GEMC1, a Novel Factor Required for Chromosomal DNA replication

By

Alessia Balestrini

A thesis submitted for the degree of Ph.D.
at
The University of London
May 2009

Cancer Research UK
London Research Institute
Clare Hall Laboratories
South Mimms
Herts EN6 3LD

and

Department of Biochemistry
University College London
WC1E 6BT
I, Alessia Balestrini, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
“Oh God thy sea is so great and my boat is so small”

Breton Fisherman's Prayer
In eukaryotic cells DNA replication begins from multiple origins. During the process of initiation, the DNA replication fork is established at each origin. In lower eukaryotes many factors required for chromosomal DNA replication have been identified. However, the regulation of DNA replication in complex multi-cellular organisms is still poorly understood.

In this thesis I report the identification of GEMC1 (GEMinin Coiled-coil containing protein 1), a novel vertebrate factor belonging to a new protein family required to initiate chromosomal DNA replication. GEMC1 contains a domain similar to Geminin, a fundamental regulator of DNA replication (McGarry and Kirschner, 1998). GEMC1 is highly conserved in vertebrates and is preferentially expressed in proliferating cells. I show that *Xenopus* GEMC1 (XlGEMC1) binds the checkpoint and replication factor TopBP1, which promotes XlGEMC1 binding to chromatin during pre-replication complex (pre-RC) assembly. Moreover, I demonstrate that XlGEMC1 directly interacts with the replication factors Cdc45 and Cdk2/CyclinE by which it is heavily phosphorylated. Phosphorylated XlGEMC1 stimulates initiation of DNA replication. Inhibition of XlGEMC1 function with XlGEMC1 neutralizing antibodies prevents DNA replication onset by blocking Cdc45 loading onto chromatin.

Inhibition of XlGEMC1 expression by morpholino antisense oligos is lethal for embryonic development. Furthermore, down-regulation of mouse GEMC1 (mGEMC1) expression by siRNA (small interfering RNA) oligos prevents initiation of DNA replication in somatic vertebrate cells. Data presented in this thesis suggest that GEMC1 promotes initiation of chromosomal DNA replication in higher eukaryotes by mediating TopBP1 and Cdk2 dependent Cdc45 recruitment onto replication origins.
Acknowledgements

I would first like to give special thanks to my supervisor Vincenzo for guidance and support. Additionally I would like to thank both Dale Wigley and Svend Petersen-Mahrt for their help as thesis committee members.

I would also like to acknowledge the excellent CRUK services that have helped make this work possible; LRI Central Cell Service (Trevor in particular), peptide synthesis, and sequencing services.

Finally, I would like to thank all those at Clare Hall and Costanzo lab members past and present. Special thanks to Alessia, Babe and Liz for constructive criticism of this thesis and EndNote expertise.
# Table of Contents

Abstract 4  
Acknowledgements 5  
Table of contents 6  
Table of figures 11  
Table of tables 13  
List Of Abbreviations 14  

1 Introduction 17  

1.1 The Eukaryotic Cell Cycle 18  

1.2 Replication Origins and Control of Initiation of DNA Replication 18  

1.2.1 Initiation of DNA replication in bacteria: the replicon model 18  
1.2.2 Initiation of DNA replication in *Simian virus* (SV40) 21  
1.2.3 Replication origins in yeast *Saccharomyces cerevisiae* 22  
1.2.4 Origins in fission yeast *Schizosaccharomyces pombe* 24  
1.2.5 Sequence independent replication initiation in higher eukaryotes 24  

1.2.5.1 *Insights from Xenopus laevis embryonic system* 25  
1.2.5.2 *Replication origin in mammalian cells* 26  

1.3 DNA Replication Licencing 28  

1.3.1 The Origin Recognition Complex (ORC) 28  
1.3.2 Cdc6 protein (Cell Division Cycle 6) 29  
1.3.3 Cdt1 (Chromatin licensing and DNA replication factor 1) 30  
1.3.4 MCM2-7 complex (mini-chromosome maintenance 2-7) 31  

1.4 Factors Involved in the Activation of Pre-replication Complex 33  

1.4.1 Control of DNA replication initiation through two protein kinases 33  

1.4.1.1 DDK (*Dbf4* dependent kinase) 33  
1.4.1.2 CDKs (*Cyclin*-Dependent Kinases) 34  
1.4.2 MCM10 (mini-chromosome maintenance 10) 36  
1.4.3 Dpb11/Cut5/TopBP1 and its interactors 37
1.4.4 Sld2 and Sld3

1.4.5 GINS complex (Go, Ichi, Nii, San; five, one, two and three in Japanese)

1.4.6 Cdc45 (cell division cycle 45-like)

1.5 How Do Eukaryotic Cells Ensure Once per Cell Cycle DNA Replication?

1.5.1 Phosphorylation of ORC

1.5.2 Cdc6 undergoes dual regulation

1.5.3 MCM2-7 complex regulation is CDK dependent

1.5.4 Cdt1 regulation and Geminin

1.6 DNA Damage Response to Maintain Genome Integrity

1.6.1 Sensing DNA damage

1.6.2 Transmission of the checkpoint signal

1.6.3 ATM-Dependent checkpoint

1.6.3.1 p53 dependent pathway

1.6.3.2 p53 independent pathway

1.6.4 ATR dependent checkpoint

1.7 ATM and ATR Control the Selection and Activation of DNA Replication Origin Firing

1.8 Conclusions

1.9 Overview of the Work Described in this Thesis

2 Materials and Methods

2.1 Chemicals and reagents

2.1.1 Suppliers of reagents

2.1.2 Suppliers of commonly used reagents are listed below

2.1.3 Bacterial media and general solutions

2.2 Bacteria strains

2.2.1 Bacteria strains

2.2.2 Bacteria storage

2.3 Molecular biology techniques
2.3.1 Plasmid minipreparation
2.3.2 Restriction digests and ligation reactions
2.3.3 Agarose gel electrophoresis
2.3.4 Purification of DNA from agarose gels
2.3.5 DNA sequencing
2.3.6 Transformation of E. coli with plasmidic DNA
2.3.7 Site direct mutagenesis
2.3.8 Amplification of constructs for expression studies
2.3.9 Expression of recombinant proteins
  2.3.9.1 MBP and GST recombinant proteins
  2.3.9.2 Histidine-tagged recombinant protein
2.3.10 Quantification of proteins
2.4 Antibodies
  2.4.1 Anti XIGEMC1 antibodies and affinity purification of anti
       XIGEMC1 policlonal antibodies
  2.4.2 Antibodies
  2.4.3 Western blotting
       2.4.3.1 Membrane stripping and re-probing
       2.4.3.2 Coomassie blue staining
  2.4.4 Antibodies cross-linking to resin
  2.4.5 Anti phospho antibodies
  2.4.6 Immunoassay
2.5 Frog techniques
  2.5.1 Preparation of interphase Xenopus egg extract
  2.5.2 Preparation of sperm chromatin
  2.5.3 cDNA expression library screening
  2.5.4 Chromosomal and single strand replication assay
  2.5.5 Chromatin binding
  2.5.6 Pull-down assays
       2.5.6.1 Into the extract
       2.5.6.2 In vitro
  2.5.7 In Vitro Transcription Translation (IVTT) and Kinase Assay
  2.5.8 Peptide arrays
2.5.9 Immunofluorescence microscopy 75
2.5.10 XIGEMC1 phosphorylation in *Xenopus* egg extract 76

2.6 *Xenopus* embryology and cell culture 76
2.6.1 Xenopus embryo manipulation and microinjection 76
2.6.1.1 Recipes 77
2.6.2 Morpholino oligonucleotide 78
2.6.3 Protein extraction from oocytes and embryos 79
2.6.4 tPARP cleavage assay 79
2.6.5 Genomic DNA preparation 79
2.6.6 Histological analyses 80
2.6.7 Cell culture and flow cytometry 80
2.6.8 siRNA Transfection 81
2.6.9 RNA isolation and RT-PCR 81

3 Results I - Role of *Xenopus* GEMC1 protein in DNA replication initiation 83

3.1 Introduction 83

3.2 Results 86
3.2.1 Identification of *Xenopus* GEMC1 protein 86
3.2.2 Characterization of XIGEMC1 structure and sequence analysis 91
3.2.3 Anti *Xenopus* GEMC1 polyclonal antibodies 96
3.2.4 Chromatin binding of *Xenopus* GEMC1 during DNA replication 103
3.2.5 Role of XIGEMC1 in DNA replication 105
3.2.6 Role of XIGEMC1 in the formation of the initiation complex 113
3.2.7 Identification of XIGEMC1 associated proteins 115
3.2.8 Interaction between *Xenopus* GEMC1 and XITopBP1 120
3.2.9 XIGEMC1 is substrate of S phase Kinases 124
3.2.10 Effect of Cdk2 phosphorylation on XIGEMC1 activity in DNA replication 133

3.3 Discussion 136
3.3.1 The XIGEMC1 coiled-coil domain shares homology with the hGeminin coiled-coil domain 136
3.3.2 Functional role of XIGEMC1 protein in DNA replication initiation 138
3.3.3 XIGEMC1 : vertebrate homologue of yeast Sld3 protein? 138
3.3.4 Investigating the interaction of XIGEMC1 and known replication factors. 142
3.3.5 XIGEMC1 and DNA synthesis 143

4 Results II- Xenopus GEMC1 in development embryos 144

4.1 Introduction 144

4.2 Results 145
  4.2.1 XIGEMC1 expression in developing embryos 145
  4.2.2 Depletion of XIGEMC1 by antisense oligos in frog embryos 148
    4.2.2.1 Specificity of XIGEMC1 morpholino knockdown 148
    4.2.2.2 XIGEMC1 deficiency is lethal for Xenopus embryogenesis 150
  4.2.3 Loss of XIGEMC1 function by antisense morpholino oligonucleotides induces apoptosis 155

4.3 Discussion 159

5 Results III–Towards the characterization of mammalian GEMC1 161

5.1 Introduction 161

5.2 Results 162
  5.2.1 First evidence for mouse GEMC1 to be required for initiation of DNA replication 162

5.3 Discussion 166
  5.3.1 From Xenopus to mammalian cells: preliminary data a mGEMC1 function in mouse cells 166

6 Conclusions 168

7 References 171
Table of Figures

Fig. 1.1 Initiation of DNA Replication at oriC 20
Fig. 1.2 SV40 core origin 21
Fig. 1.3 Mechanism of DNA replication initiation and helicase unwinding in Xenopus laevis (upper picture) and budding yeast (lower picture) 43
Fig. 1.4 Hypothetical ATM-ATR regulation of DNA replication origin firing 56
Fig. 3.1 Schematic representation of the screening for ATM-ATR targets 88
Fig. 3.2 Screening for ATM and ATR target 89
Fig. 3.3 Similarity of the coiled-coil domain of XIGEMC1 and human geminin (hGEM). 90
Fig. 3.4 Identification of the ATM, ATR target XIGEMC1 90
Fig. 3.5 XIGEMC1 coiled-coil structure 92
Fig. 3.6 Conservation of GEMC1 in vertebrates 93
Fig. 3.7 Purification of recombinant GEMC1 protein 98
Fig. 3.8 XIGEMC1 recombinant protein visualized by Coomassie blue staining and immunoblot with anti GEMC1 antisera 99
Fig. 3.9 Immunoblot of frog extract with anti GEMC1 antisera 99
Fig. 3.10 GEMC1 quantification in Xenopus egg extract 100
Fig. 3.11 Effects of recombinant XIGEMC1 on DNA replication 101
Fig. 3.12 XIGEMC1 expression in adult Xenopus tissues 102
Fig. 3.13 Chromatin binding of Xenopus XIGEMC1 during DNA replication 104
Fig. 3.14 GEMC1 does not require ORC for association with sperm chromatin 104
Fig. 3.15 Test for purified anti XIGEMC1 antibody specificity 106
Fig. 3.16 Anti XIGEMC1 antibodies abolish replication of sperm chromatin 107
Fig. 3.17 Replication activity of extract added with anti XIGEMC1 antibodies 108
Fig. 3.18 Effects of different anti xXIGEMC1 antibodies 109
Fig. 3.19 XIGEMC1 is not involved in the elongation phase of replication 111
Fig. 3.20 Nuclei assembly in the presence of anti XIGEMC1 antibodies 112
Fig. 3.21 Requirement of XIGEMC1 for the loading of Cdc45 onto chromatin 114
Fig. 3.22 GEMC1 interacting proteins in Xenopus egg extract in pull down assays 116
Fig. 3.23 GEMC1 interactions in in vivo pull down and IP 118
Fig. 3.24 GEMC1 interactions in in vitro pull down assays 119
Fig. 3.25  XIGEMC1 interacts in vivo and in vitro with xTopBP1
Fig. 3.26  XIGEMC1 requires xTopBP1 for association with sperm chromatin
Fig. 3.27  XIGEMC1 is a substrate for cyclin E/CDK2
Fig. 3.28  Sequence comparison RXL/KXL motives and S-T/P consensus sites among XIGEMC1 orthologs.
Fig. 3.29  Mapping the sites on XIGEMC1 phosphorylated by cyclinE/CDK2
Fig. 3.30  Specificity of anti phospho-Threonine 153 antibody
Fig. 3.31  In vivo phosphorylation of XIGEMC1 on Threonine 153
Fig. 3.32  XIGEMC1 phosphorylation sites for CDK2/CyclinE
Fig. 3.33  XIGEMC1-8ST/D was able to stimulate DNA replication initiation
Fig. 3.34  XIGEMC1-8ST/D shows higher affinity for TopBP1 compare to wt
Fig. 4.1  Xenopus embryo staging
Fig. 4.2  Embryonic expression of GEMC1
Fig. 4.3  MOs target sites on XIGEMC1 mRNA
Fig. 4.4  Effects of morpholino oligos on in vitro translated XIGEMC1
Fig. 4.5  Depletion of XIGEMC1 with a specific antisense MO.
Fig. 4.6  XIGEMC1-MO does not affect embryo development before mid blastula transition (MBT)
Fig. 4.7  Delayed development of Xenopus embryos after gastrulation in response to XIGEMC1-MO treatment
Fig. 4.8  XIGEMC1 depleted Xenopus embryos
Fig. 4.9  Embryos injected with GEMC1-MO present a severe tissue disorganization
Fig. 4.10  DNA content reduction in GEMC1 deficient embryos
Fig. 4.11  Inhibition of endogenous XIGEMC1 result in apoptosis
Fig. 5.1  Depletion of mGEMC1 using siRNAs
Fig. 5.2  Effects of siRNA oligos targeting mouse Cdc45
Fig. 5.3  mGEMC1 is required for initiation of DNA replication
Fig. 6.1  Model of GEMC1 function at replication origins.
Table of Tables

Table 2.1 Antibodies used in this study 68
Table 3.1 GEMC1 family and subfamilies 94
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACS</td>
<td>ARS consensus sequence</td>
</tr>
<tr>
<td>APC</td>
<td>anaphase promoting complex</td>
</tr>
<tr>
<td>ARS</td>
<td>autonomously replicating sequence</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia telangiectasia mutated protein</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5’triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>ATM and Rad3 related protein</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BRCT</td>
<td>BRCA1 C-terminal</td>
</tr>
<tr>
<td>Cdc</td>
<td>Cell Division Cycle</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>CMG</td>
<td>Cdc45, MCM2-7 and GINS</td>
</tr>
<tr>
<td>Cdt1</td>
<td>Chromatin licensing and DNA replication factor 1</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>Clb</td>
<td>B-type cyclin in S. cerevisiae</td>
</tr>
<tr>
<td>Cln</td>
<td>G1 cyclin in S. cerevisiae</td>
</tr>
<tr>
<td>CSF</td>
<td>Cytostatic factor</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DDK</td>
<td>Dbf4 dependent kinase</td>
</tr>
<tr>
<td>DHFR</td>
<td>dihydrofolate reductase</td>
</tr>
<tr>
<td>D. melanogaster</td>
<td><em>Drosophila melanogaster</em></td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide phosphate</td>
</tr>
<tr>
<td>DSB</td>
<td>double strand break</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double-stranded DNA</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>FHA</td>
<td>forkhead associated</td>
</tr>
<tr>
<td>G1</td>
<td>gap 1 in the cell cycle</td>
</tr>
</tbody>
</table>
G2  gap 2 in the cell cycle
GEMC1  GEMinin Coiled-coil containing protein
GINS  Go, Ichi, Nii, San
GST  Glutathione S-transferase
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His  Histidine
HRP  Horseradish peroxidase
IPTG  Isopropyl β-D-1-thiogalactopyranoside
kb  kilobase
kDa  kiloDalton
LB  Luria-Bertani Broth
M  mitotic phase
MBP  maltose binding protein
MBT  Mid-blastula transition
MCM  minichromosome maintenance
MO  Morpholino oligonucleotide
NAM  normal amphibian medium
NPE  nucleoplasmic extract
nt  nucleotide(s)
ORC  origin recognition complex
PAGE  Polyacrilamide gel electrophoresis
PBS  Phosphate-buffered saline
PCNA  proliferating cell nuclear antigen
PCR  polymerase chain reaction
PI3KK  phosphoinositol 3-kinase like kinase
pre-IC  preinitiation complex
pre-RC  prereplicative complex
RFC  replication factor C
RNA  ribonucleic acid
RNAi  RNA-interference
RPA  replication protein A
rpm  revolutions per minute
*S. cerevisiae*  *Saccharomyces cerevisiae*
S phase  synthetic phase
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPKs</td>
<td>S-Phase Kinases</td>
</tr>
<tr>
<td><em>S. pombe</em></td>
<td><em>Schizosaccharomyces pombe</em></td>
</tr>
<tr>
<td>SSB</td>
<td>single strand binding protein</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single-stranded DNA</td>
</tr>
<tr>
<td>SV40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>T-Ag</td>
<td>large T-antigen</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>ts</td>
<td>temperature sensitive</td>
</tr>
<tr>
<td>TOpBP1</td>
<td>Topoisomerase (DNA) II binding protein 1</td>
</tr>
<tr>
<td>WB</td>
<td>Western Blot</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
</tr>
<tr>
<td>X. laevis</td>
<td>Xenopus laevis</td>
</tr>
</tbody>
</table>
1 Introduction

One of the fundamental aims of modern biology is to understand how the genomes of eukaryotes are duplicated during each cell cycle. Cells must duplicate their chromosomes before cell division through a DNA replication pathway. Replication is a tightly regulated process such that each daughter cell receives exactly one copy of the maternal cell’s DNA content upon cell division. To guarantee the fidelity of transmission of genetic information stored in DNA, eukaryotic cells have evolved intricate mechanisms named checkpoints, DNA repair, and DNA recombination, which contribute to control and assist the faithful duplication of the genome. Therefore, understanding the mechanisms that regulate the events that control DNA replication has become of primary importance in order to ensure genome stability.

The genome of eukaryotes contains an amount of DNA that can be up to $10^5$ larger than the one in bacteria and viruses. Still DNA must be duplicated rapidly and precisely before entering mitosis. For this reason DNA replication initiates at multiple origins that are distributed along each chromosome. Their activity must be tightly regulated to ensure that the whole chromosome is replicated only once per cell cycle. The molecular mechanisms that govern the initiation of DNA replication have been investigated using several model systems. Recent studies of the assembly and activation of the complexes loaded to origins have provided new insights into eukaryotic DNA replication initiation. Although many of the factors involved in this process are conserved from yeast to mammals, the function and the order by which they are loaded to DNA are still poorly understood. In this thesis I focus on the events occurring at eukaryotic origins during DNA replication initiation describing the role of a new factor involved in this critical initial step.

Forty years ago, Jacob and colleagues proposed the origin replicon model. They postulated the existence of two important essential genetic loci that would be required for replication initiation: the “replicator” and the “initiator” (Jacob and Brenner, 1963). The “replicator” represents the genetic element required for initiation of DNA replication. The “initiator” instead is the gene that encodes the factor implicated in recognizing a specific sequence in the genome that overlaps with the
replicator. Importantly the replicon model has provided a unique framework for understanding the initiation of DNA replication in viruses, bacteria and eukaryotic chromosomes

For simplicity in this thesis I will refer to each organism using the following abbreviations: Sp (S. Pombe), Sc (S. cerevisiae), Xl (X. laevis), Dm (D. melanogaster) mouse or Mm (Mus musculus) and human or Hs (H. sapiens).

1.1 The Eukaryotic Cell Cycle

In eukaryotic cells the highly regulated series of events that leads to the passage from one cell division to the next one follows a defined pattern of distinct stages, termed the cell cycle (Murray, 2004; Nasmyth, 1996). The cell cycle is divided into four main stages, the “gap” before DNA replication (G1), the DNA synthesis phase (S), the “gap” after DNA replication (G2), and cell division that represents the mitotic phase (M). In this thesis I will discuss the S phase of the cell cycle.

1.2 Replication Origins and Control of Initiation of DNA Replication

1.2.1 Initiation of DNA replication in bacteria: the replicon model

E. coli contains a single circular bacterial chromosome that is entirely replicated in a bidirectional process (Hiraga, 1976). Moreover, bacteria contain a single and non-redundant origin of replication termed oriC (Hiraga, 1976). This site is 245 base pairs long and contains four nine-mers, which constitute a specific binding sites for DnaA, a protein essential for initiation of replication (Tomizawa and Selzer, 1979) (Fuller et al., 1984). Twenty to forty DnaA monomers bind to the origin while the DNA becomes wrapped around the oligomerized DnaA core. Once bound to oriC, DnaA initiates the melting of the origin at three 13-mers of the AT-rich region and form an “open” complex (Bramhill and Kornberg, 1988). Following melting, DnaB helicase binds to the origins in a DnaC dependent manner. The two proteins (DnaB and DnaC) form a complex stabilized by ATP. Upon delivery of DnaB helicase, DnaC
is released from the protein-DNA complex in a process dependent on ATP, which also triggers DnaB helicase activity (Wahle et al., 1989a, b). After activation, DnaB begins to unwind the dsDNA using ATP as a source of energy. Single-stranded DNA generated by the unwinding process is coated and stabilized by single strand DNA binding protein (SSB) and DNA gyrase (type II topoisomerase) acts to resolve obstructing topological structures. Finally DnaG primase binds to DnaB completing the primosome multi-enzyme complex, which stimulates primer synthesis by RNA polymerase. The primosome continually moves along the lagging strand in order to prime each Okazaki fragment, and only primes once on the leading strand. This is followed by replication of the template by DNA polymerase III holoenzyme (Fig. 1.1)

Although the proteins involved in initiating replication in eubacteria, eukaryotes and viruses such as SV40 and lambda bacteriophage are not closely related to each other, the basic factors in initiation are remarkably similar. Among these factors, it is pretty clear that DnaA conserves the predicted role of an initiator protein that recognizes and binds to the origins. DnaA characterization helped to postulate three major roles for initiator proteins that may be conserved in initiator proteins from other organisms, the ability to:

i) bind to the origins
ii) melt the DNA at the origins
iii) recruit other replication factors
(Bramhill and Kornberg, 1988)
Fig. 1.1  Initiation of DNA Replication at oriC  (Heller and Marians, 2006)
1.2.2 Initiation of DNA replication in *Simian virus* (SV40)

Simian virus (SV40) and related viruses have served as powerful model systems to understand many aspects of the molecular biology of eukaryotic DNA replication. The viral genome consists of 5 kb of circular duplex DNA molecules and a single DNA replication origin. SV40 replicates in the nucleus of the host cell (human and monkey cells) where the viral genome is complexed with histones to form a nucleoprotein structure (minichromosome) indistinguishable from cellular chromatin. Most of the enzymatic activities for DNA replication are provided from the cellular proteins, in fact just one protein involved in DNA replication, Large Tumor antigen (T antigen) is encoded by the virus. For this reason viral and cellular DNA replication show a high degree of similarity. The large T antigen represents the regulatory protein that orchestrates all subsequent events in the virus replication.

The SV40 origin is tripartite including a central core of 27bp (also known as site II) bound by SV40 T antigen, an A/T rich domain and an imperfect palindromic sequence element (site I) which are adjacent to site II and are all required for the initiation of SV40 DNA replication (Fig. 1.2). The T antigen recognizes a specific region of SV40 DNA and tightly binds four tetrameric pentamers GAGGC within the site II (Tjian, 1979). The T antigen oligomerizes into two hexamers in the presence of ATP and this multimeric complex sits over each side of palindromic region (Mastrangelo et al., 1989). This binding causes alterations in the local DNA structure including DNA melting of the site II and additional distortion of site I and the A/T rich site (Borowiec and Hurwitz, 1988; DeLucia et al., 1983).

![Fig. 1.2 SV40 core origin](image)

**Fig. 1.2 SV40 core origin**

SV40 replication origin consisting of: inverted repeats indicated with green arrows; site II containing the GAGGC pentamers repeats and the 17bp A/T rich sequence as blue square.
In addition to its function in origin recognition, T antigen also exhibits helicase activity. The unwinding reaction requires ATP hydrolysis to allow the initial opening of the duplex in the immediate vicinity of the origin and extension of the initial opening by the helicase activity (Wahle et al., 1989a, b).

A SV40 cell free system permitted the identification of additional proteins required to promote SV40 DNA replication (among which are Replication protein A, DNA polymerase \(\alpha\), \(\delta\) and many more). For this reason, SV40 DNA replication represents a valuable model system to better dissect eukaryotic replication machinery.

In summary, the initiation of SV40 and similarly \(E.coli\) replication shows a specific origin sequence, an origin recognition protein and additional proteins to be recruited to the initiation site. Unlike bacterial DnaA, T antigen manifests an additional helicase activity. The observation that SV40 T-antigen not only recognise the replication origin but also exhibits helicase activity as dual functions of a single protein contrast observations from higher eukaryotic cells in which these functions are usually accomplished by different proteins.

### 1.2.3 Replication origins in yeast \textit{Saccharomyces cerevisiae}

Much of our basic understanding of the molecular mechanism that links DNA replication initiation to cell cycle control has come from studies in yeast. In the unicellular eukaryote, budding yeast \(S. cerevisiae\), there are about 400 origins. A typical origin consists of three to four sequences of 10-15 bp spread over 100-150 base pairs (bp). The initiation of DNA replication in budding yeast takes place through a two-step process (Diffley, 1996; Newlon, 1997). The first step involves the assembly of a protein complex known as the pre-replicative complex (pre-RC) assembled over the potential replication origins. This step occurs in late M phase/early G1 phase of the cell cycle when the B cyclin-dependent kinase (the complex that maintains cells in mitosis) activity is decreased (Jorgensen and Tyers, 2004). The second step occurs at the entry into S phase when G1 cyclin dependent kinase activity increases, this phenomenon leads in turn to the activation of downstream protein kinases: Dbf4-Cdc7 and Clb5/6 (B cyclin)-Cdc28. Importantly, the activation of these protein kinases not
only triggers origin firing but also inhibits the formation of new pre-RCs until the end of mitosis (Dahmann et al., 1995; Piatti et al., 1996).

To enable DNA replication of the whole genome, eukaryotic cell DNA replication starts at multiple sites that in the 1970s where identified as Autonomously Replicating Sequence elements (ARSs). These specific genome sequences were shown to be able to allow a circular plasmid transformed in yeast *S. cerevisiae* to stably and autonomously be maintained in yeast cells without requiring integration into the chromosome (Hsiao and Carbon, 1979; Stinchcomb et al., 1979). These sequences are distributed throughout each chromosome at average intervals of about 30 kb (Beach et al., 1980).

Sequence analysis of ARS elements allowed the identification of the ARS consensus sequence: ACS (A/T)TTTA(T/C)(A/G)TTT(A/T) (Broach et al., 1983). In budding yeast ARSs are in fact considerably AT-rich sequences. *In vitro* mutation analysis of the 11 bp ACS identified three functional domains A, B and C. ACS is part of the domain A and highly conserved in all yeast origins. Despite the fact that the ACS domain has been shown to represent the ORC (Origin Recognition Complex that will be discussed in the paragraph 1.3.1) binding site, it is necessary but not sufficient to promote efficient DNA replication (Bell et al., 1993; Celniker et al., 1984). A specific area (B1) of the B domain has been shown to also be essential for ORC binding. A point mutation at that position diminishes ORC binding and replication activity (Rao and Stillman, 1995). The domain C is important for replication only in the absence of domain B (Celniker et al., 1984).

The two ARS elements more studied are ARS 1 and ARS 307. Mutational analyses performed on ARS1 and ARS307 have shown that ARSs are modular. Linker substitution analyses across a 193 bp fragment of ARS1 revealed the existence of different areas within the B domain, B1 (13bp), B2 (12bp) and B3 (18bp), which all together, in addition to A domain, contribute to ARS1 function as a replication origin (Marahrens and Stillman, 1992). A linker mutagenesis approach was also exploited to analyse the yeast replication origin ARS307. In this case the B domain was divided in 2 different sub-domains: B1 and B2 important for efficient origin function (Rao et al., 1994; Theis and Newlon, 1994). Although the similarities between these two ARSs are
low, they show functional conservation. B1 element of ARS1 is indeed able to substitute the B1 domain in ARS307 and vice versa (Rao et al., 1994). However, it is not possible to interchange sub-domains within the same ARS, supporting the idea that each sub-domain covers a different role from each other.

1.2.4 Origins in fission yeast *Schizosaccharomyces pombe*

The fission yeast *Schizosaccharomyces pombe* is distantly related to *S. cerevisiae*. Although they show a similar DNA content it contains three chromosomes compared to the 16 of the budding yeast. *S. pombe* replicators consist of AT-rich domains that are bound by ORC complex and are able to trigger autonomous replication in a plasmid (Clyne and Kelly, 1995; Dubey et al., 1996; Dubey et al., 1994). In fission yeast, replicators are larger than those of budding yeast (500-1000bp) (Maundrell et al., 1988). They contain 20-50 bp that are important for origin function but differently from *S. cerevisiae* they lack a conserved essential sequence motif such as an ACS. Genetic studies have shown a high functional redundancy of ARS elements. This flexible organization guarantees a compensatory mechanism in which ORC binding and replication onset can occur even in the event that an AT-rich sequence is deleted (Clyne and Kelly, 1995). Unlike the classical replicon model, most of the chromosomal origins analyzed in *S. pombe* do not fire in S phase, suggesting that in a similar way to higher eukaryotes the number of potential origins is greater than the actual number that is used per cell cycle (Caddle and Calos, 1994; Dubey et al., 1994).

