DOCTOR OF PHILOSOPHY

Consequences of partial chromosome re-replication in mammalian cells

Klotz-Noack, Kathleen

Award date:
2013

Awarding institution:
University of Dundee

DOCTOR OF PHILOSOPHY

Consequences of partial chromosome re-replication in mammalian

Kathleen Klotz-Noack

2013

University of Dundee

University of Dundee
College of Life Sciences
Division of Gene Regulation and Expression

PhD Thesis

Consequences of partial chromosome re-replication in mammalian cells

Kathleen Klotz-Noack

Thesis submitted for Doctor of Philosophy

July 2012

ABSTRACT

To prevent re-replication of DNA in a single cell cycle, the licensing of replication origins by Mcm2-7 is prevented during S and G2 phases. Metazoans achieve this by cell cycle regulated proteolysis of the essential licensing factor Cdt1 and formation of an inhibitory heterohexameric complex of Cdt1 with a small protein called geminin. The consequences of either stabilising Cdt1 or ablating geminin in synchronised human U2OS cells are investigated in this PhD Thesis to elucidate the possible contribution of re-replication in gene amplifications or rearrangements commonly seen in human tumours.

I show that following geminin loss, cells complete an apparently normal S-phase, but a proportion arrests at the G2/M boundary. When Cdt1 starts to accumulate in these cells, DNA re-replicates, suggesting that the key role of geminin is to prevent re-licensing in G2. Inhibition of cell cycle checkpoints in cells lacking geminin promotes progression through mitosis without detectable levels of re-replication. Checkpoint kinases thereby amplify re-replication into an all-or-nothing response by delaying geminin depleted cells in G2 phase. Comparative Genomic Hybridisation (CGH) array and Solexa Deep DNA sequencing revealed that re-replication after geminin depletion does not appear at preferential genomic regions within the human genome. This is consistent with a recent observation that G2 cells have lost their replication timing information and reduplicate their genome stochastically. In contrast, when Cdt1 is stabilised by the neddylation inhibitor MLN4924, re-replication starts directly from within S-phase raising the question whether alternative mechanisms of re-replication may cause distinct genomic consequences.

STATEMENT OF ORIGINALITY

I hereby declare that I am the author of this Thesis and that some of the results have been already published with me as first author (publication attached on CD).

The work presented in this Thesis is my original work undertaken under the supervision of Prof. J. Julian Blow and work done by others is clearly stated. To the best of my knowledge and belief, this Thesis contains no material previously published by another person and has not been previously accepted for a higher degree.

J. Julian Blow

Kathleen Klotz-Noack

DEDICATION

For my parents Brigitte and Horst Klotz whose persistence and thirst for knowledge shaped me and without whose trust, patience and love my way would not have led to the submission of a dissertation.

and

For my family Sandy, Paul and Emma without whose understanding and help the writing of this Thesis would have not been such pleasure.

WIDMUNG

Für meine Eltern Brigitte und Horst Klotz deren Unermüdlichkeit und Wissensdurst mich prägten und ohne deren Vertrauen, Geduld und Liebe mein Weg wohl nicht zur Abgabe dieser Dissertation geführt hätte.

und

Für meine Familie Sandy, Paul und Emma ohne deren Verständnis und Hilfe das Schreiben dieser Doktorarbeit nicht solchen Spass gemacht hätte.

Acknowledgements

I would like to thank Prof. Julian Blow for giving me the opportunity to work in his laboratory. The four years in his lab have been full of new experiences, excitement and growth, turning me into an independent young scientist. We went through frustration and plenty of setbacks but his constant optimism and passion for science inspired me providing enough energy to work for better times to come.

I also like to thank my lab mates Aga, Alan, Debbie, Gagan, Nicola, Niladri, Peter, Remi, Weitheng and Xinquan for the guidance and stimulating conversations during coffee breaks or whenever ideas or results needed to be discussed. Equally grateful I am to my flat mate Andrew Melvin who stood living with me for 4 years and whom I had great science chats with. Thanks to all the people working for the Division of Gene Regulation and Expression for creating and maintaining such a well equipped and well organised work environment. For the help with FACS I am very grateful to Rosemary Clarke, who always had suggestions and an open ear for problems.

I would like to thank Cancer Research UK for funding this PhD project thus giving me the chance to work in Dundee and to discover the beautiful Scottish Highlands.

Ausserdem möchte ich Iris Müller für die schönen Stunden in Dundee und das kritische Lesen dieser Doktorarbeit danken. Nicht zuletzt danke ich meinen Eltern, denen die Vollendung dieser Doktorarbeit so am Herzen liegt, für die jahrelange Unterstützung. Natürlich möchte ich mich auch bei meiner Freundin Lysann Sauer bedanken, die lange mit mir durch die Welt reiste, damit wir uns wissenschaftlich aber auch persönlich weiterentwickeln und die auch zum Lesen dieses Werkes nicht vor einer Reise zurückschreckte. Claudia Thomas danke ich für die selbstlose Hilfe in einer der härtesten Wochen meines Lebens. Abschliessend danke ich meiner Frau Sandy und unseren Kindern Paul und Emma für diese tolle Liebe und Wärme und das überwältigende Gefühl eine Mama zu sein. Danke für die Unterstützung und das Verständnis während des Schreibens dieser Arbeit.

Table of Contents

List of Figures

Chapter I. Introduction

Chapter III. Physiological consequences of geminin depletion

Chapter IV. Stabilisation and Over-expression of Cdt1 causes re-replication and inhibition of replication

List of Tables

Abbreviation

14-3-3	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein
6-DMAP	6-dimethylaminopurine
9-1-1	Rad9-Rad1-Hus1
AAA+	ATPase associated with a variety of activities
ACS	ARSs consensus sequence
And-1	acidic nucleoplasmic DNA binding protein
Apaf-1	apoptotic peptidase activating factor 1
APC	anaphase promoting complex
ARS	autonomously replicating sequences
AT	ataxia telangiectasia
ATM	ataxia-telangiectasia-mutated
ATP	Adenosine-5'-triphosphate
ATP	Adenosine-5'-triphosphate
ATR	ataxia-telangiectasia and Rad3 related
ATRIP	ATR-interacting protein
Bax	Bcl2-associated X protein
Bcl2	B-cell leukemia/lymphoma 2
BRAC1	breast cancer 1
BrdU	Bromodeoxyuridine
Brg1	Brahma-related gene 1
BSA	Bovine Serum Albumin
CAD	caspase-activated DNase
Cdc4	Cell division cycle
CDH1	cadherin 1
Cdk	Cyclin-dependent kinase
Cdt1	Cdc10 dependent transcript 1
CGH	comparative genomic hybridisation
Chk1	Checkpoint kinase 1
CRL	Cullin-RING ubiquitin ligase
CSK	cytoskeleton
Cul 1,4	Cullin
DAPI	4',6-diamidino-2-phenylindole
Ddb1	DNA damage-binding protein 1
DDR	DNA damage response
DISC	death-inducing signalling complex
Dm	*Drosophila melanogaster*
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
Drf1	diaphanous-related formin 1
DSB	double strand break
E2F1	E2F transcription factor 1
EdU	5-Ethynyl-2'-deoxyuridine
EGFR	epidermal growth factor receptor
Emi1	Early mitotic inhibitor 1
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2

EtOH	Ethanol
FADD	Fas associated via death domain
FASL	FAS ligand
FASR	FAS receptor
FC_BB	Flow cytometry blocking buffer
FC_WB	Flow cytometry wash buffer
FHA	forkhead-associated
FISH	Fluorescence in situ hybridsation
GAL	GenePix® Array List
GFP	green fluorescent protein
GINS	go-ichi-ni-san (japanese 5-1-2-3 related to subunits of the complex Sld5, Psf1, Psf2 and Psf3)
H_2AX	H2A histone family member X
H3	histone H3
HER2	human epidermal growth factor receptor 2
HU	hydroxyurea
IB	Immunoblot
ICAD	inhibitor of caspase-activated DNase
IFA	Immunofluorescence assay
IR	ionising radiation
MAP	Mitogen-activated protein
MCM	mini-chromosome maintenance
MCS	Multiple cloning site
Mdc1	mediator of DNA-damage checkpoint 1
Mdm2	transformed mouse 3T3 cell double minute 2
Mm	*Mus musculus*
MRN	Mre11-Rad50-Nbs1
MRN	Mre11-Rad50-Nbs1
MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived
N8E2	Nedd8-conjugating enzyme E2
NAD	Nicotinamide adenine dinucleotide
NAE	Nedd8 activating enzyme
Nbs	Nijmegen Breakage syndrome
Nbs1	Nijmegen breakage syndrome 1
Nedd8	Neural precursor cell expressed, developmentally down-regulated 8
OIS	oncogene induced senescence
ORC	origin recognition complex
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCNA	Proliferating Cell Nuclear Antigen
PFA	Paraformaldehyde
PI-3	phosphatidylinositol 3-kinase
PIP	PCNA interaction protein motif
PK	protein kinase
Plk3	polo-like kinase 3
Pml	promyelocytic leukemia
pre-RC	pre-replication complex

qPCR	quantitative PCR
Rad	Ras-related associated with diabetes
Ras	Rat sarcoma
Rb	retinoblastoma protein
RFC	replication factor C
RLF	replication licensing factor
RNA	Ribonucleic acid
RNAi	RNA interference
RNR	Ribonucleotide reductase
RPA	replication protein A
RT	room temperature
Sc	*Saccharomyces cerevisiae*
SCF	Skp1/Cul1-F-box E3 ubiquitin ligase
SD2	Schneider D2 cells
siRNA	small interfering RNA
Skp2	S-phase kinase-associated protein 2
Sld	synthetic lethal mutations with *dpb11–1*
Sp	*Saccharomyces pombe*
ssDNA	single strand DNA
SWI/SNF	switch/sucrose nonfermentable
TGF-β	transforming growth factor beta
TNFR1	TNF receptor 1
TNFα	tumour necrosis factor alpha
TopBP1	topoisomerase (DNA) II binding protein 1
TRADD	TNFR superfamily 1A associated via death domain
TRAIL	TNF-related apoptosis inducing ligand
UV	ultraviolet light
Xl	*Xenopus laevis*

Chapter I

Introduction

I. 1. MILESTONES IN MOLECULAR BIOLOGY- A BRIEF JOURNEY THROUGH HISTORY

The focus of this PhD Thesis is the process of deoxyribonucleic acid (DNA) replication, thus it seems natural that it starts with the discovery of DNA. It was in 1869 when Friedrich Miescher isolated nuclei from leukocytes and found an unexpected, acidic and phosphorus rich substance he called 'nuclein' (Miescher, 1871).

Although chromosomes were initially discovered by Walter Flemming in the 1870s, the chromosome theory of inheritance was a result of observations from Boveri and Sutton in 1902 suggesting that the association of paternal and maternal chromosomes in pairs and their subsequent separation during cell division may constitute the physical basis of the Mendelian law of heredity (Boveri, 1902; Sutton, 1902). In 1924 Robert Feulgen utilized microscopic dyes to show that chromosomes contain both proteins and nucleic acids (Feulgen and Rossenbeck, 1924), starting a race to identify the heredity molecule. Mainly because scientists wanted to believe that proteins are carrier of the genetic material, it was 20 years later before Oswald Avery and co-workers used infectious and non-infectious *Pneumococcus* strains and found that DNA is the transforming material (Avery et al., 1944). Avery's discovery came too early for the scientific community and its great significance was never rewarded as deserved. In contrast, when Alfred Hershey and Martha Chase in 1952 used the bacteriophage T2 to infect bacteria and showed that it is the P^{32}-labelled phage DNA which is found in the bacteria and not the S^{35}-labelled coat protein, researchers accepted that DNA is the hereditary material (Hershey and Chase, 1952). A year later, James D. Watson and Francis H. C. Crick proposed the double helix structure of the DNA molecule using x-ray diffraction data collected by Rosalind Franklin and revealed the base pairing suggesting a possible copying mechanism for the genetic material (Franklin and Gosling, 1953; Watson and Crick, 1953).

After the discovery of the DNA structure one could say the next breakthrough came with the description of the semiconservative nature of replication by Meselson and Stahl. They designed a very clever experiment where bacterial DNA was labelled

with heavy nitrogen (N^{15}), bacteria subsequently grown on medium containing N^{14} in excess and after ultracentrifugation in a Caesium Chloride gradient the density of DNA was monitored over several generations. Consistent with the semiconservative replication hypothesis the results revealed that the heavy nitrogen is distributed evenly to each daughter molecule (Meselson and Stahl, 1958).

DNA polymerase 1, the first enzyme involved in DNA replication had already been described in 1955 (Kornberg et al., 1955) but it was in 1977, after some very laborious and inefficient techniques were developed, when Frederick Sanger came up with the dideoxy chain- termination method revolutionising the method of DNA sequencing (Sanger et al., 1977). Amplification of specific DNA templates became possible with the invention of the polymerase chain reaction (PCR) by Kary Mullis in 1983 (Mullis and Faloona, 1987) thus starting a new era in molecular biology and medical diagnostics.

Thanks to those findings biologists were able to synthesise and manipulate recombinant DNA encoding proteins of interest, thus identifying their function in cellular processes and potential pathological implications in very great detail. Molecular Biology developed faster than any other field in Biology enabling scientists to sequence the whole human genome in several hours or analyse single cells by microscopy or flow cytometry, techniques which made the work presented in this Thesis a challenging and rewarding experience.

I. 2. The Mammalian Cell Cycle

A eukaryotic cell divides via alternation of two central processes: the precise doubling of the genome in S-phase and the segregation of the sister chromatids during mitosis. When cells exit mitosis they enter the G1 phase, which is characterised by cell growth and increased metabolic activity. However, the central cell cycle function is to prepare for DNA replication in S-phase and if necessary activate G1/S cell cycle checkpoints to prevent or delay progression into S-phase. Cells can be synchronised in

G1-phase by serum starvation. In response to DNA damage or other stress situations cells can irreversibly enter a senescent state, which represents an exit from the cell cycle. Alternatively, in the absence of proliferative stimuli cells can temporarily withdraw from the cell cycle and enter a G0 or quiescent state. During S-phase the initiation of DNA replication from multiple sites leads to the precise doubling of the genetic material. DNA replication can be monitored microscopically or by flow cytometry via incorporation of nucleotide analogues such a BrdU or EdU. DNA damage is detected and fixed during S-phase by activation of intra S-phase checkpoints. Cells can be synchronised in S-phase by addition of replication inhibitors such as thymidine, hydroxyurea or aphidicolin. Only after the complete genome is replicated do the cells enter G2-phase and prepare for cell division. The most important feature of the G2 phase is the G2/M checkpoint, which prevents entry into mitosis in response to DNA damage or genotoxic stress thus inhibiting proliferation of damaged cells. After a cell enters mitosis it segregates the genetic material and undergoes cytokinesis to create two identical daughter cells, which then enter into the next G1 phase.

I. 3. How to ensure complete genome duplication?

Eukaryotic genomes contain a very large amount of DNA that is divided amongst a number of chromosomes. To allow rapid duplication of the genome replication must initiate from multiple sites along each chromosome. For complete duplication eukaryotes utilise between 10^3-10^5 replication origins, that are typically spaced 30-100 kB apart (Blow and Dutta, 2005). At present it is not clear what determines the position of replication origins in animal cells. In *S. cerevisiae* replication initiation has been shown to start within or very close to autonomously replicating sequences (ARSs) (Bielinsky and Gerbi, 1999; Brewer and Fangman, 1987; Huberman et al., 1987) which contain an 11 bp ARSs consensus sequence (ACS) that is essential for origin function (Diffley, 1996). In contrast, experiments by Harland and Laskey (1980) showed that numerous DNA sequences from viruses or prokaryotes replicate

efficiently and once-per cell cycle when injected into *Xenopus laevis* eggs, arguing against the requirement for specific sequences for initiation (Harland and Laskey, 1980). This was confirmed by experiments using the *Xenopus* cell free system and the 2D gel electrophoresis approach developed by Brewer and Fangman and showed that plasmids carrying ribosomal *Xenopus* DNA initiated and terminated at multiple, random locations (Hyrien and Mechali, 1992; Mahbubani et al., 1992). Those studies have raised the 'random completion problem': to avoid under-replication a random distribution would require origins to be spaced less than 1 kb apart (Blow et al., 2001; Laskey, 1985). Further studies pointed out that such random distribution is unlikely as *Xenopus laevis* origin recognition complex (XlORC), an essential replication protein (see Section I. 5. 1), saturated sperm chromatin at about one copy per 8-15 kb (Rowles et al., 1996). DNA fiber analysis revealed that origins are spaced 5-15 kb apart and that they are activated in clusters of approximately 5-10 origins (Blow et al., 2001), suggesting some sort of sequence independent origin spacing.

In mammalian cells the DNA replicated from a single origin, the replicon, ranges in size between 20-150 kb with a maximum of ~450 kb (Jackson and Pombo, 1998). Replicon clusters contain up to 10 adjacent origins, typical encompass less than 1 Mb of DNA (Figure I. 1)(Gillespie and Blow, 2010). When replication is initiated within a cluster, the replicons become associated with multiple proteins forming the replication machinery, turning the cluster into a replication factory or foci. In a human cell approximately ~50,000 replicons within ~10,000 foci replicate the entire genome in about 9 h, suggesting that a focus needs approximately 75 min to complete replication (Maya-Mendoza et al., 2010). That suggests that there are ~1400 active foci at any time during S-phase and this is indeed quite close to the 1230 active sites observed by high-resolution light microscopy (Cseresnyes et al., 2009). Interestingly, replication factories are not activated randomly but follow a predetermined timing program.

Figure I. 1.

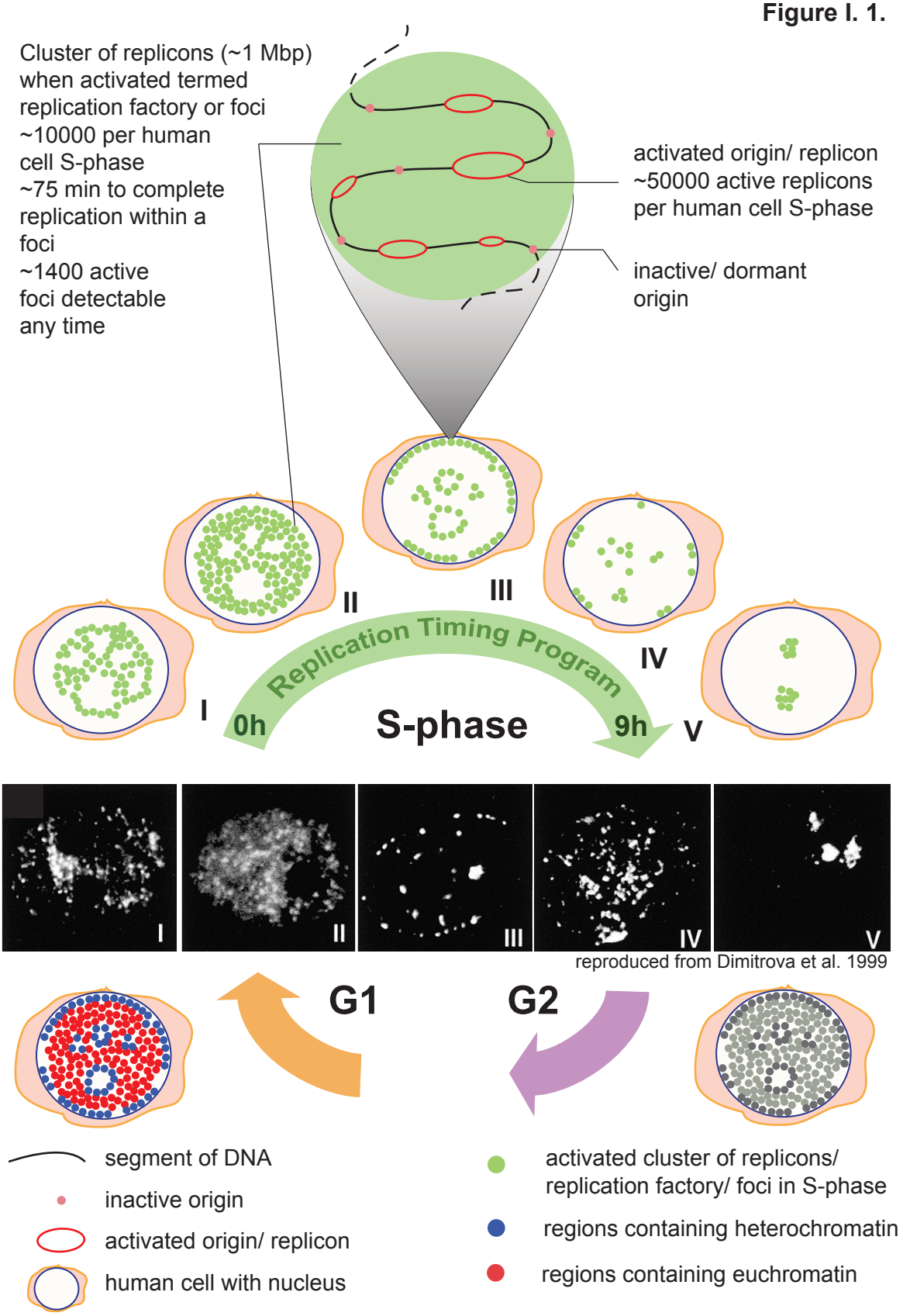
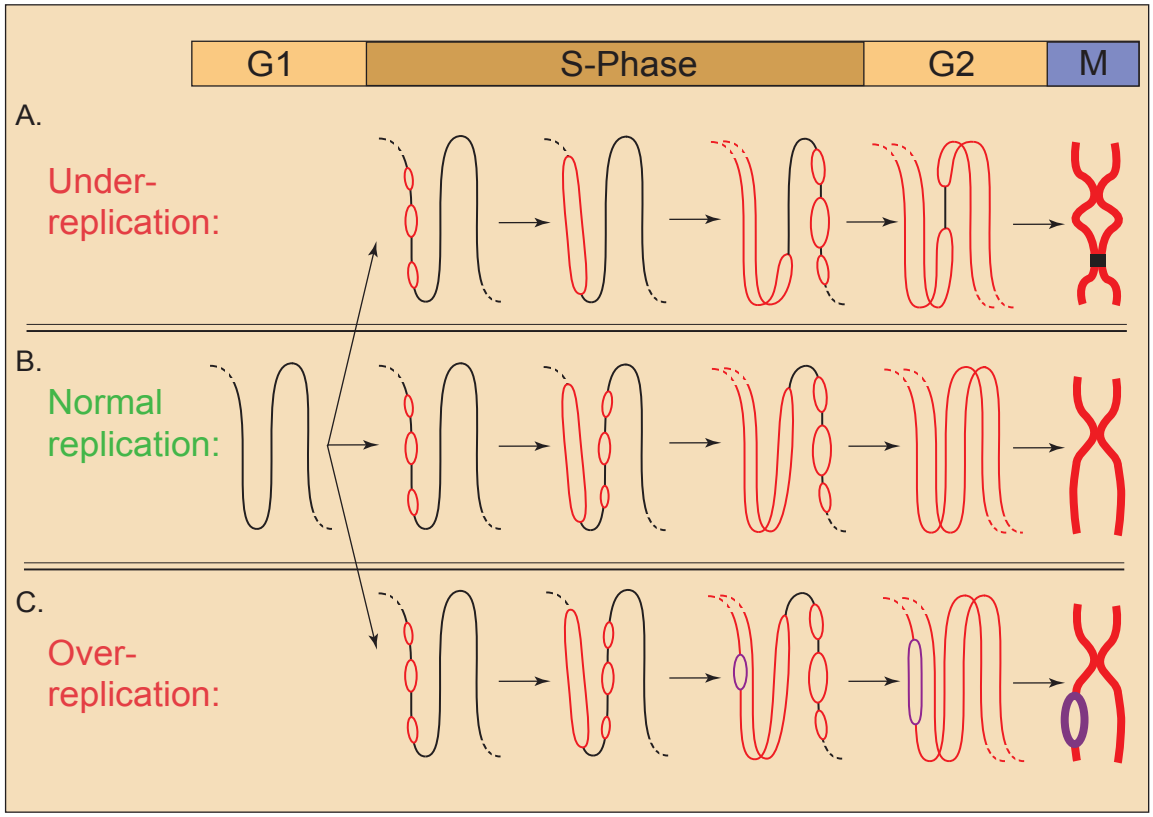


Figure I. 1. How to ensure complete genome duplication. Shown is a segment of DNA (black line) with five inactive origins and five activated origins/ replicons which form a replication bubble with fork moving bidirectional away from the origin. Those ten origins form an active cluster (green dots) of replicons. Human cells have approximately 10000 of those clusters with ~50000 replicons during there S-phase. When a cluster is enganged in sythesis it becomes associated with the replication machinery and is also termed replication factory or foci. A foci needs about 75 min to complete replication resulting in ~1400 active foci (green dots) any time during the 9h human S-phase. To ensure complete replication S-phase follows a strict spatial and temporal replication timing program estabished during G1 phase (blue and red dots) of the cell cycle and lost (grey dots) after S-phase is completed. In general euchromatin (red dot) replicates early (Pattern I and II) while heterochromatin (blue dot) replicates late (Pattern III,IV and V).

At the Timing Decision Point in early G1 phase subchromosomal domains move to reproducible positions within the nucleus thus establishing the replication timing program (Dimitrova and Gilbert, 1999). The positioning correlates with chromatin organisation and the underlying transcriptional activity, with the transcriptionally active euchromatin localised in the nuclear interior and the inactive heterochromatin in the periphery or around the nucleoli (Figure I. 1) (Goren and Cedar, 2003; Zink, 2006). Once S-phase starts the execution of the timing program drives cells through 5 discrete phases of replication: bulk nucleoplasmic, peripheral and perinuclear-perinucleolar localisation precede replication first in small and then large, intranuclear patches (O'Keefe et al., 1992). It has recently been shown that replication induced in G2 nuclei does not follow the replication timing program suggesting that replication in S-phase erases the timing determinants leaving the G2 chromatin without timing information (Lu et al., 2010).

Initiation of replication origins must occur throughout S-phase to allow rapid, spatial and temporally determined duplication of the genome. It is therefore of crucial importance that initiation is a very tightly controlled event that can only occur once per origin. Figure I. 2. shows the dramatic consequence of deregulated origin usage. If origins fail to fire, portions of the genome may remain un-replicated as cells progress into mitosis, potentially causing problems during sister chromatid segregation leading to chromosome breaks and loss of genetic information. In contrast, if an origin initiates more than once a section of the genome would be re-replicated, which may have a range of deleterious effects such as a gene amplification (Green et al., 2010), a common feature in tumourigenesis, or cell death (Klotz-Noack et al., 2012; Melixetian et al., 2004; Zhu et al., 2004). Eukaryotes developed a mechanism called ‘licensing’ of replication origins that occurs in late mitosis and early G1, and is controlled by a licensing checkpoint, to prevent cells from re-replicating DNA during a single cell cycle. This depends on the licensing machinery being inactive during S-phase and G2 to inhibit re-firing of origins.



Reproduced from Blow et al. 2011

Key:

Unreplicated DNA — Replicated DNA — Rereplicated DNA

Figure I. 2. Consequences of deregulated origin usage. A section of chromosomal DNA with three replication clusters, each replicating from three origins is shown at different stages of the cell cycle. A whole chromosom containing the section is shown in mitosis. Panel B illustrates how the correct usage of all origins mediates complete DNA duplication. Panel A demonstrates the consequence of a failure to initiate origins leading to under-replication and potentially to chromosome breaks in mitosis. In panel C an origin fires more then once, leading to rereplication of a portion of the genome causing irreversible genetic changes.

I. 4. THE REPLICATION LICENSING FACTOR

I. 4. 1. Discovery of the licensing factor

The first experimental evidence that the initiation of DNA replication is indeed a tightly regulated process came from Rao and Johnson in 1970 who fused somatic cells in different cell cycle stages together such that the cytoplasm is shared. They found an accelerated replication in G1 nuclei when fused with an S-phase cell, which replicated normally. When G1 nuclei were fused with a G2 cell, they replicated normally while the G2 nuclei failed to enter S-phase. When G2 nuclei were fused with a S-phase cell the S-phase cell replicated normally while the G2 cell did not initiate replication. This suggests that initiation of replication requires a diffusible S-phase promoting activity that G1 but not G2 cells can respond to (Rao and Johnson, 1970).

Blow and Laskey (1988) extended this using the *Xenopus* cell free system, revealing that G1, S-phase or G2 nuclei did not re-replicate after transfer into fresh G1 extract. They found that re-replication could be induced in G2 nuclei after permeabilisation of the nuclear envelope, indicating the presence of an essential licensing factor that binds to origins after nuclear envelope breakdown in late mitosis allowing initiation of replication after formation of the nuclear envelope (Blow and Laskey, 1988). They proposed that the licensing factor is removed from origins upon initiation and cannot rebind due to exclusion by the nuclear envelope. Further experiments in other *in vitro* systems confirmed that the nuclear membrane is essential in preventing re-licensing and re-firing of replication origins (Coverley et al., 1993; Crevel and Cotterill, 1991; Leno et al., 1992).

I. 4. 2. Identification of MCM2-7 as licensing factor

A screening in *S. cerevisiae* identified mutants that are defective in maintenance of minichromosomes (Maine et al., 1984). One of them was the previously identified cell division cycle 46 (Cdc46)/MCM5 accumulating in the nucleus in late mitosis and disappearing upon entry into S-phase. Six MCM proteins (MCM2-7),

displaying the same cell cycle dependent subcellular localisation as Cdc46/MCM5, were subsequently found to be essential for DNA replication (reviewed in (Chong et al., 1996). Experiments by John Diffley in 1994 showed that origins exist in either a post-replicative state, with only the origin recognition complex (ORC)(see Section I. 5. 1) bound to the origin, or in a pre-replicative state, with ORC and additional proteins bound, supporting the idea of a licensing factor.

Meanwhile, biochemical experiments in *Xenopus* extracts showed that the activation of licensing factor in metaphase extract is dependent on cyclin-dependent kinase (Cdk) activity and is inhibited by the protein kinase inhibitor, 6-dimethylaminopurine (6-DMAP) (Blow, 1993). Thus provided a clever assay for licensing activity in different chromatographic fractions of *Xenopus egg* extracts (Chong et al., 1995). First sperm was incubated in whole 6-DMAP treated extract for decondensation, isolated and incubated with different purified fractions of extract and then again incubated in 6-DMAP egg extracts. Two of the fractions (namely replication licensing factor M (RLF-M) and RLF-B) allowed DNA replication with RLF-M containing a complex of proteins that included MCM2 and MCM3 (Chong et al., 1995; Kubota et al., 1995) and RLF-B later shown to contain Cdc10 dependent transcript 1 (Cdt1) (Tada et al., 2001). Further studies identified all six MCM proteins in the RLF-M fraction (Kubota et al., 1997; Thommes et al., 1997). Microscopic analysis of human cells revealed that MCMs are equally distributed throughout the entire nucleus in G1 and early S-phase and displaced from their site on chromatin once the site has finished replication. They were not associated with DNA in G2 cells or on condensed chromosomes in early mitosis (Krude et al., 1996) fulfilling the requirements demanded for the licensing factor.

I. 4. 3. The MCM2-7 protein complex

The MCM protein complex consists of six highly related subunits MCM2- MCM7, which are conserved throughout eukaryotic evolution and are also present in *Archaea*. Despite their sequence homology each subunit possesses unique sequences conserved in all species, indicating a universal and essential function for each individual MCM. This idea is supported by experiments in *S. cerevisiae* and *S. pombe* revealing that deletion of each single MCM is lethal (reviewed by (Dutta and Bell, 1997; Kelly and Brown, 2000). All six subunits contain a 250 aa region encoding an ATPase active site (ATPase associated with a variety of activities (AAA+) domain) (Koonin, 1993) and form a 600 kDa (Fujita et al., 1997; Kubota et al., 1997) doughnut like heterohexameric complex (Figure I. 3 A). The positively charged central channel that is large enough to encircle the DNA double helix (Fletcher et al., 2003; Sato et al., 2000).

A number of observations suggest that beside their role in initiation of replication at the origins, the MCM2-7 complex functions as the replicative helicase unwinding the DNA in front of the replication forks. A MCM4, 6, 7 complex purified from HeLa cells has been shown to posses limited ATP dependent 3'-5' helicase activity *in vitro* (Ishimi, 1997), while in combination with the fork proteins Cdc45 and GINS (from the Japanese go-ichi-ni-san meaning 5-1-2-3, after the four related subunits of the complex Sld5, Psf1, Psf2 and Psf3), the MCM2-7 complex displays strong helicase activity (Moyer et al., 2006). Furthermore degradation of MCM2-7 proteins after initiation of replication rapidly inhibits replication fork progression (Labib et al., 2000). Chromatin-immunoprecipitation experiments in *S. cerevisiae* and *Xenopus* showed that MCM complexes travel with the replication fork strongly supporting the helicase hypothesis and providing an elegant solution to how origins become 'unlicensed' upon initiation to prevent re-replication (Aparicio et al., 1997; Pacek et al., 2006).

I. 4. 4. The MCM 'paradox'

The so-called 'MCM paradox' summarises observations that questioned the idea that MCM2-7 function simply as the replicative helicase. First of all it has been shown that instead of the expected two MCM2-7 per replication origin, there are a total of 10-40 molecules of MCM2-7 associated with chromatin for each origin that normally fires during S-phase (Burkhart et al., 1995; Edwards et al., 2002; Lei et al., 1996; Mahbubani et al., 1997). Interestingly not all of these complexes are in close proximity to the origins, in fact some are distributed at least 1 kb from the ORC (Edwards et al., 2002; Harvey and Newport, 2003; Ritzi et al., 1998). Supporting these findings, immunofluorescence studies showed that most MCM2-7 complexes do not co-localise with sites of replication (Dimitrova et al., 1999; Krude et al., 1996; Madine et al., 1995). The finding that the number of MCMs can be reduced to 1-2 copies per origin without inhibiting replication efficiency (Edwards et al., 2002; Mahbubani et al., 1997; Oehlmann et al., 2004) raised questions about additional functions of the excess MCM. A study suggested a role in intra-S-phase checkpoint activation by binding of MCM7 to ATR-interacting protein (ATRIP), a protein involved in activation of ataxia-telangiectasia and Rad3 related (ATR) kinase (Cortez et al., 2004). However, the main function of excess licensed origins, named dormant origins, is to fire under replication stress to ensure the complete replication of the genome.

I. 4. 4. 1. Dormant origins- an essential function for excess MCM's

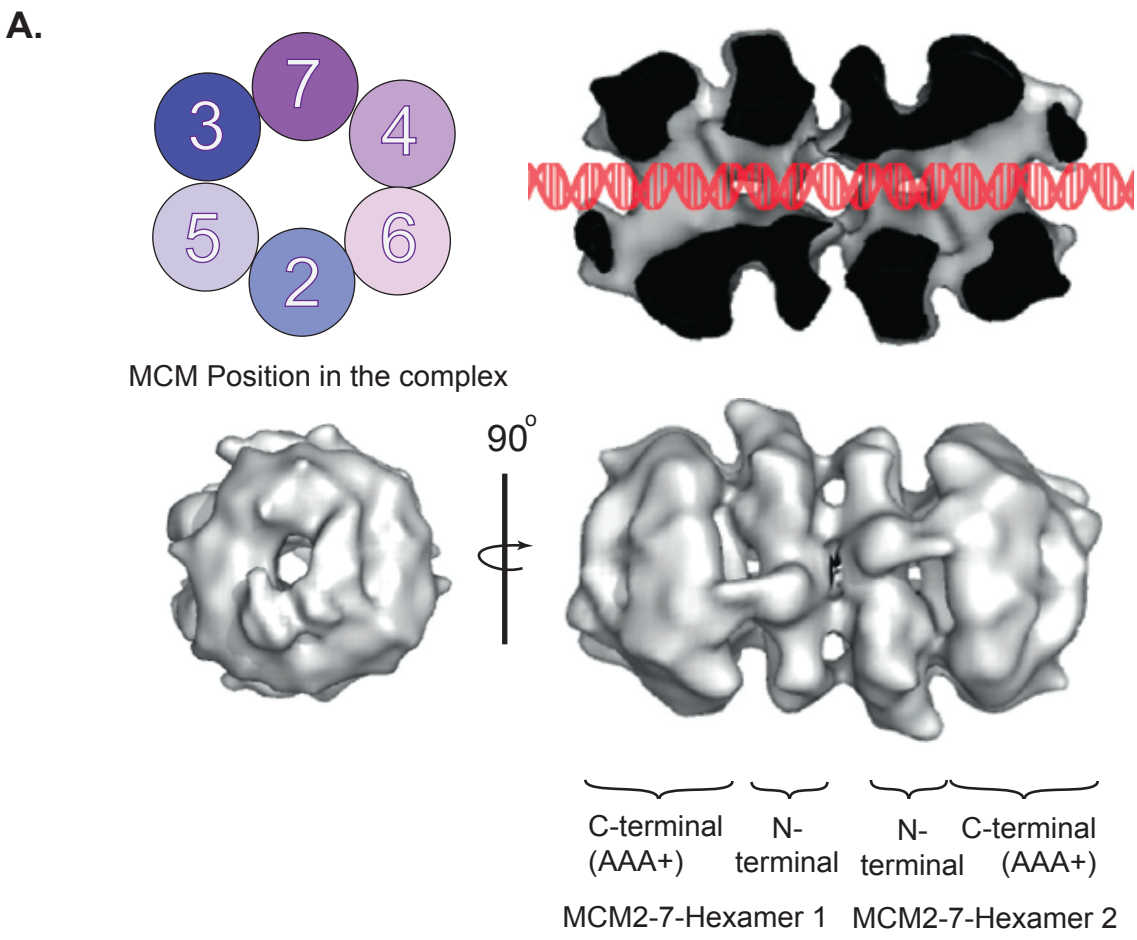
In all organisms examined to date, there is a significant 3- to 20-fold excess of MCM2-7 double hexamers over the number of origins typically active in any one S-phase. It has been reported that the excess MCMs can provide dormant origins that, in response to replicative stress, can fire and allow a complete replication of the genome (Blow et al., 2011; Ge and Blow, 2010; Ge et al., 2007; Ibarra et al., 2008; Woodward et al., 2006). As licensing can only occur in late mitosis and early G1 no MCM's can be loaded when cells encounter problems during S-phase, for example, when DNA damage or tightly bound proteins cause an irreversible fork arrest. Loading an excess

of MCM's during the licensing period can therefore provide a mechanism to rescue replication of a segment of the genome when two converging forks stall (Figure I. 7 middle panel). Mechanisms that activate dormant origins are not fully understood but the increased density of replication forks seen in active clusters by DNA fiber analysis (Ge et al., 2007) are consistent with dormant origins initiating in active replication clusters as a consequence of stochastic origin firing (Blow and Ge, 2009). In this scheme, once a chromosomal domain becomes competent to support initiation during S-phase, the origins (MCM2-7 double hexamers) that actually undergo initiation are selected by some non-deterministic process. If replication fork progression is inhibited, nearby licensed origins that would otherwise be eliminated by passive replication have an increased probability of undergoing initiation. Computer simulation of this simple model shows that it can account for the increased density of origins observed after HU treatment of U2OS cells if they contain 3-4 licensed origins for each origin that actually initiates in an unperturbed S phase (Blow and Ge, 2009).

Previous work has shown that when replication forks stall, the ATR and Chk1 kinases suppress the global rate on initiation. This provides a potential difficulty in explaining how replicative stress can lead to dormant origin activation under conditions where Chk1 is suppressing global rates of initiation. A recent study showed that when replication stress is induced by treatment of cell with low levels of hydroxyurea or aphidicolin, the DNA damage checkpoint kinases ATR and Chk1 preferentially inhibit activation of new replication factories while allowing firing of dormant origins within active clusters (Ge and Blow, 2010). A study by Thomson and colleagues revealed that modest reduction of Cdk activity inhibits activation of new replication factories without significantly affecting initiation within clusters (Thomson et al., 2010). Chk1 could directly lower Cdk activity (Figure I. 8) or alternatively could inhibit only the Cdk substrates that are essential for factory activation (Gillespie and Blow, 2010; Thomson et al., 2010). A lack of dormant origins has recently been shown to promote tumourigenesis and will be discussed in Section I. 7. 1.

I. 5. The licensing reaction and its components

Origin licensing involves the stepwise assembly of 'pre-Replicative Complex' (pre-RC) proteins onto DNA, and has been reconstituted *in vitro* with pre-RC proteins from *Xenopus* eggs (Gillespie et al., 2001) and budding yeast (Evrin et al., 2009; Remus et al., 2009) (Figure I. 3. B). ORC, the origin recognition complex (Coleman et al., 1996; Rowles et al., 1996), first binds DNA, then recruits Cdc6 (Cocker et al., 1996; Coleman et al., 1996) and Cdt1 (Maiorano et al., 2000; Nishitani et al., 2000). Two Cdt1 molecules recruit MCM2-7 proteins to ORC/Cdc6 on chromatin where they can act together to load Mcm2-7 onto DNA (Takara and Bell, 2011). Unlike MCM2-7, ORC, Cdc6 and Cdt1 are dispensable for replication after licensing is completed suggesting their only role in replication is to facilitate the loading of MCM2-7 complex (Donovan et al., 1997; Hua and Newport, 1998; Maiorano et al., 2000; Nishitani et al., 2000; Rowles et al., 1999). This reaction probably involves the opening of the MCM2-7 heterohexameric ring and clamping it around DNA (Evrin et al., 2009; Remus et al., 2009). MCM2-7 complexes are loaded onto origins as double hexamers, providing a configuration capable of initiating a pair of bidirectional replication forks (Evrin et al., 2009; Gambus et al., 2011; Remus et al., 2009).



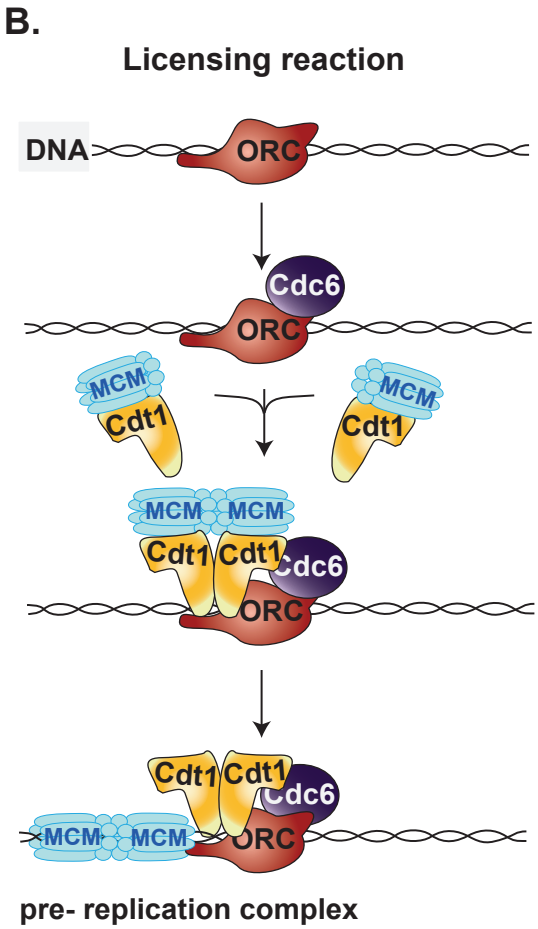


Figure I. 3. Stucture and chromatin loading of the MCM2-7 complex. The position of the MCM2-7 protein within the complex (top left) is shown as well as a three-dimensional EM reconstruction of budding yeast MCM2-7 double hexamer. Bottom shows the representations while top right shows a cross section with DNA. AAA+= AAA+ ATP binding domain. **B. Proposed model for the licensing reaction.** ORC binds to DNA and recruits Cdc6 to the origin of replication. It has recently been proposed that two Cdt1 molecules recruit two MCM2-7 complexes to ORC/Cdc6 (Takara and Bell, 2011). Subsequently the pre- RC complex is formed by the loading of the MCM2-7 double hexamer onto chromatin.

I. 5. 1. The Origin Recognition Complex (ORC)

The ORC was first identified by Bell and Stillman in *S. cerevisiae* binding to a conserved Autonomously Replicating Sequence (ARS consensus sequence (Bell and Stillman, 1992). ORC is a five- or six- subunit protein complex (ORC1-5 or ORC1-6) (Gossen et al., 1995; Rowles et al., 1996) being a conserved feature of chromosomal replication in all eukaryotes studied so far. Deletion of ORC genes is lethal in *Drosophila* and *S. pombe* (Gavin et al., 1995; Grallert and Nurse, 1996; Landis et al., 1997; Leatherwood et al., 1996) and depletion of the ORC1-5 blocks licensing and DNA replication in *Xenopus* (Coleman et al., 1996; Romanowski et al., 1996; Rowles et al., 1996) .

In *S. cerevisiae* ORC6 is not required for DNA binding but remains essential for DNA replication and cell viability (Li and Herskowitz, 1993), while it is shown to be indispensable for both processes in *D. melanogaster* (Chesnokov et al., 2001). Furthermore it has been shown that ORC6 is not required for the licensing activity of ORC1-5 in *Xenopus* (Gillespie et al., 2001) and is not part of the human ORC (Dhar et al., 2001). A crucial function of ORC is the binding and hydrolysis of ATP and studies of *S. cerevisiae* ORC (ScORC) and *D. melanogaster* ORC (DmORC) indicated that ATP binding by ORC1 is required for DNA binding (Brauchle et al., 2003). ORC1 also promotes ATP hydrolysis, which if inactivated does not interfere with DNA binding but with MCM loading (Bowers et al., 2004).

ORC1 protein has been shown to exhibit a replication independent function in controlling centriole and centrosome copy number in human cells (Hemerly et al., 2009), possibly explaining recent findings showing that ORC1 binds to chromatin independently of ORC2-5 (Sonneville et al., 2012).

I. 5. 2. The Cdc6 protein

The Cell Division Cycle 6 Protein (Cdc6) was first identified in a screen for *S. cerevisiae* mutants with defects in cell cycle progression (Hartwell et al., 1973). Cdc6

has been found in many eukaryotes and plays a crucial role in assembly of the pre-RC, where it acts after ORC and is required for MCM2-7 loading onto chromatin (Bell et al., 1995; Coleman et al., 1996; Grallert and Nurse, 1996; Leatherwood et al., 1996; Saha et al., 1998). Cdc6 is highly related to ORC1 and with a limited extent to ORC4, ORC5 and MCM2-7 proteins (Neuwald et al., 1999) and belongs to the family of AAA+ ATPase containing proteins. Mutations in this motif block MCM loading and consequently DNA replication (Perkins and Diffley, 1998; Weinreich and Stillman, 1999a). Cdc6 binding to chromatin requires ORC binding (Coleman et al., 1996), while its ATP binding and hydrolysis does not require ATP binding and hydrolysis by ORC (Randell et al., 2006). Interestingly, destabilisation of Cdc6 on chromatin after licensing seems to be essential for Cdc7 kinase mediated phosphorylation of MCM4 and therefore required for initiation of DNA replication in S-phase (Kundu et al., 2010).

Cdc6 has also been found to play an important role outside DNA replication, as a mitotic substrate of polo-like kinase1, in regulating chromosomal segregation mediated by Cdk1 and separase (Yim and Erikson, 2010). Studies in *S. pombe* and *Xenopus* egg extracts also indicated that Cdc6 is required for activation and maintenance of the ATR/ Chk1 mediated S-phase checkpoint in response to replication inhibition or presence of apparent DNA structures (Murakami et al., 2002; Oehlmann et al., 2004). In human cells mitotic entry can be inhibited by Cdc6 mediated activation of Chk1 kinase thus preventing cyclin B/ Cdk1 mediated progression through the G2/M boundary (Clay-Farrace et al., 2003).

