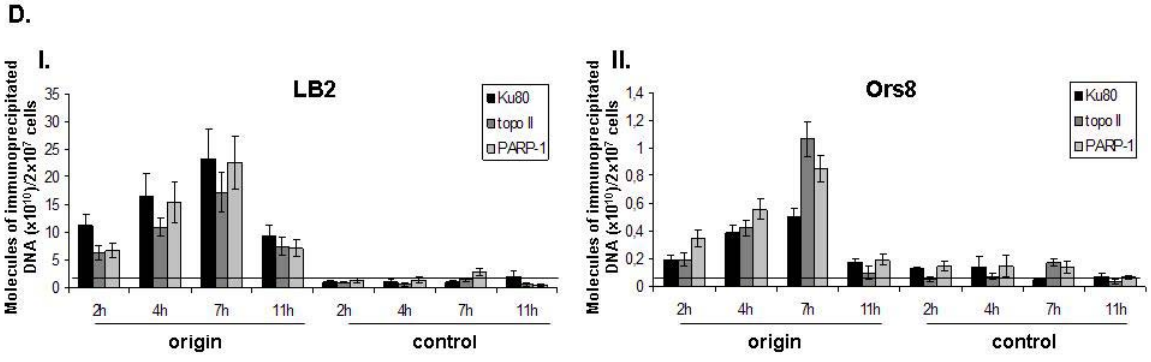


Fig.1. Ku and topo IIβ interact onto the hOrs8 origin *in vitro*. (A) EMSA showing the formation of a protein-DNA complex (indicated by an arrow) formed on hOrs8 (lane 2) in the presence of high concentration of poly-dI·dC to block non-specific binding. Addition of increasing amounts of origin probe (lanes 3-5) potently inhibits complex formation even at 10x-molar excess, unlike a non-specific oligo (lanes 6-8) of the same size and GC content, which is less effective. (B) Interference experiment showing the presence of Ku80 and topo IIβ in the complex. Addition of an anti-Ku80 (lane 5) or anti-topo IIβ (lane 7) antibody inhibits complex formation, while normal rabbit serum or an anti-ORC2 antibody has no effect. (C) EMSA using recombinant proteins. Ku binds onto the hOrs8 replication origin (lane 2) targeting topo IIβ (lane 5, protein-DNA complex of reduced electrophoretic mobility) but not 14-3-3 (lane 6). Additional controls, where Ku has been omitted from the reaction, showing that neither recombinant topo IIβ nor 14-3-3 bind onto the origin (lanes 3 and 4 respectively), and that 14-3-3 does not target topo IIβ onto hOrs8 (lane 7) are also shown, suggesting the specificity of the Ku/topo IIβ binding.

seen in Fig.1B, use of the anti-Ku and anti-topo IIβ antibodies resulted in the inhibition of the origin complex indicating the presence of these proteins in the origin-binding complex, whereas use of pre-immune rabbit serum or an antibody targeted against ORC2 had no effect on complex formation. Use of recombinant proteins showed that topo IIβ is targeted onto origin DNA *in vitro* by the Ku heterodimer (Ku70/Ku80), forming a complex of reduced electrophoretic mobility (Fig.1C, lane 5) by comparison to that formed in the presence of Ku alone (Fig.1C, lane 2). This was not found to be the case for another origin binding protein, 14-3-3 (Pearson, Zannis-Hadjopoulos et al. 1995; Todd, Cossons et al. 1998) (Ku does not target 14-3-3 and 14-3-3 does not target topo IIβ onto replication origins), or a non-origin DNA region (data not shown), suggesting that this targeting is both sequence- and protein-specific. To explore the origin association of Ku and topo IIβ *in vivo* we performed chromatin immunoprecipitation assays (ChIP) using anti-Ku and anti-topo IIβ antibodies. Both proteins were found to bind *in vivo* onto the lamin B2 and hOrs8 replication origins (Fig.2A), with some background binding detected at the non origin-containing chromosomal regions located 4kb and 2kb away from the origins, respectively. ORC2 which binds to both origins (Sibani, Price et al. 2005; Callejo, Sibani et al. 2006; Abdurashidova, Radulescu et al. 2007), was used as a positive control. In light of the recent finding that topo IIβ, in complex with Ku and PARP-1, participate in the recruitment of the transcription machinery and activation of the pS2 gene, by modifying the chromatin architecture of its promoter (Ju, Lunyak et al. 2006), we examined whether PARP-1 is also present in the protein complex formed on replication origins. Indeed, PARP-1 was found to associate with the lamin B2 and hOrs8 origins, similarly to ORC2, Ku and topo IIβ (Fig.2A). We also determined their origin

Figure 2


A.
Markers
Peak Region
Control Region
dH2O
NRS
Ku80
Topo
ORC2
PARP-1
P C P C P C P C P C P C P C
Markers
Ors8
LB2
B.
Asynchronous
Nocodazole + Mitotic shake
M phase
1 hour
2 hours
3 hours
4 hours
5 hours
Hours post-release from M phase
C.
Cyclin B
Cyclin E
Cyclin A
Ku80
TopoIIβ
PARP-1


D.
I.
LB2
Ku80
topo II
PARP-1
Molecules of immunoprecipitated DNA (x10^10)/2x10^7 cells
2h
4h
7h
11h
origin
control
II.
Ors8

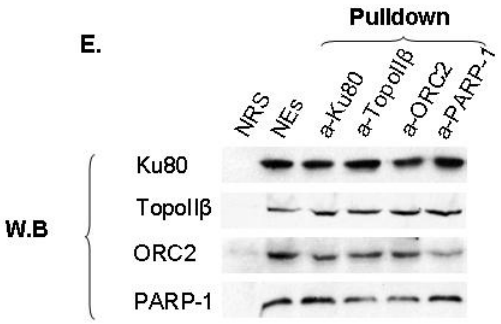


Fig. 2. Ku, topo IIβ and PARP-1 bind onto replication origins *in vivo*. (A) ChIP assay showing the association of Ku80, topo IIβ and PARP-1 onto the hOrs8 and lamin B2 origins, whereas only background binding onto the origin-lacking regions is observed. "P" corresponds to the peak (origin-containing) region and "C" to the associated control (origin-lacking) region of the respective origin loci. Immunoprecipitation with normal rabbit serum (NRS) was used as negative control, while ORC2, previously known to bind to both origins, was used as positive control. A sample with water as template was used as negative control for the real-time PCR reaction. (B) Cell cycle progression following release from M phase. Synchronization of cells in M phase was done using nocodazole treatment and mitotic shake. Upon release from M phase, cells start entering in G_1 after 2 hours and reach S phase at 10 hours. (C) Representative western blot analysis, showing the levels of the M phase-specific Cyclin B, G_1 phase-specific Cyclin E and S phase-specific Cyclin A following release from M phase. Protein levels of Ku80, topo IIβ and PARP-1 showing constant expression during G_1 phase are also included. (D) Cell cycle analysis of the Ku80, topo IIβ and PARP-1 association with hOrs8 (i) and lamin B2 (ii) using the ChIP assay. Abundance of the origin-containing or –lacking regions in the Ku80, topo IIβ and PARP-1 immunoprecipitates at various timepoints following release from M phase. Values are expressed as molecules of immunoprecipitated DNA per $2x10^7$ cells and represent the average of three experiments and one standard deviation (s.d). The horizontal bar represents background DNA immunoprecipitated by NRS. (E) Reciprocal pull-down experiment using anti-Ku80, anti-topo IIβ and anti-PARP-1, anti-ORC2 antibodies and NRS (negative control), and immunostaining with the same antibodies.

association as a function of the cell cycle. For this, cells were synchronized in M phase, using mitotic shake-off, and then released in G_1 phase by the addition of complete media. Upon this treatment, cells started entering G_1 phase after two hours and reached S phase at 10 hours, as monitored by FACS analysis (Fig.2B) and the levels of the G_2/M, G_1 and S phase Cyclins B, E and A, respectively (Fig. 2C). All three proteins (Ku, topo IIβ and PARP-1) bound to lamin B2 and hOrs8 origins during early and mid-G_1 phase, while

dissociated upon entrance into S phase, following the same pattern (Fig.2D). Furthemore, all proteins co-precipitated in pull-down experiments (Fig.2E), indicating that they associate with replication origins in close proximity or they function as a holocomplex, similarly to their role in transcription activation (Ju, Lunyak et al. 2006).

Topo II-mediated, transient dsDNA breaks during G_1 phase

We next examined whether the enzymatic activity of topo IIβ might alter the DNA topology at replication origins during pre-RC assembly. dsDNA-break formation was screened using a recently developed protocol that detects DNA break formation by combining biotin-deoxyuridine triphosphate (dUTP) labeling by terminal deoxynucleotide transferase (TdT) and subsequent ChIP analysis using an anti-biotin antibody (Fig.3A). In HeLa cells traversing through G_1 phase, putative DNA breaks were detected in both the lamin B2 and hOrs8 replication origins, but no enrichment of biotin incorporation at the non-origin containing chromosomal regions located 4kb and 2kb away, respectively (Fig.3B). Time-course DNA-break labeling indicated that the origin-specific DNA breaks occur in a biphasic and dynamic fashion. An initial break was detected early in G_1 phase (within four hours upon release) followed by a second one during mid-G_1 phase (within seven hours post-release). Generation of both breaks was transient, as they started reverting to their initial status within one hour. Interestingly, both breaks at the hOrs8 origin occured within the origin core, whereas in the lamin B2 origin only one break was created within the area covered by the pre-RC complex. Consistent with this finding is the localization of a topoisomerase II site upstream of the pre-RC area at the lamin B2 origin, which might correspond to a scaffold attachment region (Abdurashidova, Radulescu et al. 2007). To ascertain whether the origin DNA cleavages are indeed topo-II mediated, the time-course DNA-break labeling assay was repeated in the presence of the topo II inhibitor, merbarone (5-N-phenylcarboxamido-2-thiobarbituric acid), which inhibits the enzyme's cleavage activity without damaging the DNA or stabilizing intermediate DNA-topo II cleavable

Figure 3

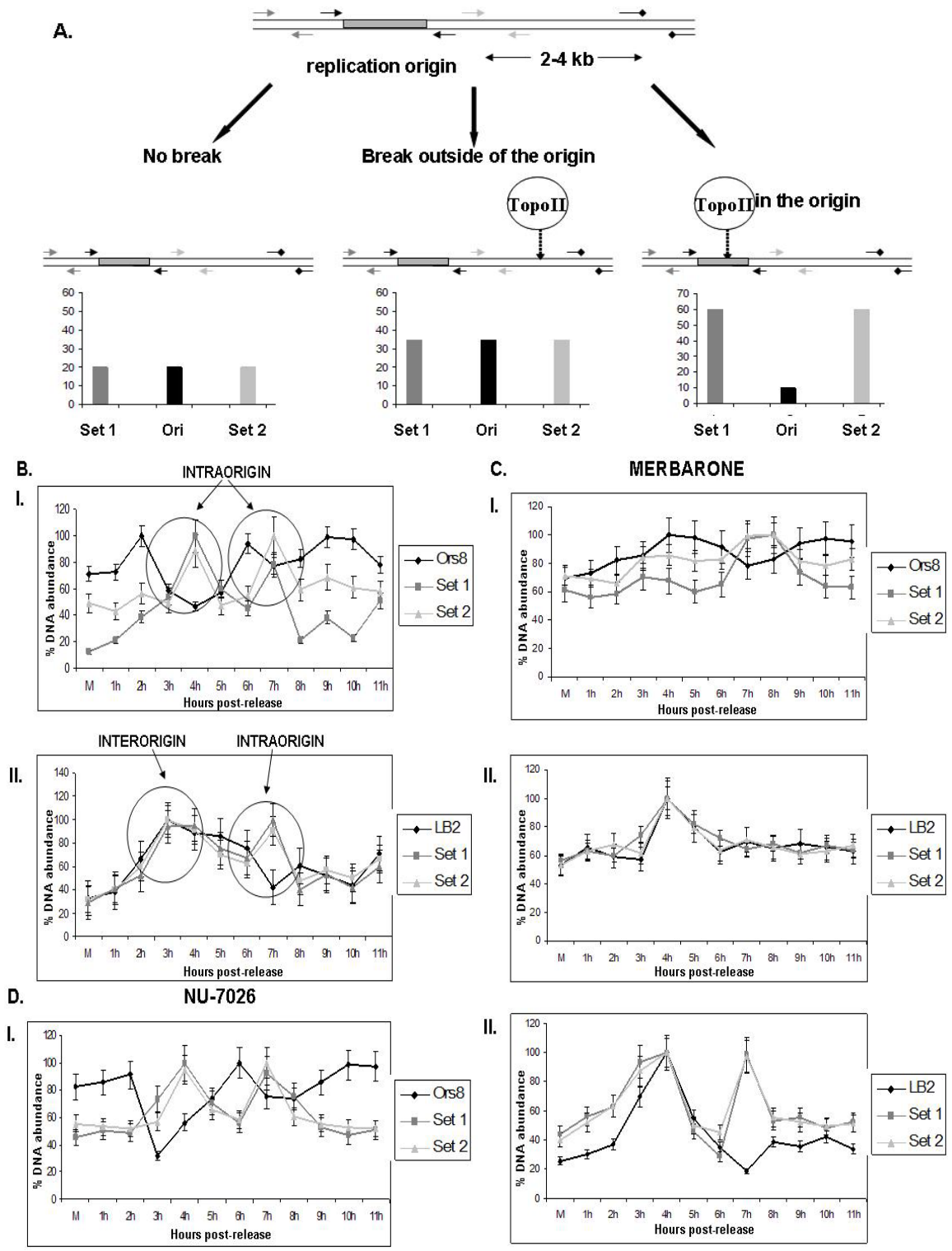


Fig. 3. dsDNA break formation during G_1 phase. (A) Schematic diagram explaining the different scenarios of the DNA break labeling assay. No break formation (left) should lead to background levels of DNA immunoprecipitated by the anti-biotin antibody. In the case of a break in proximity to the origin, but

outside of the core region (middle), increased amounts of the PCR products upstream (Set 1), downstream (Set 2) and flanking (Ori) the origin region should be produced, while an intra-origin break (right) should result in increased Set 1 and Set 2, but decreased Ori products. (B) Generation of dsDNA breaks in hOrs8 (i) and lamin B2 (ii) during G_1 phase. Encircled are the two consecutive DNA breaks formed at the two chromosomal loci, two intra-origin breaks in hOrs8, whereas one inter-origin and one intra-origin in lamin B2. Values are expressed as ratios of the individual DNA abundances at each timepoint to the maximal abundance observed during the course of the assay, following normalization with the non-origin containing regions. The error bars represent 1 s.d of three experiments. (C) Effect of merbarone on dsDNA break formation at the hOrs8 (i) and lamin B2 (ii) replication origins. Merbarone treatment results in complete (hOrs8 4h, LB2 7h) or partial (hOrs8 7h, LB2 4h) inhibition of DNA break formation. (D) DNA break labeling assay at the hOrs8 (i) and lamin B2 (ii) replication origins following NU-7026 treatment, showing that dsDNA break formation remains unaffected upon DNA-PK inhibition.

complexes. The advantage of topo II pharmacological inhibition over a RNAi approach is the ability to add the inhibitor after cell exit from G_2/M, when topo II is known to participate in chromosome condensation and segregation, thus focusing on its role in G_1 phase. Treatment with merbarone prevented the DNA breaks at the hOrs8 and lamin B2 origins (Fig.3C) indicating that the transient DNA breaks were triggered by the DNA topology machinery. Considering the presence of the binding subunit of DNA-PK at the origin area, we also examined the affect of the inhibition of the DNA-PK enzymatic activity using the DNA-PK inhibitor NU-7026. In contrast to merbarone, NU-7026 had no effect on DNA-break formation (Fig.3D), suggesting that Ku only functions in tethering topo IIβ onto replication origins, or that DNA-PK activity is only activated under stress conditions.

Functional significance of DNA-break formation

In view of the presence of topo IIβ in close proximity to the DNA synthesis start site, which suggests a role for this enzyme in the initiation step of DNA replication, we

examined the effect of topo II inhibition on pre-RC assembly. Using ChIP assays we established the kinetics of the recruitment of the pre-RC members at the Ors8 and lamin B2 replication origins during G_1 phase. Pre-RC is known to assemble onto replication origins in a stepwise manner (Sclafani and Holzen 2007); origins are initially marked by the ORC hexamer, which is recognized and bound by Cdc6 and Cdt1 during G_1-phase. Binding of the latter two proteins permits the subsequent loading of the putative DNA helicase, the minichromosome maintenance protein complex (MCM2-7), which becomes active by forming a complex with Cdc45 and GINS (Moyer, Lewis et al. 2006). Consistent with this, the hOrs8 and lamin B2 origins were initially bound by ORC2, followed by ORC4, Cdt1 and finally Cdc45 (Fig.4B and Fig.4C). The expression levels of these proteins during G_1 phase were constant suggesting that their origin-association pattern is solely due to their stepwise recruitment (Fig.4A). Treatment with merbarone blocked recruitment of ORC4/Cdt1 and Cdc45 at the hOrs8 replication origin whereas ORC2 binding was not affected. Similarly, inhibition of dsDNA-break formation led to impaired recruitment of Cdt1 and Cdc45, but not of ORC2, onto the lamin B2 origin (Fig.4C). This is in agreement with previous studies showing that Ku participates in the stepwise pre-RC assembly at a stage downstream of ORC2 (Sibani, Price et al. 2005; Rampakakis, Di Paola et al. 2008). Furthermore, merbarone treatment resulted in prolonged G_1 phase (Fig.5) supporting the notion that pre-RC assembly was affected by topo II activity. Altogether, these results suggest that dynamic changes in the DNA topology of origin-containing chromosomal regions naturally occur during pre-RC assembly and that topo II activity is required for replication initiation.

Figure 4

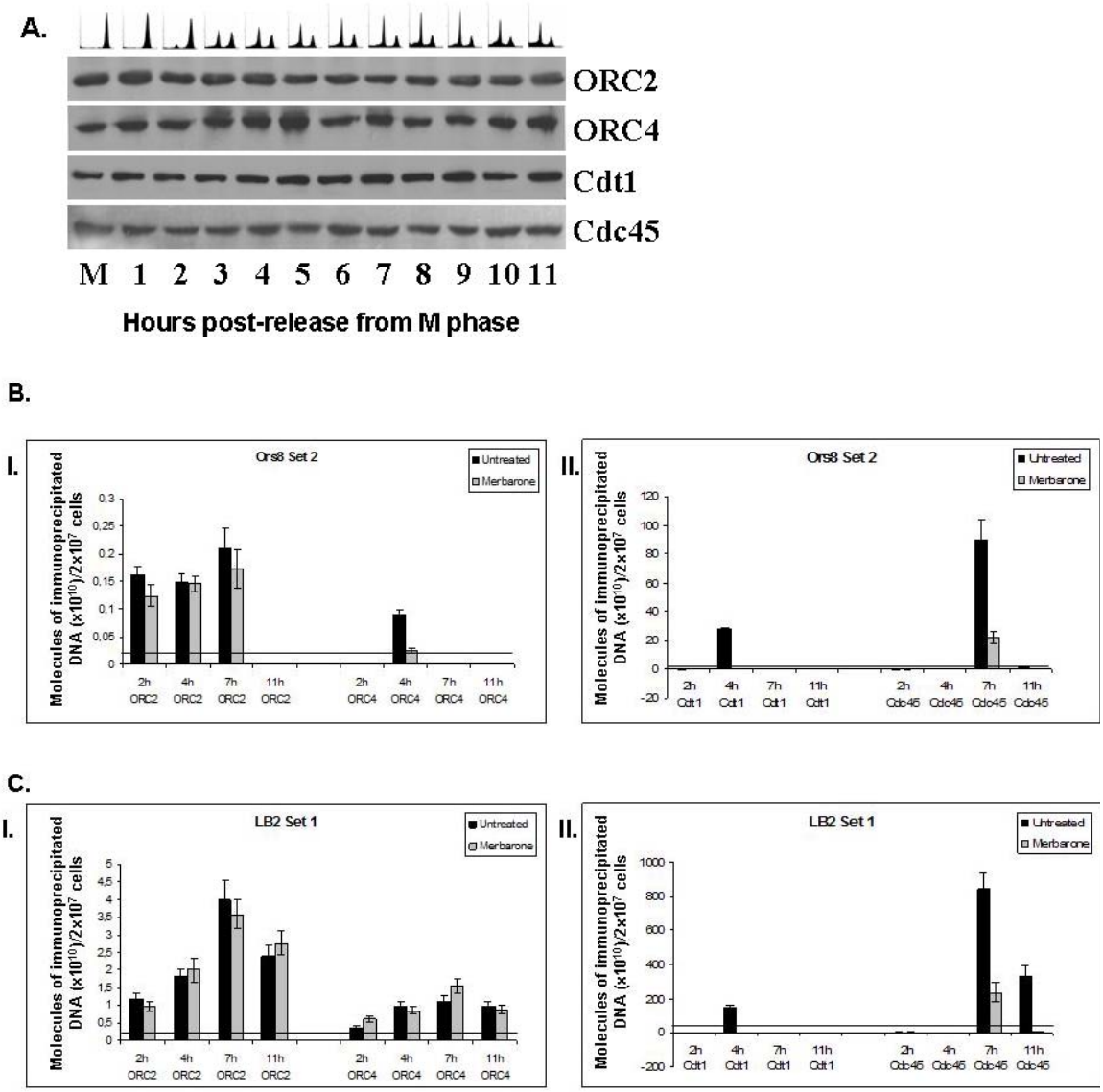


Fig. 4. Origin association of replication initiator proteins during G_1 phase. (A) Immunostaining showing the nuclear expression of ORC2, ORC4, Cdt1 and Cdc45 upon release from M phase. (B-C) ChIP assay showing the association of ORC2, ORC4, Cdt1 and Cdc45 with the hOrs8 (B) and lamin B2 (C) origins with (grey bars) or without (black bars) merbarone treatment. Abundance of immunoprecipitated origin DNA is expressed in molecules per $2x10^7$ cells and the error bars represent three experiments and 1 s.d. The horizontal bars represent background DNA immunoprecipitated by NRS and origin-lacking DNA immunoprecipitated by the anti-Ku80, -topo IIβ, -PARP-1, -ORC2, -ORC4, -Cdt1 and -Cdc45 antibodies.

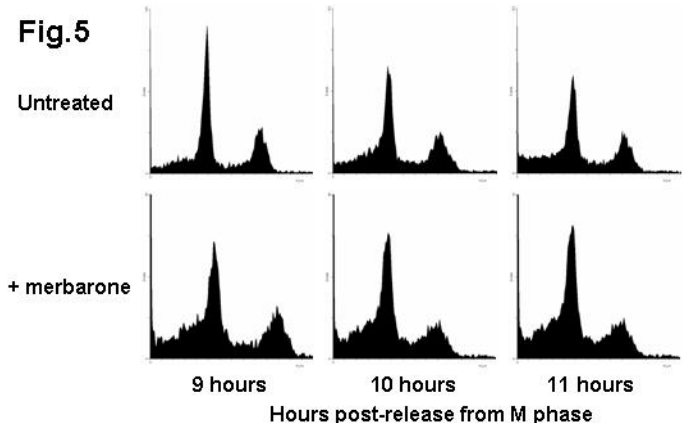


Fig. S1. FACS analysis showing the cell cycle distribution of untreated and merbarone-treated cells at 9h, 10h and 11h post-release from M phase. Untreated cells enter S phase at 10h post-release, as indicated by the reduction of the G_1 DNA content peak and the characteristic right shoulder formed, while merbarone-treated cells still remain at G_1 phase at 11 hours post-release.

DISCUSSION

High order chromatin organization is believed to play an important role in metazoan DNA replication origin specification and selection (Aladjem 2007; Rampakakis, Arvanitis et al. 2009). Previous studies implicated chromatin topology in the initiation step of replication of viral and *D.melanogaster* genomes (Tsurimoto, Melendy et al. 1990; Kawanishi 1993; Halmer, Vestner et al. 1998; Trowbridge, Roy et al. 1999; Remus, Beall et al. 2004; Simmons, Gai et al. 2004; Hu, Clower et al. 2006). In this study we examined whether this role is extended in the human genome as well. Our findings indicate that activation of the human lamin B2 and Ors8 replication origins requires the generation of topo II-dependent transient dsDNA breaks. Mammalian cells possess two isoforms of topo II, α and β, with high sequence homology (68% identity and 86% similarity) (Jenkins, Ayton et al. 1992; Austin, Sng et al. 1993). Topo IIα levels fluctuate during the cell cycle peaking in G_2/M (Heck, Hittelman et al. 1988; Kimura, Saijo et al. 1994) and thus appears to be required for chromosome condensation and segregation,

whereas topo IIβ is less well understood (Austin and Marsh 1998). Recently, it was reported that it is the topo IIβ isoform that is implicated in the initiation of DNA replication of KSHV, which utilizes the host molecular machinery in order to proliferate (Wang, Li et al. 2008). In agreement, we found that topo IIβ binds onto human replication origins suggesting that it is the DNA replication-specific isoform.