One important observation is that chromosomal replication origins appear to be organized in clusters in larger initiation areas (with high A+T composition) and that about 90% of these discrete genomic regions colocalize with active origins, possibly ensuring in this way that replication occurs at regular intervals along the chromosome (Dubey et al., 1994; Wohlgemuth et al., 1994).

1.2.5 Sequence independent replication initiation in higher eukaryotes

In higher eukaryotes, replication initiates from hundreds if not thousands of initiation sites called origins. In mammalian cells, for instance, DNA replication
initiates from about 25,000 origins spaced ~ 100 kb apart. Much has been learnt about replication initiation from studies in yeast but where does replication initiation occur in higher eukaryotes? The most striking feature emerging from metazoan analysis of replication origins is the lack of any consensus motifs. In fact, although the replication apparatus seems to be evolutionary conserved from prokaryotes to eukaryotes (Dutta and Bell, 1997), no conserved target sequence in replication appears to be maintained among species.

1.2.5.1 Insights from Xenopus laevis embryonic system

The plasmid-based ARS assay which has helped to identify several replication origins in both budding and fission yeast (Huberman, 1999), has failed in identifying metazoan replicators. In fact almost any sequence tested could initiate replication in a plasmid of at least 15kb when tested in Xenopus laevis (Mechali and Kearsey, 1984) or 25kb when tested in human cells (Krysan et al., 1993). However, the combination of two dimensional gel electrophoresis (2Dgel) techniques (Brun et al., 1995; DePamphilis, 1999) with more sensitive approaches that exploit PCR to determine the abundance of nascent strands (Vassilev and Johnson, 1989) allowed the identification of 40 origin sequences to different degrees of structural and functional detail. In particular 2D gel electrophoresis revealed that origins in metazoans were much larger than the ones in yeast whereas the second method allowed a more precise mapping of the origins to small regions of 500 bp (Giacca et al., 1994). Furthermore the 2D gel electrophoresis technique employed in Xenopus embryos was used to investigate the random nature of DNA replication initiation in early blastula. This approach enabled the discrimination of structures emerging upon replication initiation visualized as bubbles from forks whose structure is indicative of elongating replication forks. By analyzing intermediates of the chromosomal rDNA cluster in early blastula of the cell stage embryo, Hyrien and colleagues showed that initiation of DNA replication takes place in random positions within an average of 9-12 kb of one another on the chromosomal rDNA (ribosomal DNA) repeat (Hyrien and Mechali, 1993).

Differently from specific ARS elements found in budding and fission yeast, experiments performed in Xenopus embryonic systems showed that DNA replication initiation occurs regardless of sequence specificity. Injection of bacterial plasmid
DNA or strikingly SV40 DNA lacking the specific origin could be entirely replicated upon injection in frog eggs (Harland and Laskey; Mechali and Kearsey, 1984). Comparable to the results obtained in eggs, incubation of plasmid DNA or sperm nuclei in *Xenopus* cell free extract showed the same results (Blow and Laskey, 1986). It was also demonstrated that the replication efficiency relied on the size of the DNA molecule employed rather than any specific sequence (Mechali and Kearsey, 1984). Since ORC represents an essential protein for DNA replication, it could be speculated that the same ORC is able to interact functionally with any DNA sequence although a higher affinity for AT-rich domains has been observed (Kong et al., 2003). On the other hand the high concentration of initiator proteins could also be responsible for the ability to support initiation from numerous sites in *Xenopus* cell free system (DePamphilis, 1999).

The importance of chromatin arrangement in contributing towards origin firing has also been considered. This idea came from experiments performed in *Xenopus* extract where the addition of histone H1 to the extract reduced the number of replication forks by blocking the assembly of pre-replicative proteins onto chromatin (Lu et al., 1998). Evidence in support of this idea include developmental studies in *Xenopus* embryos, specifically after MBT (mid blastula transition) where Hyrien and colleagues employed 2D gel analysis to study DNA origins within the ribosomal RNA genes (Hyrien et al., 1995). Before MBT these genes are not transcribed and DNA replication was shown to initiate from different sites throughout the area. After MBT when transcription is activated a strong inhibition of DNA initiation from the rRNA transcription unit was detected. Such data support the possibility that chromatin structure is likely to be an important determinant for origin activity.

1.2.5.2 *Replication origin in mammalian cells*

In order to identify mammalian origins, studies involving mapping the actual origin sites that initiate DNA replication were performed. Such approaches allowed the identification of several mammalian origins among which the most characterized are the DHFR locus, and the β-globin locus (Vassilev and Johnson, 1989; Vassilev et al., 1990).
Early studies on the DHFR locus were performed by incorporation of radiolabel nucleotides in the Chinese hamster ovary that identified three different initiation sites (Heintz and Hamlin, 1982) indicating that replication initiates within a defined region of this locus. 2D gel electrophoresis techniques allowed the identification of bubbles and forks travelling in both directions throughout an initiation zone spanning 55 kb of DNA that lies between DHFR and 2BE2121 genes (Dijkwel and Hamlin, 1992; Vaughn et al., 1990). This result suggested that initiation from multiple sites could occur within a wide intragenic region. An additional result, in support of this observation, came from a subsequent analysis where high resolution labelling studies in an amplified cell line allowed identification of initiation in two short regions called oriβ and oriγ as preferred initiation sites. Studies of Okazaki fragments, which are found on different DNA strands on the opposite sides of the replication origins, helped to verify whether either of these two origins showed a predominant initiation activity. Between these two specific origins a 500bp region surrounding oriβ was demonstrated to account for 80% of all initiation events from DHFR locus (Burhans et al., 1986). When oriβ was placed randomly at ectopic locations in both hamster and human cells, it was able to promote DNA replication, confirming that oriβ indeed incorporates features of a replicator element (Altman and Fanning, 2001). Subsequently a third initiation site named oriβ’ was discovered 5 kb downstream of oriβ (Kobayashi et al., 1998). This origin appeared to be used at lower frequency compared to oriβ suggesting that all three elements (oriβ, oriγ, oriβ’) might contribute in the initiation events occurring in the DHFR locus although with different degrees of efficiency.

The β-globin locus represents another mammalian region where origin firing was found to be restricted to a discrete site. This locus was identified through a similar nucleotide radiolabeling approach used to discover the DHFR origin region (Kitsberg et al., 1993). Differently from the DHFR locus, one single bidirectional origin of replication was found and located upstream of the β-globin gene. To confirm the role as an origin of DNA replication, the β-globin initiation sequence was successfully shown to trigger replication initiation at ectopic sites following cloning into a simian genome (Wang et al., 2004).
In conclusion, because various origins initiate DNA replication at different times throughout S phase, we should take into consideration that other elements may play an active role in their regulation. Possible candidates are represented by: a) sequences enriched in AT; which are significantly abundant in many prokaryotic and eukaryotic origins; b) CpG islands colocalized with promoters and corresponding to open chromatin structures (removal of these regions affected efficacy of origin initiation in some cases (Paixao et al., 2004); c) Transcriptional control elements; origins in metazoans have been found to localize in regions close to sites capable of binding transcription factors; d) DNA topological state; studies have revealed that a negatively supercoiled state favours ORC binding (Remus et al., 2004) and promotes localized DNA unwinding (Williams and Kowalski, 1993).

1.3 DNA Replication Licensing

DNA replication licensing begins with the assembly of the pre-RC whose components are conserved from yeast to humans. The pre-RC complex was originally identified in yeast by genomic footprinting using a DNA I protection assay (Diffley, 1994). Licensing begins in early G1 at replicating origins, with the sequential recruitment of the ORCs, the loading factors Cdc6 and Cdt1, and the putative DNA replicative helicase MCM2-7 complexes. These processes will be discussed in more detail in the following sections.

1.3.1 The Origin Recognition Complex (ORC)

The Origin Recognition Complex is a 6 subunit complex (Orc1-6), which represents the first factor to be restricted to discrete replicating origins. It was first identified in S. cerevisiae as a complex that binds to the ARS consensus sequence (ACS) (Bell and Stillman, 1992). Further studies revealed how it is conserved across species including S. pombe, X. laevis, D. melanogaster, mouse and human. ORC serves as a platform onto which other pre-RC proteins bind during replication. Unlike yeast, higher eukaryotes do not show a unique conserved ORC DNA binding site although the DNA sequences identified contain poly-A stretches that appear similar to the S. cerevisiae ACS (Reeves and Nissen, 1990). In most organisms ORC requires
multiple subunits to bind DNA. In *S. cerevisiae* Orc1-5 have been shown to be sufficient for the association to DNA (Lee and Bell, 1997; Li and Herskowitz, 1993). Human Orc 1-5 is sufficient to support DNA replication in *Xenopus* extract depleted of XIORC, suggesting that also in higher eukaryotes Orc6 is not essential for chromatin association of the complex and DNA replication (Giordano-Coltart et al., 2005). Orc1, 4 and 5 are members of the AAA+ superfamily of ATPases and ORC binds to DNA in an ATP dependent manner. Studies in yeast and *D.melanogaster* showed that ScOrc1 and DmOrc1 ATP binding sites are required for the complex association to DNA (for review see (Bell, 2002). In contrast to this when ATP-binding sites were mutated in the human Orc1, 4 and 5, the whole complex still maintained the ability to bind to DNA but DNA replication was completely abolished (Giordano-Coltart et al., 2005). Additional studies in human cells specifically demonstrated that ATP was able to stimulate ORC DNA binding (Vashee et al., 2003).

Studies in mammalian cells suggested the association of ORC with chromatin is cell-cycle regulated and not all ORC subunits remain tightly associated to the DNA throughout the cell cycle (DePamphilis, 2003). Orc1 in fact is removed from DNA in late S phase and is kept dissociated during G2 and M phase. Further studies in human cells suggest that Orc1 could be either stable during the cell cycle (Okuno et al., 2001) or undergo proteolysis during S phase through a mechanism aimed at preventing re-replication (Kreitz et al., 2001). Such differences in Orc1 regulation could be attributed to the utilization of different tissue culture cell models, which show alternative pathways for Orc1 control. Mammalian Orc2 was found, instead, to be constitutively assembled with chromatin and more specific studies revealed that the complex Orc2-5 is maintained on the chromatin for the whole cell cycle (Natale et al., 2000).

In *Xenopus* egg extract and likely in embryos the specificity of DNA replication origin is extremely low and origins are believed to have the potential to fire from any DNA sequence. Despite this, it has been shown that ORC appears to bind DNA once every 16 kb (Rowles et al., 1996), suggesting that also in such embryonic systems there is a tightly controlled mechanism that regulates and limits ORC binding.
1.3.2 Cdc6 protein (Cell Division Cycle 6)

Cdc6 protein was first identified in *S. cerevisiae* in a screen for mutants with an altered cell division cycle (Hartwell, 1973). This protein is also conserved in *S. pombe, X. laevis* and human (Coleman et al., 1996; Saha et al., 1998). Cdc6 also belongs to the family of AAA+ATPases and shows similarity with Orc1 protein. This group includes factors that act as clamp loading proteins; Perkin and Diffley have shown that following sequence analysis Cdc6 displays some similarities to such a group of proteins (Perkins and Diffley, 1998). Moreover, ChIP analysis demonstrates that Cdc6 directly associates with DNA in *S. cerevisiae* (Tanaka et al., 1997).

Cdc6 plays a crucial role in promoting pre-RC formation. Genetic studies have shown that it associates with DNA in an ORC dependent manner and along with Cdt1 (described in the next paragraph) promotes MCM2-7 chromatin association (Aparicio et al., 1997). Mutations in the ATP binding motif strongly affect Cdc6 function, revealing their essential role for Cdc6 activity. Knock-out or mutation of the CDC6 gene in different species generates a variety of phenotypes. In *S. pombe*, cdc18/Cdc6 null mutants are lethal due to the fact that cells go through mitosis without having first replicated their DNA (Kelly et al., 1993). A lethal phenotype preceded by reduction in division is also obtained in *S. cerevisiae* upon Cdc6 deletion (Piatti et al., 1995). In higher eukaryotes *in vivo* knockout experiments for Cdc6 have not been reported, however, depletion of Cdc6 protein in *Xenopus* extracts inhibits DNA replication initiation (Coleman et al., 1996). Finally, in human cells dominant negative mutants carrying mutation in either Walker A (which abolish ATP binding) or Walker B (which affect ATP hydrolysis) motifs, block cells in S phase of the cell cycle (Herbig et al., 1999).

Cdc6 protein abundance is regulated in a cell cycle dependent manner. In budding yeast Cdc6 accumulates in early G1 phase and is absent from late G1 to the following M phase. Cdc6 is degraded after G1 phase in a manner dependent on the Cyclin Dependent Kinase (CDK). CDK phosphorylation targets the protein for ubiquitin mediated proteolysis by SCFcdc4 (Piatti et al., 1995). In *Xenopus laevis* (Pelizon et al., 2000) and in mammalian cells Cdc6 is displaced from the nucleous upon CDK phosphorylation after cells enter S phase (Petersen et al., 2000) (such regulation will be discussed in detail in the paragraph 1.5.2)
1.3.3 Cdt1 (Chromatin licensing and DNA replication factor 1)

Originally identified in yeast *S. pombe* as a gene regulated by spCdc10 transcription factor (Hofmann and Beach, 1994), Cdt1 protein shows no significant similarity to any other proteins. It is conserved in several eukaryotes including *S. cerevisiae* (Devault et al., 2002), *X. laevis* (Maiorano et al., 2000), human (Wohlschlegel et al., 2000) and Drosophila (Whittaker et al., 2000). Cdt1 has been shown to directly interact with Cdc6 in both *S.pombe* and mammalian cells (Nishitani et al., 2000). In *Xenopus* egg extract Cdt1 recruitment to chromatin is ORC dependent (Maiorano et al., 2000). Maiorano’s lab could not identify any association between Cdc6 and Cdt1 in the *Xenopus* model system (Maiorano et al., 2004). Recent studies have shown that the binding of Cdc6 to the chromatin is essential for Cdt1 function at the origins (Tsuyama et al., 2005), probably due to the fact that the pool of Cdt1 protein bound to chromatin independently of Cdc6 cannot contribute to pre-RC formation in the absence of Cdc6 (Yanagi et al., 2005). Experiments in mammalian cells demonstrate that Cdt1 and MCM2-7 binds together and this binding is more efficient in the presence of Cdc6 (Cook et al., 2004). In *S. cerevisiae*, Cdt1 levels remains constant during the cell cycle. Cdt1 sub cellular localization instead is regulated by CDK in order to have a nuclear accumulation in G1 but cytoplasmic localization in subsequent phases of the cell cycle later on (Tanaka and Diffley, 2002). In *Xenopus* extract, during the first round of DNA replication, Cdt1 is degraded by ubiquitin-mediated proteolysis and this degradation depends on the Cdk2/Cyclin complex (Arias and Walter, 2005). Furthermore in eukaryotic cells regulation of Cdt1 throughout the cell cycle is tightly dependent on its interaction with Geminin and the importance of this will be discussed in the later sections.

1.3.4 MCM2-7 complex (mini-chromosome maintenance 2-7)

Together, ORC, Cdc6 and Cdt1 promote the loading of the MCM2-7 complex to chromatin. MCM2-7 complex consists of six subunits that assemble together to form a ring shape hexamer. It was originally identified in yeast in a screening for genes regulating cell cycle progression, plasmid maintenance in cells and chromosome segregation (reviewed by (Dutta and Bell, 1997)). Deletion of any of the subunits was shown to be lethal in both *S.pombe* and *cerevisiae* and all the subunit are essential for efficient DNA replication in the *Xenopus* model system (Kearsey and Labib, 1998;
Several studies of the MCM2-7 complex suggest that this protein acts as a replicative helicase by unwinding DNA during replication (Ishimi, 1997; Koonin, 1993). Data supported by ChIP assays demonstrate a role for the MCM complex in both DNA replication initiation and elongation (Zou and Stillman, 2000). Inactivation of any MCM subunit in *S. cerevisiae* prevents any progression of the replication fork (Labib et al., 2000). Biochemical analysis in *Methanobacteriim* thermotrophicum demonstrated that the purified proteins, which form a double hexamer (a typical feature of other replicative helicases), acts as a 3’ to 5’ DNA helicase (Fletcher et al., 2003; Shechter et al., 2000). Additional evidence for a DNA unwinding activity has been provided in *Xenopus* egg extract. In this system, DNA unwinding is inhibited in the presence of specific MCM2-7 neutralizing antibodies or by inhibition of the Mcm7 subunit with a fragment of the retinoblastoma protein (Pacek and Walter, 2004). *In vitro* experiments using purified MCM4, 6, 7 complex from Hela cells shows that this minimal complex is the one that retains helicase activity but is shown to be not very processive (Ishimi, 1997). Surprisingly, despite the fact that the MCM2-7 complex is required for fork progression during S phase (Labib et al., 2000), complexes containing all six MCM proteins lack DNA helicase activity (Takahashi et al., 2005). It is likely that the helicase activity of MCM2–7 is stimulated *in vivo* by the interaction of the complex with additional factors. Supporting this idea is the finding that both Cdc45 and GINS (Kubota et al., 2003; Takayama et al., 2003), in *Xenopus* extract, are required for replication elongation. Together these two proteins may function as auxiliary components for the replicative helicase MCM2-7.

MCM2-7 is a member of AAA+ATPase family. The ATP binding sites are conserved in all six subunits although none of them taken individually are capable of ATP hydrolysis (Koonin, 1993). This observation suggests that ATP hydrolysis represents a coordinated event that requires all six subunits of the complex.

MCM2–7 proteins are released from chromatin during DNA synthesis. In *S. cerevisiae*, MCM2–7 are exported from the nucleus to the cytoplasm during S phase. This regulation seems to require a nuclear export signal (NES) in Mcm3. CDK phosphorylation of the Mcm3 portion on this region could be responsible to promote
the nuclear exclusion of MCM2-7 complex (Liku et al., 2005). This regulation appears to be specific to budding yeast since in both fission yeast and higher eukaryotes, MCM2–7 complexes are simply released in the nucleoplasm (Kearsey and Labib, 1998). In Xenopus extract and human cells, although there is a decrease in Mcm protein association with the chromatin in S phase, no nuclear export of the complex has been described. Nevertheless a subsequent MCM2-7 re-association to the chromatin requires that cells pass through mitosis first (Lei and Tye, 2001). In higher eukaryotes, Cdt1 destruction and Geminin activity suppress MCM2–7 chromatin binding in S phase once cells enter S phase (Maiorano et al., 2005).

1.4 Factors Involved in the Activation of Pre-replication Complex

CDK and DDK (Cdc7/Dbf4) represent the two conserved protein kinases required for helicase activation and replisome loading events that trigger DNA replication origin firing. They act together to allow the binding of auxiliary factors (Mcm10, Dpb11/Cut5, Sld2, Sld3, GINS) which lead to the association of the last initiator factor Cdc45 (Fig. 1.3)

1.4.1 Control of DNA replication initiation through two protein kinases

1.4.1.1 DDK (Dbf4 dependent kinase)

DDK is a complex of Cdc7 and its regulatory subunit Dbf4. Cdc7 is a serine/threonine protein kinase, conserved from yeast to humans. It was originally identified in a screening for genes that, when mutated, show cell cycle arrest. Depletion of Cdc7 inhibits the loading of Cdc45 to origins (Jares and Blow, 2000), whilst pre-RC assembly is not affected (Wohlschlegel et al., 2002). Previous data have shown that Cdc7 is required for both the initiation of early origins and also the firing of late origins suggesting an involvement of this protein in promoting initiation of individual origins (Bousset and Diffley, 1998). Consistent with this, the DDK complex has been shown to localize to chromatin. In both Xenopus and yeast, DDK binding depends on pre-RC complex formation (Hardy, 1996; Takahashi and Walter, 2005). In S.cerevisiae, Dbf4 protein associates to chromatin in an ORC dependent manner but independently of
both Cdc6 and MCM loading (Pasero et al., 1999). In human cells, Cdc7 binds chromatin before ASK (the human Dbf4 homologue) is loaded onto chromatin. The different kinetics of ASK chromatin binding suggests that it is regulated by an alternative pathway to Cdc7.

Cdc7 protein levels remain constant throughout the cell cycle whilst its kinase activity peaks at the G1/S transition (Jackson et al., 1993). The Dbf4 regulatory subunit instead is degraded in late mitosis and early G1 by the anaphase promoting complex (APC) (Ferreira et al., 2000).

In Xenopus extract (differently from budding yeast) Cdc7 acts independently of Cdk2 and must exert its function before Cdk2 itself (Walter, 2000). Consistently, only chromatin that is sequentially exposed to Cdc7 first, followed by Cdk2 is capable of efficient replication. The *S.cerevisiae* Cdc7-Dbf4 complex has been shown to phosphorylate MCM2,3,4,5 and Mcm2 protein was found to be an *in vivo* substrate (Lei et al., 1997). Similarly to yeast, the human homologue Cdc7-ASK can phosphorylate human Mcm3 protein *in vitro* (Masai et al., 2000). Although the MCM2-7 complex appears an appealing candidate for DDK function, essential Cdc7 phosphorylation sites have not been identified yet within MCM2-7 subunits. However, taken together these results lead to the speculation that Cdc7 phosphorylation modifies MCM2-7 conformation, an event that would allow initiation of DNA replication. In budding yeast the *bob-1* allele carrying a mutation in Mcm5 subunit makes both Cdc7 and Dbf4 dispensable for cell cycle progression (Fletcher et al., 2003). This mutation by itself could mimic Mcm5 conformational changing such as that induced in higher eukaryotes by Cdc7.

Vertebrates contain an additional regulator of Cdc7 named Drf1 (Dbf4-related factor1) (Montagnoli et al., 2002). In *Xenopus* embryos Drf1 expression is restricted to oogenesis and declines after gastrulation, whereas XDbf4 is expressed throughout development increasing after MBT (Mid Blastula Transition). This would limit Cdc7-Drf1 function to the early embryonic cell cycle. Consistent with this, experiments performed in *Xenopus* egg extract show that Dbf4 immunodepletion does not affect
DNA replication, conversely Drfl was found to be essential for this process (Takahashi and Walter, 2005).

1.4.1.2 CDKs (Cyclin–Dependent Kinases)

CDKs represent a family of protein kinases whose catalytic function can be activated through the association with activating cyclin proteins (Nasmyth, 1996). Cyclin dependent kinases are also essential along with DDK to trigger DNA replication initiation.

Both budding and fission yeast contain one single CDK: ScCdc28 and SpCdc2 respectively. These kinases become activated after their interaction with several B-type cyclins and are essential for the regulation of DNA replication (Stern and Nurse, 1996). Cyclin-CDK complexes are activated at different times of the cell cycle likely targeting different substrates. Despite this observation, they seem to exhibit functional redundancy.

In budding yeast Cdc28 is activated by two different cyclins during S phase; Clb5 and Clb6 (Mendenhall and Hodge, 1998). Although deletion of Clb5 or Clb6 causes slow S-phase and defects in cell cycle progression respectively, an intriguing observation was that deletion of both cyclins together was not able to completely inhibit DNA replication. This process was in fact promoted by mitotic cyclins. Analogously, in fission yeast depletion of mitotic CDK/cyclin complexes was able to support DNA replication.

In budding yeast Cdc28 is activated by two different cyclins during S phase; Clb5 and Clb6 (Mendenhall and Hodge, 1998). Although deletion of Clb5 or Clb6 causes slow S-phase and defects in cell cycle progression respectively, an intriguing observation was that deletion of both cyclins together was not able to completely inhibit DNA replication. This process was in fact promoted by mitotic cyclins. Analogously, in fission yeast depletion of mitotic CDK/cyclin complexes was able to support DNA replication.

In Xenopus egg extract, immunodepletion of Cdk2 completely abolished DNA replication (Jackson et al., 1995). Human cells contain multiple CDKs as well as multiple cyclins (reviewed by (Morgan and Kastan, 1997)). Several studies supported the idea that Cdk2-cyclinE plays a main role in inducing DNA replication although other cyclin-CDK complexes including Cdk2-cyclin A and Cdk4-cyclin D also cover important roles (Morgan and Kastan, 1997). Furthermore Cdk2-cyclin E was also found to be essential for S-phase entry in Drosophila embryos (Knoblich et al., 1994). Despite these initial results, Cdk2 knock-out mice were shown to be viable (Berthet et al., 2003). These data reveal that neither Cdk2 nor cyclin E are essential in vivo, breaking the dogma regarding the essential role of the Cdk2-Cyclin E complex for
DNA replication. Through biochemical approaches using *Xenopus* extract it was possible to show that mitotic protein kinase complexes, Cdk1-cyclinA and cyclin B also enable DNA replication whereas Cdk4-cyclin D does not (Pacek et al., 2004). This data strongly suggest that Cdk1 also plays a role in DNA replication origin firing.

Several substrates have been identified to be phosphorylated by CDKs. In particular members of the pre-RC complex such as ORC, Cdc6 are believed to be involved in the recruitment of Cdk2 to the DNA origins. In *X. laevis* CDK binding to chromatin is dependent on ORC and Cdc6 (Furstenthal et al., 2001). Consistent with these results, human Cdc6 could coimmunoprecipitate CDK (Saha et al., 1998). This interaction is mediated by a Cy-motif, which consists of a sequence composed of hydrophobic aminoacids that frequently contain a RXL cyclin consensus site, responsible for increasing the affinity of a protein for CDK (Furstenthal et al., 2001). In *Xenopus* extract this motif was shown to be essential for the Cdc6-CDK2/cyclin E interaction (Furstenthal et al., 2001). ORC complex was also reported to interact with CDKs. This interaction was verified by coimmunoprecipitation in *S.pombe* (Vas et al., 2001) and *X. laevis* (Pelizon et al., 2000).

### 1.4.2 MCM10 (mini-chromosome maintenance 10)

Mcm10 is an essential replication factor identified by two independent screenings: the first one for genes required for chromosomal DNA replication (Solomon et al., 1992), the second one for strains showing defects in plasmid stability (Maine et al., 1984). It contains a highly conserved zinc-binding motif (Merchant et al., 1997). Although *Xenopus* MCM10 is not essential for DNA synthesis of M13 ssDNA, mutation in the ScMCM10 allele induced a block of the replicative fork at sites close to origins. Moreover, in budding yeast MCM10 is essential to guarantee replication fork progression after cells have been released from Hydroxyurea (HU). In the same yeast model system, MCM10 is also required for MCM2-7 loading to chromatin (Homesley et al., 2000), conversely immunodepletion of MCM10 from *Xenopus* extract does not affect pre-RC formation but instead completely inhibits Cdc45 loading (Wohlschlegel et al., 2002). MCM10 represents a clear example of how proteins develop different functions during evolution.
In addition to its involvement in DNA elongation, MCM 10 is also required for the DNA initiation process. MCM10 does not bind to chromatin during pre-RC formation but after CDK/DDK activation at the G1/S transition in both *Xenopus* and human (Izumi et al., 2001). Its recruitment has been described as a two step mechanism occurring before and after Cdc45 association in a complex with DNA polymerase α primase. It has been proposed that MCM10 may represent a docking site of MCM2-7 complexed to DNA polymerase α-primase. In addition, MCM10 would cooperate with DNA polymerase α travelling with the replication fork up to G2 phase of the cell cycle when MCM10 is released from chromatin (Yang et al., 2005). A possible role of MCM10 in initiation can be inferred by the fact that *in vitro* experiments have shown MCM10 to stimulate phosphorylation of Mcm2 and Mcm4 by Cdc7/Dbf4 (Lee et al., 2003). Finally co-immunoprecipitation and yeast two-hybrid assays, strongly suggest that MCM10 interacts with both ORC and Mcm2 (Kawasaki et al., 2000). This interaction of MCM10 with Mcm2 factor involved in both initiation and the fork progression complex could explain how MCM10 can play roles in both replicative stages.

### 1.4.3 Dpb11/Cut5/TopBP1 and its interactors

*S. cerevisiae* Dpb11 protein has orthologues in fission yeast (Rad4), worms (Cut5), flies (Mus101), in *Xenopus* also called Cut5 and Human (TopBP1). For simplicity in this paragraph I will refer to this protein using TopBP1 name preceded by each organism abbreviation.

Although previous studies show that HsTopBP1 is unable to suppress temperature sensitive mutants of both budding and fission yeasts (Makiniemi et al., 2001) this negative result in functional complementation could be likely due to differences in sequence conservation. Nevertheless functional similarities among higher eukaryotic TopBP1 support the idea that this protein represents the yeast counterpart of TopBP1.

Fission yeast SpTopBP1 (Rad4) protein was one of the first fission yeast Rad genes to be identified in a screening for mutants showing sensitivity to radiation (Schupbach, 1971). It is composed of four conserved BRCT domains (BRCA1 C
Subsequently, a budding yeast functional homologue of SpTopBP1 was identified as a multicopy suppressor of pol2-12 and dpb2-1 mutations that inhibit the catalytic, and the second larger subunit of DNA polymerase ε (pol ε) (Araki et al., 1995). First data supporting the role of ScTopBP1 in DNA replication come from experiments performed in budding and fission yeast. ChIP analysis showed that both ScTopBP1 and ScPol ε associate with the ARSs but differently from Polε, ScTopBP1 did migrate with the replication fork. Moreover studies of DNA replication intermediates performed by 2D gel electrophoresis analysis demonstrated the importance of ScTopBP1 for DNA replication initiation (Brewer and Fangman, 1987). ScTopBP1 binding to chromatin is dependent on the MCM2-7 complex. Consistent with its suggested role in DNA replication initiation, the ScTopBP1 mutants (ScTopBP1/dpb11-1) do not affect MCM2-7 recruitment but block the complex at the origins preventing it from travelling with the replication fork. The same mutant is able to inhibit Polα and Polε binding suggesting that ScTopBP1 is essential for initiation of DNA replication (Masumoto et al., 2000). Subsequent data showed that ScTopBP1 is required for the loading of additional initiator factors. In a screening for proteins synthetically lethal with ScTopBP1/dpb11-1, additional factors including Cdc45, GINS, Sld2 and Sld3 were found to interact with ScTopBP1 protein. These factors will be described in the following paragraphs.