I. 5. 3. The Cdt1 protein

The Cdc10 dependent transcript 1 (Cdt1) protein is another key factor in assembling the pre-RC and was first identified in *S. pombe* (Sp) as a gene that is regulated by the Cdc10 transcription factor (Hofmann and Beach, 1994). The same study revealed that mutation in *SpCDT1* resulted in a failure of DNA replication and defects in the S-phase checkpoint. Nishitani *et al.* (2000) showed that SpCdt1 forms a complex with SpCdc6 and is required for MCM loading onto chromatin. Cdt1 homologues have been reported

in *Xenopus*, *Drosophila*, human cells, (Maiorano et al., 2000; Nishitani et al., 2000; Whittaker et al., 2000; Wohlschlegel et al., 2000) and later in *S. cerevisiae* and *C. elegans (Tanaka and Diffley, 2002; Zhong et al., 2003)*. Similar to the situation in *S. pombe*, the chromatin binding of *Xenopus* Cdt1 is dependent on ORC and Cdc6 binding to the DNA (Gillespie et al., 2001; Maiorano et al., 2000). Furthermore, XlCdt1 as well as *Mus musculus* Cdt1 (MmCdt1) have been shown to directly interact with MCM2-7 complexes via a domain at the C-terminus (Ferenbach et al., 2005; Yanagi et al., 2002) indicating a role in recruiting MCM2-7 to the origins. As shown in Figure I. 3, it has been shown in three independent studies that MCM2-7 complexes are loaded onto chromatin as double hexamers (Evrin et al., 2009; Gambus et al., 2011; Remus et al., 2009). Recent studies revealed that two Cdt1 molecules facilitate the loading of the MCM2-7 double hexamer, suggesting a model in which origin-bound ORC and Cdc6 recruit two Cdt1 molecules to initiate double-hexamer formation prior to helicase loading (Takara and Bell, 2011). In metazoans Cdt1 has been shown to be the key regulatory protein for the licensing machinery and its regulation will be explained in more detail in Section I. 6. 1.

I. 6. Regulation of Replication licensing

Tight regulation of the licensing system is essential to ensure that MCM2-7 complexes can only be loaded in late mitosis and early G1. In yeast, down regulation of the licensing machinery is achieved by Cdk activity and its inhibition in G2/M cells results in a full re-replication of the genome (Broek et al., 1991; Dahmann et al., 1995; Green et al., 2010; Nishitani and Nurse, 1995). In *S. cerevisiae* and *S. pombe* ORC function is inhibited by Cdk mediated phosphorylation of ORC2 (Nguyen et al., 2001; Vas et al., 2001), while Cdc6 is degraded in a Cdk dependent manner (Drury et al., 2000; Elsasser et al., 1999; Jallepalli et al., 1997). Additionally, Cdks phosphorylate MCM2-7 and Cdt1 leading to their nuclear export during S-phase, G2 and mitosis (Hennessy et al., 1990; Labib et al., 1999; Nguyen et al., 2001; Tanaka and Diffley, 2002).

However in metazoans regulation of the licensing system is more complex and although Cdk activity plays a role in the mitotic regulation of the licensing system, the main route to prevent re-licensing during S phase and G2 is by down-regulation of Cdt1 activity (Figure I. 4.). In addition, ORC and Cdc6 are also subject to cell cycle regulation, though this appears to play a relatively minor role in preventing re-replication. The experiments and results described in the next Chapters of this dissertation show how re-replication of the genome is induced by down regulation of Cdt1 activity, therefore the next sections provide insight into the regulation of the licensing system in metazoans focusing on Cdt1.

ORC. In several human cell lines the level of chromatin bound ORC1 has been shown to be cell cycle regulated with a peak in G1 and a subsequent decrease during S-phase (Kreitz et al., 2001; Mendez et al., 2002). Over-expressed ORC1 binds and is ubiquitinated by the F-box protein S-phase kinase-associated protein 2 (Skp2), a component of the Skp1/Cul1-F-box E3 ubiquitin ligase (SCF^{Skp2}), known to mediate proteasomal degradation of numerous cell cycle proteins. Interestingly, depletion of Skp2 increases endogenous levels of ORC1 suggesting a regulation via that pathway (Mendez et al., 2002). Studies in *Xenopus* extracts revealed that the affinity of ORC1 and 2 to chromatin is reduced following licensing (Rowles et al., 1999) and DNA binding of ORC1 is inhibited during metaphase by Cdk phosphorylation (Romanowski et al., 2000)

Cdc6. Although humans possess the SCF^{hcdc4} ubiquitin ligase machinery, it appears that Cdk phosphorylation in S-phase promotes nuclear export of Cdc6 (Fujita et al., 1999; Jiang et al., 1999; Petersen et al., 2000; Saha et al., 1998) instead of proteolysis such as seen in yeast. Instead Cdc6 degradation has been reported to take place in mitosis and is mediated by N-terminal destruction motifs (KEN-box and a D-box) that are recognised by the anaphase promoting complex with cadherin 1 APC^{CDH1} ubiquitin ligase (Mendez and Stillman, 2000; Petersen et al., 2000). In *Xenopus,* Cdc6

is removed from chromatin upon binding of MCM2-7 in late mitosis and early G1 and reloads in S-phase when MCM2-7 is displaced from the origin of replication and plays a role in the activation of the intra-S-phase checkpoint (Oehlmann et al., 2004).

I. 6. 1. Regulation of the licensing factor Cdt1

I. 6. 1. 1. Proteolysis

In all cell types studied so far (*S. pombe, C. elegans, Drosophila, Xenopus* and human cells), Cdt1 protein levels oscillate during the cell cycle, with high levels in late mitosis and G1, and low levels in S and G2 phase (Figure I. 4 A). Three pathways of Cdt1 degradation have been identified so far.

***Proliferating Cell Nuclear Antigen* (PCNA) dependent Cul4/Ddb1/Cdt2 ubiquitin ligase.** In *Xenopus* efficient Cdt1 ubiquitination and degradation has been shown to be mediated by chromatin-bound PCNA, which binds Cdt1 via its PCNA interaction protein (PIP) domain (Figure I. 4 B) and subsequently recruits the Cul4/ DNA damage-binding protein 1 (Ddb1)/Cdt2 ubiquitin ligase.

PCNA binds to DNA as part of the replication fork providing a mechanism to specifically degrade Cdt1 during S phase (Arias and Walter, 2005; Arias and Walter, 2006; Jin et al., 2006a). However, during the first cell cycles of the early *Xenopus* embryo, the nuclear/cytoplasmic ratio is too low for Cdt1 to be quantitatively degraded by this mechanism, so that Cdt1 levels remain high throughout the cell cycle (Kisielewska and Blow, 2012).

The PCNA/Cul4/Ddb1/Cdt2 pathway has also been shown to be crucial in *C. elegans* where siRNA mediated knockdown of Cul4 results in elevated Cdt1 levels with the consequence of extensive re-replication of DNA (Zhong et al., 2003).

In human cells it has been observed that the Cdt1 mRNA levels remained constant throughout the cell cycle (Nishitani et al., 2001) while Cdt1 levels decrease upon entry into S-phase, suggesting a post-translational regulation of Cdt1 (Nishitani et al., 2001; Wohlschlegel et al., 2000). It has later been found that the PCNA/Cul4/Ddb1

pathway is the main route for Cdt1 down-regulation during DNA replication and in response to DNA damage (Higa et al., 2003; Jin et al., 2006b; Nishitani et al., 2006; Senga et al., 2006) (Figure I. 4). When re-replication occurs the DNA damage response is activated in human and *Drosophila* cells, Cdt1 as well as Cdc6 degradation is increased providing a mechanism to inhibit re-licensing and thereby limiting the extent of re-replication (Hall et al., 2008). In contrast, the same group found that Cdt1 is protected from degradation by Mitogen-activated protein (MAP) kinase induced phosphorylation in unperturbed G2 phase and during acute cellular stress response, such as exposure to toxins, osmotic shock or inflammatory cytokines (Chandrasekaran et al., 2011). In addition to the stabilisation of Cdt1, its phosphorylation blocks MCM2-7 binding thus functionally inactivating Cdt1, providing a controlled environment to cope with stress while entering mitosis and holding enough Cdt1 to start licensing.

After DNA damage PCNA is loaded onto chromatin followed by dynamic recruitment of Cul4/Ddb1/Cdt2 and Cdt1 to the site of damage prior to Cdt1 ubiquitination and degradation (Ishii et al., 2010; Roukos et al., 2011). How Cdt1 is recruited is not yet solved but a recent study provided evidence that Cul4/Ddb1/Cdt2 is recruited to PCNA by the clamp loader complex replication factor C in complex with chromosome cohesion factor Ctf18 (Ctf18-RFC) (Shiomi et al., 2012).

The hexameric (AAA+) ATPase p97, involved in endoplasmic reticulum-associated protein degradation and other relevant cellular processes, has recently been shown to mediate the release of PCNA/Cul4/Ddb1 ubiquitinated Cdt1 from chromatin which is essential for its proteasomal degradation in response to UV irradiation (Raman et al., 2011). *C. elegans* embryos deficient of p97 (Cdc48) not only show accumulation of Cdt1 on mitotic chromatin but also a persistent chromatin association of Cdc45/GINS leading to defects in S-phase progression and a reduced DNA content (Franz et al., 2011).

Cdk dependent SCF^{Skp2} ubiquitin ligase. The Cdk dependent SCF^{Skp2} pathway provides an additional mechanism for Cdt1 degradation in S-phase and G2 (Li

et al., 2003; Nishitani et al., 2004; Sugimoto et al., 2004). This is a replication independent process that requires Cdk binding at residues 65-72 and subsequent phosphorylation of Threonine 29 (Liu et al., 2004; Takeda et al., 2005) (Figure I. 4 B).

In *Drosophila* the SCF^{Skp2} pathway has been shown to be a major pathway for Cdt1 proteolysis (Thomer et al., 2004). Direct evidence for the existence of the Cul4/Ddb1 pathway is missing but mutations of replication proteins inhibit Cdt1 degradation suggesting a role of a replication dependent mechanism (May et al., 2005). In agreement with this, abolition of Cdk phosphorylation does not fully stabilise *Drosophila* Cdt1, which contains the PIP motif required for PCNA dependent degradation.

APC/ CDH1 ubiquitin ligase. The APC/ CDH1 ubiquitin ligase is active from anaphase until late G1 and has been shown target Cdt1 for proteasomal degradation (Li and Blow, 2005; Sugimoto et al., 2008). Although APC mediated degradation of Cdt1 is not as efficient as for geminin but its impairment causes re-replication and chromosomal damage (Sugimoto et al., 2008). This pathway may prevent an excessive accumulation of Cdt1 before S-phase onset and geminin accumulation (Blow and Gillespie, 2008b) and plays a role in clearance of Cdt1 upon cell cycle exit (Sugimoto et al., 2008).

Besides the complex network of Cdt1 degradation, metazoans express a Cdt1 inactivating protein called Geminin, indicating the importance of strict regulation for Cdt1.

I. 6. 1. 2. The Cdt1 inhibitor - Geminin

Geminin is a 25 kDa protein that was initially identified using the *Xenopus* cell-free system in a screen for targets of the APC performed by McGarry and Kirschner in 1998. APC is a multi-subunit E3 ubiquitin ligase that is active from anaphase until the beginning of S-phase ensuring the exit from mitosis by ubiquitination of various substrates such as mitotic cyclins and proteins involved in spindle function and sister

chromatin cohesion (Uhlmann et al., 1999). Geminin transcription is driven by the E2F transcription factor (Yoshida and Inoue, 2004) whereas protein levels are regulated by APC, with high abundance in S-phase and G2, and low abundance upon entry into anaphase (McGarry and Kirschner, 1998)(Figure I. 4 A). Interestingly the same study revealed that geminin functions as a potent inhibitor of MCM2-7 loading in *Xenopus* egg extract. McGarry and Kirschner also reported the sequence of human geminin and Wohlschlegel et al. produced the first antibody targeting human geminin (McGarry and Kirschner, 1998; Wohlschlegel et al., 2000). However, the target of geminin inhibition was unknown until Wohlschlegel *et al.* and Tada *et al.* showed that geminin binds and inhibits Cdt1 (Tada et al., 2001; Wohlschlegel et al., 2000). Since then geminin homologues have been identified in *C. elegans* (Yanagi et al., 2005) and *Drosophila* (Quinn et al., 2001).

A number of studies have investigated the functional domains of Cdt1 responsible for geminin, MCM2-7 and DNA binding. In Figure I. 4 B those findings are taken together and presented in a schematic overview of human Cdt1 and geminin and their functional domains. The N-terminus of Cdt1 has been shown to contain the motifs required for Cdt1 degradation as well as a nuclear localisation sequence. The central region contains a coiled-coil domain that is required for interaction with geminin (Ballabeni et al., 2004; Ferenbach et al., 2005; Saxena et al., 2004) although it also has been reported that the N-terminus can weakly interact with geminin (Ferenbach et al., 2005; Lee et al., 2004; Saxena et al., 2004). Ferenbach *et al* showed that the C-terminal 377 residues are required for licensing and that the extreme C-terminus contains the domain that interacts with MCM2-7.

Figure I. 4.

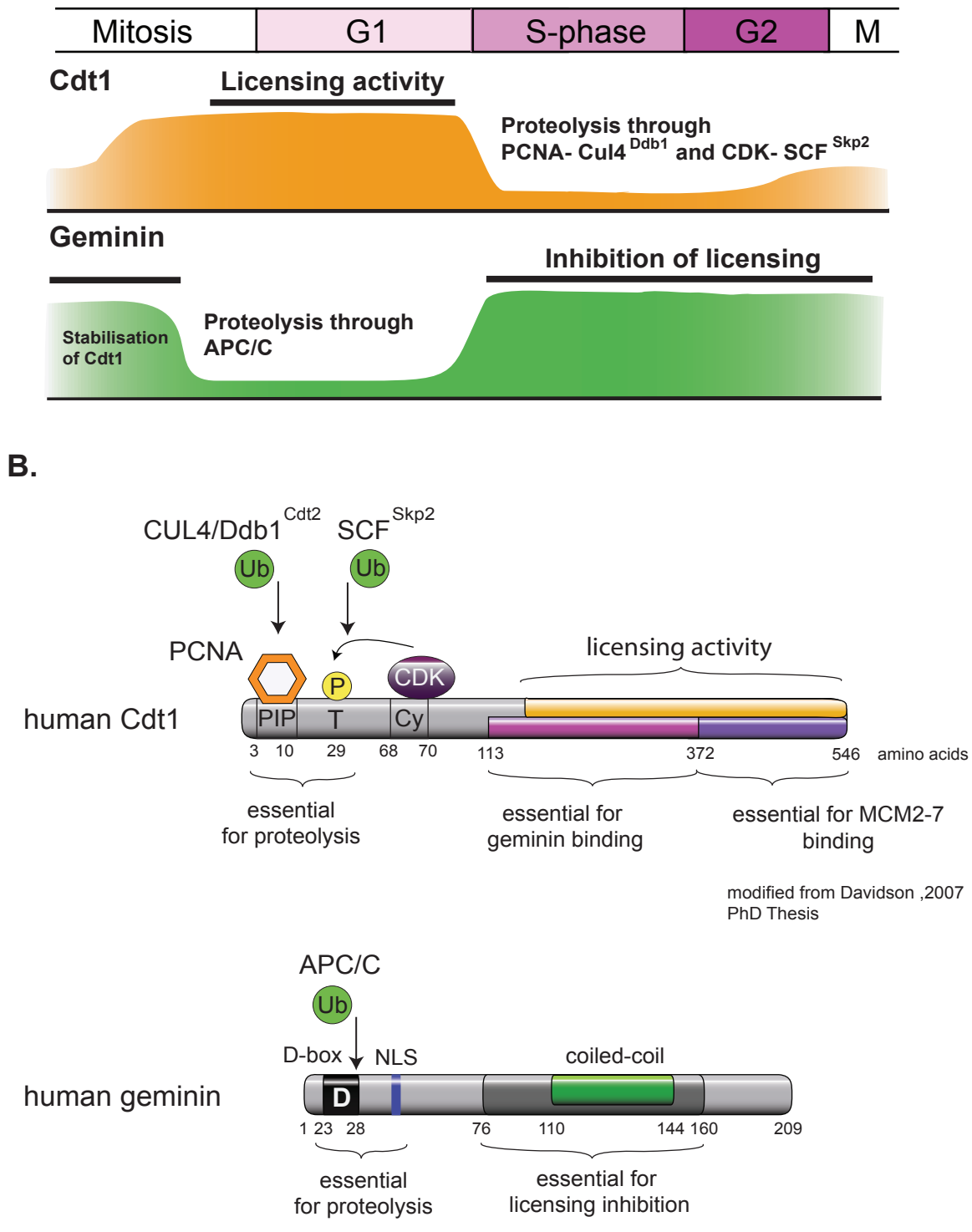


Figure I. 4. Cell cycle regulation of Cdt1 and geminin protein and a schematic representation of both proteins. A. Cell cycle phases are indicated and protein levels of Cdt1 (orange) and geminin (green) throughout the cell cycle are shown. **B.** Cdt1 and geminin protein with essential domains for poteolysis and licensing or licensing inhibition are shown. The domain architecture for Cdt1 is based on studies of Ferenbach et al.,2005 and reproduced from Davidson, 2007. PIP- PCNA binding motif, Cy- Cyclin/Cdk binding modif, T29- Cdk phosphorylation site. The geminin binding domain is pink, the MCM binding domain violet and the region required for licensing is yellow. The domain architecture for geminin is based on Thepaut et al., 2004. NLS- Nuclear localisation sequence, D-box- Destruction box required for APC/C binding. The functional essential coiled-coil domain is shown in green and the region required for licensing inhibition is grey.

While geminin degradation is dependent on a 9 aa destruction box near the N-terminus, a central 80 aa coiled-coil domain is responsible for its role as an inhibitor of DNA replication (Figure I. 4 B).

The mechanism by which geminin inhibits Cdt1 is not clear; it does not prevent binding of Cdt1 to the DNA but prevents the binding of Cdt1 to MCM2-7 (Gillespie et al., 2001; Kim et al., 2007). Several studies have shown that geminin exists as a homodimer (Ferenbach et al., 2005; Lee et al., 2004; Saxena et al., 2004; Thepaut et al., 2004) and binds Cdt1 in a ratio of 2 geminin to 1 Cdt1 forming a heterotrimer (Ferenbach et al., 2005; Lee et al., 2004). However, the ratio between geminin and Cdt1 may functions as a non-linear molecular switch between controlled licensing and complete inhibition of licensing. This is based on an accumulation of evidence that started with the observation that only ~50% of ubiquitinated geminin is degraded upon exit from metaphase in the *Xenopus* early embryo (Hodgson et al., 2002), while the remainder becomes inactivated and unable to bind Cdt1 until geminin reactivation (Li and Blow, 2004). Another study in *Xenopus* revealed that a 3:1 geminin -Cdt1 ratio is active in licensing when added to interphase egg extract, while a 4:1 ratio is not active, suggesting the presence of a licensing switch (Lutzmann et al., 2006). The same study estimated that Cdt1-geminin complexes are associated with chromatin with about 5 Cdt1 and 12 geminin molecules per origin during licensing and 20 geminin molecules after initiation. Consistent with those findings Ode and co-workers investigated an all-or-none switch for origin licensing that is based on inter-origin cooperativity. They showed that licensing is inhibited when geminin dependent Cdt1 foci are formed on chromatin. In their model geminin is recruited to chromatin via its interaction to Cdt1, followed by an interaction of geminin-Cdt1 complexes from different origins with each other thus forming the inhibitory higher ordered structure visible as foci (Ode et al., 2011).

In line with these findings a structural study revealed that the Cdt1:geminin complex can exist in two forms, a ‘permissive’ heterotrimer and an ‘inhibitory’ heterohexamer (De Marco et al., 2009) (Figure I. 5 A).

In the heterotrimeric complex a geminin dimer binds one Cdt1 molecule via two interfaces such as shown by Lee *et al*., 2004 but only in the heterohexamer a tertiary interface is formed that buries Cdt1 residues required for MCM binding, which are exposed in the permissive heterotrimer complex. The study suggests the equilibrium between licensing permissive and inhibitory complex functions as conformational switch regulating licensing activity (Figure I. 5 B). Posttranslational modifications such as ubiquitination, phosphorylation or sumoylation would be potential candidates to change the equilibrium towards one or other of these states. The previously described inactivation of geminin by APC mediated ubiquitination (Hodgson et al., 2002) or the hyperphosphorylation of Cdt1 and geminin (Ballabeni et al., 2004; Nishitani et al., 2004) in mitosis could inhibit the formation of the heterohexamer thus allowing licensing of replication origins for the next cell cycle.

A study performed by Ballabeni *et al.* suggests that in addition to inhibiting Cdt1, geminin protects Cdt1 from degradation in G2/M possibly allowing Cdt1 levels to build up for efficient reactivation of the licensing machinery in late mitosis. This points to a dual function of geminin as both a negative and positive regulator of pre-RC formation in human cells (Ballabeni et al., 2004). However, an alternative explanation for these results is that removal of geminin leads to re-replication, which causes Cdt1 degradation by a checkpoint-mediated mechanism (Hall et al, 2008).

Studies also revealed that geminin can regulate neuronal differentiation by binding to the catalytic subunit of the switch/sucrose nonfermentable (SWI/SNF) chromatin-remodeling complex, Brahma-related gene 1 (Brg1) (Seo et al., 2005). Pefani and co-workers have recently identified Idas, another geminin interaction partner. Idas is a coiled-coil protein related to geminin, that binds geminin and inhibits its interaction with Cdt1. Idas depletion causes accumulation of cells in S-phase and prevents progression into mitosis (Pefani et al., 2011).

Figure I. 5.

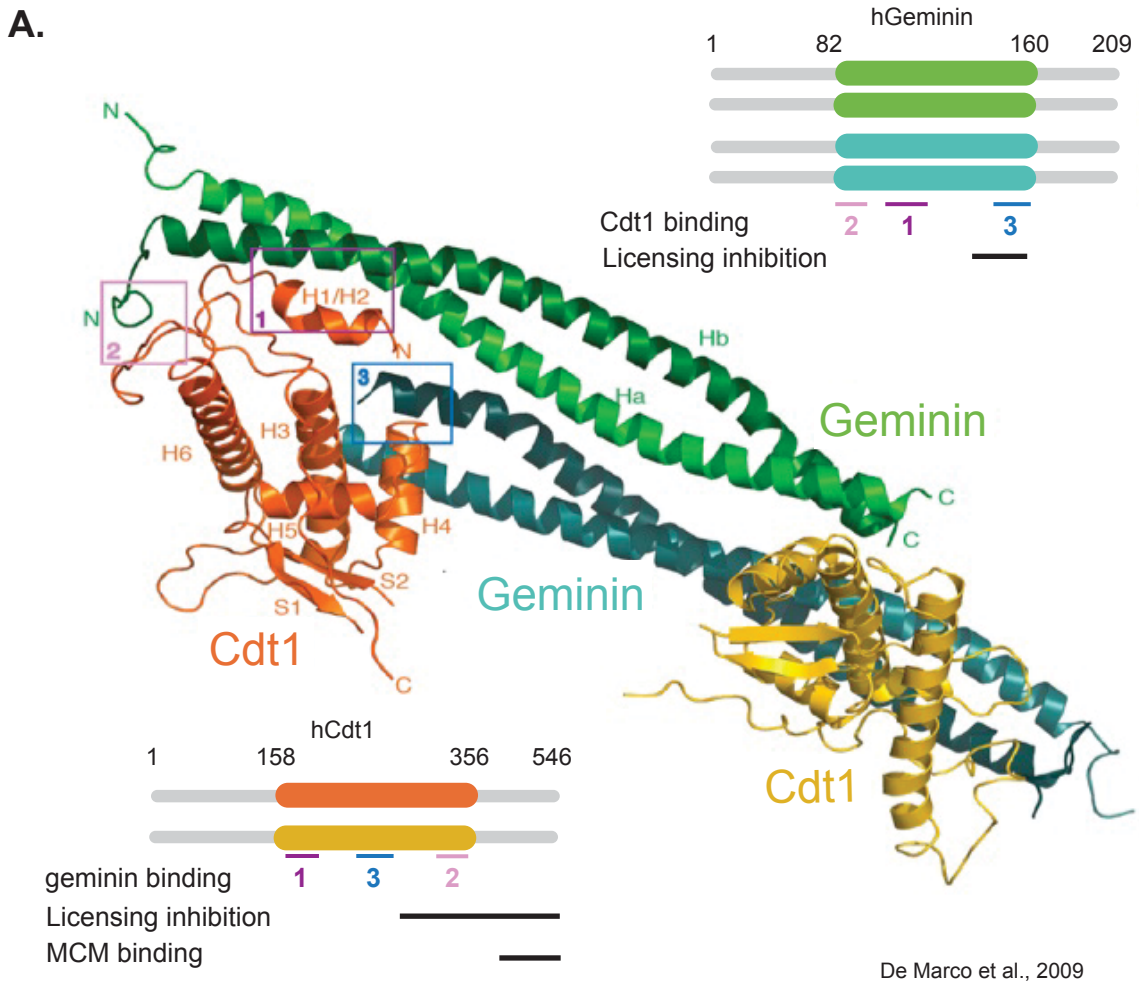
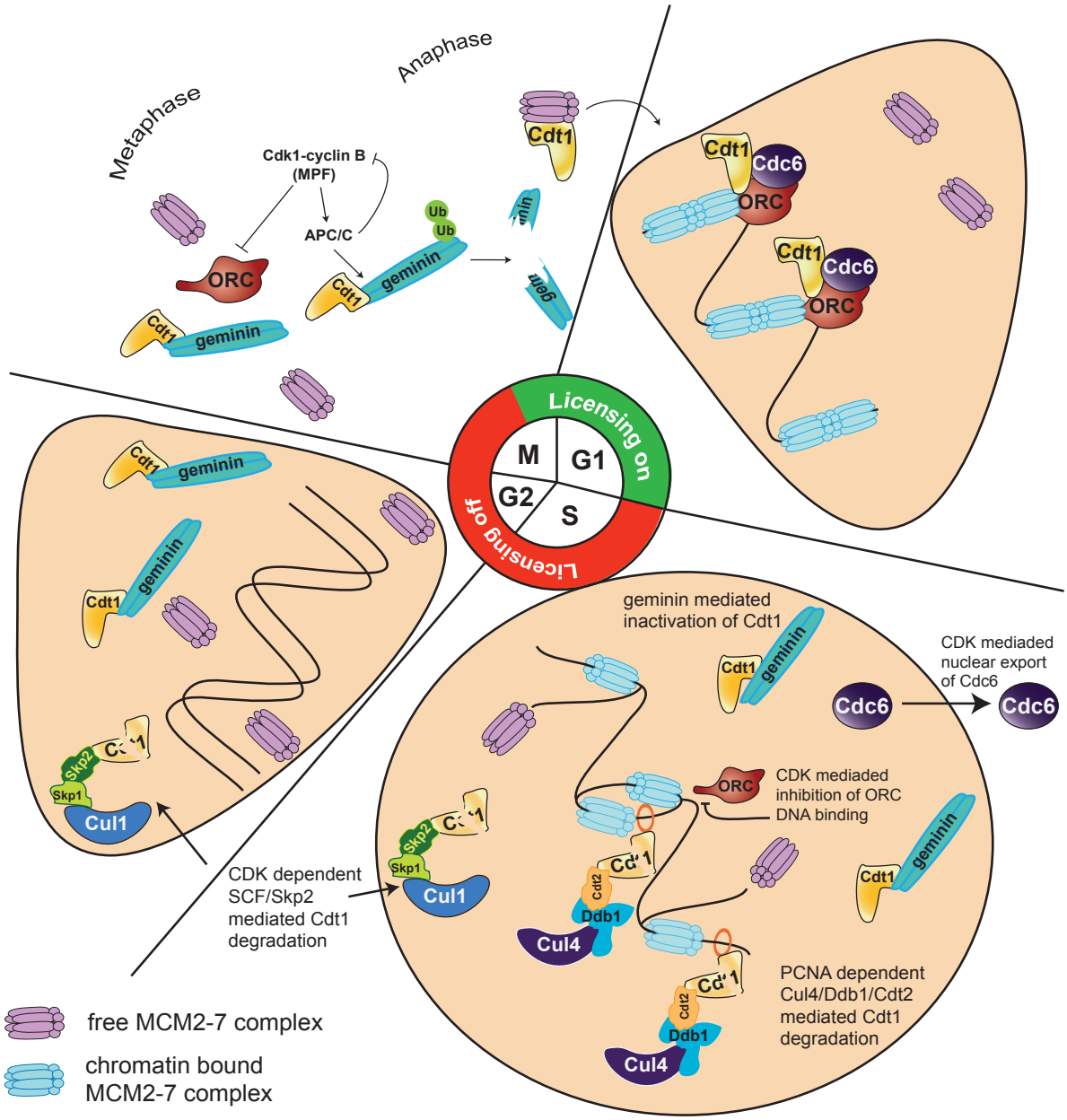


Figure I. 5. Structure of the human Cdt1:geminin complex and a proposed model for its inhibitory function. A. The 2x (Cdt1: 2x geminin) heterohexamer is shown as a cartoon representation. Cdt1 molecules in yellow and orange and geminin dimers in green and blue shades. The primary, secondary and tertiary interface regons are boxed, in a magenta, pink and blue-colored box. A schematic representation of Cdt1 and geminin proteins with the 3 interfaces marked in the sequence is also shown (additional functional regions are marked with black bars). **B.** Based on results from De Marco et al.,2009 a model for the mechanism of licensing inhibition by geminin was proposed with the heterotrimer allowing MCM chromatin loading while the heterohexamer blocks loading.

Additional regulatory roles in development, cell differentiation and cell proliferation have been suggested from the observations that geminin interacts with transcription factors of the Hox and polycomb as well as of the Six family. When geminin binds the Six3 transcription factor it is displaced from Cdt1 thus promoting proliferation of retinal precursor cells. In line with this, loss of geminin leads to expanded optic vesicles, while geminin over-expression causes forebrain and eye defects (Del Bene et al., 2004). Geminin binding to Hox transcription factors not only releases Cdt1, thus inducing licensing and therefore proliferation, but also inhibits binding of Hox to DNA thereby preventing transcription of Hox target genes (Luo et al., 2004).

Figure I. 6. shows the complex regulatory network of the replication licensing system throughout the cell cycle. The next section describes the consequences of deregulation of the licensing machinery and its implications in disease.

Figure I. 6.



I. 6. Regulation of the replication licensing system throughout the mammalian cell cycle. Shown are the four cell cycle phases with nuclei (light brown areas) and the relevant proteins. From late mitosis until late G1 the licensing machinary is active (green) and free MCMs (lila) can be loaded onto chromatin (blue) as double hexamer by sequential action of ORC, Cdc6 and Cdt1. Upon initiation MCMs travel in front of the replication fork as replicative helicase leaving the origins unlicensed. In S-phase until Anaphase the licensing system is inactivated by nuclear export of Cdc6, Cdk mediated inhibition of ORC and most impotantly down regulation of Cdt1 activity. Cdt1 is in an inhibitory complex with geminin and degraded by two ubiquitin ligases. In S-phase it is predominantly degraded in a PCNA dependent manner by the Cul1/Ddb1 ubiquitin ligase and from S-phase until Anaphase it is also degraded by the SCF/Skp2 ligase. During G2 phase Cdt1 levels start to build up and once the cells enter anaphase geminin is degraded by the anaphase promoting complex thus allowing licensing of replication origins.

I. 7. De-regulation of the Licensing Machinery

I. 7. 1. Insufficient licensing - the lack of dormant origins

Section I. 4. 4. 1. described the role of excess MCM2-7 loaded onto chromatin during licensing to provide dormant origins that can fire during replication stress such as fork stalling to ensure complete genome replication (Ge et al., 2007; Ibarra et al., 2008; Woodward et al., 2006). Minimal licensed DNA, exhibiting a ~50% reduction of chromatin bound ~MCM2-7 complexes, showed an essentially normal replication rate, average origin spacing and cell cycle checkpoint activity but a reduced long term viability. However cells with minimal licensed origin are hypersensitive to replication inhibitors such as hydroxyurea (Ge et al., 2007). This suggests that minimal licensing has no effect on 'normal' replication but reduces viability when replication stress occurs during normal cell cycles or is induced by the presence of replication inhibitors.

Mice heterozygous for an *MCM4* hypomorphic mutation that seems to destabilise MCM4 protein (*MCM4*^{Chaos3}), showed an increased rate of chromosome breakage in response to a replication inhibitor (Blow and Gillespie, 2008b; Shima et al., 2007). Chaos3 mutant mice also exhibited increased levels of micronuclei and 80% of the females died of mammary adenocarcinomas. A new paper by Kawabata and colleagues (2011) revealed that *MCM4*$^{Chaos3/Chaos3}$ mice exhibit ~60% reduced levels of chromatin bound MCM2-7 protein and a corresponding inability to activate dormant origins when challenged with aphidicolin, an inhibitor of the replicative DNA polymerases (MCM hypomorph in Figure I. 7). Strikingly, even in the absence of externally supplied replication stresses, *MCM4*^{Chaos3} mutant cells had an increased number of stalled replication forks, a small increase in DNA damage foci containing Ras-related associated with diabetes 51 protein (RAD51), Replication protein A 32 (RPA32) and RAD17, a 50% increase of Fanconi anemia group D2 (FANCD2) foci (a protein involved in resolving stalled replication intermediates) and >2-fold increase of abnormal mitoses (Kawabata et al., 2011; Klotz-Noack and Blow, 2011). This genetic instability very likely explains why the mice are tumour-prone. In a second study

homozygous transgenic *MCM2* mice (*MCM2*$^{Ires\text{-}CreERT2}$) showed a one-third reduced MCM2 level and exhibited severe deficiencies in the proliferative cell compartments of a variety of tissues. They died of T- and B- cell lymphomas at an early age (Pruitt et al., 2007). These findings demonstrate the critical importance of dormant replication origins for cells in a physiological setting, and have implications for the genetic instability commonly seen in cancer cells (Figure I. 7).

In primary cells a 'licensing checkpoint' exists in G1 phase of the cell cycle to ensure that a sufficient number of origins are licensed before entry into S-phase. It involves pathways that activate p53 and suppress retinoblastoma protein (Rb) function during G1 (Blow and Gillespie, 2008b; Liu et al., 2009; Nevis et al., 2009; Shreeram et al., 2002). In cancer these pathways are quite often disturbed and the checkpoint does not engage allowing insufficiently licensed cells to progress into S-phase but it remains unclear how sensitive the licensing checkpoint is to smaller reductions such as those seen in MCM4$^{Chaos3/Chaos3}$ cells. Neither the MCM4 nor the MCM2 hypomorphic cells display gross proliferation defects. One possibility is that dormant origin defects are seen in MCM4$^{Chaos3/Chaos3}$ and MCM2 mutant mice because the reduction is too modest to reproducibly engage the licensing checkpoint. The levels of MCM2-7 could just be enough to enter S-phase, causing replication defects that are too minor to strongly activate checkpoints, but in the long term substantial enough to cause genomic instability and cancer (Klotz-Noack and Blow, 2011)(Figure I. 7).

Figure I. 7.

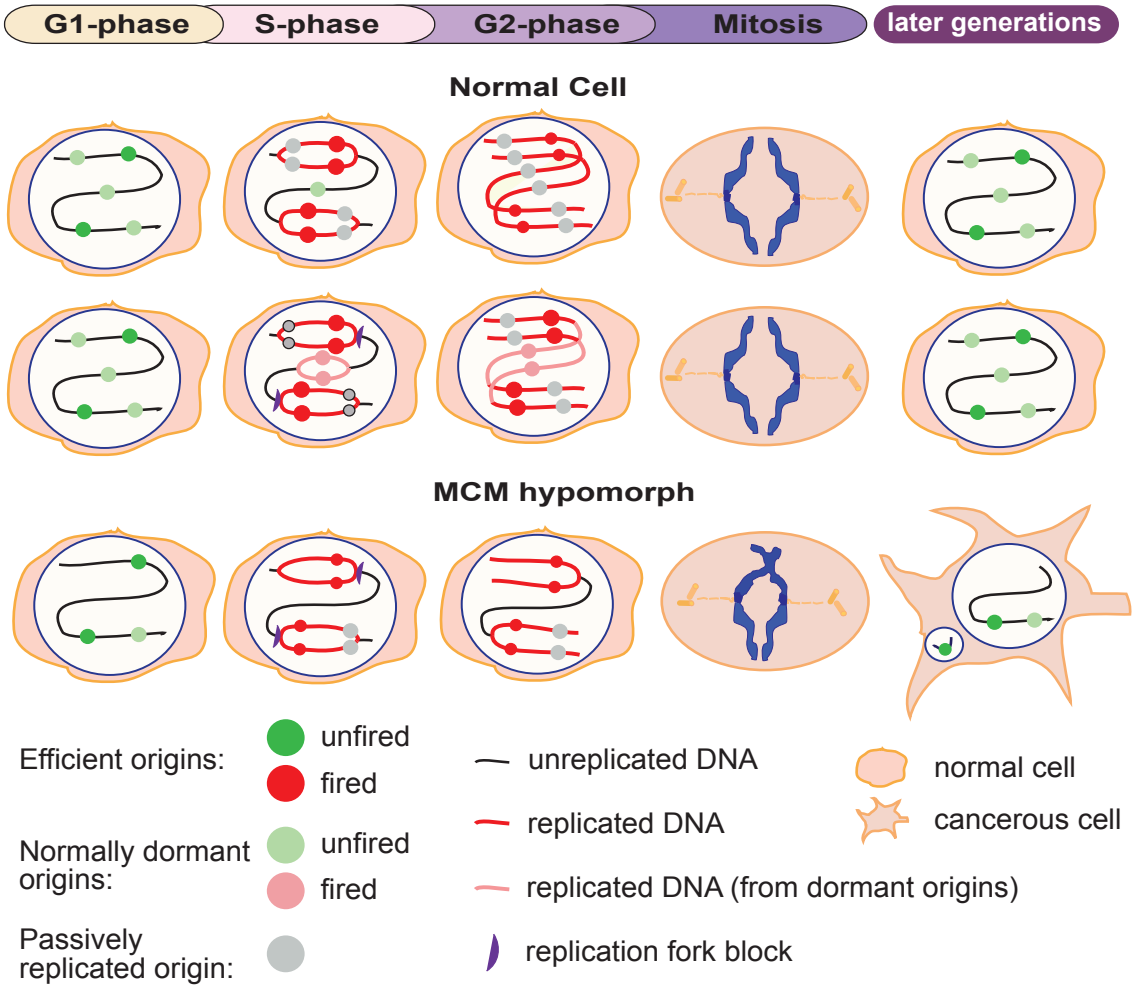


Figure I. 7. A role for dormant origins in maintaining genomic stability. A small segment of the genome is shown during the cell cycle of normal (top) and MCM hypomorphic (bottom) cells. In G1, efficient and normally dormant (unefficient) origins are licensed by loading MCM2-7 onto chromatin (green and light green dots). In S-phase, some origins fire (red dots) and replication forks move bidirectionally away from them, passively replicating other origins (grey dots) until forks stall or terminate. Fork stalling triggers the firing of some origins that usually remain dormant (light red dots) to ensure complete genome duplication. Lack of dormant origins can result in progression of cells into mitosis with unreplicated segments of the genome, leading to chromosome breakage and formation of micronuclei.

Cyclin E/Cdk2 activity drives cell cycle progression through the restriction point (R) and initiates the S-phase program (Ohtsubo et al., 1995). A wealth of experimental data has linked deregulated expression of cyclin E to human cancer exposing a very powerful oncogene (reviewed in (Donnellan and Chetty, 1999). Over-expression of cyclin E has been shown to shorten the duration of G1 and accelerate G1/ S-phase transition but leads to slowing of S-phase progression (Ohtsubo and Roberts, 1993; Resnitzky et al., 1994; Spruck et al., 1999). Interestingly, cyclin E overexpressing S-phase cells also exhibited a reduced number of BrdU and PCNA foci suggesting fewer initiation events took place possibly explaining why S-phase progression is slowed down (Ekholm-Reed et al., 2004). The same study showed a reduction of chromatin bound MCM2-7 complex components in cyclin E overexpressing cells explaining why fewer initiation events can be observed. The study implies that the shortened G1 phase and the accelerated S-phase entry caused by high cyclin E levels, interfered with proper pre-RC formation leading to insufficient origin licensing. Genetic instability, and accelerated tumourigenesis are consequences of cyclin E deregulation (Loeb et al., 2005; Spruck et al., 1999) and might be caused by insufficient origin licensing.

Licensing inhibitors have a high therapeutic potential and anticancer agents targeting ORC, Cdc6, Cdt1 or MCMs, thus preventing licensing of enough origins to allow complete genome duplication, would engage the licensing checkpoint of normal proliferating cells, whereas cancer cells would progress into S-phase trying to complete replication (Feng et al., 2003; Shreeram et al., 2002). Those cells would be hypersensitive to a range of replication inhibitors and would only partially replicate their genome while the G1 arrested ‘normal cells’ would be unaffected. Removal of the anticancer agent would enable the ‘normal cells’ to license the remaining origins and pass the checkpoint to progress into S-phase. The cancer cells that have progressed into S-phase, where further licensing is impossible are doomed to suffer the fatal consequences of partial replication ultimately leading to cell death. Finding licensing inhibitors could be an important step forward in the fight against cancer.

I. 7. 2. Uncontrolled licensing – re-replication of the genome

I. 7. 2. 1. De-regulated expression of licensing proteins

Several studies have linked inappropriate expression of MCM2-7 and other pre-RC proteins to a variety of pre-malignant dysplasias and cancer (Gonzalez et al., 2005; Hook et al., 2007; Lau et al., 2007; Williams and Stoeber, 2007; Xouri et al., 2004). In actively cycling cells, MCM2-7 are constitutively expressed and only show a cycle of chromatin binding and release (Figure I. 6). However most cells in metazoans exit the cell cycle through quiescence or differentiation and exhibit a lack of detectable MCM proteins (Madine et al., 2000; Musahl et al., 1998; Todorov et al., 1998). One could say that MCM2-7 proteins mark proliferating cells and their expression level in human tissue can be indicative for early tumour formation. Therefore Ron Laskey and colleagues have developed a cervical smear test using MCM5 antibodies which allows early detection of squamous intraepithelial lesions (Williams et al., 1998). Attempts to recover cells from a range of body fluids to detect MCMs and screen for pre-invasive stages of the most common cancer types gave promising results (reviewed in (Tachibana et al., 2005a) and led to clinical trials for cervical, lung, colon and bladder cancers (Laskey, 2005).

It is currently not understood why malignant cells express licensing proteins inappropriately but one possibility would be a failure to exit the cell cycle. Frequently an increase in Cdk activity driving cell cycle progression can be observed during malignant transformation. Consequently less cells would exit the cell cycle undergoing differentiation but instead would proliferate and express licensing proteins (Sherr, 1996). In this scenario MCM2-7 would be a powerful marker for ‘in-cycle’ cells but they would not be directly involved in malignant progression as an oncogene-induced stimulation of cell division is required.

Alternatively, a review by Blow and Gillespie (2008) discussed the deregulation of replication licensing as a consequence of oncogene induced cell proliferation. Activation of oncogenes linked to tumour formation has been shown to interfere with

mechanisms that typically shut down replication licensing in S-phase and G2. One of those oncogenes is cyclin D1, which is frequently deregulated in human cancers through mutations, alternative splicing and gene amplification. The accumulation of cyclin D1 results in an increase in cyclin D1/Cdk4 activity leading to transcriptional repression of CUL4 thus stabilising Cdt1 protein levels (Aggarwal et al., 2007; Aggarwal et al., 2010). Consequently low levels of re-replication could be detected that are sufficient to cause genetic instability but are compatible with cell survival mainly due to additional mutations in the DNA damage response.

Another strong oncogene is Ras and its expression causes a robust DNA damage response leading to oncogene induced senescence (OIS) in normal human cells (Di Micco et al., 2006). The senescent cells exhibit partially replicated DNA and segments where replication origins have fired multiple times. Interestingly the DNA damage response (DDR) is not activated in the absence of DNA replication suggesting that deregulation of the licensing machinery allowed re-firing of origins thus activating the DDR (Di Micco et al., 2006). Inactivation of the DNA damage response triggered the abrogation of OIS and promoted cell transformation in this study. It is quite likely that other oncogenes activating the pathways upstream of the retinoblastoma protein can prevent complete inactivation of the licensing machinery in S-phase and G2 thus promoting re-replication of DNA.

I. 7. 2. 2. Loss of licensing inhibitor geminin.

The importance of geminin in preventing re-replication varies between different model systems. Geminin knockdown by siRNA is sufficient to induce re-replication in several human cancer cells and a few primary human cells (Klotz-Noack et al., 2012; Melixetian et al., 2004; Zhu et al., 2004; Zhu and DePamphilis, 2009). Kristian Helin and co-workers showed that geminin knockdown in normal human and cancer derived cell lines leads to re-replication and Chk1-dependent checkpoint activation accompanied by formation of γH_2AX and RAD51 nuclear foci (Melixetian et al., 2004). They also demonstrated that abrogation of that checkpoint by caffeine or UCN01 led to

abortive mitosis and death of the re-replicating cell. A mitotic defect was also reported by Tachibana *et al.,* whose study revealed that geminin silencing causes centrosome over-duplication in normal human and cancer cell lines with the consequence of chromosome mis-segregation in mitosis (Tachibana et al., 2005c). In contrast, several studies showed that geminin depletion does not cause detectable re-replication in several other cell types such as HeLa or MCF10A cells (Kulartz and Knippers, 2004; Machida and Dutta, 2007; Zhu and DePamphilis, 2009). The cell line specific response is perhaps due to different protein levels of Cdt1 and geminin.

Loss of geminin leads to extensive re-replication of DNA in *D. melanogaster* (Mihaylov et al., 2002; Quinn et al., 2001), while it does not in *C. elegans* (Yanagi et al., 2005). It has been reported that in mice a lack of geminin resulted in pre-implantation mortality. DNA replication occurred but mitosis was not detected in the mutant embryos. The abnormal blastomeres contained damaged DNA and underwent apoptosis, likely as a consequence of the deregulation of DNA replication (Gonzalez et al., 2006; Hara et al., 2006). In *Xenopus,* depletion of geminin or inhibition of Cdt1 degradation alone leads to little re-replication but when both mechanisms are inhibited directly or by Cdt1 over-expression then substantial re-replication can be detected (Arias and Walter, 2005; Hodgson et al., 2002; Li and Blow, 2005; Maiorano et al., 2005; McGarry and Kirschner, 1998; Yoshida et al., 2005)

Knockdown of APC inhibitor early mitotic inhibitor 1 (Emi1) caused an increase of APC activity followed by decreased levels of geminin and cyclin A outside mitosis leading to re-replication even in HeLa cells (Machida and Dutta, 2007). The same study revealed that co-depletion of cyclin A and geminin causes re-replication, while in contrast the same group found that over-expression of cyclin A stimulates the re-replication induced by Cdt1 over-expression (Vaziri et al., 2003).