Targeting of topo IIβ onto chromatin is dependent on complex formation with the Ku protein, the DNA-binding subunit of DNA-PK, which has been previously linked to the initiation step of DNA replication (Sibani, Price et al. 2005; Sibani, Price et al. 2005; Burckstummer, Bennett et al. 2006; Rampakakis, Di Paola et al. 2008). PARP-1, a multi-functional protein involved in chromatin regulation, transcription regulation, DNA repair and cell death (Kim, Mauro et al. 2004), was also found to be a member of this complex. These proteins were previously shown to form an activation complex on the promoter of the pS2 gene participating in local changes of chromatin architecture and recruitment of the transcription machinery during estradiol-dependent transcriptional activation (Ju, Lunyak et al. 2006; Lis and Kraus 2006). Similarly, a topo IIβ/Ku/PARP-1 holoenzyme seems to act in origin firing, binding onto replication origins during G_1 phase and regulating the chromatin topology of the surrounding chromosomal region.

Pharmacological inhibition of the topo II activity using the specific inhibitor merbarone, resulted in suppression of DNA break formation and blocked the pre-RC assembly as well as the downstream initiator protein Cdc45. In contrast, DNA-PK inhibition had no effect on DNA-break formation suggesting that Ku functions in tethering topo IIβ onto replication origins. This would increase the sequence specificity of topo IIβ cleavage, similarly to what was shown in the case of RAG recombinases, which have similar

Table 2. Abundance of topoisomerase II sites in characterized human DNA replication origins

Replication Origin	# of topoisomerase II sites	Similarity	Publication
Human c-myc	1	12/12	Iguchi-Ariga,S.M. et al., 1988, EMBO
Human Ors8	2	10/12	Callejo M et al., 2006, JCB
Human lamin B2	2	11/12	Biamonti G et al., 1992, MCB
Human beta-globin	2	10/12, 12/12	Wang Lixin et al., 2006, MCB
Human hsp70	1	11/12	Taira T et al., 1994, MCB
Human Top1	2	10/12	Keller C et al., 2002, JBC
Human DNMT1 (C3 region)	1	11/12	Araujo FD et al., 1999, JBC
Human rDNA	5	11/12(4x), 12/12	Coffman FD et al., 2005, Cell cycle

enzymatic properties with topoisomerases (Sawchuk, Mansilla-Soto et al. 2004). Interestingly, pre-RC formation was blocked at two stages, upstream of ORC4/Cdt1 and Cdc45 recruitment respectively. On the contrary, origin-binding by ORC2 remained unaffected suggesting that topo II participates in pre-RC assembly rather than origin recognition. ORC2 targeting may instead be attributed to other accessory factors with intrinsic DNA sequence specificity such as AIF-C (Minami, Takahashi et al. 2006), Trf2 (Atanasiu, Deng et al. 2006), EBNA-1 (Schepers, Ritzi et al. 2001), HMGA1a (Thomae, Pich et al. 2008) or topo I (Abdurashidova, Radulescu et al. 2007).

Altogether, the data support that topo IIβ activity is required for the initiation of DNA replication, at least at the lamin B2 and hOrs8 replication origins studied here. Sequence analysis of eight well-characterized human replication origins identified the presence of the mammalian topoisomerase II consensus sequence (table 2), suggesting that the mechanism shown here may be more generalized and applicable genome-wide, as well as underscoring the importance of DNA topology as a new emerging important factor in mammalian origin activation.

ACKNOWLEDGMENTS

This work was supported by grants from the Canadian Institute of Health Research (CIHR) and the Canadian Cancer Society/National Institute of Cancer of Canada (CCS/NCIC) to MZH. E.R is past recipient of a McGill Faculty of Medicine internal studentship award, and a present recipient of the Roland and Marcel Gosselin Graduate Studentship and the Victor K.S. Lui Fellowship.

Preface to Chapter 3

In the previous chapter, the mechanistic involvement of the Ku protein in the initiation of DNA replication was analyzed. Ku was shown to target topoisomerase IIβ onto replication origins *in vitro* and *in vivo* and thus partipate in the regulation of the DNA topology of the surrounding chromosomal regions. Pharmacological inhibition of the enzymatic activity of topoisomerase II led to blockage of pre-replicative complex (pre-RC) assembly and prolonged G_1 phase. These results suggested the functional synergy of the Ku protein with the DNA topology machinery and shed light on the mechanism of action of Ku in this process.

In this chapter, we utilize a complementary strategy involving the RNAi inhibition of Ku expression. Similarly to the previous chapter, the effect of Ku-knockdown on the pre-RC chromatin loading and the initiation of DNA replication is studied. Furthermore, the underlying mechanism of G_1 phase prolongation following inactivation of the Ku/topo IIβ complex is examined, looking at the interaction of the replication initiation machinery with the cell cycle machinery.

Chapter 3:

Ku is involved in cell growth, DNA replication and G1/S transition

Emmanouil Rampakakis, Domenic Di Paola and Maria Zannis-Hadjopoulos

*This chapter appears in the Journal of Cell Science, 2008, **121**:590-600.*

ABSTRACT

The Ku protein (Ku80/Ku70) is involved in various genome maintenance processes such as DNA replication and repair, telomere maintenance and chromosomal stability. We previously found that Ku80 is implicated in the loading of members of the pre-Replicative Complex (pre-RC) onto replication origins. Here, we report that acute reduction of Ku80 to 10% of its normal levels leads to impaired DNA replication and activation of a replication stress checkpoint. In the absence of Ku80, decreased levels of the initiator proteins Orc1, Orc6 as well as reduced chromatin binding of Orc1, Orc4 and Cdc45 were observed, leading to decreased origin firing, whereas Orc2 and Orc3 were unaffected. Prolonged perturbation of DNA replication caused the block of cell-cycle progression in late G_1 phase with low Cdk2 activity due to increased p21 expression and decreased Cdc25A and Cdk2 levels. The data suggest the interplay between the DNA replication and cell-cycle machineries and shed light on a new role of Ku in G_1/S transition.

INTRODUCTION

Ku is a heterodimeric protein (Ku70/Ku80) that was first identified as an autoantigen in the sera of patients with autoimmune diseases (Mimori, Akizuki et al. 1981). It is a versatile guard of the genome that participates in its duplication and repair, telomeric maintenance as well as the suppression of chromosomal rearrangements (Reviewed in (Tuteja and Tuteja 2000; Zannis-Hadjopoulos, Sibani et al. 2004; Gullo, Au et al. 2006)). Ku is the DNA binding subunit and allosteric activator of the DNA Protein Kinase (DNA-PK) (West, Yaneva et al. 1998), but DNA-PK-independent roles have also been ascribed to it, such as a role in cell proliferation, as indicated by the phenotypes of knockout mice (Nussenzweig, Chen et al. 1996; Gao, Chaudhuri et al. 1998; Kurimasa, Ouyang et al. 1999). Increasing evidence has implicated Ku in DNA replication, expanding its repertoire of functions (Park, Ciccone et al. 2004; Zannis-Hadjopoulos, Sibani et al. 2004; Sibani, Price et al. 2005; Sibani, Price et al. 2005; Shimura, Martin et al. 2007). DNA replication initiates at specific chromosomal regions termed origins of replication. The ordered assembly of a pre-Replicative Complex (pre-RC) followed by its activation marks the origins for firing during S phase (Bell and Dutta 2002). In human cells the ORC hexamer (ORC1-6) binds to chromatin in late mitosis, followed by Cdc6 and Cdt1, which recruit the putative replicative helicase MCM (MCM2-7 complex), forming the pre-RC (Mendez and Stillman 2000). Subsequent activation of the pre-RC then loads Cdc45 and the ring-shaped GINS complex, which target the replicative polymerases on the DNA with the onset of S-phase (Sato, Arai et al. 1997; Sclafani 2000; Coverley, Laman et al. 2002; Woo and Poon 2003). After replication initiation, the pre-

RCs are inactivated, ensuring that the chromosomes are replicated only once per cell cycle and the DNA content of the cell remains constant (Machida, Hamlin et al. 2005).

Ku has been shown to associate with a number of human replication origins such as the ones associated with the *Lamin B2*, *c-myc*, *β-globin* (Sibani, Price et al. 2005; Sibani, Price et al. 2005) and *DNMT-1* (Araujo, Knox et al. 1999) loci, in a cell-cycle dependent manner (Novac, Matheos et al. 2001), as well as to co-fractionate with complexes competent for DNA synthesis (Vishwanatha and Baril 1990; Ruiz, Pearson et al. 1995). Recently, Ku was shown to form a complex *in vivo* with the MCM hexamer (Burckstummer, Bennett et al. 2006), and also participate in the loading of ORC onto origins of replication (Sibani, Price et al. 2005), implicating it in the pre-RC formation.

The DNA replication and cdk cycles are in direct communication, with both the activation and inactivation of the pre-RCs relying on the activity of cyclin-dependent kinases (cdks). Low levels of Cyclin E and Cdk2 activity are required for proper loading of the MCM proteins (Ekholm-Reed, Mendez et al. 2004), while Cdk2 and DDK (Dbf4-dependent kinase) (cdc7/Dbf4) kinase activities are required for the activation of the pre-RCs (Takeda and Dutta 2005) and G_1/S transition in mammalian cells (Pagano, Pepperkok et al. 1993; Tsai, Lees et al. 1993; van den Heuvel and Harlow 1993). Cyclin E was recently shown to also have a cdk-independent function in the loading of the MCM helicase during the G_0/S transition (Geng, Lee et al. 2007). Conversely, replication errors trigger changes in the string of events in the cdk cycle, either blocking the cells in specific stages or causing them to succumb to apoptosis in the case of extensive damage. Inhibition of fork progression by topoisomerase inhibitors (Downes, Clarke et al. 1994; Clifford, Beljin et al. 2003; Mikhailov, Shinohara et al. 2004), or double strand breaks

(Kastan and Bartek 2004) leads to the activation of a G_2/M checkpoint before mitotic entry, while low levels of replication initiator proteins block cells to late G_1 phase (Machida, Teer et al. 2005) or promote apoptosis (Feng, Tu et al. 2003), protecting cells from DNA replication crisis and possible aberrant genome duplication.

In the present study, we have examined the effect of Ku80 knockdown in DNA replication, cell growth and cell-cycle progression. Reduction of the Ku80 expression to 10% of its normal levels by RNAi resulted in impaired cell growth with the doubling time increasing from 30 hours to 58.8 hours. This growth defect was independent of DNA damage and apoptosis, but reliant on impaired chromatin loading of replication factors and origin activation. The Ku80 deficient cells were blocked in late G1 phase with increased levels of Cyclin E but decreased Cdk2 activity, suggesting a new role of Ku80 in G_1/S progression.

MATERIALS AND METHODS

Cell Culture, Cell-Cycle Analysis and Drug Treatments

HeLa and HCT-116 Ku80$^{+/-}$ cells were cultured in αMEM (minimum essential medium) (Invitrogen, Carlsad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), penicillin (100 μg/ml), streptomycin (100 μg/ml) and 1 mmol/L L-glutamine at 37^{0}C and 5% CO_2. Cell-cycle progression was monitored by FACS analysis; cells were washed twice in ice-cold phosphate buffered saline (PBS), resuspended in Vindelov's solution (3.4 mM Tris, 75μM Propidium Iodide, 0.1% NP-40, 700 U/L RNase A (Roche), 0.01 M NaCl) (Vindelov 1977) overnight at 4^{o}C and then analysed using a Beckman flow cytometer and the WinMDI program. For Hydroxyurea (HU) and Nocodazole (NOC) treatment, cells were grown in complete medium in the presence of 10mM HU or 100 ng/μl NOC, concentrations which are known to induce single-stranded DNA (ssDNA) and double-strand DNA breaks (DSB), respectively.

RNAi knockdown

The oligonucleotides (dsRNA) used in this study were previously described in Belenkov et al., 2002. The targeting (anti-Ku86) siRNA (5'-CAG AGA AGA UUC UUC AUG GGT T-3') and its mismatch control (5'-CAC AGG AGC UUA UUG AUA GGT T-3') were custom synthesized by Invitrogen. For RNAi knockdown, equal numbers of HeLa cells were seeded in medium lacking antibiotics 24 hours prior to transfection with 200 nM siRNA using Lipofectamine 2000 (Invitrogen) in serum-free Opti-MEM (Invitrogen). The oligonucleotide-containing Opti-MEM was removed from the cells and replaced with complete growth medium 4 hours post-transfection. The cells were grown

at 37^0C and 5% CO_2 in complete medium and harvested at 24 hours, 72 hours and 96 hours for use in the appropriate experiments.

Immunofluorescence

For immunostaining with the anti-Ku antibody, HeLa cells growing on coverslips were rinsed with PBS, treated with 0.5% Triton X-100 in PBS for 1 minute at RT and then fixed with 4% paraformaldehyde. After treatment with blocking buffer (1% BSA and 0.5% Triton X-100 in PBS) for 1 hour at RT, the cells were incubated with a mouse monoclonal anti-Ku antibody (NeoMarkers) and then with a Texas Red-conjugated goat anti-mouse IgG (Invitrogen). Co-staining with 0.1 mg/ml DAPI for 1 minute at RT was also performed to allow visualization of nuclear DNA. Fluorescence data were collected using a Nikon Eclipse E800 microscope and analyzed with the Nikon ACT-1 software.

Growth Curves

Equal densities of HeLa cells were seeded in complete medium and at the indicated time-points after siRNA treatment they were harvested and their numbers were determined using a Beckman Coulter counter.

BrdU Incorporation

Cells were pulse-labeled with 50 μM 5-bromo-2'-deoxyuridine (BrdU) (Sigma) for 1 hour, harvested, washed with PBS containing 1% BSA at 4^oC and fixed in cold (-20^0C) 70% ethanol for 1 hour. Cells were subsequently pelleted, resuspended in a 2N HCl solution containing 0.5% Triton X-100 and incubated at room temperature for 30

minutes. The acid was then neutralized with 0.1M $Na_2B_4O_7$ (pH 8.5). Following centrifugation, the cells were washed with PBS + 0.1% BSA + 0.5% Tween 20 containing a fluorescein-conjugated anti-BrdU antibody (Roche) at a concentration of 0.5 μg/10^6 cells and incubated at RT for 30 minutes. After antibody incubation, cells were washed once with PBS and analysed by FACS using a Beckman flow cytometer and the WinMDI program.

Extract Preparation, Immunoblot Analysis and Quantification

For the preparation of whole cell extracts (WCE), the cells were harvested, washed twice with ice-cold phosphate buffered saline (PBS) and resuspended in 2x packed cell volume (pcv) hypotonic buffer (20 mM HEPES-KOH pH7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 20 mM EDTA, 50 mM DTT and Complete Protease Inhibitor tablet (Roche Molecular Biochemicals, Indianapolis, IN)). Following 1 hour incubation at 4^0C in constant agitation, the cells were centrifuged at 14,000g, the supernatant was harvested and its protein concentration was determined using the Bradford Protein Assay (Biorad, Hercules, CA). Western blot analysis was carried out according to standard protocols (Sambrook, Fritsch et al. 1989). Briefly, the indicated amounts of WCE were resuspended in SDS loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), boiled for 10 minutes and loaded on an 8% SDS-PAGE gel. Following electrophoresis, the proteins were transferred onto a PVDF membrane and the membrane was immunoblotted with the indicated primary and corresponding HRP-conjugated secondary antibodies. The following antibodies were used: anti-Ku86 (sc-9034), anti-Orc1 (sc-23887), anti-Orc2

(sc-28742), anti-Orc3 (sc-21862), anti-Orc6 (sc-32735), anti-MCM7 (sc-9966), anti-Cdc45 (sc-20685), anti-PCNA (sc-56), anti-Cyclin E (sc-481), anti-Cyclin D1 (sc-753), anti-Cyclin A (H-432), anti-p53 (sc-6243), anti-p21 (sc-397), anti-cdk2 (sc-6248), anti-Cdc25A (sc-7389), anti-phospho-Chk2 (Thr68) (sc-16297-R), anti-caspase3 (sc-7148), anti-PARP-1 (sc-8007), anti-RPA70 (sc-25376) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Orc4 (Serotec), anti-actin (A 2066; Sigma), anti-phospho-Chk1 (2341; Cell Signaling), anti-phospho-Histone H2A.X (Ser139) (05-636; Upstate), anti-panH3 (30374; Upstate). Proteins were visualized using the enhanced chemiluminescence (ECL) kit according to the manufacturer's instructions (Amersham Biosciences, Arlington Heights, IL) and the signals were quantified using the ImageJ program.

Chromatin Loading

Cell fractionation and preparation of the chromatin enriched fractions was performed as described in Tatsumi et al., 2000 (Tatsumi, Tsurimoto et al. 2000). Untreated or transfected cells were harvested from 10cm dishes into ice-cold PBS, centrifuged, resuspended in 1ml of Lysis Buffer A (10 mM Hepes-KOH pH 7.9, 100 mM NaCl, 300 mM Sucrose, 0.1% Triton X-100 and Complete Protease Inhibitor tablet (Roche Molecular Biochemicals)), and lysed on ice for 10 minutes. After centrifugation at 2000g for 3 minutes at 4^0C, pellets were washed once more with ice-cold Lysis buffer A, and resuspended in Lysis Buffer B (10 mM Hepes-KOH pH 7.9, 200 mM NaCl, 300 mM Sucrose, 0.1% Triton X-100, 5 mM $MgCl_2$ and Complete Protease Inhibitor tablet (Roche Molecular Biochemicals)) containing 1000U of DNase I (Invitrogen). Following incubation at 25^{o}C for 30 minutes, the chromatin enriched fraction was isolated in the supernatant after centrifugation at 2500g for 5 minutes at 4^{o}C.

Isolation of Genomic and Nascent Strand DNA

Isolation of nascent strand DNA was performed using the λ exonuclease method as previously described (Giacca, Pelizon et al. 1997; Tao, Dong et al. 2000). Briefly, the cells were washed twice with PBS and lysed in Hirt's Lysis buffer (50 mM Tris-HCl, pH 8.0, 0.6 M NaCl, 1 mM EDTA and 0.5% SDS) (Hirt 1967). Following a 10 minute incubation at room temperature, the lysate was digested overnight with 0.1 mg/ml Proteinase K at 65^0C. The lysate was extracted twice with phenol/chloroform (1:1), the DNA was ethanol precipitated and sheared by passage through a 26G3/8 needle. 20 μg of DNA were denatured at 100^oC for 5 minutes, phosphorylated with 10 U of T4-polynucleotide kinase (New England Biolabs) for 30 minutes at 37^oC and the enzyme was then heat inactivated at 100^oC. Subsequently the samples were subjected to λ exonuclease (NEB) digestion at 37^oC for 12 hours. In order to separate nascent DNA from Okazaki fragments, the samples were subjected to electrophoresis on a 2% agarose gel. The DNA was visualized by staining with 0.01% (w/v) methylene blue (Sigma), and the origin-containing nascent DNA ranging between 350-1000 bp in size, was excised from the gel and purified using the QiaExII extraction kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. DNA was eluted with dH_2O and quantified using Real-Time PCR.

For the genomic fragmentation assay total genomic DNA from cells at the indicated timepoints was prepared after ethanol precipitation as described above. 10 μg of DNA were subjected to electrophoresis on a 2% agarose gel and stained with ethidium bromide. As a positive control for apoptosis, DNA isolated from cells treated with the

Histone Deacetylase Inhibitor (HDAC) Trichostatin A (Sigma; T 8552) for 24 hours was used, which is known to stimulate cell death (Finzer, Kuntzen et al. 2001).

Real-Time PCR quantification of DNA

Nascent strand DNA was quantified using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. PCR reactions were carried out in a total volume of 20 μl, as previously described (Sibani, Price et al. 2005). The sequences and amplification conditions for all primer sets are listed in Table 1. Non replicating genomic DNA from serum starved HeLa cells was included in each run to create a standard curve necessary for the quantification of the PCR products. A negative control without template DNA was also included with each set of reactions. The PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide and visualized with an Eagle Eye apparatus (Speed Light/BT Sciencetech-LT1000).

Table 1. Sequences and amplification conditions of primers used for the real-time PCR quantification of DNA with the LightCycler

Primer name	Sequence (5'➔ 3')	Amplicon size	$T_{Annealing}$ (C^0)
LB2-F	GGCTGGCATGGACTTTCATTTCAG	232	66
LB2-R	GTGGAGGGATCTTTCTTAGACATC		
LB2C-F	GTTACCAGTCAGGCGCATGGGCC	240	66
LB2C-R	CCATCAGGGTCACCTCTGGTTCC		
Myc11-F	TATCTACACTAACATCCCACGCTCTG	192	62
Myc11-R	CATCCTTGTCCTGTGAGTATAAATCATCG		
Myc1-F	TCTCAACCTCAGCACTGGTGACA	248	60
Myc1-R	GACTTTGCTGTTTGCTGTCAGGCT		

Names and sequences of primers used for real-time quantification of DNA with the LightCycler (Roche Diagnostics). The primer sets correspond to the amplification regions shown at the maps of the Lamin B2 and c-myc origins (see FIG 3A). 'F' and 'R' designate the forward and reverse primers respectively. The size of the PCR products in base pairs (bp) and the annealing temperature ($T_{Annealing}$) used in the PRC cycling conditions in ^{0}C is also indicated.

RESULTS

Knockdown of Ku80 leads to reduced cell proliferation

Initial characterization of human Ku80 haploinsufficient cells (HCT116) revealed a defect in cell proliferation (doubling time 20.5 hours compared to 17.7 hours for the Ku80 wild type cells) (Li, Nelsen et al. 2002). A second round of gene targeting generated Ku80 null cells which had a more severe growth defect and underwent massive apoptosis, signifying differences between the human and the murine knockout cells which are viable. To verify the validity of these results in different cell types we knocked-down the Ku80 levels in HeLa cells, using an RNAi approach, and analyzed the effect on cellular proliferation. At 96 h following the transfection of a siRNA targeting the Ku80 mRNA, the protein levels decreased to approximately 10% of the normal levels as measured both by Western Blot analysis and immunofluorescence (Fig.1A, 1B). The decrease in the levels of the Ku protein was gradual over time (Fig.1A and 1C), thus providing us with a system to study Ku80 deficiency mimicking the generation of Ku80 knockout cells. Consistent with the growth defect observed in the human heterozygous colorectal cancer cells (HCT116 $Ku80^{+/-}$), the cervical cancer cells (HeLa) depleted of Ku80 also displayed a proliferation defect (Fig.1D, compare panels vii, viii, ix with i, ii, iii and iv, v, vi), with their doubling time increasing 2-fold compared to cells transfected with a dsRNA with a scrambled sequence (hereafter referred to as Scr-siRNA) or cells treated with the transfection agent (hereafter referred to as Control) (doubling time 58.8h compared to 32.7h and 29.5 respectively) (Fig.1E). The results suggest that the role of Ku80 in cell proliferation is not cell type specific and is reminiscent of other DNA

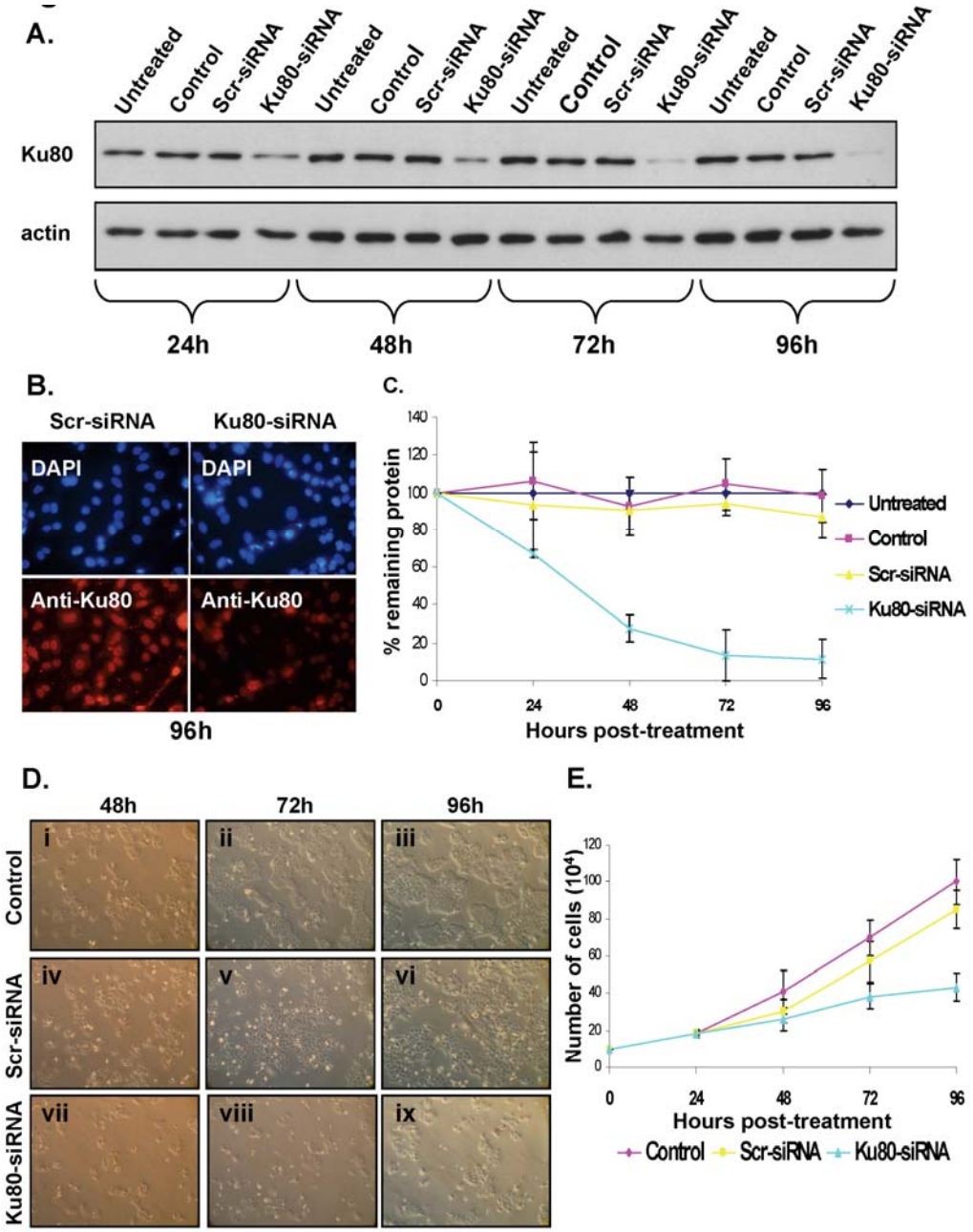


Figure 1. Reduced cell growth upon RNAi knockdown of Ku80. (A) Hela cells treated with either transfection agent (Control), a Ku80 targeting siRNA (Ku80-siRNA) or an siRNA with scrambled sequence (Scr-siRNA) were grown for 24, 48, 72 and 96 hours. Untreated cells were also included as a control. Whole cell extracts were prepared at each timepoint and immunoblotted for Ku80. Actin was used as a loading control. (B) Staining of DNA with DAPI and Ku80 by immunofluorescence, 96 hours upon transfection with Scr-siRNA or Ku80-siRNA. (C) Quantification of the Ku80 levels in each sample shown in panel A (Untreated cells: —◆— , Control cells: —■— , Scr-siRNA-treated cells: —▲— , Ku80-siRNA-treated cells: —✕—). Values are expressed as relative optical density (R.O.D) compared to the untreated cells and represent the average of three experiments and one standard deviation (S.D). (D) Representative phase contrast microscopic images of Control cells, Scr-siRNA and Ku80-siRNA transfected cells at the indicated times after transfection. (E) Growth curves of the cells shown in panel D during the time-course of the Ku80 RNAi knockdown (Control cells: —◆— , Scr-siRNA-treated cells: —■— , Ku80-siRNA-treated cells: —▲—). Values represent mean number of cells and the error bars are equivalent to one S.D.

replication proteins such as Orc2 (Dhar, Yoshida et al. 2001), Orc6 (Prasanth, Prasanth et al. 2002), Cdc6, MCM2 and Cdc45 (Feng, Tu et al. 2003).