In the Xenopus model system, immunodepletion experiments performed in egg extract showed that XlTopBP1 is essential for DNA replication initiation but is dispensable for the elongation step (Hashimoto and Takisawa, 2003). Furthermore, XlTopBP1 was shown to be dispensable for loading of pre-RC complex components ORC, Cdc6, Cdt1, MCM2-7, but it is required for the loading of initiation factors GINS, Cdc45 and the DNA polymerases Polα and Polε. This function is conserved from yeast to higher eukaryotes. Hashimoto and colleagues have also shown that in Xenopus extract XlTopBP1 binds to chromatin in two different modes. In the first mode XlTopBP1 is loaded onto chromatin prior to nuclei formation in a S-CDK independent manner. Interestingly, this binding is required for DNA replication to take place. In the second mode XlTopBP1 is recruited to chromatin in a CDK dependent mechanism. This event was shown to be non-essential for DNA replication but for the
DNA damage checkpoint (Hashimoto and Takisawa, 2003).

Several works from different model systems showed that TopBP1 is involved in many aspects of DNA metabolism. They also highlight the fact that many protein functions, originally identified in yeast, are conserved in higher eukaryotes. TopBP1 protein seems to play several important roles in the cell cycle. Clearly this factor is very interesting and further work is required to uncover its multiple functions.

1.4.4 Sld2 and Sld3

*S. cerevisiae* Sld2 and Sld3 proteins were also both identified in a screen for proteins synthetically lethal with *ScTopBP1/dpb11-1* mutants (Kamimura et al., 2001). Moreover, the fission yeast homologues Drc1 and Sld3 have been identified (Noguchi et al., 2002). Recent data strikingly show that both proteins represent the minimal CDK substrates required to promote DNA replication. Indeed phosphorylation of Sld2 and Sld3 by CDK was shown to be sufficient for origin firing (Tanaka et al., 2007; Zegerman and Diffley, 2007). Furthermore the phosphorylation of Sld2 and Sld3 stimulates the formation of complexes with the BRCT domains of ScTopBP1. This relies on the property of BRCT domain to bind phosphopeptides (Glover et al., 2004).

Sld2 was shown to form a complex with ScTopBP1 through its interaction with the two C terminal pairs of the BRCT domain. This interaction is mediated by CDK phosphorylation of Thr84. The complex ScTopBP1-Sld2 was shown to be required for replication initiation and origin firing (Kamimura et al., 1998). Importantly, Sld2 contains 11 clusters of CDK phosphorylation motifs none of which play a direct role for the interaction with ScTopBP1 but each of which is essential for regulating the phosphorylation of the specific Thr84 (Tak et al., 2006).

Similarly to Sld2, Sld3 also has to be phosphorylated by CDK, specifically on Thr600 and Ser622. This phosphorylation also mediates Sld3’s interaction with Dbp11 N-terminus BRCT domains essential to trigger replication initiation. In fact mutation of T600 and S622 to alanine are lethal (Tanaka et al., 2007). Similarly to budding yeast mutants in the Sld2 homologue (Drc1), which is phosphorylated on Thr84 failed to initiate DNA replication (Noguchi et al., 2002). Unlike Sld2, Sld3 function differs
from budding to fission yeast. In *S. cerevisiae* Sld3 binds to Cdc45 and their loading to chromatin is interdependent in G1 phase. Conversely, in fission yeast Cdc45 loading occurs in an Sld3 dependent manner but Sld3 does not require Cdc45 for loading onto chromatin. Mutations of Sld3 compromise origin unwinding (Kamimura et al., 2001). Moreover, chromatin loading of Sld3 in fission yeast depends on DDK but not on CDK (Nakajima and Masukata, 2002).

In 2006 Matsuno and colleagues isolated a putative Sld2 homologue in metazoan, called RecQl4. This protein shows a limited homology with Sld2 N terminal region while the C terminal region retains a helicase activity in contrast with yeast Sld2. RecQl4 shows an essential role in the final stage of initiation of DNA replication, promoting Polα loading and not Cdc45. The interaction with XlTopBP1 is maintained and is essential for RecQl4 to promote origin firing, although this interaction is not mediated by CDK activity (Matsuno et al., 2006).

While a putative Sld2 orthologue (RecQl4) has been identified in *Xenopus* model systems (Sangrithi et al., 2005) no Sld3 homologues have been found in metazoans yet. This may be explained by the fact that proteins involved in particular steps of chromosome DNA replication may have diverged greatly in their primary sequence during evolution. The less conserved primary structure derived from this may then be responsible for developing functional diversity.

### 1.4.5 GINS complex (Go, Ichi, Nii, San; five, one, two and three in Japanese)

GINS is a heterotetrameric complex that consists of Sld5, Psf1, Psf2, Psf3 (Kanemaki et al., 2003). The Sld5 subunit was originally identified in a screen for genes synthetically lethal with a mutated yeast ScTopBP1 gene (Kamimura et al., 1998). In the same screening that both Sld2 and Sld3 were also identified. Psf1 and Psf3 were isolated as multicopy suppressors of a temperature sensitive allele of SLD5 and PSF3 respectively. Moreover Sld5, Psf3 and Psf2 were found to co-purify with Psf1 (Takayama et al., 2003). Additional studies allowed the identification of the whole GINS complex from Xenopus (Kubota et al., 2003). In this study, Sld5 was immunoprecipitated using specific antibodies and Psf1, 2 and 3 were found to co-immunoprecipitate with it. The GINS complex is very well conserved from yeast to
higher eukaryotes and all the subunits could be identified by sequence homology.

In budding yeast, the ScGINS complex is only recruited to origins in S phase at the point of initiation of DNA replication although the complex is present throughout the whole cell cycle. In the same model system GINS is loaded onto chromatin in a DDK and CDK dependent manner (Kanemaki et al., 2003; Yabuuchi et al., 2006). Its binding to DNA also requires the pre-RC complex, ScTopBP1, Sld3 and Cdc45 (Kubota et al., 2003; Takayama et al., 2003). Both in budding and fission yeast GINS and Sc/SpTopBP1 proteins are loaded in a mutually dependent manner (Takayama et al., 2003). Furthermore in *Xenopus laevis* the GINS complex association with DNA is promoted by CDK (Kubota et al., 2003) and a stable binding to chromatin requires XITopBP1.

GINS is found on chromatin in a complex with Cdc45 and the putative DNA replication helicase MCM2-7 complex with which it interacts specifically and stably during S phase (Takayama et al., 2003). In *S. cerevisiae* GINS mediate and support the interaction between Cdc45 and the MCM2-7 complex. Therefore, the GINS complex may represent an essential factor that together with Cdc45 trigger activation of MCM2-7 helicase function. Consistent with this, in both yeast and *Xenopus*, the GINS complex moves away from the origin together with the replication fork (Calzada et al., 2005; Kanemaki and Labib, 2006). In the presence of aphidicolin, which inhibits polymerase activity, the GINS complex was found at the pause site along with MCM and Cdc45 (Pacek et al., 2006).

Evidence has shown that both GINS and Cdc45 play an essential role not merely in DNA replication initiation but also in supporting replication fork progression. Further studies will help us to better understand the fundamental role of the GINS complex for both processes.

1.4.6 **Cdc45 (cell division cycle 45-like)**

Cdc45 was isolated in a screening by complementation of cold sensitive mutants showing a phenotype typical of proteins involved in DNA replication (Moir et al., 1982). Cdc45 is widely conserved among eukaryotes and homologues have been
identified in fission yeast (Miyake and Yamashita, 1998), *Xenopus* (Mimura and Takisawa, 1998) and humans (Saha et al., 1998).

Cdc45 binding to chromatin has emerged as one of the final events that precedes DNA unwinding and origin firing. Studies in *Xenopus laevis* egg extract showed that Cdc45 interacts with DNA polα, GINS and MCM2-7 complexes (Kubota et al., 2003; Mimura et al., 2000). Studies in yeast have shown that Sld3 and Cdc45 form a complex throughout the cell cycle and this complex is essential for DNA replication initiation. It is intriguing to speculate that if an Sld3 homologue in higher eukaryotes exists it may show a similar interaction and also a similar function, regulating Cdc45 activity in DNA replication initiation. Cdc45 loading to chromatin has been shown to occur after nuclei formation correlates with the time of initiation (Aparicio et al., 1999) (Zou and Stillman, 2000) and requires MCM2-7 complex and S-CDK activity (Mimura and Takisawa, 1998; Walter and Newport, 2000). However, *in vivo* studies from cross-linking experiments have revealed that Cdc45 loads onto the origins early in G1 phase (Aparicio et al., 1997), although the binding is substantially increased by elevated CDK activity (Aparicio et al., 1997).

The exact role of Cdc45 in the assembly of the replication machinery has not been elucidated yet. Cdc45 binding to chromatin is essential for the sequential loading of replication protein A (RPA), DNA polymerase α and proliferating cell nuclear antigen (PCNA) onto chromatin (Mimura et al., 2000). In the absence of Cdc45, GINS complex also fails to associate with origins (Kubota et al., 2003).

Importantly, several works support the idea that Cdc45 plays a critical role in stimulating MCM2-7 helicase activity. Studies in yeast have shown that like the MCM2-7 complex Cdc45 also co-localizes with polymerases at the replication fork (Aparicio et al., 1999). Cdc45 immunodepletion affects unwinding of a plasmid DNA in *Xenopus* egg extract (Walter and Newport, 2000). To support the idea of Cdc45 being an essential component for replication fork progression, degradation of Cdc45 prevents replication elongation in *S.cerevisiae*. Importantly, reintroduction of Cdc45 after degradation is able to restore replication (Saha et al., 1998; Zou and Stillman, 2000). MCM2-7 and Cdc45 appear to form an extremely stable complex on chromatin that is associated with helicase activity (Kubota et al., 2003; Masuda et al., 2003; Zou
and Stillman, 1998). Recent studies pointed towards the existence of a complex formed by Cdc45, MCM2-7 and GINS (CMG) located at the sites of DNA unwinding and exhibiting helicase activity (Aparicio et al., 2006). The GINS complex is also required to induce a stable interaction of Cdc45 with chromatin during DNA replication initiation likely helping the establishment of the MCM-Cdc45 complex (Kubota et al., 2003). Finally, anti-Cdc45 antibodies are able to block DNA helicase activity in *Xenopus* egg extract (Pacek and Walter, 2004).

The MCM2-7 complex is a member of the machine responsible for DNA unwinding but more factors seem to be needed for it to exert its function. At present, GINS and Cdc45 are likely to represent the cofactors necessary for MCM2-7 activity.

![Helicase activation and Origin Unwinding](Image)

**A. Xenopus egg extracts**

**B. Budding yeast**

**Fig. 1.3 Mechanism of DNA replication initiation and helicase unwinding in *Xenopus laevis* (upper picture) and budding yeast (lower picture).**

For clarity, GINS complex and Mcm10 protein which travel with the replicative fork have been omitted from the final part of the figure (DePamphilis et al, 2006)
1.5 How Do Eukaryotic Cells Ensure Once per Cell Cycle DNA Replication?

In eukaryotic cells the genome is replicated from hundreds to thousands of replication origins. Despite this, endoreduplication (which consists of multiple rounds of DNA replication in the absence of mitosis) is a rare event. As it is essential to ensure that all DNA sequence are replicated precisely once in each cell cycle, none of the replication origins must fire more then once per cell cycle. Cells are able to accomplish this by inactivating pre-RCs after the onset of S-phase (corresponding to origin firing) while preventing the assembly of new pre-RCs until mitosis is completed and a new nuclear membrane has been generated. Many studies suggest that CDKs represent the main factors ensuring that the DNA does not undergo re-replication. Evidence for the role of CDK activity in preventing re-replication came from studies performed in budding and fission yeast where inhibition of the kinase results in an extra round of DNA replication (Dahmann et al., 1995; Moreno et al., 1994). Moreover CDK activity in G2/M prevents the association of MCM2-7 complex with DNA replication origins. In addition to their role in triggering origin firing when cells enter S phase, CDKs have a second essential role in inhibiting re-initiation of origins during S, G2 and M phase. Re-initiation is prevented by a number of mechanisms that do not allow the reassembly of pre-RCs at origins that have already fired. To achieve such regulation, CDKs phosphorylate multiple pre-RC components and negatively regulate them.

1.5.1 Phosphorylation of ORC

*S. cerevisiae* Orc2 and Orc6 as well as *S. pombe* Orc2 are phosphorylated *in vivo* by CDK (Nguyen et al., 2001; Vas et al., 2001). In both yeast models, experiments have been performed where the CDK consensus phosphorylation sites within Orc2 have been mutated. This mutation contributed to re-replication when accompanied with downregulation of Cdc6 activity and nuclear exclusion of MCM2-7 complex (Nguyen et al., 2001; Vas et al., 2001). Studies in *Xenopus laevis* have shown that ORC is released from chromatin during M phase and that exposure to high levels of CDK causes ORC release from chromatin (Hua and Newport, 1998; Rowles et al., 1999). Interestingly, CDK complexes purified with cyclin A, E and B can phosphorylate recombinant Orc1 and Orc2 (Findeisen et al., 1999). However,
inhibition of CDK activity in interphase egg extract has no effect in either the binding or release of ORC revealing that additional events may be required to induce ORC disengagement from the chromatin (Sun et al., 2002). In addition, Orc1 activity in mammalian cells seems to be regulated by two systems: CDK dependent phosphorylation and ubiquitination. In Hela cells Orc1 is degraded in S phase by ubiquitin-mediated proteolysis (DePamphilis, 2003). After DNA replication initiation the Orc1 subunit is released from the chromatin in a CDK phosphorylation-dependent manner while Orc2 and Orc3 remain on the DNA. To support the role of phosphorylation in this process, Orc1 mutants mimicking phosphorylation at a CDK consensus site accumulate in the cytoplasm (Saha et al., 2006).

1.5.2 Cdc6 undergoes dual regulation
In budding yeast Cdc6 accumulates in early G1 phase and is rapidly phosphorylated and degraded when cells enter S phase (Drury et al., 1997; Piatti et al., 1995). In both budding and fission yeasts the abundance of Cdc6/Cdc18 is also affected by degradation. This rapid degradation depends on the ubiquitin mediated proteolysis pathway (Sanchez et al., 1999). In Xenopus egg extract Cdc6 is displaced from the nucleus depending on CDK activity, however mutation of Cdc6-CDK consensus sites does not cause Cdc6 to be exported in the cytoplasm. Although such mutants accumulate in the nucleus, they do not induce endoreduplication. As such the effect of CDK phosphorylation remain unclear in this systems.

Finally, in human cells Cdc6 level remains constant during the cell cycle (Saha et al., 1998). Indeed Cdc6 regulation involves CDK dependent phosphorylation which is responsible for Cdc6 changes in cellular localization (Delmolino et al., 2001; Petersen et al., 1999). Changes in localization ensure that in G1 phase, human Cdc6 is retained in the nucleus while in S phase it is predominantly exported to the cytoplasm (Saha et al., 1998).

1.5.3 MCM2-7 complex regulation is CDK dependent
The function of the MCM2-7 complex is also controlled by CDK dependent phosphorylation. In S. cerevisiae, the level of MCM2-7 complex remains constant
throughout the cell cycle whereas its subcellular localization is regulated (Dalton and Whitbread, 1995). The complex enters the nucleus at the end of mitosis, it remains localized into the nucleus during G1 phase and is exported to cytoplasm in G2-M phase (Labib et al., 2001; Nguyen et al., 2000). Mmc2 and Mcm4 proteins have been shown to be phosphorylated in vitro and in vivo (Findeisen et al., 1999; Pereverzeva et al., 2000). It has been demonstrated that both Cdc28-Clbs (B-type cyclin activate Cdc28 in M phase) and Cdc28-Clns (G1 cyclins, activate Cdc28 in S phase) cause exit of Mcm4 from the nucleus ensuring no reassembly of MCM2-7 complex during and after replication (Labib et al., 1999). Although several pieces of data suggest that MCM2-7 is regulated in S. cerevisiae by CDK phosphorylation, no specific sites of modification in any of the subunits have been identified yet.

In other organisms no clear change in subcellular localization has been reported for the MCM2-7 complex, though other forms of regulation may be significant. In metazoans the MCM2-7 complex remains localized in the nucleus throughout the cell cycle and the observation that overexpression of Cdt1 together with Cdc6 is sufficient to trigger MCM2-7 reloading suggest that MCM2-7 activity remains unaltered throughout S phase (Arias and Walter, 2005). Consistent with this, MCM2-7 preserves the ability of binding to chromatin during the X. laevis cell cycle (Mahbubani et al., 1997). The observations raised here may suggest the idea that the inability of MCM2-7 to bind the chromatin is unlikely to be the result of posttranslational modifications but rather the result of the absence of Cdt1 and Cdc6 proteins. Availability of MCM2-7 complex at origins and their activity may be subjected to regulation by CDKs, and the method of regulation may vary between organisms.

1.5.4 Cdt1 regulation and Geminin

In S. cerevisiae Cdt1 levels remain constant throughout the cell cycle and its regulation is connected to that of MCM2-7 complex. As for MCM2-7, Cdt1 concentration into the nucleus peaks in G1 phase and becomes cytoplasmic in G2/M (Tanaka and Diffley, 2002). MCM2-7 binds to Cdt1 when dissociated from DNA and it does not accumulate in the nucleus in absence of Cdt1. Cdt1 activity in metazoans is regulated in at least two ways. Firstly by proteolysis which controls Cdt1 levels being
low in S and G2 phase, but high during M and G1 phase (Nishitani et al., 2001; Wohlschlegel et al., 2000). In this scenario, CDK dependent phosphorylation of Cdt1 has been shown to enhance its ubiquitin dependent degradation (Liu et al., 2004; Sugimoto et al., 2004).

Secondly, Cdt1 function is regulated by the interaction with a protein called Geminin that represses Cdt1 activity during S, G2 and M phase. Geminin binds tightly to Cdt1 and this association inhibits Cdt1 activity (Li and Blow, 2005) though it does not interfere with Cdt1 recruitment to chromatin (Yanagi et al., 2002). In this way, Geminin prevents the loading of MCM2-7 complex to chromatin by sequestering Cdt1 in a complex that is unable to interact with or recruit MCM2-7 (McGarry and Kirschner, 1998; Tada et al., 2001).

Metazoan studies on Geminin have revealed its importance in preventing DNA re-replication in a single cell cycle. Geminin was first identified by McGarry and Kirshner in a screening for proteins that are degraded at the metaphase/anaphase transition by APC (Anaphase Promoting Complex, an E3 ubiquitin ligase activated during mitosis that marks target proteins for degradation by the proteosome) (McGarry and Kirschner, 1998). So far Geminin has only been identified in metazoans. It is active during S, G2 and early M phase representing the major inhibitor of pre-RC formation after origin firing. It accumulates until late M phase when it becomes polyubquitinated in an APC dependent manner and targeted for degradation. In Xenopus embryos and Drosophila, however, Geminin levels are fairly constant throughout the cell cycle (Maiorano et al., 2004; Quinn et al., 2001). This is due to the fact that Geminin becomes inactivated in M phase by transient APC-dependent ubiquitination (Hodgson et al., 2002; Li and Blow, 2005). This generates a window of opportunity for Cdt1 to allow the recruitment of MCM2-7 complex to chromatin in G1 phase. Geminin is reactivated when imported into the nucleus just prior to the onset of S-phase (Hodgson et al., 2002; Li and Blow, 2005). The loss of Geminin leads to the re-replication of DNA in human cells (Melixetian et al., 2004), frog egg extract (Li and Blow, 2005) and Drosophila. In Xenopus egg extract Li and Blow showed that the mere depletion of Geminin from the extract induces only a small amount of DNA re-replication whereas when Geminin depletion is combined with proteosome inhibition by MG132 (proteosome inhibitor) up to 50% of replicated DNA undergoes re-
replication (Li and Blow, 2005).

The data showed suggest that both Cdt1 regulation systems (proteolysis and Geminin) cooperate to prevent re-replication during a single cell cycle. In metazoans the existence of a Geminin dependent mechanism that acts independently of CDK may then provide an additional level of protection from re-replication in the event that the CDK pathway is inhibited.

1.6 DNA Damage Response to Maintain Genome Integrity

Genetic information transmitted from one cell to its daughter cell must be faithfully and completely duplicated during DNA replication (Kelly and Brown, 2000). Cells are constantly exposed to DNA lesions and to ensure a faithful duplication of the DNA they have developed a complex pattern of responses collectively called the DNA damage response (Zhou and Elledge, 2000). The mechanisms responsible for regulating these processes preserve the integrity of the genome and the characteristic ploidy of every organism. Cell cycle checkpoints represent the pathways relaying signals triggered by aberrant DNA structures that ultimately prevent or slow down cell-cycle progression. Once activated they can generate three different outcomes: cell-cycle arrest, DNA repair and in the case of unreparable damage, apoptosis (programmed cell death).

DNA damage checkpoints can be separated into two broad groups on the basis of the aberrant DNA structures that trigger signalling. First, checkpoints that do not require an active replication fork, which are predominantly induced by double strand breaks (DSBs) or radiomimetic agents during G1 or S phase. They prevent the firing of all replication origins when damage is detected in G1 phase or the firing of late origins when the damage is instead detected in S phase. Consequently, DNA replication is inhibited until the damage is repaired. Secondly checkpoints requiring an active replication fork result in the inhibition of S phase or subsequent mitosis. Importantly, as previously described, DNA replication requires a stepwise assembly of protein complexes at the initiation sites to establish the pre-RC. Activation of the pre-RC is regulated by the protein kinases CDK (Cdk2) and DDK (Cdc7). Kinase
activation coincides with the loading of additional factors leading to DNA unwinding, Cdc45 association to chromatin and polymerase recruitment as considered in detail in previous sections (Bell and Dutta, 2002; Diffley, 2004). Although any of these steps could be targets for checkpoint signaling, to date all the checkpoint pathways identified result ultimately in the inhibition of CDK and DDK kinases responsible for the activation of the pre-RC by preventing the chromatin loading of Cdc45 (Costanzo et al., 2000; Costanzo et al., 2003).

In the following three sections I will briefly discuss the eukaryotic checkpoint network. As a signal transduction system this network consist of three main players: sensors for DNA damage; the mediators that amplify and convert a sensor input into a transmissible signal and transmitters that transfer the signal to the end targets.

1.6.1 Sensing DNA damage

In order to maintain genomic stability cells require careful monitoring of DNA replication. An important role is carried out by damage sensor proteins. Sensor proteins are crucial for relaying the presence of DNA damage to the checkpoint machinery activating the necessary signalling cascade. There are several key sensor proteins that will be discussed here as general sensors of DNA damage.

One group of proteins central to the sensor system is the phosphatidylinositol-3 kinase-like kinases (PIKKs). This group of proteins includes the ataxia telangiectasia mutated protein (ATM), ATM and Rad3 related protein (ATR) and the catalytic subunit of DNA protein kinase (DNA-PKcs). These proteins appear to sense DNA-damage in a lesion-specific manner.

ATM protein kinase is ubiquitously expressed and it localises predominantly in the nucleus (Rotman and Shiloh, 1999). Unlike many proposed sensor proteins ATM does not appear to have a specific interacting partner that directly binds to DNA. However, the MRN complex, composed of Mre11, Nbs1, and Rad50 proteins is important for ATM activation (Lee and Paull, 2004). MRN complex displays various DNA-binding capabilities including the ability to assemble linear single-stranded DNA fragments together with ATM to form signalling complexes (Costanzo et al.,
2004). It is likely that the MRN complex provides the DNA-binding platform for the sensor activation of ATM in response to DNA damage.

ATM mainly is activated by ionising radiation-induced strand breaks, while ATR function appears to be important in the regulation of responses to UV irradiation or replicative stress (Abraham, 2001) (Shiloh, 2001). Furthermore, unlike ATM, ATR exhibits a specific DNA-binding partner protein known as ATR-interacting protein, ATRIP. In mammalian cells ATRIP colocalises with ATR into nuclear foci upon DNA damage or inhibition of replication, and is phosphorylated by ATR (Cortez et al., 2001). The single-stranded binding protein, replication protein A (RPA) is required for ATRIP to bind irradiation-induced sites of DNA damage. For this reason the recruitment and activation of ATR is thought to require regions of single-stranded DNA (Zou and Elledge, 2003).

In addition, two further potential sensor complexes are thought to play a role in sensing DNA damage outside of S phase; the 9-1-1 complex made up of RAD9, RAD1 and HUS1 and the RAD17-RFC complex. The presence of RPA on ssDNA is thought to promote RAD17-RFC binding to gapped and primed ssDNA facilitating in this way the binding of the 9-1-1 complex (Zou et al., 2003).

### 1.6.2 Transmission of the checkpoint signal

Sensing DNA damage represents the first step of a DNA damage response. How do cells generate the signal that can be transmitted to regulators of cell cycle progression? To this end cells need to amplify the signal responsible for checkpoint activation. These signal transduction mechanisms rely on the activation of effectors protein kinases by phosphorylation. Mediators represent a class of proteins responsible to convert the sensor input in the activation of these effector kinases. In humans two of the most important effector proteins of DNA damage are the protein kinases CHK1 and CHK2.

CHK2 protein structure is highly conserved and consists of regulatory domain known as an activation loop and at least one SQ/TQ residue-rich domain, which represent the favoured consensus site for ATM/ATR kinase. CHK2 protein is rapidly
phosphorylated by ATM upon DNA damage or inhibition of DNA replication (Matsuoka et al., 1998). This is then followed by further auto-phosphorylation (Lee and Chung, 2001). The ‘mediator of DNA damage checkpoint’ protein, MDC1, is thought to transduce the DNA damage signalling from CHK2 via phosphorylation dependent recognition of specific regions in CHK2. Though evidence also exists for the importance of 53BP1 as mediator. Both MDC1 and 53BP1 are phosphorylated by ATM. Both proteins demonstrate physical and functional interaction with phosphorylated H2AX (phosphorylated form denoted by γH2AX), which is phosphorylated by ATM following ionising radiation, and by ATR-ATRIP following UV irradiation. Strong support of 53BP1’s mediatory role comes from the fact that in response to ionising radiation, 53BP1 binds ATM and this binding is necessary for subsequent ATM-dependent phosphorylation of CHK2 (DiTullio et al., 2002). The role of MDC1 may be somewhat more complex since MDC1 also interacts with the MRN complex and strengthens ATM binding to double-strand break lesions (Lukas et al., 2004).

CHK1 appears to be the principle recipient of ATR kinase activity, and human Claspin is thought to be a mediator for this signalling cascade (Chini and Chen, 2003). Following DNA damage or replication stress, Claspin associates with ATR, the 9-1-1 complex and CHK1, and is required for CHK1 phosphorylation.

Since my thesis will predominantly analyze and discuss the DNA replication initiation pathway with some connection to DNA damage checkpoints, in these paragraphs I will briefly describe DNA damage checkpoints that operate in S phase and do not require an active replication fork.

1.6.3 ATM-Dependent checkpoint

Double strand breaks trigger ATM activation and downstream signaling that induce p53-dependent and p53-independent responses.

1.6.3.1 p53 dependent pathway

p53 is a transcriptional regulator that acts as a tumor suppressor and plays roles in maintaining genome stability. In the absence of DNA damage p53 protein is
unstable and a poor activator of transcription. DNA damage induces p53 protein stabilization raising its level (Siliciano et al., 1997). The activation of p53 depends on ATM and ATR phosphorylation that are reinforced by CHK1 and CHK2 activity. p53 in turn activates a critical transcriptional target p21Cip/Waf which inhibits Cdk2/CyclinE protein kinase activity and causes G1 arrest (Massagué, 2004).

1.6.3.2 p53 independent pathway

Both IR-induced DSBs in mammalian cells and the addition of DSBs to Xenopus egg extract trigger a p53-independent, ATM-dependent activation checkpoint (Costanzo et al., 2004). This pathway results in the inhibition of Cdk2 kinase activity by phosphorylation of Cdk2 Tyr15 (Costanzo et al., 2000). As previously mentioned, Cdk2/cyclinE inhibition prevents the loading of Cdc45 onto chromatin and blocks subsequent origin activation (Costanzo et al., 2000). In mammalian cells, DSBs trigger proteolysis of Cdc25A (the phosphatase essential for activation of Cdk2 kinase in G1 phase). ATM-dependent activation of Chk2 leads to phosphorylation and proteolysis of Cdc25A resulting in inhibition of Cdk2 (Sorensen et al., 2003). In Xenopus the Cdc25 regulatory mechanism has not been completely characterized. ATM activation “mediator” proteins (Mdc1, 53BP1) are also involved in the ATM-dependent signaling pathway (Kastan and Bartek, 2004).

1.6.4 ATR dependent checkpoint

The ATR dependent checkpoint is triggered by ssDNA coated by RPA generated in G1. A way to generate this structure in Xenopus cell free extract is by treatment with a drug called etoposide (topoisomerase II inhibitor) (Costanzo et al., 2003). This drug traps DNA topoisomerase covalently bound to the 5’ end of the cleaved DNA, resulting in a significant accumulation of ssDNA followed by subsequent accumulation of DSBs. Such structures are responsible for ATR activation, which ultimately results in the inhibition of Cdc7-Dbf4 protein kinase. Cdc7 activity is down regulated, possibly by dissociation of Dbf4. In turn Cdc45 chromatin loading is inhibited and as a consequence also the activation of the pre-RC is prevented (Costanzo et al., 2003). Interestingly, upon ATR activation, the other Cdc7 subunit Drf1 binds to chromatin suggesting that following DNA damage, exchange of regulatory subunit may play a role in the checkpoint response (Dierov et al., 2004;
1.7 **ATM and ATR Control the Selection and Activation of DNA Replication Origin Firing in Higher Eukaryotes**

The position of replication origins and the time at which these origins are activated represent the main factors affecting the time required for replication to be completed. In mammalian cells, some chromatin loci have been found to show a reproducible initiator activity like the β-globin (Huberman, 1998) and DHFR loci (Dijkwel et al., 2002). However in higher eukaryotes sequence-dependent initiation does not appear to be absolutely required.