I. 7. 2. 3. Stabilisation or over-expression of Cdt1

Cdt1 is an oncogene and its over-expression in cells injected into nude mice results in tumour formation (Arentson et al., 2002). Cdt1 over-expression is sufficient to

induce re-replication in *Drosophil*a, *Xenopus*, and humans (Arias and Walter, 2005; Li and Blow, 2005; Maiorano et al., 2005; Thomer et al., 2004; Vaziri et al., 2003). Disruption of the PCNA dependent degradation pathway by siRNA targeting CUL4, Ddb1 or Cdt2 induces re-replication in *C. elegans* and several human cancer cell lines (Jin et al., 2006b; Lee et al., 2007a; Lovejoy et al., 2006; Sansam et al., 2006; Zhong et al., 2003). When both Cdt1 degradation pathways (Section I. 6. 1. 1) are inhibited by addition of a Nedd8 activating enzyme inhibitor called MLN4924, the activity of CUL1 and CUL4 E3 ligases is reduced and Cdt1 is stabilised (Figure IV. 9). Several studies have shown that MLN4924 treatment induces re-replication within S-phase, senescence and apoptosis emphasising the importance of proper Cdt1 regulation (Klotz-Noack et al., 2012; Lin et al., 2010; Milhollen et al., 2011; Soucy et al., 2009).

In *Xenopus* egg extracts addition of recombinant Cdt1 in G2 phase of the cell cycle leads to re-initiation of DNA synthesis and substantial re-replication (Li et al, 2005; Maiorano et al, 2005). High levels of Cdt1 also cause activation of the DNA damage response, inhibition of re-replication and appearance of small double stranded DNA fragments (Davidson et al., 2006). This study showed that DNA fragments only appear when uncontrolled re-replication takes place but not after a single round of re-replication being explained in a fork collision model (Figure VI. 3.). Briefly, when origins re-fire multiple times the replication bubbles within existing bubbles are formed and the replication forks chase one another along the DNA. The rear forks are likely to be faster than the front forks as newly synthesised DNA behind replication forks is in an immature form (Davidson et al., 2006). When both rear forks catch up with the front forks causing a head-to-tail fork collision and double stranded DNA fragments would be released. It would be very interesting to know whether this sort of collision can occur in human cells as it would be tempting to assume that gene amplifications such as seen in many human tumours could be the attempt of a cell to resolve structures caused by re-replication induced DNA fragments.

Deregulation of MCM2-7 and Cdc6 causes re-replication on a single budding yeast origin leading to gene amplification. The re-replication induced gene

amplifications are in head-to-tail orientation and are mediated by nonallelic homologous recombination between repetitive elements (Green et al., 2010). Re-replication generates slowed or stalled forks and DNA damage (Green and Li, 2005) and the homologous recombination most likely serves as repair mechanism to resolve the DNA structure cause by fork stalling and breakage within the re-replication bubble (Green et al., 2010). As a consequence the re-replicated segment appears in head-to-tail orientation adjacent to the parental locus. Head-to-tail gene amplifications are a common feature of many tumours and suggest that re-replication could be an early step in malignant transformation.

I. 8. Cell Cycle Checkpoints- Responding to environmental insults

The maintenance of genomic stability is the basis of cellular survival and environmental insults can lead to cell cycle arrest, cellular senescence, cell death and cancer cell transformation. The integrity of our genome is constantly challenged by exogenous and endogenous stress such as ionising radiation, ultraviolet radiation, carcinogenic agents, as well as cellular stress caused by replication errors and toxic by-products of cellular metabolism. DNA damage is a common event; therefore organisms have evolved several cell cycle checkpoint pathways, which prevent cell cycle progression upon detection of DNA damage or factors not favouring proliferation. The first cell cycle checkpoints exist between G1 and S-phase, sensing environmental conditions that would not favour progression into S-phase including transforming growth factor beta (TGF-β), differentiation factors, growth factor withdrawal, senescence or DNA damage. Progression into S-phase is ultimately blocked by inhibition of cyclin D/ Cdk4/6 and cyclin E/Cdk2 activity.

Another checkpoint in G1 phase of the cell cycle is the licensing checkpoint responding to a reduced number of licensed origins in primary cells. Consequently cells are delayed in G1 phase and cannot enter S-phase, thus lingering at a cell cycle stage where further licensing can still take place (Blow and Gillespie, 2008b). However,

cancer cells do not respond with activation of the licensing checkpoint and progress into S-phase with to few origins, leading to defects in replication and ultimately to cell death (Shreeram et al., 2002).

Once cells are in S-phase, various sensory proteins can detect DNA damage thus triggering the activation of the intra-S-phase checkpoint. Subsequently S-phase progression is blocked by inhibition of origin firing, chromatin remodelling, transcription of replication factors and stimulation of ribonucleotide reductase activity (Labib and De Piccoli, 2011). Furthermore the intra-S-phase checkpoint stabilises replication forks to prevent irreversible fork collapse allowing time to resolve the damage. The G2/M checkpoint guarantees that cells do not progress into mitosis with DNA damage or un-replicated DNA mainly by inhibition of cyclin B/Cdk1 kinase activity via inactivation of Cdc25 phosphatase and activation of Wee1 kinase as well as activation of p53. The final checkpoint in the cell cycle is the mitotic spindle checkpoint that engages when the spindle is not properly attached to the kinetochores or when chromosomes are not correctly aligned at the metaphase plate and prevents onset of anaphase by inhibiting the APC. Spindle checkpoint defects can cause chromosome missegregation and aneuploidy and can be directly linked to cancer formation and genetic disabilities such as Trisomy 21. The focus of this Thesis is the process of DNA replication, therefore the next section will provide details about the Intra-S-phase and the DNA damage checkpoint response, its sensors, transducers and effectors and the consequences of its abrogation.

I. 9. The DNA Damage and S- phase Checkpoints

As described above, cells monitor the state of their DNA throughout the entire cell cycle to minimise the danger of passing damaged genetic material to daughter cells. The DNA damage pathways are linked to DNA repair and apoptotic pathways and can promote the resolution of damage and the subsequent re-entry into the cell cycle or in the event of irreversible damage can trigger apoptosis. One could say two main pathways; the DNA damage checkpoint and the S-phase checkpoint control the

maintenance of DNA. The DNA damage checkpoint recognises and responds to defective DNA and the S-phase checkpoint monitors the fidelity of copying DNA, eventually activating the DNA damage response.

I. 9. 1. The S-phase or replication checkpoint

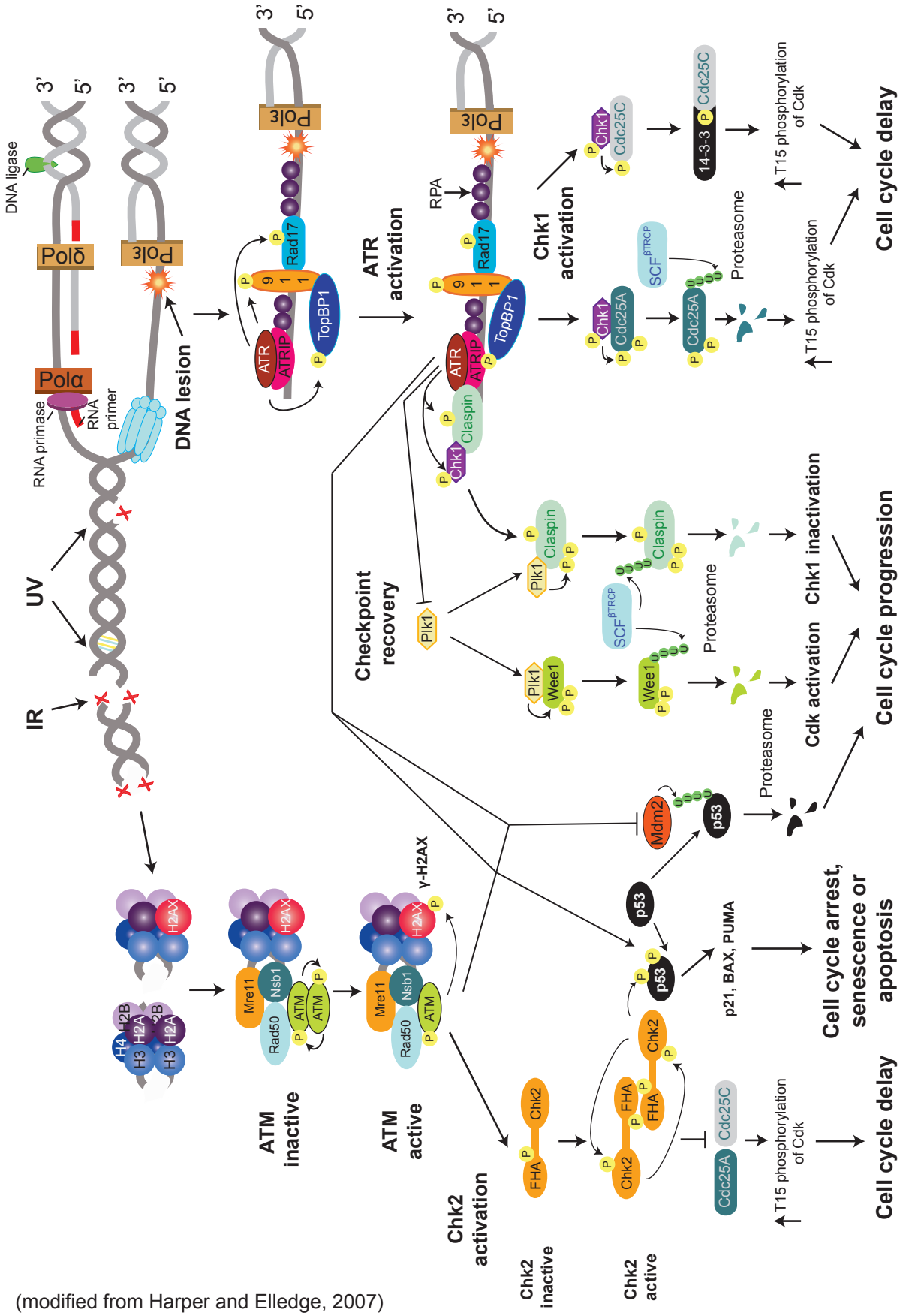
The existence of a S-phase checkpoint became apparent when cells treated with ionizing radiation rapidly blocked DNA synthesis. The response was too quick to be caused by a failure in the G1/S-phase transition and could only be the result of immediate inhibition of origin initiation in regions where replication forks had not yet been established (Larner et al., 1994). This response is defective in cells lacking ATM kinase (Labib and De Piccoli, 2011). Interestingly, studies in yeast revealed that Rad53, the yeast homolog of the ATM downstream kinase Chk2, delays late origin firing when DNA synthesis is defective at early forks after treatment with the ribonucleotide reductase (RNR) inhibitor hydroxyurea (Santocanale and Diffley, 1998; Shirahige et al., 1998). Recent work showed that Chk2/Rad53 down regulates the activity of the replication promoting kinase Cdc7 by phosphorylation of its subunit Dbf4 (Duch et al., 2011; Weinreich and Stillman, 1999b) as well as by an inhibitory phosphorylation of synthetic lethal mutations with *dpb11–1* 3 (Sld3), a replication regulator that recruits the essential fork protein Cdc45 (Lopez-Mosqueda et al., 2010; Zegerman and Diffley, 2010). In higher eukaryotes such as *Xenopus* or human cells Cdc7 exists in two complexes as Cdc7/Dbf4 and Cdc7/Drf1 and induction of replication stress by treatment with HU or etoposide does not inhibit Cdc7 activity in a variety of human cancer cell lines (Tenca et al., 2007). Consistently, in *Xenopus* cell free extract total Cdc7 activity is not altered in the presence of DNA double strand breaks or upon treatment with aphidicolin (Petersen et al., 2006; Yanow et al., 2003). It is currently unclear what function Cdc7 activity has in response to replication stress and why it is differentially regulated in yeast and higher eukaryotes.

The main function of the intra- S-phase checkpoint is to activate ATM/ ATR and there downstream kinases Chk2 and Chk1 to delay S-phase, activate DNA repair,

prevent late origins from firing, and stabilise the replisome and the stalled forks. Each of those functions helps to eventually overcome the replication stress and resume replication of the complete genome. Whereas Chk2/ Rad53 seems to be of major importance in the yeast S-phase response it is not clear whether the same pathways are active in human cells as Chk1 kinase and not Chk2 kinase has been shown to prevent activation of additional replication clusters but allowing activation of dormant origins within active clusters to complete replication of that region (Ge and Blow, 2010; Karnani and Dutta, 2011). Karnani and co-workers provided evidence that the intra- S-phase checkpoint inhibits origin firing after the loading of MCM10 but before recruitment of Cdc45 and And-1 and suggested the loading could be inhibited by a local reduction of Cdk2 activity or the increased H3 lysin 4 trimethylation (Karnani and Dutta, 2011).

S-phase checkpoint activation is a consequence of slowed replication fork progression. This can either be a consequence of deoxyribonucleotide triphosphate (dNTP) depletion, inhibition of DNA polymerases, other replication fork proteins or aberrant DNA structures such as nicks, crosslinks, or single and double strand breaks. S-phase checkpoint activation results in stabilisation of replication forks to prevent irreversible fork collapse and in the event of encountering aberrant DNA structures activation of the DNA damage response. The DNA damage response can be divided into the double strand break (DSB) response activated by irradiation, DSB inducing agents, or forks encountering certain repair or recombination intermediates and into the single-strand DNA response. Both pathways are explained in more detail in the next section and are illustrated in Figure I. 8.

Figure I. 8.



(modified from Harper and Elledge, 2007)

Figure I. 8. The DNA damage response. A schematic of the DNA damage pathways discussed in Section I. 9. is shown, focusing on the ATM mediated double strand break response and the ATR mediated single strand break or replication stress response. The red X's represent DNA strand breaks and the blue and yellow lines DNA crosslinks. The yellow circles containing 'P' represent phosphate, while green filled circles containing 'U' represent ubiquitin. For detailed desciption see text.

I. 9. 2. The ATM kinase activity – Double strand break response

The ataxia-telangiectasia-mutated (ATM) and ataxia-telangiectasia and Rad3 related (ATR) kinases are members of the phosphatidylinositol 3-kinase (PI-3) superfamily and phosphorylate their targets on serine or threonine residues. The ATM kinase (Tel1 in budding and fission yeast) is a 3056 amino acid protein that predominantly exists as an inactive dimer that auto-phosphorylates and dissociates turning into an active monomeric complex when recruited to DNA double strand breaks (Bakkenist and Kastan, 2003) (Figure I. 8). Lee and Paull demonstrated in 2005 that DSBs are sensed by the Mre11-Rad50-Nijmegen Breakage syndrome protein (Nbs) (MRN) complex, which then recruits ATM to the broken DNA molecule (Lee and Paull, 2005). One of the first and most important targets of ATM at the site of damage is the phosphorylation of the adjacent histone variant H_2AX (Ser139) which can also be phosphorylated by ATR and DNA protein kinase (PK) (Rogakou et al., 1998). The phosphorylated H_2AX recruits mediator of DNA-damage checkpoint 1 (Mdc1), which acts to amplify H_2AX phosphorylation possibly by inhibiting its de-phosphorylation and recruitment of more MRN and ATM complexes (Stucki and Jackson, 2006). Thus the H_2AX phosphorylation spreads along chromatin resulting in DNA damage foci formation and the recruitment of many DNA damage response proteins facilitating an efficient activation of DNA repair and cell cycle checkpoints.

Another important target of the ATM kinase is its downstream kinase checkpoint kinase 2 (Chk2) (Rad53 in budding yeast). Chk2 is constitutively expressed throughout the cell cycle (Lukas et al., 2001) and is mainly inactive in absence of DNA damage. ATM phosphorylates Chk2 at Threonine 68 (Ahn et al., 2000; Melchionna et al., 2000) leading to homo-dimerisation via its forkhead-associated (FHA) domains and auto-phosphorylation of its kinase activation loop (Ahn et al., 2004). Targets thought to be exclusive for Chk2 kinase are breast cancer 1 (BRCA1) at Serine 988, E2F transcription factor 1 (E2F1), promyelocytic leukemia (Pml) and polo-like kinase 3 (Plk3) proteins that are involved in DNA repair, damage induced transcription, apoptosis and cell cycle arrest (Bartek and Lukas, 2003). Other downstream targets of

Chk2 are tumour suppressor p53 at Serine 20 and proto-oncogenes Cdc25A/C but since those targets are shared with the ATR downstream kinase Chk1 more details will be presented in the next section. It should also be stated that p53 is directly activated by ATM/ATR mediated phosphorylation of Serine 15 which inhibits the binding of the p53 repressor transformed mouse 3T3 cell double minute 2 (Mdm2) (Figure I.8).

I. 9. 3. The ATR kinase activity – Single strand DNA response

ATM kinase is activated in response to DSB whereas the second major DNA damage signalling pathway is activated by the related ATR kinase that responds to single stranded DNA after UV radiation or replication stress. ATR (Rad3 in fission yeast and Mec1 in budding yeast) is a 2644 amino acid protein whose depletion causes cessation of proliferation and cell death even in absence of exogenous genotoxic agents (Brown and Baltimore, 2003; Cortez et al., 2001). The importance of the ATR signalling cascade is underlined by the fact that ATR and downstream kinase Chk1 knockout mice are embryonic lethal (Brown and Baltimore, 2000; de Klein et al., 2000; Liu et al., 2000; Takai et al., 2000).

Single stranded DNA coated by the single-strand binding protein replication protein A (RPA) is the primary signal or a boost for the ATR checkpoint responds. When replication forks encounter small lesions or nicks they stall, while the MCM replicative helicase continues to unwind the DNA, a process essential for downstream signalling as it leads to the generation of single stranded (ss) DNA (Figure I. 8) (Byun et al., 2005). Single stranded DNA is also generated when synthesis of lagging and leading strand become uncoupled (Sogo et al., 2002). When DNA-protein intermediates or DNA crosslinks generate DNA double strand breaks a crosstalk between ATM and ATR signalling can be mediated by collaboration of Mre11 with exonuclease 1 thus leading to generation of long ssDNA tails (Nakada et al., 2004). The ssDNA is recognised and bound by RPA which in turn recruits the regulatory subunit of ATR, the ATRIP (Zou and Elledge, 2003). The RPA-ssDNA complexes also facilitate the loading of clamp loader Rad17 onto chromatin, which subsequently loads

the PCNA like protein complex Rad9-Rad1-Hus1 (9-1-1) onto DNA (Zou et al., 2003). Rad17 and Rad9 are phosphorylated by ATR while the loading of Rad17 and 9-1-1 is largely ATR independent. Interestingly 9-1-1 has been shown to stimulate ATR activity in yeast (Majka et al., 2006), indicating that Rad17 and 9-1-1 loading onto DNA are early events in ATR signalling. Moreover phosphorylation of Rad17 and 9-1-1 by ATR are important for ATR stimulation and downstream signalling.

Another important player in the ATR signalling pathway is topoisomerase (DNA) II binding protein 1 (TopBP1) (Cut5 in fission yeast and Dpb11 in budding yeast) a protein essential for replication initiation and the DNA damage response (Garcia et al., 2005). TopBP1 binds to the phosphorylated 9-1-1 complex (Delacroix et al., 2007; Lee et al., 2007b) on chromatin and interacts with ATRIP to stimulate ATR kinase activity (Kumagai et al., 2006).

The major kinase downstream of activated ATR is Chk1 whose expression in largely restricted to S-phase and G2 (Lukas et al., 2001). Chk1 activation is dependent on the presence of phosphorylated Claspin (Mrc1 in yeast)(Kumagai and Dunphy, 2000) a replication fork protein that travels with the replication fork. In the presence of phosphorylated Claspin the ATR kinase activates Chk1 via phosphorylation of Serine 317 or 345. The Chk1 signalling cascade can be inactivated via Plk1 mediated phosphorylation of Claspin and its subsequent ubiquitination by $SCF^{\beta TRCP}$ leading to its degradation (Mamely et al., 2006; Peschiaroli et al., 2006). Most Chk1 signalling targets are shared with the Chk2 kinase and therefore will be highlighted in the next section.

I. 9. 4. Chk1/ Chk2 signalling - Common targets and cellular consequences

The main targets of Chk1/Chk2 kinases are p53 and Cdc25A and Cdc25C. The Chk1 but mainly Chk2 mediated phosphorylation of p53 (Ser20) stabilises the protein and activates the transcription of Cdk inhibitor p21, apoptosis promoting factor Bcl2-associated X protein (BAX), regulatory signalling protein tyrosine 3-

monooxygenase/tryptophan 5-monooxygenase activation protein (14-3-3) and many more. The p53 mediated expression of p21 has a strong inhibitory effect on cell cycle progression in most cell cycle phases; it inhibits progression into S-phase by blocking Cdk2/ cyclin E activity, prevents origin initiation in S-phase via inhibition of Cdk2/ cyclin A and supresses entry into mitosis by inhibition of Cdk1/ cyclin B.

Similar effects on cell cycle progression can be observed by the Chk1 and Chk2 mediated inhibitory phosphorylation of the phosphatase Cdc25, which promotes cell cycle progression by removing the inhibitory phosphorylation on Cdk1/2 (Thr15). Chk1 and Chk2 kinase both rapidly inhibit Cdc25C function by phosphorylation of Serine 216 (Matsuoka et al., 1998; Peng et al., 1997; Sanchez et al., 1997) thereby creating a binding site for 14-3-3 which reduces Cdc25C activity and mediates it nuclear exclusion (Nyberg et al., 2002). The consequence of Cdc25C inactivation is a G2/M delay as Cdk1/ cyclin B cannot promote mitotic entry. Both kinases but mainly Chk2 also mediate the rapid phosphorylation of Cdc25A, thereby priming it for ubiquitination and degradation (Mailand et al., 2000). Loss of Cdc25A prevents S-phase entry by inhibition of cyclin E association with phosphorylated Cdk2 (Thr15). Chk1 has also been shown to play a major role in the intra S-phase checkpoint responds as it inhibits activation of replication factories during replication stress but allows firing of dormant origin within active clusters to ensure completed replication after fork stalling or collapse (Ge and Blow, 2010). This is consistent with data from Thomson and colleagues (2010) revealing that lowering Cdk activity inhibits new factory activation but does not affect number of forks within a factory. Cdk's could have specialized substrates within a factory that need to be phosphorylated to activate it and others that need to be phosphorylated to initiate origins. Alternatively, high Cdk activity could be needed to initiate the first origin within a factory, which causes a change propagating throughout the factory allowing the remaining origin to fire with low levels of Cdk. In the study of Ge and co-workers (2010) a decrease in total Cdk activity in response to replication stress could not be detected, therefore Chk1 most likely inhibits specific Cdk substrates required for factory activation or origin initiation (Ge and Blow, 2010).

I. 9. 5. DNA damage response defects and cancer

This section serves to briefly underline the pathological consequences of a defective DNA damage response due to the mutation or absence of the key players. The absence of ATM for instance leads to the disease ataxia telangiectasia (AT) which is characterised at a cellular level by gross chromosomal rearrangements and increased sensitivity to irradiation and on an organismal level by immune deficiency, cerebellar degeneration and increased predisposition to cancer (Lavin and Shiloh, 1997; Savitsky et al., 1995). Other diseases exhibiting the same symptoms as AT also result from defects in the double strand break response. Examples are the Nijmegan breakage syndrome and the "AT-like disorder" caused by mutations in the MRN complex genes *NBS1 and MRE11* (Carney et al., 1998; Stewart et al., 1999; Varon et al., 1998). Further downstream in the cascade is the tumour suppressor p53 whose gene TP53 is mutated or deleted in about 50% of human tumours. People inheriting only one functional copy of TP53 exhibit a high predisposition to tumours in early adulthood, a disease known as Li-Fraumeni syndrome (Malkin et al., 1990). It is currently unclear whether the cancer predisposition is a consequence of defects in p53 mediated cell cycle arrest or its pro-apoptotic function but it is likely to be both.

A serious medical condition is the hereditary breast-ovarian cancer syndrome, which is a result of gene mutations in the DNA repair protein BRCA1 and accounts for 50% of all inherited cases of breast and ovarian cancer.

Chk1 mutations are extremely rare and have only been observed in carcinomas of the colon, stomach and endometrium, while Chk2 qualifies as tumour suppressor and mutations have been found in carcinomas of the breast, colon, lung, bladder, ovary, and vulva as well as in sarcomas lymphomas and patients with Li-Fraumeni syndrome (Bartek and Lukas, 2003). This section described mutations in the DNA damage response pathways found in cancer but it should be stated that according to the Weinberg model six capabilities have to be acquired by a cell to cause cancer; self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion/metastasis

(Hanahan and Weinberg, 2000). The mutations described can only account for one or two of the first three points and therefore cannot solely be made responsible for the tumour formation.

One central capability in tumour formation is the ability to escape apoptosis as irreversible genomic insults such as deregulated DNA synthesis or irreversible DNA damage should always result in cell death. Deregulation of the licensing machinery has been shown to induce DNA damage checkpoints in humans, *Xenopus*, *Drosophila melanogaster* and *S. cerevisiae* and in human cells has been associated with re-replication induced apoptosis (Archambault et al., 2005; Green and Li, 2005; Klotz-Noack et al., 2012; Li and Blow, 2005; Melixetian et al., 2004; Mihaylov et al., 2002; Vaziri et al., 2003; Zhu et al., 2004; Zhu and DePamphilis, 2009; Zhu and Dutta, 2006). The present work aims to investigate consequences of re-replication in human tissue culture cells and should therefore provide insight into the process of programmed cell death.

I. 10. APOPTOSIS

Our understanding of the process of programmed cell death was initially inspired by J. F. R. Kerr and colleagues providing an electron microscopic tissue study illustrating the morphological changes cells undergo during the course of apoptosis. (Kerr et al., 1972). The initial changes start with cell shrinkage, chromatin condensation and loss of contact to neighbouring cells. Later in the process membrane blebbing and nuclear fragmentation occurs and the cell dis-aggregates into apoptotic bodies, which are phagocytosed by macrophages. They also suggested the term apoptosis derived from the Greek ‘falling off”. Investigations of the nematode *C. elegans* whose organism is composed of 1090 somatic cells of which 131 undergo apoptosis at particular and fixed time points during development provided further insight into the genetic regulation of programmed cell death and were awarded with a Nobel Prize in 2002 for Sydney Brenner, H. Robert Horvitz and John E. Sulston.

Apoptosis is induced either via the intrinsic or mitochondrial pathway due to irreversible DNA damage, acute cellular stress by growth factor withdrawal or hypoxia or it is induced via the extrinsic or death receptor pathway where cytokines such as TNF-related apoptosis inducing ligand (TRAIL), tumour necrosis factor alpha (TNFα) or FAS ligand (FASL) are release by cytotoxic T-cells, natural killer cells or macrophages to kill cells (e.g. after virus infection). When the ligands bind to the appropriate receptor (FasL-FAS receptor (FASR) and TNFα-TNF receptor 1 ((TNFR1)) a recruitment of adapter proteins (FASL-FASR-Fas associated via death domain (FADD) and TNFα-TNFR1- TNFR superfamily 1A associated via death domain (TRADD)-FADD) is initiated, forming the death-inducing signalling complex (DISC). DISC triggers the transformation of procaspase-8 into active Caspase-8, which can activate Caspase-3 to execute apoptosis.

Caspase-3 is also activated via the intrinsic pathway that is more relevant to the present work as it is induced by DNA damage. Increase in p53 results in the expression of the pro-apoptotic protein Bax, Bax activating protein p53 upregulated modulator of apoptosis protein (PUMA) and Noxa, a protein that interacts with anti-apoptotic B-cell leukemia/lymphoma 2 (Bcl-2) family members resulting in Caspase-9 activation (Oda et al., 2000). When Bax forms a homodimer in the mitochondrial membrane, cytochrome C is released and associates with apoptotic peptidase activating factor 1 (Apaf1) and procaspase-9 forming the ‘apoptosome’ that mediates the activation of Caspase-3.

Execution of apoptosis is then triggered by the Caspase-3 induced cleavage of the heterodimer inhibitor of caspase-activated DNase (ICAD)/CAC releasing the endonuclease caspase-activated DNase (CAD) (Sakahira et al., 1998), which facilitates the fragmentation and degradation of genomic DNA. Caspase-3 activation also leads to cleavage of DNA repair protein PARP possibly to prevent depletion of NAD and ATP from cells.

Apoptosis is an essential process in any organism to remove damaged cells and the capability to bypass cell death is one step towards oncogenic transformation.

I. 11. Aims of this Thesis

Gene amplification and chromosomal DNA rearrangements have been observed in a variety of cancer cells (Lengauer et al., 1998). Certain cancers have high frequencies of specific gene amplifications, such as human epidermal growth factor receptor 2 *(HER2)/* v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 *(ERBB2)* amplification in breast cancer, v-myc myelocytomatosis viral related oncogene, neuroblastoma derived *gene (MYCN)* amplification in neuroblastomas and epidermal growth factor receptor *(EGFR)* amplification in gliomas (Hanby, 2005; Pession and Tonelli, 2005; Vogt et al., 2004). It is not fully understood how these changes arise, but it is likely that DNA strand breaks and DNA replication defects are initiating events (Albertson, 2006; Myllykangas and Knuutila, 2006).

De-regulation of the replication licensing machinery promotes partial re-replication of the genome (Blow and Dutta, 2005), resulting in the production of aberrant DNA structures (Davidson et al., 2006; Green et al., 2006; Tanny et al., 2006). However, the long-term genetic consequences of partial chromosome re-replication are unknown.

The aim of this thesis is to examine this in detail. We have determined the physiological and genomic long term consequences of human tissue culture cells that suffered partial DNA re-replication. We have deregulated the replication licensing system by geminin depletion or overexpression of Cdt1 and have investigated whether this leads to gene amplification or gross chromosome re-arrangement.

These findings have provided information about whether partial re-replication is a plausible initiating factor for gene amplification and rearrangement in human cancers.

Chapter II

Materials and Methods

II. 1. CELL CULTURE, SYNCHRONISATION AND DRUG TREATMENT

U2OS (ATCC, Cat. No. HTB-96, Lot. 7658494) and HeLa cells (kindly provided by Prof. Swedlow at passage 5) were grown in DMEM (Invitrogen, Cat No. 12491-023) and supplemented with 10% FBS (Invitrogen) and 100 U/ml Penicillin and 100 µg/ml Streptomycin (Invitrogen, Cat. No. 15070-063) at 37°C and 5% CO_2. For the cell cycle synchronisation at early S-phase, a double Thymidine block was carried out. The cells were treated with 2.5 mM Thymidine (Sigma, Cat. No. T1895) for 14-16 h, then washed twice with 8 ml 1x PBS, released for 10-12 h (transfections at 10 h post release from 1st Thymidine block) and incubated again with 2.5 mM Thymidine for 14-16 h.

For inhibition of ATR, ATM and DNA-PK, Caffeine (Calbiochem, Cat. No. 205548) was freshly prepared at a 100 mM stock solution in H_2O and cells were treated with 5 mM Caffeine for the indicated time. The ATM kinase inhibitor KU55933 (Tocris Bioscience, Cat. No. 3544) was prepared at a 10 mM stock solution in DMSO and applied to cells in a final concentration of 10 µM for the indicated time. The Chk1 inhibitor UCN-01 (Calbiochem, Cat. No. 539644) was prepared at a 0.5 mg/ml stock in DMSO and used at a final concentration of 300 nM. The Nedd8 activating enzyme inhibitor MLN4924, was provided by Dimitris Xirodimas and Philip Cohen at a 10 mM stock in DMSO and diluted as indicated.

II. 2. TRANSFECTION

II. 2. 1. RNAi transfection

Cells were transfected using Lipofectamine™ RNAiMAX (Invitrogen™, Cat.No. 13778) at least 24 h post seeding at 25-50% confluency. Forward transfection was performed according to the manufacturer's instructions with slight alterations. Briefly, geminin siRNA (*5' AACUUCCAGCCCUGGGGUUAU 3',* Dharmacon) and control siRNAi *(5' CGUACGCGGAAUACUUCGA 3'*, Dharmacon) in a concentration range of 2.5-25 nM and 10 nM sip53 (ON-TARGET plus SMART pool Human TP53, (Dharmacon, Cat No. L-003329-00-0005)) was added to the corresponding volume of

OPTI-MEM® I + GlutaMAX™I (Invitrogen™, Cat. No. 51985-026). Lipofectamine™ RNAiMAX was added in a volume, specified in the manufacturer's instructions and depending on the scale of the experiment, to OPTI-MEM® I. The reactions were combined, incubated for 15-30 min at RT and subsequently added to the cells in the specified volume of OPTI-MEM® I. After 4-6 h the OPTI-MEM® I was replaced by complete DMEM (if synchrony was required, 2.5 mM Thymidine was also added) and cells were grown for the indicated time.

II. 2. 2. Plasmid transfection

At 80% confluency 10 cm dishes of U2OS cells were transfected with 6 µg (pEGFP-N1) or 24 µg plasmid (Fl-Cdt1-GFP, Cdt1$^{(34-546)}$-GFP, Cdt1$^{(1-372)}$-GFP) using Lipofectamine™ 2000 (Invitrogen™, Cat. No. 11668) according to the manufacturer's instructions. Transfections were performed in OPTI-MEM® I for 6 h then medium was changed to DMEM and cells were grown for the indicated time.

II. 3. CDT1 CONSTRUCT DESIGN

The IMAGE clone of human Cdt1 cDNA (Accession No. BC009410) was obtained in a pOBT7 vector from www.geneservice.co.uk. Initially the cDNA was re-cloned into the EcoRI/ XhoI site of pcDNA3 (Figure IV. 1). Full-length Cdt1 (amino acids 1-546), Cdt1$^{34-546}$ (amino acids 34-546) and Cdt1$^{1-372}$ (amino acids 1 - 372) were PCR amplified from pcDNA3-Cdt1 and cloned into the EcoRI/ KpnI site of pEGFP-N1 (Figure IV. 2). The primers are listed in Table II. 1. All restriction enzymes and T4 ligase with their recommended buffers were obtained from New England BioLabs®. PCR purification and gel extraction was performed using QIAquick® PCR Purification Kit (QIAGEN®, Cat. No. 28104) and QIAquick Gel Extraction Kit (QIAGEN®, Cat. No. 28704) according to the manufacturer's instructions. One Shot® TOP10 chemically competent *E.coli* cells (Invitrogen™, Cat. No. C4040-03) were used for plasmid transformations (according to the manufacturer's protocol). DNA was prepared using

QIAprep® Spin Miniprep Kit (QIAGEN®, Cat. No. 27106) or QIAGEN® Plasmid Maxi Kit (QIAGEN®, Cat. No. 12163).

Table II. 1. Cdt1 primer

Primer Name	Sequence
Full length-Cdt1_fw	5' TCCGAATTCATGGAGCAGCGCCGCGTCACC 3'
Full length- Cdt1_rv	5' CCCGGTACCGCTCCCAGCCCCTCCTCAGC 3'
$Cdt1^{34-546}$_fw:	5' AGCGAATTCATGCCCGCACTCCGCGCCCCG 3'
$Cdt1^{1-372}$_rv:	5' ATCGGTACCGAAATCAGGTTGCGGGCC 3'

(All oligonucleotides were made by Operon)

To verify correct positioning and sequence of the Cdt1 constructs the DNA was sequenced by the University of Dundee DNA sequencing service.

II. 4. PROLIFERATION AND CLONOGENIC ASSAY

To determine the cell growth, the control and geminin depleted cells were released from the double Thymidine block, trypsinised and equal numbers of cells were re-plated. For the proliferation assay, cells were harvested at 24, 48, 72, 96 and 120 h post release and counted using the automated cell counter Countess® (Invitrogen, Cat. No. C10227).

For the clonogenic assay 500 cells were plated in triplicate into 6 well dishes. After 10 days the colonies were washed twice with 1x PSB, fixed in ice-cold 70% ethanol for 20 min at -20°C and stained with crystal violet solution (0.5% crystal violet w/v, 25% methanol) for at least 2 h. After thorough washing and drying the colonies appearing as violet dots on the plate were counted by eye.

II. 5. WHOLE CELL LYSATES, CHROMATIN FRACTIONATION, IMMUNOBLOTTING AND ANTIBODIES

II. 5. 1. Whole cell lysates

Cells were trypsinised, washed twice in 1x PBS, snap frozen in liquid nitrogen and stored at -80°C before preparing whole cell lysates. The pellets were resuspended in 50-200 µl radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 7.4; 150 mM NaCl; 1% NP-40 (IGEpal CA-630); 1 mM EDTA; 0.5% Na-deoxychelate) containing freshly added protease inhibitors (1 mM PMSF; 0.1 mM NaOVan; 0.1 mM NaF and 1 µg/ml leupeptin, aprotinin and pepstatin) and incubated on ice for 10 min. An additional lysis was carried out by sonication (Diagenode Bioruptor®, Cat. No. UCD-200 TM) twice for 10 min in intervals of 30 s (310 W). For clearing the lysates the samples were centrifuged at 14000xg for 10 min at 4°C. Protein determination was carried out using the Bio-Rad protein assay (Bio-Rad, Cat. No. 500-0006) and protein concentration quantified using a BSA standard curve ranging from 0-1 mg/ml. Equal protein amount and equal volume were loaded unless otherwise stated.

II. 5. 2. Chromatin fractionation

For chromatin fractionation the cells were trypsinised and washed twice with 1x PBS. The cell pellets were loosened and carefully resuspended in 50-150 µl freshly prepared, ice-cold cytoskeleton (CSK) buffer (10 mM Hepes pH 7.4; 300 mM Sucrose; 100 mM NaCl, 3 mM $MgCl_2$; 0.5% Triton-X-100; 1 mM PMSF, 0.1 mM NaOVan, 1 µg/ml leupeptin, aprotinin and pepstatin). Samples were placed on ice for 15 min and subsequently centrifuged for 5 min at 5000xg. The supernatant containing the cytoplasmic fraction was collected into a new tube and snap frozen in liquid nitrogen. The pellets were resuspended and washed twice in 1 ml CSK buffer before snap freezing in liquid nitrogen. At this step chromatin pellets were either stored at -80°C or resuspended in 50- 150 µl RIPA buffer (depending on the volume of CSK buffer used for extraction) and protein concentration was measured as described for whole cell lysates in section *II. 5. 1.*

II. 5. 3. Immunoblotting and antibodies

SDS-PAGE was performed using NuPAGE® precast gels (Invitrogen™, Cat. No. NP0321, 2, 3 or WG1403) and PageRuler™ pre-stained Protein Ladders (Fermentas, Cat. No. #SM1811, #SM0671) as molecular weight standard. Samples were mixed with 6x SDS loading buffer (375 mM Tris-HCl pH 6.8, 17% w/v SDS, 28% glycerol, 0.2% bromophenol blue, 14% β-mercaptoethanol) and incubated at 95°C for 5 min prior to loading onto gels. Protein separation was performed at 170 V in 1x NuPAGE MOPS SDS running buffer (Invitrogen™, Cat. No. NP0001-02) for approximately 1¼ h. Proteins were transferred using either the Invitrogen XCell II Blot Module (Invitrogen™, Cat. No. 1301637-056) with NuPAGE Transfer Buffer (20x) (Invitrogen™, Cat. No. NP0006-1) containing 10% methanol for 2 h at 30 V or using a standard immunoblot tank with Transfer buffer 2 (200 mM glycine, 25 mM Tris-HCl, 3.5 mM SDS, 20% methanol) at 40 V overnight.

Table II. 2. Antibodies

Antibody Name	Company	Cat. No.	Dilution
Primary antibody			
Actin (ACTN05)	Neomarker	MS-1295	1:1000, 5% milk solution
Cdt1	Zoi Lygerou		1:3000, 5% milk solution
* cleaved PARP (Asp214)	Cell Signalling	♯9541	1:1000, 5% milk solution
Cyclin E (HE12)	Santa Cruz Biotechnology	sc-247	1:1000, 5% milk solution
Geminin (FL-209)	Santa Cruz Biotechnology	sc-13015	1:500, 5% BSA solution
MCM2 (BM28)	BD Transduction Lab.		1:1000, 5% BSA solution
PCNA (PC10)	Santa Cruz Biotechnology	sc-56	1:1000, 5% milk solution
* phosphor-Chk1	Cell Signaling	♯2341	1:1000, 5% BSA solution
phospho-Histone H2AX (S139)	Upstate	S139	1:500, 5% BSA solution
* phospho-Histone H3	Cell Signalling	♯9701	1:500, 5% BSA solution
* phospho-p53 (Ser15)	Cell Signalling	♯9284	1:1000, 5% BSA solution
Secondary antibody			
* anti-rabbit IgG HRP	Cell Signalling	♯7074	1:1000, 5% milk solution
anti-mouse HRP	Sigma	A5278	1:10000, 5% milk solution
anti-rabbit IgG HRP	Sigma	A0545	1:50000, 5% milk solution

* marks primary antibodies which have been used with the Cell Signalling anti-rabbit IgG HRP

To check equal loading and transfer quality, the PVDF membrane (GE Healthcare, Cat. No. RPN303F) was stained with naphthol blue black (amido black) (Sigma, Cat. No. N3393). Membranes were blocked with the selected blocking solution for 1 h at RT and then incubated with the indicated primary antibodies according to the manufacturer's instructions (Table II. 2). Subsequently they were washed three times for at least 5 min in PBS/0.2%Tween and incubated with the appropriate secondary antibody for 1 h. The membranes were washed again and proteins of interest visualized using two enhanced chemiluminescent substrates depending on the signal strength (SuperSignal West Pico substrate (for strong signals), ThermoScientific, Cat. No. 34080; SuperSignal West Femto substrate (for weak signals), ThermoScientific, Cat. No. 34096).

II. 6. FLOW CYTOMETRY

II. 6. 1. Propidium iodide cell cycle

For propidium iodide (PI) cell cycle analysis, cells were trypsinised, washed in 1x PBS, resuspended in 70% ice-cold ethanol and incubated at -20°C for at least 30 min. Afterwards cells were washed twice in Flow cytometry wash buffer (FC_WB) (1x PBS containing 1% BSA and 0.2% TX-100) followed by an incubation in 0.25-1.0 ml PI staining solution (50 μg/ml Propidium iodide, 50 μg/ml RNase, 0.1 % Triton-X-100 made up in PBS) for 15-30 min at RT. The DNA content of each cell was measured by the propidium iodide staining using the Flow Cytometer FACS Calibur™ (Becton Dickinson) running with the CellQuest data acquisition software. Analysis was carried out using the FlowJo 8.8.4 software.

II. 6. 2. 2D BrdU/ Propidium iodide

For 2D BrdU/PI flow cytometry, cells were cultured with freshly prepared 20 μM BrdU (Sigma, Cat. No. B5002) for 30 min.

To track replicating or re-replicating cells pulse-chase experiments were carried out. For the chase, the 20 μM BrdU was removed, cells washed 3 times with 1x PBS and cultured with 100 μM Thymidine for 30 min to prevent further BrdU incubation. Subsequently the Thymidine was diluted to a final concentration of 10 μM.

The cells were trypsinised, washed in 1x PBS, fixed with 70% ice-cold ethanol and stored at -20°C. Cells were washed twice in FC_WB and incubated in 2 M HCl plus 0.2% TX-100 for 30 min at RT. Afterwards the cells were washed with FC_WB and blocked in Flow cytometry blocking buffer (FC_BB) (1x PBS containing 5% BSA and 0.2% TX-100) for 1 h. The solution was replaced by FC_BB containing mouse anti-BrdU antibody (final concentration of 0.5 μg/ml) (BD Bioscience, Cat. No. 347580) and incubated for 1 h at RT. Cells were washed twice in FC_WB and incubated in FC_BB containing rabbit anti-mouse Alexa Fluor 488 $F(ab)_2$ fragments (final concentration 2 μg/ml) (Invitrogen™, Cat. No. A21204) for 1 h at RT. Finally the cells were washed twice in FC_WB, stained for DNA content and analysed as described above.

II. 6. 3. 3D GFP/ EdU/ 7AAD

GFP transfected cells were pulsed with 10 μM Click-iT® EdU for 1 h or 2 h, washed and trypsinised. The cells were fixed in 4% paraformaldehyde (PFA) for 20 min and washed with 1x PBS containing 1% BSA. For the time course experiment, cells were stored in PBS containing 1% BSA until all samples were collected. The cells were permeabilised with 1x PBS containing 1% BSA and 0.3% TX-100 for 30 min, washed twice in FC_WB and blocked in FC_BB2 (PBS, 3% BSA, 0.1% TX-100) for 1 h. The primary mouse anti-GFP antibody (diluted 1:500, Roche, Cat. No. 11814460001) was incubated in FC_BB2 for 1 h. Cells were washed twice in FC_BB2 and incubated with Alexa Fluor 488 F $(ab)_2$ fragment of rabbit anti-mouse (2 μg/ml) (Invitrogen™, Cat. No.

A21204) for 1 h. Afterwards the Click-iT EdU (Invitrogen™, Cat. No. A10202) reaction with the Alexa Fluor 647 azide was performed according to the manufacturer's instructions and then cells were stained for their DNA content with 7AAD (20 µg/ml) (Sigma, Cat. No. A9400-1MG). Analysis was done as described above.

II. 7. FLUORESCENCE ACTIVATED CELL SORTING (FACS)

To collect re-replicating (>4C) and control cells (2C), the cells were trypsinised, washed in 1x PBS and subsequently incubated in DMEM media containing 15 µg/ml Hoechst 33342 (Invitrogen, Cat. No. H1399) for 30 min at 37°C. Cells were filtered and immediately sorted according to their DNA content (performed by facility manager Dr. Rosemary Clarke) using the Enterprise argon ion laser at 345nm (UV) in the FACS Vantage (with DIVA upgrade) (Becton Dickinson). Cells were also sorted to collect GFP/ GFP-Cdt1 transfected cells using the Enterprise argon ion laser at 488nm.

II. 8. MICROSCOPY

II. 8. 1. GFP-Cdt1 localisation

Cells were plated onto poly-L-Lysine coated coverslips (Cat. No. 354085, BD Biosciences) at 50% confluency and grown for at least 24 h. At 80-90% confluency cells were transfected with the pEGFP-N1 or pEGFP-Cdt1 plasmids as described in section II. 2. 2. After 24 h the coverslips were washed in 1x PBS containing 0.1% TX-100, fixed with 4% PFA for 20 min, washed again and incubated with 1 µg/ml 4'-6-diamidino-2-phenylindole (DAPI) (Cat. No. D9542-1MG, Sigma Aldrich) for 5 min. The coverslips were transferred onto microscope slides (Cat. No. 48312, VWR) with the cells facing down into Vectashield mounting media (Cat. No. H1000, Vector Laboratories). The coverslips were fixed onto the slide with nail polish. Microscopic analysis was performed using the Nikon TE-200 DIC microscope with a Nikon 40x/1.2, S fluor, CFI/60 objective and a camera (CoolSNAP HQ2, Roper Scientific). After

deconvolution with softWoRx (Applied Precision) the images were processed with OMERO 4.3.0 (Dundee University).