Decreased DNA replication in Ku80 knocked-down cells

Deregulated expression of Ku80 has been reported to affect both the initiation and progression of S phase, either dependently or independently of the DNA-PK activity. On one hand, Ku80 haploinsufficient cells display decreased origin activation and delayed G_1/S transition after synchronization in late G_1 phase (Sibani, Price et al. 2005), suggesting a role in replication initiation. On the other hand, application of genotoxic stress to cells revealed a role of Ku in S phase progression which seems to be different from its initiation role in the absence of cellular stress; Ku was implicated in the maintenance of the proliferating cell nuclear antigen (PCNA) on chromatin following ionizing radiation (IR) and chromosomal double-strand break (DSB) induction, and this role was independent of the DNA-PK kinase activity (Park, Ciccone et al. 2004). Similarly, deceleration of DNA replication caused by the polymerase inhibitor aphidicolin induced transient DNA breaks, which were shown to be repaired with the action of DNA-PK (Shimura, Martin et al. 2007).

To determine whether the deregulated expression of Ku80 affected the efficiency of DNA replication, the cells were pulse-labeled with the nucleotide analogue bromodeoxyuridine (BrdU) and its incorporation was measured by FACS analysis, using an anti-BrdU antibody. The total rate of DNA replication was found to be similar for the Control cells, Scr-siRNA and Ku80-siRNA transfected cells at 24 hours post-transfection when the protein levels were not significantly changed, while at 72 hours the replication

efficiency of the Ku80-siRNA transfected cells was approximately 41% of the control levels and dropped to background levels at 96 hours (Fig.2A). A quantification of the FACS profiles is shown in Fig.2B.

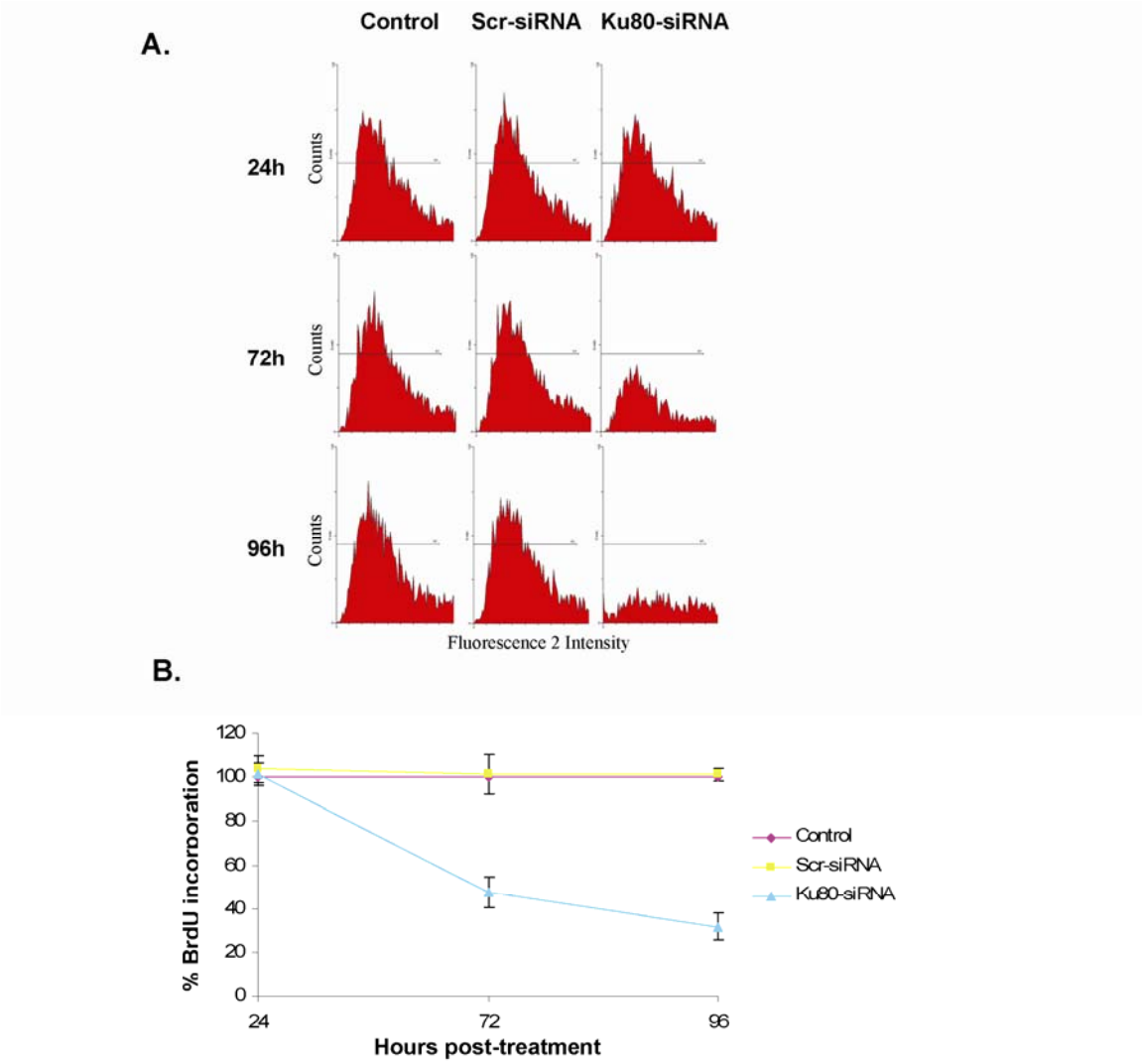


Figure 2. Time-course of BrdU incorporation as a function of Ku80 knockdown

(A) Representative FACS analysis of BrdU incorporation by logarithmically growing Control cells, Scr-siRNA and Ku80-siRNA transfected cells upon Ku80 silencing for 24, 72 and 96 hours. (B) Quantification of BrdU incorporation by each sample shown in panel A at the indicated times after transfection. Values are expressed as BrdU incorporation per 10,000 cells relative to Control cells and are means of three individual experiments ± 1 S.D.

The observed reduction of BrdU incorporation indicates a decreased replication rate but does not distinguish between an impairment in the initiation of replication or in fork progression. To distinguish between these two possibilities, we analyzed the activities of two well-characterized replication origins, namely the Lamin B2 (Giacca, Zentilin et al. 1994; Abdurashidova, Deganuto et al. 2000) and c-myc (Tao, Dong et al. 2000) origins [FIG 3A], by measuring the nascent strand abundance at these chromosomal regions, as previously described (see Materials and Methods). Similarly to the total replication rate, origin firing from both the Lamin B2 (Fig.3B) and c-myc (Fig.3C) origins was reduced approximately 2-fold at 72 hours post- siRNA transfection (nascent DNA strand abundance in Ku80-siRNA treated cells 46% and 41% of Scr-siRNA treated cells in the two origins, respectively), while it approached background levels at 96 hours (2-fold enrichment of the origin-containing over the origin-lacking regions compared to a 16.5-fold enrichment, in Ku80-siRNA vs Scr-siRNA treated cells). The similarity in the patterns of origin firing and total replication rate suggests that it is the initiation step that is affected by the Ku80 knockdown. This was further confirmed by two lines of evidence: a) the total level of PCNA, the processivity factor for the replicative polymerase δ, was decreased in the Ku80 knocked-down cells (Fig.3D) by comparison to the control cells, suggesting the existence of a smaller number of active replication forks instead of impaired fork progression; and, b), the total level of serine-139 phosphorylated H2AX histone variant (γH2AX), one of the first molecules to appear following double strand break formation (Rogakou, Pilch et al. 1998), related to the Ku80 role in replication elongation (Park, Ciccone et al. 2004; Shimura, Martin et al. 2007), was undetectable even 96 hours post-siRNA transfection, while it was present in hydroxyurea- and nocodazole-treated cells (Fig.3E). Altogether, these data indicate that low

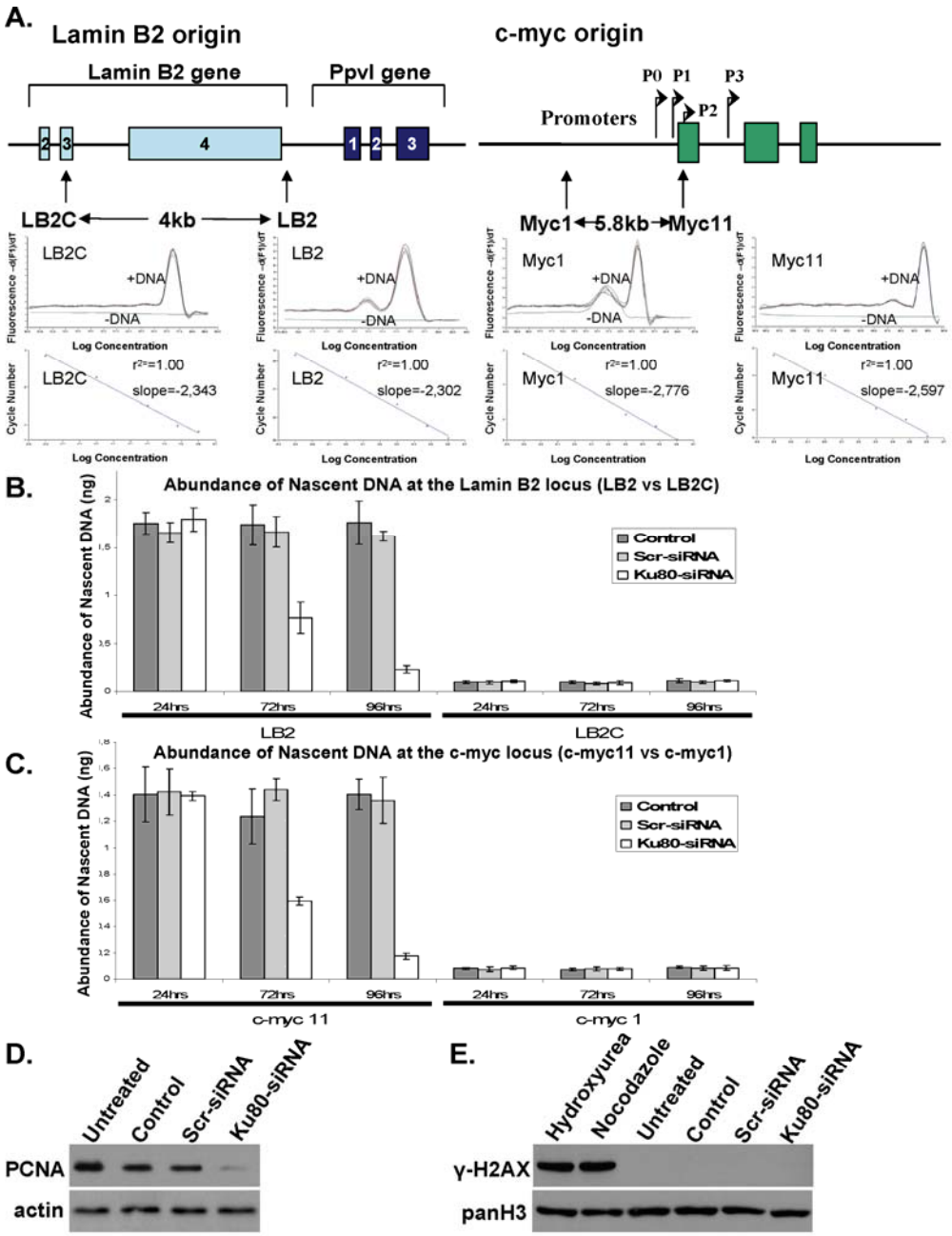


Figure 3. Origin activation and DNA synthesis as a function of Ku80 knockdown

(A) Maps of the Lamin B2 (Left) and c-myc (Right) origin loci and melting curves of the primers corresponding to the origin-containing (LB2 and Myc11 respectively) and origin-lacking (LB2C and Myc1 respectively) amplicons. The location of the amplicons relative to the gene exons as well as their distance in kb is indicated on the maps. (B and C) Histogram plots of the Lamin B2 and C-Myc origin activities throughout the silencing time-course, as measured by nascent DNA strand abundance. The LB2 and Myc11 regions lie within the origins while the LB2C and Myc1 are distal origin-lacking regions. Values are expressed as ng of nascent DNA and represent three experiments ± 1 S.D. (D) Representative Western Blot analysis of the PCNA protein expression at 72 hours post transfection with Ku80 siRNA. Actin was used as a loading control. (E) Representative Western Blot analysis of the phosphorylation levels of the H2AX histone variant at 96 hours of Ku80 RNAi. As positive controls, extracts from cells treated with the drugs Hydroxyurea (inhibitor of ribonucleotide reductase) and Nocodazole (inhibitor of microtubule formation) were used. Immunostaining with a PanH3 antibody was used as a loading control.

levels of Ku80 are responsible for the observed impaired origin firing and decreased replication rate.

Reduced chromatin loading of DNA replication licensing factors

Previous studies have implicated Ku80 in the assembly and/or stabilization of the pre-RC onto origins of DNA replication. Ku was found to form a complex with the MCM helicase *in vivo* (Burckstummer, Bennett et al. 2006) as well as to interact with the Orc subunits 3, 4 and 6 on the Lamin B2, β-globin and c-myc origins of replication (Sibani, Price et al. 2005). Based on these observations, we analyzed whether the improper expression or loading of DNA replication licensing factors at 72 hours post-siRNA treatment might be responsible for the observed decrease in origin activation. Neither the protein levels nor the chromatin loading of the Orc 2 and 3 subunits, which were previously shown to bind to origins, one independently and the other dependently of Ku80, respectively, were found to be altered (Fig.4A-D). In contrast, the levels of Orc1 and Orc6 were decreased in the absence of Ku80 (Fig.4A), Orc1 by 2.7-fold in the Ku80-siRNA- vs Scr-siRNA-treated cells and Orc6 by 4.7-fold (Fig.4B). Furthermore, the chromatin association of Orc1 and Orc4 were similarly decreased in the absence of Ku80 (Fig.4C), Orc1 by 2-fold and Orc4 by 2.4-fold, respectively (Fig.4D). The loading of Orc6 onto chromatin was unaffected (Fig.4C and 4D), possibly because Orc6 is implicated in multiple roles besides DNA replication, such as chromosome segregation and cytokinesis (Prasanth, Prasanth et al. 2002).

We also examined the levels of MCM7 after the Ku80 knockdown. The MCM7 protein appeared as a triplet in our western blots (Fig.4A), when utilizing extracts from

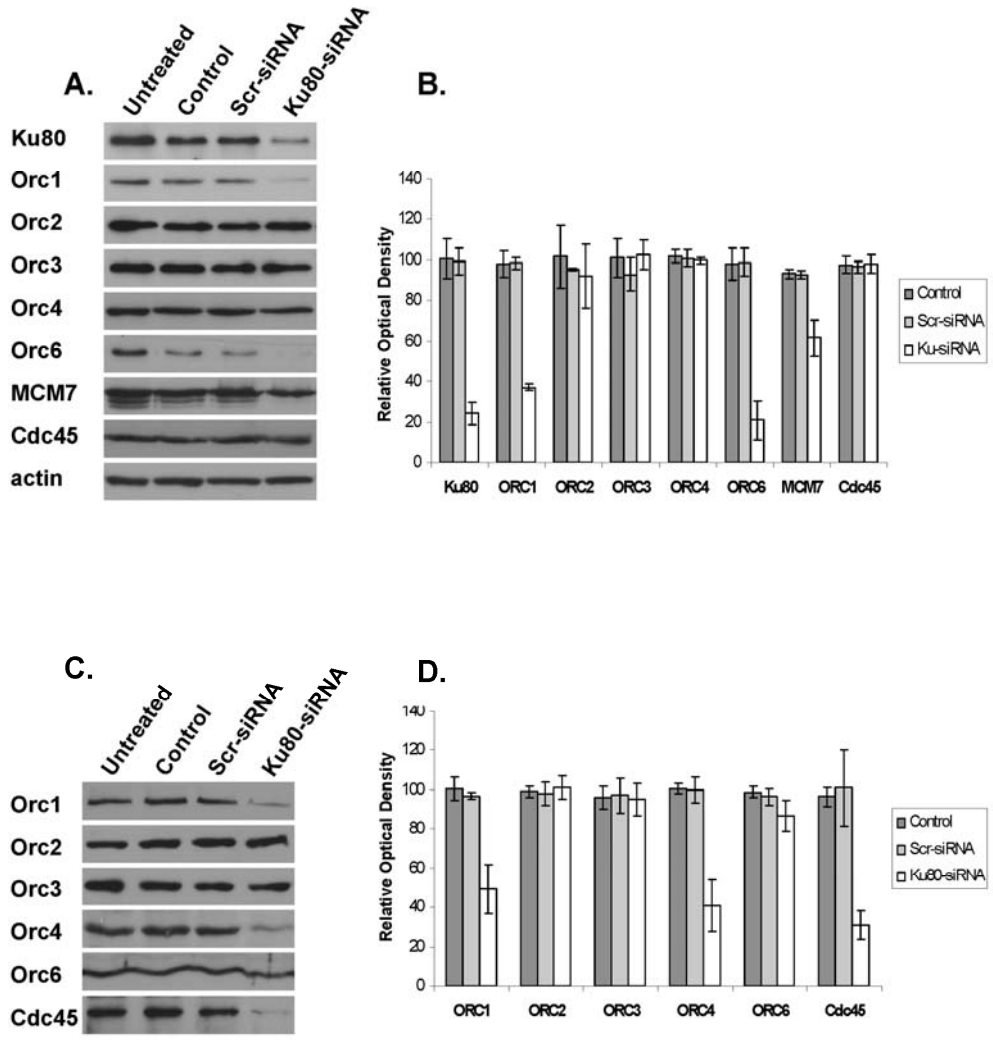


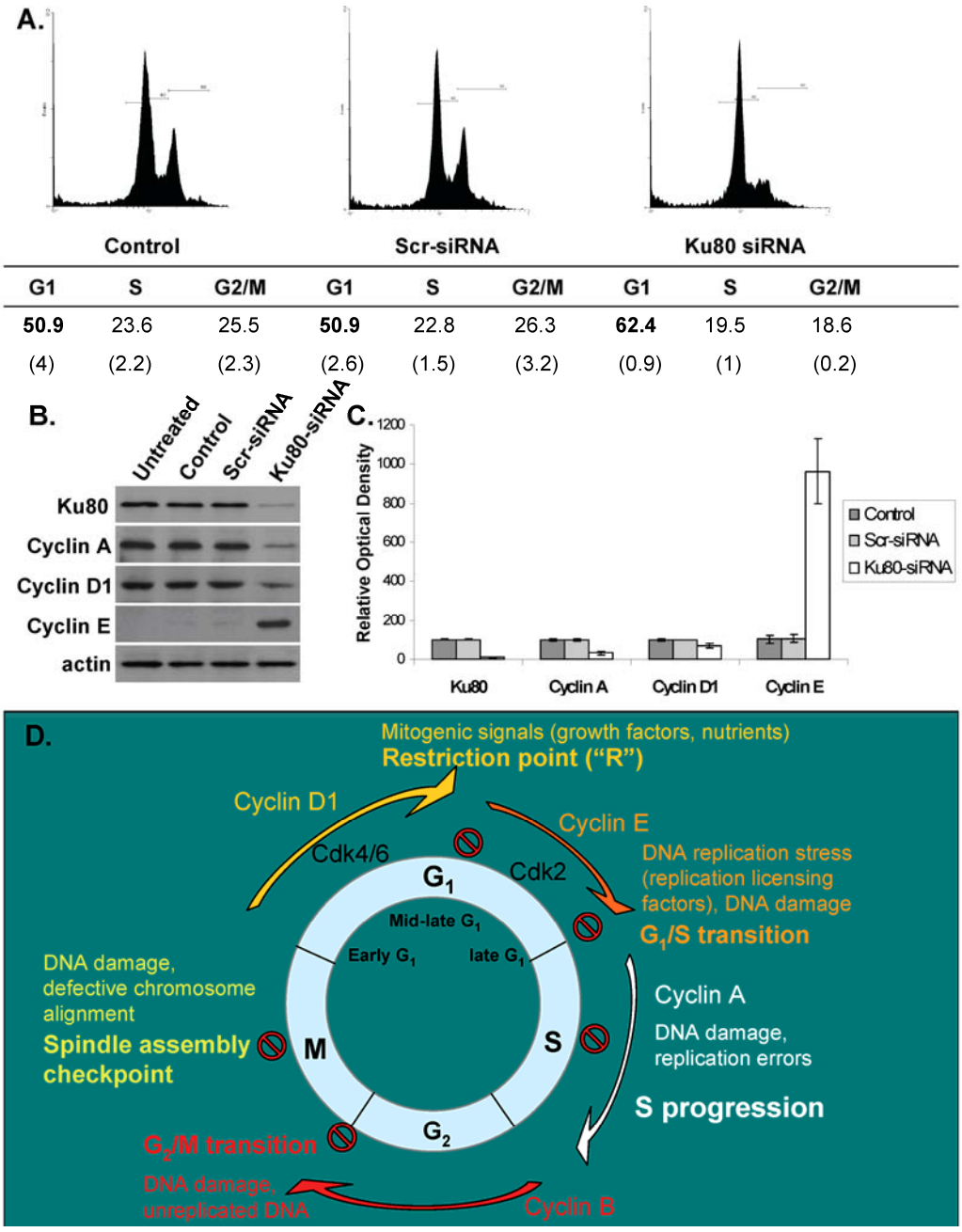
Figure 4. Expression and Chromatin loading of DNA replication licensing factors

(A) Representative Western Blot analyses of Ku80, Orc1, Orc2, Orc3, Orc4, Orc6, MCM7 and Cdc45 protein levels in whole cell extracts prepared from Untreated, Control, Scr-siRNA and Ku80-siRNA transfected HeLa cells at 72 hours post transfection. Actin was used as a loading control. (B) Relative optical densities (R.O.D) of Ku80, Orc1, Orc2, Orc3, Orc4, Orc6, MCM7 and Cdc45 immunoreactivities. Values are expressed as ratios of the individual R.O.Ds to the one corresponding to Untreated cells, and the error bars represent one S.D of three experiments. (C) Representative Western Blot analyses of Ku80, Orc1, Orc2, Orc3, Orc4, Orc6, MCM7 and Cdc45 protein levels in the chromatin bound fraction prepared from Untreated, Control, Scr-siRNA and Ku80-siRNA transfected HeLa cells 72 hours post transfection. Orc2, whose association with chromatin is essentially the same throughout the cell cycle, was used as loading control. (D) Quantification of the immunoreactivity of the proteins shown in panel C after Ku80 knockdown for 72 hours. Signal intensities (R.O.Ds) are means ± 1 S.D.

control cells and Scr-siRNA transfected cells while the Ku80-siRNA transfected cells consistently exhibited reduced levels of the two lower bands. Since MCM4 and the MCM4, 6, 7 complex have been shown to be phosphorylated (Ishimi, Komamura-Kohno et al. 2000), the triplet bands may correspond to differentially phosphorylated forms of MCM7. The phosphorylation status of the MCM helicase is thought to change its conformation and facilitate the loading of subsequent factors, such as Cdc45 (Masai, Taniyama et al. 2006), which are required for origin activation (Pacek and Walter 2004). As seen in Fig.4, upon treatment with the Ku80-siRNA the cells exhibit a decrease in the loading of Cdc45 onto the chromatin by 3.3-fold compared to Scr-siRNA treated cells (Fig.4C and 4D), providing an explanation for the observed decrease in origin activation.