This observation gives rise to the realistic possibility that a sequence specific replication origin is not necessary as long as mechanisms that regulate origin firing exist. *Xenopus laevis* represents an optimum model system to study the regulation of replication initiation. In the *Xenopus* system, a genome of 6,000 Mbp is replicated in a very short time and more than 200,000 replication origins have to fire to allow the complete replication. A mechanism that regulates origin firing becomes extremely important in particular during the first 12 embryonic cell cycles (early embryonic cell cycle) when each cycle lasts 15-20 minutes before each cleavage. Only after mid blastula transition, when the embryonic cell cycle length gets close to resemble the one of somatic cells, the sites of initiation DNA replication become restricted to specific DNA sequences (Hyrien et al., 2003). In such an embryonic system, replication origin distribution cannot be random despite the absence of sequence specificity (Hyrien and Mechali, 1993). In this scenario (also known as the random-completion problem) (Hyrien et al., 2003), we can assume that any replication sequences bigger than 20 kb would generate inter-origin fractions too long to be replicated. This would cause the formation of portions of unreplicated DNA persisting till the end of the S phase (Laskey, 1985). Therefore, despite the lack in sequence specificity a sequence-independent mechanism should exist to regulate origin spacing. The rate of DNA replication has been determined to be in the order of of 0.5 Kb /min (around 10 nucleotides/second) (Mahbubani et al., 1992). Considering a time of 20 minutes occurring for S phase to be completed, this would imply that each fork is able to travel
for 10 Kb covering a distance of 20 kb when a bidirectional fork is activated per replication origin. Lucas and colleagues analysed DNA replication origin firing of different plasmid sizes in Xenopus egg extract, a system that closely mimics replication of Xenopus sperm nuclei. Using this approach, they first manage to confirm that replication starts in a sequence independent manner. Then performing 2D gel electrophoresis and electro-microscopy analysis they observed that plasmids shorter than 15 kb could support just one initiation event compared to the multiple initiations observed for plasmids longer than 20 kb. More studies performed in Xenopus extract showed that in any DNA template that can support DNA replication the origin firing occurs at random sequences (Hyrien and Mechali, 1993; Lucas et al., 2000) (Blow et al., 2001). In 2001 Blow and colleagues by using DNA fiber techniques to examine the distribution of replication origins on sperm nuclei replicating in Xenopus egg extract, demonstrated that DNA origins were grouped into small clusters firing at approximately the same time. They also show that instead different clusters could be activated at different times during S-phase (Blow, 2001).

Studies in eukaryotic cells, have shown that an excess amount of MCM2-7 complexes are loaded to chromatin compared to ORC (Rowles and Blow, 1997). This results in a number of MCM2-7 complexes higher then the number actually engaged for origin firing. Moreover, once MCM2-7 complex has been recruited to chromatin both Cdc6 and ORC become dispensable for any subsequent origin firing (Hua et al., 1997). In physiological conditions S-phase kinases activated at the S phase onset would be able to trigger the unregulated MCM2-7 dependent melting of multiple DNA replication origins. How can eukaryotes control the distance and timing of their randomly sequenced origins?

In 2004 three studies suggested the existence of a dynamic mechanism which would allow regulation of origin spacing through the diffusion of an inhibitory signal engaged from active replicons (Marheineke and Hyrien, 2004; Shechter et al., 2004b; Sorensen et al., 2004).

In accordance with this model, replication intermediates and ssDNA coated by RPA generated from stochastically melted DNA bubbles, would be able to trigger a DNA damage response locally activated at the sites of origin firing. ATM and ATR
down-regulate Cdk2 and Cdc7 respectively in response to DNA damage (Fig. 1.4) (Abraham, 2001; Costanzo et al., 2003). In this way activated ATM and ATR kinases would prevent neighbouring origins from firing (Marheineke and Hyrien, 2004; Shechter et al., 2004a). Consistently, both caffeine (a non specific ATM-ATR inhibitor) and UCN-01 (a specific Chk1 inhibitor) lead to a rapid increase of Cdk activity followed by a higher density of origins fired (Lehmann, 1972; Shechter et al., 2004b). Studies in the SV40 viral model system revealed that inhibition of Chk1, ATM or ATR function stimulate DNA replication initiation of both cellular and viral origins, in the absence of stalled replication forks and DNA damage (Miao et al., 2003). Furthermore, Sorensen and colleagues proposed that in the absence of DNA damage an ATR/9-1-1/Claspin/Chk1 pathway controls Cdc25A and Cdk activity in mammalian cells (Sorensen et al.).

An intriguing observation is that in an unperturbed cell cycle, ATM, ATR and consecutively some downstream components of the DNA damage checkpoint response are active at a low level to guarantee origin firing regulation. This would suggest that DNA damage in S-phase results in an amplification of an already activated ATM-ATR regulated pathway. Consistent with this, Chk1 protein kinase has been found to be phosphorylated in a normal cell cycle followed by hyperphosphorylation upon DNA damage (Jiang et al., 2003).

This emerging role of such protein kinases may be important for the developing of new anticancer drugs that specifically target such kinases.
**Fig. 1.4 Hypothetical ATM-ATR regulation of DNA replication origin firing**

At entry in S-phase, some origins are stochastically fired after the action of SPKs while a ATM-ATR feedback from active replicons results in the inhibition of the origin firing on adjacent origins
1.8 Conclusions

In prokaryotes and eukaryotes, the first step towards the initiation of DNA replication is the binding of origins by an initiator protein. This event takes place in G1 phase of the cell cycle. The initiator factor represents the first component of the pre-replicative complex which provides a landing platform for the recruitment of additional factors at the DNA replication origins. In eukaryotes the initiator factor is ORC. ORC binding is followed by the association of Cdc6, Cdt1 and MCM2-7 complex (representing the presumptive replicative helicase). At the onset of S phase the replicative DNA helicase is activated. This activation step requires the combined function of two protein kinases CDK and DDK. In addition to them at least six auxiliary factors are also involved in the helicase activation: Mcm10, TopBP1, Sld2, Sld3, GINS and Cdc45. Finally, DNA polymerases are recruited to allow the complete generation and progression of the replication fork. The process that allows DNA replication to initiate is well conserved across all species. Protein kinases CDK and DDK play a pivotal role in promoting the switch from G1 to S phase after pre-RC has been assemble. At the heart of this complicated process is the phosphorylation of proteins Sld2 and Sld3 representing the minimal CDK substrates essential to promote origin firing. Sld2 and Sld3 have been identified so far in budding and fission yeast. An Sld2 orthologue has been discovered in X.leavis (Matsuno et al., 2006) while the identification of Sld3 orthologue in higher eukaryotes is still ongoing.

I have also described some of the regulatory mechanisms that ensure DNA replication to occur once per cell cycle. This is accomplished by inactivating pre-RCs after S phase begins and preventing new pre-RCs formation until mitosis is completed. These events are driven by CDK acting on ORC, Cdc6, MCM2-7 and Cdt1. In metazoans Geminin protein also collaborates with CDK in regulating Cdt1 activity.

Finally, I highlighted the mechanism that is believed to dynamically regulate origin firing in metazoan eukaryotic DNA. Two checkpoint protein kinases ATM and ATR are proposed to regulate both the selection and the timing of origin firing. They would act from stochastically activated origins downregulating the activity of CDKs and DDKs of adjacent origins.
1.9 Overview of the Work Described in this Thesis

Most of the basic biochemical mechanisms of DNA replication have been conserved from bacteria to human but the evolution of eukaryotic cells resulted in many changes concerning the regulatory mechanisms of DNA replication. Although recent studies have unveiled the identity and the molecular function of new factors involved in the initiation of DNA replication, still more unknown factors are likely to be involved in this process. This thesis discusses the identification and characterization of a new component of the replication initiation machinery; XlGEMC1. *Xenopus laevis* cell free extract was used as main model system to perform biochemical characterization of XIGEMC1 (chapter 2). Moreover I report studies in *Xenopus* embryos that reveal a role of XIGEMC1 in development (chapter 3). Finally, preliminary data in mammalian cells are also discussed to assess the functional conservation of GEMC1 protein (chapter 4) in higher eukaryotes.
2 Materials and Methods

2.1 Chemicals and reagents

2.1.1 Suppliers of reagents

Unless stated, all the chemicals were obtained from Sigma-Aldrich, BDH Laboratory Supplies (UK) or Fisher Scientific (UK). Standard solutions of 0.5 M EDTA, 1M Tris-EDTA (TE), 1M MgCl₂, 5M NaCl, Phosphatase Buffered Saline (PBS) and Tris Borate EDTA (TBE), Fetal calf serum (FCS). Dulbecco’s modified Eagle’s medium (DMEM) was prepared by Cancer Research UK London Research Institute (LRI) Central Services. All other stock solutions were made according to standard methods (Sambrook et al.).

2.1.2 Suppliers of commonly used reagents are listed below:

Agarose, ammonium persulfate (APS), ethidium bromide, sodium dodecyl sulfate (SDS) and N,N,N,N’-tetra-methyl-ethylenediamine (TEMED) were obtained from Bio-Rad (UK). Ultra Pure ProtoGel Acrylamide was obtained from National Diagnostics (UK). Complete Protease Inhibitor Cocktail Tablets were obtained from Roche (UK). All primers were produced by Sigma-Genosys (UK).

2.1.3 Bacterial media and general solutions

*Luria-Bertani Broth (LB)*

1% w/v bacto-tryptone (DIFCO)

0.5% w/v yeast extract (DIFCO)

0.1 M NaCl

pH adjusted to ~7

*LB agar*

LB broth + 2% (w/v) Bacto agar
**SOC**

2% w/v bacto-tryptone  
0.5% w/v yeast extract  
10mM NaCl  
2.5mM KCl  
10mM MgCl₂  
10mM MgSO₄  
20mM glucose  
pH adjusted to ~7

**PBS (Phosphate Buffered Saline)**

0.13M NaCl  
7mM Na₂HPO₄  
3mM NaH₂PO₄  
pH adjusted to 7.5  
Routinely, a 10x stock solution was prepared and diluted in water before use.

**TBS (Tris Buffered Saline) and TBST**

10mM Tris-base  
150mM NaCl  
0.1% Tween-20 (only for TBST)  
pH adjusted to 7.5 with HCl  
Routinely, a 10x stock solution was prepared and diluted in water before use.

**TE (Tris-EDTA)**

1mM Tris-Cl pH 7.5  
0.1mM EDTA pH 8.0  
Routinely, a 10x stock solution was prepared and diluted in sterile water before use.
2.2 Bacteria strains

2.2.1 Bacteria strains

*From Invitrogen company*

Library efficiency DH5α Competent Cells

MAX Efficiency DH5α Competent Cells

One shot TOP10

One shot BL21-A1

*From Stratagene*

BL21 Codon Plus (DE3-RIL)

XL1-Blue Supercompetent Cells

2.2.2 Bacteria storage

*E. coli* strains were stored at 4°C on solid medium containing the appropriate antibiotic, for up to 1 week. For long term storage glycerol stocks were made (overnight culture was frozen in ethanol/dry ice in the presence of 30% glycerol) and stored at -80°C.

2.3 Molecular biology techniques

2.3.1 Plasmid minipreparation

For plasmid minipreparation a QIAGEN miniprep kit was used according to the manufacturer’s instruction.

2.3.2 Restriction digests and ligation reactions

DNA was digested in a final volume of 20 µl at 37°C for 1 h. All the restriction enzymes were from New England Biolabs, and digestions were performed in appropriate buffers, supplied by the manufacturer with the enzyme. All digestions were analysed by agarose gel electrophoresis.

Ligation reactions were performed using Quick T4 DNA ligase (New
England Biolabs) as recommended by the manufacturer:

- 10 µl 2X Quick Ligation Buffer
- 50 ng vector DNA
- 3X molar excess of insert DNA
- 1 µl of Quick T4 DNA Ligase (New England Biolabs)
- dH₂O to 20 µl

Ligation was carried out for 5 minutes at room temperature.

2.3.3 Agarose gel electrophoresis

Horizontal agarose gels were routinely used for the separation of DNA fragments. All agarose gels were 0.8% w/v agarose (SseKem LE, Cambrex) in 1xTBE (89 mM Tris-Base, 89 mM Boric acid 1mM EDTA pH8.0). The samples were loaded in 1x loading dye (6x stock: 0.25% bromophenol blue; 0.25 xylene cyanol FF; 30% v/v glycerol). Gels also contained 1µg/ml ethidium bromide to allow visualisation of the DNA under UV light. Gels were run at ~6V/cm of the distance between the two electrodes. 1Kb ladder (New England Biolabs) was used for fragment size determination.

2.3.4 Purification of DNA from agarose gels

Following agarose gel electrophoresis, DNA gel slices were excised under UV light. DNA was extracted from these gel slices using Qiaquick columns (Qiagen) following the gel extraction protocol supplied by the manufacturer. Purified DNA was eluted from the columns using 30-50 µl deionized water.

2.3.5 DNA sequencing

Sequencing reactions were carried out using the BigDye Terminator v.3.0 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. The Cancer Research UK Sequencing Service was used for analysis of the sequencing reactions. In all cases, both strands were sequenced. Sequences were analyzed using Sequencher v4.5 software (Genecodes).
2.3.6 Transformation of E. coli with plasmid DNA

Plasmid transformation into *E. coli* 100µl of competent cells were mixed with transformation DNA and incubated on ice for 30min. The cells were then heat-shocked at 42°C for 30 sec, and cooled on ice. 1ml of SOC was then added and the tubes were incubated at 37°C with shaking for 1 hour. Lastly, the cells were spun down and plated onto selective plates.

2.3.7 Site directed mutagenesis

Point mutations in XIGEMC1 ORF were introduced by using the QuikChange site-direct mutagenesis kit, PfuTurboDNA polymerase and temperature cycler following the instructions of the manufacture’s. The presence of the point mutation was then confirmed by DNA sequencing.

The following primers were used to mutate XIGEMC1 S/T-P to alanine:

Fw_T153A 5’- CCATTCCAGCAGCAATTCTGCTCTGGGAGTAAGGCAAG -3’
Rw_T153A 5’- CTTGGCCTTTAATTCCAGGAGCAATTGCTCTGGGATAATGG-3’
Fw_S177A 5’- CTGTGAAGCCAGTGTCACGTTGATAGGAAG-3’
Rw_S177A 5’- CTTCCTCTACAACCTGGAGGGGAACCTGGGCTACACAG-3’
Fw_S215A 5’- CACCCAAGAAGCAAGCGTCCCTCTCTGTGCTGCAAG-3’
Rw_S215A 5’- GATCCCAAGGAGAAGGGCCGCTCTTTGCTTGGCTG-3’
Fw_T226A 5’- GTTCTTGGCCACCTGGCCCTGGGACACAGCC -3’
Rw_T226A 5’- GGCTGTGTCAGGGGCAAGTTGGCAGAAAC-3’
Fw_S239A 5’- GCCACCTCTTGTAGCCCTCAGACACCTCCAATGTG-3’AGCTC
Rw_S239A 5’- GAGCTACATTGGAGAGGTGGCGAAGGCTACAGAG-G3’GTGGC
Fw_S255A 5’- GAAAGAGACTGCCGACCTTTTCTCTCCTTCTTC-3’
Rw_S255A 5’- GGGAGAGAGAGGTGGGCGCAGTCTCACTTTTAC-3’
Fw_S259A 5’- GCCAGCAATCCTGCTCGGCTACACAGGACACACCACC-3’
Rw_S259A 5’- GGTGTGTTAGGTGGGCAAGAGTGGGGCTGGAG-3’
Fw_T294A 5’- CTCCCCACCTCCAGCCCTTGATGTGGCCTCC-3’
Rw_T294A 5’- GGAGCCACATCAGGGGCTGGGATGGGAGA-3’
The following primers were used to mutate XIGEMC1 S/T-P to glutamate:

**Fw_T153E5’**- CCATTCAGCAGCAATTCTGTAGCCTGGGGAGTAAGGGCAAAG-3’
**Rw_T153E** 5’- CTTGCGCTTACTCCAGGCTGGAATGCTGCTGAATGG-3’
**Fw_S177E** 5’- CTGTGAAGCCCAGCAGCTTTGAGAGGAAG-3’
**Rw_S177E** 5’- CTTCTCTAAACTGGCTGCCAGCTTTGCTACAG-3’
**Fw_S215E** 5’- CTGTGAAGCCCAGCAGCTTTGAGAGGAAG-3’
**Rw_S215E** 5’- CTTCTCTAAACTGGCTGCCAGCTTTGCTACAG-3’
**Fw_T226E** 5’- GTTCTGCCACCAGCTGGACATGGACACAGCC-3’
**Rw_T226E** 5’- GGCTGTGTCCAGGCTTGCTTTGGCTG-3’
**Fw_S239E** 5’- GCCAACCTCTTTGTAGCCTCAGTCCTTCCTCCAAATGT-3’AGCTC
**Rw_S239E** 5’- GAGCTACATTTGGGAAGGGCTGAGCTTCAAGAGAGCACC-3’GTGGC
**Fw_S255E** 5’- GGGAGAAAGAGAGTGTCGGCGCAGCTCTACTCTTTTCCC-3’
**Rw_S255E** 5’- GGGAGAAAGAGAGTGTCGGCGCAGCTCTACTCTTTTCCC-3’
**Fw_S259E** 5’- GCCAGCGCCTCTCAGGCCCCACCTACCAACACC-3’
**Rw_S259E** 5’- GGTGTTGGTAGGGTGCTGACAGAGTAGGGCTGCGG-3’
**Fw_T294E** 5’- CTCCCACTTACCACAGCTGGCTGTGAGGTGGGA-3’
**Rw_T294E** 5’- GGAGGCCACATCAGCTGGCTGTGAGGTGGGA-3’

The following primers were used to mutate XIGEMC1 cyclin binding site R163NL to alanine ANA:

**Fw_R163NL/ANA**
5’- GGCCAAGAGGGCCAGGCAAAACGCGTATGGGGAAAECTCACTGCC-3’
**Rw_R163NL/ANA**
5’- GGCAGTGAGTTCATCCAGCCGCTGCTGTGAGGTGGGA-3’

**2.3.8 Amplification of constructs for expression studies**

To clone the full-length of XIGEMC1 cDNA into pMAL-c4X (Invitrogen) and pGEX-6P-1 (GE-healthcare) expression vectors conventional Polymerase Chain Reaction (PCR) was performed with appropriate primers that incorporated convenient restriction enzyme sites. *Xenopus laevis* egg cDNA library built in a modified pCS2 expression vector was used as template for amplifying XIGEMC1 cDNA. *Pfu Turbo* Polymerase (Stratagene) was used as follows:
5 µl 10x *Pfu turbo* buffer
0.4 mM of each dNTP
125 ng forward primer
125 ng reverse primer
1 µl *Pfu turbo* Polymerase
50 ng DNA template
dH₂O to 50 µl

For the amplification of XIGEMC1 cDNA, PCR reactions were usually performed with the following cycling parameters:

1 cycle: 5 min at 95°C
30 cycles: 30 sec at 95°C
30 sec at the primer annealing temperature
2 min at 72°C (or 1 min per each Kb of DNA to amplify)
1 cycle: 10 min at 72°C

The resulting PCR product was analysed by agarose gel electrophoresis, and extracted from the gel using Qiaquick columns (Qiagen), according to the manufacturer’s protocol. The purified DNA was then digested with the appropriate restriction enzymes and the resulting fragments were subcloned in frame (as described in the next paragraph)

### 2.3.9 Expression of recombinant proteins

#### 2.3.9.1 MBP and GST recombinant proteins

MBP and GST tagged XIGEMC1 recombinant proteins were generated as follow. Full length XIGEMC1 was amplified by PCR using 5’ primer containing a restriction site for BamHI:

5’ GGATCCAAACACTATTCTAACTTGCCAAGACGAG 3’

and 3’ primer containing a restriction site for Sall:

5’ GTCGACGCTCATTCAGACTGCTTGGGCACCCAAGTGAACTTCC 3’.

The PCR product was cloned into pCR-BluntII TOPO vector (Invitrogen). The
fragment from cDNA amplified was subsequently digested with BamHI-SalI and BamHI-NotI and subcloned respectively into pMAL-c4X vector (Invitrogen) and pGEX-6P-1 vector (GE-healthcare). The vectors (3 µg) were digested using the same restriction enzymes of the respective inserts, the DNA were analysed and purified from agarose gel (eluted in 30 µl). Ligation reactions were performed using Quick T4 DNA ligase (New England Biolabs) as previously described (paragraph 2.3.2). Ligation was carried out for 5 minutes at room temperature. 2µl from the whole reaction were used for transformation of chemical competent MAX Efficiency DH5α competent cells. The resulting clones were analysed by restriction digest and sequencing.

MBP and GST tagged GEMC1 were expressed in BL21-A1 (Invitrogen) and BL21 Codon Plus (DE3-RIL) (Stratagene) cells and purified on amylose resin or Glutathione Sepharose 4B resin according to manufacture protocols.

2.3.9.2 Histidine-tagged recombinant protein

6xHis-XIGEMC1 was generated using Gateway technology (Invitrogen). Full length ORF of GEMC1 was generated by PCR as previously described using the following primers:

5’ primer 5’CACCAACACTATTCTAACCTTGCCAAGACGAGTACTTTGC 3’ and
3’ primer 5’ TCATTCAGACTGCTTGGGCACCCAAGTGAACTTCC 3’.

The amplified PCR product was cloned into the pENTR vector (Invitrogen) by Gateway technology and finally transferred to destination vectors with Histidine-tag (pDEST17, Invitrogen). Recombinant protein was expressed in BL21-CodonPlus (DE3)-RIL cells (Stratagene) and purified on a Ni-NTA column (Qiagen) in denaturing conditions according to the manufacturer protocol.

Baculovirus encoding 6xHis-TopBP1 (a gift from H. Takisawa) and 6xHis-Cdc45 (a gift from J. Gautier) were used to infect Sf9 cells, which were expanded in large Petri dishes using complete GRACE medium (Invitrogen) according to manufacturer protocols. The recombinant proteins were purified on Ni-NTA column (Qiagen) following the instructions of the supplier.
2.3.10 Quantification of proteins

Protein concentrations were determined using Bradford methods (Bradford, 1976). Protein samples were mixed with Bradford’s reagent (Biorad) and the absorbance at 595 nm was measured on a spectrophotometer. Protein absorbances were converted to mg/ml concentrations using a standard curve constructed by measuring the absorbances of a range of bovine serum albumin (BSA) concentrations.

2.4 Antibodies

2.4.1 Anti XlGEMC1 antibodies and affinity purification of anti XlGEMC1 polyclonal antibodies

Rabbit *Xenopus* GEMC1 antisera were generated by Harlan UK and Biogenes (Berlin, Germany) against recombinant 6His-tagged XlGEMC1 protein expressed in bacteria. 16 rabbits were injected. Serum from each rabbit was collected and screened for the ability to recognize recombinant and endogenous XlGEMC1. IgGs specific for XlGEMC1: XlGEMC1 Ab-1 (Harlan, Animal ID: XZLB, PAS n° 117775), XlGEMC1 Ab-2 (Harlan, Animal ID: DUDG, PAS n° 450), XlGEMC1 Ab-3 (Biogenes, Animal ID: 2814) were further purified using NHS Hi-Trap affinity columns (GE Healthcare).

The columns were washed with 10 volumes of coupling buffer (200mM NaHCO$_3$/ 500mM NaCl pH 7.8). All the antigens were dialysed in coupling buffer. Following this, the appropriate ligand was bound to the column by recirculation of 5mg of recombinant protein (MBP-XlGEMC1) over the column for 2 hrs at RT. In order to deactivate any excess groups that have not coupled ligand and to wash out not-bound ligand, the columns were washed in buffer A (500mM NaCl/ 500mM Ethanolamine, pH 8.3) and buffer B (100mM CH$_3$COONa/ pH 4.0). After columns preparation, a 1:10 dilution (in PBS) of rabbit serum was recirculated over the column over night at 4°C. Then, the columns were washed with 20 volumes of 10mM Tris/ 500mM NaCl/ pH 7.5 and eluted with 100mM Glycine pH 2.25. 500 µl fractions were collected in 50µl of 1M Tris pH 8.0 in order to neutralise the acidic eluate and preserve antibody activity. The purified antibodies so purified were then dialysed in PBS and stored at 4°C. The procedure was repeated for 3 different sera to obtain 3
different batches of anti XlGEMC1 (Ab-1, Ab-2 and Ab3).

2.4.2 Antibodies

Table 2.1 lists the antibodies that were used in this study.

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Secondary antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen/Name</td>
<td>Provider</td>
</tr>
<tr>
<td>Xenopus Cdc7</td>
<td>J.Walter</td>
</tr>
<tr>
<td>Xenopus Cdc45</td>
<td>H.Takisawa</td>
</tr>
<tr>
<td>Xenopus Cdk2</td>
<td>J.Gannon</td>
</tr>
<tr>
<td>Xenopus Cdt1</td>
<td>J.Walter</td>
</tr>
<tr>
<td>Xenopus cyclinE</td>
<td>J.Gannon</td>
</tr>
<tr>
<td>Xenopus Orc1</td>
<td>J.Gannon</td>
</tr>
<tr>
<td>Xenopus Orc2</td>
<td>J.Gannon</td>
</tr>
<tr>
<td>Xenopus TopBP1</td>
<td>H.Takisawa</td>
</tr>
<tr>
<td>Mcm7(sc9966)</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Histone H2B (07-371)</td>
<td>Upstate</td>
</tr>
<tr>
<td>Xenopus Sld5</td>
<td>H.Takisawa</td>
</tr>
<tr>
<td>Mouse Cdc45 (sc20685)</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Mouse HRP-</td>
<td>Abcam</td>
</tr>
<tr>
<td>GADPH</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1 Antibodies used in this study
2.4.3 Western blotting

*Xenopus* proteins (extract and chromatin bound) and around 30 µg of NIH 3T3 total protein were loaded onto either 10% SDS PAGE gel or Criterion XT precast gel 4-12% Bis-Tris (Biorad) along with 10 µl of Marker precision plus protein standard (Biorad). Separated proteins were transferred to Nitrocellulose transfer membrane Protran (Whatman) overnight at 200 mA using a Biorad wet transfer apparatus. Membranes were washed, and blocked for 1 hour with 3% (w/v) non-fat powder milk in TBS-T (50 mM Tris.HCl, pH 7.4 and 150 mM NaCl.+0.1% Tween) at room temperature. Antibodies were prepared at dilutions indicated in table 2.1 in 3% milk in TBS-T. Membranes were incubated overnight at 4°C with primary antibodies, followed by 3 washes with TBS-T. Immunoreactive proteins were detected using HRP-conjugated secondary antibodies (dilution indicated in Table 2.1) in 5% milk TBS-T. Membranes were washed 3 times and antibody complexes detected using ECL substrate (Amersham Pharmacia), and visualized on Hyperfilm ECL (Amersham).

2.4.3.1 Membrane stripping and re-probing

For re-probing membranes, bound antibodies were removed by incubation with Stripping Buffer (100mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH6.7) for 30 minutes at 50°C. The membranes were washed three times for 10 minutes in TBS-T, followed by blocking and probing as described above.

2.4.3.2 Coomassie blue staining

After separation of loaded protein samples, polyacrylamide gels were incubated in Coomassie blue stain (0.5% Coomassie blue, 45% methanol, 10% acetic acid) for 30 minutes and destained with destain solution (25% methanol, 7% acetic acid) for 1 hour.

2.4.4 Antibodies cross-linking to resin

To covalently immobilize the antibodies on resin the seize X Protein A immunoprecipitation Kit (Pierce) was used. 300 µg of purified anti XIGEMC1 antibodies were cross-linked to 300 µl of immobilized Protein A disuccinimidyl suberate (DSS) as cross-linker. The manufacturer’s instructions were followed.
2.4.5 Anti phopho antibodies

N terminus 20-mer XIGEMC1 peptides containing phosphorylated threonine 153 at position 8 (CPFSSNST(p)PGSKAKRARRNL) produced by the LRI peptide facility were conjugated to KLH (Pierce) according to manufacturer instructions and injected in 2 rabbits (Harlan). IgGs specific for phospho-T153 were affinity purified against the phospho-peptide by using the SulfoLink resin (Pierce). Briefly, 5 mg of phospho peptide T-153 and 5 mg of un-phosphorylated peptide T-153 were dissolved in 3 ml of coupling buffer (50 mM Tris pH 8.5, 5 mM EDTA-Na), transferred to a column and incubated over night at 4°C under rotator. After incubation, the columns were washed with 2 ml of coupling buffer and 15 ml of wash solution (1.0 M NaCl, 0.05% NaN3). Both rabbit sera (776 and 778) were filtered through 0.22 µm Millex GP filter unit (Millipore). 5 ml of each serum were first loaded onto phospho-T153 column and incubated overnight with rotation. Antibodies were eluted by adding 10 ml of 0.1 M Glycine pH 2.5. 500 µl of eluted fractions were collected in eppendorf tube pre filled with 50 µl of 1 M Tris-HCl pH8.0. The eluted IgGs were sequentially loaded onto non-phospho-T153 column to eliminate non-phospho -153 specific IgGs.

2.4.6 Immunoassay

ELISA technique was used to test the specificity of the anti XIGEMC1 phospho-T153 antibodies. 10 mg of phospho-T153 peptide (hapten) were covalently coupled to 40 mgs of KLH/Ovalbumin (carrier) by using MBS cross-linker following the manufacturer’s instructions. The hapten-carrier conjugated complex was diluted 1:1000 and immobilized on an high protein-binding capacity polystyrene 96 well (Corning Costar, eBioscience) following an incubation of 2 hours at 37°C. Complex bound to the solid support was subsequently blocked with 1% milk in PBS for 30 minutes. Serial dilutions of purified XIGEMC1 phospho-T153 antibody (1:100, 1:300, 1:900) were added to the microtiter in the presence of Buffer, non phospho peptide or
phospho T-153 peptide (100 µg/ml final concentration) to compete with the antibody for the interaction with the hapten. After 1 hour incubation at room temperature, the titer was washed in PBS 0.1% Tween, incubated with anti rabbit LICor 1:5000 for one hour and read on Licor scanner using 800 nm as wavelength.