II. 8. 2. BrdU replication timing pattern

Cells were plated onto poly-L-Lysine coated coverslips (BD Biosciences, Cat. No. 354085) at 30% confluency, synchronised (section II.1) and transfected with geminin RNAi as described in section II. 2. 1. The geminin depleted and control cells were released into S-phase and 50 µM BrdU was added to the cell culture plates at 3.5, 5.5, 7.5, 9.5, 13.5, 15.5, and 17.5 h post Thymidine release for 30 min to label S-phase cells. At the relevant time points 4, 6, 8, 10, 14, 16, 18 h post release the coverslips were washed and fixed with 70% ethanol for at least 20 min or stored at 4°C until all time points were collected. The ethanol was removed and the cells were washed in 1x PBS. Subsequently the DNA was denatured in 2 M HCl containing 0.2% TX-100 for 30 min and washed 3 times in immunofluorescence assay wash buffer (IFA_WB) (1x PBS containing 1%BSA and 0.2% TX-100). Afterwards cells were blocked for 1 h in immunofluorescence assay blocking buffer (IFA_BB) (1x PBS containing 5% BSA and 0.2% TX-100) followed by incubation for 1 h at RT with mouse anti-BrdU antibody (0.5 µg/ml) (BD Bioscience, Cat. No. 347580). Cells were washed twice in IFA_WB and incubated in IFA_BB containing Alexa Fluor 488 $F(ab)_2$ fragment of rabbit anti-mouse (2 µg/ ml) (Invitrogen™, Cat. No. A21204) for 1 h at RT. Finally cells were washed twice in IFA_WB and stained with DAPI 1 µg/ml (Sigma, Cat. No. D9542-1MG) for 5 min. Cells were mounted as described above. Microscopic analysis was performed using the DeltaVision DV3 Deconvolution Microscope (Applied Precision) with a 60x NA 1.42 Plan Apochromat objective (Model 1-U2B933, Olympus) and a camera (CoolSNAP HQ2, Roper Scientific). After deconvolution with softWoRx (Applied Precision) the images were processed with OMERO 4.3.0 (Dundee University). Between 37 and 60 cells per time point and sample were analysed and the replication pattern was selected based on the work of O'Keefe *et al.* (1992) according to the following characteristics. Type I, a faintly punctate labelling throughout

euchromatic regions; type II, complete diffuse labelling of euchromatic regions with lack of nucleolar labelling; type III, intense labelling of the peripheral ring, possibly with some perinucleolar labelling; type IV, mainly labelling of small-speckled heterochromatic foci within the nuclear interior or at the periphery, with some of the speckled foci forming chain-like structures; type V, predominant labelling of large internal replication foci and at the periphery of the nucleus (Thomson *et al.*, 2010)

II. 9. DNA ISOLATION

To analyse genomic alterations by microarray analysis or deep sequencing, the DNA from rereplicated (>4C) and control (2C) sorted cells was isolated using the DNeasy Blood & Tissue kit (QIAGEN®, Cat. No. 69504). The isolation was performed according to the manufacturer's protocol with exceptions at the 2nd wash, which was performed with 80% ethanol and then DNA was eluted in H_2O. The DNA quality was checked by agarose gel electrophoresis and DNA quantity was determined using the NanoDrop® Ultraviolet (UV) Spectrophotometer.

II. 10. AGAROSE GEL ELECTROPHORESIS

The sizes of PCR and ligation products as well as the quality of microarray and deep sequencing DNA samples were checked using agarose gel electrophoresis. Depending on the size of the DNA, 0.8-1% agarose gels were prepared using standard melting point agarose (Cambrex Bioscience, Cat. No. 50004) in 0.5x TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH 8.3). The visualisation was done using SYBR®-safe DNA gel stain (Invitrogen™, Cat. No. S33102) (1:10000) in molten agarose. The DNA samples were diluted in 6x DNA loading buffer (0.25% (w/v) bromophenol blue, 0.25% xylene cyanol, 40% (w/v) sucrose) and run in 0.5x TBE for 45-60 min at 90V. A 1 kb DNA ladder (0.5 µg) (Fermentas, Cat. No ＃SM1331) was used as DNA size standard. Gels were visualised using the GeneFlash bio imager (Syngene).

II. 11. HUMAN GENOME COMPARATIVE GENOMIC HYBRIDISATION (CGH) MICROARRAY

II. 11. 1. Details and Specifications

Comparative genomic hybridisation was the first tool to detect potential genomic alterations, such as amplifications, in response to re-replication induced by geminin depletion. In this Thesis two Microarray formats, Sure Print G3 Human CGH microarray containing 8 arrays on one slide and Human Genome CGH Microarray containing 4 arrays on one slide, were used (Table II. 3.) to analyse the genomic DNA of > 4 copy (C) geminin depleted cells compared to 2C control cells. I performed the Sure Print G3 8x60K microarray, while Agilent Technologies performed the 4x44K microarray.

Table II. 3. CGH microarray specifications

Features	**Specifications**	
Name	Sure Print G3 Human CGH Microarray	Human Genome CGH Microarray
Format	8x60K	4x44K
Arrays per slides	8	4
Amount of DNA per sample and per Array	250 ng	500 ng
DNA concentration	32 ng/µl	62.5 ng/µl
Agilent product number	G4450A	G4426B
Distinct biological features	55,077	42,494
Replicated biological features (X5)	1,000	301
Internal quality control features	3,886	2,118
Composition	Content sourced from- UCSC hg18 (NCBI Build 36), March 2006	
Probe Spacing	41 kb overall median probe spacing (33 kb in Refseq genes)	43 kb overall median probe spacing (24 kb in Refseq genes)

II. 11. 2. Sure Print G3 8x60K microarray - Experimental procedure

For the 8x60K microarray format 250 ng of >4C geminin depleted DNA and 250 ng of 2C DNA was used for each array (setup see in Figure V. 1). The arrays were done in biological triplicates (A, B, C) with A and B used as experimental triplicates (1,2,3) and C (1,2) as duplicate to fill the 8 arrays on the slide. Each sample (A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2) contained 250 ng sample DNA (>4C) and 250 ng control DNA (2C).

To generate short DNA fragments that improve labelling and hybridisation quality, the control and sample DNA needed was heat-fragmented for 10 min at 95°C, kept one ice for 3 min and spun at 6000xg for 30 s to collect the sample. In the mean time the Agilent ULS-labelling (Cat. No. 5190-0419) master mix was prepared according to Table II. 4.

Table II. 4. Preparation of labelling master mix

Components	Per reaction (µl)	Per 8x60K slide (µl) including excess
Nuclease-free water	0.75	7.5
ULS-Cy3 or ULS-Cy5	0.25	2.5
10x labelling solution	1	10
Final volume of labelling master mix	**2**	**20**

The ULS-Cy3 labelling mix was added to the sample DNA (>4C) while the ULS-Cy5 mix was added to the control DNA (2C). The DNA-labelling mix was incubated at 85°C for 30 min, stored on ice for 3 min and subsequently spun for 30 s at 6000xg.

Unbound ULS-Cy3 and 5 was removed using the Agilent KREApure column (Cat. No. 5190-0418). The column material was mixed, the storage buffer removed by centrifugation (1 min, 16,000xg) and the column washed with 300 µl nuclease free water. Residual water was removed by filter paper and then the column was transferred into a clean 1.5 ml tube, the labelled DNA added and spun at 16,000xg for 1 min to collect the purified labelled DNA. The labelling efficiency was determined using 1.5 µl sample on the NanoDrop ND-1000 UV Spectrophotometer. The

experiment was discarded when the labelling efficiency differed from the recommended 0.75-2.5% for Cy5 and 1.75- 3.0% for Cy3. The appropriate ULS-Cy3 sample DNA and ULS-Cy5 control DNA (total volume 17 µl) were combined. Labelled DNA was stored in the dark at 4°C overnight.

To obtain a 100x solution the lyophilised CGH blocking agent (supplied with Agilent Oligo CGH hybridisation kit, Cat. No. 5188-5220) was resuspended in 135 µl nuclease-free water and left at RT for 60 min. In the mean time the labelled DNA was concentrated to 9 µl. The hybridisation master mix was prepared as listed in Table II. 5.

Table II. 5. Preparation of hybridisation master mix

Components	Per reaction (µl)	Per 8x60K slide (µl) including excess
Human Cot-1 DNA (1.0mg/ml)	2.0	20
100x CGH blocking agent	0.5	5
2x CGH hybridisation buffer	22.5	230
Final volume of hybridisation master mix	**25**	**255**

The hybridisation mix was added to the labelled DNA, the sample mixed by pipetting up and down and spun to collect the sample. The sample was incubated at 95°C for 3 min, then immediately transferred to 37°C for 30 min and spun to remove residual liquid from the wall. The hybridisation sample mixture was supplemented with 11 µl of Agilent-CGH Block (supplied with the ULS-labelling kit), mixed and spun down.

The hybridisation chamber and gasket slides were assembled as described and illustrated in the Microarray Hybridisation Chamber User Guide (Cat. No. G2534-90001). The gasket slide was placed on the chamber base and 42 µl sample were dispensed on the gasket. The microarray was placed “active side” down onto the gasket and the chamber cover was placed onto the sandwiched slides. Chamber base and cover with the sandwiched slides were clamped and the mobility of bubbles was assessed before the slide was placed into the hybridisation oven (Cat. No. G2545A) at 65°C. The hybridisation rotor was set at 20 rpm for 40 h.

The hybridisation chamber was disassembled in Oligo aCGH Wash Buffer 1 (Cat. No. 5188- 5221) at RT. The array was carefully placed in a slide rack and immediately transferred into fresh stirring Oligo array CGH (aCGH) Wash Buffer 1 for 5 min. Subsequently the array was incubated in stirring Oligo aCGH Wash Buffer 2 (Cat. No. 5188- 5222) at 37°C for 1 min. The array was slowly removed from Oligo aCGH Wash Buffer 2 and scanned immediately using the InnoScan900AL scanner.

II. 11. 3. Sure Print G3 8x60K Microarray - Analysis

The scanned 8x60K array was further analysed using the Mapix® (4.1.0) software to save the acquired images in TIFF format thereby saving all scan and analysis parameters. Furthermore Mapix® was used to analyse the image by determining the position of each spot automatically using a grid (GAL file for 8x60K array, provided by Norman Pratt, Department of Human Genetics, Ninewells Hospital, Dundee). This GAL file contains all the information for each spot on the array eg. spot position, spacing, diameter and identification (chromosomal location, gene name if applicable, control type etc.). Mapix used the photometric results from the grid to extract the data and display the results in table format (txt.). The table contains the information from the GAL file and the photometric information such as the Cy5 and Cy3 median of a spot, the Cy5 and Cy3 median of background, the median of a spot minus background, the Cy5/Cy3 ratio etc.

In case of the 8x60K array performed first, whole genome plots of the Cy3/Cy5 ratios performed by Nexus 4 software showed very poor reproducibility between biological (A, B, C) and even technical (1, 2, 3) replicates. Therefore the Cy5 and Cy3 median minus background values were used to generate quality control plots of two technical replicates for each wavelength and their Cy3/Cy5 ratios (Figure V. 2, 3, 4). Discussion with Nick Schurch from the Data Analysis Group, University Dundee led to the idea of excluding invalid data points based on their poor Cy5/Cy5 correlation. Reproducibility between technical replicates is a basic necessity in microarray experiments, and Cy5/Cy5 ratios between technical replicates should be around one.

Values smaller 0.7 or greater 1.3 were excluded from the analysis using the IF command in Excel.

=IF(logical_test,[value_if true],[value_if_false])

=IF(AND($cy5_{replicate\ 1}/cy5_{replicate\ 2} < 1.3$, $cy5_{replicate\ 1}/cy5_{replicate\ 2} > 0.7$), $cy5_{replicate\ 1}/cy5_{replicate\ 2}$, 0)

Using this command all invalid data points were labelled with a zero and the filter tool "Does Not Equal" zero could be used to hide all invalid data points.

The remaining valid data points of the 8x60K array were sorted according to chromosome number and chromosomal location using the information provided by the GAL file.

The 8x60K microarray was normalised using the designated normalisation probes provided on the array. The probes were filtered for invalid data points as described above and the median Cy3/Cy5 ratio for normalisation probes for each array was calculated. Subsequently all valid Cy3/Cy5 data points for each array were divided by the median Cy3/Cy5 ratio of their normalisation probes.

The >4C/2C ratio of every experiment was used to generate whole genome plots with KaleidaGraph 4.0 which provided information about possible amplified regions in the genome of geminin depleted and re-replicated cells (Figure V. 6, 7, 8).

II. 11. 4. 4x44K human genome microarray

Agilent Technologies, Germany, performed the heat fragmentation, the labelling and hybridisation for the 4x44K microarray slide as described above according to their manual. For each array 500 ng geminin depleted (>4C) and 500 ng control (2C) input DNA was used. The 4x44k slide setup is illustrated in Figure V. 9. All DNA samples are from one biological experiment but used as technical quadruple. The >4C DNA was Cy3 labelled in array 1 and 2 and Cy5 labelled in array 3 and 4, while control 2C DNA was Cy5 labelled in array 1 and 2 and Cy3 labelled in array 3 and 4. This dye change

was done to obtain a better control for the labelling quality and increase the chance to receive valid data even if one colour gives bad signal.

The array was scanned using the Agilent C Scanner and the output text file generated using the Agilent Feature Extraction Software 10.7.3 providing normalised signal intensities for each wavelength. In contrast to the 8x60K microarray the reproducibility between technical replicates was very good and filtering of data points was not necessary (Figure V. 10). Therefore all data points of the 4x44K were sorted according to chromosome number and chromosomal location using the information provided by the GAL file and whole genome plots were generated with KaleidaGraph 4.0 providing information about possible amplified regions in the genome of geminin depleted and re-replicated cells (Figure V. 11, 12).

II. 12. SOLEXA SEQUENCING, DATA REDUCTION AND PROCESSING

The >4C and 2C DNA from geminin depleted and control cells was sequenced at the GenePool Next-Generation Sequencing facility in Edinburgh (http://genepool.bio.ed.ac.uk/). The six samples (3 biological replicates for each treatment) produced more than 82×10^6, 50 bp long, single-end, sequenced reads. Quality scores were >38/40 along the entire length of the sequences across all samples, and reads needed no clipping prior to alignment. The reads were aligned to the reference human genome (GRCh37/hg19) with the Bowtie short read aligner (v0.12.3). The resulting alignment was filtered for reads that were unique matches to a position in the genome, allowing for up to two mismatches in the sequence alignment. ~80% of the reads fulfilled these criteria. A further $42x10^5$ reads were excluded because they were mapped to the human mitochondrial chromosome ($37x10^5$ reads) or to the Y chromosome ($5x10^5$ reads; as the U2OS is a female cell line). Therefore a total of ~70×10^6 usable mapped reads remained covering 3.04×10^9 bases (average depth=0.02). Data from the biological replicates were combined and then initially binned into 10 kb bins, resulting typically in 10s of reads per bin.

Data binning was also increased to 100 kb bins to ensure that the random counting errors for each bin were normally distributed, before the ratio of each of the bins was taken. There were large regions of several chromosomes composed of bins with read counts per bin that were more than an order of magnitude less than the average across the genome (~10^2 reads/100kb c.f. ~10^4 reads/100kb across most of the genome). The low numbers of counts in these bins produced a significant increase in the variance of the ratios in these regions and produced values for the ratios strongly depending on the binning factor chosen. The origins of the low read counts in these bins were not clear from this data. There was no correlation for any of the classes of repeat annotated by RepeatMasker with the low count bins. Instead we hypothesised that they may be associated with mutations in the U2OS genome relative to the reference sequence.

Of the high-quality reads that mapped to the female core genome (ignoring reads mapping to the Y chromosome or mitochondria), 89.9% (+-0.2%) mapped uniquely to a single location in both the control and geminin-depleted samples. To investigate the re-replication of repetitive DNA, ratios of geminin-depleted/control ratios were examined for the non-unique mapping data. These ratios were almost identical to the ratios observed in the unique mapping data. The average difference between the ratio for non-unique reads minus the ratio for unique reads was consistent with zero (-0.0001 ± 0.0007) for the 10 kb binned-data, which corresponds to 0.13% ± 0.06% as a fraction of the ratio.

Chapter III.

Results:

Physiological consequences of geminin depletion in U2OS cancer cells

III. 1. INTRODUCTION

It has already been described in Chapter I. of this Thesis that the correct regulation of the licensing machinery is crucial to prevent re-replication of genomic DNA. In metazoans the loading of mini-chromosome maintenance 2-7 (MCM2-7) complex onto chromatin is restricted to late mitosis and G1 phase of the cell cycle. The key regulator is the Cdc10 dependent transcript 1 (Cdt1) protein and its activity is restricted to G1 and mitosis by proteolysis via at least three distinct pathways and by formation of a heterohexameric (2x (2xgeminin+1xCdt1)) inhibitory complex with a protein called geminin. Geminin is expressed from late G1 until anaphase where it is degraded or inactivated by the anaphase-promoting complex. Geminin depletion leads to premature endoreduplication and failure to form pluripotent cells during mammalian development (Gonzalez et al., 2006), centrosome over-duplication (Tachibana et al., 2005b) and re-replication of DNA in various human cell lines (Klotz-Noack et al., 2012; Melixetian et al., 2004; Zhu et al., 2004).

Interestingly, a study by Ballabeni and colleagues demonstrated that geminin is a negative and a positive regulator of pre-replication complex (pre-RC) formation in human cells. They revealed that during G2/M when Cdt1 levels have to increase for MCM2-7 complex loading geminin-bound Cdt1 is protected from degradation. Geminin depletion during mitosis leads to reduced loading of MCM2-7 proteins and defects in DNA replication (Ballabeni et al., 2004).

In this Chapter the physiological consequences of re-replication induced by geminin depletion in synchronised U2OS cancer cells were elucidated. Synchronisation was required to avoid defects in pre-RC formation and replication when geminin is depleted during mitosis. Initially the long term consequences of an acute loss of geminin were investigated in detail. Furthermore the work reveals precise information about a restricted cell cycle phase that allows re-firing of replication origins and provides insight into the role of checkpoint kinases in enhancing re-replication.

III. 2. How to deplete geminin in synchronised U2OS cancer cells?

Taking into account the data of Ballabeni and colleagues a synchronisation protocol was designed to deplete geminin in G1/ S-phase of the cell cycle. As shown in Figure III. 1, cell cycle profiles of asynchronous U2OS human osteosarcoma cells treated with 2.5 mM Thymidine for 16 h showed an enrichment of cells in early S-phase. The cells were released and profiles monitored after 10 and 12 h (data not shown). At 12 h post Thymidine release the cells were synchronised again in early S-phase with Thymidine. After releasing the cells from the second Thymidine block, this time point was designated as 'T0'. More time points were taken while cells progressed synchronously through S phase (T4, T6, T8), G2/M phase (T12, T14) into G1 phase of the subsequent cell cycle (T18).

On the basis of these cell cycle profiles the geminin RNAi transfection protocol was designed (Figure III. 2). Transfections were performed 10 h post release from first Thymidine block as most cells have finished S-phase. At this time point the geminin levels were very high and Cdt1 levels started to increase again. At 6 h post transfection, a second Thymidine block was carried out for 16 h. At this time point, geminin siRNA treated cells already showed a decrease in geminin and Cdt1 levels by immunoblotting (Figure III. 2). However, an impairment of DNA replication in the subsequent S-phase was never detected by flow cytometry. In accordance with its known cell cycle regulation Cdt1 levels were low at the time of the second Thymidine release (T0) in geminin RNAi treated cells and control cells. Geminin levels were high in cells treated with non-targeting siRNA and efficiently decreased in geminin siRNA treated cells. This protocol was established to guarantee experimental conditions in which the cells undergo S-phase and G2/M without geminin as inhibitor of origin licensing.

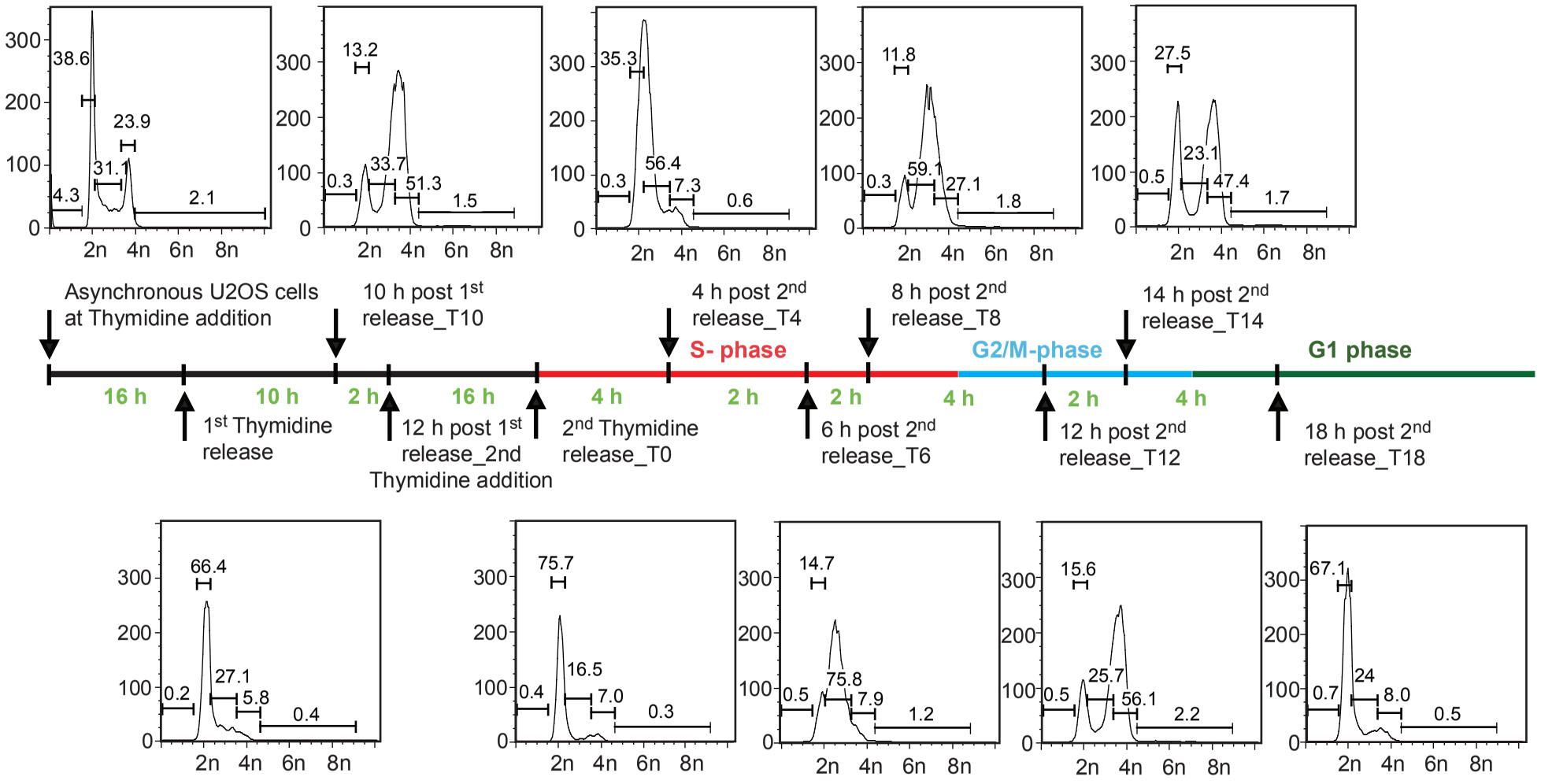


Figure III. 1. A Schematic of the U2OS synchronisation protocol and the relevant cell cycle stages. Asynchronous U2OS cells were treated with 2.5 mM Thymidine for 16 h and subsequently released for 10 h. Twelve hours post 1st release Thymidine was added again for 16 h. U2OS cells were then released from the double Thymidine block and the cell cycle progression was monitored by flow cytometry for the subsequent 18 h.

Figure III. 1.

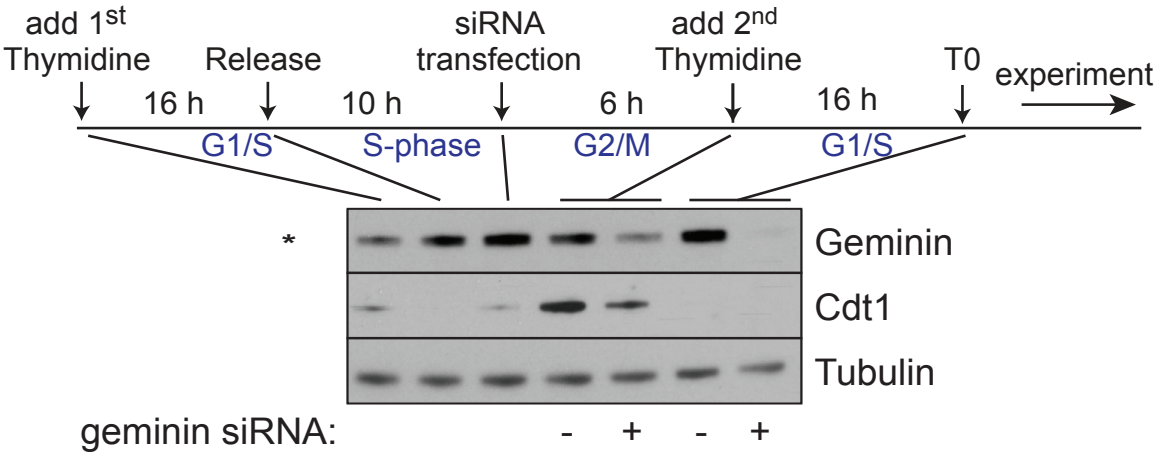


Figure III. 2. Schematic for double thymidine synchronisation and RNAi transfection and the corresponding Cdt1 and geminin levels. At the indicated time points during the synchronisation protocol, whole cell extracts were prepared and immunoblotted for geminin, Cdt1 and tubulin. Cells treated with 15 nM control siRNA (-) and cells treated with 15 nM geminin siRNA (+).

* The synchronisation protocol was established by me during the course of my PhD but this Cdt1, geminin blot was kindly provided by Debbie McIntosh as part of the JCS paper revision.

III. 3. TRANSIENT GEMININ DEPLETION IS LETHAL

III. 3. 1. Geminin depletion blocks cell proliferation

To study the long-term consequences of geminin depletion, U2OS human osteosarcoma cells were synchronized and transfected (as in Figure III. 2) with increasing amounts of geminin siRNA. The cell proliferation was severely inhibited for up to 120 h even at very low concentrations (2.5 nM) of geminin siRNA (Figure III. 3 A). This observation was strengthened by the results of clonogenic assays showing that siRNA concentrations as low as 2.5 nM geminin siRNA decreased colony numbers nearly to background levels (Figure III. 3 B, C).

III. 3. 2. Geminin depletion results in re-replication and cell death

In agreement with previous studies (Melixetian et al., 2004; Zhu et al., 2004) we observed by flow cytometry that geminin RNAi induced substantial levels of re-replication, with typically 15 - 35% of cells exhibiting a >4C DNA content 48 h after Thymidine release (Figure III. 4 A). At longer times, the number of re-replicated cells (>4C DNA content) decreased with an associated increase in cell death (<2C DNA content) (Figure III. 4 B). These results suggest that re-replication induced by a lack of geminin ultimately results in apoptosis in U2OS cells, as it has been reported in other cancer cell lines (Zhu et al., 2004; Zhu and DePamphilis, 2009).

To investigate possible molecular mechanisms leading to cell death, low concentrations of geminin RNAi (2.5 nM) were applied to the cells and re-replication as well as cell death were monitored over several days. In parallel immunoblotting of whole cell extracts was performed and revealed a significant increase of apoptosis marker cleaved Poly (ADP-ribose) polymerase (PARP) and phosphorylated p53 at Serine 15 in geminin depleted cells as early as 48 h post Thymidine release (Figure III. 5 A). The levels of re-replication increased to 12% at 96 h, but dropped again at 120 h accompanied by a substantial increase in cell death (Figure III. 5 B).

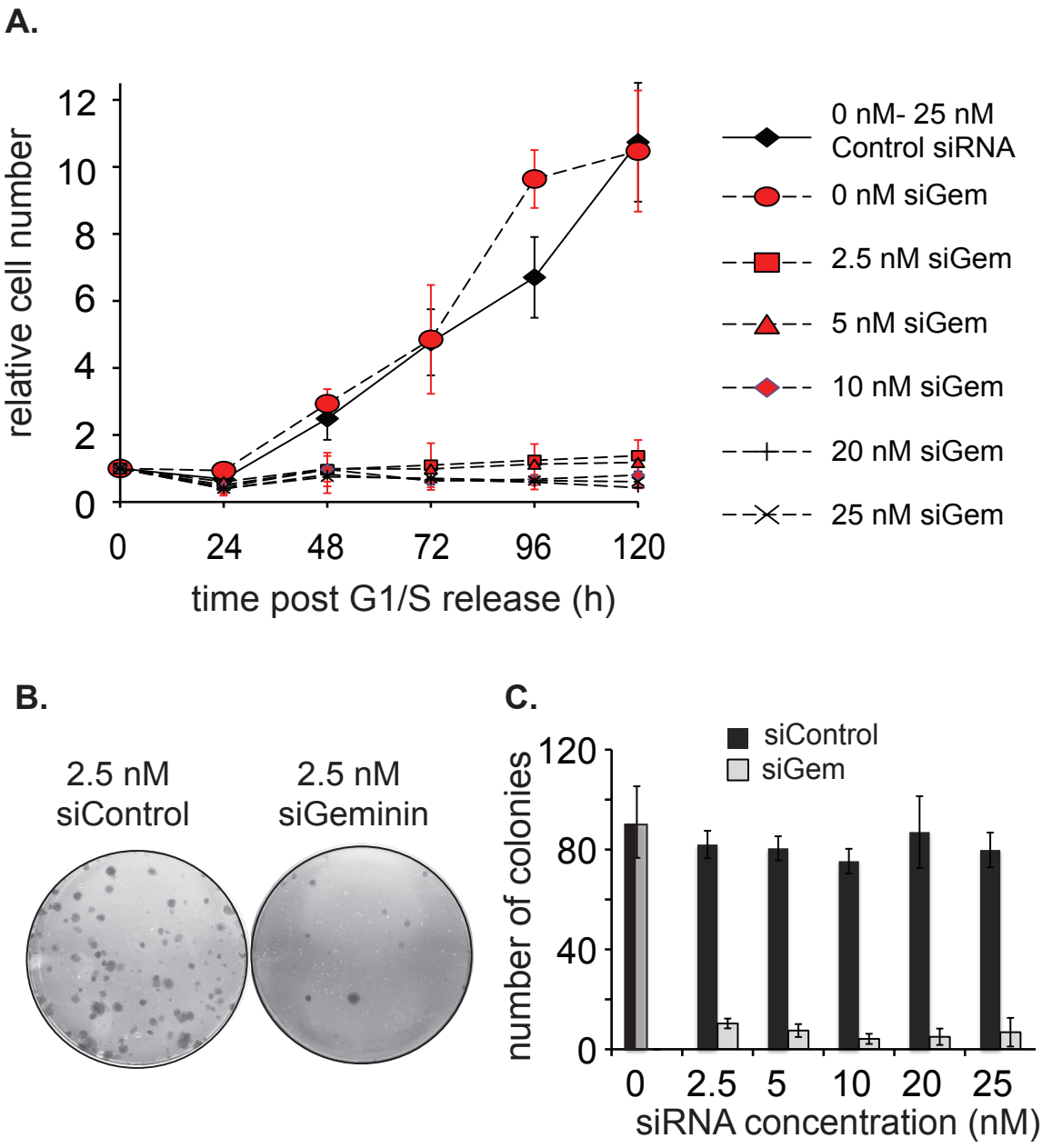


Figure III. 3. Geminin depletion causes inhibition of proliferation. Cells were released from a double thymidine block after prior treatment with either control or geminin RNAi, as in Figure III. 2. **A.** Graph shows the cell number at various times after transfection with different concentrations of control or geminin RNAi. **B.** Example well of clonogenic assays for 500 cells plated at release from double Thymidine block after treated with 2.5 nM control or geminin siRNA. **C.** Clonogenic assay of cells treated with increasing amounts of geminin or control RNAi. The graph shows the results of two biological experiments in triplicates, with standard error.

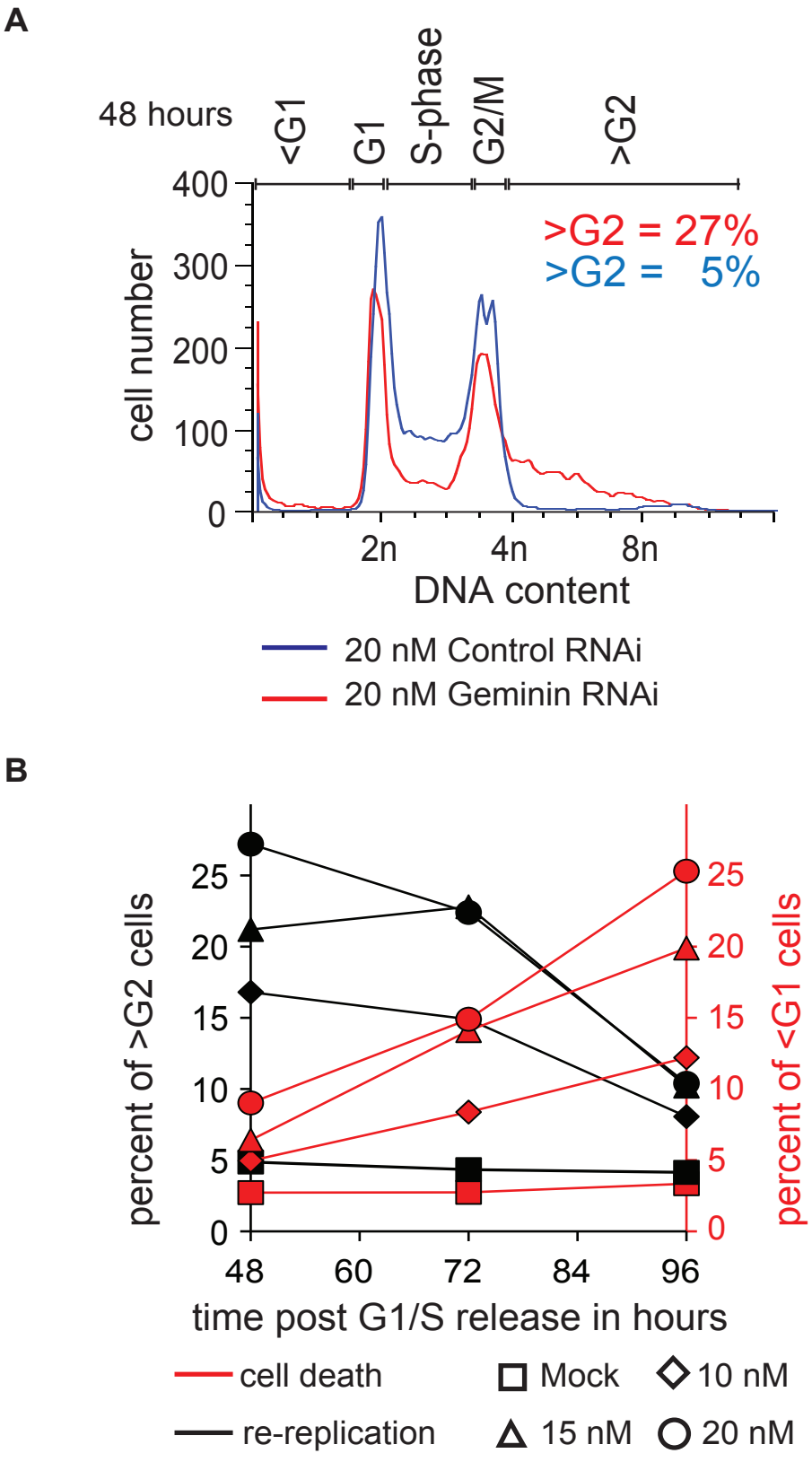


Figure III. 4. Geminin depletion causes dose dependent levels of re-replication and cell death. A. DNA content of cells treated with 20 nM geminin or control RNAi is shown at 48 h after release from double Thymidine block. **B.** Titration of geminin siRNA at different time points after release from 2nd Thymidine block; cultures were analysed by flow cytometry, and the percentage of cells with <G1 or >G2 DNA content is illustrated.

In parallel, phosphorylated H2A histone family member X (H_2AX) was detected by immunoblotting (Figure III. 5. A), which is most likely due to fragmentation of the genome during apoptosis. The presented data suggests that under these experimental conditions, cell death is an inevitable consequence of re-replication in U2OS cells.

III. 3. 3. Cells with a >4C DNA content form no colonies

One of the main objectives of this project was to study the long-term consequences of re-replication and potential underlying genomic alterations. Therefore experiments were designed to obtain cells with >4C DNA content. Geminin depleted >4C and control 4C cells were isolated by FACS 120 h post Thymidine release. This time point was chosen as previous results (Figure III. 5 B) showed that maximum cell death had taken place by that time. The sorted cells (Figure III. 6 B) were re-plated as populations of 50,000 cells in 24 well dishes. In Figure III. 6 B, the sorted population of control 4C cells attached and grew normally on the plate over a period of 168 h, while the geminin depleted >4C cells attached to a lesser extent and did not proliferate but died instead. Moreover, it should be stated that a geminin depleted single >4C cells plated individually into 96 well plates were unable to form colonies while a single 4C control cell did (data not shown). This suggests that re-replication to an extent detectable by flow cytometry, causes cell damage that ultimately leads to cell death. Therefore it was not possible to study long term consequences or genomic alterations of clones derived from cells with >4C DNA content.

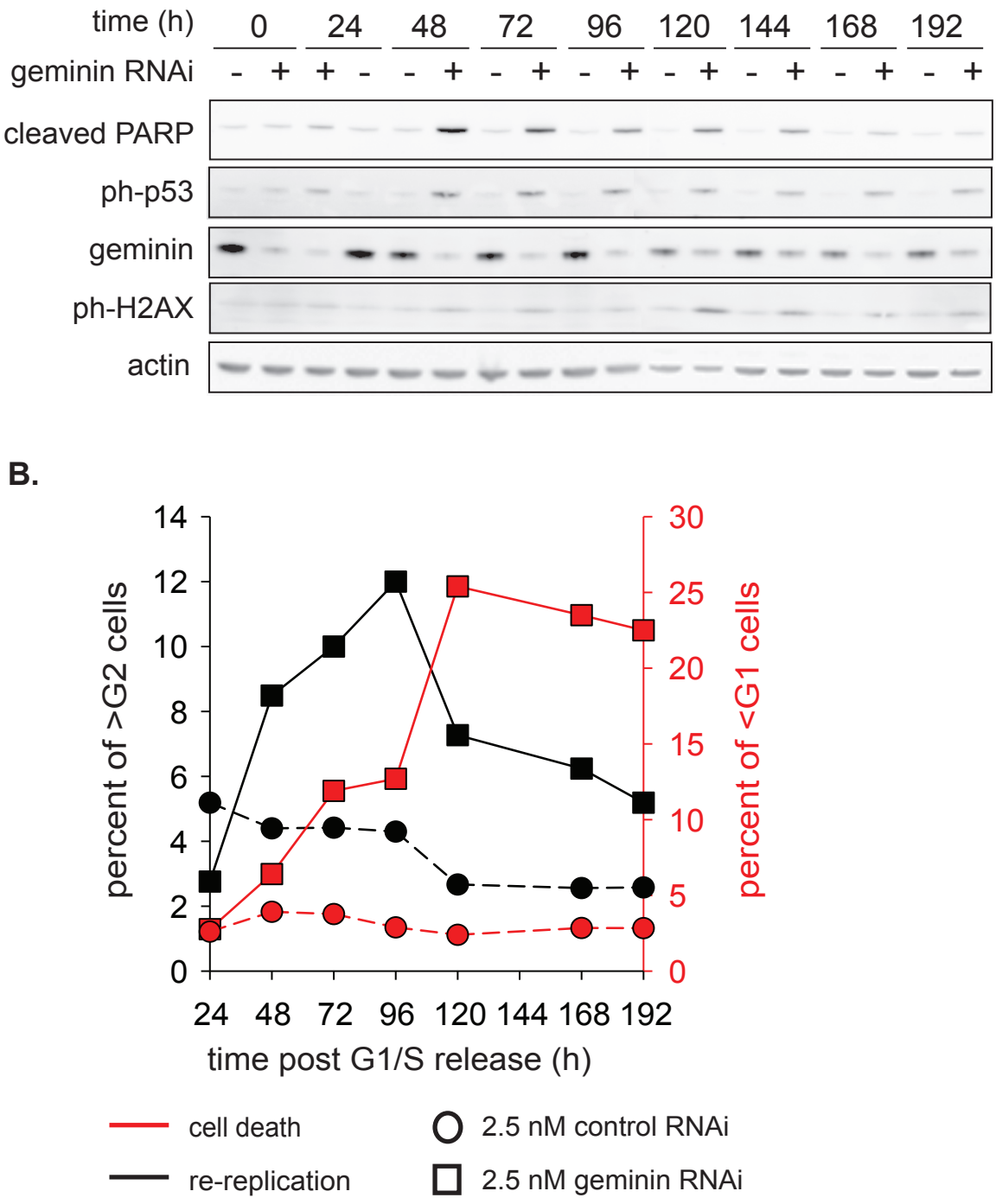


Figure III. 5. DNA damage and apoptosis in geminin depleted cells. U2OS cells were released from a double Thymidine block after prior treatment with either control or geminin RNAi, as in Figure III. 2. **A.** At different times after 2nd Thymidine release whole cell extracts were prepared, seperated by SDS-PAGE and immunblotted for the indicated protein. **B.** At different times after release, cells were analysed by flow cytometry and percentage of cells with either <G1 DNA content or >G2 DNA content was measured.

Figure III. 6.

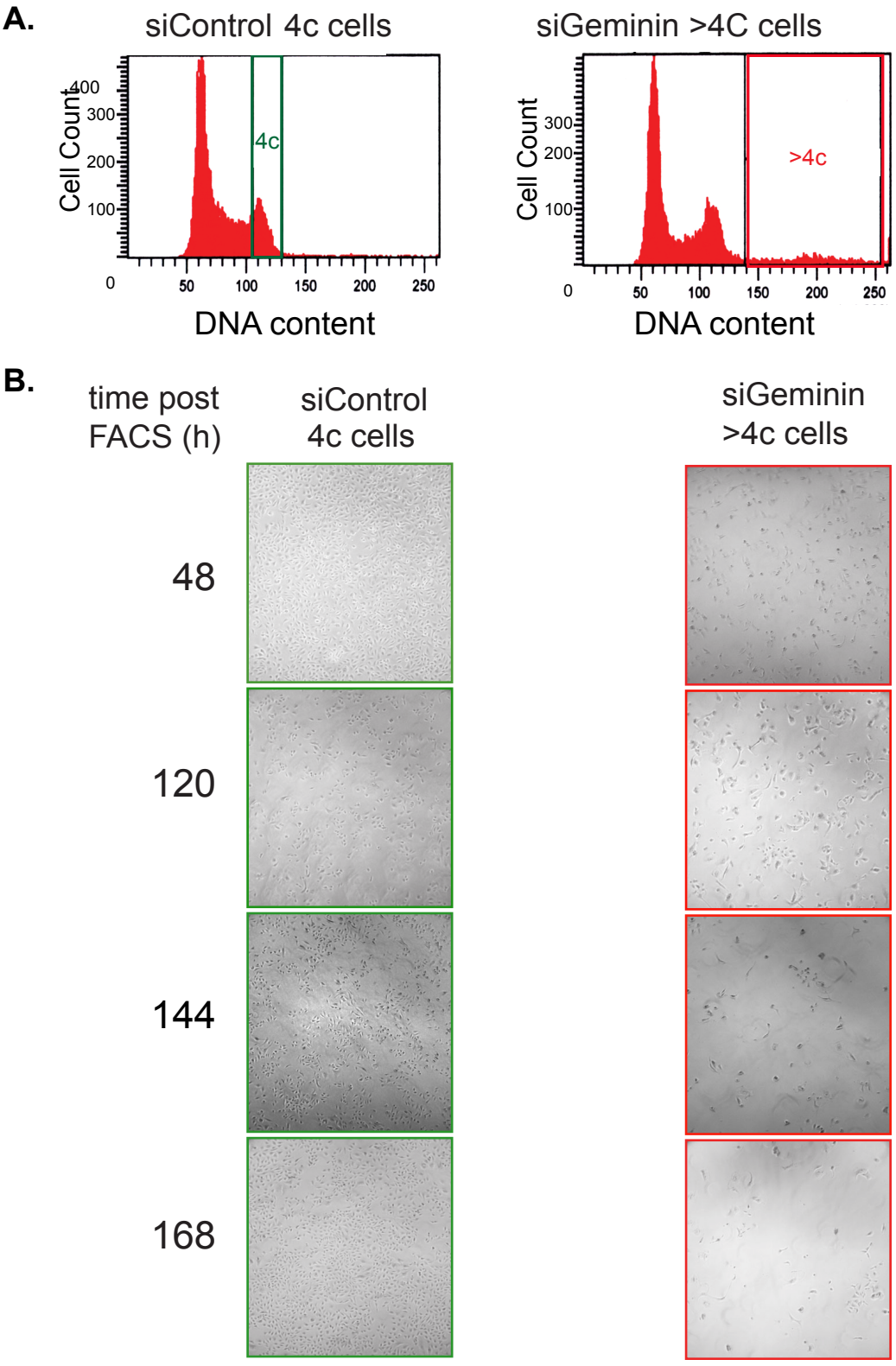


Figure III. 6. Cells fail to survive re-replication. During Thymidine synchronisation, U2OS cells were treated with 2.5 nM geminin or control RNAi, and grown for 120 h after thymidine release (so the bulk of apoptosis in the geminin-depleted culture was over). Cultures were washed to remove apoptotic cells and stained with Hoechst 33342 for FACS. For Geminin RNAi treated cultures, cells with >4c DNA were collected, whereas for control cultures cells with a 4c DNA content were collected. 50,000 cells were re-plated into 24 well dishes and cultures monitored microscopically over the next 168 h (48 h, 120 h, 144 h and 168h are shown). After 96 h control cells were confluent and had to be diluted 1:6 explaining the difference between 48 and 120 h.Over time no cells with an altered DNA content could be recovered. **A.** FACS profiles plus sort windows. **B.** Examples of cultures 48, 120, 144 and 168 h after FACS.

III. 4. GEMININ DEPLETION DOES NOT SIGNIFICANTLY ALTER S-PHASE PROGRESSION

Given the dramatic consequences of re-replication, the objective of the next part of the project was to investigate the initiation of re-replication and how cellular processes such as DNA replication, mitosis and checkpoint activation are affected by geminin depletion. To study whether geminin depletion alters cell cycle progression, U2OS cells were synchronised and transfected with 15 nM geminin RNAi as described in Figure III. 2. The cells were released from the 2nd Thymidine block and then cultured for different times, subsequently whole cell lysate were analysed by Western Blot (Figure III. 7 A). In parallel cells were incubated for different time periods and then pulsed with 20 μM BrdU for 30 min just prior to cell cycle analysis by flow cytometry (Figure III. 7 B). As shown in panel B, cells progress through S-phase (T4 and T10), G2/M (T14 and T16) and then into the following cell cycles (T29, T38 and T48). During the first 10 h of S-phase there was no significant difference in BrdU intensity or DNA content between control and geminin depleted cells. After 14-16 h about 50-60% of control cells were BrdU negative and had a G2/M DNA content. Many of these cells were passing through mitosis as indicated by increased phosphorylated histone H3 levels (Figure III. 7 A). Whilst the majority of geminin depleted cells also exhibited a near-G2/M DNA content after 14-16 h of Thymidine release (Figure III. 7 B), a significant proportion remained BrdU positive. This was associated with a reduced staining for phosphorylated H3 and an increase in phosphorylated p53 and cleaved PARP (Figure III. 7 A). At later times, control cells entered again G1 and S phase, while geminin depleted cells were either enriched at G2/M or exhibited a >4C DNA content.

The flow cytometry results indicate that at least until late S-phase, geminin depletion seems not to significantly alter S-phase progression.