Cell-cycle analysis

We next examined whether the cell-cycle distribution of the cells was affected by the Ku80 deficiency and if such a change might account for the reduced chromatin loading of the licensing factors and decline in origin activation. Interestingly, 72 hours after the anti-Ku80 siRNA treatment, the cell-cycle distribution of the cells remained unaffected (data not shown), while at 96 hours a significant accumulation of cells at the G_1 phase was observed, with 62.4% of total cell population having a G_1 phase DNA content compared to 50.9% in Ku80-siRNA-treated cells vs the Scr-siRNA-treated and Control cells ($P<0.05$, Student's *t* test; n=4) (Fig.5A). This suggests that the cell cycle block did not play a causative role, but was rather a consequence of the DNA replication defect. The accumulation of the cells at the G_1 phase was also confirmed at the molecular level, as depicted by the Cyclin A, Cyclin D1 and Cyclin E levels (Fig.5B), which were



G1	S	G2/M	G1	S	G2/M	G1	S	G2/M
50.9	23.6	25.5	**50.9**	22.8	26.3	**62.4**	19.5	18.6
(4)	(2.2)	(2.3)	(2.6)	(1.5)	(3.2)	(0.9)	(1)	(0.2)

Figure 5. Cell-cycle analysis of Ku80 knockdown cells
(A) Asynchronous cultures of Control, Scr-siRNA and Ku80-siRNA transfected HeLa cells were harvested after 96 hours of Ku80 silencing and stained with propidium iodide for monitoring of their cell-cycle distribution. Representative flow cytometric analysis is shown (Panel A, top). The table (Panel A, bottom) shows the means (n=3) of the quantification of the percentage of cells present in each phase of the cell cycle. In brackets one standard deviation of three individual experiments is shown. (B) Western Blot analysis of the Cyclin A, Cyclin D1 and Cyclin E protein levels in whole cell extracts prepared from Untreated, Control, Scr-siRNA and Ku80-siRNA transfected HeLa cells. (C) Quantification of the relative optical densities of Cyclin A, Cyclin D1 and Cyclin E. Values are expressed as average of three experiments ± 1 S.D. (D) Schematic presentation of the Cyclin/cdk cycle and the various checkpoints. Silencing of Ku80 blocks the cells at the G_1/S transition point with high levels of Cyclin E (Orange Arrow).

appropriately decreased by 3-fold and 1.5-fold or increased by 9-fold, respectively (Fig.5C). In mammalian cells, two major types of cyclins, the D-type and E-type cyclins, are required for progression through G_1 phase. D-type cyclins are active at mid-G_1 and regulate the activity of Cdk4 and Cdk6 in response to mitogens, mainly through regulating the retinoblastoma (Rb) pathway (Reviewed in (Sherr 1994)), while E-type cyclins are accumulated later, just prior to the the G_1/S transition, regulating it through regulation of the Cdk2 activity (Reviewed in (Hwang and Clurman 2005)). Cyclin A, on the other hand, is expressed at the onset of S phase and participates in the activation of DNA synthesis as well as S-phase and G_2-phase progression (Pagano, Pepperkok et al. 1992; Coverley, Laman et al. 2002). Taken together, these results suggest that the Ku80-depleted cells were blocked after the G_1 restriction point, at the G_1/S border just before the activation of DNA synthesis (Fig.5D).

Activation of a DNA replication stress checkpoint

In view of the observed accumulation of cells in late G_1, we examined the underlying mechanism that prevents cells from entering S phase. Since the Cyclin E-Cdk2 complex is the major regulator of the G_1/S transition (Sherr 1994), we checked its activation status. The Cdk2 activity is regulated by the periodic expression of Cyclin E, the presence of the cyclin-dependent kinase inhibitors (CKIs) of the CIP/KIP family, which includes p21, p27 and p57 (Morgan 1995), as well as an inhibitory phosphorylation at Tyr15, which is removed by the action of the Cdc25A phosphatase (Costanzo, Robertson et al. 2000; Falck, Mailand et al. 2001). Surprisingly, we observed a triple block of Cdk2 activity (Fig.6A), namely, decreased levels of the Cdc25A

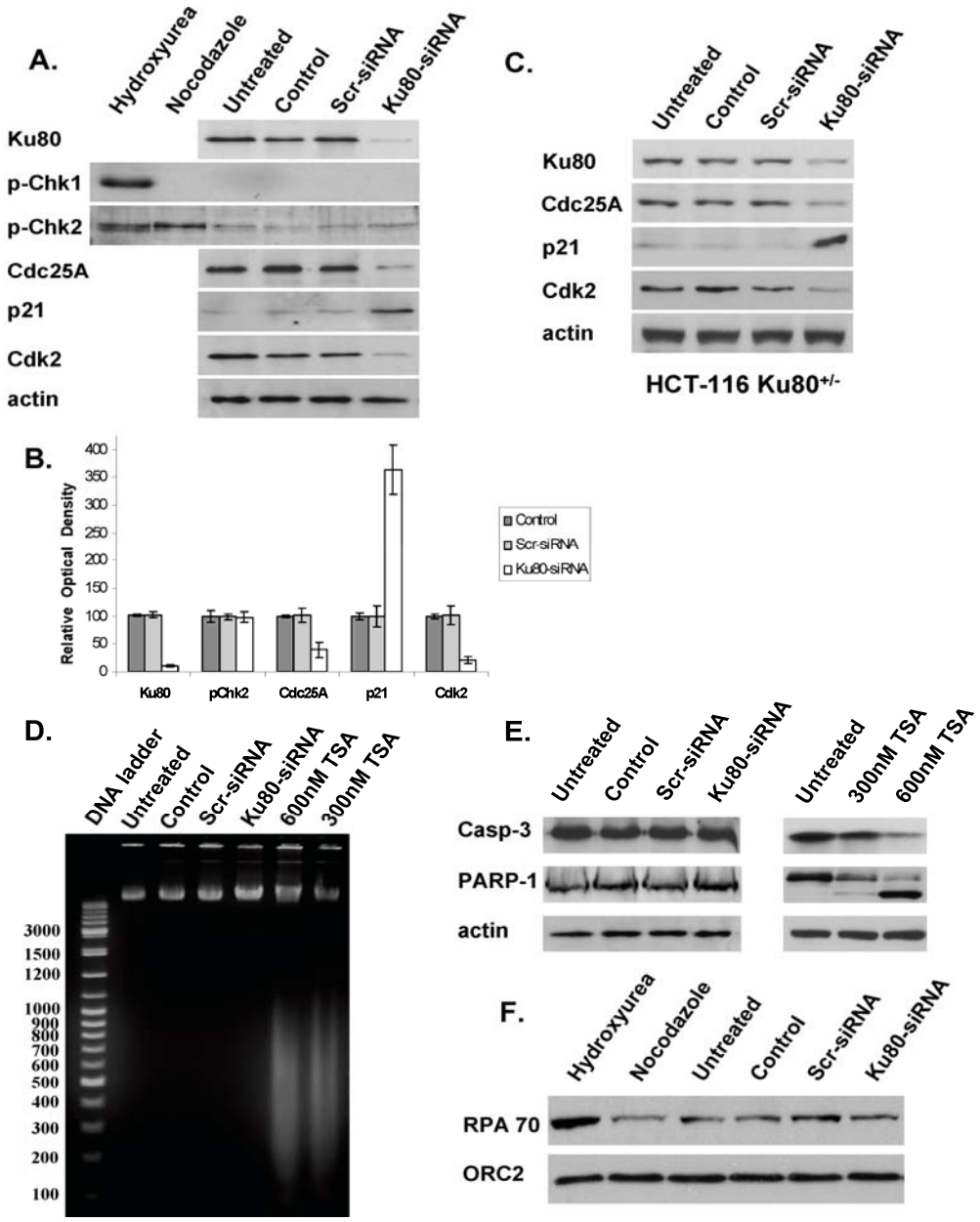


Figure 6. Activation of a replication stress checkpoint and analysis of apoptosis and ssDNA levels. (A) Protein expression of the cell-cycle regulators p-Chk1, p-Chk2, Cdc25A, p21 and Cdk2 in whole cell extracts prepared at 96 hours from Untreated, Control, Scr-siRNA and Ku80-siRNA transfected HeLa cells. Actin was used as loading control. Extracts from cells treated with the drugs Hydroxyurea (10mM) and Nocodazole (100ng/μl) were used as positive controls for the activational phosphorylation of Chk1 and Chk2. (B) Quantification of the protein levels of the cell-cycle regulators shown in panel A. Values represent the average of three experiments and the error bars correspond to one standard deviation. pChk1 levels were not included in the bar graph, since no detectable levels were observed. (C) Protein expression of the Cdk2 regulators, Cdc25A, p21 and Cdk2 in whole cell extracts prepared after Ku80 RNAi in HCT-116 Ku80$^{+/-}$ cells. (D) Genomic DNA was isolated from Untreated, Control, Scr-siRNA and Ku80-siRNA transfected HeLa cells after 96 hours of Ku80 silencing and was subjected to electrophoresis in order to determine its integrity. Extracts from cells treated with the Histone Deacetylase (HDAC) inhibitor Trichostatin A (TSA), which is known to induce apoptosis, were used as positive controls. (E) Representative Western Blot analysis of the protein levels of the full-length PARP-1 and the full-length precursor form of caspase 3 which are cleaved during apoptosis. (F) Chromatin association of the ssDNA binding protein RPA70, 96 hours post transfection.

phosphatase and the Cdk2 kinase (2.5-fold and 4.7-fold decrease, respectively, Fig.6B) and increased expression of p21 (3.6-fold increase, Fig.6B). In contrast, the level of Cdc25B was not affected (data not shown). Similar results were also obtained with the p53-containing HCT-116 Ku80$^{+/-}$cells (Fig.6C), excluding the possibility of a cell type-specific mechanism. Altogether, the data suggest a potent inactivation of the Cdk2 kinase activity, which ensures that the cells do not proceed to S-phase. The Cyclin D1 degradation (Fig.5B and 5C) also contributes to the p21-mediated inhibition of Cyclin E/Cdk2 through a mechanism termed CKI exchange, whereby less p21 is sequestered away from the Cyclin E/Cdk2 complex. Indeed, this has been shown to be the initiatory fast step of G_1 arrest, which is followed by the more time-consuming transcriptional activation of p21 (Agami and Bernards 2000).

p53 is a well-known inducer of p21 expression upon DNA damage, thus we tested for its involvement in the p21 induction. HeLa cells contain no detectable p53 protein although they contain p53 mRNA that is translationally active, as they are HPV-18 positive and their p53 is highly unstable due to the presence of the E6 oncoprotein (Scheffner, Munger et al. 1991). Upon Ku80 silencing no stabilization of p53 was observed in these p53-devoid cells (data not shown), suggesting that p21 expression is induced by a p53-independent mechanism. p21 expression was previously shown to be induced via a DNA damage- and p53-dependent-manner as well as a DNA damage- and p53-independent-manner (Macleod, Sherry et al. 1995).

The ATM-Chk2 pathway is considered the predominant pathway that is activated upon DNA damage leading to phosphorylation of Cdc25A and subsequent degradation (Costanzo, Robertson et al. 2000; Falck, Mailand et al. 2001). Again no activation of

Chk2 was observed after Ku80 knockdown (Fig.6A, 6B), suggesting a DNA damage-independent mechanism. These results are in agreement with the findings of another study, whereby silencing of Orc2 also led to p21 induction independently of p53 stabilization and Chk2 activation (Machida, Teer et al. 2005), suggesting that defects in the loading of initiator proteins and DNA damage act upon the Cyclin/cdk machinery via different pathways.

DNA damage and apoptosis are not induced in the absence of a genotoxic stress

Ku has been extensively shown to be implicated in double strand break (DSB) repair by Non Homologous End Joining (NHEJ) as well as telomere protection and maintenance (Reviewed in (Collis, DeWeese et al. 2005)). In response to these stimuli, cells have evolved cell-cycle checkpoints which are activated in order for DNA repair mechanisms to take place or, in the case of extensive damage, they are driven to apoptosis (de Lange 2002; Zhou, Wang et al. 2002). In view of the multifunctional nature of Ku80, we examined whether silencing of its expression gave rise to apoptotic stimuli or damaged DNA, which might be responsible for the reduced growth rate and cell-cycle checkpoint activation. As can be seen in Fig.6, apoptosis, as measured by genomic DNA fragmentation (Fig.6D), PARP-1 cleavage and caspase-3 activation (Fig.6E), was not induced after Ku80 knockdown. Since human Ku80-null cells are not viable, the survival of the Ku80 knockdown cells can be explained either by the presence of residual Ku80 protein (10% remaining protein at 96 hours) or the unstable nature of p53 in HeLa cells (Scheffner, Munger et al. 1991). Alternatively, apoptosis may be induced at a later stage as a result of the G_1 cell-arrest, beyond the 96 hours examined in this study.

Furthermore, no activation of the DNA-damage repair machinery, which detects both DSBs and short telomeres (Reviewed in (Downs, Nussenzweig et al. 2007; Verdun and Karlseder 2007)) was observed even after 96h of Ku silencing; phosphorylation of the H2AX histone variant (γH2AX), p53 stabilization and activation of Chk2 (p-Chk2) represent some of the primary molecular events upon DNA-damage (Matsuoka, Huang et al. 1998; Rogakou, Pilch et al. 1998; Chaturvedi, Eng et al. 1999; de Lange 2002), none of which were observed here (Fig.3E, Fig.6A). Similarly no activation of the Chk1 kinase (pChk-1) (Fig.6A) or increase in the levels of the chromatin bound single-stranded DNA (ssDNA) binding protein RPA70 was detected, indicating the absence of DNA single strand break (SSB) formation (Fig.6F). Collectively, these results support our previous findings that impaired loading of licensing factors and not DNA damage is responsible for the inactivation of cdk2 and block of the cells at the G_1/S border.

DISCUSSION

In this study we have shown that depletion of the Ku80 protein by RNAi severely impairs the growth rate of HeLa cells, with their doubling-time increasing from 30h to 58.8h. This is in agreement with previous studies, showing that human HCT-116 $Ku80^{+/-}$ cells have a growth defect (Sadji, Le Romancer et al. 2000; Li, Nelsen et al. 2002) and that the proliferative state of established cell lines compared to normal tissues is correlated with increased levels of Ku expression (Cai, Plet et al. 1994). We found that this growth defect was not associated with DNA damage caused by the lack of Ku's repair activity, but was due to decreased DNA synthesis. Since Ku has been previously implicated in the repair of DSBs caused either by IR, radiomimetic drugs such as bleomycin, or replication fork blockage (Collis, DeWeese et al. 2005; Shimura, Martin et al. 2007), this finding was surprising and suggests that in the absence of a genotoxic stress no significant DNA damage was induced and thus the action of DNA-PK was not needed.

The knockdown of Ku80 was followed by a 59% decrease in the total rate of DNA replication at 72 hours post-treatment with the siRNA oligonucleotides, further decreasing to background levels at 96 hours. We also observed a similar pattern in the frequency of initiation events at replication origins, indicating that the Ku deficiency affected the initiation step of DNA synthesis. In eukaryotic cells, initiation of DNA replication relies on a two-step process: 1) the ordered assembly of a pre-Replicative Complex onto the origin of DNA replication during late M and G_1 phase, consisting of the ORC hexamer (Orc1-6), Cdc6, Cdt1 and the putative replicative helicase MCM (MCM2-7) and, 2) the activation of the pre-RC with the action of two kinases,

CyclinE/Cdk2 and Cdc7/Dbf4, which leads to the loading of additional factors such as Cdc45, MCM10 and the GINS complex (Bell and Dutta 2002). Subsequent recruitment of the DNA primase and the replicative DNA polymerases results in origin firing. We therefore analyzed whether the expression and chromatin loading of replication licensing factors were responsible for the DNA replication defect. In the absence of Ku80, Orc2 and Orc3 were loaded normally onto chromatin, but Orc1 and Orc4 were not, resulting in impaired formation of the ORC complex. Among the six human Orc subunits (Orc 1-6), Orc 2, 3, 4 and 5 form a stable core complex, while Orc1 and Orc6 are weakly bound (Dhar and Dutta 2000; Vashee, Simancek et al. 2001) and are thought to regulate the function of the ORC hexamer. We found that the knockdown of Ku80 affected the chromatin association of both core- and regulatory-subunits, but the protein levels of only the latter. It might be possible that in the absence of a fully-formed ORC core these subunits are unstable and, therefore, targeted for proteolysis, although a transcriptional regulation cannot be excluded. Another interesting observation was the presence of MCM7 as a triplet throughout our study and the disappearance of the two lower bands upon Ku80 silencing. Although a post-translational modification of MCM7 has not been previously reported, MCM2, MCM4, MCM5 as well as the MCM4,6,7 complex have been shown to be phosphorylated (Ishimi, Komamura-Kohno et al. 2000) and this modification is thought to change the MCM complex conformation, leading to the recruitment of additional factors such as Cdc45 (Masai, Taniyama et al. 2006). Utilization of the ELM server (Puntervoll, Linding et al. 2003) predicted five PIKK phosphorylation sites, suggesting that a post-translational modification of MCM7 could occur and thus the triplet might represent differentially phosphorylated forms of MCM7.

As might be expected, low levels of Cdc45, a downstream licensing protein necessary for origin firing (Pacek and Walter 2004), were also detected on total chromatin, further supporting the hypothesis that impaired loading of DNA replication licensing factors is responsible for the observed decreased growth rate and replication initiation.

Although the cell-cycle distribution of the Ku80 deficient and control cells was the same at 72 hours when DNA replication was found to be impaired, these cells accumulated in G_1 phase 24 hours later. The Ku knockdown cells were blocked very late in G_1 phase, close to the G_1/S transition point, but before origin activation, as evidenced by the high levels of Cyclin E and low levels of Cyclin A. Both Cyclin E and Cyclin A are co-factors of Cdk2, but they have distinct roles: Cyclin E opens a "window of opportunity" for replication complex assembly, while Cyclin A is implicated in the activation of the assembled replication complexes and the inhibition of the assembly of new complexes after origin firing (Coverley, Laman et al. 2002). Thus, in agreement with this statement and as we also observed, in the absence of sufficient replication complexes, the Ku80-silenced cells would be blocked in G_1 phase with the "window" open in order for more complexes to be assembled onto the origins. Progression into S phase without the appropriate number of activated replication origins would lead to an increase of the average replicon size, resulting in stalled replication forks and chromosomal instability (Tanaka and Diffley 2002; Ekholm-Reed, Mendez et al. 2004). Our findings are in agreement with another recent study, where the levels of Orc2 were knocked down and the cells also arrested in late G_1 phase (Machida, Teer et al. 2005), suggesting the existence of a mechanism that senses pre-RC levels and controls S-phase entry.

In response to genotoxic stress that produces damaged or unreplicated DNA, eukaryotic cells have evolved checkpoint responses, which lead to DNA repair, cell-cycle arrest or apoptosis (Sagata 2002; Massague 2004). Two large protein kinases, the ataxia telangiecstasia-mutated (ATM) and related protein (ATR) are responsible for the induction of such checkpoints in response to DNA damage. ATM is activated by stimuli that induce DSB, such as ionizing radiation, and acts through the ATM/Chk2/p53 pathway, while ATR is activated by the presence of ssDNA, which is induced by UV radiation or stalled DNA replication forks and acts through the Chk1/Chk2 downstream kinases (Fisher and Mechali 2004). When we examined the activation status of these checkpoints in the Ku80-silenced cells, we observed that neither of them was induced; no phosphorylation of the Chk1 or Chk2 kinases was detected and the highly unstable p53 protein of the HeLa cells was not stabilized. Interestingly, Orc2 RNAi also did not activate these DNA damage checkpoints (Machida, Teer et al. 2005), in agreement with our findings. Instead, what we observed was a triple block acting upon the Cdk2 activity. High levels of the Cdk2 inhibitor p21 in combination with low levels of the Cdk2-activating Cdc25A phosphatase and low Cdk2 levels provided a potent barrier against Cdk2 activity and G_1/S progression.

Since Cdk2 has been implicated in the activation of the pre-RC and entry into S phase, it might be argued that the decreased levels of DNA replication may simply reflect low levels of Cdk2 activity. However at 72 hours, when initiation of DNA replication was already impaired, none of the blocks in the Cdk2 activity was observed, negating that possibility. Alternatively, our data support another model that was recently proposed, namely the existence of a new type of checkpoint, which prevents cell-cycle progression

into S-phase in the absence of sufficient initiator proteins by blocking the activity of the cdks responsible for G_1/S transition (Machida and Dutta 2005). Ku binds onto origins in proximity to Orc2 and aids the ORC assembly, either by modifying the DNA environment or by stabilizing the interactions between the different subunits. Depletion of Ku, similar to depletion of other initiator proteins, leads to incomplete ORC formation and defective recruitment of downstream licensing factors onto chromatin, thus activating this checkpoint. Additional studies are necessary to substantiate the validity of this pathway, which represents an interesting perspective.

In conclusion, the results presented here further support the interplay between the DNA replication and cdk-cycle machineries. This is the first study implicating Ku in G_1/S progression via regulation of Cdk2 activity, providing a mechanism whereby the appropriate amount of pre-RCs is loaded onto chromatin before entering the S phase, thus preventing genomic instability.

ACKNOWLEDGEMENTS

This work was supported by a grant (MOP-69029) from the Canadian Institutes of Health Research (CIHR). ER was a recipient of a doctoral studentship from McGill University Cancer Consortium Training Grant in Cancer Research of the CIHR and a McGill Internal Medicine studentship.

Preface to Chapter 4

In chapters 2 and 3 the regulation of the initiation of DNA replication during G_1 and G_1/S transition respectively was examined, revealing a new interesting role for DNA topology and the cell cycle machinery in fine-tuning origin activation. In this chapter, the regulation of the third phase of the initiation of DNA replication is examined, namely the recruitment of the replictive machinery. This issue is addressed by determining the chromatin structure and the specific epigenetic events that take place during activation of two human replication origins, lamin B2 and hOrs8. Comparative analysis of the early-firing lamin B2 and the late-firing Ors8 is also performed, discussing how chromatin structure may affect replication timing.

Chapter 4:

Dynamic changes in the chromatin structure during replication origin activation

Rampakakis E, Di Paola D, Chan MK, and Zannis-Hadjopoulos M

This chapter has been submitted for consideration for publication in the Journal of Molecular Biology.

ABSTRACT

Genome duplication relies on the timely activation of multiple replication origins throughout the genome during S phase. Each of these sites is marked by the assembly of a multiprotein pre-replication complex (pre-RC), which needs to access replication origins on the DNA through the barrier of specific chromatin structures. Inheritance of the genetic information is further accompanied by maintenance and inheritance of the epigenetic marks, which are accomplished by the activity of histone and DNA modifying enzymes traveling with the replisome. Here, we studied the changes in the chromatin structure of three replication origins, the human lamin B2 and ors8 and the monkey ors8, during their activation by measuring their abundance in post-translationally modified histone H3. The data show that dynamic changes within the levels of acetylated, methylated and phosphorylated histone H3 occur during the initiation of DNA replication, which differ between early- and late-firing origins as well as between human- and monkey-derived cell lines. These results suggest that specific histone modifications are associated with origin firing, temporal activation and replication fork progression and underscore the importance of species specificity.

INTRODUCTION

Replication of both the eukaryotic genome and epigenome rely on the initiation of DNA replication from specific chromosomal regions, termed origins, at specific times during S phase. Replication origins are marked by the presence of DNA consensus sequences and the binding of the Origin Recognition Complex proteins (ORC1-6), which leads to the assembly of a pre-Replicative Complex (pre-RC) (DePamphilis, Blow et al. 2006; Sclafani and Holzen 2007). Although DNA replication in lower eukaryotes initiates from well characterized consensus sequences, metazoan origins exhibit a certain degree of degeneracy. As a result, both origin selection and temporal activation are characterized by plasticity; during the development of *Xenopus* and *Drosophila* embryos there is a dramatic change in origin usage from widespread random initiations to specific initiation sites, which correlates with the onset of zygotic transcription and global chromatin remodeling (Shinomiya and Ina 1991; Hyrien and Mechali 1993; Hyrien, Maric et al. 1995; Maric, Levacher et al. 1999; Sasaki, Sawado et al. 1999). The human β-globin locus replicates early in S phase in pre-erythroid cells that express globin, but late in non-erythroid cells which don't express globin. This origin plasticity is more likely due to reversible changes in chromatin structure as opposed to the rigidity of the genetic code (Aladjem 2007; Jorgensen, Azuara et al. 2007).