2.5 Frog techniques

2.5.1 Preparation of interphase Xenopus egg extract
S-phase extracts was essentially prepared as previously described (Newport and Forbes, 1987) with some modifications brought by Kubota and Takisawa (Kubota and Takisawa, 1993). Briefly, freshly laid Xenopus eggs were collected in 0.6% NaCl; those that appeared degenerative were removed. Unfertilized eggs were dejellyed for 5 minutes in dejelllying buffer consisting of 5 mM dithiothreitol, 110 mM NaCl, and 20 mM Tris-HCl at pH 8.5, washed in 1/4 MMR (MMR: 0.4 M NaCl, 2 mM KCl, 1.0 mM MgSO4, 2.0 mM CaCl2, 0.1 mM EDTA, and 25 mM Hepes-KOH at pH 7.5), and activated with 0.5 µg/ml calcium ionophore A23187 in MMR. Activated eggs were then washed 3 times with 1/5 MMR, followed 3 washes with ice-cold S buffer (0.25 M sucrose, 50 mM KCl, 2.5 mM MgCl2, 2 mM β-mercaptoethanol, 15µg/ml leupeptin, and 50 mM Hepes-KOH at pH 7.5). The washed eggs were packed into tubes by brief centrifugation for several seconds at 6000 rpm. All excess buffer was removed and the eggs were crushed by centrifugation at 15,000 g for 10 min at 4°C. The resulting cytoplasm layer (middle layer) between the lipid cap and pellet was collected and mixed with cytochalasin B (40 µg/ml final concentration). Samples were centrifuged at 70000 rpm for 15 minutes at 4°C to remove residual debris. The egg extracts were supplemented with 30 mM creatine phosphate, 150 µg/ml creatine phosphokinase and 40 µg/ml of cycloheximide (inhibitor of protein biosynthesis) The extract obtained was used either immediately or supplemented with 3 % glycerol and stored in liquid nitrogen.

2.5.2 Preparation of sperm chromatin
Demembranated sperm chromatin for replication assay was prepared from
testes of male frogs primed with 50U Folligon seven days in advance. Testes were rinsed three times in cold MMR buffer (100 mM NaCl, 2 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 0.1 mM EDTA, 5mM Hepes pH7.8), twice in cold NPB buffer (250 mM sucrose, 15mM Hepes pH 7.4, 1 mM EDTA, 0.5 mM spermidine trichloride ,0.2 mM spermidine, tetrachloride, 1 mM DTT) and finely chopped with a razor. The obtained material was homogenized in a homogeniser, filtered through 25 µm Nylon membrane and centrifuged at 3000 rpm for 10 min at 4°C in HB-4 swing-out rotor. The pellet was resuspended in 1 ml of NPB buffer (250 mM sucrose, 15 mM Hepes pH 7.4, 1mM EDTA, 0.5 mM spermidine trichloride ,0.2 mM spermidine, tetrachloride, 1 mM DTT) at room temperature and 50 µl of 10 mg/ml lysolecithin was added and samples were incubated at room temperature for 5 min. Sperm demembranation was tested by mixing 1 µl of sample with 1 µl of Hoesht stain (1µl/ml). Following demembranation greater then 95%, 10 ml of cold NPB buffer supplemented with 3% BSA was added to the 1 ml sample and centrifuged at 3000 rpm for 10 min at 4°C in HB-4 swing –out rotor. Obtained pellet was resuspended in 500 µl of cold NPB buffer supplemented with 0.3% BSA and 30% glycerol. The sperm density was then counted, adjusted to 10⁷ sperms/ml and aliquots were quickly frozen in liquid nitrogen.

2.5.3 cDNA expression library screening

For the screening performed in this thesis, I used a full-length *Xenopus laevis* egg cDNA library. cDNAs were built in a modified pCS2 expression vector. The library was donated by Tony Hyman (Max Planck Institute of Molecular and Cellular Biology and Genetics, Dresden, Germany). Bacterial cells were transformed with *Xenopus* cDNA and individually arrayed in 384-well plates. Pools of cells corresponding to rows and columns from each plate were grown. Plasmid DNA was purified from each pool using a Qiagen miniprep kit and 35S-methionine (Promix, Amersham) labelled recombinant proteins were generated using a SP6 quick-coupled transcription-translation reticulocyte lysate (TNT, Promega). After the translation was completed, 2 µl of the 35S -labelled lysates were added to 2µl egg extracts previously treated with or without DSBs (50 ng/µl) in the presence of Ku55933 (20 µM) or caffeine (2 mM). Samples were incubated for 30 minutes at 23 °C. The reactions were stopped by adding 8µl of Leammli buffer. Samples were run on a 10% SDS–PAGE. Proteins were identified by isolating their corresponding cDNA by intersecting pools
from rows and columns of the plate. XIGEMC1 cDNA sequence was determined by sequencing both strands.

2.5.4 Chromosomal and single strand DNA replication assay

Replication of sperm DNA in egg extracts was measured by monitoring the incorporation of $\alpha^{32}$P-dATP. Purified anti XIGEMC1 antibodies to inhibit DNA replication (80 ng/µl) and rec-XIGEMC1 protein (100 ng/µl) were pre-incubated with extract on ice for 30 minutes. Sperm nuclei at the concentration of 3000 nuclei/µl were added to interphase extracts and replicated in the presence of $[\alpha^{32}]$P dATP at 23°C. The replication reactions were stopped at 120 min with Stop buffer (1% SDS, 80 mM Tris, pH 8, 8 mM EDTA). The samples were digested with 1 mg/ml proteinase K for 1 hour at 37°C, DNA was extracted with phenol/chloroform/isoamylalcohol and precipitated with cold 70% ethanol. The total incorporated radiolabelled DNA was detected by autoradiography. Samples were treated with RNase (0.6 mg/ml) to degrade any trace of RNA and purified DNA was then separated from unincorporated label by electrophoresis through a 0.8% agarose gel. The gel was fixed in 30% TCA for 20 min, dried and exposed for autoradiography. For quantification of DNA replication, the gel was exposed to a phosphoscreen, the signal was obtained with a PhosphorImager and quantified by ImageQuant software. Replication of single-stranded DNA of M13 phage was performed in a similar manner using 12.5 ng/ml of DNA per reaction.

Single stranded DNA was incubated with 20 µl of extract in the presence of buffer, preimmune IgG or affinity purified anti XIGEMC1 antibody (80 ng/µl). DNA replication was monitored using $\alpha^{32}$P-dATP. Reactions were stopped by adding 10 volumes of stop buffer (80 mM Tris-HCl pH 8.0, 8 mM EDTA, 1% SDS. DNA was then isolated and run on 1.2% agarose gel, which was dried and exposed.

2.5.5 Chromatin binding

For all chromatin binding experiments sperm nuclei (3000 nuclei/µl) were added to 50 µl of egg extracts for the indicated times (see figure legends). To isolate the chromatin samples were diluted with 10 volumes of EB buffer (100 mM KCl, 2.5 mM MgCl$_2$, 50 mM HepesKOH, pH 7.5) containing 0.25% Nonidet P-40 (NP-40) and layered onto 150 µl of a 30% sucrose cushion made of the same buffer lacking in NP-40. Chromatin was spun at 10,000xg at 4°C for 5 minutes using a swing-out bucket.
rotor, washed with 500 µl EB buffer and spun again at 10000xg for 5 minutes, in a fixed-angle rotor. The pellet was resuspended in 40 µl of Laemmli loading buffer, shaken for 20 minutes in Thermomixer (800 rpm) at room temperature to allow the chromatin pellet to completely dissolve and loaded onto a 10% SDS–PAGE. Bound proteins were analysed by western blotting with specific antibodies, as indicated in the figure legend.

### 2.5.6 Pull-down assays

#### 2.5.6.1 Into the extract

For pull down assays, MBP or MBP-GEMC1 recombinant protein (3 µg) were incubated with 200 µl of extract at 23 °C for 30 minutes. 60 µl of amylose resin were added to the reactions that were then diluted with 700 µl of PBS 0.4% Triton X-100. After 2 hours incubation at 4°C, beads were harvested, extensively washed with PBS supplemented with 600 mM NaCl and 4% Triton X-100 and processed for SDS_PAGE. Beads were resuspended in 100µl of Laemmli buffer and boiled for 5 minutes. 20 µl (Fig 3.22a) or 15 µl (Fig.3.22b) of samples were loaded on SDS-PAGE gel and Western blotting was performed using antibodies against MCM7, Cdc45, CyclinE, Cdc7, Cdt1, Orc1 and Sld5.

#### 2.5.6.2 In vitro

$^{35}$S-labeled GEMC1 (3 µl of IVTT) was added to 100 µl of conjugation buffer (PBS with 0.5% Triton X-100) in the presence or absence of 6xHis-Cdc45 (2 µg) conjugated with 10 µl of Ni-NTA agarose (Qiagen). After 2 hours of incubation at 4°C, beads were recovered and washed 5 times with wash buffer (50 mM imidazole, 50 Hepes-KOH pH 7.5, 600 mM NaCl, 0,5 % Triton). Washed resin was resuspended in 30 µl of Laemmli buffer, and boiled. 15 µl of samples were run on 10%SDS-PAGE gel, processed for Western blotting and autoradiography. 6xHis-TopBP1 recombinant protein was expressed in baculoviruses infected Sf9 cells by the LRI (London Research Institute) cell culture facility. 6xHis-TopBP1 50 ng/µl was incubated in 100 µl of EB 0.5% Triton buffer in the presence of MBP, MBP-xGEMC1 or MBP-xGEMC1-8ST/D proteins pre-bound to 20 µl of amylose resin. Samples were incubated for 45 minutes at 4 °C. Beads were collected and extensively washed with EB buffer supplemented with 0.5% Triton X-100. 40 µl of Laemmli buffer was added, samples were boiled and 20 µl were processed for SDS-PAGE and WB.
2.5.7 In Vitro Transcription Translation (IVTT) and Kinase Assay

$^{35}$S-labeled XIGEMC1 and XIGEMC1 R198NL proteins were synthesized using Quick Couple SP6 kit. This system couples for transcription and translation of genes cloned downstream the SP6 RNA polymerase promoter. 1.0µg of circular plasmid DNA containing SP6 promoter was added to an aliquot of the TNT Quick Master Mix (40 µl) in the presence of 2 µ of $[^{35}\text{S}]$ methionine (1,000Ci/mmol at 10mCi/ml) and incubated in a 50µl reaction volume for 90 minutes at 30°C. In vitro Kinase assays were performed by incubating 5 µl of in vitro translated $^{35}$S-labeled XIGEMC1 and XIGEMC1 R198NL in 20 µl of kinase buffer (80 mM β–glycerolphosphate, 0.5 mM EGTA, 100 µM ATP and 2.5 mM MgCl$_2$) in the presence or absence of 100 nM recombinant Cyclin E/Cdk2 or Cyclin A/Cdk2 (a gift from T. Hunt) at 23 °C for 30 minutes.

2.5.8 Peptide arrays

Briefly, 20 mer peptides deriving from XIGEMC1 sequence spanning from amino acid residue N to C and advancing by 2 residues were arrayed on nitrocellulose membranes (LRI protein chemistry facility). XIGEMC1 peptide arrays were incubated with EB kinase buffer with or without Cdk2/CyclinE complex in the presence of 50 µM ATP and 10 µCi of γ-32P-ATP at 30°C for 60 minutes. Membranes were then washed with EB buffer and exposed for autoradiography.

2.5.9 Immunofluorescence microscopy

Sperm nuclei (4,000/µl) were incubated in the egg extract containing 10 µM of Cy3-dCTP (Amersham Biosciences) for appropriate times at 23°C. Reactions were stopped by pipetting 1µl samples onto glass slides spotted with 1µl of fixing solution (3% formaldehyde, 2/~g/ml HOECHST dye 33342, 80 mM KCl, 15 mM NaCl, 50% glycerol, and 15 mM Pipes at pH 7.2) containing 0.1 mg/ml 3,3 diethylthiadicarbocyanine iodide membrane dye (Di3C18). The fixed samples, covered with glass coverslips were observed using fluorescence microscopy, and fluorescence images were captured with the OpenLab imaging program (Improvision).
2.5.10  XIGEMC1 phosphorylation in Xenopus egg extract

20 µl Xenopus egg extract were treated or not with 2 µl λ protein phosphatase (λ PPase, NEB) (400 U/µl) supplemented with 1X λ PPase reaction buffer (50mM Tris- HCl pH 7.5, 100 mM NaCl, 2 mM DTT, 0.1 mM EGTA, 0.01% Brij) and 2mM MnCl. The reactions were incubated for 30 minutes at 30 °C in the presence or not of 20 mM sodium orthovanadate. Phosphorylation of endogenous XIGEMC1 at Threonine 153 was detected by immunoblotting with anti XIGEMC1 pospho-Threonine 153 antibodies. For the phosphorylation of in vitro-translated XIGEMC1 in Xenopus egg extract 2µl of wild-type 35S-labeled XIGEMC1 and XIGEMC1 mutated in the cyclin binding site (35S-XIGEMC1-ANA) were added to 4 µl of interphase extract in the presence or absence of 400 U/µl of λ protein phosphatase. Samples were incubated 30 minutes at 30 °C with 1X λ PPase reaction buffer and 2mM MnCl. The reactions were stopped by adding loading buffer and analyzed by SDS/PAGE and autoradiography.

2.6  Xenopus embryology and cell culture

2.6.1  Xenopus embryo manipulation and microinjection

Xenopus embryos were obtained by in vitro fertilization. Eggs were collected by manual pression into a dry glass petri-dish and fertilized by rubbing minced and homogenized fragment of Xenopus testis over them. Sperm is obtained by crushing a testis in 1X high-salt MBS using a pellet mixer (Anachem). Testes were kept for up to 24 hours at 4°C in 60% (v/v) Leibovits medium (Gibco). After contact with sperm eggs were flooded with 10% normal amphibian medium (NAM) for 30 minutes at room temperature. The first sign of fertilization is a contraction of the pigmented animal hemisphere to less than one half of the eggs. A fertilized egg is more elastic and due to the thickening of the vitelline membrane is also more resistant to deformation than an unfertilized egg when squeezed gently. In approximately 20 minutes, eggs had rotated within the jelly coat so that the animal hemisphere was facing upward as indication of a successful fertilization. Eggs were then dejellyed by swirling in a solution with 2% cysteine hidrocloride pH8.0 in deionized water for 10 minutes, followed by 3 washes with 10% NAM. Successfully fertilized eggs were selected on a shape and consistency.
criteria by lightly squeezing them with an hair loop mounted in beeswax at the tip of a cut–off Pasteur pipette, transferred to 75% NAM, 2% Ficoll 400 and injected at the one cell stage into the animal pole with 97 ng of control MOs and specific GEMC1 MOs. Injection was performed by using 30 µl Drummond micropipette needles (Fisher) mounted on Narishige IM-300 microinjector. Embryos are incubated in 75 % NAM + 2 % (w/v) Ficoll during injection. The higher salt concentration helps to stimulate healing of the embryo after injection and the increased viscosity of the Ficoll prevents loss of embryo contents through the injection wound. After injection, embryos were maintained at 16°C in 75 % NAM + 2 % (w/v) Ficoll for 3 hours. Embryos cultured beyond stage 8 were transferred to Petri dishes containing 10 % NAM and maintained at 22-23 °C.

2.6.1.1 Recipes

**1X MBS (for 1 litre)**
100 ml 10 X MBS salts
7 ml 0.1 M CaCl2
H2O to 1 litre

**1/10th X MBS (for 1 litre)**
100 ml 1X MBS
H2O to 1 litre

**1X MBS High salt (for 500 ml)**
As for 1X MBS but with 2 ml 5M NaCl and 350µl 1M CaCl2

**10 XMBS salts 1 litre**
51.3g NaCl
0.75g KCl
2g MgSO4
2g NaHCO3
pH to 7.6

**10XNAM Salts (500 ml)**
32.5g NaCl
0.75g KCl  
1.2g CaNO₃  
1.25g MgSO₄  
1ml 0.5M EDTA  

*IXNAM (for 1 litre)*  
100 ml 10X salts  
2 ml 1M HEPES-NaOH pH pH 7.6  
10 ml 0.1 M NaHCO₃  

*1/10XNAM (for 100 ml)*  
1ml 10X NAM  
200µl 1M HEPES pH 7.5  

*75% NAM (for 200 ml)*  
150ml 1XNAM  
H₂O to 200ml  

*75%NAM+4% Ficoll*  
as above but with 8g Ficoll  

*Cysteine*  
2% cysteine in water (or 1/15XNAM) PH to 7.5-8 with NaOH  

### 2.6.2 Morpholino oligonucleotide

MOs were obtained from Gene Tools (Philomath, OR). Stock solutions (1 mM in water) were vortexed and heated at 65°C for 5 min before dilution. Diluted MO solutions were heated to 65°C for 5 min and cooled to room temperature before injection. The following antisense MOs designed to act against XIGEMC1 were used:  

\[
5\text{'}-\text{CTTGGCAAGTTAGAATAGTGTTCAT} 3\text{'} \quad (\text{GCC1.1})
\]

\[
5\text{'}-\text{GTTCCTGGCAGGCAGGCACCTTTTCATTG} 3\text{'} \quad (\text{GCC1.2})
\]

A random standard control morpholino (CONMO) was also used as a negative control (5\text{'}-\text{CCTCTTACCTCAGTTACAATTTATA-3'}) to assay for any non-specific effects of the injection or possible cytotoxicity associated with morpholinos. Morpholino antisense oligo conjugated in 3’ to a fluorescein tracer was used to verify injection success.
2.6.3 Protein extraction from oocytes and embryos

Oocyte and embryo proteins were extracted by using extraction and acetone precipitation technique. Twenty embryos were resuspended in 200 µl of extraction buffer (80 mM Sodium glycerophosphate, 50 mM Sodium fluoride, 20 mM EGTA, 0.2% NP-40, 10 µg/ml LPC, 10µg/ml Cytochalasin and 1mM DTT), homogenized in ice and spun 10 minutes at 10,000 rpm at 4°C. Supernatants were recovered, mixed to 3 volumes of acetone and spun at 10,000 rpm at 4°C. Sample pellets were resuspended in 200 µl of 2X Sample buffer, boiled for 5 minutes at 100 °C and left for 1-2 days at 4 °C shaking to dissolve.

2.6.4 tPARP cleavage assay

Apoptotic cell death in embryos was assayed for caspase activity by monitoring the cleavage of recombinant poly ADP-ribose polymerase (PARP) substrate, as previously shown (Hensey and Gautier, 1997). Three embryos were collected at stage 37, snap frozen in liquid nitrogen and homogenized in caspase extraction buffer (80 mM β–glicerophosphate, 15 mM MgCl₂, 20 mM EGTA, 10 mM DTT). The homogenate was centrifuged 3 times at 4°C, to remove particulate matter. 8 µl of each homogenate was mixed with 3 µl of 35S-labelled PARP and incubated at 30°C for 15 minutes. Samples were denatured and run on a 12% SDS-PAGE gel, fixed and exposed.

2.6.5 Genomic DNA preparation

Genomic DNA was obtained from 5 stage-30 embryos, using the QIAamp DNA Mini Kit (QIAGEN) following the protocol for DNA purification from tissues. Embryos were resuspended in 100 µl of ATL buffer in the presence of 20 µl proteinase K (Qiagen) and incubated at 56 °C overnight shaking to allow complete tissue lysis. RNA degradation was achieved by incubating lysate with 4 µl of RNA (100 mg/ml) for 2 minutes at room temperature. Samples were mixed with 200 µl of buffer AL, incubated at 70 °C for 10 minutes, supplemented with 200 µl of 100% ethanol and spun through a QIAamp Mini spin column at 8,000 rpm to achieve binding of the DNA to QIAamp membrane. Bound DNA was washed with 500µl of AW1 and AW2 (composition unknown)buffer and eluted with 200 µl of Buffer AE (10 mMTris HCl pH 9.0, 0.5 mM EDTA). Genomic DNA was quantified using NanoDrop (NanoDrop technologies)
spectrophotometer based on $A_{260\text{nm}}$ measurement.

2.6.6 Histological analyses

Embryos were fixed in 4% paraformaldehyde in PBS, embedded in wax and transversally sectioned at a thickness of 10 µm. They were stained with Hematoxylin and eosin. Essentially, the Hematoxylin component stains the nuclei blue/black, with good intranuclear detail, whilst eosin stains cell cytoplasm and most connective tissue fibres in varying shades and intensities of pink, orange and red. Staining was performed by using the automated stainer available at CRUK LRI Histology Service. Sections were de-waxed in xylene for 5 minutes twice, dehydrated in 100% IMS and 3 minutes in 70% IMS twice. Samples were washed with tap water, stained in Harris Haematoxylin for 8 minutes (RA LAMB 5898), treated with 1% acid alcohol (1% HCl, 70% ethanol) and stained in 1% Eosin (BDH Eosin yellowish 341973R) for 5-10 minutes. Stained samples were quickly rinsed, dehydrated, mounted on a glass slide and allowed to dry at room temperature.

2.6.7 Cell culture and flow cytometry

NIH 3T3 cells (Mouse embryonic fibroblast) were grown in DMEM medium supplemented with 10% heat-inactivated donor calf serum and antibiotics. For FACS analysis cells were treated with 10 µM BrdUTP (Sigma) for 45 minutes, harvested, washed in PBS and fixed in cold 70% ethanol. Cells were spun off and washed twice in PBS. Pellets were re-suspended in 2 M hydrochloric acid and incubated 30 minutes at room temperature. To neutralize the samples, acid was removed by centrifugation and cells washed twice with PBS and once with neutralization buffer (PBS, 0.1% BSA, 2% Tween 20). Re-suspended pellets were stained with 50 µl FITC conjugated rabbit anti-mouse (DAKO) and treated with 50 µl ribonuclease (100 µg/ml, Sigma) for 15 minutes at room temperature. Finally, samples were incubated with 200 µl of Propidium Iodide (50 µg/ml) for 30 minutes at room temperature and analyzed by FACS. siRNA for Cdc45 and mGEMC1 were obtained from Invitrogen (mCdc45 and Gm606 SMART pool) and used following manufacturer instructions.
2.6.8 siRNA Transfection

siRNA oligonucleotides (Invitrogen) were synthesized to the following target sequences:
mCdc45: UUAUGCUUGAACCCAAACUGAAUGC;
mGEMC1: AACUUGAUGACACUCUACCAGCUAA; negative scrambled control (mock) was purchased from Invitrogen. NIH 3T3 cells were transfected with siRNAs at a final concentration of 50 nM (mGEMC1) or 30 nM (mCdc45) with lipofectamine RNAiMAX (Invitrogen) according to the instructions of the manufacturer. Transfections were performed up to two times at 24-h intervals for both mGEMC1 and mCdc45 depletion. After 48 hours cells were harvested, lysed and 10 μg of total protein was run on SDS-PAGE gel. mCdc45 depletion was assessed by immunoblotting with anti mouse Cdc45 antibodies. Loading control was performed by immunoblotting with antibodies against GAPDH protein.

2.6.9 RNA isolation and RT-PCR

RNA from NIH 3T3 cells was isolated using the RNeasy Micro kit (Qiagen) following the manufacturer’s instructions. NIH3T3 collected with a cell scraper from the plates and lysed with buffer RLT directly in the eppendorf tubes. The lysate was homogenized by using Qishredder spin columns (Qiagen). Samples were applied to the RNeasy MinElute spin column. RNA binds to the silica membrane in the tube. Traces of DNA that were removed by DNase treatment on the RNeasy MinElute spin column. DNase and any contaminants were washed away, and total RNA was eluted in 30 μl of RNase-free water.

A mouse GEMC1 (mGEMC1) cDNA clone spanning the entire predicted coding sequence was isolated by RT-PCR using the Qiagen one step RT-PCR kit as follows:

10 μl of 5x Qiagen One step TR-PCR Buffer
2 μl of dNTPs Mix (10mM each dNTP)
0.6 μM of Rw Primer
0.6 μM of Fw Primer
2 μl of Qiagen OneStep RT-PCR Enzyme Mix
5 units of RNase inhibitor
50 ng of purified RNA
RNase-free water up to 50 µl

For the reverse transcription reaction (1) and cDNA amplification (2) the following cycling parameters were used:

(1) 30 min at 50 °C
(2) 1 cycle: 15 min at 95 °C
30 cycles: 1 min at 94 °C
30 sec at the primer annealing temperature
1 min at 72 °C
1 cycle: 10 min at 72 °C

Primers used for RT-PCR in this study are listed below:

Fw_mGEMC1: 5’-AGAACAGCATTCTGCCTTGC-3’
Rw_mGEMC1: 5’-CTAGGTCTGCTTAGGGACCCA-3’

Fw_mGADPH: 5’-ACCCAGAAGACTGTGGATGG-3’
Rw_mGADPH: 5’-CCCTGTTGCTGTAGCCCTAT-3’
3 Results I - Role of *Xenopus* GEMC1 protein in DNA replication initiation

3.1 Introduction

From unicellular organisms to the most complex multicellular organisms, DNA replication is an essential process that needs to be tightly controlled in order to preserve genetic integrity. The accuracy of these events is crucial to multicellular organisms in which any changes to the genome may potentially give rise to cancers that threaten the life of the entire organism.

While in prokaryotes, replication begins from a single site and continues until it terminates at the end of the genome, in eukaryotic cells replication initiates from multiple locations referred to as replication origins. The time required to complete replication depends on the number and the timing of origin activation. In budding yeast these two parameters are rigidly defined at the nucleotide sequence level since specific origins fire at specific times (Raghuraman et al., 1997). Differently from yeast, in metazoans DNA replication can initiate from virtually any sequence. The mechanisms that control origin firing are still unclear.

In metazoans, origin firing is dynamically regulated during S phase. *Xenopus laevis* is a vertebrate experimental model system that shows a high level of homology with humans. Eggs of *X. laevis* are a valuable source of the molecular components involved in DNA replication (Laskey et al., 1983; Mechali et al., 1983). Cell-free extracts support the assembly of demembranated sperm nuclei into interphase nuclei (Lohka and Masui, 1983) (Newmeyer et al., 1986). These extracts also support the efficient semiconservative replication of demembranated sperm nuclei (Blow et al., 1987).

DNA replication begins in late M phase/G1 when the ORCs bind directly to chromosomal origins (Bell and Dutta, 2002; Kelly and Brown, 2000). Once bound, ORC recruits Cdc6 and Cdt1, which in turn allow the assembly of the mini-chromosome maintenance (MCM)2-7 complex onto DNA. Recruitment of MCM2-7, the presumptive replicative helicase (Labib et al., 2001) completes the assembly of the
pre-replication complex (pre-RC). At the G1/S transition, the pre-RC is converted to a pre-initiation complex (pre-IC) through the activation of two S phase protein kinases: CDK2 and the Cdc7-Dbf4. These protein kinases cooperate to recruit the initiation factor Cdc45 (Jares and Blow, 2000; Mimura et al., 2000). In addition to S phase CDK (S-CDK) and Cdc7-Dbf4, three other factors, MCM10, the GINS complex and Xmus101 have been shown to promote Cdc45 recruitment onto the DNA (Wohlschlegel et al., 2002). Binding of Cdc45 protein is critical for origin unwinding and recruitment of the single-stranded DNA binding protein (RPA), followed by DNA polymerase α (Walter, 2000). In the budding yeast *Saccharomyces cerevisiae* a complex composed of Sld2 (RecQ4 in *X. laevis*) and Sld3 with ScTopBP1 is essential to trigger DNA replication initiation. To date, Sld3 homologues have not been identified in multicellular eukaryotes. Indeed, even within fungi, Sld3 is poorly conserved suggesting rapid evolution. Sld2 and Sld3 interact with ScTopBP1 protein and S-CDK phosphorylation is necessary for this interaction. Emerging evidence suggests that these two proteins represent the minimal set of S-CDK substrates required for DNA replication (Tanaka et al., 2007; Zegerman and Diffley, 2007). However, the possibility that S-CDK phosphorylation of additional factors contributes to efficient DNA replication, cannot be ruled out.

To ensure that DNA replication takes place once per cell cycle and that it is followed by mitosis, multicellular eukaryotes have evolved a mechanism in which after cells enter S-phase the protein Geminin sequesters Cdt1 in an inactive complex that is unable to interact with and recruit MCM2-7 (McGarry and Kirschner, 1998). In this way it prevents the reassembly of the pre-RC complex (Tada et al., 2001; Thepaut et al., 2004). The formation of new pre-RCs occurs when Geminin and mitotic cyclin are degraded by the anaphase-promoting complex (APC) during mitosis. The degradation of Geminin by APC allows Cdt1 to promote the reloading of MCM 2-7 complex onto the chromatin and rebuild a new replication complex (McGarry and Kirschner, 1998).

Timing of initiation of DNA replication depends on S phase kinases activity, CDK2 and Cdc7, at discrete origins (Kelly and Brown, 2000). In the presence of DNA damage, two different pathways prevent origin firing through ATM and ATR dependent inhibition of Cdk2 and Cdc7-Dbf4 (Abraham, 2001; Shechter et al., 2004b). In the absence of DNA damage, the rate of origin firing is regulated by caffeine.
sensitive ATM-ATR pathways. ATM and ATR pathways operate during an unperturbed cell cycle to regulate initiation of DNA replication and the progression of DNA synthesis. Several data Marheineke and Hyrien, 2004; Sorensen et al., 2004; Miao et al., 2003) indicate that during normal DNA replication, in absence of DNA damage, both in *Xenopus* extract and human cell lines: 1. The DNA-damage checkpoint kinases ATM, ATR, and Chk1 are active during S phase, even in the absence of induced DNA damage, and function to downregulate the Cdk2 and Cdc7 kinases, slowing down the rate of DNA replication by blocking origin firing. 2. Inhibition of ATM, ATR, and Chk1 kinases speeds up the rate of DNA replication. 3. ATM and ATR are activated by feedback from active replicons, mediated in part by a signal from RPA-bound ssDNA and the Rad9-Rad1-Hus1 complex. This data support the concept that dynamic regulation of origin selection appears to be essential for the duplication of large metazoan genomes and for the concurrent maintenance of genome integrity.
3.2 Results

3.2.1 Identification of Xenopus GEMC1 protein

Although many proteins involved in the initiation of DNA replication have been identified, their function is still poorly understood and it is likely that additional proteins are required to control origin firing. In order to identify ATM and ATR direct or indirect targets regulating origin firing I utilized a screening system that scores for ATM and ATR phosphorylated proteins using a *Xenopus laevis* expression library (McGarry and Kirschner, 1998). This approach is based on the ability of several (but not all) proteins to undergo a mobility shift upon phosphorylation. Small pools of *Xenopus* cDNAs were translated in a reticulocyte lysate system in the presence of $^{35}$S methionine. Radiolabelled proteins were incubated in interphase extract in the presence (or absence) of double stranded DNA templates that mimic double strand breaks (DSBs) and subsequently separated on SDS-PAGE gel to monitor differences in migration pattern. (Fig. 3.1) Activation of ATM and ATR (Costanzo et al., 2004) caused the appearance of slower-migrating forms of $^{35}$S labelled proteins. An example of 3 pools undergoing posttranslational modification is shown in figure 3.2.