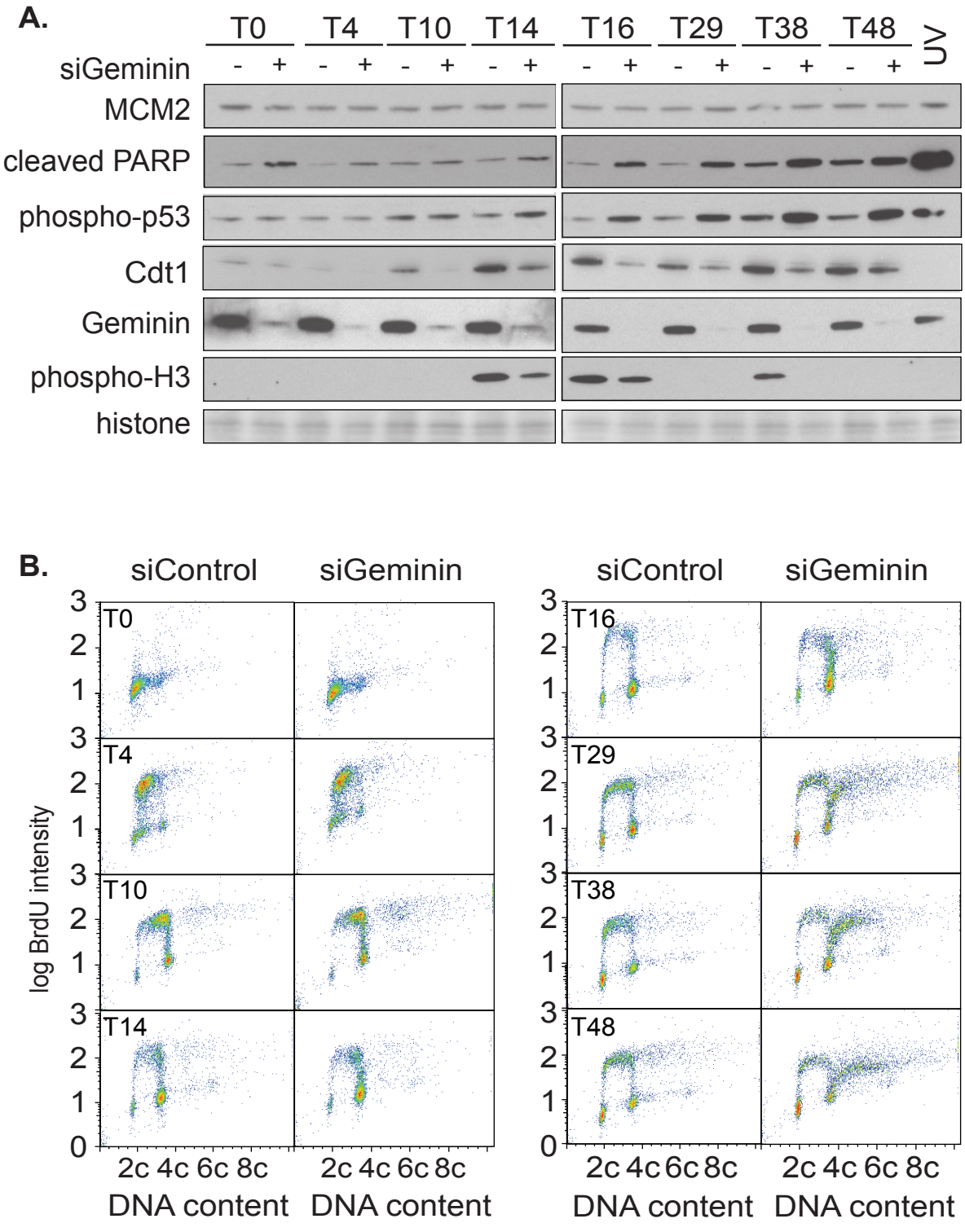


Figure III. 7. Geminin depletion does not alter S-phase progession and induces re-replication earliest in late S-phase or G2. Cells were released from a double Thymidine block after prior treatment with either control or geminin RNAi, as in Figure III. 2. **A.** Western blot analysis of whole cell extracts at different times after Thymidine release. Geminin RNAi: +; control RNAi: -. As DNA damage positive control, cells were treated with 120 mJ UV. The membrane was stained with amido black to show equal loading of histones. **B.** At different times after release from double Thymidine block, cells were pulsed with BrdU and analysed by flow cytometry to determine the cell cycle progression based on the BrdU staining intensity and the propidium iodide intensity.

To strengthen this observation the chromatin association of replication proteins such as MCM2, Proliferating Cell Nuclear Antigen (PCNA) and Cdt1 during S-phase was elucidated after geminin depletion. Therefore geminin was depleted again in synchronised U2OS cells and supernatant and chromatin fractions from time points T0, T4, T8, T10, T14 and T18 were collected (Figure III. 8). In agreement with the flow cytometry results no increase of MCM2, PCNA or Cdt1 was detected on S-phase chromatin in geminin depleted cells in comparison to control cells, which would be expected if re-replication would occur already in the first S-phase in geminin depleted cells. Instead it is possible that the low levels of Cdt1 during the first S phase (Figure III. 7 A; Figure III. 8) limit the amount of re-licensing that occurs in the absence of geminin. However, geminin depleted cells showed a defect in progression into mitosis, associated with continued BrdU incorporation and checkpoint activation, which is potentially the result of a small number of origins re-firing.

During G2 phase and mitosis, Cdt1 levels start to rise again. It should be noted that the Cdt1 accumulation in the whole cell extracts (Figure III. 7 A) and the supernatant fractions (Figure III. 8) was lower in the geminin depleted cells than in control cells. The lower Cdt1 levels in the geminin depleted cells are likely a consequence of increased Cdt1 proteolysis (Ballabeni et al., 2004), which could be a result of re-replication induced activation of the DNA damage response (Hall et al., 2008). In contrast, on late S-phase and G2 phase chromatin the increase in Cdt1 binding was equal in geminin depleted and control cells (Figure III. 8). Consequently, cells lacking geminin failed to restrain Cdt1 activity at the G2/M border and could therefore load MCM2-7 complexes onto DNA inducing massive re-replication.

Consistent with this interpretation, at 18 h after Thymidine release, MCM2 chromatin association was approximately equal in geminin depleted and control cells, although many of the geminin depleted cells were still in G2 as indicated by the reduced phosphorylated histone H3 level (Figure III. 8).

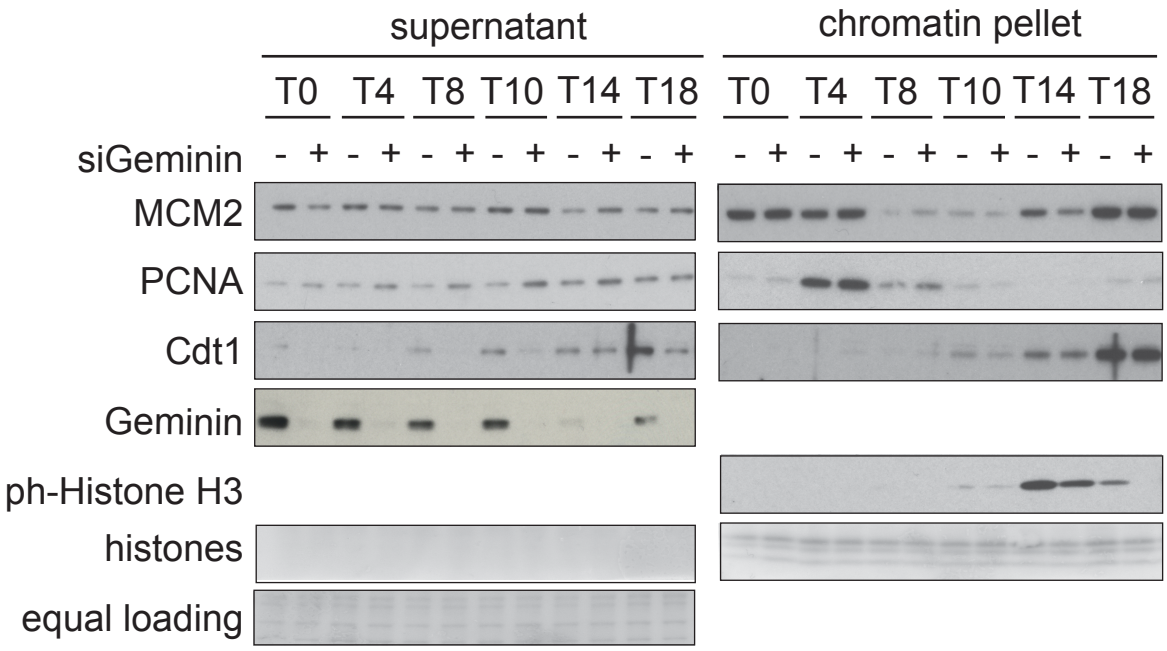


Figure III. 8. Chromatin proteins during S-phase in geminin depleted cells. Cells were released from a double Thymidine block after prior treatment with either control (-) orgeminin (+) RNAi, as in Figure III. 2. At different times after release, cells were treated with CSK buffer for chromatin fractionation and seperated into supernatant and pellet fractions. Samples were then run on SDS-PAGE and immunoblotted for the indicated proteins. The lower part of the membrane was stained with amido black to show equal loading.

III. 5. Geminin depletion does not significantly alter replication timing

Another way to study and compare S-phase progression of control and geminin depleted cells is to monitor the replication timing pattern. Therefore the cells were released from a double Thymidine block after prior treatment with either non-targeting or geminin RNAi. At indicated times cells were pulsed with BrdU and subsequently fixed (Figure III. 9 A). For each time point at least 37 cells were analysed and grouped into the five different timing patterns in accordance with previous nomenclature (O'Keefe et al., 1992). In the first pattern replication occurs throughout the nucleus, specifically in regions of euchromatin with a distinct lack of peripheral heterochromatin and nucleolar labelling. In the second pattern the sites of replication are larger and more discrete, they are localised more towards the periphery of the nucleus with fewer interior sites. In the third pattern the sites are predominantly at the nuclear periphery and the perinucleolar regions. In the fourth pattern the sites become larger in size and fewer in number they are located throughout the interior and at few discrete sites at the periphery (O'Keefe et al., 1992). Pattern five is characterised by labelling of large internal replication foci and at the periphery of the nucleus (Thomson et al., 2010). Figure III. 9 B shows the percentage of control or geminin depleted cells within the different replication patterns at times across S-phase (T4, T6, T8, T10), G2/M (T14, T16) and G1 (T18) of the cell cycle. A microscopic example of how the BrdU staining is categorised into the five patterns is given in Figure III. 9. C. There was no significant difference in the temporal progression through those patterns between control and geminin depleted cells. This suggests that extensive re-firing of replication origins does not occur in this phase of the cell cycle. Only during late S or G2, in the time points T14, T16 and T18, was a difference observed, with slightly fewer unlabelled cells in the geminin-depleted samples, which could mean that cells either finish S-phase slower or especially in T18 start to replicate again.

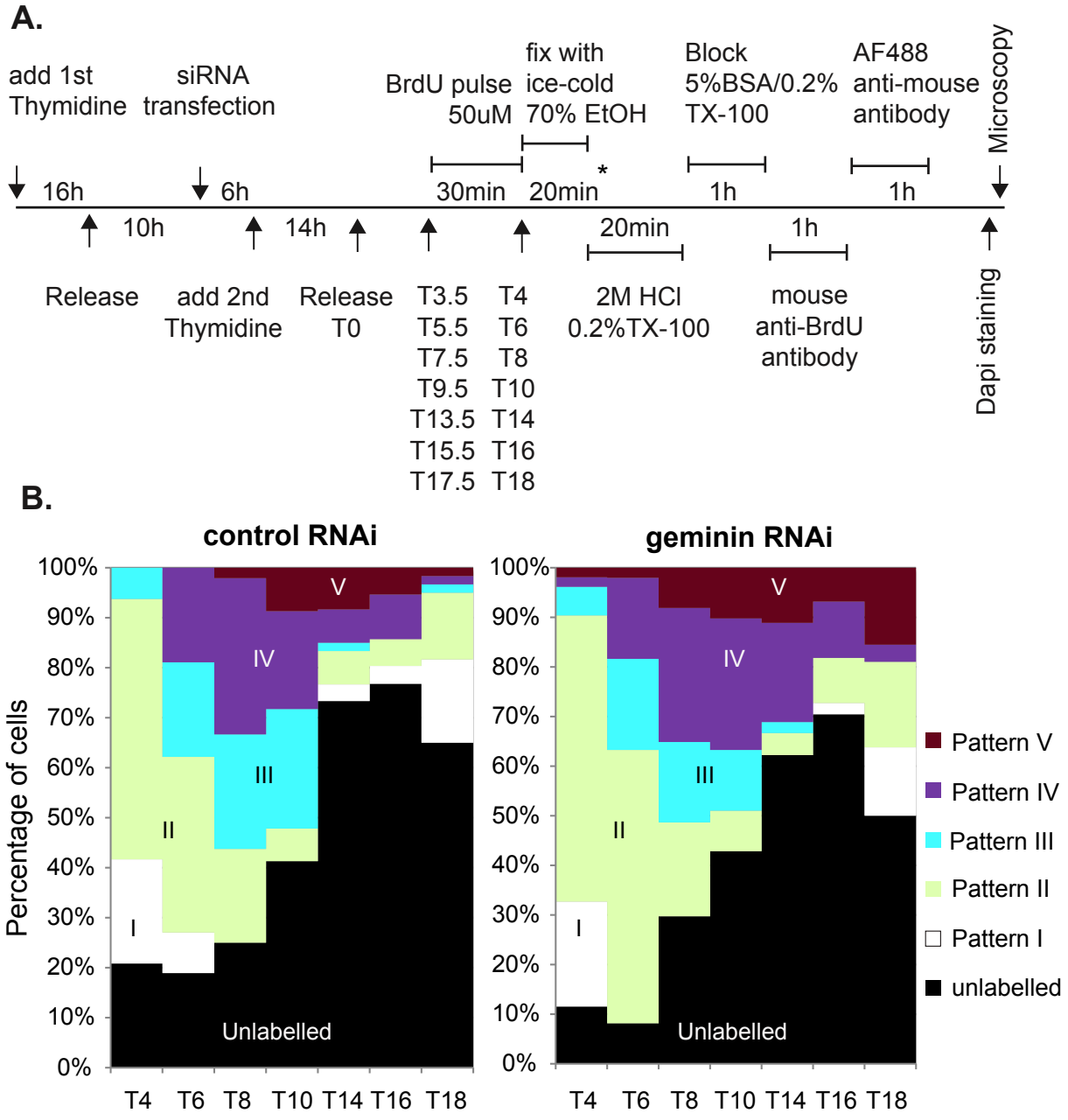
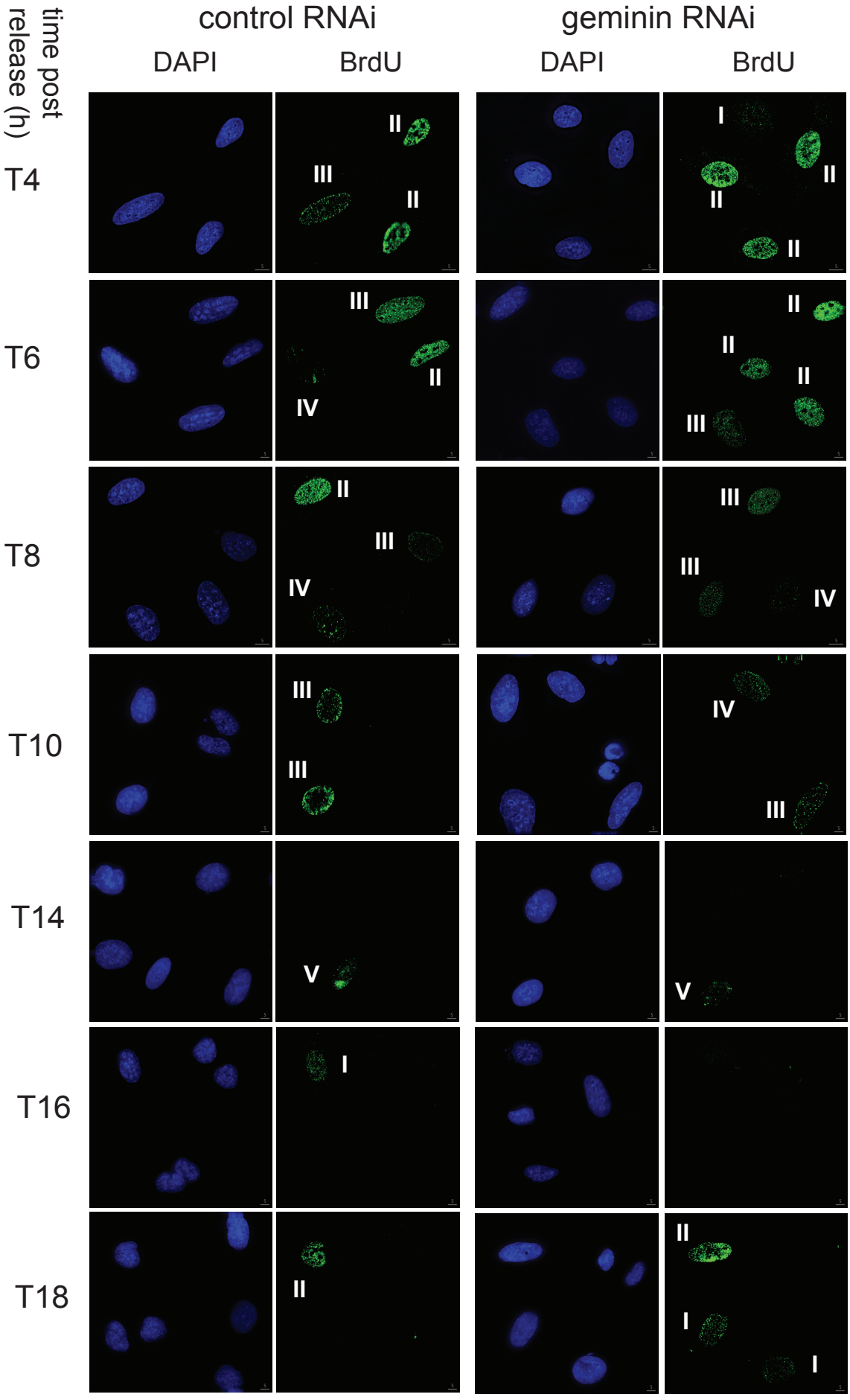


Figure III. 9. Replication timing pattern in synchronised geminin depleted cells. Cells were released from a double Thymidine block after prior treatment with either control or geminin RNAi, as in Figure III. 2. and pulsed with BrdU for 30min at indicated times to monitor replication timing pattern. **A.** Schematic of the experiment. **B.** At each time point at least 37 cells were analysed for their replication timing pattern according to O' Keefe et al.,1992. The percentage of cells falling into each of the 5 patterns is illustrated. **C.** For each time point representive images of U2OS cells with their timing pattern are shown, see Materials and Methods Section II. 9. 2. for description of the different replication timing patterns.

*Samples were incubated in EtOH for at least 20min or stored until all timepoints were collected

Figure III. 9. C

C.



III. 6. PROGRESSION THROUGH MITOSIS IS INHIBITED IN GEMININ DEPLETED CELLS THAT UNDERGO RE-REPLICATION.

The results so far showed that geminin depletion neither alters S-phase progression and chromatin association of replication proteins within the first S-phase, nor changed the replication timing pattern. However, geminin-depleted cells remain longer in G2 and after some time a large proportion of these cells acquired a >4C DNA content. Therefore it was interesting to ask, whether geminin depleted cells that fail to enter mitosis are doomed to undergo re-replication from the late S-phase or G2 stage. To address this, control and geminin depleted cells were released from the 2nd Thymidine block for 14 h. At this time point cells were mainly in G2/M with 12% (control) and 17% (geminin depleted) still in late S-phase. Thymidine was then added again for 10 h, allowing those cells that had completed S-phase to progress through mitosis and G1 but preventing any further DNA replication or re-replication (Figure III. 10). Moreover we analysed the cell cycle profile after the 3rd Thymidine release (T24). About 83% of the control cells progressed through mitosis and G1 and then accumulated in early S-phase, while the 12% that were in late S-phase at the time of 3rd Thymidine addition, arrested in late S-phase. In the population of geminin depleted cells only 62% progressed through mitosis and G1, while 33% remained with a ~4C DNA content. Since only 17% of 33% were incorporating detectable amounts of BrdU at the time of the 3rd Thymidine addition, this suggests that a significant proportion of the geminin depleted cells were prevented from entering mitosis even though they had essentially finished synthesising DNA. When the cells that had passed through mitosis and had been arrested in early S-phase (79% control cells, 58% geminin depleted cells) were released from the 3rd Thymidine block for 5h, almost all of them progressed into S-phase and incorporated BrdU. At the same time, almost two thirds of the geminin depleted cells that had arrested in G2/M (19% of the total) started to re-replicate.

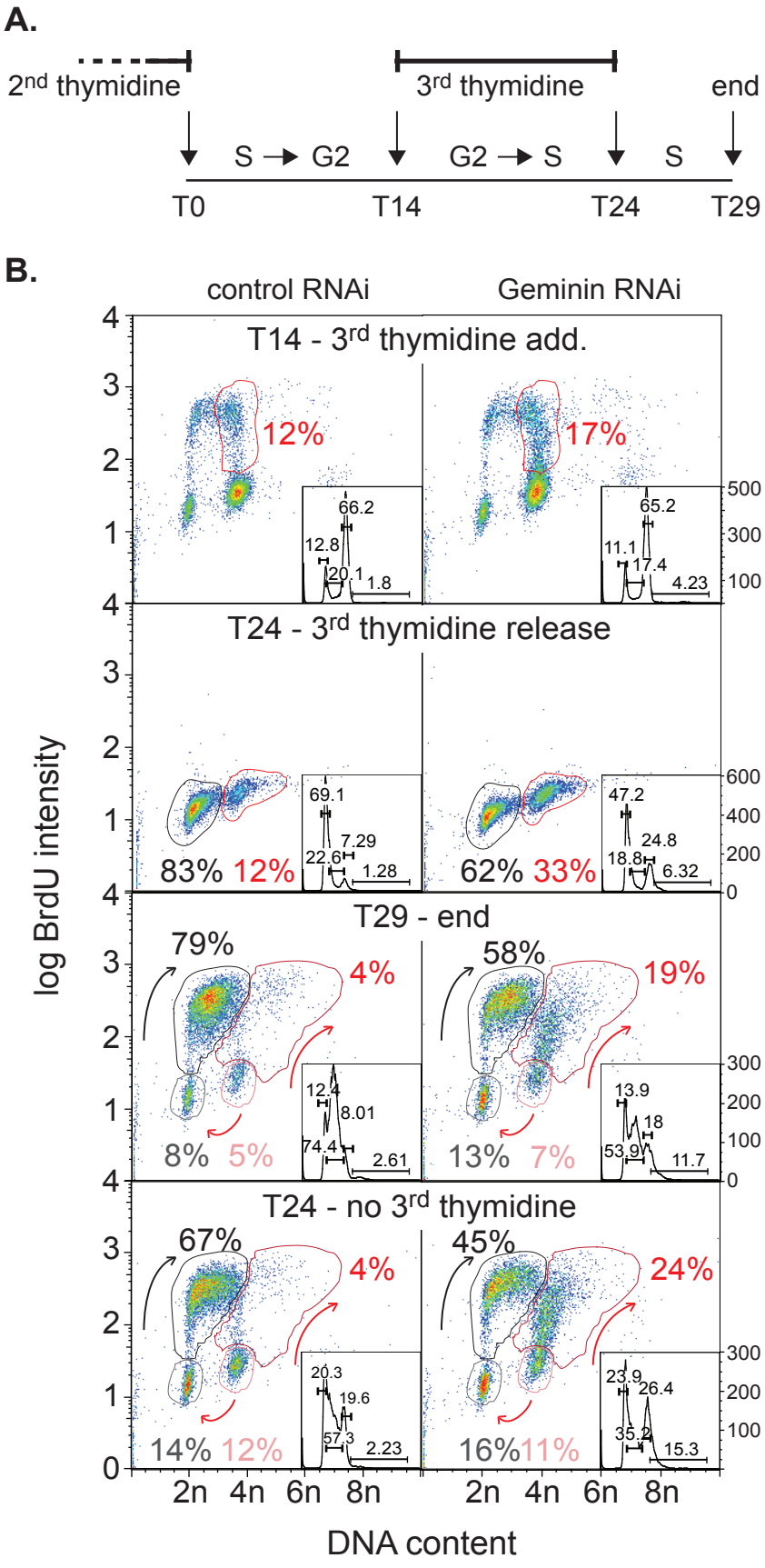


Figure III. 10. Geminin depletion prevents passage through mitosis followed by rereplication. Cells were released from a double Thymidine block after prior treatment with either control or geminin RNAi, as in Figure III. 2. 14 h later cells were optionally given a 3rd thymidine block for 10 h, and then released for a further 5 h. **A.** Schematic of experiment. **B.** At different times during the procedure, batches of cells were given a BrdU pulse and then analysed by flow cytometry. Arrows show the movement of cells suggested by the data.

In comparison, geminin depleted and non-arrested cells that were not given a 3rd Thymidine treatment (Figure III. 10 B 'T24 – no 3rd thymidine') showed a very similar final cell cycle profile and underwent similar levels of re-replication.

Taken together, these results suggest that geminin depletion causes a subset of cells to arrest in the late S/G2 phase state without progressing into mitosis, and that these cells subsequently start to re-fire replication origins leading to re-replication of their DNA.

III. 7. RE-REPLICATION OCCURS FROM G2 PHASE

Observations from numerous experiments including the time course (Figure III. 7 and 10) suggested that geminin depletion causes re-replication in cells arrested in a G2-like state. The cells appeared to stop the incorporation of BrdU and later on start to incorporate again, moving into a state that could be mistaken as late S-phase by flow cytometry as substantial re-replication has to occur before cells clearly acquire a >4C DNA content. In order to confirm that re-replication can occur in cells in G2 phase that have effectively ceased DNA synthesis, we carried out a pulse chase experiment, where control and geminin depleted cells were pulse labelled with BrdU 12 h after the 2nd Thymidine release (T12) (Figure III. 11 A). At this time the majority of cells had progressed through S-phase and were BrdU negative, with only 30 – 35% of cells still incorporating significant quantities of BrdU (Figure III. 11 B). The BrdU was then removed and the cells chased for 36 h to allow sufficient time for a measurable degree of re-replication to occur. The results showed that after the chase, more than half (22%) of the cells with a >4C DNA content (39%) were BrdU negative. This shows that cells in G2 can subsequently start to re-replicate. It can not be completely excluded that a small proportion of cells start to re-replicate from late S-phase, but it is more likely that there is a common mechanism in which cells stop incorporating BrdU and enter G2 before re-replication occurs.

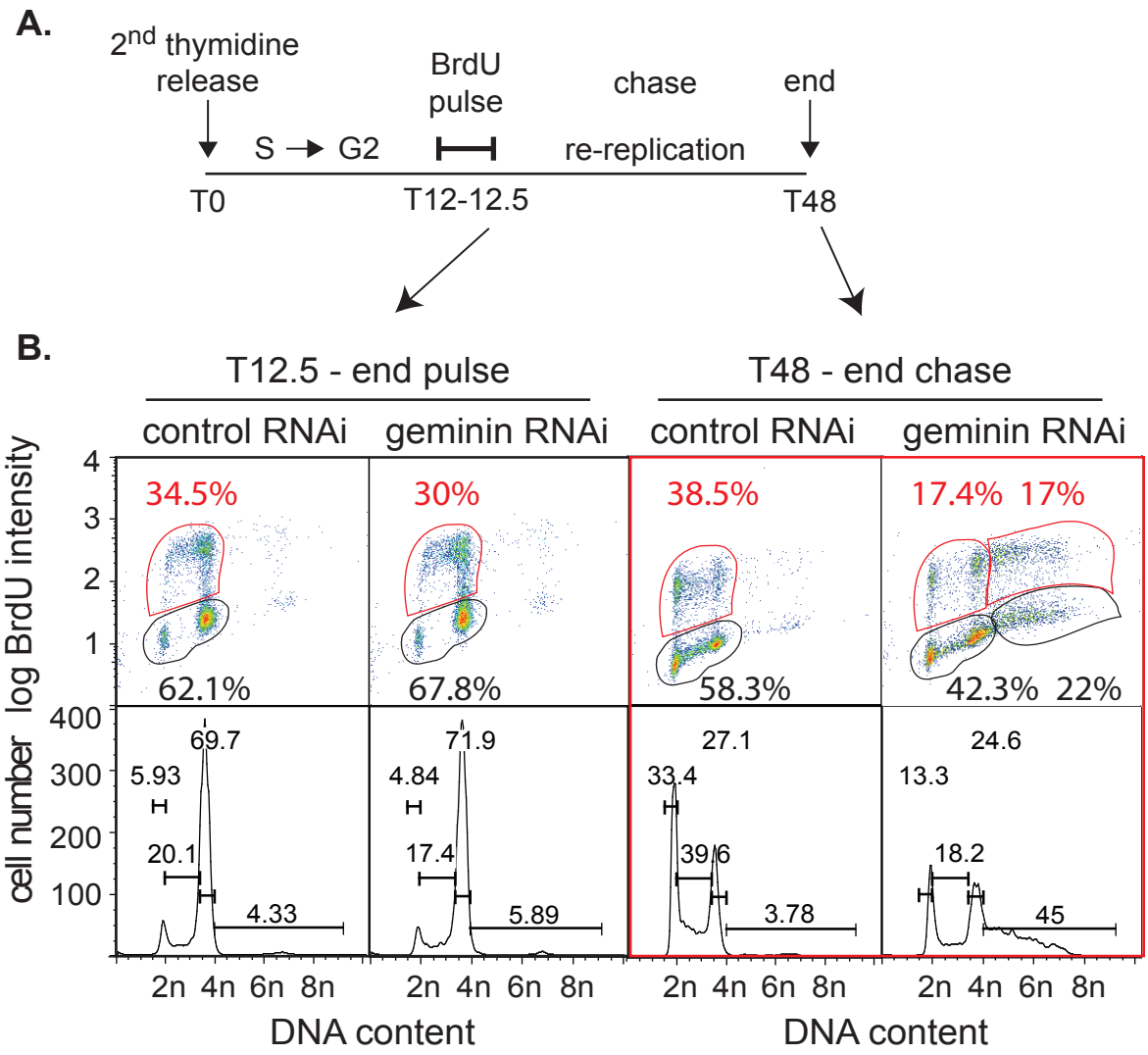


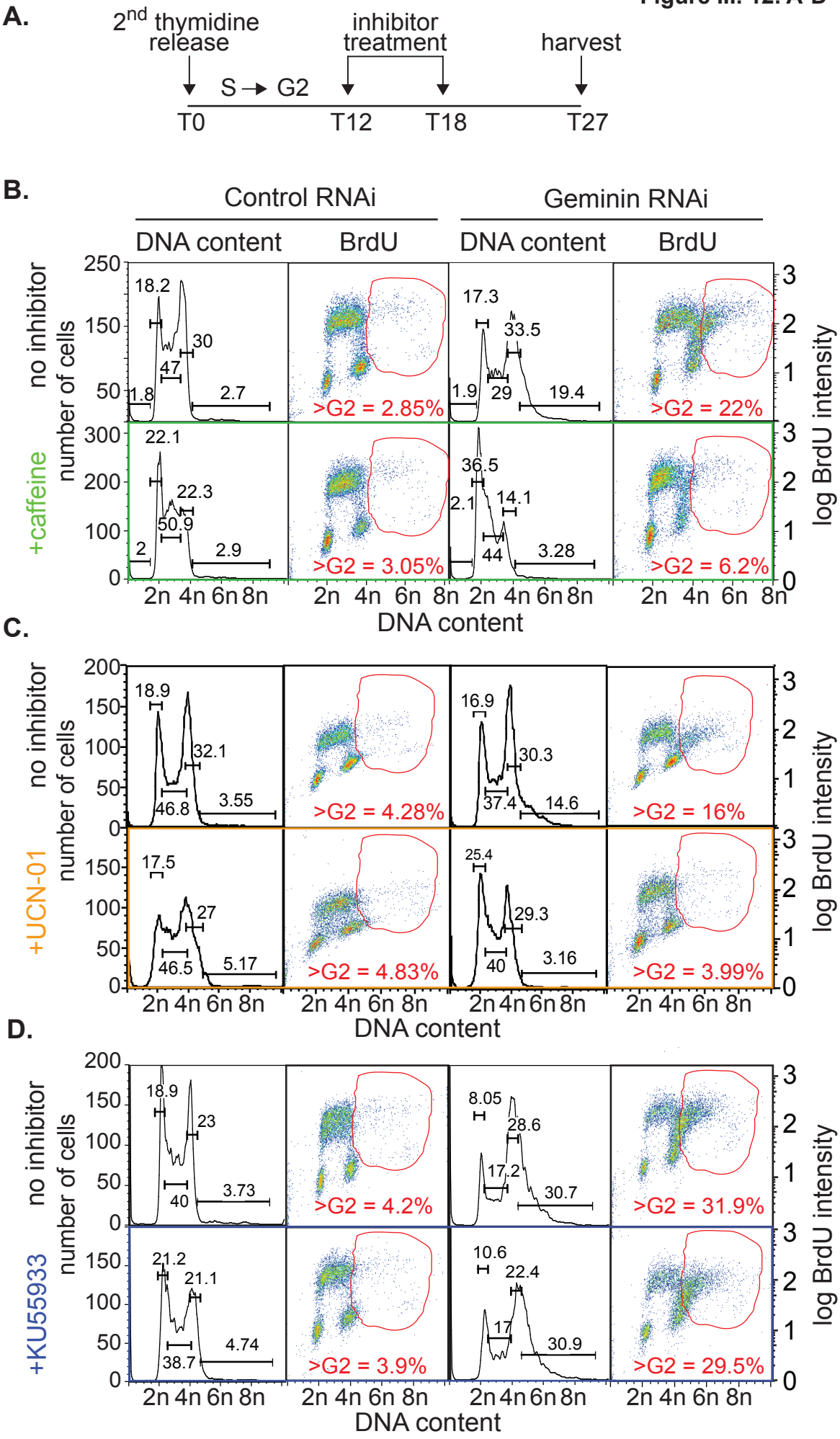
Figure III. 11. Assessment of cells spontaneously re-replicating from G2. Cells were released from a double Thymidine block after prior treatment with either control or geminin RNAi, as in Figure III. 2. Twelwe hours later cells were pulsed with BrdU for 30 mins. BrdU was then removed and cultures continued for a further 35.5 h. **A.** Schematic of experiment. **B.** At different times during the procedure, batches of cells were analysed by flow cytometry. Red labelled cells are BrdU positive and black labelled cells are BrdU negative.

III. 8. THE G2/M CHECKPOINT PROMOTES RE-REPLICATION OF GEMININ DEPLETED CELLS

Several studies have investigated the role of the G2/M checkpoint in re-replication induced by geminin depletion. Melixetian *et al.*, 2004 have shown that re-replicated HCT116 cells can re-enter mitosis if the ATR checkpoint pathway is abolished but subsequently undergo mitotic catastrophe and cell death. Consistent with this study, Zhu *et al.* (2004) demonstrated that the level of re-replication decreased upon checkpoint abrogation accompanied by a significant increase in mitotic cells (Melixetian et al., 2004; Zhu et al., 2004). However, the increase of Cdt1 protein that occurs during G2 may make cells particularly dependent on geminin to restrain re-licensing of replicated DNA during G2.

The results so far suggest that geminin depletion causes cells to arrest in G2, precisely the cell cycle phase where they are most reliant on geminin to prevent re-replication. Therefore we investigated whether the G2/M checkpoint activity actually enhances re-replication in geminin depleted cells. The synchronised re-replication assay was used to address this question by treating cells with checkpoint inhibitors 12 – 18 h after release from the 2nd Thymidine block, during the time when geminin depleted cells accumulate in G2 (Figure III. 12 A). This allows geminin depleted cells to pass through mitosis and re-enter G1. The checkpoint inhibitors were withdrawn after 18 h and the incubation continued for a further 9 h to allow cells to progress into the subsequent S-phase. After this time (27 h) cells were labelled with BrdU and analysed by flow cytometry. Treatment of geminin depleted cells with the ATM/ATR and DNA PK inhibitor caffeine significantly reduced both the number of cells arrested in G2 and the number of re-replicating cells with >4C DNA content. Consequently the percentage of geminin depleted cells in G1 and S-phase was dramatically increased, though they showed a slight delay in S-phase progression compared to control cells (Figure III. 12 B, F).

Figure III. 12. A-D



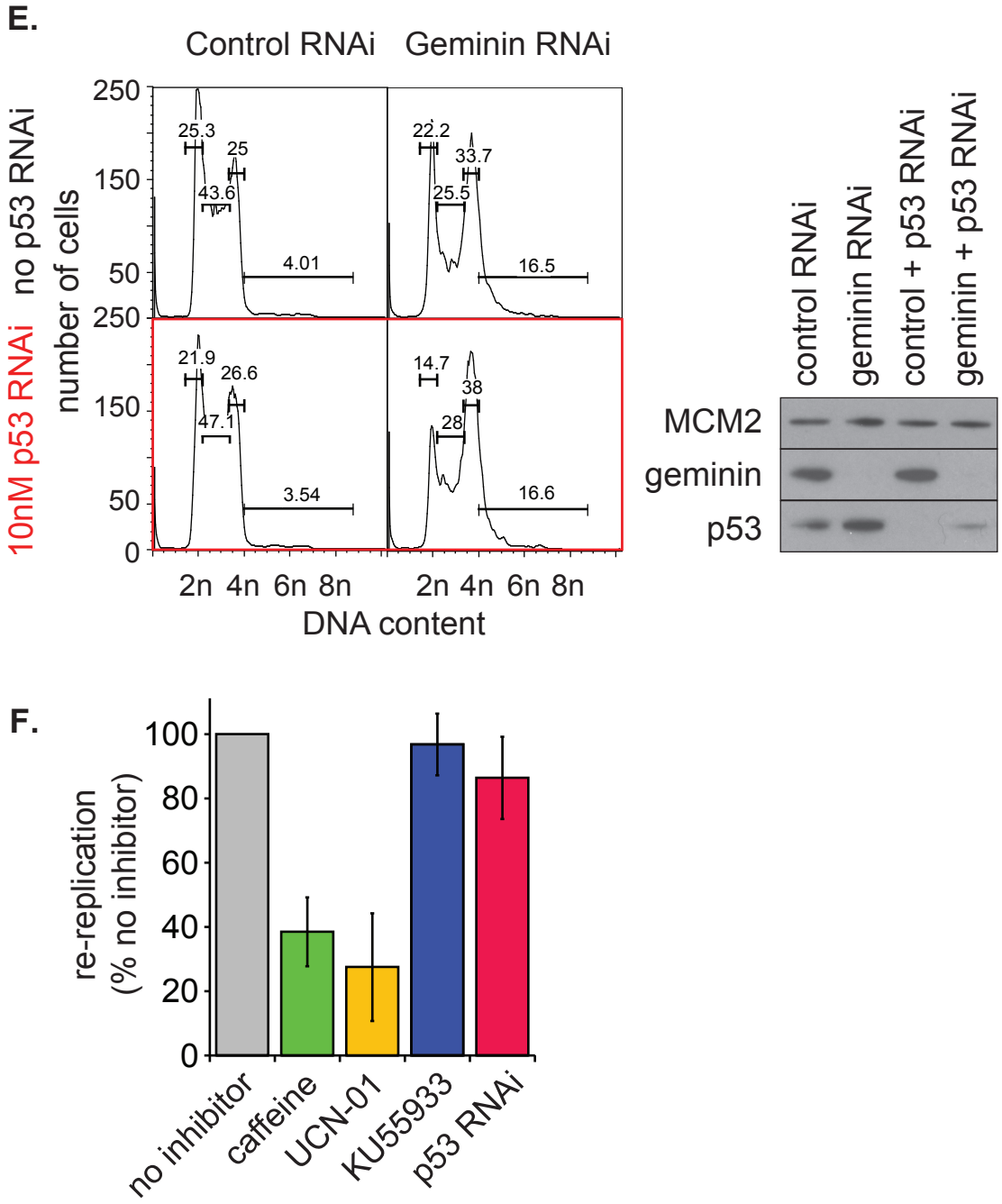


Figure III. 12. The G2/M checkpoint promotes re-replication. Cells were released from a double Thymidine block after prior treatment with either control or geminin RNAi, and optionally p53 RNAi, as in Figure III.2. Twelve hours after release, cells were optionally treated with caffeine, UCN-01 or KU55933 for 6 h. 27 h after Thymidine release, cells were pulsed with BrdU and analysed by flow cytometry. **A.** Schematic of experiment. **B.** Flow cytometry profiles of geminin RNAi and control RNAi cells plus and minus caffeine treatment. **C.** Flow cytometry profiles of geminin RNAi and control RNAi cells plus and minus UCN-01 treatment. **D.** Flow cytometry profiles of geminin RNAi and control RNAi cells plus and minus KU55933 treatment. **E.** Flow cytometry profiles of geminin RNAi and control RNAi cells plus and minus p53 RNAi and demonstration of geminin and p53 knock-down by immunoblotting. **F.** The suppression of re-replication observed in geminin RNAi seen with different inhibitor treatments or with additional p53 knockdown, expressed as a percentage of the amount of re-replication in geminin RNAi cells without inhibitor treatment (average of 3 independent experiments is shown).

Similar results were observed when checkpoint kinase 1 (Chk1) was inhibited by UCN-01 treatment (Figure III. 12 C, F). In contrast, addition of ATM inhibitor KU55933 or co-depletion of p53 showed no or very little impact on the levels of re-replication (Figure III. 12 D, E, F).

III. 9. SUMMARY

Geminin depletion caused severe levels of DNA re-replication and inhibition of proliferation in U2OS cancer cells. However, S-phase progression and the replication timing pattern were not significantly altered in geminin depleted cells but due to the G2/M checkpoint activity some cells arrest in G2 and are unable to enter mitosis. ATR and Chk1, but not ATM, are the major checkpoint kinases responsible for the G2/M arrest of geminin depleted cells. It is currently unclear what is responsible for the activation of these kinases, but low levels of re-replication is a likely cause. In the absence of geminin, many of these G2-arrested cells then undergo further re-replication to gain a >4C DNA content. ATR and Chk1 therefore promote DNA re-replication in geminin depleted cells by preventing mitotic entry and delaying them in G2. At this cell cycle stage cells are particularly dependent on geminin to prevent re-licensing of replicated DNA. The checkpoint mediated cell cycle arrest thereby amplifies the effect of a small amount of re-replication, creating an 'all or nothing' effect (see Discussion). Once re-replication has taken place the >4C cells are not able to form colonies and are doomed to undergo apoptosis as a consequence of the genomic damage they suffered.

III. 10. FUTURE DIRECTIONS

This is the first study to show that geminin depletion in synchronised cells causes re-replication from G2. The data provided is based exclusively on the U2OS cell line. Given that tumour-derived cell lines vary greatly in their ability to re-replicate and the general resistance of 'normal' cells to re-replication, the findings would be strengthened

be re-capitulating some of the key cell cycle or and checkpoint results in other cell lines. During the course of this project, the re-replication assay has been done on HeLa cervical cancer cells and HCT116 colon cancer cells. HeLa cells showed no re-replication detectable by flow cytometry in response to geminin depletion whereas HCT116 re-replicated massively consistent with previous reports (Zhu et al., 2004). However, HCT116 cells do not respond to double Thymidine synchronisation, which complicated the investigation of re-replication from G2 phase. A possible approach would be to subject HCT116 cells to counterflow centrifugation elutriation and collect a pure G1 population to transfect them with geminin RNAi once they reached G2/M. This way the progression to subsequent S-phase could be studied in a more or less synchronised population to identify the origin of re-replication. Another promising candidate for reproducing the U2OS results are Saos2 cells, as they are from similar tumour type as U2OS and synchronise well with Thymidine.

Chapter IV

Results:

Stabilisation and over-expression of Cdt1 results in re- replication and inhibition of replication

IV. 1. INTRODUCTION

Deregulation of the licensing machinery can be achieved by other ways in addition to geminin depletion. Geminin's main function is to inhibit origin re-licensing in S-phase and G2 phase by formation of a heterohexameric inhibitory complex with licensing factor Cdt1 (Section I. 6. 1. 2). Cdt1 is expressed throughout the cell cycle but its activity is limited to late mitosis and G1 by geminin inhibition and by proteolysis via two Cullin-Ring and the APC ubiquitin ligases (Section I. 6. 1. 1). Overexpression of Cdt1 can therefore neutralise the inhibitory effect of geminin simply by overwhelming the system and titrating out the geminin. Similarly the degradation machinery can only remove a limited amount of Cdt1 protein and its over-expression has been shown to induce re-replication of genomic DNA in several model organisms (Arias and Walter, 2005; Li and Blow, 2005; Maiorano et al., 2005; Thomer et al., 2004; Vaziri et al., 2003). Alternatively to Cdt1 over-expression, the protein can also be stabilised by inhibition of its degradation machinery. Recent studies revealed that treatment of cells with the Nedd8 activating enzyme inhibitor MLN4924 stabilises Cdt1 in S-phase and causes re-replication, apoptosis and senescence (Lin et al., 2010; Milhollen et al., 2011; Soucy et al., 2009).

In this Chapter the physiological consequences of Cdt1 overexpression or stabilisation were investigated to further characterise the mechanism of re-replication in human cells. The previous Chapter showed that geminin depletion did not alter the initial S-phase progression but induced re-replication from G2 phase of the cell cycle. The hypothesis is that this is due to low levels of Cdt1 during 'normal' S-phase, and that over-expression or stabilisation of Cdt1 could lead to a different phenotype being most likely visible directly during S-phase progression. Consequently, Cdt1 was over-expressed as GFP-fusion protein or stabilised by MLN4924 treatment to study changes in the DNA content during cell cycle progression. It is important to gain further insight into the deregulation of the licensing machinery especially if it leads to re-replication via

several pathways in distinct cell cycle phases, which may result in different genomic alterations.

IV. 2. CDT1 OVEREXPRESSION INHIBITS DNA REPLICATION

Previous studies have shown that besides deregulation of the licensing machinery by geminin depletion, over-expression or stabilisation of licensing factor Cdt1 can also cause re-replication (Arias and Walter, 2005; Li and Blow, 2005; Maiorano et al., 2005; Nishitani et al., 2004; Thomer et al., 2004; Vaziri et al., 2003). We were interested whether re-replication in U2OS cells caused by Cdt1 stabilisation is initiated via the same mechanism that is seen following loss of geminin (Section III. 9). Initially, human Cdt1 was overexpressed using GFP-Cdt1 fusion proteins to study how Cdt1 stabilisation causes re-replication.

IV. 2. 1. Cloning of human Cdt1 and GFP-Cdt1 construct design

Human *CDT1* (Accession BC009411) was obtained in a pOBT7 backbone from www.geneservice.co.uk. and transferred into pcDNA3 (Figure IV. 1). GFP-Cdt1 fusion constructs were designed according to the previous results of Ferenbach and colleagues (Figure IV. 2 A) (Ferenbach et al., 2005). Beside full-length Cdt1, truncated constructs were generated to gain insight in possible functions during re-replication. In detail, $Cdt1^{34-546}$ lacks the N-terminal region required for its proteolysis and therefore should stabilise the protein and induce higher levels of re-replication than the full-length protein. $Cdt1^{1-372}$ lacks the C-terminal region essential for MCM2-7 binding and loading onto chromatin and therefore could only promote re-replication indirectly by titrating out geminin and overwhelming the Cdt1 degradation machinery (Figure IV. 2 A). All constructs can still bind and titrate out geminin but only full-length -Cdt1 and $Cdt1^{34-546}$ can actively load MCM2-7 complex onto chromatin. The nuclear localisation of the Cdt1-GFP fusion proteins was verified microscopically (Figure IV. 3) and the sequences were confirmed using the College of Life Sciences sequencing service.