Recent studies showed that chromatin structure could indeed affect origin selection, activation and temporal program. Thus, treatment of HeLa cells with the histone deacetylase (HDAC) inhibitor trichostatin A (TSA), led to a more dispersive pattern of initiation site selection as well as an earlier activation of the late-firing β-globin origin (Kemp, Ghosh et al. 2005). SIR2, a histone deacetylase specific for acetyl-lysines K9 and

K14 of histone H3 and K16 of histone H4, inhibits activation of some origins, but not others (Pasero, Bensimon et al. 2002; Pappas, Frisch et al. 2004), by promoting an unfavorable chromatin structure for pre-RC assembly (Crampton, Chang et al. 2008). Deletion of the Rpd3 HDAC in budding yeast promoted an earlier activation of many late-firing replication origins (Vogelauer, Rubbi et al. 2002; Aparicio, Viggiani et al. 2004), while deletion of the silent chromatin proteins Sir3 and Ku advanced the replication timing of certain origins (Stevenson and Gottschling 1999; Cosgrove, Nieduszynski et al. 2002).

Overall, these data suggested a role for epigenetic control of origin firing. To date, however, the chromatin events regulating the initiation of DNA replication are not well understood. Histone acetylation seems to be a clear regulator of origin activation in some cases, but it is neither the sole nor a necessary determinant of origin firing (Prioleau, Gendron et al. 2003). The methylation status of histone H3 also seems to be an important molecular event during the initiation of DNA replication (Stedman, Deng et al. 2004; Zhou, Chau et al. 2005), but it alone is not sufficient to dictate the spatial and temporal regulation of chromosomal domains (Wu, Terry et al. 2005). It is, therefore, likely that multiple histone modifications collaborate to specify the replication program observed *in vivo*. This hypothesis is supported by evidence demonstrating that a number of chromatin modifying enzymes associate with the DNA polymerase processivity factor PCNA (Groth, Corpet et al. 2007; Moldovan, Pfander et al. 2007), which are rapidly accumulating (Esteve, Chin et al. 2006; Jorgensen, Elvers et al. 2007).

In the present study we examined the chromatin structure of three replication origins, as evidenced by post-translational modifications (PTMs) of histone H3. Previous

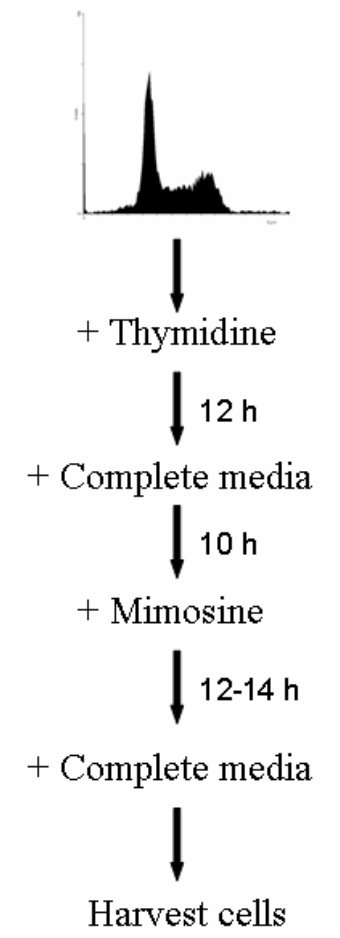
studies with regards to epigenetic control of replication origins have provided significant data (Prioleau, Gendron et al. 2003; Stedman, Deng et al. 2004; Zhou, Chau et al. 2005), but were limited because of their static nature, looking at origin chromatin structure in cells that were asynchronous or synchronized at the G_1 and G_2/M phases of the cell cycle. Here, we examined real-time chromatin changes, which permit the identification of dynamic, reversible histone modifications within initiation sites. The data show that late-firing origins have a compact chromatin structure during S phase, which opens only transiently during their activation and reverts rapidly to its initial status. The origin-specific increase in acetylated histone H3 at K9 and K14 as well as methylated histone H3 at K4, but not in adjacent non-origin containing chromosomal regions during origin firing, suggests the spatiotemporal-specific targeting of H3 acetylases and H3 K4 methylases in late-firing origins. In contrast, early-firing origins display histone modifications that are associated with open chromatin structure, which is only passively affected during their activation by passage of the replication fork. Finally, transient H3 PTM fluctuations in the levels of phosphorylated H3 at serine 10 (monkey replication origin), methylated H3 at K9 and acetylated H3 at K9 and K14 (early-firing origins) were observed, which were associated with both origin- and non-origin containing regions, suggesting that they are related to histone maturation during fork progression. Overall, the data indicate that dynamic changes in the chromatin structure occur during origin activation and underscore the need for a finer identification of chromatin modifiers that transiently interact with replication origins in order to elucidate their regulation.

RESULTS AND DISCUSSION

Replication timing of the lamin B2, hors8 and mors8 replication origins

To evaluate the chromatin structure during origin activation two different cell lines were used, the human cervical cancer HeLa cell line and the Green African Monkey kidney fibroblast CV-1 cell line. The cells were synchronized to G_1/S by treatment of the cells with thymidine and mimosine and subsequent released into S phase by addition of complete media, as shown in Fig. 1A. Mimosine is an inhibitor of the serine hydroxymethyltransferase (SHMT) expression (Lin, Falchetto et al. 1996; Oppenheim, Nasrallah et al. 2000; Perry, Sastry et al. 2005), which is involved in the conversion of serine to glycine and 5,6,7,8-tetrahydrofolate, used in purine production during synthesis

A.



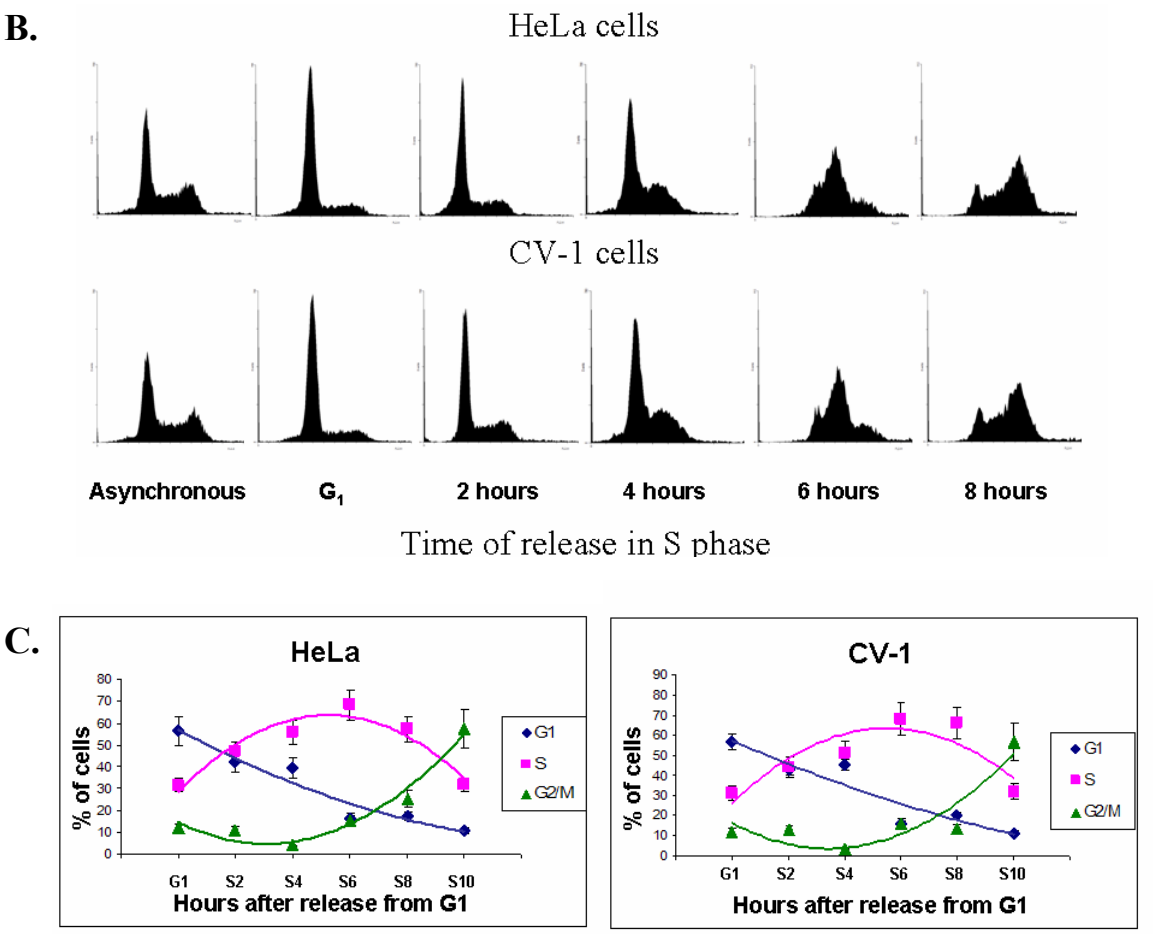


Figure 1: Cell cycle synchronization and release in S phase. (A) Synchronization protocol used for G_1/S blockage. For synchronization to late G_1 phase, asynchronous HeLa and CV-1 cells, cultured in alpha minimum essential medium (α-MEM) (Invitrogen, Carlsad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), penicillin (100 µg/ml), streptomycin (100 µg/ml) and 1 mmol/L L-glutamine (complete medium) at 37^0C and 5% CO_2, were subjected to a double thymidine/mimosine block as previously described (Sibani, Price et al. 2005). In brief, cells were cultured in complete medium in the presence of 2mM thymidine (Sigma, St.Louis, MO) for 12 hours (h), released for 10 h in pre-warmed complete medium without thymidine, and then incubated for 12-14 h in complete medium containing 400uM mimosine (Sigma). For S phase synchronization, the cells were released from the G_1/S block into pre-warmed complete medium and harvested every 2 hours in order to be used for downstream applications. **(B) Cell cycle progression of HeLa and CV-1 cells following G_1/S release.** Representative FACS analyses of asynchronous, G_1/S and S phase cells. Both cell lines traverse through S phase with the same kinetics, entering G_2/M after 10 hours. Cells were fixed in 70% ice-cold ethanol, washed twice with ice-cold phosphate buffered saline (PBS) and resuspended in Vindelov's solution (3.4 mM Tris, 75µM Propidium Iodide, 0.1% NP-40, 700 U/L RNase A (Roche), 0.01 M NaCl) (Vindelov 1977) overnight at 4^oC. Analysis was performed using a Beckman flow cytometer and the WinMDI program. **(C) Quantification of the cell cycle distribution of HeLa and CV-1 cells during S phase progression.** Values are expressed as percentages of cells in each phase and the error bars are equivalent to 1 standard deviation (s.d).

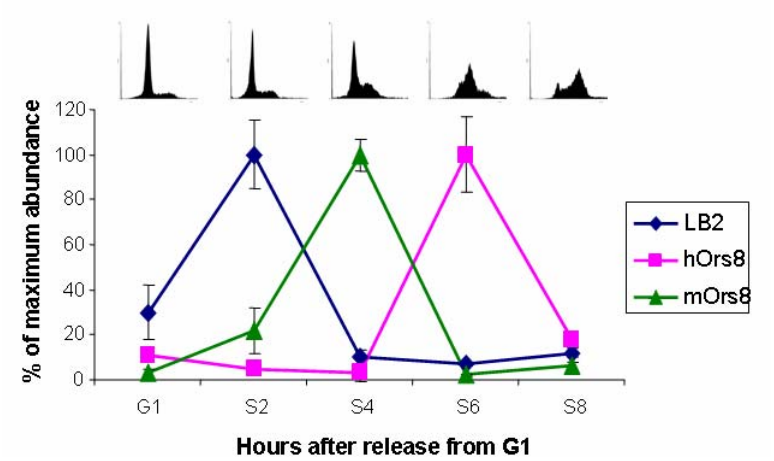


Figure 2: Origin activation as a function of S phase progression. Histogram plots of the LB2, hors8 and mors8 origin activities throughout S phase, as measured by nascent-DNA-strand abundance. Values are expressed as % percentage of the maximum nascent-DNA-strand abundance at each locus and represent three experiments ± 1 s.d. Cell synchronization was monitored by FACS (upper panel). Isolation of nascent strand DNA was performed using the λ exonuclease method as previously described (Giacca, Pelizon et al. 1997; Tao, Dong et al. 2000). Briefly, at the indicated time points following release from late G_1 synchronization, the cells were washed twice with PBS and lysed in Hirt's Lysis buffer (50 mM Tris-HCl, pH 8.0, 0.6 M NaCl, 1 mM EDTA and 0.5% SDS) (Hirt 1967). Following a 10 minute incubation at room temperature, the lysate was digested overnight with 0.1 mg/ml Proteinase K at 65^0C, while nucleic acids were extracted by the standard phenol/chloroform method and sheared by passage through a 26G3/8 needle. Twenty micrograms (μg) of DNA were denatured at 100^oC for 5 minutes, phosphorylated with 10 U of T4-polynucleotide kinase (New England Biolabs) for 30 minutes at 37^oC and digested with λ exonuclease (NEB) overnight at 37^oC. In order to separate the nascent DNA from Okazaki fragments, the samples were subjected to electrophoresis on a 2% agarose gel, the DNA was visualized by staining with 0.01% (w/v) methylene blue (Sigma), and the origin-containing nascent DNA ranging between 350-1000 bp in size, was excised from the gel and purified using the QiaExII extraction kit (QIAGEN, Valencia, CA), as per the manufacturer's instructions. DNA was eluted with dH_2O and quantified by Real-Time PCR, using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals), as per the manufacturer's instructions. The sequences and amplification conditions for all primer sets are listed in Table 1. Non-replicating genomic DNA from serum-starved HeLa cells was included in each reaction set to create a standard curve necessary for the quantification of the PCR products. A negative control without template DNA was also included with each set of reactions. The PCR products were resolved by electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualized with an Eagle Eye apparatus (Speed Light/BT Sciencetech-LT1000).

of RNA primers in DNA replication (Rosenblatt and Fenton 2001). Following this treatment both the HeLa and CV-1 cells were synchronized at the G_1/S transition and upon release they traversed through S phase in 8 hours (Fig.1B), entering G_2/M in 10 hours (Fig.1C). The bell-shaped distribution of the cells in S-phase indicates a good cell cycle progression of both cell lines upon release from G_1/S (Fig.1C).

The ors8 replication origin, originally isolated from early-S-phase monkey kidney (CV-1) cells (mors8) has been shown to replicate early in those cells (Kaufmann, Zannis-Hadjopoulos et al. 1985; Zannis-Hadjopoulos, Frappier et al. 1988), but its human homologue (hors8) replicates late in S phase (Callejo, Sibani et al. 2006), while the lamin B2 (LB2) origin is early-firing in human cells (Biamonti, Giacca et al. 1992). To verify the temporal activation of these origins in our system we measured the nascent DNA strand abundance at these chromosomal regions as a function of S phase progression. In agreement with previous studies the LB2 origin was found to be activated early (within the first two hours upon S phase entry), while the human ors8 (hors8) fired late in S phase (six hours upon S phase entry), by comparison to its monkey counterpart (mors8), whose maximal activation occurred within the first half of S phase (with four hours upon S phase entry) (Fig.2).

Chromatin structure of human replication origins during activation

Origin activation involves loading of the pre-RC during late-M and G_1 phases followed by the replication machinery during S phase. Histone modifications can affect the chromatin accessibility to various DNA-binding factors, hence representing an important barrier to the initiation of replication. The role of chromatin structure with

regards to pre-RC assembly has been extensively studied (Aggarwal and Calvi 2004; Stedman, Deng et al. 2004; Zhou, Chau et al. 2005; Crampton, Chang et al. 2008). To investigate the chromatin dynamics that regulate the recruitment of the replicative polymerases during S phase, we used a chromatin immunoprecipitation (ChIP) assay, using antibodies targeted against acetylated histone H3 at K9+K14 (Ac K9+K14), methylated H3 at K4 (Me K4), methylated H3 at K9 (Me K9), and phosphorylated H3 at Serine 10 (Phospho-S10). Chromatin was immunoprecipitated and the levels of origin-DNA were determined by real-time PCR. In order to also identify reversible histone modifications we looked at real-time chromatin changes by performing ChIP at three different time points per origin, before, during and after their activation. The results show that the early-firing LB2 origin is associated with high levels of the "activating" PTMs, H3 Ac K9+K14 and H3 Me K4, but low levels of the "silencing" H3 Me K9 before activation, indicating an overall open chromatin structure (Fig.3A, bar set LB2 BA). The late-firing hors8 origin on the other hand retained a more compact conformation until its activation, as evidenced by low levels of Ac K9+K14 and Me K4 and increased Me K9 levels (Fig.3B, bar set hors8 BA). Upon firing, both origins were subject to transient chromatin remodeling. Thus, during its activation, LB2 became enriched in H3 Ac K9+K14 and H3 Me K4 and began reverting to its initial status two hours later, with an associated increase in H3 Me K9 (Fig.3A, bar sets LB2 A and AA). A similar oscillation in the H3 Ac K9+K14 and H3 Me K4 levels was observed during hors8 firing, which was also accompanied by a temporary decrease in H3 K9 methylation (Fig.3B, bar sets hors8 A and AA). Notably, none of the two origins were found to be associated with H3

Phospho-S10 (Fig. 3A and 3B, white bars), suggesting that H3 phosphorylation is not a critical determinant of the initiation of DNA replication.

The chromatin structure at two non-origin containing chromosomal regions, the first located 4kb upstream of the LB2 origin (LB2C) and the second 2kb downstream of the hors8 origin (hors8+2kb), was also examined to analyze whether the observed dynamic epigenetic changes were origin-specific or not, rather than being due to propagation of the chromatin organization during replication fork passage (Groth, Corpet et al. 2007). As shown in Fig.3A, LB2C undergoes similar epigenetic alterations as the LB2 origin during origin activation, namely a transient increase in H3 Ac K9+K14 and Me K4 levels followed by an increase in H3 Me K9 post activation. The similarity of the chromatin dynamics at the LB2 and LB2C regions can be explained by the maturation mechanism that takes place during epigenetic maintenance, which occurs at the replication fork; progression of the replisome through the high-order chromosomal structure involves: i) the transient disruption of nucleosomes ahead of the replication fork and their transfer onto nascent DNA (parental histone segregation), and ii) the transfer of newly synthesized histones that participate in the assembly of new nucleosomes (*de novo* histone deposition). Previous studies suggest that newly replicated chromatin contains a range of H3 and H4 post-translational modifications indicative of transcriptional activity (Jackson, Shires et al. 1976; Allis, Chicoine et al. 1985; Benson, Gu et al. 2006), which are removed during chromatin maturation (Annunziato and Seale 1983), but lack modifications that correlate with heterochromatic silencing (Benson, Gu et al. 2006). In agreement, our results indicate that newly replicated DNA is packaged with histones that are enriched in H3 Ac K9+K14 and Me K4 but poor in H3 Me K9. Considering that the

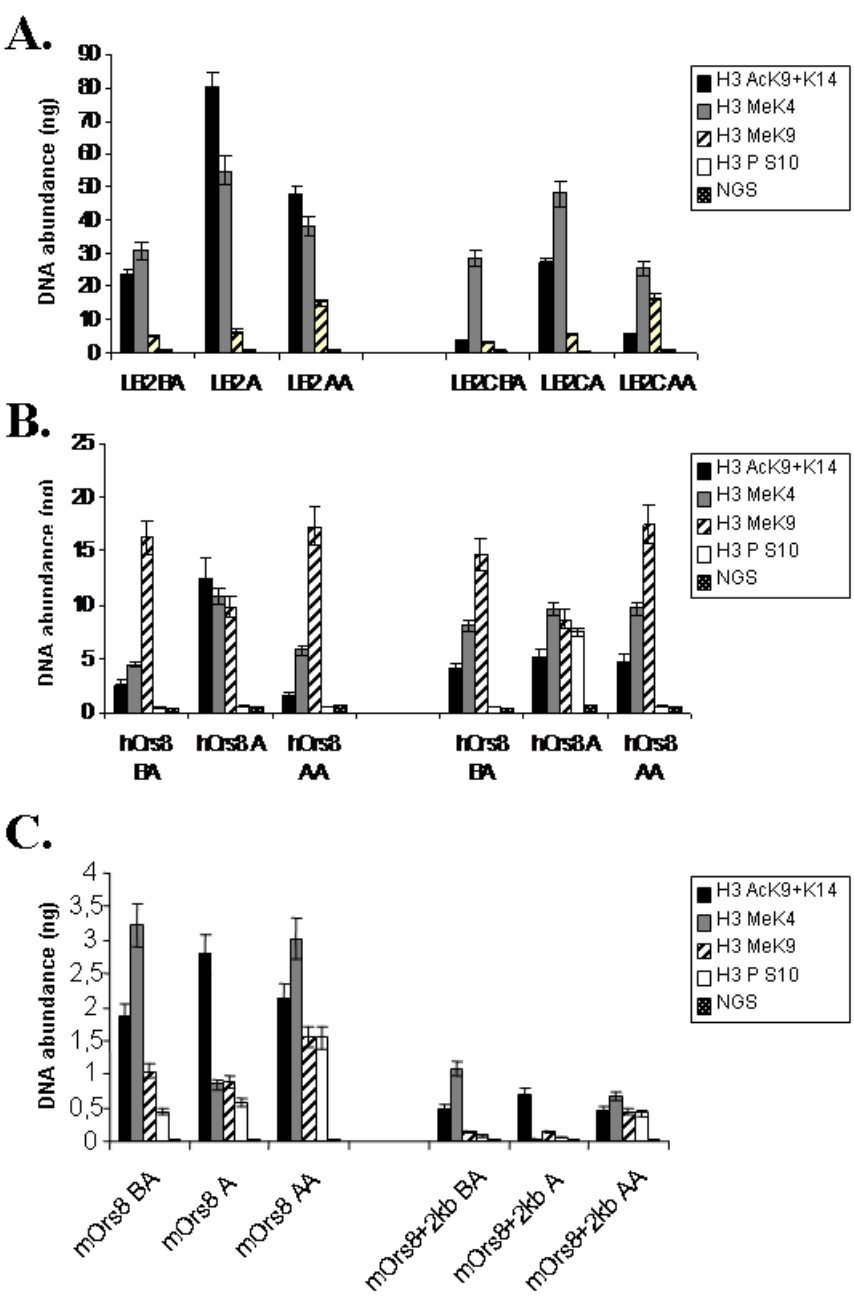


Figure 3. Chromatin immunoprecipitation assay of post-translationally modified histone H3 during origin activation. Abundance of DNA immunoprecipitated with anti-acetylated histone 3 (K9+K14) (black bars), anti-tri-methylated histone 3 (K9) (grey bars), anti-tri-methylated histone 3 (K4) (striped bars), anti-phosphorylated histone 3 (S10) (white bars) or pre-immune serum (checked bars) at the LB2 (A), hors8 (B) and mors8 (C) in HeLa and CV-1 cells is shown. For each immunoprecipitate, the abundance of the origin-containing (LB2, hors8 and mors8) and origin-lacking (LB2C, hors8+2kb and mors8+2kb) regions was determined. Normal goat serum (NGS) was used as a negative control. Each bar represents the average of at least three experiments and 1 s.d. For the ChIPs, sheared chromatin lysates from $2x10^7$ cells were pre-cleared by incubation with 50 µl of Protein G or Protein A agarose (Roche Molecular Biochemicals) to reduce background DNA precipitation caused by nonspecific binding to the beads, as previously described (Sibani, Price et al. 2005). Pre-cleared lysates were incubated overnight with either 20 µg of anti-acetylated histone 3 (K9+K14) (Upstate; 06-599), or anti-tri-methylated histone 3 (K9) (Upstate; 07-442), anti-tri-methylated histone 3 (K4) (Upstate; 07-473), anti-phosphorylated histone 3 (S10) (Upstate; 05-817) or pre-immune serum with constant shaking. Protein G or A was added and incubated at 4°C for one hour. The pelleted beads were washed successively with 1 ml Lysis Buffer for 15 minutes at 4°C, followed by 1 ml of WB1 (50mM Tris-HCl pH 7.5, 500mM NaCl, 0.1% NP40, 0.05% sodium deoxycholate, complete protease inhibitors tablet (Roche Molecular Biochemicals)), 1ml of WB2 (50mM Tris-HCl pH 7.5, 0.1% NP40, 0.05% sodium deoxycholate, complete protease inhibitors tablet) and 1 ml sterile TE lacking any protease inhibitors. The beads were subsequently resuspended in 200 µl TE/1% SDS, incubated at room temperature for 15 minutes and centrifuged at 1000g for 1 minute. Half of the supernatant was then incubated overnight at 65°C to reverse the crosslinks, followed by digestion with 100 µg of Proteinase K at 55°C for two hours. The DNA was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA), and eluted in 100µl 10 mM Tris-HCl (pH 8.0). The remaining half of the supernatant was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

mean speed of replication fork progression is approximately 1 kb/min (Daboussi, Courbet et al. 2008), it would require 5 minutes to transmit the nascent-chromatin epigenetic marks from the LB2 origin to the non-origin-containing LB2C region, which would explain the reason why these marks resemble each other at both regions. In contrast, the non-origin-containing hors8+2kb region resembles the hors8 origin only in the H3 Me K9 levels, while the "activating" PTMs do not follow the same pattern, remaining constant during the course of origin firing (Fig.3B). These findings suggest first, that the "activating" histone marks associated with H3 maturation at the LB2 origin are locus-specific and are established after nucleosome assembly, since they are not observed at the hors8+2kb locus; and second, that late-firing origins maintain a compact chromatin structure during S phase, which transiently opens up during activation by the timely and targeted recruitment of chromatin modifiers in order for the replicative machinery to access the DNA for replication. Following origin firing they rapidly revert to their initial status, obtaining again a closed chromatin structure. We are in favor of a model that involves synthesizing and/or targeting different chromatin regulators at specific time-points during S phase in order to designate origin activation and temporal regulation. Such a model would explain why most heterochromatin is duplicated late in S phase, with certain exceptions (Kim, Dubey et al. 2003; Prioleau, Gendron et al. 2003), while euchromatin is early-replicating (Woodfine, Fiegler et al. 2004; Jeon, Bekiranov et al. 2005).