To isolate candidate substrates with possible involvement in DNA replication, I performed a bio-informatic search to identify similarities with known replicative factors. This search led to the identification of an open reading frame which was named XlGEMC1 (GEMinin Coil-coiled containing protein) containing a region similar to the Geminin coiled-coil domain (McGarry and Kirschner) and containing some of the residues critical for Geminin function (Saxena et al., 2004). Figure 3.3 highlights the conserved regions of XIGEMC1 when aligned with human Geminin. cDNA coding for XIGEMC1 protein was sequenced. Sequences for XIGEMC1 orthologues were identified using NCBI databases. Multiple alignments of XIGEMC1 with its orthologues allowed the identification of a very well conserved 5’ region (the aminoacid sequence MNTIL), which was presumed to contain the starting codon of Xenopus GEMC1 ORF (Fig. 3.6)

Translated XIGEMC1 underwent a caffeine (non specific ATM-ATR inhibitor)sensitive mobility shift in egg extract supplemented with DSBs whereas no effect was observed upon treatment with ATM specific inhibitor (Ku55933). These
data suggest that XIGEMC1 phosphorylation might depend on ATR kinase activity which is also activated by DSBs. Radiolabelled protein was hyper-phosphorylated in the presence of active ATM and ATR and this phosphorylation was abolished by phosphates treatment (Fig. 3.4).
Fig. 3.1 Schematic representation of the screening for ATM-ATR targets

Xenopus cDNAs transcribed and translated in reticulocyte lysates in the presence of $^{35}$S-methionine were mixed with egg extracts that were untreated, treated with linear DNA (DSBs) with (+) or without (−) caffeine (2 mM) and run on SDS–PAGE. Differences in migration patterns (red) following ATM and ATR activation were monitored.
**Fig. 3.2 Screening for ATM and ATR target**

cDNA expression screening to identify ATM and ATR targets. Pools of *Xenopus* cDNAs derived from a library from maternal mRNA. Clones individually arrayed in 384 well-plates were transcribed and translated in reticulocyte lysates in the presence of $^{35}$S labelled methionine (Smith et al., 2009). Lysates were mixed with egg extracts supplemented with (+) or without (-) linear DNA (DSBs) and run on 10% SDS-PAGE. An example of 3 pools (K,G,F) of translated proteins is shown in the figure. Each pool contains a different shifting clone (K11, G10, F5). On average we identified 1 to a maximum of 3 shifting clones every 384 well plate. The position of the clones in the 384 well plates was derived by intersecting the pools corresponding to rows and columns of each plate.
Fig. 3.3. Similarity of the coiled-coil domain of XIGEMC1 and human geminin (hGEM).

Alignment of aminoacid sequence of human geminin coiled-coil domain (residues 92-142) and XIGEMC1 coiled-coil domain (residues 110-158). Identical aminoacids are indicated between the two sequences. % corresponding to sequence identity and similarity (Positive) are also shown.

![Sequence Alignment](image)

Fig. 3.4 Identification of the ATM/ATR target, XIGEMC1

$^{35}$S-XIGEMC1 in extract that was untreated (-) or supplemented with DSBs (+) in the presence or absence of caffeine (caff., 2 mM), specific ATM inhibitor (Ku55933, 20 µM) or λ phosphatase (λPP, 1 unite). $^{35}$S-XIGEMC1 phosphorylation ATM and ATR dependent was visualized as band shift on SDS-PAGE gel.
3.2.2 Characterization of XlGEMC1 structure and sequence analysis

The secondary and tertiary structure of XlGEMC1 protein was predicted. To better understand XlGEMC1 properties, I used a coiled-coil prediction software and a 3D protein structure homology-modeling server (Pyre). Consistent with our first observation, the XlGEMC1 coiled-coil domain showed a high structural similarity with hGeminin Coil-coiled domain (Fig. 3.5a, 5b).

By comparing the 3D structure of hGeminin and XlGEMC1 coiled-coil domains, a flexible region between aa 127 and 132 was identified in XlGEMC1 protein. An important observation is that the afore mentioned region could be responsible for the destabilization of XlGEMC1 protein coiled-coil domain (the implication of this will be discussed further in the discussion section).

XlGEMC1 protein sequence and homology with its orthologues was then analyzed. GEMC1 is highly conserved in vertebrate organisms as close homologues can be found in H. sapiens (HsGEMC1), M. musculus (MmGEMC1), R. norvegicus (RnGEMC1) and Xenopus laevis (XIGEMC1), (Fig. 3.6). Coiled-coil- domains show around 80% similarity suggesting the existence of a new family of coiled-coil containing proteins. A further database search with the full-length Xenopus GEMC1 sequence reveled that it is conserved in 12 different species.

Furthermore, the GEMC1 family can be divided into 2 subfamilies (group I and group II) with proteins sharing less than 50% sequence identity among subfamilies. 6 species (Xenopus laevis, Mus musculus, Rattus norvegicus, Homo sapiens, Dani orerio, Pan troglodytes) contained proteins belonging to both groups, while the remaining species were found to contain proteins belonging to only one of those groups (table 3.1). It is likely that these missing genes are still to be found.
Fig. 3.5 XIGEMC1 coiled-coil structure
Analysis of XIGEMC1 and H-Geminin aminoacid with a) coiled-coil prediction software and b) 3D structure prediction server (PHYRE). A flexible domain between aa 127 and 132 (open circle) interrupts XIGEMC1 coil-coiled structure.
Fig. 3.6 Conservation of GEMC1 in vertebrates

XIGEMC1p primary sequence was compared to the sequences of vertebrate homologous (R. Norvegicus RnGEMC1, M. Musculus MmGEMC1, H. Sapiens HsGEMC1 and X. laevis XIGEMC1). Aminoacid identity in all species is in green, in more than 2 species is in yellow. Similarity is indicated in blue. Line indicates coiled-coil domain.
Table 3.1 GEMC1 family and subfamilies

GEMC1 family consists of 19 different proteins from 12 species (listed in the first column on the left side). GEMC1 family can be divided into 2 Subfamilies (Group I and Group II). Information about taxonomy are from NCBI databases.

<table>
<thead>
<tr>
<th>Species</th>
<th>Group I</th>
<th>Group II</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Xenopus laevis</strong></td>
<td>GCC1</td>
<td>LOC100158359 (169217660)</td>
</tr>
<tr>
<td><strong>Mus musculus</strong></td>
<td>LOC239789 (71274117)</td>
<td>EG622408/LOC622408 (85702306)</td>
</tr>
<tr>
<td><strong>Rattus norvegicus</strong></td>
<td>RGD1563170 (10493437)</td>
<td>LOC688802 (109464391)</td>
</tr>
<tr>
<td><strong>Homo sapiens</strong></td>
<td>LOC647309 (169165690)</td>
<td>CH471123/HCG_40453 (119575305)</td>
</tr>
<tr>
<td><strong>Canis lupus familiaris</strong></td>
<td>LOC608255 (74003612)</td>
<td></td>
</tr>
<tr>
<td><strong>Ornithorhynchus anatinus</strong></td>
<td>LOC100081633 (14941433)</td>
<td></td>
</tr>
<tr>
<td><strong>Monodelphis domestica</strong></td>
<td>LOC100016728 (126338613)</td>
<td></td>
</tr>
<tr>
<td><strong>Danio rerio</strong></td>
<td>LOC100148564 (189529048)</td>
<td>LOC100150074 (189523996)</td>
</tr>
<tr>
<td><strong>Equus caballus</strong></td>
<td>LOC100062400 (194223887)</td>
<td></td>
</tr>
<tr>
<td><strong>Pan troglodytes</strong></td>
<td>LOC741674 (114591160)</td>
<td>LOC738862 (114600275)</td>
</tr>
<tr>
<td><strong>Macaca mulatta</strong></td>
<td>LOC706081 (109077247)</td>
<td></td>
</tr>
<tr>
<td><strong>Monodelphis domestica</strong></td>
<td>LOC100031826 (126316689)</td>
<td></td>
</tr>
<tr>
<td><strong>Tetraodon nigroviridis</strong></td>
<td>GSTEN:00021169:G:001 (47216516)</td>
<td></td>
</tr>
</tbody>
</table>
The structural similarity between *Xenopus* GEMC1 and human Geminin strongly supported the idea that GEMC1 protein was involved in chromosomal DNA replication. Intrigued by such possibility, I decided to focus my attention on the characterization of the physiological role of GEMC1 in DNA replication. I also decided not to investigate the effect of ATM and ATR on GEMC1 activity.

In this chapter I will describe the experiments performed in *Xenopus* egg extract to study XIGEMC1 function in DNA replication.
3.2.3 Anti *Xenopus* GEMC1 polyclonal antibodies

To investigate the function of GEMC1 in *Xenopus* egg extract, polyclonal antibodies against *Xenopus* GEMC1 protein were raised in rabbits. The GEMC1 full-length open reading frame was amplified from the *Xenopus* cDNA library. PCR product was subcloned into a pENTR vector (Invitrogen) by Gateway technology and finally transferred to destination vectors with a hexahistidine tag to the N-terminus (pDEST17, Invitrogen) (see Materials and Methods). The fusion protein was expressed very efficiently in bacteria though the majority of the protein was insoluble. For this reason I purified 6His-GEMC1 in denaturing conditions by using 8M Urea buffer. Figure 3.7 shows that the purification on Ni-NTA column yielded a preparation of a relatively pure recombinant protein migrating at 47 kDa, higher than the predicted molecular weight (38kDa). Anti XIGEMC1 antibodies recognized both GST and MBP-GEMC1 recombinant proteins (Fig. 3.8). Likewise, polyclonal antibodies recognised a band migrating at an apparent molecular weight of 47 kDa. In Figure 3.9 shows that the preimmune sera did not recognise any band of the same molecular weight (MW) of XIGEMC1, confirming the specificity of the antibody produced.

By comparing the amount of XIGEMC1 into the extract with known amounts of recombinant MBP-GEMC1 protein the concentration of endogenous XIGEMC1 was estimated to be approximately 55 nM (Fig. 3.10).

Due to similarity with Geminin, recombinant XIGEMC1 protein was tested for its ability to inhibit DNA replication in the same way that Geminin does. Thomas J. McGarry and Marc W. Kirschner showed that the binding of the MCM complex was strongly inhibited when Geminin was added to the extract at physiological concentration (McGarry and Kirschner, 1998). Egg extract was incubated with 55nM of recombinant XIGEMC1 protein (rec-XIGEMC1) and replication was monitored as previously described. No significant inhibition of chromosomal DNA replication was observed when physiological amounts of XIGEMC1 were added to egg extract. Despite this, DNA replication inhibition could be observed at much higher doses (Fig. 3.11).
I then verified the expression pattern of XIGEMC1 protein in embryonic cells and somatic cells. Proteins extracted from each tissue in adult *Xenopus* frog were isolated and prepared for protein extraction (see Material and Methods). XIGEMC1 is ubiquitously expressed in eggs and in most adult *Xenopus* tissues. The expression profile revealed that XIGEMC1 is particularly abundant in highly proliferating tissues such as the skin and the gut but was also present in ovary, brain and lungs (Fig. 3.12).
Fig. 3.7 Purification of recombinant XIGEMC1 protein

Recombinant XIGEMC1 was produced in bacteria and purified on nickel resin. Sample of purified protein was run on SDS-PAGE gel and stained by Comassie blue.
**Fig. 3.8** XIGEMC1 recombinant protein visualized by Coomassie blue staining and immunoblot with anti GEMC1 antisera

Recombinant XIGEMC1 (rec-XIGEMC1) fused to Maltose Binding Protein (MBP-XIGEMC1) and glutathione S-transferase (GST-XIGEMC1) were monitored with coumassie stain (lane 1-3) or WB (lane 2-4) using anti XIGEMC1 antibodies.

**Fig. 3.9** Immunoblot of frog extract with anti GEMC1 antisera

Total of 0.5µl of Xenopus egg extract was run per lane and immunoblotted to test for the presence of anti-XIGEMC1 antibodies in the sera. The pre immune serum (prebleed) did not recognise any protein with significant specificity. The antibody recognizes a single band of a molecular weight of 47 kDa.
Fig. 3.10 GEMC1 quantification in *Xenopus* egg extract

WB comparing the amount of XIGEMC1 presents in 0.5 µl extract and 1, 4, 7, 10, 30 and 70 ng of rec-XIGEMC1 as indicated.
**Fig. 3.11 Effects of recombinant XlGEMC1 on DNA replication**

DNA replication assay showing incorporation of a $^{32}$P-dATP in sperm nuclei replicated in egg extracts that were untreated or supplemented with 55 nM recombinant MBP-XIGEMC1 (rec-XIGEMC1).
**Fig. 3.12 XlGEMC1 expression in adult Xenopus tissues**

WB analysis of XlGEMC1 protein expression in various tissues. The WB contained 15 µg per line of total protein isolated from the various tissues and was probed with polyclonal antibody, anti XlGEMC1
3.2.4 Chromatin binding of Xenopus GEMC1 during DNA replication

In order for DNA replication to initiate, multi-protein complexes such as ORC1-6 and MCM 2-7 need to be loaded onto chromatin to assemble replication origins. Origin firing is then triggered following the action of Cdk2/CycE and Cdc7/Dbf4 that promote the loading of Cdc45 (Diffley, 2004; Diffley and Labib, 2002; Takisawa et al., 2000). To gain mechanistic insights into how XIGEMC1 is involved in DNA replication it was essential to examine whether XIGEMC1 itself associates with chromatin. A time course of chromatin binding of XIGEMC1 was compared to the binding of three crucial proteins involved in pre-RC and initiation complex formations: Orc2, MCM7 and Cdc45. XIGEMC1 was able to bind chromatin at a very early stage of DNA replication with kinetics similar to ORC1-6 (Fig. 3.13).

Upon addition of sperm nuclei to the extract, Orc2 (a component of the ORC origin recognition complex) bound to chromatin within 15 minutes. Chromatin binding of Mcm7 was established within 30 min. Following licensing, nuclear structures were formed around the chromatin within 30 min (data not shown), thus allowing initiation of DNA replication. Chromatin binding of XIGEMC1 was detected before the initiation of DNA replication. I showed that XIGEMC1 chromatin binding is independent of the MCM2-7 complex as it can also be detected in extracts treated with recombinant Geminin (Wohlschlegel et al., 2000) (Fig. 3.13). I then considered the role of S-Cdk activity with regards to the chromatin association of XIGEMC1. XIGEMC1 loading does not require Cdk2 activity as demonstrated by the use of the CDK inhibitor roscovitine (Luciani et al., 2004; Meijer et al., 1997), which suppresses initiation of DNA replication and inhibits Cdk2 dependent Cdc45 loading (Fig 3.13). These results show that XIGEMC1 is recruited to chromatin upstream and independently of the MCM 2-7 and Cdc45.

Figure 3.13 also shows that XIGEMC1 remains bound to chromatin throughout S phase and its association gradually increases. This behaviour may also be indicative of a role during replication elongation. Surprisingly, as shown in Figure 3.14, Orc2 depletion from the egg-extract did not affect XIGEMC1 binding to chromatin. This result implies that chromatin recruitment of XIGEMC1 does not require any of the pre-RC complex proteins and suggests the possibility that XIGEMC1 binds chromatin autonomously.
Fig. 3.13 Chromatin binding of *Xenopus* XlGEMC1 during DNA replication
Chromatin binding of Orc2, MCM7, Cdc45 and XlGEMC1 at the indicated times after addition of sperm nuclei (3000 n/µl) to interphase egg extracts supplemented with buffer (Control), 80 nM Geminin (GEM) or 500 mM roscovitine (ROSC).

Fig. 3.14 GEMC1 does not require ORC for association with sperm chromatin
Eggs extract that either contained (mock) or lacked Orc2 (∆Orc2), was mixed with sperm chromatin and incubated for different time points. The chromatin was then isolated and probed by immunoblotting for the presence of Orc2 and GEMC1.
3.2.5 Role of XIGEMC1 in DNA replication

The ability of XIGEMC1 to bind chromatin raised the possibility that it could be involved in DNA replication. To investigate such a role several trials to immunodeplete XIGEMC1 from the extract were attempted. Different batches of polyclonal antibodies were generated but unfortunately none of them were able to deplete XIGEMC1 from the extract. A way to overcome this problem is to incubate the extract with function-blocking antibodies that inhibit the targeted protein activity (Emanuele and Stukenberg, 2007). To this end purified anti XIGEMC1 antibodies were purified (Materials and Methods). Figure 3.15 shows the specificity of the purified antibodies for XIGEMC1p (Ab-1) in the extract. Affinity-purified antibodies were incubated in egg extract and DNA replication was monitored by incorporation of \( [\alpha-^{32}P] \) dATP into replicated DNA. These antibodies completely inhibited chromosomal DNA replication whereas control IgGs did not have any effect (Fig. 3.16a,b; Fig. 3.17). DNA replication inhibition was rescued by out-competing anti XIGEMC1 neutralizing antibodies with an excess of recombinant XIGEMC1 (Fig. 3.16a,b).

I had to rule out the possibility that DNA replication inhibition was due to a non specific effect of the antibodies. Replication assays were performed using several batches of polyclonal anti XIGEMC1 neutralizing antibodies produced in different rabbits ((Ab-2, Ab-3) (see Materials and Methods)). Importantly, figure 3.18 shows that the effect on DNA replication was reproducible.
Fig. 3.15 Test for purified anti XIGEMC1 antibody specificity
Total of 1µl of *Xenopus* egg extract was run on SDS-PAGE gel and immunoblotted with two different dilutions of purified anti XIGEMC1 antibody
Fig. 3.16 Anti XIGEMC1 antibodies abolish replication of sperm chromatin

a) DNA replication assay showing incorporation of a $^{32}$P-dATP in sperm nuclei replicated in egg extracts that were untreated (Un), treated with control (Mock IgG) or anti XIGEMC1 immunoglobulins (XIGEMC1 IgG) (upper panel) in the presence of buffer or an excess of recombinant XIGEMC1 (rec-XIGEMC1)(lower panel) as indicated. DNA was isolated, run on agarose gel that was dried and exposed. The experiment shows a typical result. b) Quantification of the experiment shown in B. Each error bar refers to three independent experiments. Error bars indicate SD. p<0.001 compared with the untreated sample.
**Fig. 3.17 Replication activity of extract added with anti XIGEMC1 antibodies**

Time course of DNA replication showing incorporation of $\alpha^{32}$P-dCTP in sperm nuclei added to egg extract and isolated at the indicated times. Extracts were treated with mock (Mock IgG) or anti XIGEMC1 immunoglobulins (Anti XIGEMC1 Ab-3). DNA was isolated, run on agarose gel that was dried and exposed.
Fig. 3.18 Effects of different anti XIGEMC1 antibodies

DNA replication assay showing incorporation of α\textsuperscript{32}P-dATP in sperm nuclei replicated in egg extracts that were treated with mock (Mock IgG) or different batches of anti XIGEMC1 immunoglobulins (Anti XIGEMC1 Ab-1 and Ab-2) produced in different rabbits.
To verify whether XIGEMC1 is specifically involved in origin dependent DNA replication and to exclude its role in DNA elongation a ssDNA (single strand DNA) replication assay was used. ssM13 DNA was used as a template to monitor replication of ssDNA that does not require the formation of a replication fork. ssDNA replication does not require origins and consequently initiation factors (Mechali and Harland, 1982). RNA primase-DNA polymerase machinery is required for the elongation reaction. Aphidicolin, a specific DNA polymerase α inhibitor, completely abolishes complementary-strand synthesis (Mechali and Harland, 1982). Anti XIGEMC1 neutralizing antibodies did not affect ssDNA replication again indicating that this effect was specific for origin dependent DNA replication (Fig. 3.19) These results strongly suggest that the XIGEMC1 protein is required for origin dependent initiation steps of DNA replication.

In order to exclude any effects on nuclear membrane formation, the generation of such structure was analyzed in the presence or absence of XIGEMC1 antibodies. Eggs of *Xenopus laevis*, and the extract derived from eggs, are capable of assembling functional nuclei that then undergo a single complete round of DNA replication. Noticeably, efficient replication is observed only when DNA is assembled into nuclei. (Blow and Laskey, 1986; Blow and Sleeman, 1990; Newport and Forbes, 1987; Sheehan et al., 1988). Moreover assembly of nuclei from sperm chromatin or purified DNA requires both soluble and vesicular components (membrane vesicle) (Lohka and Masui, 1984; Newport and Forbes, 1987; Sheehan et al., 1988). The most obvious sign that a nuclear envelope has been assembled around the sperm chromatin is the change in nuclear morphology seen during incubation in the extract. These changes can be seen by staining the sperm DNA with fluorescent dye. In addition, nuclear envelopes can also be visualized by using fluorescence staining (DiOC₆(3)) which specifically interacts with membrane phospholipids. Figure 3.20 shows that nuclear membrane formation, which is required for chromosomal DNA replication is not affected by anti XIGEMC1 antibodies.
Fig. 3.19 XIGEMC1 is not involved in the elongation phase of replication

Quantification of single stranded M13 phage DNA replication in egg extracts that were untreated (Un), treated with mock (Mock IgG) or anti XIGEMC1 immunoglobulins (XIGEMC1 IgG). Each error bar refers to three independent experiments. Error bars indicate SD. p<0.001 with untreated sample.
Fig. 3.20 Nuclei assembly in the presence of anti XlGEMC1 antibodies

Hoechst (left) and (DiOC₆(3)) (Right) stained nuclei assembled in extracts that were treated with mock (Mock IgG) and anti XlGEMC1 (XlGEMC1 IgG) immunoglobulins. Scale bars, 10 µm
3.2.6 Role of XIGEMC1 in the formation of the initiation complex

The replication defect observed in the presence of anti XIGEMC1 antibodies suggested a defect at or before replication initiation. The molecular step during origin dependent DNA replication regulated by XIGEMC1 was investigated. To this end the recruitment of pre-RC proteins as well as proteins involved in origin initiation was investigated in the presence or absence of neutralizing XIGEMC1 antibodies. Although they did not affect XIGEMC1 binding to chromatin anti XIGEMC1 antibodies prevented Cdc45 loading onto DNA. The loading of other essential proteins such as XITopBP1, Cdc7, ORC1-6 and MCM2-7 complex was instead unaffected by anti XIGEMC1 neutralizing antibodies, confirming that their effect was specific (Fig. 3.21a, b). This data suggest that following its binding to chromatin, XIGEMC1 is required to promote Cdc45 loading onto replication origins.
**Fig. 3.21 Requirement of XIGEMC1 for the loading of Cdc45 onto chromatin**

Chromatin binding of **a)** Orc2, Mcm7, Cdc45 and XIGEMC1 and **b)** XITopBP1, Orc1, Cdc7 and Cdc45 at the indicated times after addition of sperm nuclei (3000 n/µl) to interphase egg extracts supplemented with mock (Mock IgG) or anti XIGEMC1 immunoglobulins (XIGEMC1 IgG).
3.2.7 Identification of XIGEMC1 associated proteins

To gain details about the molecular mechanism underlying XIGEMC1 function several experiments were performed to identify XIGEMC1 binding partners.

I. Pull Down assay

Recombinant XIGEMC1 fused to MBP tag was incubated in egg extract and pulled down with amylose resin that specifically binds the MBP tag. Proteins interacting with XIGEMC1 were resolved on SDS-page gel. XIGEMC1 was able to co-precipitate Mcm7, Cdc45, CyclinE and Cdk2 (Fig 3.22a, 23a). This experiment suggested that XIGEMC1 interacts (directly or indirectly) with specific factors required for DNA replication initiation. XIGEMC1 did not interact with other factors required for DNA replication such as Cdc7 and Xenopus Sld5, a component of the GINS complex (Fig 3.22b). These interactions were resistant to very stringent washing conditions (NaCl 500-700 mM), implying a strong binding affinity of the identified proteins to XIGEMC1. Surprisingly, although similar to Geminin, XIGEMC1 was not able to interact with the main Geminin partner Cdt1 (Fig. 3.22a). Indeed the aminoacid residues required for Cdt1 binding were not present in XIGEMC1 (Saxena et al., 2004). Cdc6 protein was also not detected in the XIGEMC1 precipitates (data not shown). The XIGEMC1 interaction with the ORC complex was not easily reproducible most likely due to a weak or transient interaction of the two proteins.
Fig. 3.22 XIGEMC1 interacting proteins in *Xenopus* egg extract in pull down assays

WB using antibodies against a) XIGEMC1, Orc1, Mcm7, Cdt1, Cdc45, CyclinE, and Cdk2 and b) Sld5, Cdc7 on pull downs performed with amylose resin that was untreated (Mock), pre-bound to MBP (MBP) or to XIGEMC1 fused to MBP (rec-xXIGEMC1) and subsequently incubated in egg extract. * indicates non-specific band.
II Immunoprecipitation assay (IP)

In order to confirm the pull down results, immunoprecipitation of XIGEMC1 protein was performed. Unfortunately the band corresponding to XIGEMC1 co-migrates in SDS-PAGE gel with the heavy chain of IgGs. To overcome this problem anti XIGEMC1 purified antibody was cross-linked to immobilized protein A. In this way the antibody did not detach from the beads and did not interfere with the XIGEMC1 signal once immunoblotted. Figure 3.23a shows Mcm7 proteins immunoprecipitating with XIGEMC1 protein. The XIGEMC1–Cdc45 interaction could not be reproduced. This result gave rise to the possibility that Cdc45 bound to XIGEMC1 may occur only on the chromatin or that anti XIGEMC1 antibodies interfere with the binding with Cdc45.

To show interaction with Cdk2 we pull down p13sic1 complex that preferentially binds to active Cdk2–Cyclin-E and Cdc2–Cyclin-B complexes with high affinity (Sudakin et al., 1997) (Gabrielli et al., 1992). Interphase extracts prepared from cycloheximide-treated extracts lack Cyclin B (Lohka and Maller, 1985) (Murray and Kirschner, 1989) therefore, only Cdk2–Cyclin E binds to p13sic1 beads (Moreno et al., 1989) (Tang and Reed, 1993). In a pull down assay with p13sic1 cross-linked to agarose beads, XIGEMC1 protein was found to co-immunoprecipitate with Cdk2 kinase Fig. 3.23b.

III In vitro pull down

In order to verify whether any of the previously described interactions were direct, in vitro assays were utilized using recombinant proteins. Recombinant XIGEMC1 was able to directly interact with Cdc45 (Fig. 3.24a) and Cdk2/CyclinE complex (Fig. 3.24b).
Fig. 3.23 GEMC1 interactions in in vivo pull down and IP

a) WB using antibodies against Mcm7 and XIGEMC1 on egg extract immunoprecipitates performed using buffer, anti XIGEMC1 (XIGEMC1 IgG) or mock immunoglobulins (Mock IgG).

b) Pull down of Cdk2 by p^{13}Suc1 agarose resin and WB with antibodies anti XIGEMC1 and Cdk2.
Fig. 3.24 XIGEMC1 interactions in *in vitro* pull down assays

**a)** Pull-downs with nickel resin of histidine tagged recombinant Cdc45 (6xHis-Cdc45) incubated with $^{35}$S-labelled XIGEMC1 produced in reticulocyte lysates ($^{35}$S-XIGEMC1). Reactions were separated on SDS-PAGE and nitrocellulose membrane that was exposed for the $^{35}$S signal and probed by WB with anti Cdc45 antibodies. **b)** WB using antibodies against *Xenopus* Cdk2 on pull downs performed with amylose resin that was untreated (-), pre-bound to MBP (MBP) or to XIGEMC1 fused to MBP (rec-XIGEMC1) and incubated with recombinant Cdk2/Cyclin E complex.
3.2.8 Interaction between Xenopus GEMC1 and XITopBP1

An essential component of the DNA replication machinery is the checkpoint and replication factor TopBP1 (Takisawa et al., 2000). In budding yeast, ScTopBP1 has been shown to play a pivotal role in the loading of the GINS complex (Takayama et al., 2003) and DNA polymerases onto replication origins (Masumoto et al., 2000). The fission yeast protein SpTopBP1 is a functional homolog of ScTopBP1 and both mutants display similar phenotypes of abnormal cell division with incomplete DNA replication, suggesting that SpTopBP1 and ScTopBP1 are required for DNA replication itself (Saka and Yanagida, 1993; Saka et al., 1994; Araki et al., 1995). Human TopBP1 (HsTopBP1) and Drosophila TopBP1 (DmTopBP1) share sequence similarities with XITopBP1 (Yamamoto et al., 2000; Yamane et al., 1997) and HsTopBP1 is also involved in DNA replication and the DNA damage response (Makiniemi et al., 2001) (Yamane et al., 2002). Some mutants of DmTopBP1 display mutagen sensitivities and defects in DNA synthesis suggesting that HsTopBP1 and DmTopBP1 are the functional homologues of yeast ScTopBP1. In 2003 Hashimoto et al. showed that XITopBP1 is essential for DNA replication initiation by promoting Cdc45 loading to chromatin. They also demonstrated that XITopBP1 binds to chromatin at two distinct moments: i) before origin initiation in a S-CDK and pre-RC-independent manner; ii) after origin initiation in a S-CDK pre-RC dependent manner. Strikingly the small quantity of XITopBP1 bound prior to initiation was essential and sufficient to support full replication. Finally recombinant HsTopBP1 binds DNA in vitro in the absence of any other chromatin binding mediator protein such as ORC (Yamane and Tsuruo, 1999). Noticeably XITopBP1 shares with XIGEMC1 important features in the DNA replication pathway. Both proteins load to chromatin at a very early stage of Pre-RC formation, possibly in an ORC independent manner and are essential for Cdc45 association with chromatin.