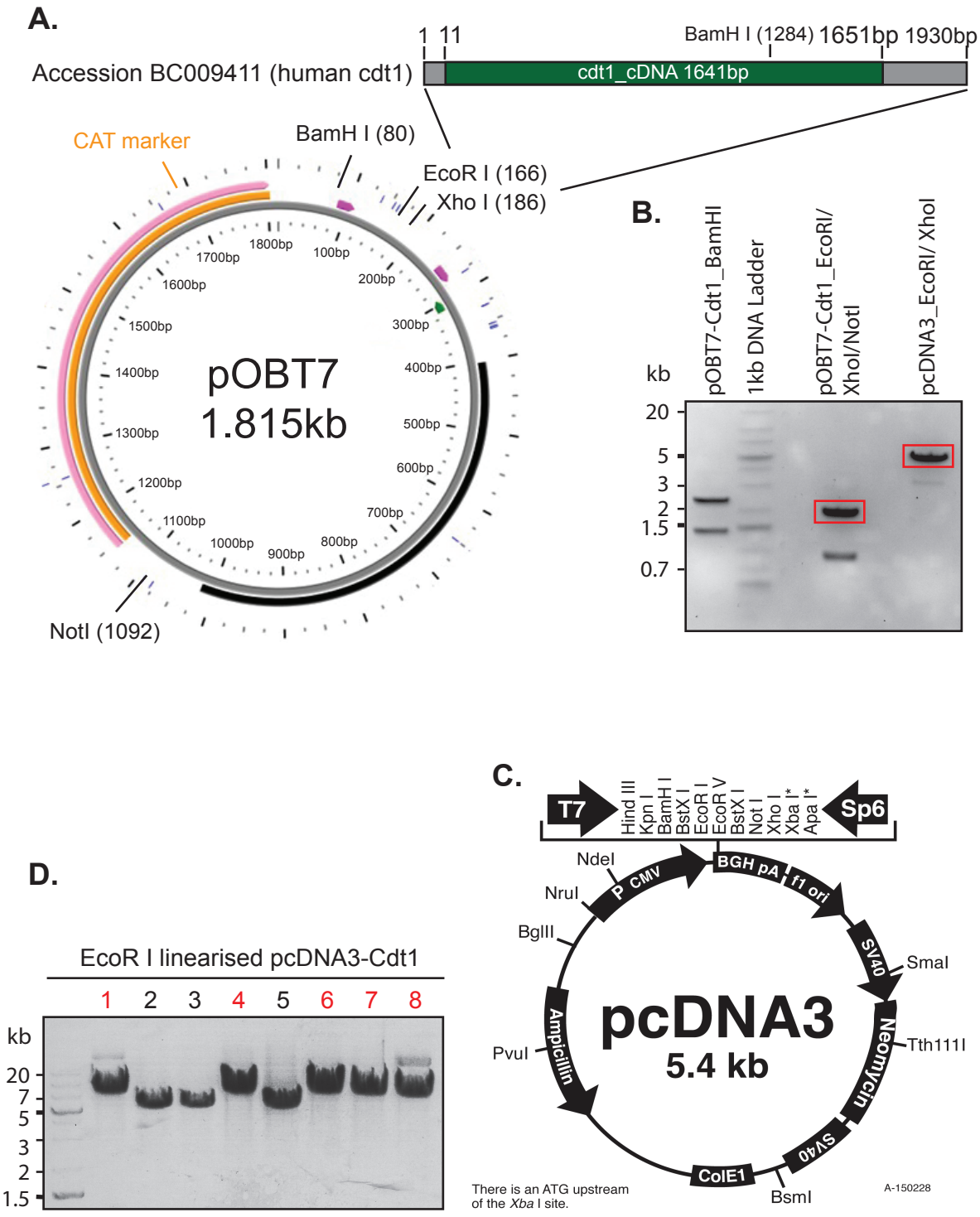


Figure IV. 1. Cloning of human Cdt1 into pcDNA3. Cdt1 was obtained in a pOBT7 vector and cloned into the EcoRI/ XhoI site of pcDNA3. **A.** pOBT7 map with relevant restriction sites and the Cdt1 construct. **B.** Agarose gel with the Cdt1 and linearised pcDNA3 restriction fragments (red box). **C.** pcDNA3 vector map. **D.** Agarose gel with EcoR I linearised plasmid DNA of eight colonies. Lane 1,4,6,7,8 shows pcDNA3 containing Cdt1 cDNA.

Figure IV. 2.

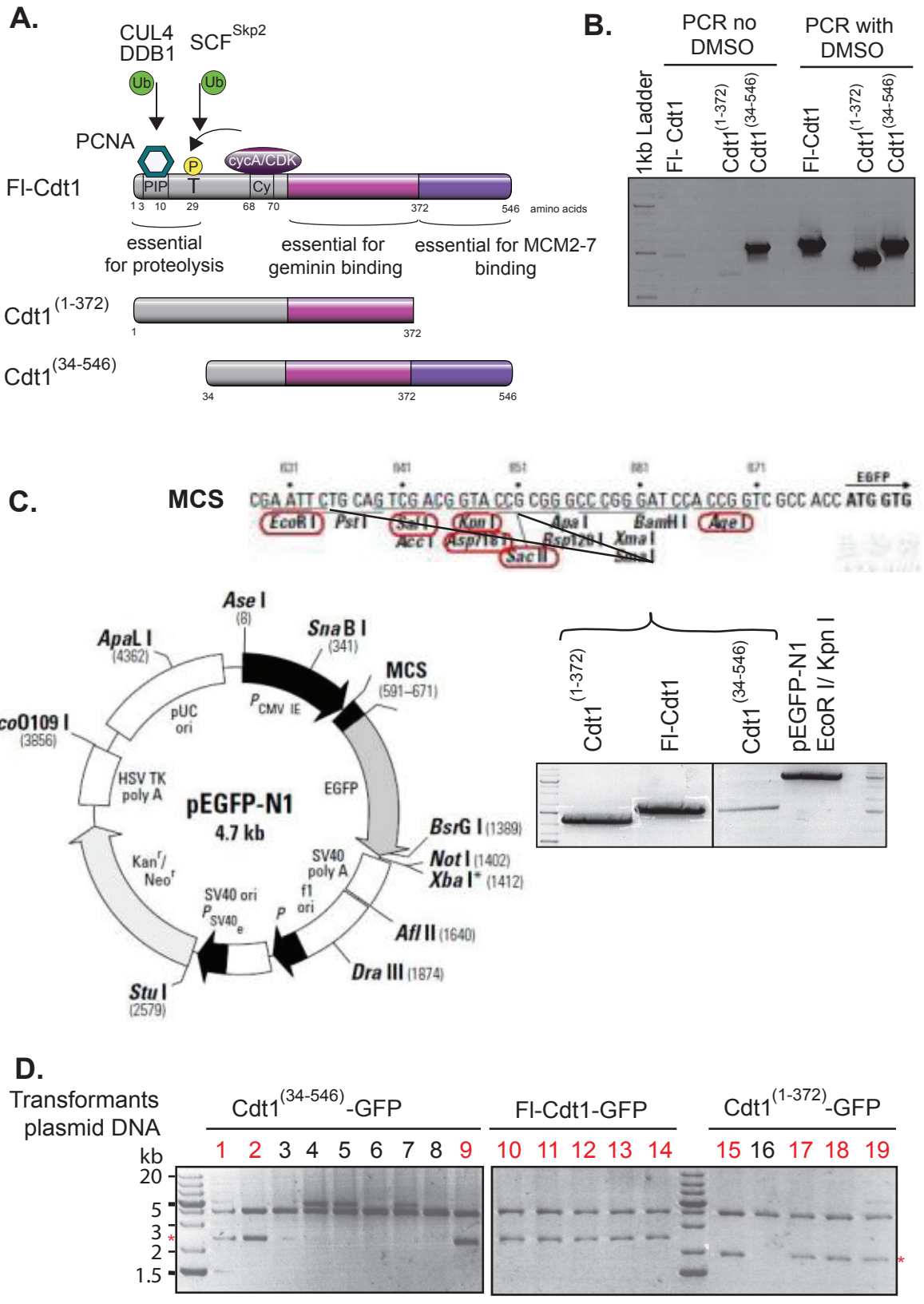


Figure IV. 2. PCR amplification of Cdt1 constructs and cloning into pEGFP-N1. Three Cdt1 constructs were PCR amplified from pcDNA3-Cdt1. PCR products were purified, cut with EcoRI/ KnpI and run on an Agarose gel with EcoRI/KnpI restricted pEGFP-N1. All fragments were gel purified and the Cdt1 constructs transferred into pEGFP-N1 via T4 ligase reaction. **A.** Functional domains of human Cdt1 and constructs used in this study. **B.** PCR reaction products with and without addition of DMSO. **C.** Vector map of pEGFP-N1 with MCS and PCR fragments before ligation. **D.** Transformants after ligation. Plasmid DNA was cut with EcoRI/NotI to check Cdt1-GFP presence. * Fl-Cdt1-GFP, Cdt1$_{(34-546)}$-GFP and Cdt1$_{(1-372)}$-GFP fragments.

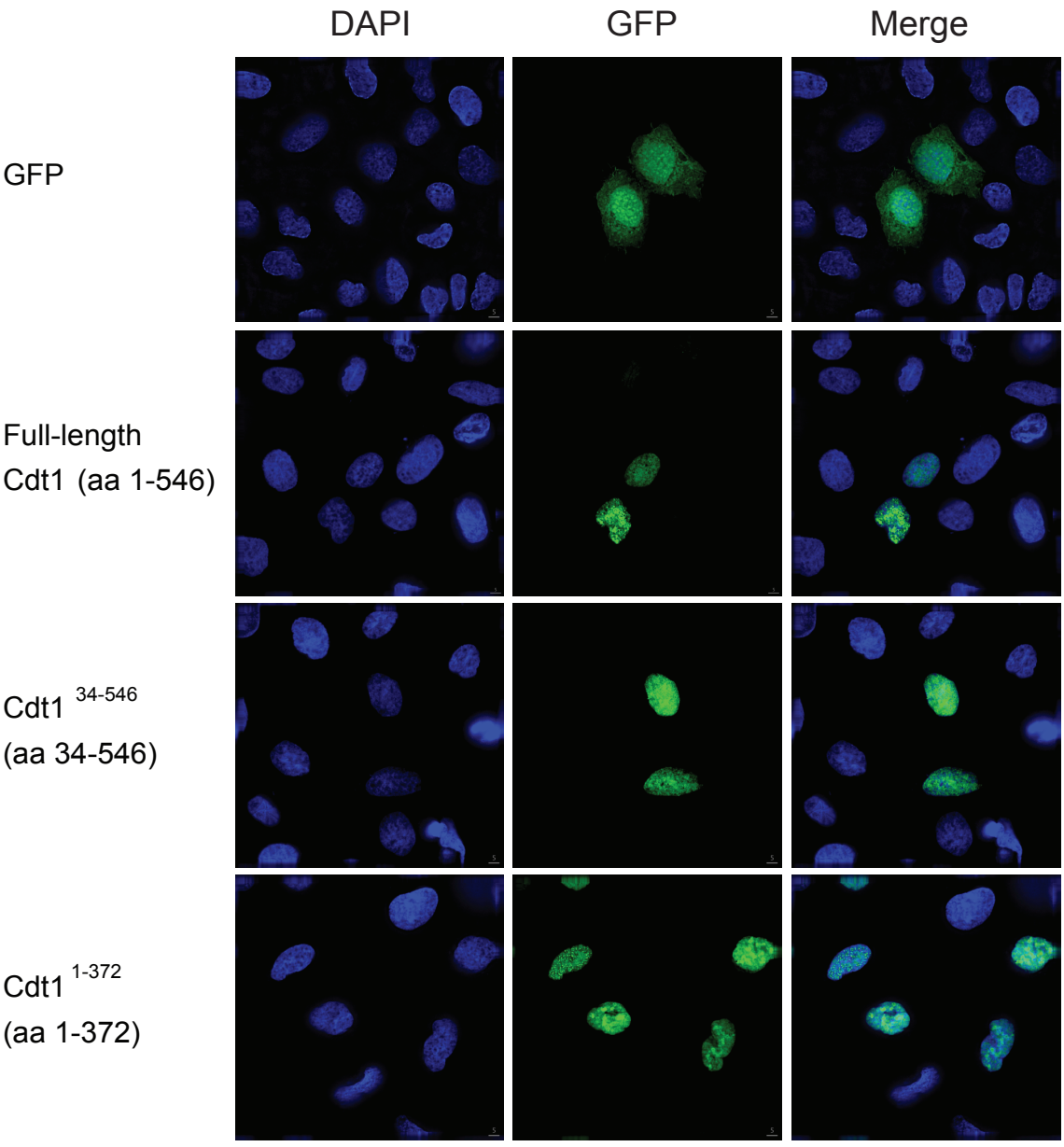


Figure IV. 3. Localisation of Cdt1 constructs. U2OS cells were transfected with Cdt1-GFP constructs or pEGFP-N1 for 24 h and then monitored by microscopy. The left panel shows the DAPI (nuclear) staining for all samples while the middle panel shows the GFP signal. On the right site the merge of both signals is shown.

IV. 2. 2. Cdt1 overexpression blocks replication in asynchronous U2OS and HeLa cancer cells

HeLa (cervical cancer cells) and U2OS (human osteosarcoma cells) cancer cells were transfected with empty pEGFP-N1 vector or one of the Cdt1-GFP fusion constructs and grown for 48 h. Two hours prior harvesting the cells were pulsed with the Thymidine analogue EdU (5-Ethynyl-2'-deoxyuridine) to monitor DNA replication. To investigate the impact of Cdt1 over-expression on DNA replication a flow cytometry assay was performed to determine the DNA content and EdU incorporation (Section II. 7. 3) (Figure IV. 4 A, Figure IV. 5 A). However due to a very low transfection efficiency no clear effect could be detected by analysing the entire cell population. Therefore green fluorescent protein (GFP) positive and GFP negative cells were considered separately (Figures IV. 4 and 5). When cells were transfected with control plasmids expressing GFP alone, the DNA content and EdU intensity was similar in GFP negative (black frame) and GFP positive (green frame) cells, suggesting that the levels of DNA replication are not significantly altered by GFP over-expression. In contrast the cells transfected with any of the Cdt1-GFP constructs exhibited significantly less EdU staining, and accumulated in G1 and early S-phase of the cell cycle (Figure IV. 4 and IV. 5). The GFP-negative cells in these populations provide an internal control for these experiments. Similar results were obtained in U2OS (Figure IV. 4 and IV. 5) and HeLa cell lines (Figure IV. 4. and IV. 5).

To investigate the inhibition of replication in more detail, cells were transfected as described above but 24 h post transfection EdU was added for 24 h to monitor DNA replication over a full cell cycle (Figure IV. 6). As expected around 90% of control cells (GFP negative and empty vector) incorporated significant amounts of EdU. In contrast only 25-30% of cells transfected with full-length Cdt1-GFP and ($Cdt1^{1-372}$)-GFP and 49% of cells transfected with ($Cdt1^{34-546}$)-GFP showed incorporation of EdU. Re-replication of DNA was never observed under these experimental conditions.

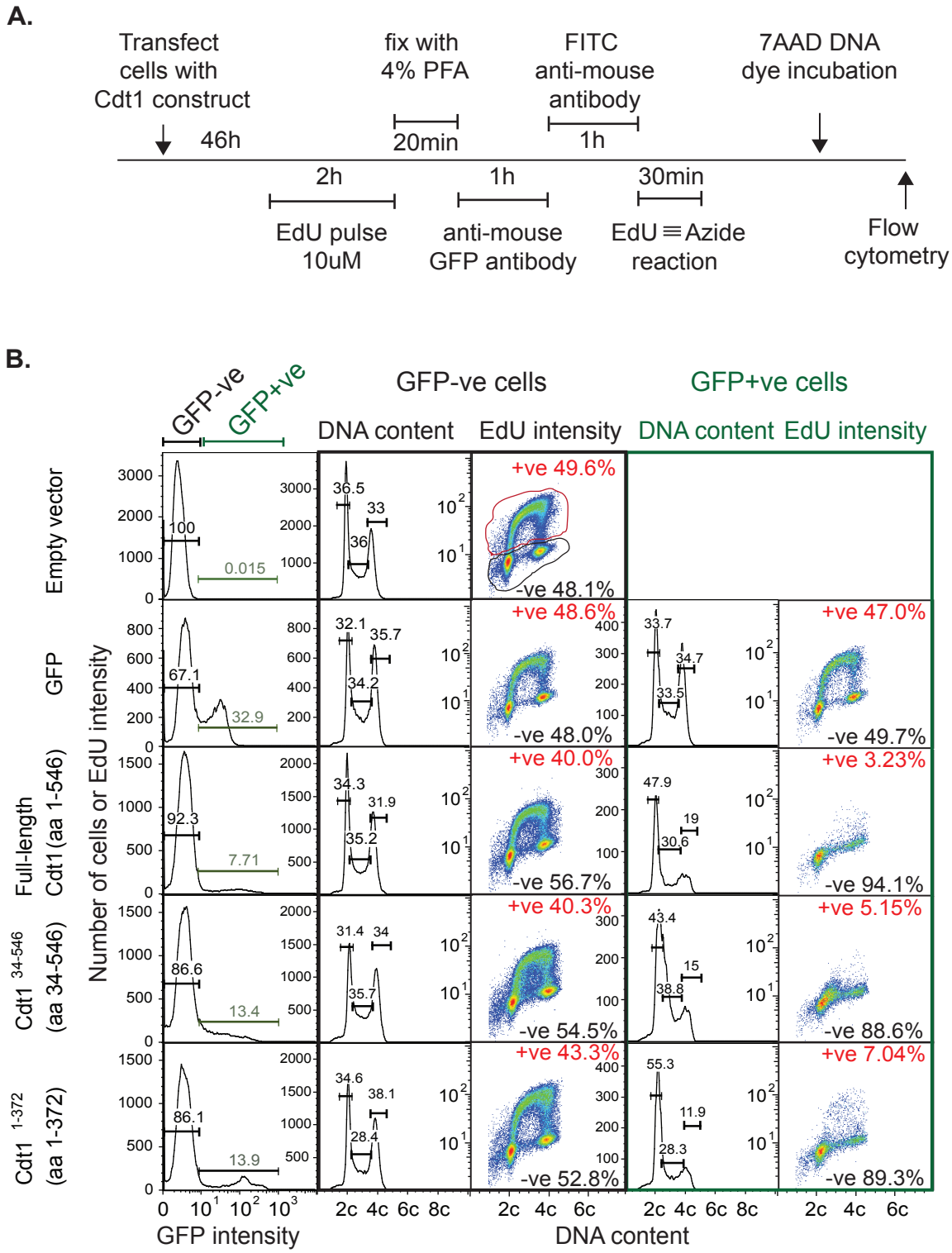
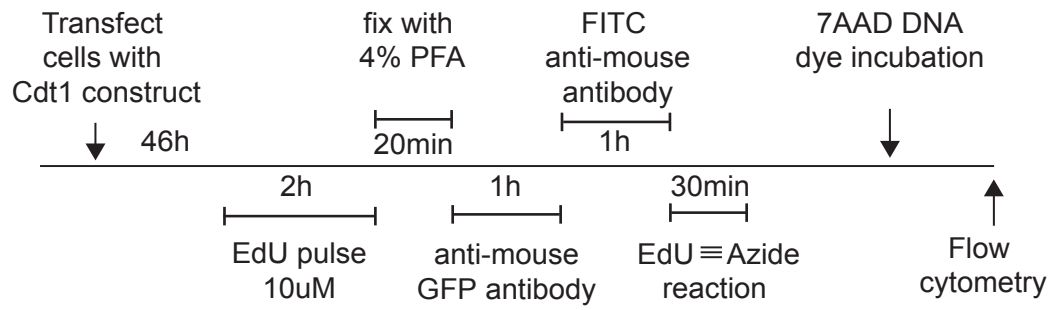


Figure IV. 4. Cdt1 over-expression blocks S-phase progression in U20S cells. U2OS cellswere transfected with empty expression vector or constructs containing GFP, full-length GFP-Cdt1, GFP-Cdt1$_{(34\text{-}546)}$ or GFP-Cdt1$_{(1\text{-}372)}$. 48 h later, cells were pulsed with EdU for 20 min and were then stained with anti-GFP antibodies, DNA content, and for incorporated EdU. **A.** Schematic of the experiment. **B.** Cells were then analysed by flow cytometry. Cells were separated by the GFP intensity (left columns) into GFP-ve (non-transfected or non-expressers, black frame) and GFP+ve categories (green frame). DNA content frequency graphs and EdU versus DNA content is shown for each category.

Figure IV. 5.

A.



B.

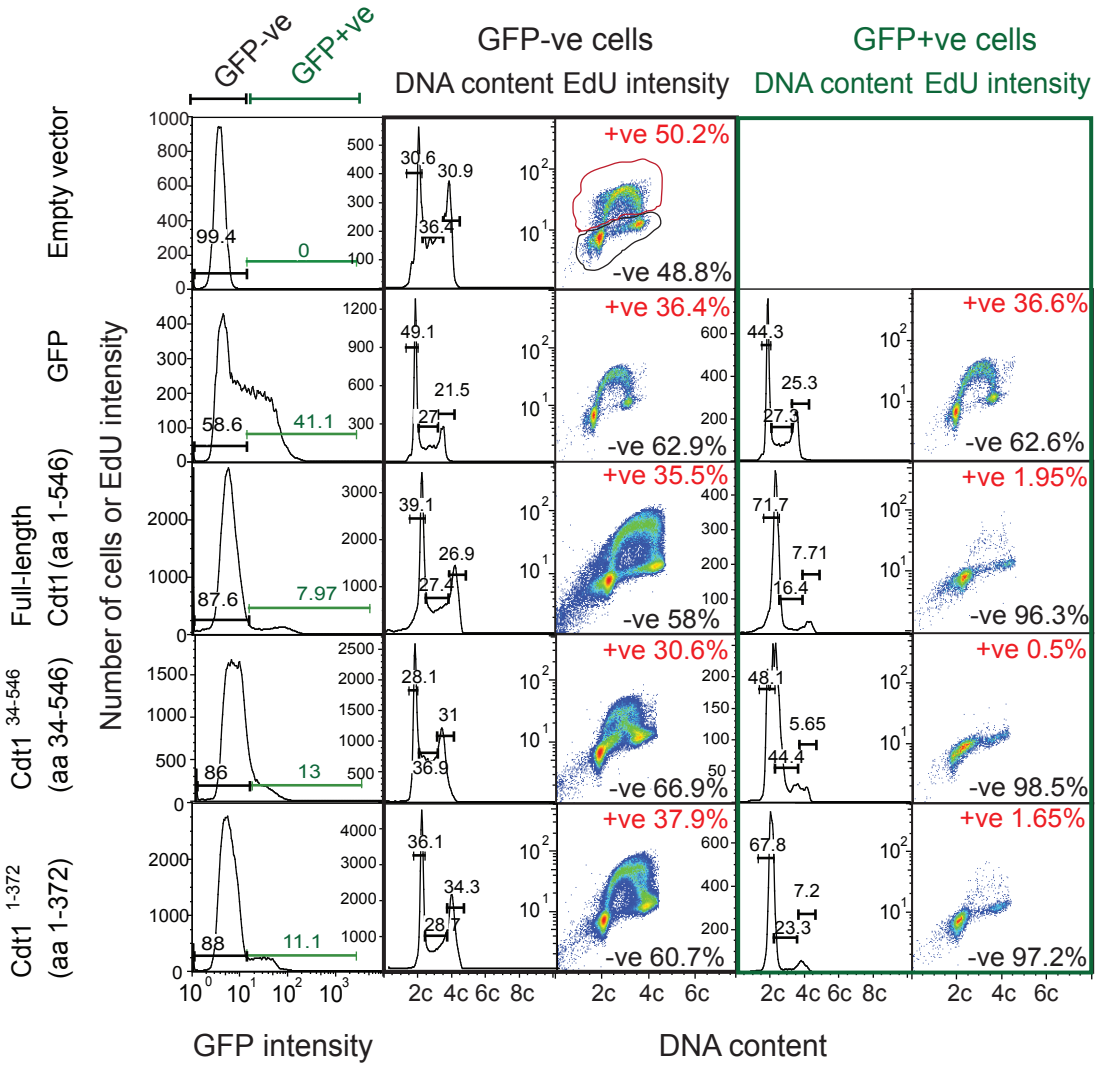
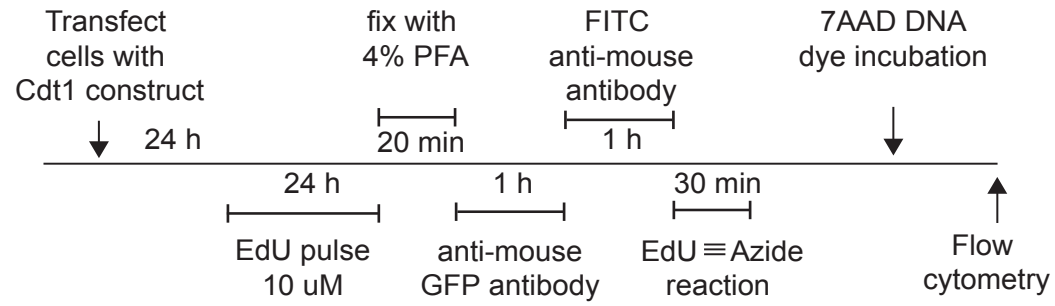


Figure IV. 5. Cdt1 over-expression blocks S-phase progression in HeLa cells. HeLa cellswere transfected with empty expression vector or constructs containing GFP, full-length GFP-Cdt1, GFP-Cdt1$_{(34-546)}$ or GFP-Cdt1$_{(1-372)}$. 48 h later, cells were pulsed with EdU for 20 min and were then stained with anti-GFP antibodies, DNA content, and for incorporated EdU. **A.** Schematic of the experiment. **B.** Cells were then analysed by flow cytometry. Cells were separated by the GFP intensity (left columns) into GFP-ve (non-transfected or non-expressers, black frame) and GFP+ve categories (green frame). DNA content frequency graphs and EdU versus DNA content is shown for each category.

A.



B.

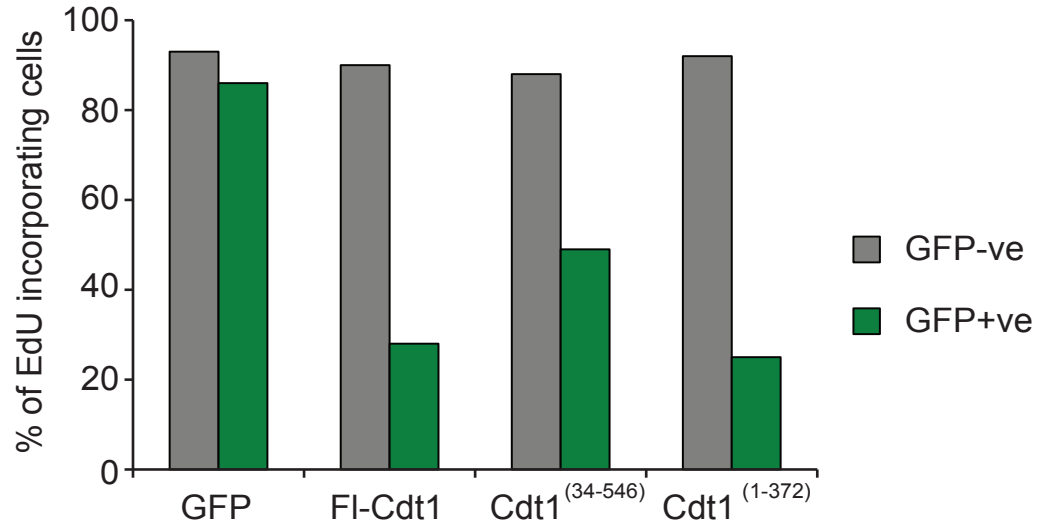


Figure IV. 6. Cdt1 over-expression inhibits DNA replication in a full U20S cell cycle. U2OS cells were transfected with pEGFP-N1 or full-length GFP-Cdt1, GFP-Cdt1$_{(34-546)}$ or GFP-Cdt1$_{(1-372)}$. 24 h later, cells were pulsed with EdU for 24 h and were then stained with anti-GFP antibodies, DNA content, and for incorporated EdU. **A.** Schematic of the experiment. **B.** Cells were then analysed by flow cytometry to monitor replication efficiency in a full cell cycle. Cells were separated by the GFP intensity into GFP-ve (non-transfected orrnon-expressers, grey bars) and GFP+ve (green bars).

IV. 2. 3. S- phase progression is blocked by Cdt1 over-expression

To further investigate the block in DNA replication seen in Cdt1 over-expressing cells, an experiment was designed to analyse S-phase progression in synchronised U2OS cells. The ($Cdt1^{34-546}$)-GFP construct, showing the weakest phenotype (Figure IV. 6) was selected and transfected into synchronised U2OS cells 10 h after Thymidine release (Figure IV. 7 A). Six hours post transfection the cells were treated again with Thymidine for 14 h and subsequently released into S-phase to monitor cell cycle progression via EdU incorporation at the indicated time points (Figure IV. 7 B). Whereas 50-80% of control cells advanced into S-phase, less than 5% of the ($Cdt1^{34-546}$)-GFP positive cells showed EdU incorporation after 6 h.

IV. 2. 4. Checkpoint activation is induced by Cdt1 over-expression

The eukaryotic cell cycle is tightly regulated by cell cycle checkpoints. These checkpoints control whether the processes in one cell cycle phase are accurately completed before entering the next phase. Many proteins such as cyclin E, H_2AX, Chk1 or p53 are regulated by these checkpoints and become inhibited or activated to stop cell cycle progression or induce apoptosis. To test for activation of cell cycle checkpoints, cells from the previously described experiments were analysed (Figure IV. 4 and IV. 7). Therefore cells were handled as described before and GFP positive and negative cells were separated by FACS (Figure IV. 8 and IV. 7 C). Whole cell lysates were prepared and analysed for the expression of indicated proteins. As shown in Figures IV. 8 and IV. 13, the Cdt1-GFP fusion protein levels were roughly 100 times higher than endogenous Cdt1 protein levels. Cyclin E levels fluctuate periodically through the mammalian cell cycle peaking in late G1 and early S-phase (Dulic et al., 1992). Therefore the high cyclin E protein levels (Figure IV. 8) correlate well with the increased percentage of Cdt1-GFP positive cells in G1/ early S-phase of the cell cycle observed by flow cytometry (Figure IV. 4 and 5).

Figure IV. 7.

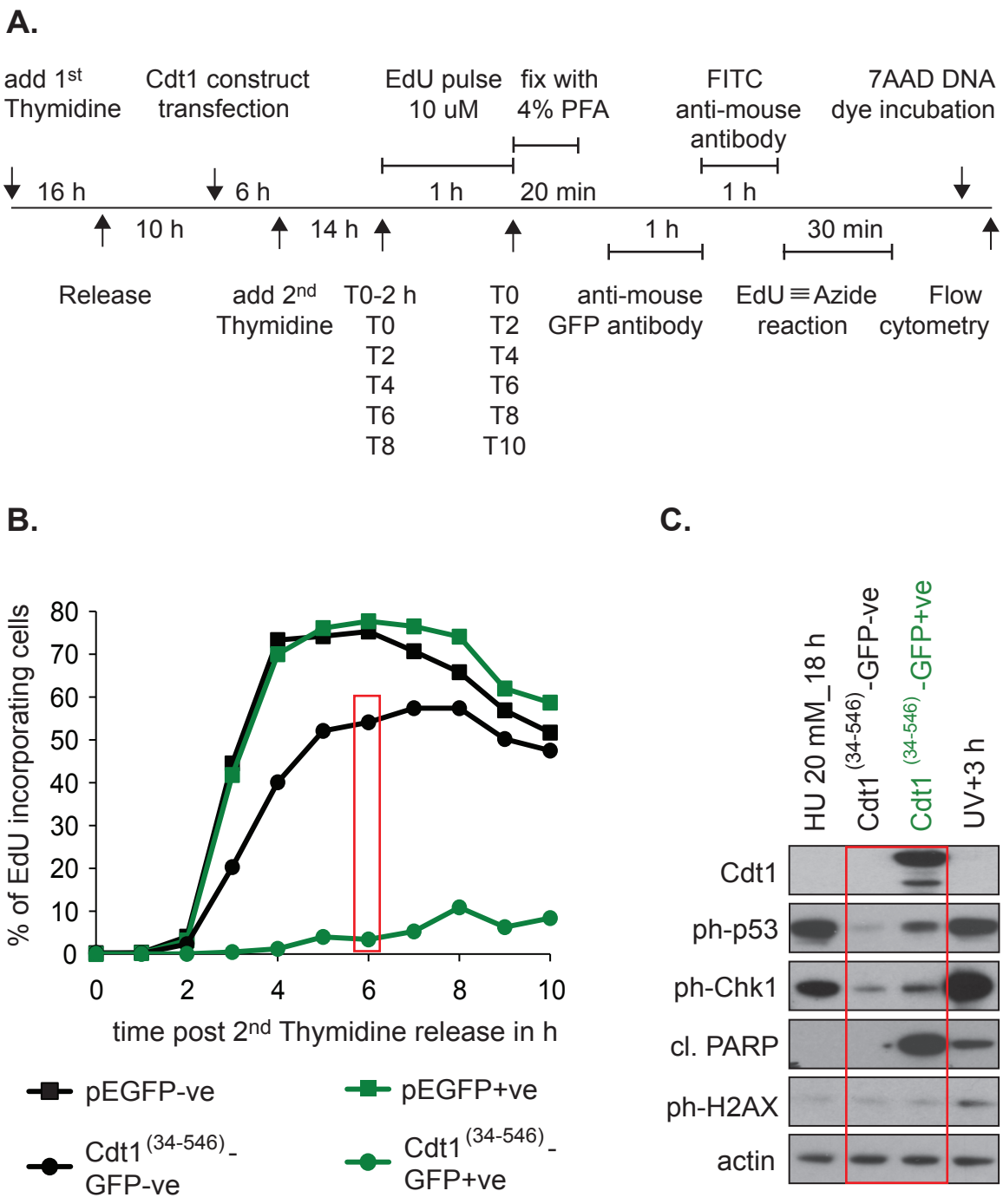


Figure IV. 7. Cdt1 over-expression blocks S-phase progression in synchronised U20S cells. U2OS cells were synchronised with 2.5 mM Thymidine for 16 h, released into S-phase and transfected with pEGFP-N1 or GFP-Cdt1$_{(34-546)}$. 6 h later, cells were treated again with Thymidine for 14 h and subsequently S-phase progression was monitored at different times by 20 min pulses with EdU. Cells were stained with anti-GFP antibodies, DNA content, and for incorporated EdU. **A.** Schematic of the experiment. **B.** Cells were then analysed by flow cytometry and gated for the GFP intensity into GFP-ve (non-transfected or non-expressers, black line) and GFP+ve categories (green line).**C.** The 6 h time point (red frame) of Cdt1(34-546)-GFP was also subjected to FACS and sorted for GFP-ve and +ve cells which were then used to prepare whole cell lysates and run on SDS-PAGE to immunoblot for the indicated proteins. As controls, extracts were prepared from U2OS cells 3 h after treatment with UV (120 mJ/cm) or 18 h after treatmentwith 20 mM hydroxyurea.

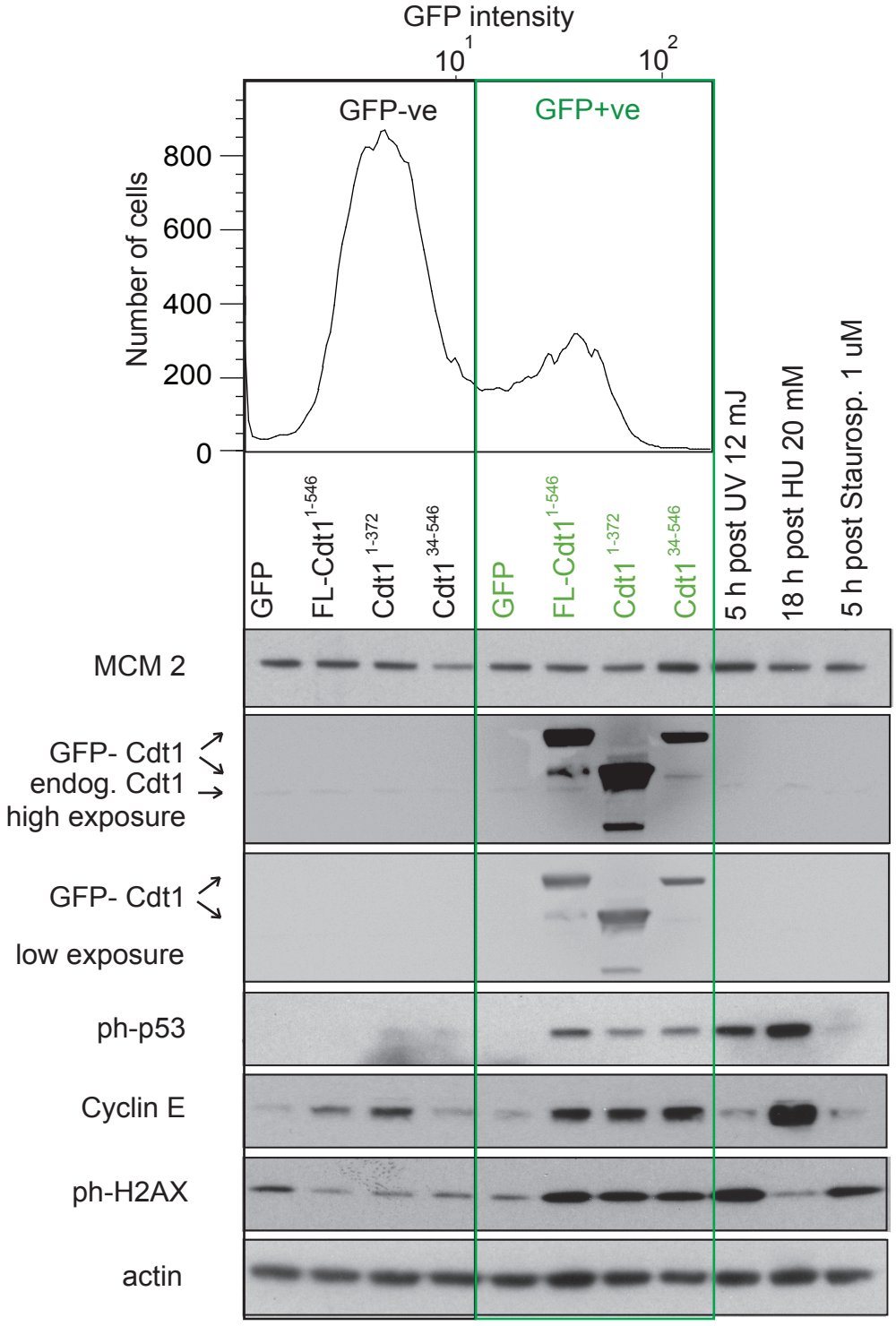


Figure IV. 8. Checkpoint activation following Cdt1 over-expression. U2OS cells were transfected with empty pEGFP-N1 or full-lenght Cdt1-GFP, Cdt1(1-372)-GFP or Cdt1(34-546)-GFP. 48 h later, cells were sorted according to GFP expression into GFP-ve (non-transfected or non-expressers) and GFP+ve categories (green frame). Whole cell extracts were then prepared and subjected to SDS-PAGE and immunoblotted for the indicated proteins. As controls, extracts were prepared from U2OS cells 5 h after treatment with UV (120 mJ/cm), 18 h after treatment with 20 mM hydroxyurea, or 5 h after treatment with 1 µM staurosporine.

The Cdt1 over-expressing cells also showed a rapid activation of Chk1 and p53 (Figure IV. 8 and IV. 7 C). Moreover 48 h post transfection (not 26 h post transfection Figure IV. 7 C) high levels of the DNA damage marker γ - H_2AX were observed (Figure IV. 8). The high H_2AX levels could also indicate fragmentation of the genome during apoptosis as the apoptosis marker cleaved PARP rapidly increased in Cdt1 over-expressing cells (Figure IV. 7 C)

In summary, these experiments revealed that Cdt1 over-expression led to checkpoint activation, inhibition of DNA replication and G1/ early S-phase arrest rather then re-replication of DNA.

IV. 3. NEDD8 ACTIVATING ENZYME INHIBITOR MLN4924 CAUSES RE-REPLICATION

Stabilisation of Cdt1 by interference with its degradation might be a less severe approach then over-expression of Cdt1 and thus might be a better tool to study Cdt1 induced re-replication. As described in detail in Section I. 6. 1. 1 mammalian Cdt1 degradation in S-phase is mediated by two ubiquitin ligases: a PCNA dependent one in a complex with Cullin 4 (CUL4) (Arias and Walter, 2006; Nishitani et al., 2006; Senga et al., 2006; Zhong et al., 2003) and a second dependent on SCF-Skp2 in complex with CUL1 (Li et al., 2003; Nishitani et al., 2006; Sugimoto et al., 2004). Cullin activity itself is dependent on its modification by Nedd8, which can be blocked by the small molecule inhibitor MLN4924 (Soucy et al., 2009) (Figure IV. 9 A).

IV. 3. 1. Overview of MLN4924 action on U2OS cells

MLN4924 is a small molecule inhibitor of Nedd8 activating enzyme (NAE) with a potential antineoplastic activity. The drug acts as an inhibitor of Cullin-RING ubiquitin ligase (CRL) activity by interfering with the neddylation of the Cullins (Figure IV. 9 A). For CRL activation Nedd8 reacts initially with ATP to bind to NAE, which in turn transfers Nedd8 to the Nedd8-conjugating enzyme E2 (N8E2).

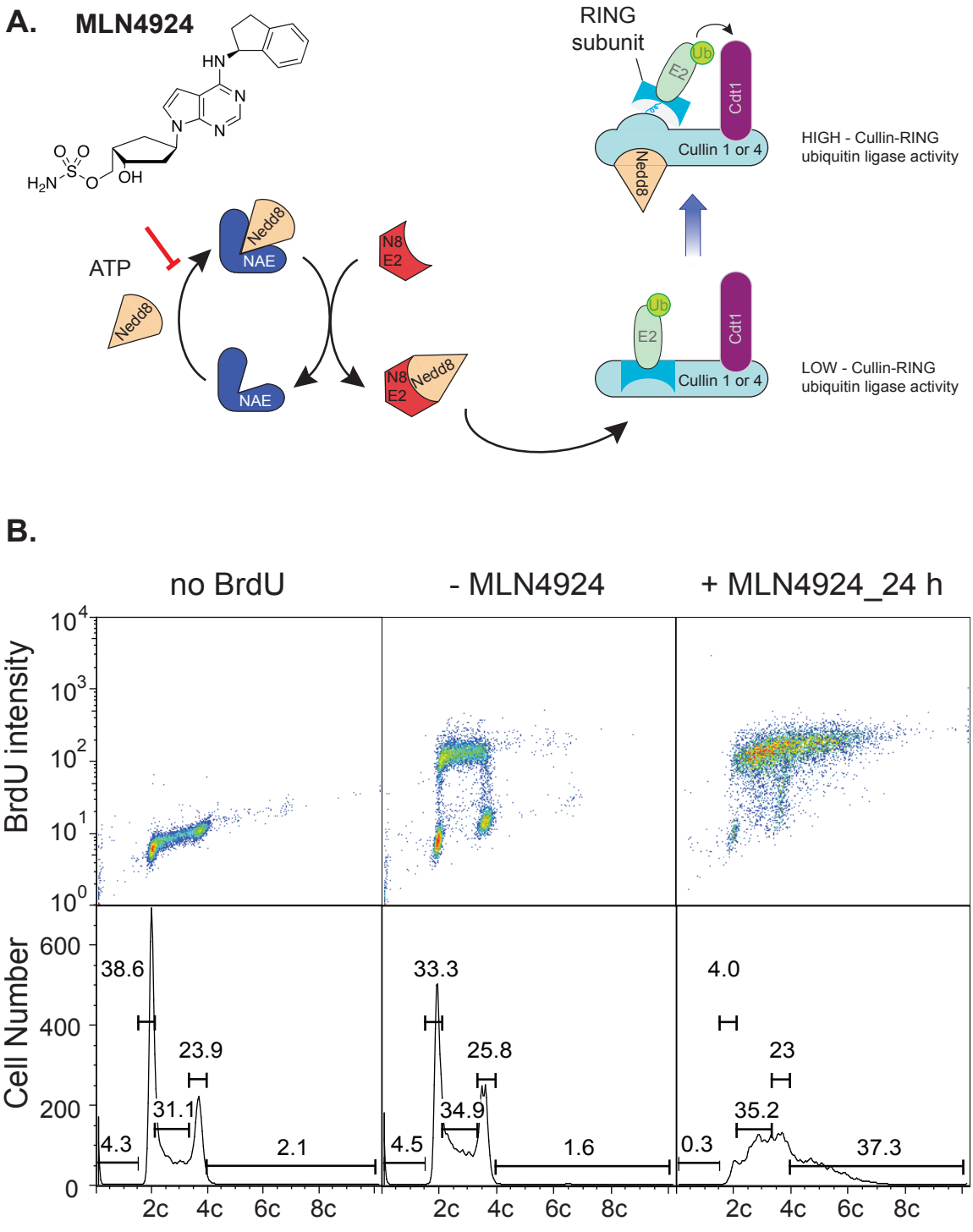


Figure IV. 9. Nedd8 activating enyme inhibitor MLN4924 on U2OS cells. A. MLN4924 inhibits Cullin-RING ubiquitin ligase (CRL) activity by interfering with the neddylation of the Cullins. Nedd8 activation of Cullins starts when Nedd8 reacts with ATP followed by binding to the Nedd8 activating enzyme (NAE). Without MLN4924 Nedd8 gets transfereed to the Nedd8-conjugating enzyme E2 (N8 E2) which transfers Nedd8 to the CRLs. CRL enzymes are important for the ubiquitination of protein substrates such as Cdt1 which subsequently becomes degraded. Without Nedd8 CRL activity is low but once Nedd8 is attached to the Cullins the conformation of the RING subunit changes and allows the ubiquitin to be transfered to the target protein. **B.** U2OS cells were treated with 1 μM MLN4924 for 24 h and pulsed with BrdU the resulting changes in the cell cycle profile were monitored by flow cytometry.

N8E2 transfers Nedd8 to the non-activated CRL, thus mediating a change in conformation of the RING subunit allowing the transfer of ubiquitin to CRL target proteins such as Cdt1. MLN4924 addition causes an immediate inhibition of Nedd8 binding to NEA and consequently low activity of CRLs (Soucy et al., 2009).

In agreement with previous studies (Lin et al., 2010; Milhollen et al., 2011; Soucy et al., 2009) addition of 1 μM MLN4924 to asynchronous U2OS cells for 24 h led to a significant increase in cells exhibiting a >4C DNA content (Figure IV. 9 B). In contrast with geminin induced re-replication, the BrdU profiles revealed a loss of a distinct G1 and G2/M cell populations.

IV. 3. 2. Time and dose dependency of MLN4924 induced re-replication

To analyse the mechanism by which MLN4924 induces re-replication, U2OS cells were treated with increasing levels of MLN4924 for 24 h and analysed by immunoblot or flow cytometry after pulsing cells with BrdU. Substantial re-replication of DNA was seen at concentrations of 0.5 - 2 μM MLN4924 causing Cdt1 levels to rise 2 - 5 fold (Figure IV. 10). This was associated with an enrichment of cells in S-phase, and a loss of G1 or G2/M cells. The lack of G2 cells is in marked contrast with previous results in geminin-depleted cells suggesting that unlike geminin depletion, Cdt1 stabilisation induces re-replication directly from S-phase without entering G2/M or a subsequent G1 phase. An increase in phospho-p53 and weak up-regulation of cleaved PARP was also detected at concentrations of 0.5 – 2 μM MLN4924 (Figure IV. 10 A) indicating a DNA damage response and an increase in apoptosis. Interestingly, concentrations higher than 1 μM led to an accumulation of cells in early S-phase with reduced BrdU intensity, similar to the results we obtained with massive Cdt1 over-expression (Section IV. 2).