Comparison between the chromatin dynamics of the hors8 and the mors8

The ors8 locus has been identified as a replication initiation site in both human (Callejo, Sibani et al. 2006) and monkey (Kaufmann, Zannis-Hadjopoulos et al. 1985) cells. Interestingly, although mors8 replicates in the first half of S phase in CV-1 cells, hors8 is late-firing in human cells (Fig.2). We examined whether this phenomenon is associated with differential epigenetic marks imprinted on these respective chromosomal regions in human and monkey cells. Indeed, the results show that the mors8 behaves as an early-firing origin (Fig.3C, left panel), being subject to a transient opening (enrichment in H3 Ac K9+K14) during its activation and compacting thereafter (decrease in H3 Ac K9+K14 and increase in H3 Me K9), due to replication fork progression, as indicated by the same pattern of imprinting at its associated negative (non-origin-containing) region (Fig.3C, right panel). Surprisingly, unlike LB2, the mors8 locus is also subject to a temporary decrease in H3 Me K4 as well as, a post-activation increase in H3 Phospho-S10 at both the origin and the control region, indicating that newly synthesized histones in monkeys are poor in the activating H3 Me K4 mark, as well as, the presence of a H3 kinase activity at the monkey replisome.

Altogether, the data indicate that different epigenetic events take place in early- and late-firing origins, which differ between humans and monkeys. The latter is also evident in the epigenetic programs of HeLa and CV-1 cells during S phase (Fig. 4). With the exception of histone H3 acetylation which remained relatively constant, the levels of all the other histone H3 PTMs fluctuated during S phase, exhibiting different patterns in HeLa (Fig.4A and Fig.4C) and CV-1 cells (Fig.4B and Fig.4D). These data indicate that PTMs are highly regulated throughout S phase and suggest that different chromatin

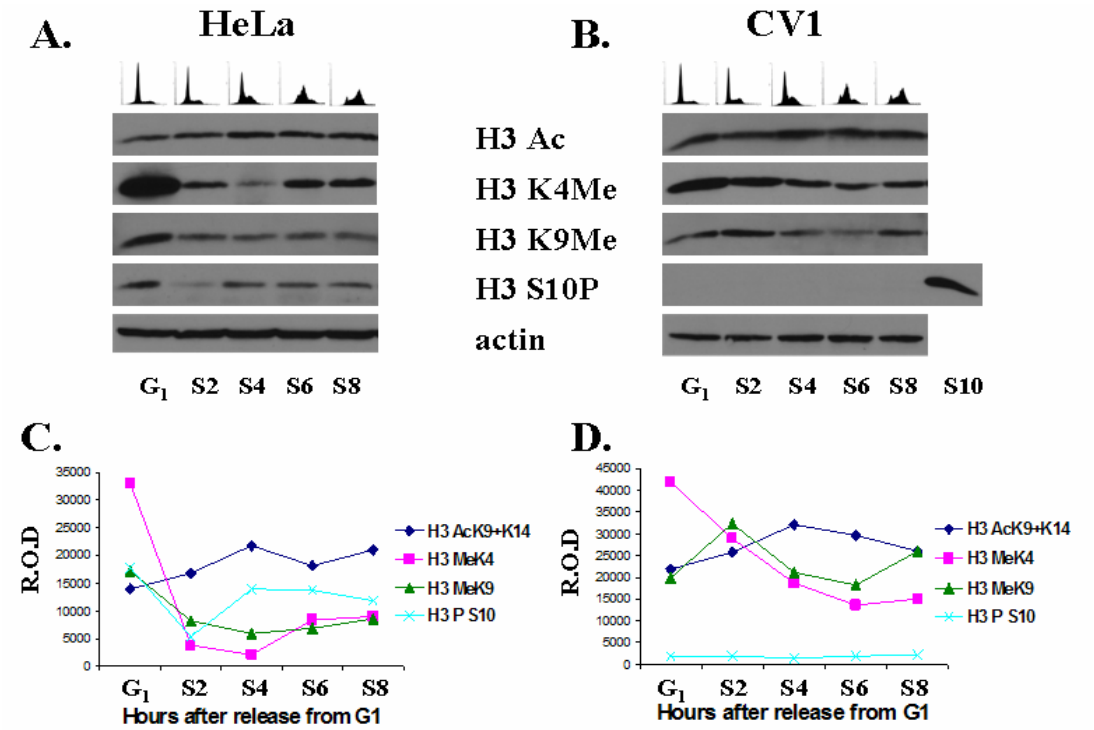


Figure 4. Western blot analysis and quantification of post-translationally modified histone H3 during S phase. Representative Western blot analyses of acetylated histone 3 (K9+K14), tri-methylated histone 3 (K9), tri-methylated histone 3 (K4) and phosphorylated histone 3 (S10) in nuclear extracts prepared from G_1/S synchronized or S phase (S2-S8) Hela (A) and CV-1 (B) cells. Actin was used as a loading control. (C-D) Relative optical densities (R.O.D) of histone H3 Ac K9+K14 (rhombic line), Me K4 (squared line), Me K9 (triangle line) and Phospho-S10 (crossed line) immunoreactivities. Values were normalized with the R.O.D value of the actin band within the actin lane. The experiment was done in duplicate. For histone 3 S10 phosphorylation extracts prepared 10 hours after release from G_1/S were included as positive controls. At this timepoint cells have entered G_2/M and therefore the mitosis-specific Histone 3 serine-10 phosphorylation (Paulson and Taylor 1982) is enhanced. Nuclear cell extracts (NEs) were prepared as previously described (Dignam, Lebovitz et al. 1983) and the protein concentration of each extract preparation was determined using the Bradford Protein Assay (BioRad, Hercules, CA). Western blot analysis was carried out according to standard protocols (Sambrook, Fritsch et al. 1989). Briefly, the indicated amounts of NEs or Chromatin Immunoprecipitates were resuspended in SDS loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), boiled for 10 minutes and loaded on an 8% SDS-PAGE gel. Following electrophoresis, the proteins were transferred onto a PVDF membrane and the membrane was immunoblotted with the indicated primary and corresponding HRP-conjugated secondary antibodies. The following antibodies were used: anti-acetylated histone 3 (K9+K14) (Upstate; 06-599), anti-tri-methylated histone 3 (K9) (Upstate; 07-442), anti-tri-methylated histone 3 (K4) (Upstate; 07-473), anti-phosphorylated histone 3 (S10) (Upstate; 05-817), anti-actin (A 2066; Sigma). Proteins were visualized using the enhanced chemiluminescence (ECL) kit according to the manufacturer's instructions (Amersham Biosciences, Arlington Heights, IL) and the signals were quantified using the ImageJ program.

modifying activities take place at different timepoints. Temporal regulation of both the expression and targeting of specific chromatin modifiers to origins might be responsible for the differential timing of origin activation (Fig. 2) as well as for the dynamic changes in the chromatin structure during S phase, both locally (Fig. 3) and globally (Fig. 4). Identification of the chromatin modifying enzymes that are implicated in origin firing as well as replication fork progression will require further rigorous and fine analyses that will enable detection of transient interactions and enzymatic activities during the cell cycle.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Canadian Institute of Health Research (CIHR). E.R was a recipient of a doctoral studentship from the McGill University Faculty of Medicine, the Rolande and Marcel Gosselin Graduate Studentship, and the Victor KS Lui Fellowship.

Chapter 5:

General Discussion

The heterodimeric Ku protein (Ku70/Ku80) is a highly abundant nuclear protein present in all prokaryotic and eukaryotic organisms, suggesting a conserved function. It was initially purified and studied for its role in DNA end binding and non-homologous end joining (NHEJ), but later was identified to have roles in V(D)J recombination, telomere maintenance, transcriptional regulation and DNA replication (reviewed in chapter 1, section 5.3, pages 39-53). With regard to DNA replication, Ku was first purified as the origin binding activity (OBA) from HeLa cells (Ruiz, Pearson et al. 1995) and was subsequently shown to specifically bind replication origins *in vivo* and *in vitro* using electromobility shift assays (Ruiz, Matheos et al. 1999), DNA footprinting (Schild-Poulter, Matheos et al. 2003) and chromatin immunoprecipitation assays (Sibani, Price et al. 2005). Origin association of Ku was found to be cell-cycle regulated, peaking in G_1 and decreasing thereafter (Novac, Matheos et al. 2001). *In vitro* DNA replication of Ku-immunodepleted extracts was impaired relative to normal extracts (Ruiz, Matheos et al. 1999). This deficiency was rescued by the addition of affinity purified OBA. *In vivo*, Ku deficiency was shown to affect the initiation step of DNA replication resulting in decreased origin usage and delayed G_1/S transition (Sibani, Price et al. 2005) through a mechanism involving the recruitment or stabilization of the ORC (Sibani, Price et al. 2005). However, despite this knowledge, the mechanism by which Ku acts at origins remains unknown.

In this thesis the molecular mechanism of action of Ku in the initiation of DNA replication was examined. The origins I chose to examine here were the well characterized human lamin B2 and Ors8 as well as the monkey homologue of the latter (mOrs8). Ku was found to target topo IIβ onto the hOrs8 replication origin *in vitro* and

form a complex with it both *in vivo* and *in vitro*. Interestingly, PARP-1 was also found to be a member of this complex. A complex of the same composition was previously shown to bind onto the pS2 gene and participate in the recruitment of the transcription machinery and gene activation by modifying the chromatin architecture of its promoter (Ju, Lunyak et al. 2006). I therefore hypothesized that this Ku/topo IIβ/PARP-1 complex plays a similar role during the initiation of DNA replication. To test this hypothesis, cells synchronized in M phase were released into the cell cycle and collected at various timepoints in G_1 phase so as to differentiate between the role of these proteins in replication initiation and chromosome segregation. Applying a protocol that combines DNA-break labeling with biotin-deoxyuridine triphosphate (dUTP) and subsequent ChIP analysis using an anti-biotin antibody, it was indeed found that transient, origin-specific dsDNA breaks occur during G_1 phase. Generation of these DNA breaks in both the lamin B2 and hOrs8 origins was biphasic, occurring during early- and mid-G_1 phase. However, although both breaks at the hOrs8 replication origin occured within the origin core, in the lamin B2 origin only one break was created within the area covered by the pre-RC complex and the other one occurred in close proximity to, but outside the origin region. The full significance of this finding cannot be determined at the present time and it will be interesting to determine whether it is associated with the differential activation timing of the two origins, lamin B2 being early-firing and hOrs8 being late-firing. Using a topoisomerase II-specific inhibitor, DNA-break formation was inhibited, suggesting that the transient DNA-breaks are due to the enzymatic activity of topo II. Since Ku represents the DNA-binding subunit and allosteric activator of the DNA-PK, the effect of the DNA-PK inhibitor NU-7026 on DNA topology was also examined. In contrast to

merbarone, NU-7026 had no effect on DNA-break formation (Fig.3D), suggesting that either Ku only functions in tethering topo IIβ onto replication origins, or that DNA-PK activity is only activated under stress conditions. The next logical question was: what is the physiological significance of these transient DNA-breaks? To address this, the impact of merbarone on pre-RC assembly was determined. Origin association of ORC4, Cdt1 and Cdc45 but not ORC2 was found to be inhibited suggesting that the changes in the DNA topology facilitate pre-RC formation at the level of ORC assembly and Cdt1 and Cdc45 recruitment. Altogether, these results suggest that dynamic changes in the DNA topology of origin-containing chromosomal regions naturally occur during pre-RC assembly and that the Ku-binding and topo IIβ-enzymatic activities are required for replication initiation. The remaining puzzle is exactly how does Ku bind onto origins in a sequence-specific manner? Resolution of the crystal structure of the Ku heterodimer on DNA showed that it is a molecule with dyad symmetry and has a preformed ring that encircles duplex DNA making no contacts with DNA bases (Walker, Corpina et al. 2001). How does it then recognize specific internal sequences? One possible mechanism involves the formation of the Ku heterodimer upon recognition of specific DNA sequences. Ku70 and Ku80 are known to dimerize in the absence of DNA stabilizing each other, however the presence of a small fraction of free Ku70 and Ku80 molecules cannot be excluded, especially since Ku70- and Ku80-independent functions have been reported. Another model involves the extrusion of specific internal sequences to hairpins or cruciforms, which are then recognized by Ku. In support of this, Ku is known to have affinity for similar secondary structures (Dynan and Yoo 1998). Finally, the Ku ring may be somehow topologically linked onto DNA and, upon translocation, pause at specific

DNA sequences. This is a very attractive model for the role of Ku in DNA replication, considering the findings of this thesis. Binding of the Ku/topoIIβ/PARP-1 complex onto DNA cruciforms, known to extrude at many replication origins during their activation, and subsequent transient DNA break formation due to the topo IIβ enzymatic activity provides a window of opportunity for the internalization of the Ku ring. Translocation of the Ku heterodimer and pausing at specific DNA sequences mediated by the SAP domain of the Ku70 carboxyterminus might then lead to replication origin binding (Aravind and Koonin 2000).

In chapter 3, a complementary approach was followed to determine the role of Ku in the initiation of DNA replication. Using RNAi, the level of Ku80 was knocked-down to approximately 10% of its normal expression. This resulted in a significant increase of the doubling time of HeLa cells from 30 hours to 58.8 hours, independently of DNA damage and apoptosis. Instead, the decreased Ku levels resulted in decreased DNA synthesis and impaired origin activation. This was found to be due to impaired chromatin loading of replication factors, in agreement with the findings in chapter 1, where Ku was shown to participate in pre-RC assembly. Interestingly, the prolonged inhibition of the initiation of DNA replication resulted in the activation of a cellular checkpoint, blocking cells in late G1 phase. Checkpoint activation involved inhibition of Cdk2 activity, the Cdk responsible for G_1/S transition, through decreased levels of the Cdc25A phosphatase and the Cdk2 kinase and increased expression of p21. Overall, these data implicate Ku in the initiation of DNA replication through regulation of pre-RC assembly and support the interplay between the DNA replication and cdk-cycle machineries. Similar results showing the activation a G_1/S checkpoint upon acute reduction of the levels of replication

initiator proteins were also reported by other groups as well (Machida, Teer et al. 2005; Lau and Jiang 2006). Currently, characterization of this new cellular checkpoint, which senses the levels of replication initiator proteins and blocks cells in late G_1 phase, so as to prevent chromosomal instability due to insufficient active replication origins, is undergoing. Preliminary data suggest that this replication licensing checkpoint may be deregulated during carcinogenesis, thus representing a tumorigenesis barrier (Feng, Tu et al. 2003; Lau and Jiang 2006).

Another level of regulation of the initiation of eukaryotic DNA replication following pre-RC assembly is the recruitment of the replicative machinery during S phase. DNA replication is biphasic, occurring either early or late during S phase. Accordingly, certain replication origins are early-firing while others are late-firing, a property which has been previously proposed to be dependent on chromatin structure (Aladjem 2007; Jorgensen, Azuara et al. 2007). To date, however, the chromatin events regulating the initiation of DNA replication are not well understood. In chapter 4, the chromatin structure of three replication origins was examined, as evidenced by post-translational modifications (PTMs) of histone H3. For this purpose, chromatin changes were followed in real-time, thus permitting the identification of dynamic, reversible histone modifications within sites of initiation of DNA replication. The data indicated that, as would be expected, late-firing origins have a compact chromatin structure during S phase, which opens only transiently during their activation and reverts rapidly to its initial status. The origin-specific increase in acetylated histone H3 at K9 and K14 as well as methylated histone H3 at K4 suggested the spatiotemporal-specific targeting of H3 acetylases and H3 K4 methylases in late-firing origins. In contrast, early-firing origins

displayed histone modifications that are associated with open chromatin structure (high levels of H3 Ac K9+K14 and H3 Me K4 but low levels of the "silencing" H3 Me K9). Finally, transient H3 PTM fluctuations in the levels of phosphorylated H3 at serine 10 (early-firing monkey replication origin), methylated H3 at K9 and acetylated H3 at K9 and K14 (early-firing origins) were observed, which were associated with both origin- and non-origin containing regions, suggesting that they are related to histone maturation during fork progression. Altogether, the data indicated that distinct epigenetic events take place in early- and late-firing origins, which differ between humans and monkeys, and that temporal regulation of both the expression and targeting of specific chromatin modifiers to origins might be responsible for the differential timing of origin activation.

In conclusion, the work described in this thesis provides novel insights into the mechanism of action of Ku in the initiation of DNA replication, as well as into the regulation of the activation timing of replication origins. Future work addressing the targeting of the Ku/topoIIβ/PARP-1 complex onto replication origins as well as its deregulation during human disease would yield a more comprehensive view of Ku's role in the initiation of DNA replication. Finally, identification of the chromatin modifying enzymes that bind onto replication origins as well as travel with the replication fork will shed light on the epigenetic regulation of DNA replication.

General References

Abdurashidova, G., M. Deganuto, et al. (2000). "Start sites of bidirectional DNA synthesis at the human lamin B2 origin." Science 287(5460): 2023-6.

Abdurashidova, G., S. Radulescu, et al. (2007). "Functional interactions of DNA topoisomerases with a human replication origin." Embo J 26(4): 998-1009.

Agami, R. and R. Bernards (2000). "Distinct initiation and maintenance mechanisms cooperate to induce G1 cell cycle arrest in response to DNA damage." Cell 102(1): 55-66.

Aggarwal, B. D. and B. R. Calvi (2004). "Chromatin regulates origin activity in Drosophila follicle cells." Nature 430(6997): 372-6.

Ahnesorg, P., P. Smith, et al. (2006). "XLF interacts with the XRCC4-DNA ligase IV complex to promote DNA nonhomologous end-joining." Cell 124(2): 301-13.

Aitken, A., H. Baxter, et al. (2002). "Specificity of 14-3-3 isoform dimer interactions and phosphorylation." Biochem Soc Trans 30(4): 351-60.

Aladjem, M. I. (2007). "Replication in context: dynamic regulation of DNA replication patterns in metazoans." Nat Rev Genet 8(8): 588-600.

Allis, C. D., L. G. Chicoine, et al. (1985). "Deposition-related histone acetylation in micronuclei of conjugating Tetrahymena." Proc Natl Acad Sci U S A 82(23): 8048-52.

Alvarez, D., O. Novac, et al. (2002). "14-3-3 sigma is a cruciform DNA binding protein and associates in vivo with origins of DNA replication." J Cell Biochem 87(2): 194-207.

Annunziato, A. T. and R. L. Seale (1983). "Histone deacetylation is required for the maturation of newly replicated chromatin." J Biol Chem 258(20): 12675-84.

Aparicio, J. G., C. J. Viggiani, et al. (2004). "The Rpd3-Sin3 histone deacetylase regulates replication timing and enables intra-S origin control in Saccharomyces cerevisiae." Mol Cell Biol 24(11): 4769-80.

Aparicio, O. M., A. M. Stout, et al. (1999). "Differential assembly of Cdc45p and DNA polymerases at early and late origins of DNA replication." Proc.Natl.Acad.Sci.USA 96: 9130-9135.

Araujo, F. D., J. D. Knox, et al. (1999). "Identification of initiation sites for DNA replication in the human *dnmt1* (DNA-methyltransferase) locus." J.Biol.Chem. 274: 9335-9341.

Aravind, L. and E. V. Koonin (2000). "SAP - a putative DNA-binding motif involved in chromosomal organization." Trends Biochem Sci 25(3): 112-4.

Aravind, L. and E. V. Koonin (2001). "Prokaryotic homologs of the eukaryotic DNA-end-binding protein Ku, novel domains in the Ku protein and prediction of a prokaryotic double-strand break repair system." Genome Res 11(8): 1365-74.

Arosio, D., S. Costantini, et al. (2004). "Fluorescence anisotropy studies on the Ku-DNA interaction: anion and cation effects." J Biol Chem 279(41): 42826-35.

Atanasiu, C., Z. Deng, et al. (2006). "ORC binding to TRF2 stimulates OriP replication." EMBO Rep 7(7): 716-21.

Austin, C. A. and K. L. Marsh (1998). "Eukaryotic DNA topoisomerase IIβ." BioEssays 20: 215-226.

Austin, C. A., J. H. Sng, et al. (1993). "Novel HeLa topoisomerase II is the II beta isoform: complete coding sequence and homology with other type II topoisomerases." Biochim Biophys Acta 1172(3): 283-91.

Austin, R. J., T. L. Orr-Weaver, et al. (1999). "Drosophila ORC specifically binds to ACE3, an origin of DNA replication control element." Genes Dev 13(20): 2639-49.

Barbic, A., D. P. Zimmer, et al. (2003). "Structural origins of adenine-tract bending." Proc Natl Acad Sci U S A 100(5): 2369-73.

Bassing, C. H., W. Swat, et al. (2002). "The mechanism and regulation of chromosomal V(D)J recombination." Cell 109 Suppl: S45-55.

Bell, S. P. and A. Dutta (2002). "DNA replication in eukaryotic cells." Annu Rev Biochem 71: 333-74.

Bell, S. P., R. Kobayashi, et al. (1993). "Yeast origin recognition complex functions in transcription silencing and DNA replication." Science 262: 1844-1849.

Bell, S. P. and B. Stillman (1992). "ATP-dependent recognition of eukaryotic recognition of origins of DNA replication by multiprotein complex." Nature 357: 128-134.

Benson, L. J., Y. Gu, et al. (2006). "Modifications of H3 and H4 during chromatin replication, nucleosome assembly, and histone exchange." J Biol Chem 281(14): 9287-96.

Biamonti, G., M. Giacca, et al. (1992). "The gene for a novel human lamin maps at a highly transcribed locus of chromosome 19 which replicates at the onset of S-phase." Mol Cell Biol 12(8): 3499-506.

Blow, J. J. and A. Dutta (2005). "Preventing re-replication of chromosomal DNA." Nat Rev Mol Cell Biol 6(6): 476-86.

Botchan, M. (2007). "Cell biology: a switch for S phase." Nature 445(7125): 272-4.

Boulikas, T. (1996). "Common structural features of replication origins in all life forms." Journal of Cellular Biochemistry 60: 297-316.

Boulton, S. J. and S. P. Jackson (1996). "*Saccharomyces cerevisiae* Ku70 potentiates illegitimate DNA double-strand break repair and serves as a barrier to error-prone DNA repair pathways." EMBO Journal 15: 5093-5103.

Branzei, D. and M. Foiani (2008). "Regulation of DNA repair throughout the cell cycle." Nat Rev Mol Cell Biol 9(4): 297-308.

Brown, S. W. (1966). "Heterochromatin." Science 151(709): 417-25.

Brylawski, B. P., S. M. Cohen, et al. (2000). "On the relationship of matrix association and DNA replication." Crit Rev Eukaryot Gene Expr 10(1): 91-9.

Burckstummer, T., K. L. Bennett, et al. (2006). "An efficient tandem affinity purification procedure for interaction proteomics in mammalian cells." Nat Methods 3(12): 1013-9.

Burma, S. and D. J. Chen (2004). "Role of DNA-PK in the cellular response to DNA double-strand breaks." DNA Repair (Amst) 3(8-9): 909-18.

Cai, Q. Q., A. Plet, et al. (1994). "Chromosomal location and expression of the genes coding for Ku p70 and p80 in human cell lines and normal tissues." Cytogenet Cell Genet 65(4): 221-7.

Callejo, M., S. Sibani, et al. (2006). "Identification and functional analysis of a human homologue of the monkey replication origin ors8." J Cell Biochem 99(6): 1606-15.

Camara-Clayette, V., D. Thomas, et al. (1999). "The repressor which binds the -75 GATA motif of the GPB promoter contains Ku70 as the DNA binding subunit." Nucleic Acids Res 27(7): 1656-63.

Camara-Clayette, V., D. Thomas, et al. (1999). "The repressor which binds the -75 GATA motif of the GPB promoter contains Ku70 as the DNA binding subunit." Nucleic Acids Res. 27: 1656-1663.

Cao, Q. P., S. Pitt, et al. (1994). "DNA-dependent ATPase from HeLa cells is related to human Ku autoantigen." Biochemistry 33(28): 8548-57.

Carpenter, P. B., P. R. Mueller, et al. (1996). "Role for a *Xenopus* Orc2-related protein in controlling DNA replication." Nature 379: 357-360.

Celli, G. B., E. L. Denchi, et al. (2006). "Ku70 stimulates fusion of dysfunctional telomeres yet protects chromosome ends from homologous recombination." Nat Cell Biol 8(8): 885-90.

Cervantes, R. B. and V. Lundblad (2002). "Mechanisms of chromosome-end protection." Curr Opin Cell Biol 14(3): 351-6.

Chakhparonian, M. and R. J. Wellinger (2003). "Telomere maintenance and DNA replication: how closely are these two connected?" Trends Genet 19(8): 439-46.