Further experiments were carried out to investigate the relationship between XITopBP1 and XIGEMC1. XIGEMC1 was found to interact in vitro with recombinant XITopBP1 (Fig. 3.25). This interaction could also be detected in vivo in XIGEMC1 immunoprecipitates (Fig. 3.25). Depletion of XITopBP1 from egg extract inhibited XIGEMC1 loading onto chromatin during the early stages of pre-RC formation (Fig. 3.26). This suggests that XITopBP1 is directly required for XIGEMC1 binding to chromatin. A late and XITopBP1 independent binding of XIGEMC1 to chromatin was
also observed to take place after DNA replication initiation (70 min after nuclei addition) (Fig. 3.26), suggesting that XIGEMC1 has additional binding partners on the DNA that are not directly involved in replication origin firing. The fact that recombinant HsTopBP1 binds DNA \textit{in vitro} (Yamane and Tsuruo, 1999) could also explain how XIGEMC1 recruitment to chromatin occurs independently from ORC.
Fig. 3.25 XIGEMC1 interacts in vivo and in vitro with xTopBP1

a) WB using antibodies against TopBP1 on pull-downs performed with amylose resin that was untreated (Buffer), pre-bound to MBP (MBP) or to XIGEMC1 fused to MBP (rec-XIGEMC1) and incubated with extract (+ Extract) or with recombinant XITopBP1 (rTopBP1). Coomassie blue staining of MBP and MBP-GEMC1 is shown.

b) WB of XITopBP1 and XIGEMC1 on egg extract immunoprecipitates performed using buffer, anti XIGEMC1 (XIGEMC1 IgG) or mock immunoglobulins (Mock IgG).

122
Fig. 3.26 XlGEMC1 requires XlTopBP1 for association with sperm chromatin.

Chromatin binding of TopBP1, Orc1, Cdc45 and XlGEMC1 at the indicated times after addition of sperm nuclei (3000 n/µl) to interphase egg extracts that were mock (Mock) or TopBP1 (∆TopBP1) depleted.
3.2.9 XIGEMC1 is a substrate of S phase kinases

The interaction of XIGEMC1 with Cdk2/CyclinE prompted us to test whether XIGEMC1 was also a substrate for Cdk2. CDKs play crucial roles in cell cycle regulation. They consist of a small kinase subunits which is inactive until complexed to a regulatory subunit called cyclin. In Xenopus egg extract, complete DNA replication still occurs even when protein synthesis is blocked by cycloheximide (Harland and Laskey, 1980; Blow and Laskey, 1988) meaning that all proteins essential for DNA replication are present in the extract, and all replication control is by post-translational modification. Extracts contain approximately 600 nM Cdc2 (Cdk1) and 60 nM Cdk2 (Kobayashi et al., 1991; Kobayashi et al., 1994). Cyclins A and E can both associate with Cdk2 (Pagano et al., 1992; Pines and Hunter, 1990, 1991) and appear to regulate S-phase progression in mammalian cells (Girard et al., 1991; Zindy et al., 1992; Ohtsubo and Roberts, 1993). On exit from mitosis, cyclins A and B, but not cyclin E, are degraded (Minshull et al., 1990; Fang and Newport, 1991; Gabrielli et al., 1992; Rempel et al., 1995). Hence, in interphase Xenopus extracts treated with protein synthesis inhibitors such as cycloheximide, cyclin A is absent and Cdk2 complexed to cyclin E is the only active S phase kinase. Consistent with this, in the presence of cycloheximide DNA replication is inhibited by the CDK inhibitor p21Cip1 (Adachi and Laemmli, 1994; Shivji et al., 1994; Strausfeld et al., 1994), or immunodepletion of either Cdk2 (Fang et al., 1994) or cyclin E1 (Jackson et al., 1995). The analysis of the primary structure of GEMC1 revealed the presence of 8 minimal CDK phosphorylation motives S/T-P (Songyang et al., 1994). In order to ascertain whether XIGEMC1 is phosphorylated by Cdk2, $^{35}$S labelled XIGEMC1 was generated and its phosphorylation pattern was analyzed both in vitro (in vitro kinase assay) and in vivo (in Xenopus egg extract). XIGEMC1 was heavily phosphorylated in vitro by Cdk2/CyclinE and Cdk2/CyclinA (Fig. 3.27a). Indeed, upon the addition of Cdk2/CyclinE and Cdk2/CyclinA recombinant complexes a shift in the electrophoretic mobility of radiolabelled XIGEMC1 was observed. A similar phosphorylation event was induced by incubation of XIGEMC1 protein in interphase egg extract. Addition of $^{35}$S XIGEMC1 to an extract in interphase induced a rapid mobility shift, which presumably reflects phosphorylation by cyclinE/Cdk2 (Fig. 3.27b). Lambda phosphatase treatment and roscovitine were able to suppress such modification (Fig. 3.27b). These results indicate that XIGEMC1, similar to other replication proteins (Diffley, 2004) (Mimura et al., 2004) is a Cdk2 target. The ability of cyclin–Cdk
complexes to select their specific substrates is determined in part by the binding of the cyclin the substrate. The crystal structure of human cyclin A–Cdk2 bound to the inhibitor/substrate p27 Kip1 defined a region of the cyclin A protein that interacts directly with p27 (Russo et al., 1996). This region contains the Met-Arg-Ala-Ile-Leu (MRAIL) motif conserved among cyclin A and cyclin E homologues in many organisms and forms a hydrophobic binding pocket that interacts with an Arg-X-Leu (RXL) peptide within p27. The RXL motif itself is conserved among many cyclin E and cyclin A substrates, including p21, E2F, and p107 (Adams et al., 1996; Chen et al., 1996), suggesting that the RXL motifs are a signature for cyclin–Cdk2 targets. Intriguingly RXL and KXL motives were found to be highly conserved among GEMC1 protein sequences from *Xenopus, H. sapiens* (HsGEMC1), *M. musculus* (MmGEMC1) and *R. norvegicus* (RnGEMC1) (Fig. 3.28). For this reason RXL, KXL cyclin binding sites were individually mutated to Alanine. Upon mutation of R163NL to A163NA (Alanine-X-Alanine) XlGEMC1 phosphorylation by Cdk2/CyclinE complex was abolished (Fig. 3.27a). Importantly, the cyclin-binding site was also required for the interaction between GEMC1 and Cdk2/CyclinE complex as this was abolished by the A163NA mutation (Fig. 3.27c). However, mutation of the cyclin-binding site did not affect the interaction with Cdc45, suggesting that a different domain is required for its binding (Fig. 3.27c).
**Fig. 3.27 XlGEMC1 is a substrate for cyclin E/CDK2**

**a)** Autoradiograph of $^{35}$S-labelled XlGEMC1 ($^{35}$S-GEMC1) or $^{35}$S-labelled XlGEMC1 mutated in the cyclin binding site ($^{35}$S-XlGEMC1 RN1/ANA) produced in reticulocyte lysates and incubated with buffer (-), recombinant Cdk2/Cyclin E (left panel) or Cyclin A (right panel) complex. 

**b)** Autoradiograph of $^{35}$S-labelled GEMC1 ($^{35}$S-XlGEMC1) or $^{35}$S-labelled XlGEMC1 mutated in the cyclin binding site ($^{35}$S-XlGEMC1 RN1/ANA) incubated in egg extract that were treated with buffer (Buffer, lane 1), with lambda phosphatase ($\lambda$PP, lane 2), Roscovitine (rosc, lane 4) or left untreated (lane 3). 

**c)** WB using antibodies against Cdc45, CyclinE, and Cdk2 on pull downs performed with amyllose resin that pre-bound MBP fused to GEMC1 (rec-GEMC1) or to XlGEMC1 mutated in the cyclin binding domain (XlGEMC1 RN1/ANA) and incubated in egg extract.
XlGEMC1 contains 8 minimal CDK phosphorylation motives S/T-P (Songyang and Liu, 2001) none of which are remarkably conserved. (Fig. 3.28, green open square).

Several attempts to identify cyclinE/Cdk2 phosphorylation site/s were performed:

---

**Fig. 3.28** Sequence comparison RXL/KXL motives and S-T/P consensus sites among XlGEMC1 orthologs.

Alignment of XlGEMC1 and its putative orthologs. Marked in Red are the RXL-KXL cyclin binding motives and in Green are the S-T/P consensus sites for CDK2 kinase.
To identify the major Cdk2 phosphorylation site(s) I performed a phosphorylation assay using XIGEMC1 peptide arrays. The peptide array used contains 20mer peptides derived from the XIGEMC1 sequence, shifting by 2 aa towards the carboxy terminus. XIGEMC1 spotted peptide array was incubated with $^{32}$P γATP in the presence or absence of CDK2/CyclinE recombinant protein (see Materials and Methods). The appearance of radioactive signal allowed the identification of peptides most efficiently phosphorylated by Cdk2: from peptide 75 to 82 (Fig. 3.29). Interestingly, peptides showing a strong CDK2 dependent phosphorylation are all characterized by the presence of both R163NL cyclin binding motif and an upstream T153 phosphorylation consensus site for CDK. Recent results have shown that RXL motif located at a minimal distance from the S-T/P consensus sites is essential for optimal phosphorylation by CDK (Wohlschlegel et al., 2001). Our finding suggested that TP at position 153 might represent the main phosphorylation site for CDK. An antibody against phosphorylated Threonine 153 (anti P-Thr 153) was generated and its specificity was successfully tested in an elisa assay (Fig. 3.30). The anti P-Thr 153 antibody was able to recognize endogenous XIGEMC1 phosphorylated on Threonine 153 in interphase egg extract, indicating that XIGEMC is phosphorylated on Threonine 153 in vivo (Fig. 3.31).
Fig. 3.29 Mapping the sites on XIGEMC1 phosphorylated by cyclinE/CDK2

GEMC1 spotted peptide array consists of 20 aa peptide fragments shifting 2 aa towards the carboxy terminus was exposed to kinase buffer containing $^{32}$PαATP in the presence or absence of CDK2/CyclinE recombinant complex. Through phospho-image analysis a series of spot intensity changes in the presence of CDK2 were identified (these spots contain both TP and RNL sites)
Fig. 3.30 Specificity of anti phospho-Threonine 153 antibody

Chemiluminescence based elisa assay (see Methods) showing the specificity of anti phospho-Threonine 153 immunoglobulins at the indicated dilutions incubated with immobilized peptide bearing the phospho-Threonine 153 in the presence of buffer (Un), an excess of competing phospho-Threonine 153 peptide (P-Thr-153 pept) or an excess of unphosphorylated Threonine 153 peptide (Thr-153 pept).

Fig. 3.31 In vivo phosphorylation of XI GEMC1 on Threonine 153

WB of egg extract using antibodies against phosphorylated Threonine 153 of XI GEMC1. Samples were untreated (-), incubated with lambda phosphatase (λPP) or lambda phosphatase plus phosphatase inhibitors (λPP+i).
II Point mutation of S/T-P CDK phosphorylation motives to Alanine

Following the previous result a T153A mutant was generated and tested for its ability to be phosphorylated by cyclinE/Cdk2 and CyclinA/Cdk2 complexes. Contrary to our predictions the XIGEMC1 mobility shift was not affected by either kinase (Fig. 3.32a). Moreover no changes in phosphorylation patterns were observed when the 8 S/T-P sites were individually mutated to alanine. These results suggest that XIGEMC1 can be phosphorylated on multiple sites by Cdk2 or that different S/T-P sites become accessible for Cdk2 phosphorylation when the main CDK consensus site/s is/are mutated. Instead mutation to alanine of all the S/T-P motives completely abolished Cdk2 dependent phosphorylation (in vitro and in extract) (Fig. 3.32b) to the same degree as cyclin binding site A163NA mutant.

As I detected multiple roscovitine sensitive phosphorylated forms of XIGEMC1 protein in egg extract (Fig. 3.32b) it is likely that these sites are also phosphorylated by Cdk2.
Fig. 3.32 XIGEMC1 phosphorylation sites for CDK2/CyclinE

(a) Autoradiograph of $^{35}$S-labelled XIGEMC1 (^{35}$S-GEMC1) and $^{35}$S-labelled XIGEMC1 proteins containing single serine or threonine to alanine substitutions at the indicated residues (T153A; S177A; S215A; T226A; S239A; S255A; S259A and T294A) produced in reticulocyte lysates and incubated with buffer (-), recombinant Cdk2/CyclinA (Cdk2/A) or Cdk2/CyclinE complexes (Cdk2/E); schematic representation of the mutated sites. 

(b) Autoradiograph of $^{35}$S-labelled XIGEMC1 containing multiple serine or threonine to alanine substitutions at the indicated residues (T153A; S177A; S215A; T226A; S239A; S255A; S259A and T294A) produced in reticulocyte lysates and incubated with buffer (-), recombinant Cdk2/CyclinA (Cdk2/A) or Cdk2/CyclinE complexes (Cdk2/E)
3.2.10 Effect of Cdk2 phosphorylation on XIGEMC1 activity in DNA replication

I next assessed the relevance of XIGEMC1 phosphorylation by Cdk2 for DNA replication. Due to the abundance of XIGEMC1 in egg extract and its tight interaction with other essential replication factors I could not replace endogenous XIGEMC1 with XIGEMC1 mutated on Cdk2 sites. Therefore I supplemented egg extract with recombinant XIGEMC1 bearing putative Cdk2 serine-threonine residues mutated to glutamate (XIGEMC1-8ST/E) to mimic constitutively phosphorylated protein. In this case XIGEMC1-8ST/E was able to stimulate DNA replication initiation (Fig. 3.33a). The stimulation of DNA replication was accompanied by an increased loading of Cdc45 onto chromatin (Fig. 3.33b). This indicates that XIGEMC1-8ST/E enhances origin firing by promoting Cdc45 binding to chromatin. The stimulation of DNA replication was similar to the one induced by caffeine, which is known to increase Cdk2 activity by inhibiting the ATM/ATR checkpoint that represses Cdk2 dependent origin firing (Shechter et al., 2004b). Intriguingly, XIGEMC1-8ST/E showed higher affinity for XITopBP1 compared to wild type XIGEMC1 (Fig. 3.34), suggesting that the increased loading of Cdc45 is directly mediated by the increased binding of XIGEMC1-8ST/E to XITopBP1. Overall these data indicate that phosphorylation of XIGEMC1 drives initiation of DNA replication by mediating XITopBP1 and Cdk2 dependent loading of Cdc45 onto replication origins.
a) Stimulation of DNA replication measured at 30 minutes from the addition of sperm nuclei to egg extract induced by 200 ng/µl wild type recombinant XIGEMC1 (rec-XIGEMC1) and 200 ng/µl XIGEMC1-8ST/E carrying the serine and threonine residues phosphorylated by Cdk2 mutated to glutamate to mimic constitutively phosphorylation. Recombinant XIGEMC1 proteins used in this experiment were defective for the Cyclin binding domain to avoid titration of endogenous Cdk2/Cyclin E complex. b) Chromatin binding of Cdc45 at the indicated times after addition of sperm nuclei (3000 n/µl) to interphase egg extracts supplemented with buffer (Buffer) or 200 ng/µl of glutamate substituted XIGEMC1 defective for the Cyclin binding domain (XIGEMC1-8ST/E). Histone H2B was used as loading control. OD indicates optical density of each lane.

Fig. 3.33 XIGEMC1-8ST/D was able to stimulate DNA replication initiation
Fig. 3.34 XIGEMC1-8ST/D shows higher affinity for TopBP1 compared to wt

WB of TopBP1 on pull downs performed with amylose resin that was pre-bound to MBP (MBP), to MBP fused to wild type XIGEMC1 (rec-XIGEMC1) or to glutamate substituted XIGEMC1 (rec-XIGEMC1-8ST/E) and incubated with purified recombinant TopBP1.
3.3 Discussion

The aim of the work described in this thesis was to characterize the function of a novel vertebrate protein using the cell-free replication system of *Xenopus laevis* egg extract as a biochemical model system. In the next chapters I will show that experiments performed on *Xenopus* embryos and mammalian cells contributed to validate the results described here using the extract system. The experiments undertaken involved:

- isolation of a candidate protein (XlGEMC1) playing a role in chromosomal DNA replication based on the analysis of conserved domain architecture
- examination of the role of XlGEMC1 in the initiation of DNA replication which unveiled the intriguing possibility for XlGEMC1 to represent a functional homolog of yeast Sld3 in vertebrates (these data and candidate protein isolated will be discussed together in this chapter).
- study of XlGEMC1 downregulation in embryo development (discussed in chapter 4)
- Preliminary observations of mGEMC1 knock-out effect in mammalian cells (discussed in chapter 5)

3.3.1 The XlGEMC1 coiled-coil domain shares homology with the hGeminin coiled-coil domain

In the presence of DNA damage arising prior to initiation of DNA replication and pre-RC assembly, ATM and ATR have been shown to prevent origin firing through inhibition of Cdk2 and Cdc7-Dbf4 (Abraham, 2001; Costanzo et al., 2003; Shechter et al., 2004b). In addition to their role in DNA damage checkpoint, ATM and ATR have also been proposed to operate during an unperturbed cell cycle to regulate initiation of DNA replication by monitoring the selection and timing of origin firing (Shechter and Gautier, 2005). My first aim was to ascertain whether any of the positive clones emerging from the screening (described in this chapter) could be a novel candidate involved in DNA replication. The mentioned screening was meant to identify novel factors directly or indirectly phosphorylated by ATM-ATR. These two protein kinases are well known to be involved in cell cycle checkpoint and DNA repair pathways. ATM and ATR phosphorylated proteins from a *Xenopus laevis* expression library were isolated. ATM and ATR were activated in egg extract as a result of the incubation with linear double strand molecules (DSBs), which mimic DNA double
strand break damage (Heald et al., 1996; Lustig et al., 1997). Mobility shift changing of
$^{35}$S labeled proteins was used as readout to scores ATM and ATR phosphorylated
proteins. Since several positive clones were isolated, primary sequences from the
candidates were examined by using BLAST (Basic Local Alignment Search Tool),
looking for any homology with proteins known to cover a role in DNA replication.
Among several candidates, I identified XIGEMC1 protein.

The XIGEMC1 protein has a coiled–coil domain showing a primary,
secondary and tertiary structural similarity with the hGeminin coiled-coil domain. The
presence of the coiled-coil domain indicated that XIGEMC1 could play a role
comparable or related to Geminin in DNA replication. In addition to the structure of a
typical coiled-coil domain (characterized by repeating pattern of seven residues:
abcdefg)(Saxena et al., 2004), XIGEMC1 also shows an aminoacid composition that
suggests further similarities to Geminin. A conserved positively charged amino acid
Lys109 surrounded by polar residues as well as charged residues at the e and g
positions could contribute to the determination of a structural uniqueness by stabilizing
the coiled-coil structure (Akey et al., 2001). Moreover the presence of a negatively
charged surface could be responsible for protein-protein interaction based on strong
electrostatic interaction as shown for Geminin and Cdt1.

Although XIGEMC1 has a domain that shares similarity with Geminin, in
contrast to Geminin, it does not bind Cdt1 and it does not inhibit DNA replication
when recombinant protein is added to interphase extract (at endogenous concentration).
Moreover XIGEMC1 does not undergo any M phase degradation as a result of APC-
mediated ubiquitination. These data are supported by the fact that XIGEMC1 does not
contain any destruction box consensus sequence of mitotic cyclins like those observed
in the amino terminal portion of Geminin. In fact XIGEMC1 protein levels appear to be
stable throughout the cell cycle (data not shown) raising the possibility that a different
pathway or alternative post translation modifications regulate XIGEMC1 stability. The
observation that XIGEMC1 does not behave similarly to Geminin in this respect could
be explained by the fact that the N terminal region of XIGEMC1 coiled-coil domain is
not conserved. It is also possible that the presence of a loop in the coiled-coil structure
could be responsible for the destabilization of the Geminin like coiled-coil domain in
XIGEMC1. Two bulky amino acids (Phe126 and Phe131) contained in this flexible
region could in fact produce packing instability in the interface affecting the whole domain structure (O'Shea et al., 1991). It is conceivable that this domain represents a signature motif typical of replication proteins that have then acquired different functions during evolution.

3.3.2 **Functional role of XlGEMC1 protein in DNA replication initiation**

As discussed above, XlGEMC1 shares various aspects of sequence and structural homology with the human replication protein Geminin. In subsequent experiments I sought to examine whether XlGEMC1 might also share a functional role in DNA replication.

A variety of model systems have highlighted several proteins including Cdk2/CyclinE, Cdc7/Dbf4, Sld2, Sld3, TopBP1, Mcm10 and GINS that are all required for Cdc45 to be stably loaded onto S-phase chromatin, although the contribution of each protein to the initiation process is still unclear (Mendez and Stillman, 2003; Kubota et al., 2003; Van Hatten et al., 2002). Binding of Cdc45 protein is critical for origin unwinding, recruitment of the single stranded DNA binding protein RPA and the loading of DNA polymerase α (Mimura and Takisawa, 1998; Tanaka and Nasmyth, 1998). XlGEMC1 was found to be essential for Cdc45 binding to chromatin in vertebrates (this point will be discussed more in detail in the next paragraph). XlGEMC1 directly interacts with essential replication factors such as TopBP1, Cdc45 and Cdk2/CyclinE. This suggests that XlGEMC1 is involved in the initiation step of DNA replication.

3.3.3 **XlGEMC1 : vertebrate homologue of yeast Sld3 protein?**

In *S. cerevisiae* a complex formed by Sld2, Sld3 and ScTopBP1 is essential to trigger DNA replication initiation (Tanaka et al., 2007; Zegerman and Diffley, 2007). Emerging evidence shows that Sld2 and Sld3 proteins represent the minimal set of S-phase Cdk substrates required for DNA replication (Tanaka et al., 2007; Zegerman and Diffley, 2007). To date, an Sld2 homologue in vertebrates that shows a weak sequence similarity with the Sld2 N terminal region, has been identified and corresponds to RecQL4 protein (Matsuno et al., 2006; Sangrithi et al., 2005). Instead Sld3 homologues have not been identified in other eukaryotes by screening for sequence homology. Indeed, even within fungi, Sld3 is very poorly conserved (Tanaka et al., 2007;
Zegerman and Diffley, 2007), suggesting rapid evolution of this protein.

In the following paragraph I will highlight data, which support the idea that XIGEMC1 plays a different role from Geminin. The data support the possibility that GEMC1 may represent a functional homologue of Sld3 protein in higher eukaryotes.

Geminin plays an essential role in regulating the pre-RC complex formation. Geminin inhibits inappropriate origin firing once cells enter S phase. XIGEMC1 and Geminin demonstrate significant differences since XIGEMC1 does not interact with Cdt1 protein, which represents, the major Geminin interactor. Furthermore XIGEMC1 levels are not cell cycle regulated as previously discussed. Finally, XIGEMC1 inhibition by specific neutralizing antibodies does not induce any re-replication phenomenon, a typical effect of Geminin down-regulation (Melixetian et al., 2004), but it affects DNA replication initiation by preventing Cdc45 loading to chromatin.

Sld3 protein interacts with Cdc45 and is required for its chromatin binding in both budding and fission yeast (Kamimura et al., 2001) (Nakajima and Masukata, 2002). Importantly XIGEMC1 directly co-immunoprecipitates Cdc45 in vitro. The physical interaction between XIGEMC1 and Cdc45 together with the data mentioned above suggests that XIGEMC1 plays a direct role in the loading of Cdc45 onto chromatin. In addition, Sld3 associates with the origin during G1-phase, and is then displaced without being incorporated into the replisome differently from Cdc45 whose origin signal increases as cells enter S-phase and the protein moves with the replisome (Kanemaki and Labib, 2006). Likewise XIGEMC1 binds to chromatin at a very early stage of DNA replication, specifically in a both MCM2-7 and CDK independent manner. As previously mentioned in paragraph 3.2.7 an interaction between XIGEMC1 and XITopBP1 was observed in both interphase Xenopus egg extract and directly in vitro, explaining the direct dependency of XIGEMC1 on XITopBP1 for recruitment to chromatin. XITopBP1 has been shown to bind to chromatin in both a S-CDK dependent and a S-CDK independent manner (shown to be sufficient to support full replication) (Hashimoto and Takisawa, 2003). An interesting observation is that XITopBP1 could also bind to chromatin in the presence of Geminin, which inhibits MCM2-7 binding (data not shown). It is reasonable to think that the portion of XITopBP1 protein essential to promote DNA origin firing is the one, which mediates
XIGEMC1 recruitment to chromatin. Unfortunately, the experimental inability to immunodeplete XIGEMC1 from the extract did not allow me to address this point any further.

Previous works in yeast have also shown that Sld3 as well as Sld2 must bind to ScTopBP1 to promote subsequent origin unwinding and replisome assembly (Tanaka et al., 2007; Zegerman and Diffley, 2007). This docking presumably occurs at sites marked as origins of DNA replication by the origin-recognition complex. Antibodies raised toward XIGEMC1 did not work for immunofluorescence, therefore I could not verify any specific co-localization of XIGEMC1 protein with replication factors. Nevertheless, the data presented support the idea that XIGEMC1 is in fact recruited to replication origins.

I could not detect significant amino acid similarity between yeast Sld3 and XIGEMC1 proteins, but an important feature is that both XIGEMC1 and Sld3 are phosphorylated by S-phase CDKs. One of the major roles of CDKs is to promote chromosomal DNA replication (Fang and Newport, 1991; Jackson et al., 1995). However, the way in which CDKs promote DNA replication has been a long-standing question. The essential CDK substrates important for DNA replication have not been uncovered yet. Recently, Sld2 and Sld3 proteins were identified as essential substrates of CDKs in the initiation step of DNA replication in budding yeast. Zegerman and Diffley and Tanaka et al., showed that the CDK phosphorylation of Sld3 on two amino-acid residues (Thr600, Ser622) and of Sld2 on a single residue (Thr84) enhance the formation of complexes with ScTopBP1 (S.cerevisiae TopBP1) protein and this interaction was shown to be essential to initiate DNA replication. They demonstrated that in a S. cerevisiae haploid strain, fusion protein between Sld3-2A (carrying an alanine mutation of the 2 CDK consensus sites Thr600, Ser622) and the C-terminal half of ScTopBP1 (expressed as the only copy of Sld3) when combined to a phospho-mimic SLD2 T84D, was indeed able to initiate DNA synthesis even in the absence of S-phase CDK activities in G1 phase (Zegerman and Diffley, 2007). In parallel Tanaka and colleagues isolated a dominant JET-1 mutation of CDC45 (Jumping CDK Essentailty with Sld2 (Two)-11D to initiate DNA replication) that was able to induce untimely DNA replication in the absence of CDK activity when the phospho mimetic Sld2 (Sld2-11D) was expressed. The work of Tanaka et al. demonstrated that JET-1 protein appeared to enhance the interaction between Sld3 and ScTopBP1 through the
interaction between Sld3 and Cdc45. They suggest that CDKs regulate the initiation step of DNA replication by controlling Cdc45 association with chromatin indirectly and, in turn, the recruitment of the replication machinery composed of Cdc45 itself, GINS and Pole to the putative helicase MCM2-7 (Tanaka et al., 2007). The phosphorylated form of XIGEMC1 shows a strikingly higher affinity for XITopBP1 protein \textit{in vitro} giving a good indication that similarly to Sld3, CDK2 dependent regulation of XIGEMC1 could represent a mechanism of initiation control in eukaryotes. At this stage I tried to understand the relevance of such CDK phosphorylation for XIGEMC1 function. To this end I examined whether CDK dependent phosphorylation of XIGEMC1 was able to influence DNA replication similarly to Sld3 phospho-mutants. Addition of a phospho-mimic rec-XIGEMC1-8ST/D to the extract was indeed able to stimulate origin firing by promoting both earlier and more efficient Cdc45 loading to chromatin. This result partially resembles the one obtained in yeast by Sld3-Sld2, supporting the hypothesis that XIGEMC1 dependent regulation by CDK in the initiation of DNA replication might be a conserved mechanism.

A peptide array allowed the identification of Threonine 153 as the major residue phosphorylated by Cdk2. Cdk2/CycE was also found to strongly interact with XIGEMC1 \textit{in vitro} and \textit{in vivo} arguing that XIGEMC1 represents a direct substrate of the kinase. XIGEMC1, like Sld2 and Sld3 proteins, contains several CDK phosphorylation consensus sites that induce a hyperphosphorylated pattern both \textit{in vitro} and \textit{in vivo}, indicating that XIGEMC1 is an excellent substrate of S-phase CdK. Although Threonine 153 was found to be the major phosphorylated site, an XIGEMC1 mutant lacking Threonine 153 was still phosphorylated \textit{in vivo}. It was observed that the combined mutations to alanine of the additional seven Cdk sites completely suppressed XIGEMC1 phosphorylation. This could be explained by a high redundancy of the Cdk phosphorylation sites that renders none of these sites ultimately essential for XIGEMC1 function when singularly mutated. An additional possibility would be that simultaneous phosphorylation of CDK consensus sites are essential for XIGEMC1 interaction with XITopBP1. It is tempting to speculate that as for Sld2, multiple phosphorylation of XIGEMC1 by Cdk2 could work in concert to render the phosphorylation of the main CDK consensus site (likely represented by Thr153) more accessible to CDKs. Multi-site phosphorylation might set a high threshold of CDKs
activity which guarantees XIGEMC1-XITopBP1 interaction. An important implication of this is that the high threshold of CDK activity may be also important to prevent premature replication (Tak et al., 2006). Although evidence suggests that CDK-dependent phosphorylation of XIGEMC1 plays an important role in regulating XIGEMC1 function in DNA replication, a precise functional relevance for each CDK-consensus site in the complex formation between XIGEMC1 and XITopBP1 has not been addressed in this thesis.

### 3.3.4 Investigating the interaction of XIGEMC1 and known replication factors.

In *Xenopus* both Cdk2 and Cdc7 are required for the initiation of DNA replication (Bell and Dutta, 2002; Takisawa et al., 2000). The role of CDK activity with regard to XIGEMC1 function has been discussed in the previous section. However I also sought to consider any functional interaction between XIGEMC1 and Cdc7. XIGEMC1 phosphorylation was shown to be entirely dependent on CDKs since Roscovitine treatment in both *in vitro* kinase assay and in *Xenopus* extract by itself was sufficient to completely abolish recombinant $^{35}$S XIGEMC1 phosphorylation. Mutation in the RXL cyclin consensus site of XIGEMC1 also affected XIGEMC1 phosphorylation. Moreover, although XIGEMC1 recruitment to chromatin takes place independently of Cdk2, the kinase activity has been shown to be essential for XIGEMC1 interaction with XITopBP1. My experiments showed that XIGEMC1 does not co-precipitate Cdc7 in a pull down assay. Consistent with this, purified antibodies against XIGEMC1 were shown to affect DNA replication without interfering with Cdc7 loading to chromatin. These data suggest the idea that XIGEMC1 does not play any role downstream of Cdc7.