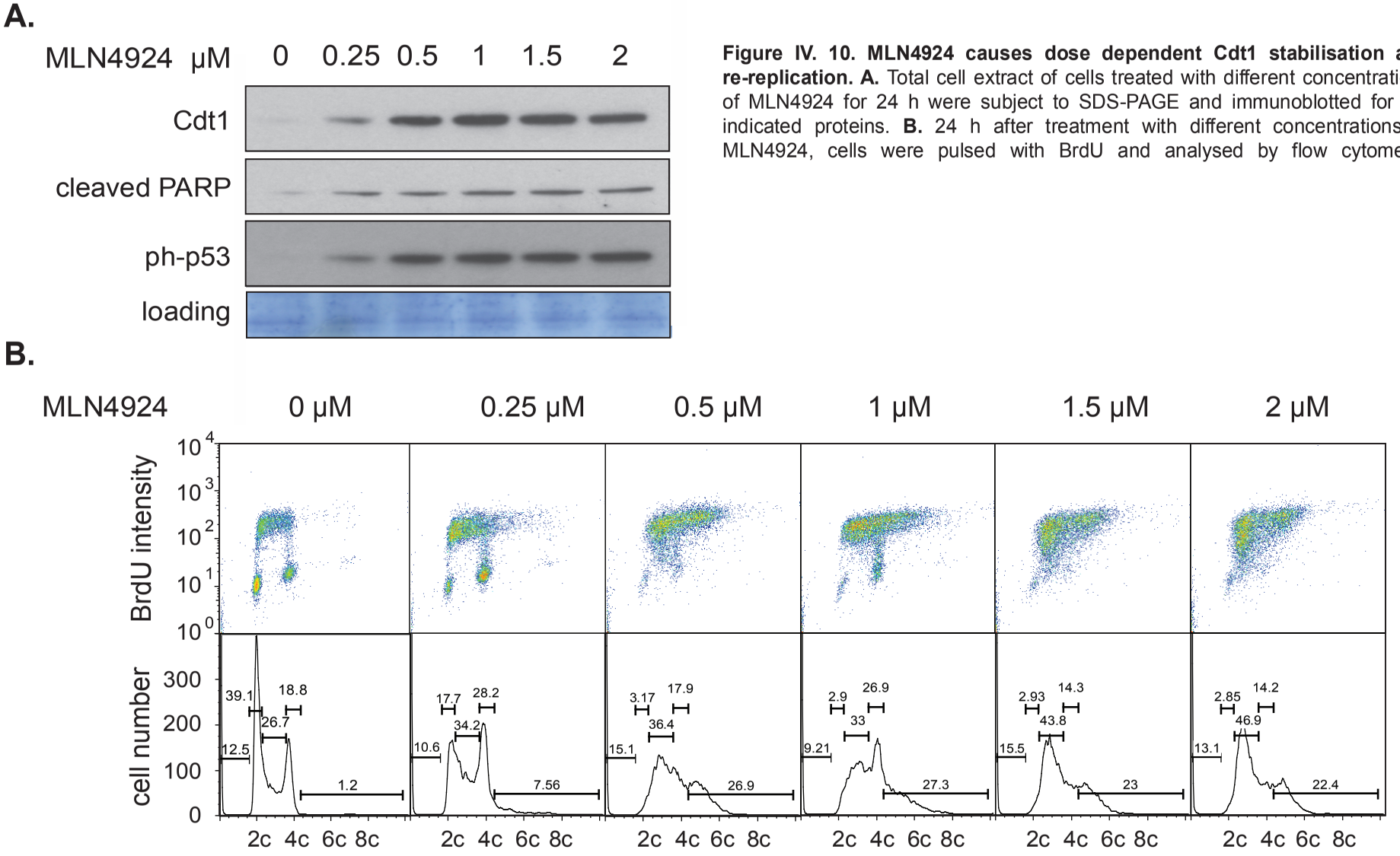


Figure IV. 10. MLN4924 causes dose dependent Cdt1 stabilisation and re-replication. A. Total cell extract of cells treated with different concentrations of MLN4924 for 24 h were subject to SDS-PAGE and immunoblotted for the indicated proteins. **B.** 24 h after treatment with different concentrations of MLN4924, cells were pulsed with BrdU and analysed by flow cytometry.

Figure IV. 10.

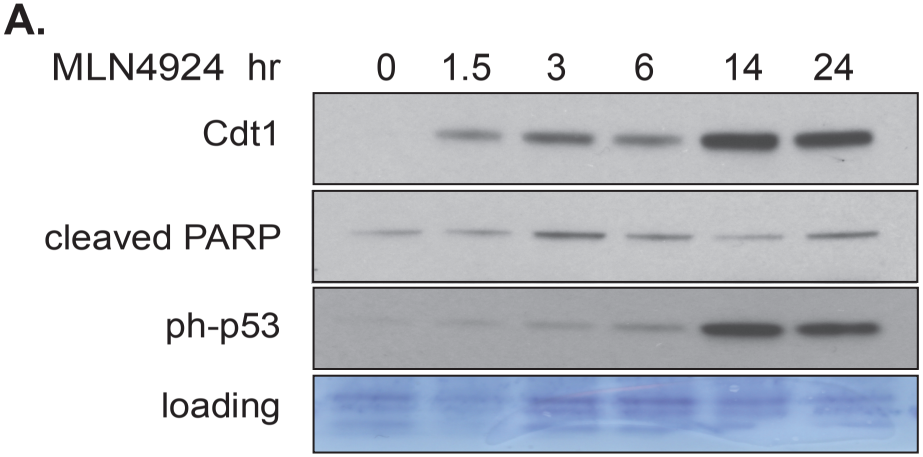


Figure IV. 11. MLN4924 causes a time dependent stabilisation of Cdt1 and re-replication A. Total cell extract of cells treated with 1 μM MLN4924 for different times were immunoblotted for the indicated proteins **B.** At different times after treatment with 1 μM MLN4924, cells were pulsed with BrdU for 30 min and analysed by flow cytometry.

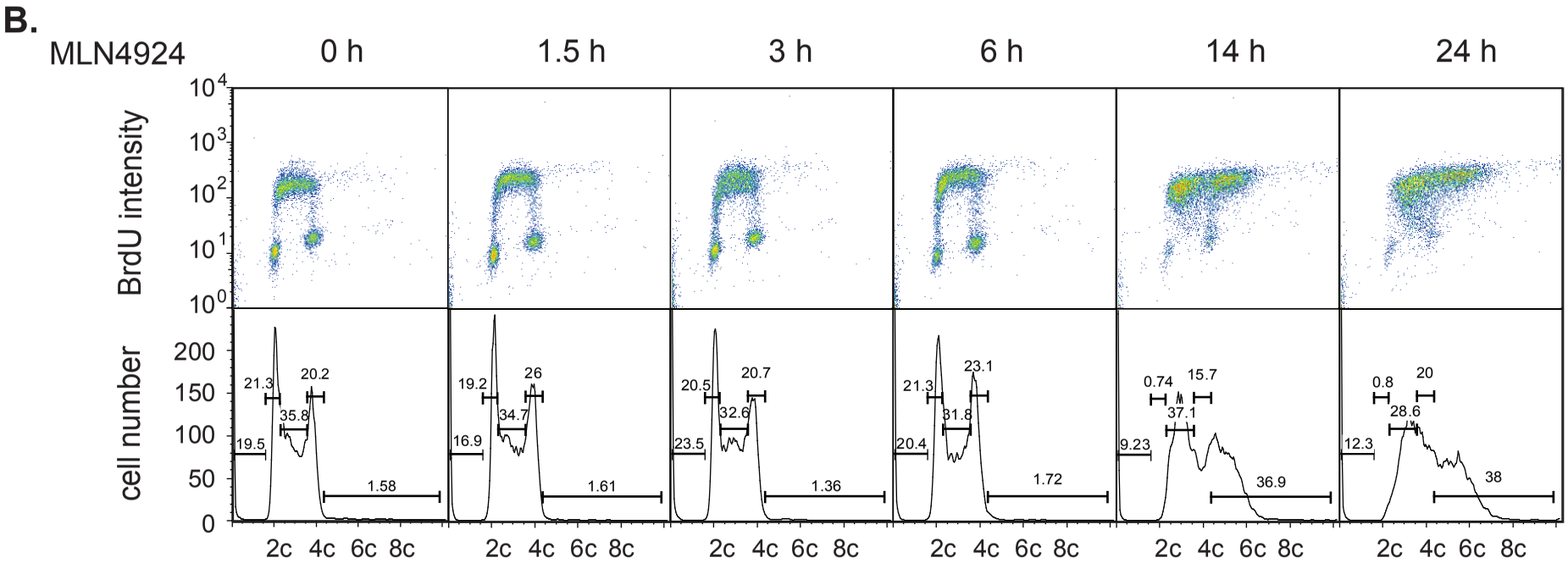


Figure IV. 11.

In order to provide further evidence that cells re-replicating in response to MLN4924 do not enter G2 phase, cells were treated with 1 µM MLN4924 for different times and pulsed with BrdU for 30 min prior harvesting (Figure IV. 11 B). Re-replication was detectable in cells treated with MLN4924 for 14 h and 24 h where they appear to continuously incorporate BrdU as they acquired >4C DNA content without passing through a G2 phase where no BrdU incorporation occurs. A strong increase of Cdt1 and phospho-p53 protein level was detected 14 h and 24 h post drug addition (Figure IV. 11 A).

IV. 3. 3. MLN4924 treatment results in loading of licensing and replication proteins onto chromatin

MLN4924 mediated stabilisation of Cdt1 causes re-replication most likely starting in S-phase. To investigate the mechanism behind this re-replication event, U2OS cells were synchronised with a double Thymidine block and then released into S-phase in the presence or absence of the drug. Prior to harvesting, cells were pulsed with BrdU for flow cytometry or trypsinised and subjected to CSK extraction based chromatin fractionation. Cells were collected after 3, 6 and 9 h where no measurable degree of re-replication was detectable by flow cytometry (Figure IV. 12 A). A DNA content of >4C within the cells was not seen before 14 h in previous experiments (Figure IV. 11). Cells re-firing origins within the first 9 h of S-phase would need more time to re-replicate to an extent detectable as a >4C DNA content providing the possibility to analyse chromatin composition at “the start” of re-replication. Immunoblots of whole cell lysate, supernatant and chromatin fraction showed a significant increase in Cdt1 levels throughout S-phase when MLN4924 was added (Figure IV. 12 B). The presence of Cdt1 on chromatin was accompanied by increased loading of MCM2 and PCNA. The increase of replication proteins on chromatin during S-phase upon drug treatment strongly suggests that re-replication already starts in S-phase.

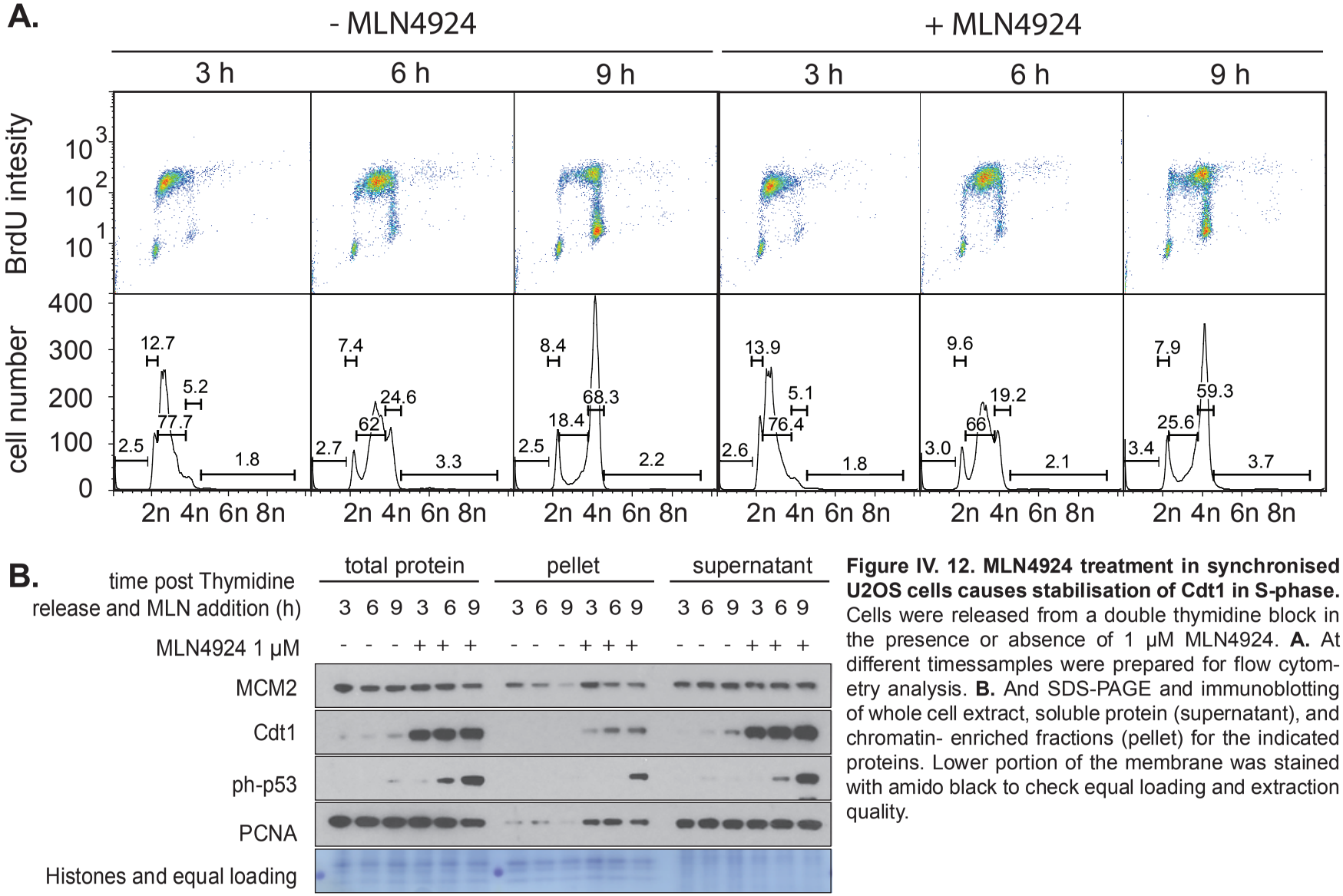
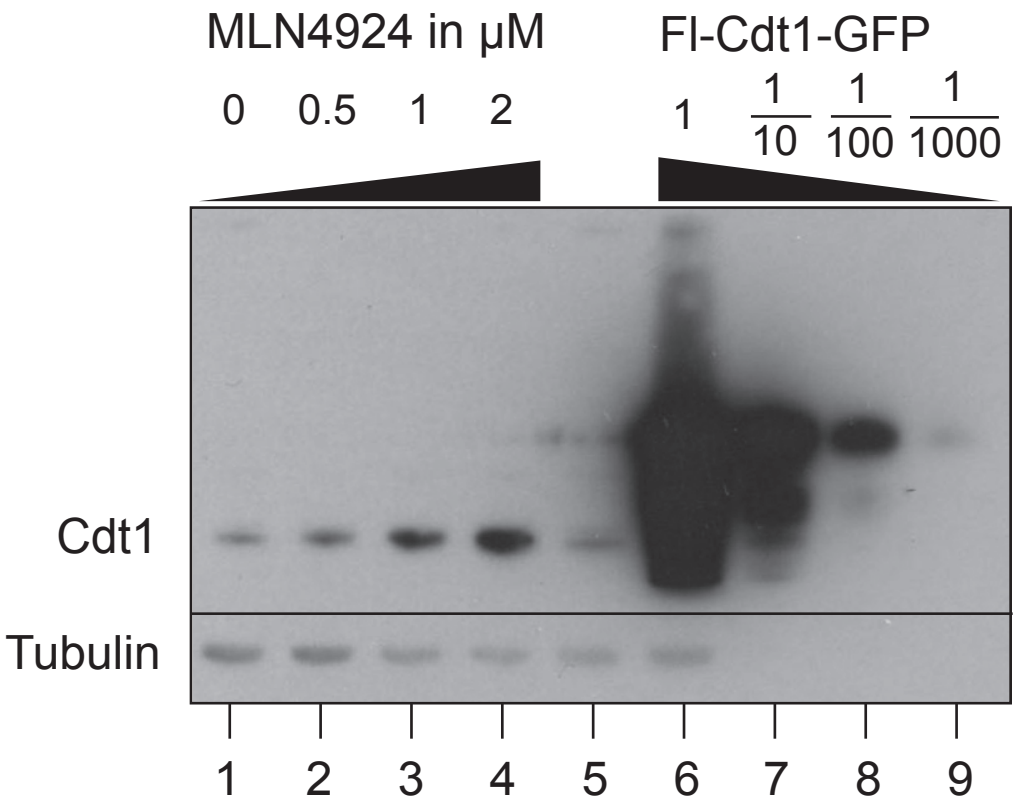


Figure IV. 12. MLN4924 treatment in synchronised U2OS cells causes stabilisation of Cdt1 in S-phase. Cells were released from a double thymidine block in the presence or absence of 1 µM MLN4924. **A.** At different timessamples were prepared for flow cytometry analysis. **B.** And SDS-PAGE and immunoblotting of whole cell extract, soluble protein (supernatant), and chromatin- enriched fractions (pellet) for the indicated proteins. Lower portion of the membrane was stained with amido black to check equal loading and extraction quality.

In contrast the recruitment of replication factors onto chromatin in S-phase was never observed in geminin depleted cells (Chapter III) indicating that re-replication cannot start efficiently before G2 due to a lack of Cdt1.

IV. 4. COMPARISON OF CDT1 OVER-EXPRESSION AND MLN4924 TREATMENT

Cdt1 over-expression caused a strong inhibition of DNA replication, while MLN4924 led to substantial levels of re-replication. How can these two very different phenotypes caused by stabilisation of the same protein be explained? In order to address this question, U2OS cells were treated with 0, 0.5, 1 and 2 μM MLN4924 or transfected with full-length Cdt1-GFP (Figure IV. 13). To compare Cdt1 expression levels whole cell lysates were prepared, normalised as shown by the equal tubulin signals and blotted for Cdt1. Consistent with previous results, MLN4924 caused a 2-10 fold increase in Cdt1 levels. In contrast, even a 100 times diluted full-length Cdt1-GFP extract exhibited higher levels of Cdt1 than lysates from cells treated with 2 μM MLN4924. It can be concluded that the very high levels of Cdt1 expression driven by the GFP expression vector causes very strong checkpoint activation and inhibition of DNA synthesis, whilst the lower levels of Cdt1 expression caused by MLN4924 allows significant re-replication to occur.



* this experiment was designed by me and Julian Blow for revision of Klotz-Noack et al.,2012 and performed by Debbie McIntosh due to my maternity break.

Figure IV. 13. Comparison of Cdt1 levels after MLN4924 treatment with Cdt1 over-expression. Cells were treated with either 0, 0.5, 1 or 2 µM MLN4924 and harvested 24 hr later (lanes 1-4). In parallel, cultures were transfected with constructs to express either GFP (lane 5) or full-length Cdt1-GFP (lanes 6-9), and after 24 h, GFP-expressing cells were isolated by FACS. Whole cell extracts were prepared and immunoblotted for Cdt1 and tubulin. To compare Cdt1 expression in MLN49234-treated cells and GFP-expressing cells, extract volumes were normalised to give equal tubulin signals (lanes 1-6). In order to assess the degree of GFP-Cdt1 overexpression, extracts of Cdt1-GFP expressing cells were diluted 10-, 100- and 1000-fold (lanes 7-9).

IV. 5. SUMMARY

In mammalian cells Cdt1 functions as a key regulator of the licensing machinery. Cdt1 levels increase in G2/M to mediate efficient loading of MCM2-7 complex onto chromatin after progression into anaphase. It is crucial that Cdt1 levels decline in late G1 and S-phase, as cells should not be able to load MCM2-7 complex once replication has started. In this Chapter of the thesis a number of different GFP-tagged Cdt1 constructs binding all to geminin but maintain different degrees of cell cycle regulation and licensing activity were massively over-expressed (>100 fold) in U2OS cells. Expression of all GFP-fusion proteins led to activation of the DNA damage checkpoint response and inhibition of EdU incorporation and S-phase progression. In contrast, when Cdt1 was stabilised with the 'neddylation' inhibitor MLN4924 the cells started to re-replicate their DNA directly from S-phase without entering G2/M or a subsequent G1 phase. This difference in response is likely due to the very different levels of Cdt1 over-expression in these treatments.

IV. 6. FUTURE DIRECTIONS

In order to investigate the relationship between the Cdt1 expression level and the resulting phenotype, the protein could be expressed with an inducible expression system like the Tet-ON or Tet-OFF system from Clontech or RheoSwitch from NEB. These would allow the induction of Cdt1 expression to different levels at specific times. Previous studies in the Blow lab showed that in *Xenopus* egg extracts addition of recombinant Cdt1 initially induced re-licensing and re-replication but at high levels inhibit DNA synthesis. The inhibition was accompanied by activation of the DNA damage response and appearance of DNA fragments caused by head-to-tail replication fork collision (Davidson et al., 2006). This could explain the rapid inhibition of DNA replication that occurred when Cdt1 is overexpressed to very high levels in mammalian cells.

Experiments showing the existence of re-replication induced DNA fragments in Cdt1 over-expressing cells could therefore provide evidence for head-to-tail fork collision as trigger for replication inhibition. Personal communication from Dimitris Xirodimas (Macromolecular Biochemistry Research Center, Montpellier) revealed that MLN4924 treatment of MCF7 cancer cells caused G1/ S-phase arrest such as seen when Cdt1 is over-expressed. Treatment of different cell lines with MLN4924 could therefore provide a good way to study the head-to-tail fork collision model.

In contrast to geminin induced re-replication within G2 phase (Section III. 9), MLN4924 treatment induces re-replication in S-phase. It is impossible to show low levels of re-replication in S-phase by flow cytometry as it is indistinguishable from 'normal' replication. However, BrdU labelling of synchronised MLN4924 treated S-phase cells followed by DNA isolation and Cesium Chloride centrifugation could yield fractions with heavy-heavy DNA thus providing evidence for re-replication in S-phase. Alternatively an elegant approach established by Dorn and colleagues using dual colour DNA fibre analysis could be used on MLN4924 treated S-phase cells. They pulsed cells with CldU (green) for 30 min and subsequently incubated them in fresh medium for 10 min before treatment with a second colour (IdU-red) for 10 min. Re-replication in response to Cdt1 over-expression caused 3-4 fold increase in yellow fibre tracks, which only appear if DNA is replicated in presence of CldU and IdU (Dorn et al., 2009).

The next Chapter reveals how re-replication induced by geminin depletion alters the genome. Solexa deep sequencing of >4C DNA from geminin depleted cells generated information about the sites of re-replication. It would be interesting to compare the genetic alterations of MLN4924 treated S-phase cells with geminin depleted >4C cells. It is likely that re-replication induced in G2 will give different sites of amplification than re-replication induced upon S-phase entry.

Chapter V

Results:

Genomic consequences of geminin depletion in U2OS cancer cells

V. 1. INTRODUCTION

Over the past few years great breakthroughs have been made in the field of cancer biology due to the availability of high-resolution detection of copy number changes. Platforms like deep sequencing or comparative genomic hybridisation enable researchers to compare the genetic composition of 'normal' cells versus cancer cells and provide knowledge about genetic alterations acquired by tumour cells during or before malignant transformation. Plenty of data for numerous cancers is available indicating that the genomic variations range from point mutations and single base deletion over deletions, translocations or amplifications of chromosomal regions up to loss or gain of whole chromosomes.

Re-replication as studied in Chapter III and IV of this thesis leads to amplification of genomic DNA and could therefore be an early or initial step in tumour formation. Consistent with this, recent data from *S. cerevisiae* shows that re-replication of a single origin induced by deregulation of MCM2-7 and Cdc6 leads to an increase in gene copy number of one or more (Green et al., 2010). Interestingly, the amplicons are bound by repetitive elements, which may be required for the homologous recombination of the amplicon in a head-to-tail orientation into the endogenous locus.

An important remaining question is where does re-replication start and whether it occurs at preferential sites within the genome or whether it is a stochastic event that takes place randomly across the genome. CGH analysis of geminin depleted *Drosophila* S2 cells revealed that re-replication occurs preferentially on heterochromatic regions (Ding and MacAlpine, 2010), while a study in mammalian cells showed re-replication on early replication origins such as Lamin B rather than in centromeric (often heterochromatin) or telomeric regions (Zhu et al., 2004). A third study revealed that re-replication from G2 phase occurs randomly without preference for early or late firing origins (Lu et al., 2010).

Given the controversial results in the field, this Chapter investigated the genetic composition of geminin depleted cells after substantial re-replication. Comparative genomic hybridisation and deep sequencing were applied to identify the sites of re-replication in cells with a >4C DNA content.

V. 2. COMPARATIVE GENOMIC HYBRIDISATION OF GEMININ DEPLETED VS. CONTROL CELLS

The results described in Chapter III of this Thesis showed that depletion of geminin leads to re-replication in human U2OS cells. To test if the re-replication occurs at preferential sites within the genome, we initially performed a CGH approach. Two different Agilent CGH microarray formats named (8x60K and 4x44K) were used during the course of this study. Both arrays use 60mer oligonucleotides, which are deposited uniformly onto specially prepared glass slides. The probes cover the entire human genome but probe spacing and therefore data resolution is different between the two arrays. The 8x60K microarray slide consists of 8 arrays providing 5.5 x 10^4 data points covering the entire human genome (3.04 x 10^9 bases) with 41 kb overall median probe spacing. The 4x44K microarray was performed by Agilent in Germany and consists of 4.2 x 10^4 data points with 43 kb overall median probe spacing (see Section II. 12 for specifications). To induce re-replication U2OS cells were released from a double Thymidine block after prior treatment with either control or geminin RNAi and grown for 48 h (see Chapter III). Subsequently cells were stained with Hoechst 33342 and subjected to FACS to isolate cells with a >4C DNA content in the geminin depleted and cells with a 2C DNA content in the control.

V. 2. 1. 8x 60K Sure Print G3 Human CGH Microarray

V. 2. 1. 1. Array setup and Quality Control

The DNA was isolated and quality as well as quantity were validated by agarose gel electrophoresis and NanoDrop® UV Spectrophotometer.

DNA from three biological replicates (A, B, C) was labelled with Cy3 for DNA from geminin depleted cells and Cy5 for DNA from control cells in three (A and B) or two (C) technical replicates. The Cy3 and Cy5 labelled DNA from each replicate was mixed and transferred onto the microarrays (for overview see Figure V. 1). Details about the data processing can be found in Methods Section II. 12. 3. To test the variability between technical replicates within these microarrays the Cy3/Cy5 ratio of all technical replicates was plotted against each other. The graphs in Figure V. 2 show that many data points did not correlate in the technical replicates. To further investigate why the data quality was so poor, plots for each wavelength were generated (Figure V. 2). The Cy3 median minus background values showed a good correlation between technical replicates except the data from array A1 where a loss of sample during the array assembly was observed (Figure V. 3). In contrast the Cy5 median minus background values showed poor correlation between the replicates especially among the low intensity values (Figure V. 4). This clearly shows that the low reproducibility between technical replicates is due to poor Cy5 signal quality.

Figure V. 1.

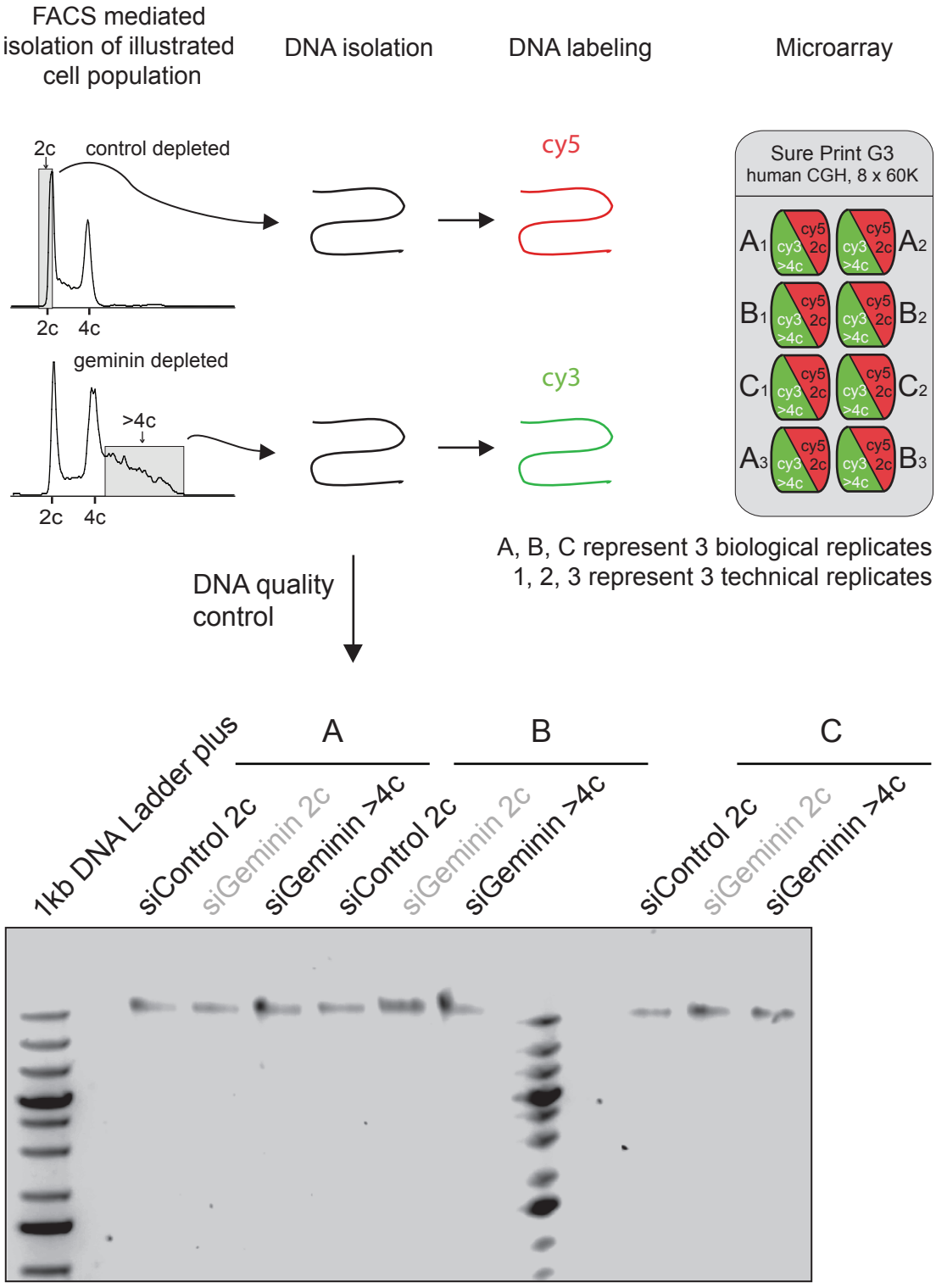


Figure V. 1. Array setup for preparation of 8x60K microarray. U2OS cells were released from a double thymidine block after prior treatment with either control or geminin RNAi, as in Figure III. 2. 2. 48 h later, cells were labeled with Hoechst 33342 for 30 min and indicated populations (2c, >4c) were seperated by FACS. Subsequently the DNA from each population of cells was isolated, quantified by agarose gel electrophorese and Nano Drop UV spectro-photometer and cy5 (2c) or cy3 (>4c) labeled according to Agilent instructions. 250ng input DNA was used per sample and per array.

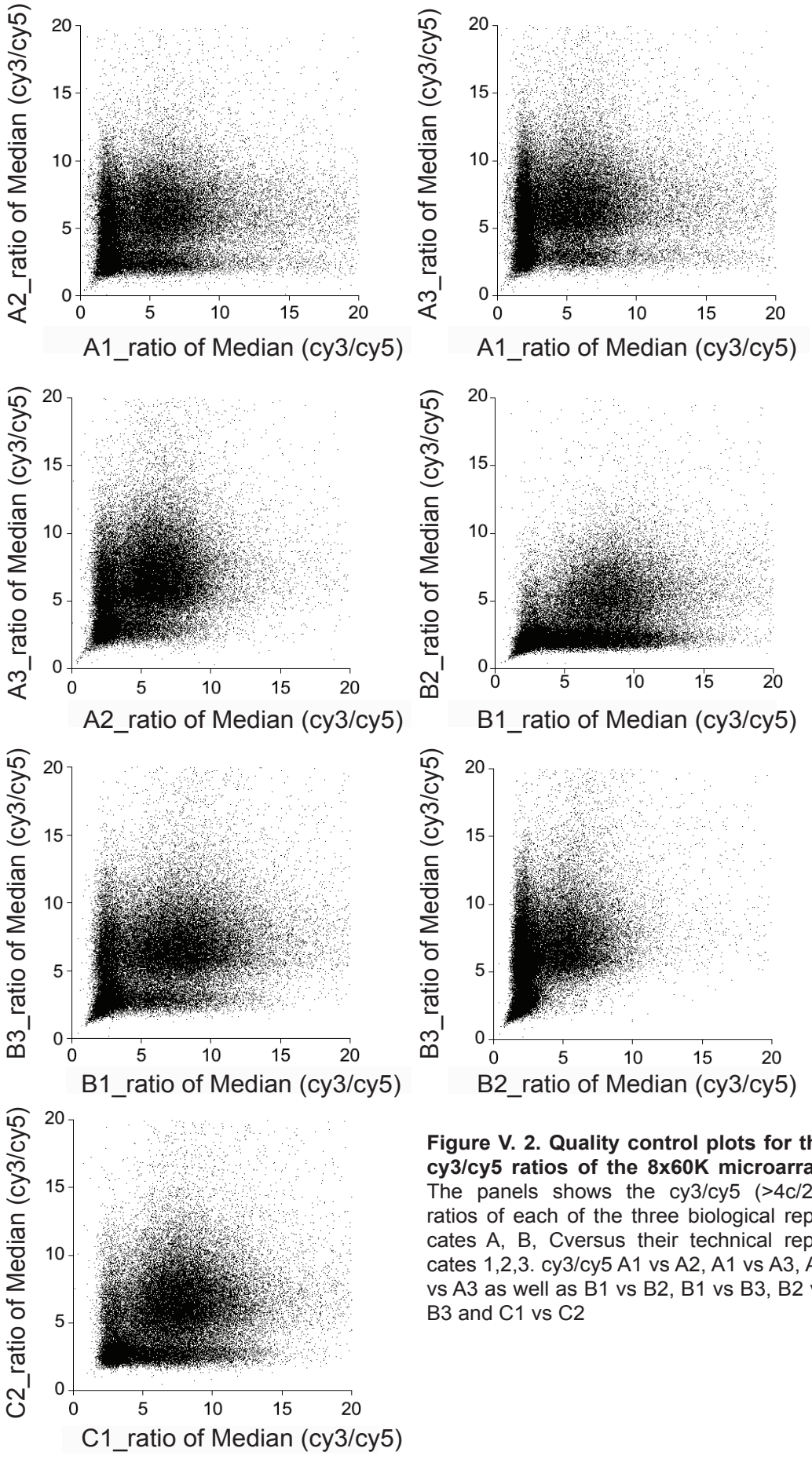


Figure V. 2. Quality control plots for the cy3/cy5 ratios of the 8x60K microarray. The panels shows the cy3/cy5 (>4c/2c) ratios of each of the three biological replicates A, B, Cversus their technical replicates 1,2,3. cy3/cy5 A1 vs A2, A1 vs A3, A2 vs A3 as well as B1 vs B2, B1 vs B3, B2 vs B3 and C1 vs C2

Figure V. 3.

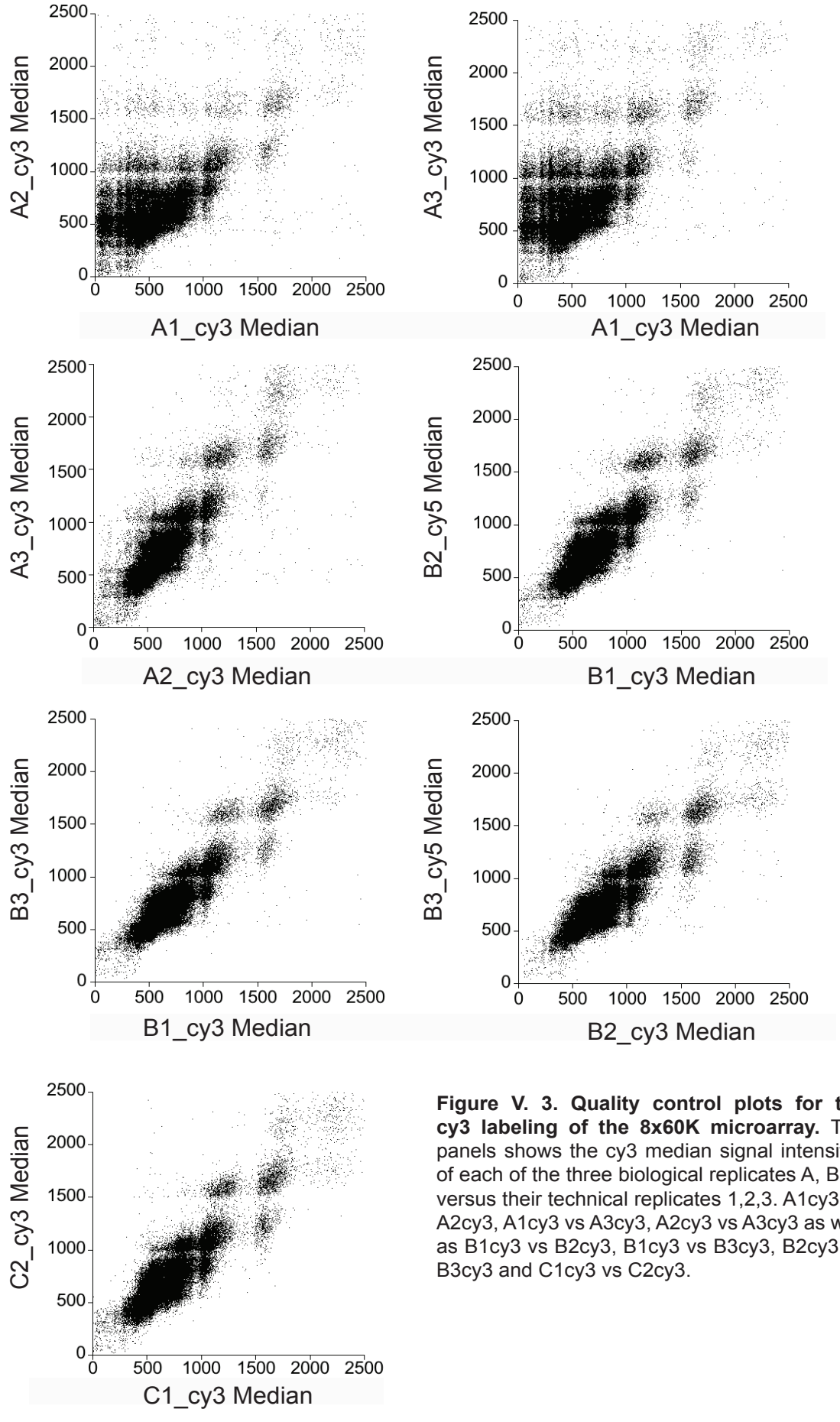


Figure V. 3. Quality control plots for the cy3 labeling of the 8x60K microarray. The panels shows the cy3 median signal intensitys of each of the three biological replicates A, B, C versus their technical replicates 1,2,3. A1cy3 vs A2cy3, A1cy3 vs A3cy3, A2cy3 vs A3cy3 as well as B1cy3 vs B2cy3, B1cy3 vs B3cy3, B2cy3 vs B3cy3 and C1cy3 vs C2cy3.

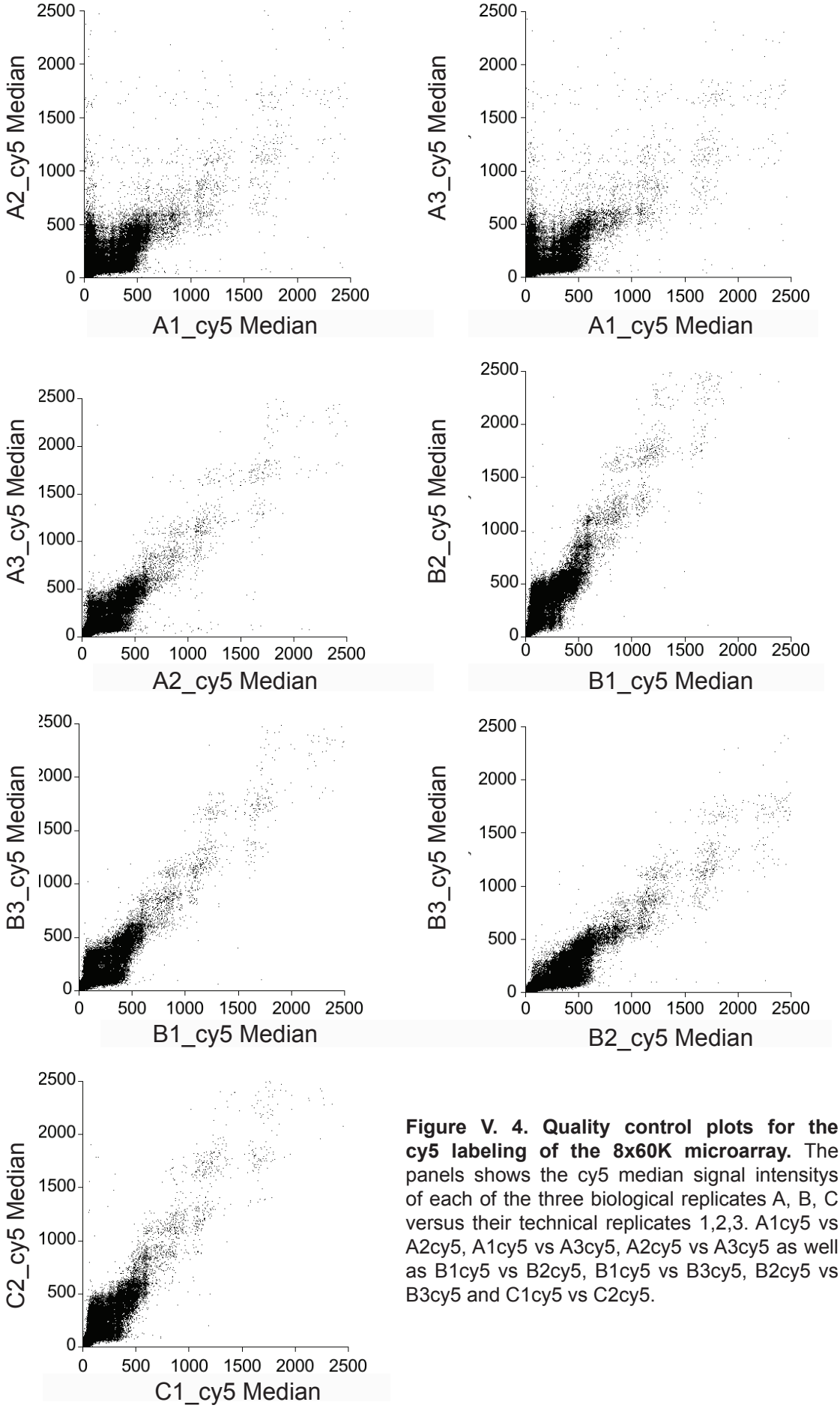


Figure V. 4. Quality control plots for the cy5 labeling of the 8x60K microarray. The panels shows the cy5 median signal intensitys of each of the three biological replicates A, B, C versus their technical replicates 1,2,3. A1cy5 vs A2cy5, A1cy5 vs A3cy5, A2cy5 vs A3cy5 as well as B1cy5 vs B2cy5, B1cy5 vs B3cy5, B2cy5 vs B3cy5 and C1cy5 vs C2cy5.

V. 2. 1. 2. Filtering of valid data points and normalisation

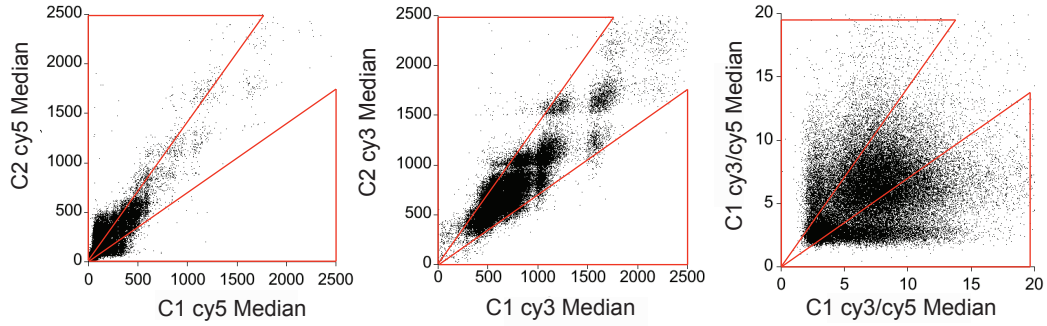
To rescue some data from this microarray experiment all unreliable (poorly correlated) points were extracted and excluded from the data set. In the best case the Cy5/Cy5 ratio between technical replicates should be around one. In contrast our results showed many outliers and in collaboration with Nick Schurch from the Data Analysis Group of the University of Dundee, the following criteria for validity of data had been set. If the Cy5/Cy5 ratio between technical replicates was greater than 1.3 or smaller than 0.7, the data point was invalid and assigned with a '0' in the excel sheet. If it was within the defined range it was assigned with the median signal intensity value. Subsequently a filter was programmed, excluding all rows containing a '0'. All remaining data points therefore were valid and used for further analysis (Figure V. 5).

A more detailed description how invalid data points were excluded from further analysis can be found in Section II. 12. 3.

To account for the generally lower Cy5 labelling standard probes provided on the array were used for normalisation, the median Cy3/Cy5 ratios of those probes were calculated for each array (between 2.1 and 2.6) and subsequently the valid Cy3/Cy5 ratios for each array were normalised by that value.

Figure V. 5.

A.



General idea to mark invalid data

=IF(logical_test,[value_if_true], [value_if_false])

The logical test is based on the required reproducibility between two technical replicates on one array. Figure V. 3 and 4 as well as the plots above show that the cy5 rather then the cy3 labeling causes the poor reproducibility between cy3/cy5 ratios of technical replicates shown in Figure V. 2. Therefore the data was filtered based on the cy5/cy5 ratio between two technical replicates. If the ratio of for example C1cy5/C2cy5 is greater then 1.3 or smaller then 0.7 the data point is invalid and assigned with a 0. If the ratio is within the range it is assigned with the median signal intensity value.

Example for C1 and C2

=IF(AND(C1cy5/C2cy5<1.3,C1cy5/C2cy5>0.7),C1cy5/C2cy5,0)

C1cy5/C2cy5	filter
0.6048	0
1.175	1.175

Excluding invalid data points

The data was then filtered simply by selecting the filter column with its zeros and value-sand set the filter on “Does Not Equal” 0 thus excluding all invalid data points resulting in the quality plots below. About 50% of the ~50000 data points were valid and used togenerate the cy3/cy5 ratio (>4c/ 2c) plots for each array covering whole human genome (Figure V. 6-8).

B.