Champoux, J. J. (2001). "DNA topoisomerases: structure, function, and mechanism." Annu Rev Biochem 70: 369-413.

Chaturvedi, P., W. K. Eng, et al. (1999). "Mammalian Chk2 is a downstream effector of the ATM-dependent DNA damage checkpoint pathway." Oncogene 18(28): 4047-54.

Chuang, R. Y. and T. J. Kelly (1999). "The fission yeast homologue of Orc4p binds to replication origin DNA via multiple AT-hooks." Proc.Natl.Acad.Sci.USA 96: 2656-2661.

Clifford, B., M. Beljin, et al. (2003). "G2 arrest in response to topoisomerase II inhibitors: the role of p53." Cancer Res 63(14): 4074-81.

Collis, S. J., T. L. DeWeese, et al. (2005). "The life and death of DNA-PK." Oncogene 24(6): 949-61.

Cook, J. G., D. A. Chasse, et al. (2004). "The regulated association of Cdt1 with minichromosome maintenance proteins and Cdc6 in mammalian cells." J Biol Chem 279(10): 9625-33.

Cosgrove, A. J., C. A. Nieduszynski, et al. (2002). "Ku complex controls the replication time of DNA in telomere regions." Genes Dev 16(19): 2485-90.

Costanzo, V., K. Robertson, et al. (2000). "Reconstitution of an ATM-dependent checkpoint that inhibits chromosomal DNA replication following DNA damage [In Process Citation]." Mol Cell 6(3): 649-59.

Coverley, D., H. Laman, et al. (2002). "Distinct roles for cyclins E and A during DNA replication complex assembly and activation." Nat Cell Biol 24: 24.

Crampton, A., F. Chang, et al. (2008). "An ARS element inhibits DNA replication through a SIR2-dependent mechanism." Mol Cell 30(2): 156-66.

Cuvier, O. and T. Hirano (2003). "A role of topoisomerase II in linking DNA replication to chromosome condensation." J Cell Biol 160(5): 645-55.

Cvetic, C. and J. C. Walter (2005). "Eukaryotic origins of DNA replication: could you please be more specific?" Semin Cell Dev Biol 16(3): 343-53.

d'Adda di Fagagna, F., M. P. Hande, et al. (2001). "Effects of DNA nonhomologous end-joining factors on telomere length and chromosomal stability in mammalian cells." Curr Biol 11(15): 1192-6.

Daboussi, F., S. Courbet, et al. (2008). "A homologous recombination defect affects replication-fork progression in mammalian cells." J Cell Sci 121(Pt 2): 162-6.

Davey, M. J., C. Indiani, et al. (2003). "Reconstitution of the Mcm2-7p heterohexamer, subunit arrangement, and ATP site architecture." J Biol Chem 278(7): 4491-9.

Davey, M. J., D. Jeruzalmi, et al. (2002). "Motors and switches: AAA+ machines within the replisome." Nat Rev Mol Cell Biol 3(11): 826-35.

de Lange, T. (2002). "Protection of mammalian telomeres." Oncogene 21(4): 532-40.

de Vries, E., W. van Driel, et al. (1989). "HeLa nuclear protein recognizing DNA termini and translocating on DNA forming a regular DNA-multimeric protein complex." J. Mol. Biol. 208: 65-78.

DePamphilis, M. (1996). Origins of DNA Replication. DNA Replication in Eukayotic Cells. M. DePamphilis. Cold Spring Harbor,NY, Cold Spring Harbor Laboratory Press: 45-86.

DePamphilis, M. L. (1993). "Eukaryotic DNA replication: Anatomy of an origin." Annu. Rev. Biochem. 62: 29-63.

DePamphilis, M. L., J. J. Blow, et al. (2006). "Regulating the licensing of DNA replication origins in metazoa." Curr Opin Cell Biol 18(3): 231-9.

Dhar, S. K. and A. Dutta (2000). "Identification and characterization of the human ORC6 homolog." J Biol Chem 275(45): 34983-8.

Dhar, S. K., K. Yoshida, et al. (2001). "Replication from oriP of Epstein-Barr virus requires human ORC and is inhibited by geminin." Cell 106(3): 287-96.

Difilippantonio, M. J., J. Zhu, et al. (2000). "DNA repair protein Ku80 suppresses chromosomal aberrations and malignant transformation." Nature 404(6777): 510-4.

Dignam, J. D., R. M. Lebovitz, et al. (1983). "Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei." Nucleic Acids Res 11(5): 1475-89.

Djeliova, V., G. Russev, et al. (2001). "Dynamics of association of origins of DNA replication with the nuclear matrix during the cell cycle." Nucleic Acids Res 29(15): 3181-7.

Donaldson, A. D. and J. J. Blow (1999). "The regulation of replication origin activation." Curr.Opin.Genet.Dev. 9: 62-68.

Downes, C. S., D. J. Clarke, et al. (1994). "A topoisomerase II-dependent G2 cycle checkpoint in mammalian cells." Nature 372(6505): 467-70.

Downs, J. A. and S. P. Jackson (2004). "A means to a DNA end: the many roles of Ku." Nat Rev Mol Cell Biol 5(5): 367-78.

Downs, J. A., M. C. Nussenzweig, et al. (2007). "Chromatin dynamics and the preservation of genetic information." Nature 447(7147): 951-8.

Dynan, W. S. and S. Yoo (1998). "Interaction of Ku protein and DNA-dependent protein kinase catalytic subunit with nucleic acids." Nucleic Acids Res. 26: 1551-1559.

Ekholm-Reed, S., J. Mendez, et al. (2004). "Deregulation of cyclin E in human cells interferes with prereplication complex assembly." J Cell Biol 165(6): 789-800.

Espejel, S., S. Franco, et al. (2002). "Mammalian Ku86 mediates chromosomal fusions and apoptosis caused by critically short telomeres." Embo J 21(9): 2207-2219.

Esteve, P. O., H. G. Chin, et al. (2006). "Direct interaction between DNMT1 and G9a coordinates DNA and histone methylation during replication." Genes Dev 20(22): 3089-103.

Falaschi, A., G. Abdurashidova, et al. (2007). "Molecular and structural transactions at human DNA replication origins." Cell Cycle 6(14): 1705-12.

Falck, J., N. Mailand, et al. (2001). "The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis." Nature 410(6830): 842-7.

Falzon, M. and E. L. Kuff (1989). "Isolation and characterization of a protein fraction that binds to enhancer core sequences in intracisternal A-particle long terminal repeats." J Biol Chem 264(36): 21915-22.

Feldmann, H., L. Driller, et al. (1996). "HDF2, the second subunit of the Ku homologue from Saccharomyces cerevisiae." J.Biol.Chem. 271: 27765-27769.

Feng, D., Z. Tu, et al. (2003). "Inhibiting the expression of DNA replication-initiation proteins induces apoptosis in human cancer cells." Cancer Res 63(21): 7356-64.

Finzer, P., C. Kuntzen, et al. (2001). "Inhibitors of histone deacetylase arrest cell cycle and induce apoptosis in cervical carcinoma cells circumventing human papillomavirus oncogene expression." Oncogene 20(35): 4768-76.

Fisher, D. and M. Mechali (2004). "Sleeping policemen for DNA replication?" Nat Cell Biol 6(7): 576-7.

Fosse, P., B. Rene, et al. (1991). "Sequence requirements for mammalian topoisomerase II mediated DNA cleavage stimulated by an ellipticine derivative." Nucleic Acids Res 19(11): 2861-8.

Friedman, K. L., J. D. Diller, et al. (1996). "Multiple determinants controlling activation of yeast replication origins late in S phase." Genes Dev. 10: 1595-1607.

Fu, H., L. Wang, et al. (2006). "Preventing gene silencing with human replicators." Nat Biotechnol 24(5): 572-6.

Gao, Y. J., J. Chaudhuri, et al. (1998). "A targeted DNA-PKcs-null mutation reveals DNA-PK-independent functions for KU in V(D)J recombination." Immunity 9: 367-376.

Gavin, K. A., M. Hidaka, et al. (1995). "Conserved initiator proteins in eukaryotes." Science 270: 1667-1671.

Gell, D. and S. P. Jackson (1999). "Mapping of protein-protein interactions within the DNA-dependent protein kinase complex." Nucleic Acids Res 27(17): 3494-502.

Geng, Y., Y. M. Lee, et al. (2007). "Kinase-independent function of cyclin E." Mol Cell 25(1): 127-39.

Giacca, M., C. Pelizon, et al. (1997). "Mapping replication origins by quantifying relative abundance of nascent DNA strands using competitive polymerase chain reaction." Methods 13(3): 301-12.

Giacca, M., L. Zentilin, et al. (1994). "Fine mapping of a replication origin of human DNA." Proc. Nat'l. Acad. Sci. U.S.A. 91: 7119-7123.

Giffin, W., W. R. Gong, et al. (1999). "Ku antigen-DNA conformation determines the activation of DNA-dependent protein kinase and DNA sequence-directed repression of mouse mammary tumor virus transcription." Mol.Cell.Biol. 19: 4065-4078.

Giffin, W., J. Kwast-Welfeld, et al. (1997). "Sequence-specific DNA binding and transcription factor phosphorylation by Ku autoantigen/DNA-dependent protein kinase." J.Biol.Chem. 272: 5647-5658.

Giffin, W., H. Torrance, et al. (1996). "Sequence-specific DNA binding by Ku autoantigen and its effects on transcription." Nature 380(6571): 265-8.

Gilbert, D. M. (2004). "In search of the holy replicator." Nat Rev Mol Cell Biol 5(10): 848-55.

Gomez-Llorente, Y., R. J. Fletcher, et al. (2005). "Polymorphism and double hexamer structure in the archaeal minichromosome maintenance (MCM) helicase from Methanobacterium thermoautotrophicum." J Biol Chem 280(49): 40909-15.

Gossen, M., D. T. Pak, et al. (1995). "A Drosophila homolog of the yeast origin recognition complex." Science 270(5242): 1674-7.

Grainge, I., M. Bregu, et al. (2007). "Unlinking chromosome catenanes in vivo by site-specific recombination." Embo J 26(19): 4228-38.

Griffith, J. D., L. Comeau, et al. (1999). "Mammalian telomeres end in a large duplex loop." Cell 97(4): 503-14.

Groth, A., A. Corpet, et al. (2007). "Regulation of replication fork progression through histone supply and demand." Science 318(5858): 1928-31.

Gullo, C., M. Au, et al. (2006). "The biology of Ku and its potential oncogenic role in cancer." Biochim Biophys Acta 1765(2): 223-34.

Haber, J. E. (2000). "Partners and pathwaysrepairing a double-strand break." Trends Genet 16(6): 259-64.

Halmer, L., B. Vestner, et al. (1998). "Involvement of topoisomerases in the initiation of simian virus 40 minichromosome replication." J.Biol.Chem. 273: 34792-34798.

Halmer, L., B. Vestner, et al. (1998). "Involvement of topoisomerases in the initiation of simian virus 40 minichromosome replication." J Biol Chem 273(52): 34792-8.

Hande, P., P. Slijepcevic, et al. (1999). "Elongated telomeres in scid mice." Genomics 56(2): 221-3.

Harris, R., D. Esposito, et al. (2004). "The 3D solution structure of the C-terminal region of Ku86 (Ku86CTR)." J Mol Biol 335(2): 573-82.

Heck, M. M., W. N. Hittelman, et al. (1988). "Differential expression of DNA topoisomerases I and II during the eukaryotic cell cycle." Proc Natl Acad Sci U S A 85(4): 1086-90.

Hiasa, H. and K. J. Marians (1994). "Topoisomerase IV can support oriC DNA replication in vitro." J Biol Chem 269(23): 16371-5.

Hirt, B. (1967). "Selective extraction of polyoma DNA from infected mouse cell cultures." J Mol Biol 26(2): 365-9.

Hofmann, J. F. and D. Beach (1994). "cdt1 is an essential target of the Cdc10/Sct1 transcription factor: requirement for DNA replication and inhibition of mitosis." Embo J 13(2): 425-34.

Hu, Y., R. V. Clower, et al. (2006). "Cellular topoisomerase I modulates origin binding by bovine papillomavirus type 1 E1." J Virol 80(9): 4363-71.

Hwang, H. C. and B. E. Clurman (2005). "Cyclin E in normal and neoplastic cell cycles." Oncogene 24(17): 2776-86.

Hyrien, O., C. Maric, et al. (1995). "Transition in specification of embryonic metazoan DNA replication origins." Science 270(5238): 994-7.

Hyrien, O. and M. Mechali (1993). "Chromosomal replication initiates and terminates at random sequences but at regular intervals in the ribosomal DNA of Xenopus early embryos." Embo J 12(12): 4511-20.

Ishimi, Y., Y. Komamura-Kohno, et al. (2000). "Inhibition of Mcm4,6,7 helicase activity by phosphorylation with cyclin A/Cdk2." J Biol Chem 275(21): 16235-41.

Jackson, V., A. Shires, et al. (1976). "Modifications to histones immediately after synthesis." J Mol Biol 104(2): 471-83.

Jeanson, L. and J. F. Mouscadet (2001). "Ku represses the HIV-1 transcription: Identification of a putative Ku binding site homologous to the MMTV NRE1 sequence in the HIV-1 LTR." J Biol Chem 30: 30.

Jeanson, L. and J. F. Mouscadet (2002). "Ku represses the HIV-1 transcription: identification of a putative Ku binding site homologous to the mouse mammary tumor virus NRE1 sequence in the HIV-1 long terminal repeat." J Biol Chem 277(7): 4918-24.

Jeggo, P. A., M. Hafezparast, et al. (1992). "Localization of a DNA repair gene (XRCC5) involved in double-strand-break rejoining to human chromosome 2." Proc Natl Acad Sci U S A 89(14): 6423-7.

Jenkins, J. R., P. Ayton, et al. (1992). "Isolation of cDNA clones encoding the beta isozyme of human DNA topoisomerase II and localisation of the gene to chromosome 3p24." Nucleic Acids Res 20(21): 5587-92.

Jeon, Y., S. Bekiranov, et al. (2005). "Temporal profile of replication of human chromosomes." Proc Natl Acad Sci U S A 102(18): 6419-24.

Jorgensen, H. F., V. Azuara, et al. (2007). "The impact of chromatin modifiers on the timing of locus replication in mouse embryonic stem cells." Genome Biol 8(8): R169.

Jorgensen, S., I. Elvers, et al. (2007). "The histone methyltransferase SET8 is required for S-phase progression." J Cell Biol 179(7): 1337-45.

Ju, B. G., V. V. Lunyak, et al. (2006). "A topoisomerase IIbeta-mediated dsDNA break required for regulated transcription." Science 312(5781): 1798-802.

Ju, B. G., D. Solum, et al. (2004). "Activating the PARP-1 sensor component of the groucho/ TLE1 corepressor complex mediates a CaMKinase IIdelta-dependent neurogenic gene activation pathway." Cell 119(6): 815-29.

Kastan, M. B. and J. Bartek (2004). "Cell-cycle checkpoints and cancer." Nature 432(7015): 316-23.

Kaufmann, G., M. Zannis-Hadjopoulos, et al. (1985). "Cloning of nascent monkey DNA synthesized early in the cell cycle." Mol. Cell. Biol. 5: 721-727.

Kawanishi, M. (1993). "Topoisomerase I and II activities are required for Epstein-Barr virus replication." J Gen Virol 74 (Pt 10): 2263-8.

Kelly, T. J. and G. W. Brown (2000). "Regulation of chromosome replication." Annu Rev Biochem 69: 829-80.

Kemp, M. G., M. Ghosh, et al. (2005). "The histone deacetylase inhibitor trichostatin A alters the pattern of DNA replication origin activity in human cells." Nucleic Acids Res 33(1): 325-36.

Kim, D., H. Ouyang, et al. (1995). "A constitutive heat shock element-binding factor is immunologically identical to the Ku autoantigen." J Biol Chem 270(25): 15277-84.

Kim, M. Y., S. Mauro, et al. (2004). "NAD+-dependent modulation of chromatin structure and transcription by nucleosome binding properties of PARP-1." Cell 119(6): 803-14.

Kim, S. M., D. D. Dubey, et al. (2003). "Early-replicating heterochromatin." Genes Dev 17(3): 330-5.

Kimura, K., M. Saijo, et al. (1994). "Growth state- and cell cycle-dependent fluctuation in the expression of two forms of DNA topoisomerase II and possible specific modification of the higher molecular weight form in the M phase." J Biol Chem 269(2): 1173-6.

Knuth, M. W., S. I. Gunderson, et al. (1990). "Purification and characterization of proximal sequence element-binding protein 1, a transcription activating protein related to Ku and TREF that binds the proximal sequence element of the human U1 promoter." J Biol Chem 265(29): 17911-20.

Kodym, R. and E. Horth (1995). "Determination of radiation-induced DNA strand breaks in individual cells by non-radioactive labelling of 3' OH ends." Int J Radiat Biol 68(2): 133-9.

Koike, M. (2002). "Dimerization, translocation and localization of Ku70 and Ku80 proteins." J Radiat Res (Tokyo) 43(3): 223-36.

Koike, M., T. Awaji, et al. (1999a). "Differential subcellular localization of DNA-dependent protein kinase components Ku and DNA-PKcs during mitosis." J Cell Sci 112(Pt 22): 4031-9.

Kuhn, A., T. M. Gottlieb, et al. (1995). "DNA-dependent protein kinase: a potent inhibitor of transcription by RNA polymerase I." Genes Dev 9(2): 193-203.

Kulartz, M., S. Kreitz, et al. (2003). "Expression and phosphorylation of the replication regulator protein geminin." Biochem Biophys Res Commun 305(2): 412-20.

Kurimasa, A., H. Ouyang, et al. (1999). "Catalytic subunit of DNA-dependent protein kinase: impact on lymphocyte development and tumorigenesis." Proc Natl Acad Sci U S A 96(4): 1403-8.

Lallev, A., B. Anachkova, et al. (1993). "Effect of ionizing radiation and topoisomerase II inhibitors on DNA synthesis in mammalian cells." Eur J Biochem 216(1): 177-81.

Laroche, T., S. G. Martin, et al. (1998). "Mutation of yeast Ku genes disrupts the subnuclear organization of telomeres." Curr.Biol. 8: 653-656.

Laskey, R. A. and M. A. Madine (2003). "A rotary pumping model for helicase function of MCM proteins at a distance from replication forks." EMBO Rep 4(1): 26-30.

Lau, E. and W. Jiang (2006). "Is there a pre-RC checkpoint that cancer cells lack?" Cell Cycle 5(15): 1602-6.

Lehman, J. A., D. J. Hoelz, et al. (2008). "DNA-dependent conformational changes in the Ku heterodimer." Biochemistry 47(15): 4359-68.

Li, G., C. Nelsen, et al. (2002). "Ku86 is essential in human somatic cells." Proc Natl Acad Sci U S A 99(2): 832-7.

Li, L. L. and N. H. Yeh (1992). "Cell cycle-dependent migration of the DNA-binding protein Ku80 into nucleoli." Exp Cell Res 199(2): 262-8.

Lienard, P., C. De Mees, et al. (2006). "Regulation of the alpha-fetoprotein promoter: Ku binding and DNA spatial conformation." Biochimie 88(10): 1409-17.

Lin, C. M., H. Fu, et al. (2003). "Dynamic alterations of replication timing in mammalian cells." Curr Biol 13(12): 1019-28.

Lin, H. B., R. Falchetto, et al. (1996). "Mimosine targets serine hydroxymethyltransferase." J Biol Chem 271(5): 2548-56.

Lis, J. T. and W. L. Kraus (2006). "Promoter cleavage: a topoIIbeta and PARP-1 collaboration." Cell 125(7): 1225-7.

Liu, D., J. Bienkowska, et al. (1995). "Crystal structure of the zeta isoform of the 14-3-3 protein." Nature 376: 191-194.

Ma, Y., U. Pannicke, et al. (2002). "Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination." Cell 108(6): 781-94.

Machida, Y. J. and A. Dutta (2005). "Cellular checkpoint mechanisms monitoring proper initiation of DNA replication." J Biol Chem 280(8): 6253-6.

Machida, Y. J., J. L. Hamlin, et al. (2005). "Right place, right time, and only once: replication initiation in metazoans." Cell 123(1): 13-24.

Machida, Y. J., J. K. Teer, et al. (2005). "Acute reduction of an origin recognition complex (ORC) subunit in human cells reveals a requirement of ORC for Cdk2 activation." J Biol Chem 280(30): 27624-30.

Macleod, K. F., N. Sherry, et al. (1995). "p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage." Genes Dev 9(8): 935-44.

Mahajan, K. N., S. A. Nick McElhinny, et al. (2002). "Association of DNA polymerase mu (pol mu) with Ku and ligase IV: role for pol mu in end-joining double-strand break repair." Mol Cell Biol 22(14): 5194-202.

Malkas, L., R. Hickey, et al. (1990). "A 21S enzyme complex from HeLa cells that functions in simian virus 40 DNA replication in vitro." Biochemistry 29: 6362-6374.

Maric, C., B. Levacher, et al. (1999). "Developmental regulation of replication fork pausing in Xenopus laevis ribosomal RNA genes." J Mol Biol 291(4): 775-88.

Masai, H., C. Taniyama, et al. (2006). "Phosphorylation of MCM4 by Cdc7 kinase facilitates its interaction with Cdc45 on the chromatin." J Biol Chem 281(51): 39249-61.

Massague, J. (2004). "G1 cell-cycle control and cancer." Nature 432(7015): 298-306.

Matheos, D., O. Novac, et al. (2003). "Analysis of the DNA replication competence of the xrs-5 mutant cells defective in Ku86." J Cell Sci 116(Pt 1): 111-24.

Matheos, D., M. T. Ruiz, et al. (2002). "Ku antigen, an origin-specific binding protein that associates with replication proteins, is required for mammalian DNA replication." Biochim Biophys Acta 1578(1-3): 59.

Matsuoka, S., M. Huang, et al. (1998). "Linkage of ATM to cell cycle regulation by the Chk2 protein kinase." Science 282(5395): 1893-7.

McBlane, J. F., D. C. Van Gent, et al. (1995). "Cleavage at a V(D)J recombination signal requires only RAG1 and RAG2 proteins and occurs in two steps." Cell 83: 387-395.

McClendon, A. K., A. C. Rodriguez, et al. (2005). "Human topoisomerase IIalpha rapidly relaxes positively supercoiled DNA: implications for enzyme action ahead of replication forks." J Biol Chem 280(47): 39337-45.

McGarry, T. J. and M. W. Kirschner (1998). "Geminin, an inhibitor of DNA replication, is degraded during mitosis." Cell 93(6): 1043-53.

Mendez, J. and B. Stillman (2000). "Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis [In Process Citation]." Mol Cell Biol 20(22): 8602-12.

Mikhailov, A., M. Shinohara, et al. (2004). "Topoisomerase II and histone deacetylase inhibitors delay the G2/M transition by triggering the p38 MAPK checkpoint pathway." J Cell Biol 166(4): 517-26.

Mimori, T., M. Akizuki, et al. (1981). "Characterization of a high molecular weight acidic nuclear protein recognized by autoantibodies in sera from patients with polymyositis-scleroderma overlap." J Clin Invest 68(3): 611-20.

Mimori, T. and J. A. Hardin (1986). "Mechanism of interaction between Ku protein and DNA." J Biol Chem 261(22): 10375-9.

Minami, H., J. Takahashi, et al. (2006). "Binding of AlF-C, an Orc1-binding transcriptional regulator, enhances replicator activity of the rat aldolase B origin." Mol Cell Biol 26(23): 8770-80.

Mo, X. and W. S. Dynan (2002). "Subnuclear Localization of Ku Protein: Functional Association with RNA Polymerase II Elongation Sites." Mol Cell Biol 22(22): 8088-99.

Moldovan, G. L., B. Pfander, et al. (2007). "PCNA, the maestro of the replication fork." Cell 129(4): 665-79.

Morgan, D. O. (1995). "Principles of CDK regulation." Nature 374(6518): 131-4.

Moyer, S. E., P. W. Lewis, et al. (2006). "Isolation of the Cdc45/Mcm2-7/GINS (CMG) complex, a candidate for the eukaryotic DNA replication fork helicase." Proc Natl Acad Sci U S A 103(27): 10236-41.

Myung, K., G. Ghosh, et al. (2004). "Regulation of telomere length and suppression of genomic instability in human somatic cells by Ku86." Mol Cell Biol 24(11): 5050-9.

Narayana, A., N. Khodarev, et al. (1998). "Synchronous block in DNA synthesis initiation with change in chromatin topology mediated by VP16." DNA Cell Biol 17(7): 613-9.

Natale, D. A., A. E. Schubert, et al. (1992). "DNA helical stability accounts for mutational defects in a yeast replication origin." Proc. Nat'l Acad. Sci. USA 89: 2654-2658.

Nieduszynski, C. A., Y. Knox, et al. (2006). "Genome-wide identification of replication origins in yeast by comparative genomics." Genes Dev 20(14): 1874-9.

Nishitani, H., Z. Lygerou, et al. (2000). "The Cdt1 protein is required to license DNA for replication in fission yeast [see comments]." Nature 404(6778): 625-8.

Novac, O., D. Matheos, et al. (2001). "In vivo association of Ku with mammalian origins of DNA replication." Mol Biol Cell 12(11): 3386-401.

Nussenzweig, A., C. H. Chen, et al. (1996). "Requirement for Ku80 in growth and immunoglobulin V(D)J recombination." Nature 382: 551-555.