Similar to Sld3, XIGEMC1 does not seem to interact with GINS as concluded from pull-down assay. Also in yeast no coprecipitation of GINS and Sld3 was observed (Kanemaki and Labib, 2006; Yabuuchi et al., 2006), although a genetic interaction between the two proteins was detected (Takayama et al., 2003). However, these data do not rule out the possibility that an interaction between XIGEMC1 and GINS (as well as Cdc7) takes place on the chromatin. In budding yeast the binding of the GINS complex to chromatin requires Sld3 (Takayama et al., 2003). Differently from Sld3, the binding of GINS to chromatin was not significantly affected (not shown) in the presence of neutralizing antibodies towards XIGEMC1.
3.3.5 XI-GEMC1 and DNA synthesis

I have investigated the role for XI-GEMC1 in origin independent DNA chain elongation by using complementary-strand DNA synthesis assay. In *Xenopus* egg extract, priming and elongation of a complementary strand from single-stranded circular DNA templates occurs with high efficiency (Mechali and Harland, 1982). Replication of ssDNA takes place independently of origin firing, DNA is not unwound and replication forks are not generated. The presence of anti XI-GEMC1 neutralizing antibodies was shown not to affect ssDNA replication. This data suggests that XI-GEMC1 is dispensable for complementary strand synthesis using an M13 ssDNA template and for the elongation phase of DNA replication. However, the assay provides only a limited model for replication elongation and as such we cannot exclude the possibility that XI-GEMC1 may cover additional functions during elongation beyond its role in DNA replication initiation.

XI-GEMC1 was initially identified through screening for direct and indirect substrates of ATM/ATR kinase activity. The XI-GEMC1 protein displays five S-T/Q consensus sites for ATM/ATR phosphorylation and is indeed hyper-phosphorylated in the presence of DSBs following activation of ATM/ATR responses. Furthermore, this work has shown that XI-GEMC1 interacts with XI-TopBP1, which is a known key participant in the S-phase DNA damage checkpoint response of eukaryotic cells (Hashimoto et al., 2006; Navadgi-Patil and Burgers, 2008; Yan and Michael, 2009).

Further studies will be required to assess whether XI-GEMC1 also plays a role in DNA damage checkpoint pathways.
4 Results II- *Xenopus* GEMC1 in developing embryos

4.1 Introduction

The use of frog embryos has provided many insights into developmental and cell biology (Danilchick et al., 1991) (Gamse and Sive, 2000). *Xenopus* embryos offer several advantages:

- They develop externally so that development can be followed continuously
- Hundreds of oocytes can be generated per ovulation
- The large size of the embryos allows microinjection techniques followed by *in situ* hybridization or immunohistochemical analysis (Gamse and Sive, 2000)

The introduction of antisense morpholino oligonucleotides (MOs) has provided a new alternative to study loss of gene function in *Xenopus* and also Zebrafish embryos (Heasman et al., 2000) (Nasevicius and Ekker, 2000). Morpholino oligos strongly and specifically bind to their complementary site in a strand of mRNA. MOs are designed so that they interact with the 5’ UTR of mRNA or in a coding region close to the ATG. MOs inhibit translation by sterically blocking the translation initiation complex and can specifically knock down the expression of many target sequences completely (Khokha et al., 2002).

In order to validate the biochemical results obtained in *Xenopus* egg extract, it was important to achieve GEMC1 depletion. In this chapter I describe the work to study the biological function of GEMC1 in *Xenopus* embryo development. My data show that morpholino-mediated knockdown of GEMC1 is responsible for specific defects in morphology, cell proliferation and activation of an apoptotic program.
4.2 Results

4.2.1 XlGEMC1 expression in developing embryos

In *Xenopus* oocytes, after fertilization, the first twelve cell divisions are rapid and synchronous (Graham, 1966). They are characterized by alternating S and M phase, without gap phase G1 or G2 phases. These cycles are regulated by maternally supplied products, because they occur in the absence of transcription. The midblastula transition (MBT) begins after the twelfth cleavage (Newport and Kirschner, 1982). It is characterized by cell cycle lengthening, loss of cell cycle synchrony, activation of zygotic transcription and appearance of cell motility. After this phase, the embryos go through several different stages (Fig. 4.1). The process of gastrulation occurs over a period of several hours (ending at stage 12). It entails an extremely complex set of movements, the first step being to achieve morphological boundaries. Shortly after the end of gastrulation, during neural development, the neural plate becomes progressively more prominent on the dorsal side of the embryo. This will successively generate the central nervous system in the adult frog.

Examining the temporal expression of XlGEMC1, between fertilization and swimming tadpole stage, the protein was found to be expressed throughout development. XlGEMC1 was detected in unfertilized eggs (as demonstrated by the presence in frog extract) and expressed at relatively constant levels throughout cleavage, gastrulation, neurulation and the tadpole stage (Fig. 4.2). Since transcription of zygotic genes does not start until the midblastula transition (Stage 8-9), XlGEMC1 mRNA as well as the protein itself are both maternally inherited.

Several attempts to identify the expression level of XlGEMC1 in embryonic tissue were carried out. Previous WB data showing the presence of XlGEMC1 protein in all adult frog tissues and in all *Xenopus* embryo developmental stages suggested the possibility that XlGEMC1 could be distributed in every embryonic tissue. Unfortunately no purified antibody gave a clear signal for immunohistological analysis.
Fig. 4.1 Xenopus embryo staging
Images representing *Xenopus* embryo staging series based on morphological criteria
**Fig. 4.2 Embryonic expression of GEMC1**

Equal amounts of protein extracts prepared from different stages were separated by electrophoresis followed by WB with anti XIGEMC1 antibody. The protein is detected from stage 1 to stage 40 and the level of expression does not change over the embryo development. Drawings of the embryonic stages are shown above the western blot.
4.2.2. **Depletion of XlGEMC1 by antisense oligos in frog embryos**

4.2.2.1 **Specificity of XlGEMC1 morpholino knockdown**

To confirm the results obtained in egg extract, XlGEMC1 protein was depleted from *Xenopus* embryos by selectively inactivating XlGEMC1 expression with morpholino antisense oligos (MOs). Morpholinos have been shown to be reliable for the analysis of gene function in *Xenopus laevis* (Ekker and Larson, 2001; Heasman, 2002). They provide advantages over siRNA and phosphorothioates including excellent sequence specificity and complete resistance to nucleases (Summerton and Weller, 1997). They are stable and effective quite late in development (untill the early tadpole stage in Xenopus) (Nutt et al., 2001). Two 25mer morpholino oligonucleotides (MO-GEMC1-A, MO-GEMC1-B) were designed within the region surrounding the start codon ATG (-80 bp to +30 bp relative ot the A of the start codon) (Fig. 4.3) according to the criteria described on the Gene Tools Web site ([www.gene-tools.com](http://www.gene-tools.com)). The specificity of GEMC1-MOs was examined in the *in vitro* transcription and translation system. As shown in Figure 4.4, the 48 kDa XIGEMC1 protein was synthesized when buffer and control Morpholino (a MO with a scrambled sequence) were added into the *in vitro* transcription and translation system. XIGEMC1 was drastically reduced when either MO-GEMC1-A or MO-GEMC1-B were added to the reaction, demonstrating the efficacy of their translation inhibition.
Fig. 4.3 MOs target sites on XlGEMC1 mRNA
Schematic diagram illustrating the morpholino oligonucleotides (MO-GEMC1-A; MO-GEMC1-B) repressing XIGEMC1 mRNA

Fig. 4.4 Effects of morpholino oligos on in vitro translated XIGEMC1

$^{35}$S labelled XIGEMC1 ($^{35}$S-XIGEMC1) translated in reticulocyte lysates that were untreated (Un), incubated with control scrambled morpholino oligos (C-MO) or morpholino oligos complementary to XIGEMC1 (XIGEMC1-1 MO and XIGEMC1-2 MO). A mixture of these 2 oligos was injected in *Xenopus* embryos.
**4.2.2.2 XIGEMC1 deficiency is lethal for Xenopus embryogenesis**

Fertilized embryos were injected with 10 µM of XIGEMC1 MOs before the first cleavage occurred. The high dose injected was chosen to increase the chances of knocking down the protein from all embryonic compartments. When injected into developing embryos, XIGEMC1-MO oligos led to complete inhibition of XIGEMC1 expression by stage 37 (Fig. 4.5). Injection of control MO oligos with a scrambled sequence (C-MO) did not affect XIGEMC1 levels. Morpholino antisense oligo conjugated in 3’ to a fluorescein tracer was used to verify injection success. By following the developmental processing no morphological anomaly was observed before MBT (Fig. 4.6). This is most likely due to XIGEMC1 already present in the unfertilized eggs. This observation might explain why XIGEMC1 protein knockdown occurs very late in development (stage 37). Consistent with this, there was no overt defects in germ layer formation, gastrulation or neurulation. Most of the embryos injected with XIGEMC1-MO oligos showed severe delay in embryo development. The most significant effects were detected in concert with the down-regulation of XIGEMC1 proteins level after the neurula stage, even though a developmental delay could also be observed at an earlier time (Fig. 4.7, 8). By stage 24 the developmental delay is clearly detectable. This effect can be due to an initial MOs dependent reduction in XIGEMC1 protein which cannot be detected by WB. In addition, we observed poor yolk resorbption and no distinct anatomical structure such as the gut, the optic vesicle or the tail in the 90% of the embryos in the XIGEMC1 depleted embryos (Fig. 4.8). Thus complete loss of XIGEMC1 was ultimately lethal to the embryo, suggesting that XIGEMC1 is required for normal development from neurula to tadpole. Equivalent dose of control morpholino as well as non-injected embryos did not affect the timing of developmental event such as neurulation. Moreover the same control did not exhibit many noticeable defects distinct from a very low frequency of minor defects likely due to the injection procedure. The majority of control embryos (not injected and C-MO) correctly developed as did XIGEMC1-MOs embryos at stage 18-22.
**Fig. 4.5 Depletion of XlGEMC1 with a specific antisense MO.**

WB of XlGEMC1 extracted from *Xenopus* embryos that were untreated (Un), injected with control (C-MO) or morpholino oligos complementary to XlGEMC1 DNA sequence (XlGEMC1-MO) taken at the indicated stages. Lower panel shows coumassie staining of total embryo lysate.
Fig. 4.6 XIGEMC1-MO does not affect embryo development before mid blastula transition (MBT)

Embryos not injected, injected with control morpholino (C-MO) or XIGEMC1 morpholino (XIGEMC1-MO) were photographed at MBT (stage 7-8)
Fig. 4.7 Delayed development of *Xenopus* embryos after gastrulation in response to XlGEMC1-MO treatment

Embryos were either not injected or injected with morpholino (C-MO, XlGEMC1-MO). Note how after gastrula stage (stage 12) XlGEMC1 embryos delay the development during neurula stage. Arrows represent the most severe delays.
Fig. 4.8 XlGEMC1 depleted Xenopus embryos

Morphology of *Xenopus* embryos that were untreated (Un), injected with control (C-MO) or anti XlGEMC1 morpholino oligos (XlGEMC1-MO) taken at the indicated stages.
4.2.3 Loss of XIGEMC1 function by antisense morpholino oligonucleotides induces apoptosis

Embryos at stage 32-34 were transversally sectioned (Material and Methods). Sections at the head level were then stained with hematoxylin and eosin. Hematoxylin colours basophilic structures (predominantly those containing nucleic acids) such as the ribosomes and the chromatin-rich cell nucleus with a blue-purple hue. Eosin instead colours eosinophilic structures mostly contained in the cytoplasm (generally composed of intracellular or extracellular proteins) that appear as a bright pink dye. Figure 4.9. Histological analysis revealed a dramatic decrease in cell density and cell adhesion when XIGEMC1 expression was inhibited (Fig. 4.9). This was also accompanied by a severe decrease in DNA content (Fig. 4.10). By stage 30, GEMC1–MO injected embryos contained 30% less DNA than control embryos.

Embryonic death with a dramatic disruption of intercellular contacts is frequently characteristic of apoptosis. Apoptosis was verified by biochemical assay for caspase activity. Embryo extracts from stage 32-34 were tested for their ability to cleave the well-characterized caspase substrate tPARP (Hensey and Gautier, 1997). Radiolabelled $^{35}$S tPARP was generated and incubated in the presence of embryo extract. Extract of non-injected embryos and C-MO did not cause cleavage of tPARP (Fig. 4.11). XIGEMC1–MO treated embryos, however, did cause tPARP cleavage, visualized in SDS-PAGE gel as two small bands compared to the original uncut band. Since the decrease of DNA content was detected earlier than the apoptotic phenotype, it is likely due to deficient proliferation and not to an increased apoptosis.
Fig. 4.9 Embryos injected with GEMC1-MO present a severe tissue disorganization. No defined anatomical structure can be recognized in most cases.

Sections of fixed *Xenopus* embryos injected with control (C-MO) or anti XlGEMC1 morpholino oligos (XlGEMC1-MO) and stained with ematossilin/eosin. Area in the rectangle shows 5x magnification.
**Fig. 4.10 DNA content reduction in GEMC1 deficient embryos**

DNA content reduction in embryos that were uninjected (Not injected), injected with control morpholino oligos (C-MO) or anti XlGEMC1 morpholino oligos (XlGEMC1-MO).

**Fig. 4.11 Inhibition of endogenous XlGEMC1 results in apoptosis**

Cleavage of $^{35}$S labeled PARP induced by lysates of embryos that were uninjected (Not injected), injected with control morpholino oligos (C-MO) or anti XlGEMC1 morpholino oligos (XlGEMC1-MO).
Altogether these results indicate that XI-GEMC1 is essential for normal cell proliferation during development as expected for a protein essential for DNA replication. Other studies have in fact shown how replication proteins (MCM5 and GINS) play a crucial role in ensuring efficient cell cycle progression during vertebrate development (Ryu et al., 2005; Walter et al., 2008).
4.3 Discussion

This section discusses the results of work undertaken in Xenopus embryos. The purpose of these experiments was to make use of a model system in which we could verify whether XIGEMC1 protein was an essential factor for development and whether XIGEMC1 function in the developing embryo could be linked to cell proliferation as expected from a protein involved in DNA replication.

This chapter will discuss studies examining the function of XIGEMC1 during development in Xenopus laevis frog, via morpholino-mediated knockdown of protein translation. XIGEMC1 depleted embryos were found to have lethal phenotype. This effect could be observed long after MBT probably due to the maternal content of XIGEMC1 already present in unfertilized oocytes. At stage 37 tadpoles lacking in XIGEMC1 were characterized by severe anatomical defect (Fig. 4.8 and 9). The morpholino dependent effect appears to affect the whole embryo body. This finding is to some degree expected as genes important for DNA replication, cell cycle control, and cell division are generally thought to be expressed ubiquitously within developing tissues. However, Brian E. Walter and Jonathan J. Henry have shown completely opposing outcomes while studying the role of replicative proteins in the Xenopus embryo system. They examined the expression of a variety of cell cycle regulatory and DNA replication genes (e.g., cdk2, cyclin E, cdc45, PCNA, Sld5, Psf1, Psf2, and Psf3) (Walter and Henry, 2004) via whole mount in situ hybridization. This study revealed that the transcriptional regulation of the above mentioned factors occurs in a tissue-specific manner and does not necessarily correlate with embryonic patterns of cell proliferation (Vernon and Philpott, 2003). Although many of these genes are expressed together in neural tissues, each one exhibits unique spatial domains within other tissues, suggesting that different replication factors are utilized in different developmental regions and could have other functions (Walter et al., 2008). Unfortunately, I could not optimize conditions to perform immunohistochemical and in situ hybridization analysis due to the failure of anti XIGEMC1 antibodies to show any specific signal in immuno staining. Despite this an evident non-tissue specificity of the developmental defects was observed. In summary, figure 8 highlights several phenotypic outcomes of XIGEMC1 deprived embryos: tail defects, optic vesicle impairment, shortened axis, epidermal destruction. µ
Interestingly, histological analysis of morpholino treated embryos revealed significant changes in cell proliferation and tissue organization when compared to various controls. Furthermore, there was a remarkable increase in the level of apoptosis as assessed by the cleavage of recombinant ADP-ribose polymerase (PARP) as a marker for caspase activity. The apoptotic phenotype was preceded by a decrease in DNA content, this is in agreement with the idea that cell proliferation is mainly affected.

A possible explanation would be that activation of apoptosis might be a direct consequence of a replication defect. Uncoupling of DNA replication from the cell-division cycle may be an important trigger of apoptosis. In S. cerevisiae it has been shown that mutations in initiation proteins that attenuate both the initiation of DNA replication and checkpoints also induce features of apoptosis similar to those observed in metazoans (Weinberger et al., 2005). A well described example of an apoptotic phenotype is the production of reactive oxygen species (ROS) and the activation of a metacaspase, Yca1p (Burhans et al., 2003; Madeo et al., 2002; Weinberger et al., 2005). The apoptotic phenotype could be a direct effect of DNA damage combined to the incapability of DNA replication to completely replicate the genome. This effect could also be aggravated by defective checkpoints of S phase. Alternatively defects in DNA replication initiation, might activate DNA-damage responses as shown in yeast and mammals (Jacobson et al., 2001; Kim et al., 2002; Liang et al., 1999). Why defects in initiation cause these effects is still poorly understood.
5 Results III–Towards the characterization of mammalian GEMC1

5.1 Introduction

Most of the proteins involved in the licensing step of DNA replication (ORC, Cdc6 and MCM) are conserved in eukaryotic organisms and have recognizable ancestors in archaea. This is not always the case for the proteins acting at the G1–S transition. Yeast ScTopBP1 and Sld2 proteins are, respectively, related to mammalian TopBP1 and RecQ4L but the latter are much larger and contain additional domains that could serve other cellular functions. Sld3 has no evident homologues outside fungi, at least at the amino acid sequence level. In contrast, the GINS complex is highly conserved and the mammalian paralogues of its four subunits have been identified by sequence homology (Kubota et al., 2003; Takayama et al., 2003). A large body of genetic and biochemical evidence has now emerged to support a model in which the initiation of DNA replication in eukaryotic cells is controlled in a stepwise manner. As replication has been studied in numerous eukaryotic organisms, it has become clear that factors identified in one organism are also found in other organisms even though their structure and function might not be as well conserved.

In this chapter I show some evidence that the GEMC1 homologue in mouse is required for efficient DNA replication.
5.2 Results

5.2.1 First evidence for mouse GEMC1 to be required for initiation of DNA replication

To confirm that XI GEMC1 has a role in DNA replication in mammalian organisms, the mouse homologue of GEMC1 (mGEMC1), annotated as hypothetical model gene Gm606, was identified. mGEMC1 expression in NIH 3T3 mouse fibroblast cells was confirmed by RT-PCR (Fig. 5.1). To define mGEMC1 expression, we designed two PCR primers used to amplify the corresponding cDNA sequence by RT-PCR (Real Time PCR)(see materials and methods). To address the role of mouse GEMC1 during cell cycle progression, the consequences of loss of mGEMC1 function were investigated by using RNA interference. RNA interference (RNAi), is a technique in which exogenous, double-stranded RNAs (dsRNAs) of 21-25 nucleotides (called siRNAs), that are complimentary to known mRNAs, are introduced into a cell to specifically neutralize the target mRNA, thereby diminishing or abolishing gene expression (Elbashir et al., 2001). The technique has proven to be effective in several organisms including mammalian cell cultures (Bantounas et al., 2004). Small interfering RNA (siRNA) duplexes were designed to silence the expression of mGEMC1 in cells. NIH 3T3 cells were transfected twice at 24-h intervals with GEMC1 siRNA and control siRNA (scrambled siRNA) and harvested at 48 hours. RT-PCR performed on purified total RNA allowed the efficacy of siRNA treatment to be assessed. A decrease in the levels of mGEMC1 mRNA was obtained in cells treated with siRNA oligos. No variation in the control mRNA GADPH was observed supporting the specificity of the siRNA treatment. Cell cycle profiles of siRNA treated cells were determined by flow cytometry. Propidium-iodide staining showed that depletion of mGEMC1 increased the percentage of cells in G1 phase (G1 peak) (Fig. 5.3a). To establish whether the mGEMC1 depleted cells were in active DNA synthesis, they were labeled with Bromodeoxyuridine (BrdU) for 45 minutes before harvest. The incorporation of BrdU was monitored by two-color flow cytometry analysis of propidium-iodide-stained cells that were immunostained by anti-BrdU-FITC antibodies. As shown in Figure 5.3b mGEMC1 down-regulation causes 83% of cells to accumulate at the G1-S transition, compared with 52% of the control population. As a consequence, a reduced percentage of cells (12%) progressed into S phase, compared with 40% in the control. The extent of inhibition was similar to the one obtained with inhibition of Cdc45 (replication initiation factor) expression (Fig.
Endonucleases activated during apoptosis cleave sections of internucleosomal DNA and cause extensive DNA fragmentation (Arends et al., 1990; Nagata, 2000). As a result of DNA fragmentation apoptotic cells emerge with a deficit in DNA content, which when stained with a DNA-specific fluorochrome can be recognized by flow cytometry as cells having fractional DNA content. On the DNA content frequency histograms they often form a characteristic, "sub-G1" peak indicative of cell death and apoptosis (Hotz et al., 1994; Nicoletti et al., 1991). No floating cells typical of cells undergoing apoptosis were observed. Consistent with this, by FACS profile, no sub-G1 populations were identified in siRNA transfected cells (Fig. 5.3a).
Fig. 5.1 Depletion of mGEMC1 using siRNAs.
RT-PCR of mouse GEMC1 mRNA (mGEMC1) using mouse NIH 3T3 cells that were untreated (Un), treated with control (C) or mouse GEMC1 siRNA (mGEMC1).

b) Quantification of RNA levels in (a). Error bar indicate SD. p<0.001 compared with the untreated sample.

Fig. 5.2 Effects of siRNA oligos targeting mouse Cdc45
WB of NIH 3T3 fibroblasts using anti Cdc45 antibodies. Cells were treated with control scrambled siRNA oligos (Mock siRNA) or siRNA oligos targeting Cdc45 mRNA (Cdc45 siRNA).
Fig. 5.3 mGEMC1 is required for initiation of DNA replication

a) DNA content analysis by flow cytometry. Sub-G1 population was obtained as a measure of apoptosis. b) Flow cytometry detection of BrdU incorporation. NIH 3T3 cells transfected with the indicated siRNAs were labeled with BrdU for 45 min prior to fixation. BrdU intensity is represented in the logarithmic y-axis and DNA content on the linear. Rectangles in pink indicate BrdU incorporating cells.
5.3 Discussion

5.3.1 From Xenopus to mammalian cells: preliminary data of mGEMC1 function in mouse cells

Work from various labs have shown that depletion of proteins involved in DNA replication causes accumulation in the G1-S transition with a reduced percentage of cells progressing into S phase. This was shown for TopBP1 protein (Jeon et al., 2007) and the hGINS complex (Aparicio et al., 2009) both of which are essential factors for Cdc45 recruitment to replication origins. Knock-down of Cdc45 itself is enough to induce cell cycle arrest in G1 phase (Bauerschmidt et al., 2007). Therefore, if mGEMC1 is a main player in the regulation of DNA initiation in mammalian cells, one would expect to observe a phenotype similar to those described with an accumulation of cells in G1 phase upon mGEMC1 down-regulation. For this purpose siRNA oligos generated against mGEMC1 were transfected in NIH 3T3 cells. FACS analysis of cells transfected with mGEMC1 siRNA showed an increase in G1 arrested cells when compared to controls. To date, these data represent the only evidence showing a potentially conserved molecular function of GEMC1 in mammalian cells, further studies will be required to clarify and characterize the role of GEMC1 in higher eukaryotes. It is, for example, extremely important to perform a functional rescue experiment by expressing mGEMC1 that contains silent mutations to avoid siRNA targeting. Rescue of the observed knockdown phenotype would help to validate the siRNA selectivity and the phenotype specificity for the target gene. The anti mGEMC1 antibody whose production is in progress will also help to validate the siRNA knockdown efficiency and specificity. It would also be interesting to perform a chromatin binding assay to assess defects in the binding of any of the replication proteins in siRNA mGEMC1 as well as to identify more mGEMC1 interactors.

I specially looked at the gene expression profile of hGEMC1 using GEO (Gene Expression Omnibus). GEO is a public repository that archives and freely distributes microarrays and other forms of high-throughput data submitted by the scientific community. It allows to search individual gene expression and molecular abundance profiles of a gene of interest. Contrary to expectations for a protein essential for cell proliferation, no convincing or highly reproducible up-regulation of
hGEMC1 gene expression was observed in a variety of cancers analyzed with the exception of some specific cases of prostate cancer, where hGEMC1 expression tends to increase as tumor develops from being clinically localized to metastatic. Although these pieces of information can be quite useful, I have some reservations for the reliability of this outcome. In fact only one experiment concerning this particular kind of cancer was reported in GEO database, limiting the results to a very low number of samples; Furthermore, not all samples analyzed show a high hGEMC1 profile expression.

Despite weak evidence supporting the expression hGEMC1 in cancer cells it would be worth bearing in mind that the function of hGEMC1 in the cell cycle might not be exclusively limited to replication promotion. hGEMC1 function could also be relevant to additional processes including DNA damage checkpoints making it more difficult to predict a specific outcome in cancer cells. In this scenario in fact hypomorphic mutations responsible for protein deregulation (where a protein loses some functions and retains some others) would be more likely to be accountable for a cancer predisposition.
6 Conclusions

The requirement for faithful duplication of genetic information in proliferating cells places DNA replication at the heart of the cancer problem. Initiation of DNA replication is one of the major control points in the mammalian cell cycle and the target of many gene products that are mis-regulated in cancer (Hanahan and Weinberg, 2000). Impressive progresses have been made in the last few years in identifying the proteins that are recruited to origins and the order in which they are recruited. However, their biochemical activities are still poorly understood.

The aim of this thesis was to characterize the novel replication factor XlGEMC1 in order to gain more insights into the mechanisms that regulate DNA replication in eukaryotic cells. Experiments have been predominantly performed in *Xenopus laevis* egg extract and results were validated by further experiments in *Xenopus* embryos and mouse cells.

XlGEMC1 is a novel vertebrate factor belonging to a new protein family required to initiate chromosomal DNA replication, no evident sequence homology has been identified within any yeast protein. Overall the data discussed here suggest a model in which XlGEMC1 mediates XlTopBP1 dependent loading of Cdc45 onto replication origins (Fig. 6.1)
Fig. 6.1 Model of GEMC1 function at replication origins (see text for explanation).
We show that XlGEMC1 is required for the loading onto chromatin of Cdc45, an essential step for replication origin firing. In addition, XlGEMC1 was found to directly interact with Cdc45 and XlTopBP1. These data strongly suggest that XlGEMC1 links Cdc45 to replication origins through its direct interaction with Cdc45 and XlTopBP1. Importantly, XlGEMC1 interacts with Cdk2 by which it is heavily phosphorylated in vivo. This constitutively phosphorylated XlGEMC1 shows an increased affinity for TopBP1 and enhances Cdc45 loading onto replication origins. Remarkably, the afore mentioned effect is highly reminiscent of the one induced by Sld3 following Cdk dependent phosphorylation in yeast, which enables its binding to ScTopBP1 protein thus promoting initiation of DNA replication. Consistent with the findings obtained in egg extract the inhibition of XlGEMC1 expression in vivo in developing Xenopus embryos arrests cell proliferation and embryo development. Likewise, inhibition of XlGEMC1 expression in mammalian cells leads to immediate and complete cell cycle arrest by efficiently blocking S-phase onset. Further studies including rescue of the siRNA phenotype, chromatin binding, protein-protein interaction will be required to specifically assess mGEMC1 protein role during DNA replication in mammalian cells. Moreover, XlGEMC1 undergoes multiple phosphorylation events and constitutively phosphorylated XlGEMC1 is by itself sufficient to stimulate origin firing. This last data strongly reinforce the argument that XlGEMC1 could represent the functional homologue of Sld3 and shows that GEMC1 is a unique essential target mediating Cdk2 dependent regulation of DNA replication.

In conclusion GEMC1 may not be the only protein with these properties as it is possible that other factors are able to mediate the interaction between TopBp1, Cdc45 and Cdk2 at replication origins. Despite this, the study of XlGEMC1 will lead to a better understanding of normal and uncontrolled cell cycle progression in vertebrate organisms. Such advances in our knowledge of replication pathway and its integration with the cell cycle machinery for instance could help to identify new therapeutic strategies that target particular aspects of DNA replication, including the events involved in the initiation step of DNA replication.
References


Dijkwel, P.A., Wang, S., and Hamlin, J.L. (2002). Initiation sites are distributed at frequent intervals in the Chinese hamster dihydrofolate reductase origin of replication but are used with very different efficiencies. Mol Cell Biol 22, 3053-3065.


Jares, P., and Blow, J.J. (2000). Xenopus cdc7 function is dependent on licensing but not on XORC, XCdc6, or CDK activity and is required for XCdc45 loading. Genes Dev 14, 1528-1540.


Labib, K., Kearsey, S.E., and Diffley, J.F. (2001). MCM2-7 proteins are essential components of prereplicative complexes that accumulate cooperatively in the nucleus during G1-phase and are required to establish, but not maintain, the S-phase checkpoint. Mol Biol Cell 12, 3658-3667.


double-strand break recognition by Nbs1 with its H2AX-dependent chromatin retention. EMBO J 23, 2674-2683.


Piatti, S., Lengauer, C., and Nasmyth, K. (1995). Cdc6 is an unstable protein whose de novo synthesis in G1 is important for the onset of S phase and for preventing a 'reductional' anaphase in the budding yeast Saccharomyces cerevisiae. EMBO J 14, 3788-3799.


adjoining part of geminin interact with two sites on Cdt1 for replication inhibition. Mol Cell 15, 245-258.