Ochem, A. E., D. Skopac, et al. (1997). "Functional properties of the separate subunits of human DNA helicase II/Ku autoantigen." J. Biol. Chem. 272: 29919-29926.

Okumura, K., S. Takagi, et al. (1994). "Autoantigen Ku protein is involved in DNA binding proteins which recognize the U5 repressive element of human T-cell leukemia virus type I long terminal repeat." FEBS Lett 356(1): 94-100.

Oppenheim, E. W., I. M. Nasrallah, et al. (2000). "Mimosine is a cell-specific antagonist of folate metabolism." J Biol Chem 275(25): 19268-74.

Pacek, M. and J. C. Walter (2004). "A requirement for MCM7 and Cdc45 in chromosome unwinding during eukaryotic DNA replication." Embo J 23(18): 3667-76.

Pagano, M., R. Pepperkok, et al. (1993). "Regulation of the cell cycle by the cdk2 protein kinase in cultured human fibroblasts." J Cell Biol 121(1): 101-11.

Pagano, M., R. Pepperkok, et al. (1992). "Cyclin A is required at two points in the human cell cycle." Embo J 11(3): 961-71.

Paillard, S. and F. Strauss (1991). "Analysis of the mechanism of interaction of simian Ku protein with DNA." Nucleic Acids Res 19(20): 5619-24.

Pappas, D. L., Jr., R. Frisch, et al. (2004). "The NAD+-dependent Sir2p histone deacetylase is a negative regulator of chromosomal DNA replication." Genes Dev 18(7): 769-81.

Park, S. J., S. L. Ciccone, et al. (2004). "A positive role for the Ku complex in DNA replication following strand break damage in mammals." J Biol Chem 279(7): 6046-55.

Pasero, P., A. Bensimon, et al. (2002). "Single-molecule analysis reveals clustering and epigenetic regulation of replication origins at the yeast rDNA locus." Genes Dev 16(19): 2479-84.

Patel, S. S. and K. M. Picha (2000). "Structure and function of hexameric helicases." Annu Rev Biochem 69: 651-97.

Paulson, J. R. and S. S. Taylor (1982). "Phosphorylation of histones 1 and 3 and nonhistone high mobility group 14 by an endogenous kinase in HeLa metaphase chromosomes." J Biol Chem 257(11): 6064-72.

Pearson, C. E., M. Ruiz, et al. (1994). "Cruciform DNA binding protein in HeLa cell extracts." Biochemistry 33: 14185-14196.

Pearson, C. E., M. Zannis-Hadjopoulos, et al. (1995). "A novel type of interaction between cruciform DNA and a cruciform binding protein from HeLa cells." EMBO Journal 14: 1571-1580.

Pearson, C. E., H. Zorbas, et al. (1996). "Inverted repeats, stem-loops, and cruciforms: Significance for initiation of DNA replication." J. Cell. Biochem. 63: 1-22.

Perkins, G. and J. F. Diffley (1998). "Nucleotide-dependent prereplicative complex assembly by Cdc6p, a homolog of eukaryotic and prokaryotic clamp-loaders." Mol Cell 2(1): 23-32.

Perry, C., R. Sastry, et al. (2005). "Mimosine attenuates serine hydroxymethyltransferase transcription by chelating zinc. Implications for inhibition of DNA replication." J Biol Chem 280(1): 396-400.

Pflumm, M. F. and M. R. Botchan (2001). "Orc mutants arrest in metaphase with abnormally condensed chromosomes." Development 128(9): 1697-707.

Prasanth, S. G., K. V. Prasanth, et al. (2002). "Orc6 involved in DNA replication, chromosome segregation, and cytokinesis." Science 297(5583): 1026-31.

Price, G. B., M. Allarakhia, et al. (2003). "Identification of a cis-element that determines autonomous DNA replication in eukaryotic cells." J Biol Chem 278(May 30): 19649-19659.

Prioleau, M. N., M. C. Gendron, et al. (2003). "Replication of the chicken beta-globin locus: early-firing origins at the 5' HS4 insulator and the rho- and betaA-globin genes show opposite epigenetic modifications." Mol Cell Biol 23(10): 3536-49.

Puntervoll, P., R. Linding, et al. (2003). "ELM server: A new resource for investigating short functional sites in modular eukaryotic proteins." Nucleic Acids Res 31(13): 3625-30.

Purugganan, M. M., S. Shah, et al. (2001). "Ku80 is required for addition of N nucleotides to V(D)J recombination junctions by terminal deoxynucleotidyl transferase." Nucleic Acids Res 29(7): 1638-46.

Quinn, L. M., A. Herr, et al. (2001). "The Drosophila Geminin homolog: roles for Geminin in limiting DNA replication, in anaphase and in neurogenesis." Genes Dev 15(20): 2741-54.

Rampakakis, E., D. N. Arvanitis, et al. (2009). "Metazoan origins of DNA replication: Regulation through dynamic chromatin structure." J Cell Biochem.

Rampakakis, E., D. Di Paola, et al. (2008). "Ku is involved in cell growth, DNA replication and G1-S transition." J Cell Sci 121(Pt 5): 590-600.

Randell, J. C., J. L. Bowers, et al. (2006). "Sequential ATP hydrolysis by Cdc6 and ORC directs loading of the Mcm2-7 helicase." Mol Cell 21(1): 29-39.

Raval, P., A. N. Kriatchko, et al. (2008). "Evidence for Ku70/Ku80 association with full-length RAG1." Nucleic Acids Res 36(6): 2060-72.

Remus, D., E. L. Beall, et al. (2004). "DNA topology, not DNA sequence, is a critical determinant for Drosophila ORC-DNA binding." Embo J 23(4): 897-907.

Ribes-Zamora, A., I. Mihalek, et al. (2007). "Distinct faces of the Ku heterodimer mediate DNA repair and telomeric functions." Nat Struct Mol Biol 14(4): 301-7.

Rivera-Calzada, A., J. D. Maman, et al. (2005). "Three-dimensional structure and regulation of the DNA-dependent protein kinase catalytic subunit (DNA-PKcs)." Structure 13(2): 243-55.

Rivera-Calzada, A., L. Spagnolo, et al. (2007). "Structural model of full-length human Ku70-Ku80 heterodimer and its recognition of DNA and DNA-PKcs." EMBO Rep 8(1): 56-62.

Rogakou, E. P., D. R. Pilch, et al. (1998). "DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139." J Biol Chem 273(10): 5858-68.

Rowles, A., J. P. J. Chong, et al. (1996). "Interaction between the origin recognition complex and the replication licensing system in Xenopus." Cell 87: 287-296.

Roy, R., B. Meier, et al. (2004). "Separation-of-function mutants of yeast Ku80 reveal a Yku80p-Sir4p interaction involved in telomeric silencing." J Biol Chem 279(1): 86-94.

Ruiz, M. T., D. Matheos, et al. (1999). "OBA/Ku86: DNA binding specificity and involvement in mammalian DNA replication." Mol.Biol.Cell 10: 567-580.

Ruiz, M. T., D. Matheos, et al. (1999). "OBA/Ku86: DNA binding specificity and involvement in mammalian DNA replication." Mol Biol Cell 10(3): 567-80.

Ruiz, M. T., C. E. Pearson, et al. (1995). "Cofractionation of HeLa cell replication proteins with *ors*-binding activity." Journal of Cellular Biochemistry 58: 221-236.

Sadji, Z., M. Le Romancer, et al. (2000). "Human colon carcinoma cell-line HCT116 transfected by antisense cDNA as a tool to study the Ku86 involvement in cell proliferation." Cell Signal 12(11-12): 745-50.

Sagata, N. (2002). "Molecular biology. Untangling checkpoints." Science 298(5600): 1905-7.

Sakaguchi, A. and A. Kikuchi (2004). "Functional compatibility between isoform alpha and beta of type II DNA topoisomerase." J Cell Sci 117(Pt 7): 1047-54.

Sambrook, J., E. F. Fritsch, et al. (1989). Molecular Cloning, Cold Spring Harbor Laboratory Press.

Sasaki, T. and D. M. Gilbert (2007). "The many faces of the origin recognition complex." Curr Opin Cell Biol 19(3): 337-43.

Sasaki, T., T. Sawado, et al. (1999). "Specification of regions of DNA replication initiation during embryogenesis in the 65-kilobase DNApolalpha-dE2F locus of Drosophila melanogaster." Mol Cell Biol 19(1): 547-55.

Sato, N., K. Arai, et al. (1997). "Human and Xenopus cDNAs encoding budding yeast Cdc7-related kinases: in vitro phosphorylation of MCM subunits by a putative human homologue of Cdc7." Embo J 16(14): 4340-51.

Sawchuk, D. J., J. Mansilla-Soto, et al. (2004). "Ku70/Ku80 and DNA-dependent protein kinase catalytic subunit modulate RAG-mediated cleavage: implications for the enforcement of the 12/23 rule." J Biol Chem 279(28): 29821-31.

Saxena, S. and A. Dutta (2005). "Geminin-Cdt1 balance is critical for genetic stability." Mutat Res 569(1-2): 111-21.

Scheffner, M., K. Munger, et al. (1991). "The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines." Proc Natl Acad Sci U S A 88(13): 5523-7.

Schepers, A., M. Ritzi, et al. (2001). "Human origin recognition complex binds to the region of the latent origin of DNA replication of Epstein-Barr virus." Embo J 20(16): 4588-602.

Schild-Poulter, C., D. Matheos, et al. (2003). "Differential DNA binding of ku antigen determines its involvement in DNA replication." DNA Cell Biol 22(2): 65-78.

Schvartzman, J. B. and A. Stasiak (2004). "A topological view of the replicon." EMBO Rep 5(3): 256-61.

Sclafani, R. A. (2000). "Cdc7p-Dbf4p becomes famous in the cell cycle." J Cell Sci 113 (Pt 12): 2111-7.

Sclafani, R. A. and T. M. Holzen (2007). "Cell cycle regulation of DNA replication." Annu Rev Genet 41: 237-80.

Shakibai, N., V. Kumar, et al. (1996). "The Ku-like protein from *Saccharomyces cerevisiae* is required *in vitro* for the assembly of a stable multiprotein complex at a eukaryotic origin of replication." Proc.Natl.Acad.Sci.USA 93: 11569-11574.

Sherr, C. J. (1994). "G1 phase progression: cycling on cue." Cell 79(4): 551-5.

Shi, L., D. Qiu, et al. (2007). "Dynamic binding of Ku80, Ku70 and NF90 to the IL-2 promoter in vivo in activated T-cells." Nucleic Acids Res 35(7): 2302-10.

Shimura, T., M. M. Martin, et al. (2007). "DNA-PK is involved in repairing a transient surge of DNA breaks induced by deceleration of DNA replication." J Mol Biol 367(3): 665-80.

Shinomiya, T. and S. Ina (1991). "Analysis of chromosomal replicons in early embryos of Drosophila melanogaster by two-dimensional gel electrophoresis." Nucleic Acids Res 19(14): 3935-41.

Sibani, S., G. B. Price, et al. (2005). "Decreased origin usage and initiation of DNA replication in haploinsufficient HCT116 Ku80+/- cells." J Cell Sci 118(Pt 15): 3247-61.

Sibani, S., G. B. Price, et al. (2005). "Ku80 binds to human replication origins prior to the assembly of the ORC complex." Biochemistry 44(21): 7885-96.

Simmons, D. T., D. Gai, et al. (2004). "Assembly of the replication initiation complex on SV40 origin DNA." Nucleic Acids Res 32(3): 1103-12.

Soulas-Sprauel, P., P. Rivera-Munoz, et al. (2007). "V(D)J and immunoglobulin class switch recombinations: a paradigm to study the regulation of DNA end-joining." Oncogene 26(56): 7780-91.

Spagnolo, L., A. Rivera-Calzada, et al. (2006). "Three-dimensional structure of the human DNA-PKcs/Ku70/Ku80 complex assembled on DNA and its implications for DNA DSB repair." Mol Cell 22(4): 511-9.

Stedman, W., Z. Deng, et al. (2004). "ORC, MCM, and histone hyperacetylation at the Kaposi's sarcoma-associated herpesvirus latent replication origin." J Virol 78(22): 12566-75.

Stellwagen, A. E., Z. W. Haimberger, et al. (2003). "Ku interacts with telomerase RNA to promote telomere addition at native and broken chromosome ends." Genes Dev 17(19): 2384-95.

Stevenson, J. B. and D. E. Gottschling (1999). "Telomeric chromatin modulates replication timing near chromosome ends." Genes Dev. 13: 146-151.

Sun, H., J. K. Karow, et al. (1998). "The Bloom's syndrome helicase unwinds G4 DNA." J Biol Chem 273(42): 27587-92.

Taccioli, G. E., T. M. Gottlieb, et al. (1994). "Ku80: product of the XRCC5 gene and its role in DNA repair and V(D)J recombination." Science 265(5177): 1442-5.

Taghva, A., Y. Ma, et al. (2002). "Analysis of the kinetic and equilibrium binding of Ku protein to DNA." J Theor Biol 214(1): 85-97.

Takeda, D. Y. and A. Dutta (2005). "DNA replication and progression through S phase." Oncogene 24(17): 2827-43.

Takeda, D. Y., Y. Shibata, et al. (2005). "Recruitment of ORC or CDC6 to DNA is sufficient to create an artificial origin of replication in mammalian cells." Genes Dev 19(23): 2827-36.

Tanaka, S. and J. F. Diffley (2002). "Deregulated G1-cyclin expression induces genomic instability by preventing efficient pre-RC formation." Genes Dev 16(20): 2639-49.

Tao, L., Z. Dong, et al. (2000). "Major DNA replication initiation sites in the c-myc locus in human cells." J Cell Biochem 78(3): 442-457.

Tatsumi, Y., T. Tsurimoto, et al. (2000). "Association of human origin recognition complex 1 with chromatin DNA and nuclease-resistant nuclear structures." J Biol Chem 275(8): 5904-10.

Teo, S. H. and S. P. Jackson (2001). "Telomerase subunit overexpression suppresses telomere-specific checkpoint activation in the yeast yku80 mutant." EMBO Rep 2(3): 197-202.

Thomae, A. W., D. Pich, et al. (2008). "Interaction between HMGA1a and the origin recognition complex creates site-specific replication origins." Proc Natl Acad Sci U S A 105(5): 1692-7.

Ting, N. S., Y. Yu, et al. (2005). "Human Ku70/80 interacts directly with hTR, the RNA component of human telomerase." Nucleic Acids Res 33(7): 2090-8.

Todd, A., N. Cossons, et al. (1998). "Human cruciform binding protein belongs to the 14-3-3 family." Biochemistry 37: 14317-14325.

Todd, A., S. Landry, et al. (1995). "Deletion analysis of minimal sequence requirements for autonomous replication of *ors8*, a monkey early-replicating DNA sequence." Journal of Cellular Biochemistry 57: 280-289.

Torrance, H., W. Giffin, et al. (1998). "Sequence-specific binding of Ku autoantigen to single-stranded DNA." J.Biol.Chem. 273: 20810-20819.

Toth, E. C., L. Marusic, et al. (1993). "Interactions of USF and Ku antigen with a human DNA region containing a replication origin." Nucl. Acids Res. 21: 3257-3263.

Trowbridge, P. W., R. Roy, et al. (1999). "Human topoisomerase I promotes initiation of simian virus 40 DNA replication in vitro." Mol.Cell.Biol. 19: 1686-1694.

Tsai, L. H., E. Lees, et al. (1993). "The cdk2 kinase is required for the G1-to-S transition in mammalian cells." Oncogene 8(6): 1593-602.

Tsurimoto, T., T. Melendy, et al. (1990). "Sequential initiation of lagging and leading strand synthesis by two different polymerase complexes at the SV40 DNA replication origin." Nature 346(6284): 534-9.

Tsuyama, T., S. Tada, et al. (2005). "Licensing for DNA replication requires a strict sequential assembly of Cdc6 and Cdt1 onto chromatin in Xenopus egg extracts." Nucleic Acids Res 33(2): 765-75.

Tuteja, N., R. Rahman, et al. (1994). "Human DNA helicase II: a novel DNA unwinding enzyme identified as the autoantigen." EMBO J. 13: 4991-5001.

Tuteja, R. and N. Tuteja (2000). "Ku autoantigen: a multifunctional DNA binding protein." Crit. Rev. Biochem. Mol. Biol. 35: 1-33.

Uematsu, N., E. Weterings, et al. (2007). "Autophosphorylation of DNA-PKCS regulates its dynamics at DNA double-strand breaks." J Cell Biol 177(2): 219-29.

van den Heuvel, S. and E. Harlow (1993). "Distinct roles for cyclin-dependent kinases in cell cycle control." Science 262(5142): 2050-4.

Van Gent, D. C., J. F. McBlane, et al. (1995). "Initiation of V(D)J recombination in a cell-free system." Cell 81: 925-934.

van Steensel, B., A. Smogorzewska, et al. (1998). "TRF2 protects human telomeres from end-to-end fusions." Cell 92(3): 401-13.

Vashee, S., P. Simancek, et al. (2001). "Assembly of the human origin recognition complex." J Biol Chem 276(28): 26666-73.

Verdun, R. E. and J. Karlseder (2007). "Replication and protection of telomeres." Nature 447(7147): 924-31.

Vindelov, L. L. (1977). "Flow microfluorometric analysis of nuclear DNA in cells from solid tumors and cell suspensions. A new method for rapid isolation and straining of nuclei." Virchows Arch B Cell Pathol 24(3): 227-42.

Vishwanatha, J. K. and E. F. Baril (1990). "Single-stranded DNA-dependent ATPase from HeLa cells that stimulates DNA polymerase α-primase activity: purification and characterization of the ATPase." Biochemistry 29: 8753-8759.

Vogelauer, M., L. Rubbi, et al. (2002). "Histone acetylation regulates the time of replication origin firing." Mol Cell 10(5): 1223-33.

Walker, J. R., R. A. Corpina, et al. (2001). "Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair." Nature 412(6847): 607-14.

Wallisch, M., E. Kunkel, et al. (2002). "Ku antigen supports termination of mammalian rDNA replication by transcription termination factor TTF-I." Biol Chem 383(5): 765-71.

Wang, H., R. Fang, et al. (2004). "Positive and negative modulation of the transcriptional activity of the ETS factor ESE-1 through interaction with p300, CREB-binding protein, and Ku 70/86." J Biol Chem 279(24): 25241-50.

Wang, Y., H. Li, et al. (2008). "Kaposi's sarcoma-associated herpesvirus ori-Lyt-dependent DNA replication: involvement of host cellular factors." J Virol 82(6): 2867-82.

Wang, Y., H. Li, et al. (2008). "Kaposi's Sarcoma-Associated Herpesvirus ori-Lyt-dependent DNA Replication: Involvement of Host Cellular Factors in the Replication." J Virol.

Ward, G. K., R. McKenzie, et al. (1990). "Dynamic distribution and quantification of DNA cruciforms in eukaryotic nuclei." Exp. Cell Res. 188: 235-246.

Ward, G. K., A. Shihab-El-Deen, et al. (1991). "DNA cruciforms and the nuclear supporting structure." Exp. Cell Res. 195: 92-98.

West, R. B., M. Yaneva, et al. (1998). "Productive and nonproductive complexes of Ku and DNA-dependent protein kinase at DNA termini." Mol.Cell.Biol. 18: 5908-5920.

West, S. C. (2003). "Molecular views of recombination proteins and their control." Nat Rev Mol Cell Biol 4(6): 435-45.

Weterings, E. and D. J. Chen (2007). "DNA-dependent protein kinase in nonhomologous end joining: a lock with multiple keys?" J Cell Biol 179(2): 183-6.

Weterings, E., N. S. Verkaik, et al. (2003). "The role of DNA dependent protein kinase in synapsis of DNA ends." Nucleic Acids Res 31(24): 7238-46.

Williams, D. L. and D. Kowalski (1993). "Easily unwound DNA sequences and hairpin structures in the Epstein-Barr virus origin of plasmid replication." J Virol 67(5): 2707-15.

Woo, R. A. and R. Y. Poon (2003). "Cyclin-dependent kinases and S phase control in mammalian cells." Cell Cycle 2(4): 316-24.

Woodfine, K., H. Fiegler, et al. (2004). "Replication timing of the human genome." Hum Mol Genet 13(2): 191-202.

Wu, R., A. V. Terry, et al. (2005). "Differential subnuclear localization and replication timing of histone H3 lysine 9 methylation states." Mol Biol Cell 16(6): 2872-81.

Wu, X. T. and M. R. Lieber (1996). "Protein-protein and protein-DNA interaction regions within the DNA end-binding protein Ku70-Ku86." Molecular & Cellular Biology 16: 5186-5193.

Yabuta, N., N. Kajimura, et al. (2003). "Mammalian Mcm2/4/6/7 complex forms a toroidal structure." Genes Cells 8(5): 413-21.

Zannis-Hadjopoulos, M., L. Frappier, et al. (1988). "Effect of anti-cruciform DNA monoclonal antibodies on DNA replication." EMBO J. 7: 1837-1844.

Zannis-Hadjopoulos, M. and G. B. Price (1999). "Eukaryotic DNA replication." J. Cell. Biochem. Supplements 32/33: 1-14.

Zannis-Hadjopoulos, M., S. Sibani, et al. (2004). "Eukaryotic Replication Origin Binding Proteins." Frontiers in Bioscience 9: 2133-2143.

Zannis-Hadjopoulos, M., W. Yahyaoui, et al. (2008). "14-3-3 cruciform-binding proteins as regulators of eukaryotic DNA replication." Trends Biochem Sci 33(1): 44-50.

Zhang, H. and J. Tower (2004). "Sequence requirements for function of the Drosophila chorion gene locus ACE3 replicator and ori-beta origin elements." Development 131(9): 2089-99.

Zhang, Z., W. Hu, et al. (2004). "Solution structure of the C-terminal domain of Ku80 suggests important sites for protein-protein interactions." Structure 12(3): 495-502.

Zhong, W., H. Feng, et al. (2003). "CUL-4 ubiquitin ligase maintains genome stability by restraining DNA-replication licensing." Nature 423(6942): 885-9.

Zhou, J., C. M. Chau, et al. (2005). "Cell cycle regulation of chromatin at an origin of DNA replication." Embo J 24(7): 1406-17.

Zhou, X. Y., X. Wang, et al. (2002). "Ku affects the ATM-dependent S phase checkpoint following ionizing radiation." Oncogene 21(41): 6377-81.

Publications by the author (Emmanouil Rampakakis)

Rampakakis, E. and Zannis-Hadjopoulos, M. Transient dsDNA breaks during pre-RC assembly. PNAS (Under Review).

Rampakakis, E., Di Paola, D., Chan, M.K., Zannis-Hadjopoulos, M. Dynamic changes in the chromatin structure during replication origin activation. Journal of Molecular Biology (Under Review).

Di Paola, D., **Rampakakis, E.,** Chan, M.K., Arvanitis, D., Zannis-Hadjopoulos, M. Searching for tumor-specific origins. Nucleic Acids Research (Under Review).

Rampakakis, E.*, Arvanitis, D.*, Di Paola, D., Zannis-Hadjopoulos, M. (2009). Metazoan Origins of DNA Replication: Regulation through dynamic chromatin structure. Journal of Cellular Biochemistry (Epub ahead of print) (*equal authorship).

Rampakakis, E., Di Paola, D., Zannis-Hadjopoulos, M. (2008). Ku is involved in cell growth, DNA replication and G1-S transition. J Cell Sci. 2008 Mar 1; 121(Pt 5):590-600.

Rampakakis, E.*, Sibani, S.*, Di Paola, D., Zannis-Hadjopoulos, M. (2008). Fine mapping and functional activity of the adenosine deaminase origin in murine embryonic fibroblasts. J Cell Biochem. 2008 Jun 1; 104(3):773-784 (*equal authorship).

Abstracts by the author

Zannis-Hadjopoulos, M. and **Rampakakis, E.** Inhibition of Ku80 leads to DNA replication shock and cell-cycle progression block. Weizmann Conference, Jerusalem, Israel, October, 2008.

Zannis-Hadjopoulos, M., **Rampakakis, E.,** Di Paola, D. Initiation of DNA replication and carcinogenesis. McGill Oncology Research Retreat, Montreal, Canada, March 2008.

Rampakakis, E., Di Paola, D., Zannis-Hadjopoulos, M. Inhibition of Ku80 leads to DNA replication shock and cell-cycle progression block. 10th International Symposium on Molecular Medicine, Hersonissos – Crete, Greece, October 2007.

Rampakakis, E., Di Paola, D., Zannis-Hadjopoulos, M. Ku80 is critical for origin firing and Cyclin/Cdk cycle progression. Cold Spring Harbor Meeting on Eukaryotic DNA Replication and Genome Maintenance, N.Y, USA, September 2007.

Di Paola, D., **Rampakakis, E.,** Zannis-Hadjopoulos, M. Searching for a tumor-specific origin. Cold Spring Harbor Meeting on Eukaryotic DNA Replication and Genome Maintenance, N.Y, USA, September 2007.

Rampakakis, E., Vassilacopoulou, D., Vassiliou, AG., Fragoulis, EG. Identification of neuronal type L-Dopa decarboxylase mRNA in human whole blood. 55th meeting of the Hellenic Society of Biochemistry and Molecular Biology, Athens, Greece, 2003.