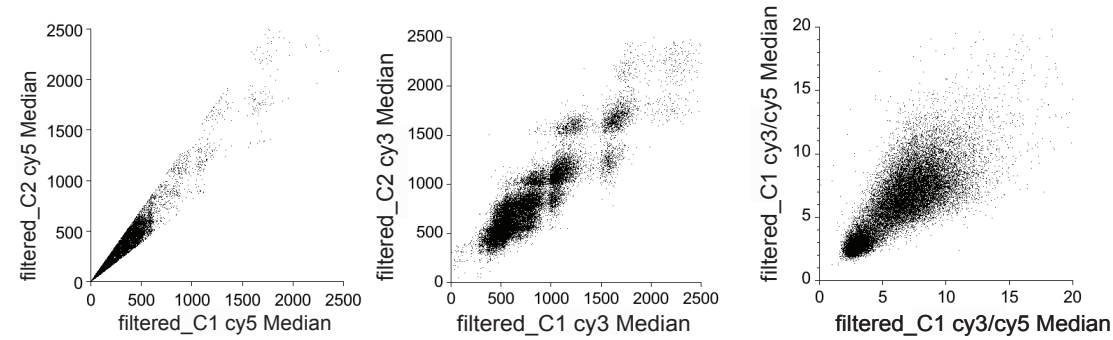


Figure V. 5. Method to exclude invalid data points. This Figure descibes how we dealt with the poor correlation between two technical replicates due to poor cy5 labeling in order to gain knowledge from this cost intensive experiment. We excluded invalid data points based on the criteria described in the text. **A.** Shows the cy3 vs cy3, cy5 vs cy5 and cy3/cy5 vs c3/cy5 plots from array C1 vs C2. The red triangles mark the areas containing anti-correlating data points between two technical replicates. **B.** Shows the plots of A. after filtering invalid data points. Those data points were used for further analysis.

V. 2. 1. 3. Whole genome analysis of geminin depleted vs. control

By plotting the ratios for geminin depleted/ control cells (>4C/2C) over the whole genome (Figure V. 6-8) the data looked very promising. Only specific regions within the whole genome seemed to be amplified reproducibly between technical and biological replicates (compare Figures V. 6-8). This would suggest that re-replication in geminin depleted cells takes place at specific regions. In those areas up to a 4-fold increase in copy number were observed, which were surrounded by areas showing no amplification at all (values around 1).

However, before these regions of amplification were further analysed by fluorescence *in situ* hybridsation (FISH) or quantitative PCR (qPCR) analysis, the data should be reproduced by an additional microarray, as the technical problems with the array that necessitated the removal of many data points raised doubts about the reliability of the data. No obvious reason for the low quality of Cy5 signal intensity could be found as the labelling quality as well as the concentration of the Cy5 labelled DNA sample was well in the range demanded by the manufacturer. Therefore Agilent Technologies kindly offered to run a 4x44K human genome array with >4C geminin depleted and 2C control DNA in their research facility in Germany to check reproducibility of this low quality array.

Figure V. 6. Whole Genome data for Array A2 and A3. The >4c/2c ratio of geminin depleted vs control depleted cells is shown for Array A2 and A3. A black dot represents one of the remaining 28349 valid data points that represent distinct biological features covering the whole human genome (array contains a total of 55077 distinct biological features).

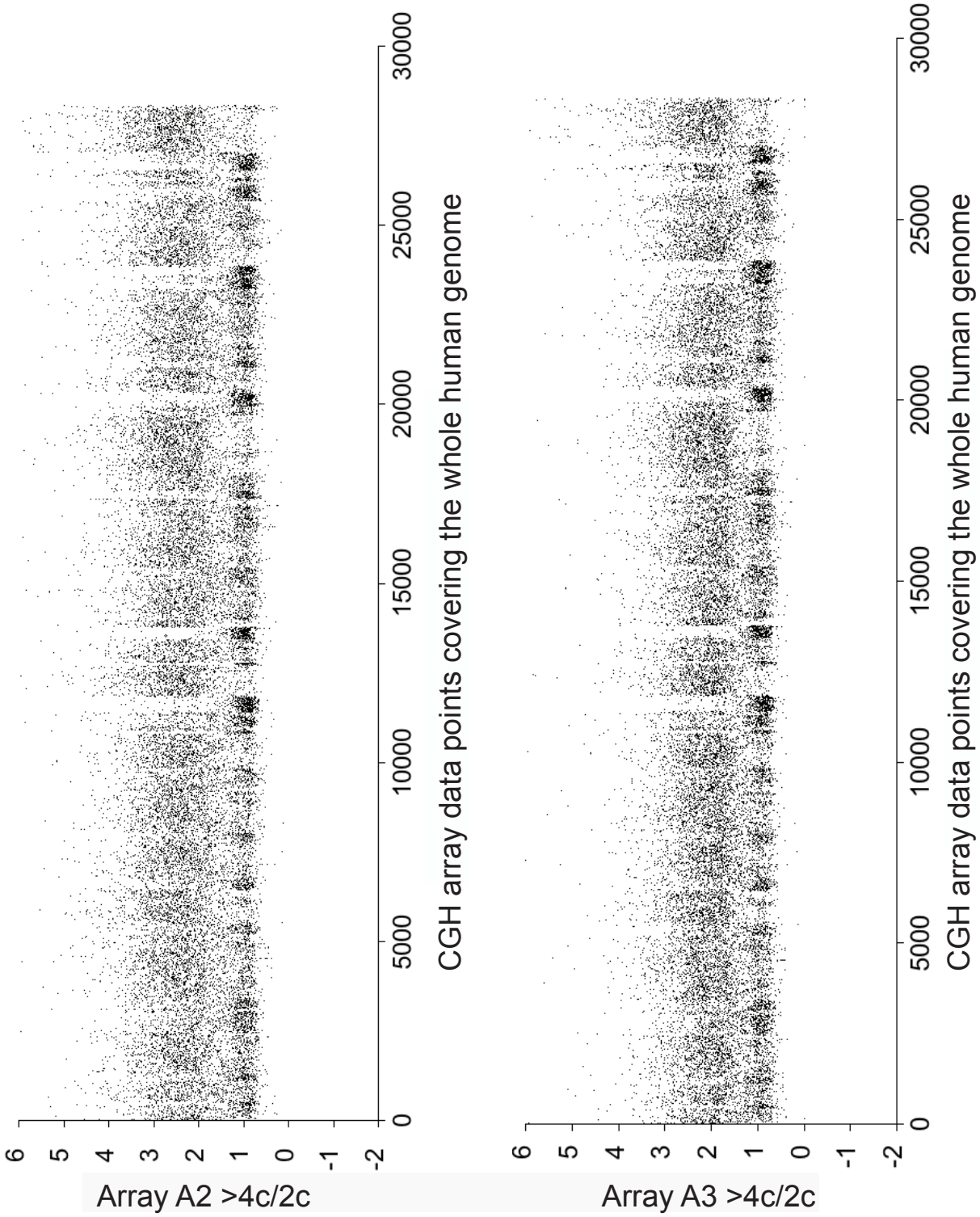


Figure V. 7. Whole Genome data for Array B1 and B3. The >4c/2c ratio of geminin depleted vs control depleted cells is shown for Array B1 and B3. A black dot represents one of the remaining 26944 valid data points that represent distinct biological features covering the whole human genome (array contains a total of 55077 distinct biological features).

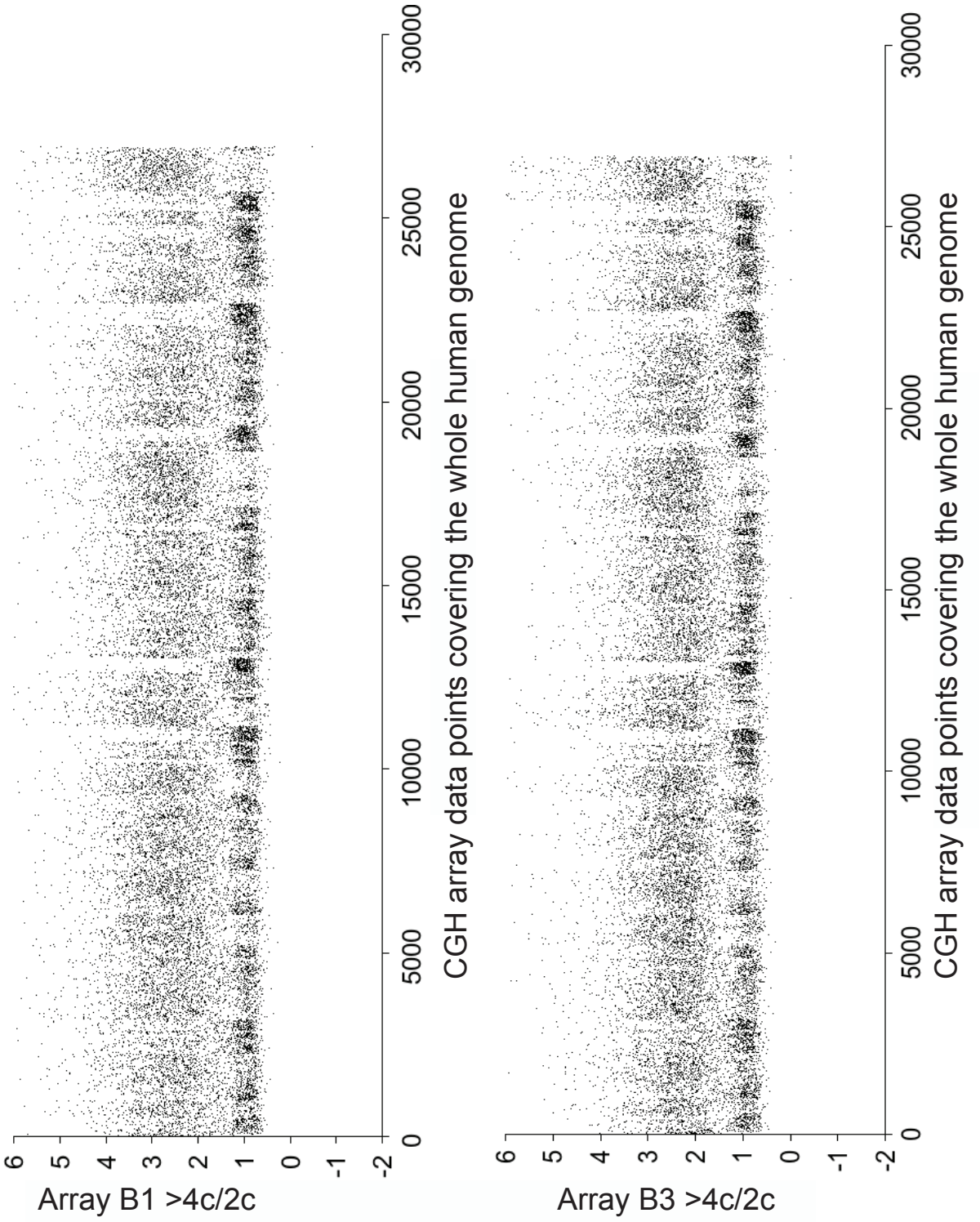
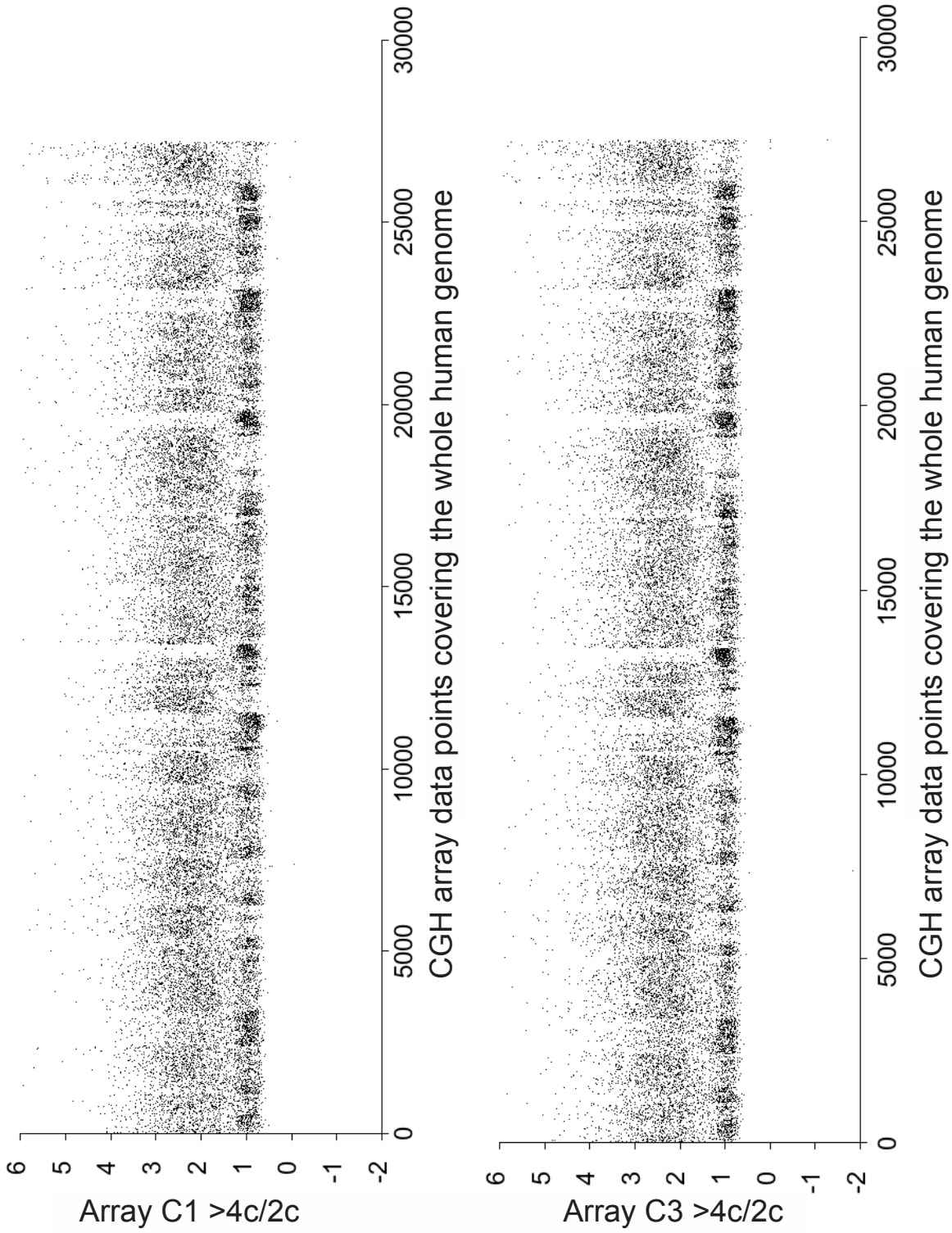


Figure V. 8. Whole Genome data for Array C1 and C2. The >4c/2c ratio of geminin depleted vs control depleted cells is shown for Array C1 and C2. A black dot represents one of the remaining 27219 valid data points that represent distinct biological features covering the whole human genome (array contains a total of 55077 distinct biological features).



V. 2. 2. 4x 44K Human Genome CGH Microarray

V. 2. 2. 1. Array setup and Quality Control

Because the problems we encountered with the 8x60K array were technical only, one biological experiment was performed for the 4x 44K array to generate DNA for four technical replicates (A1, A2, A3 and A4) (Figure V. 9). DNA was handled as described is Section V. 2. For each sample and each array, 500 ng input DNA was send to Agilent technologies. To obtain a better control for the labelling quality the array was setup as a dye swap experiment: for array A1 and A2 the >4C DNA was Cy3 labelled while for array A3 and A4 it was Cy5 labelled and vice versa.

The Cy3/Cy5 or Cy5/Cy3 ratios of array A1 vs. A2 and A3 vs. A4 showed a good correlation with only a few outliers (Figure V. 10. upper panel), indicating good data quality. In agreement the plots of single wavelengths against each other (Cy3 vs. Cy3 and Cy5 vs. Cy5) also exhibited a highly correlated data distribution (Figure V. 10 middle and lower panels). In contrast to the 8x 60K array it was not necessary to filter out invalid data points.

V. 2. 2. 2. Geminin depleted vs. control whole genome analysis

In contrast to the 8x 60K results, the whole genome analysis of >4C/ 2C on the 4x 44K array revealed no obvious areas of amplification. On all 4 arrays the ~ 44000 data points are distributed closely to the baseline of around one (Figure V. 11 - 12). This suggests that re-replication does not occur preferentially at specific origins and that re-firing of replication origins in G2 phase of the cell cycle occurs stochastically across the entire genome.

However, given the fact that both microarray experiments gave opposite results, a third and different approach was chosen to allow a thorough conclusion.

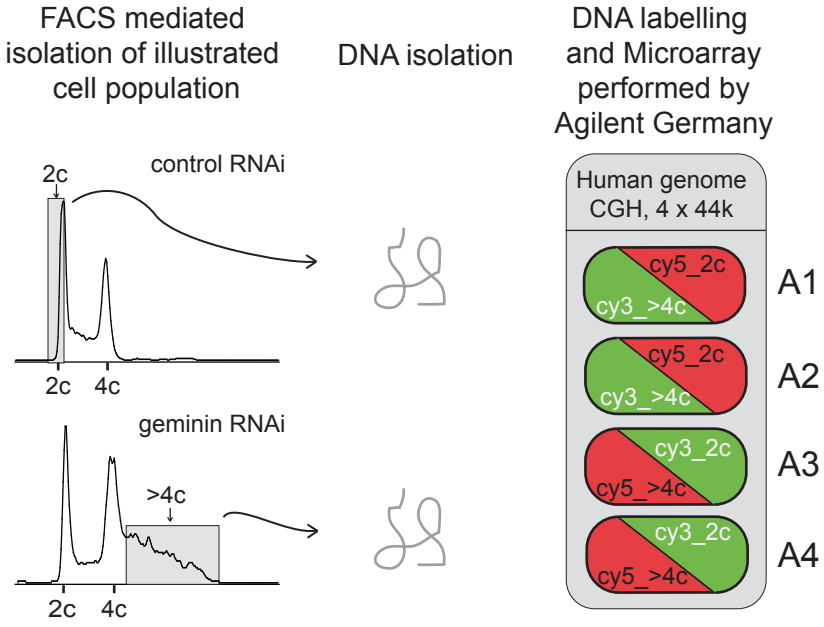


Figure V. 9. Array setup for preparation by Agilent, Germany. U2OS cells were released from a double Thymidine block after prior treatment with either control or geminin RNAi, as in Figure IV. 2. 2. 48h later, cells were labeled with Hoechst 33342 for 30 min and indicated populations (2c, >4c) were seperated by FACS. Subsequently the DNA from each population of cells was isolated and quantified by agarose gel electrophorese and Nano Drop UV spectrophotometer. 500 ng input DNA was used per sample and per array.

Figure V. 10.

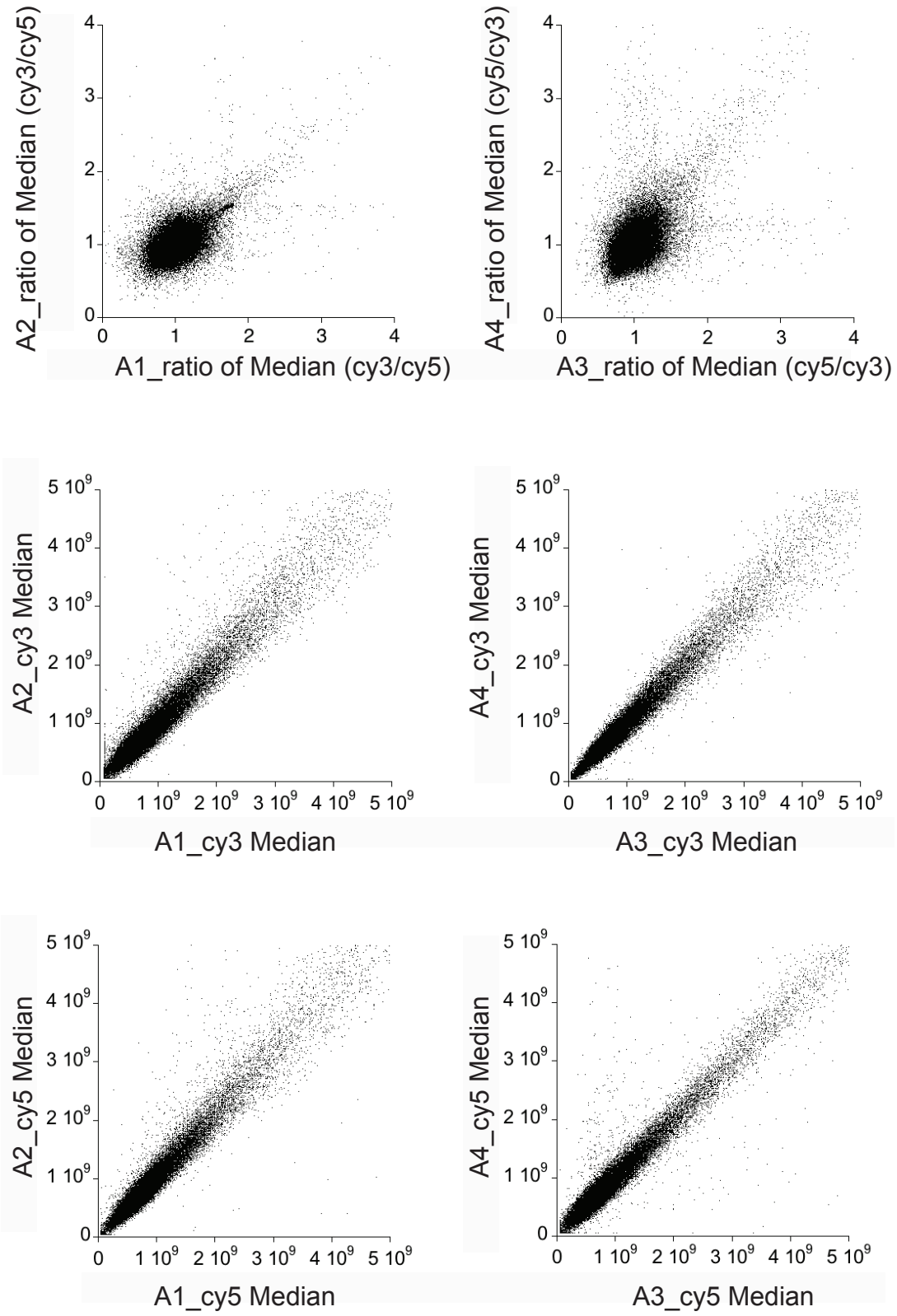


Figure V. 10. Quality control plots for the 4x44K microarray performed by Agilent. The upper panel shows the cy3/cy5 (>4c/2c) ratio of Array 1 (A1) versus Array 2 (A2) and the cy5/cy3 (>4c/2c) ratio of A3 versus A4. The middle (cy3) and bottom (cy5) panels look at the single colors against each other to check technical reproducibility between the arrays.

Figure V. 11. Whole Genome data for Array 1 and Array 2. The >4c/2c ratio of geminin depleted vs control depleted cells is shown for Array A1 and A2. A black dot represents one of the 42494 sequences that represent distinct biological features covering the whole human genome.

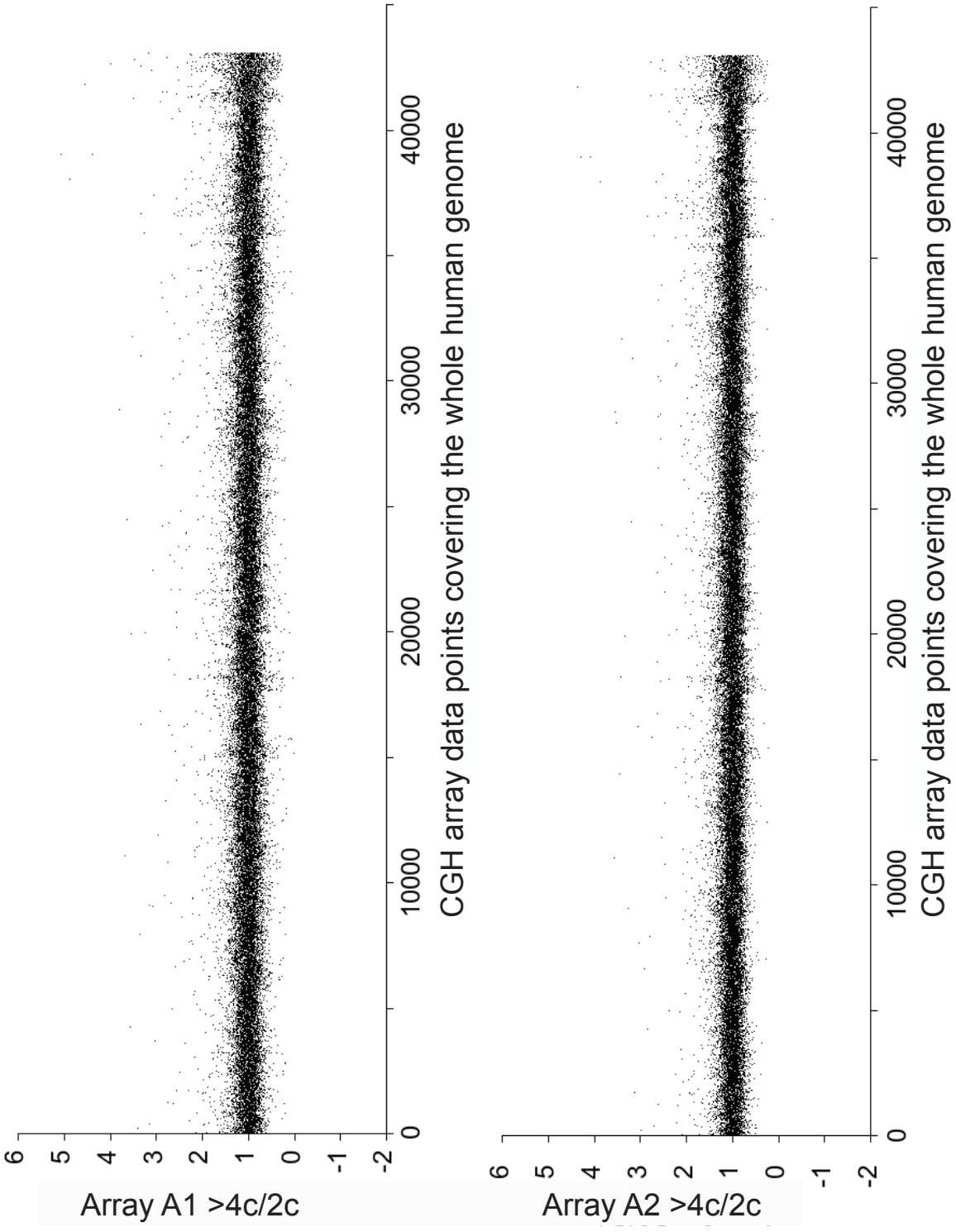
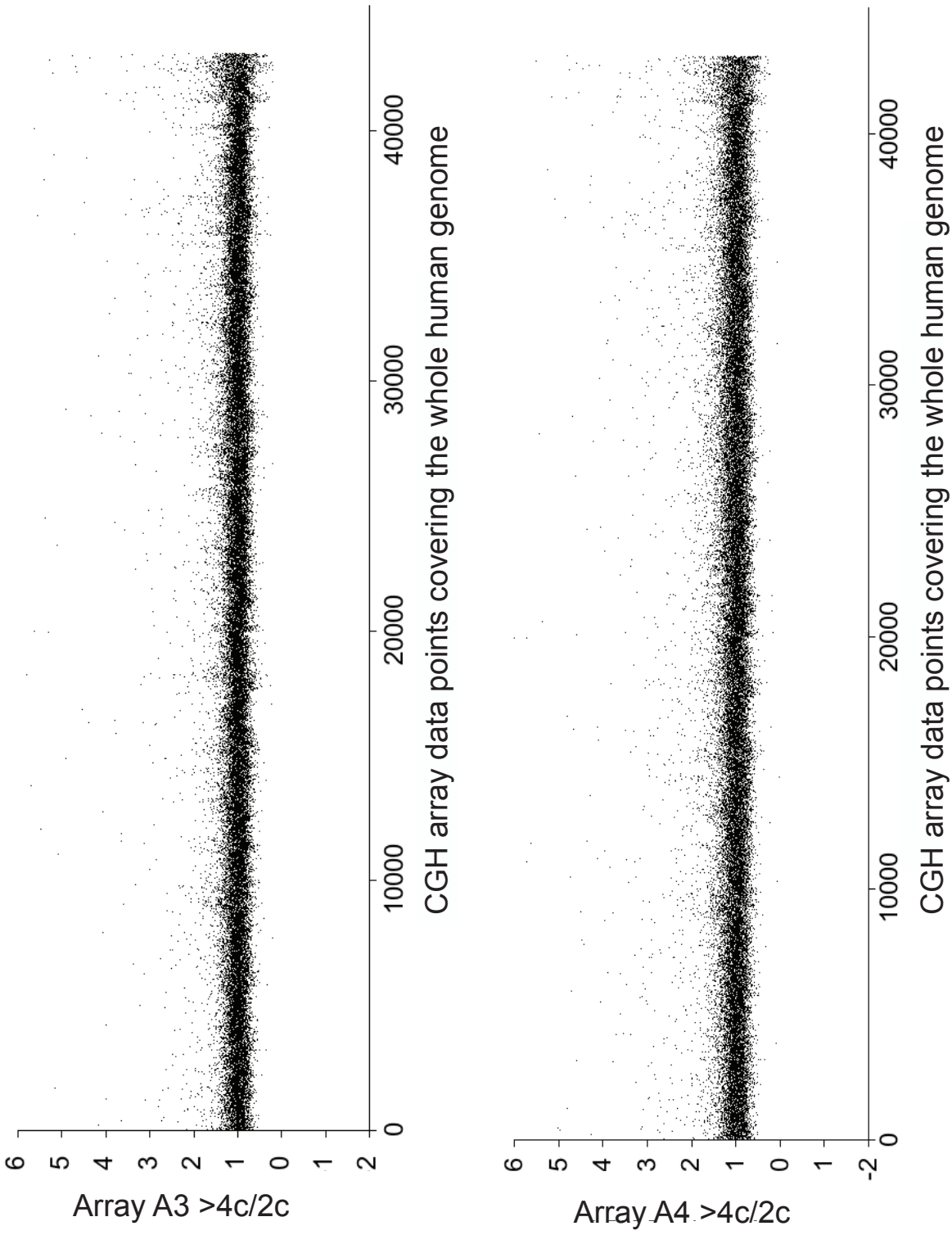


Figure V. 12. Whole Genome data for Array A3 and Array A4. The >4c/2c ratio of geminin depleted vs control depleted cells is shown for Array A3 and A4. A black dot represents one of the 42494 sequences that represent distinct biological features covering the whole human genome.



V. 3. SOLEXA DEEP SEQUENCING OF GEMININ DEPLETED VS. CONTROL CELLS

Solexa deep DNA sequencing was chosen as an alternative approach to investigate copy number changes in geminin depleted U2OS cells. Although deep sequencing is fairly costly, it provides ~70x 10^6 usable mapped reads compared to ~50x 10^3 probes from the microarray data. Thus the data resolution across the whole genome and therefore the reliability is much higher using the deep sequencing technique.

FACS was used to collect either 2C (control depleted) cells or >4C (geminin depleted) cells 48 h after double Thymidine release. The genomic DNA was isolated from biological triplicates and the six samples were analysed by deep DNA sequencing (http://genepool.bio.ed.ac.uk).

Figure V. 13. A shows the sequencing data as a circle plot, where each circle represents DNA from a single chromosome, shown as a running 1 Mb weighted-average (red line). The deviation from exact circularity, which reflects differences in abundances of the sequenced DNA, is only slight. As an example, chromosome 10 is shown in more detail in Figure V. 13 B. In addition to the 1 Mb weighted-average data (red), data grouped with a 100 kb weighted-average (blue) is also shown. The abundance ratio typically remains very close to 1 with only minor deviations. Small-scale (~10%) increases in the ratio are observed for some chromosomes for a few Mb surrounding the centromeres (notably chromosomes 4, 7, 10, 18 & 19) and telomeres (notably chromosomes 6, 10, 11, 12, 19 & 21). The raw data shows some strong, narrow (<10kb), 'spikes' which are associated with satellite repeats and are also seen in other genomic sequencing datasets (van Koningsbruggen et al., 2010). Analysis of the ~10% of sequencing reads that mapped to the repetitive chromosomal sequences gave almost identical ratios between control and geminin depleted samples (see Methods Section II. 13).

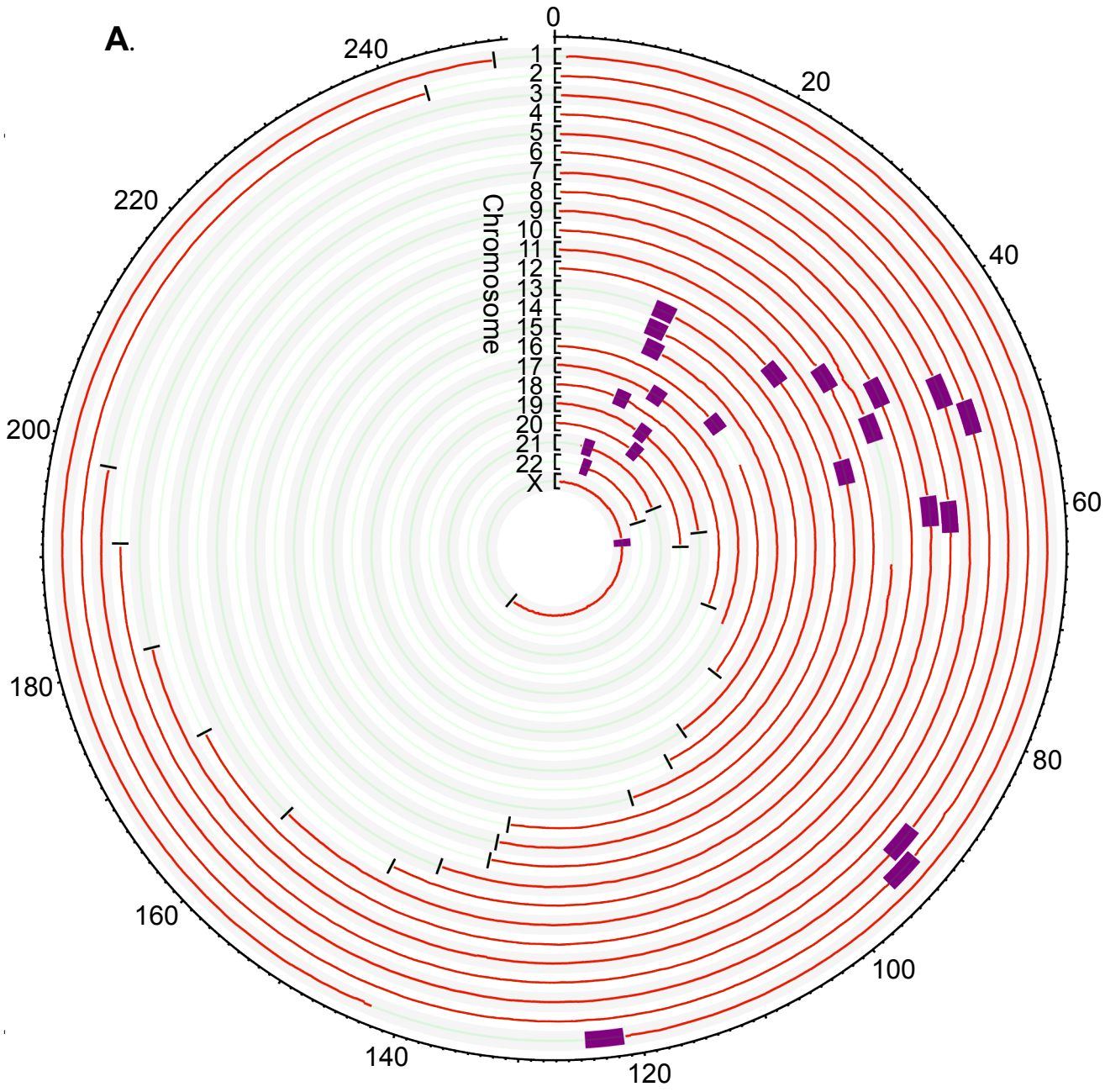
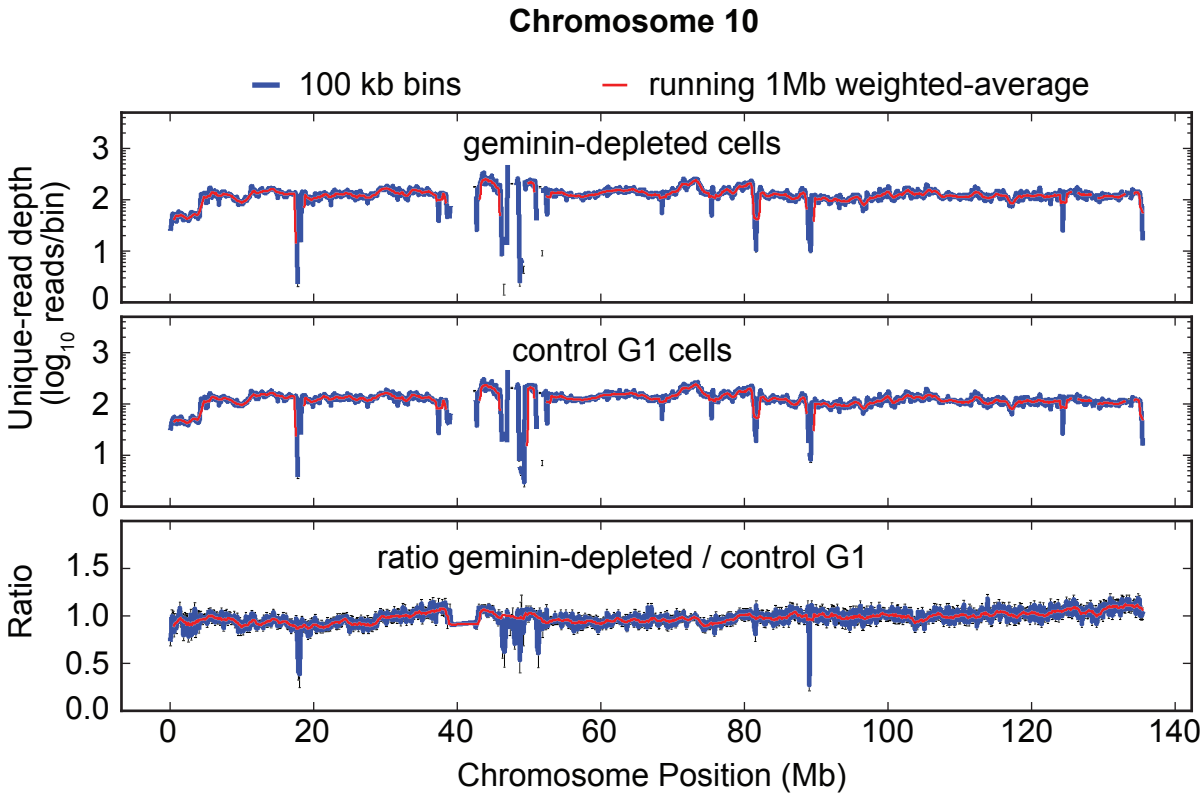


Figure V. 13. Re-replication occurs throughout the entire genome. Cells were released from a double Thymidine block after prior treatment with either control or geminin RNAi, as in Figure III. 2. 48 h after release from the 2nd Thymidine block, cells were sorted by FACS according to their DNA content. Cells with a 2c (G1) DNA content were collected from the control RNAi sample, and cells with a >4c (>G2) DNA content were collected from the geminin RNAi sample. DNA was isolated from the two samples and subjected to deep DNA sequencing. A total of ~70x10 usable mapped reads covering the whole genome (3.04x 10 bases) could be generated by sequencing. The number of reads in 1 Mb segments of the genome were derived for the two samples, and the ratioof read numbers in geminin-depleted/ control G1 samples were calculated. **A.** Geminin-depleted >G2 / control G1 ratio for all 23 chromosomes. Black brackets show the ratio scale from 0 to 2; the faint green line shows a ratio of 1. Purple boxes denote centromeres. Ticks around the ring show chromosome position in Mb. **B.** Expanded data for chromosome 10 as exemplar. The number of reads per segment is compared for 1 Mb (red lines) and 100 kb (blue lines) bins. Data for geminin depleted cells with >G2 DNA content (top), control G1 cells (middle), and their ratios are shown (bottom).

Figure V. 13. B.

B.



This indicates that repetitive DNA is not preferentially re-replicated in geminin depleted cells. Apart from the slight enrichment at centromeres and telomeres, the data therefore provide no evidence that any particular regions of the genome are preferentially re-replicated in response to geminin depletion. Consistent with the data from the 4x 44K microarray this implies that most re-replication occurs stochastically across the genome, rather than preferentially at specific loci.

V. 4. SUMMARY

In this part of the Thesis the genomic consequences of geminin induced re-replication have been investigated by comparative genomic hybridisation microarrays and Solexa deep sequencing. The aim was to determine whether re-replication occurs on preferential sites or randomly across the genome. CGH analysis of geminin depleted (>4C) versus control (2C) DNA revealed contrary results between two independent microarray experiments. The self-made 8x 60K microarray showed a low quality of Cy5 labelling resulting in a loss of ~45% of the data points. The remaining data indicated large regions of 2-4 fold amplifications across the genome. However, a 4x 44K microarray performed by Agilent failed to reproduce those findings and instead showed no preferred sites of re-replication suggesting that re-firing of replication origins in G2 phase occurs stochastically across the human genome. In agreement with the Agilent data, deep DNA sequencing revealed no preferential re-replication of specific genomic regions after geminin depletion.

V. 5. FUTURE DIRECTIONS

Chapters III and IV of this PhD thesis investigated two distinct mechanisms inducing re-replication in U2OS cancer cells. Geminin depletion causes cells to re-fire replication origins after checkpoint mediated G2 arrest while MLN4924 mediated

stabilisation of Cdt1 seems to induce re-replication directly from within S-phase of the cell cycle. Microarray analysis and deep sequencing of geminin depleted cells showed that re-replication occurs stochastically across the entire genome rather than preferential on specific loci. It would be interesting to analyse the genomic consequences within the MLN4924 treated U2OS cells with by deep sequencing. Such analysis could clarify whether this second mechanism is as well random or locus specific. One approach could be to treat asynchronous cells with MLN4924 for 24 h and isolate the DNA for sequencing. Alternatively cells could be released from a double Thymidine block in the presence or absence of MLN4924 for approximately 18 h. The isolated DNA from the control population (no treatment) would have a mainly 2C DNA content as the majority of the cells would have replicated their genome normally and progressed into the next G1 phase. That would make it easier to compare with MLN4924 treated cells, which presumably have suffered massive re-replication of either specific or random loci.

Cdt1 over-expression or MLN4924 treatment of MCF7 cells (personal communication with Dimitris Xirodimas) causes inhibition of replication and G1/S-phase arrest. Deep sequencing of DNA from Cdt1 over-expressing cells released from a double Thymidine block for 6 h could provide evidence for head to tail fork collision as it would show if multiple re-initiation events took place in certain regions and it would also show if re-replication initiated according to the replication timing program on early firing origins.

Chapter VI

Discussion

Preventing re-replication of DNA in a single cell cycle is crucial for the maintenance of genetic stability, and defects in the regulation of the replication licensing system may contribute to genetic instability commonly seen in cancer cells (Blow and Gillespie, 2008a). In mammalian cells many regulatory mechanisms prohibit relicensing of replication origins once cells have entered S-phase, the most important of which are down-regulation of Cdt1 activity by proteolysis or Cdt1 inhibition via formation of a heterohexameric complex with geminin (Arias and Walter, 2007; Blow and Dutta, 2005; DePamphilis et al., 2006). Previous work has shown that inhibition or loss of geminin (Li and Blow, 2005; Melixetian et al., 2004; Zhu et al., 2004) or the over-expression of Cdt1 (Li and Blow, 2005; Maiorano et al., 2005; Nishitani et al., 2004; Thomer et al., 2004; Vaziri et al., 2003) promotes re-replication of chromosomal DNA.

The results presented in this thesis describe how loss of geminin and stabilisation of Cdt1 induce re-replication of chromosomal DNA via distinct mechanisms.

Geminin depletion in U2OS cells does not affect progression through the first S-phase but triggers activation of the G2/M checkpoint. Cells arrested in G2 then undergo re-firing of origins randomly distributed across the genome, ultimately leading to inhibition of proliferation and cell death. Activation of the G2/M checkpoint amplifies small defects caused by geminin depletion, thereby creating an ‘all or nothing’ response to re-replication.

In contrast, stabilisation of Cdt1 by inhibition of its proteolysis causes re-replication accompanied by checkpoint activation. In parallel, association of replication proteins such as PCNA, MCM2 and Cdt1 onto S-phase chromatin was observed, suggesting that in contrast to geminin depletion, re-initiation of replication origins takes place in S-phase leading to immediate re-replication of DNA. Very high levels of Cdt1 over-expression mediated by transfection of Cdt1 expression constructs led to strong checkpoint activation and inhibition of replication with cells arresting in early S-phase.

PART VI. 1. PHYSIOLOGICAL CONSEQUENCES OF CHROMOSOMAL RE-REPLICATION

VI. 1. 1. Re-replication is lethal

Even when cells were treated with low concentrations of geminin siRNA, proliferation was decreased at least 10 fold compared to control cells. This is most likely a consequence of the 10-fold increase in cell death that can be observed after geminin depletion. A correlation between re-replication and apoptosis was observed in this study, suggesting that as a first response to geminin depletion cells start to re-replicate DNA, while later on the levels of re-replication decline to the same extent than the levels of cell death increase. Two previous studies have already pointed out that geminin depletion causes re-replication and checkpoint activation in the presence of functional p53 (Melixetian et al., 2004; Zhu et al., 2004). However both studies only showed apoptosis in geminin depleted HCT116 cells after checkpoint abrogation. In this study activation of apoptotic signalling cascades can be detected without checkpoint abrogation as early as 16 h post 2nd Thymidine release in geminin depleted cells, as evident by increased levels of cleaved PARP. Consequently the appearance of apoptotic cells (<2C DNA content) can be observed between 72 h and 96 h post 2nd Thymidine release in geminin depleted populations. In agreement with these findings a recent study from the DePamphilis lab revealed that geminin depletion causes proliferation inhibition and selective killing of cancer cells due to massive re-replication, while non-cancer cells do not re-replicate and subsequently grow and survive (Zhu and DePamphilis, 2009).

Cdt1 stabilisation by inhibition of proteolysis by the Nedd8- activating enzyme inhibitor MLN4924 caused substantial amounts of re-replication apparently starting in S-phase. The time frame of this project did not allow long term analysis of cells treated with MLN4924, but previous studies showed induction of senescence (Jia et al., 2011; Lin et al., 2010) and selective killing (Lin et al., 2010; Milhollen et al., 2011; Soucy et

al., 2009) of cancer cells re-replicating upon MLN4924 treatment, making this drug a promising anti-cancer agent in clinical trials.

Although we were not able to recover cultures of geminin depleted cells that underwent re-replication to study specific genetic alterations, we cannot exclude that cells with only marginally reduced levels of geminin, might undergo only low amounts of re-replication and therefore might be able to survive. However re-replication detectable by FACS seems to be a cellular insult that does not allow cell survival.

VI. 1. 2. Role of Cdt1 levels in initiation of re-replication

During anaphase and G1 phases, Cdt1 activity is high and geminin activity is low allowing origin licensing to take place (Figure VI. 1). During S-phase Cdt1 is degraded or inactivated by forming an inhibitory complex with geminin. Both Cdt1 proteolysis and inhibition by geminin contribute to the prevention of re-licensing of replication origins after initiation of DNA replication. We show that synchronised U2OS cells progressing through their first S-phase in the absence of geminin exhibit no large-scale defect in DNA replication or replication timing. In agreement with data provided by Ballabeni and colleagues (Ballabeni et al., 2004) we showed a decrease of Cdt1 levels upon geminin depletion. Interestingly this decline is accompanied by a small increase in soluble PCNA levels in geminin depleted cells, suggesting that the lack of geminin, the low levels of Cdt1 or the presence of geminin unbound Cdt1 directly or indirectly mediate an accumulation or stabilisation of PCNA. This is a novel observation and cannot be explained by the current literature.

The decrease in Cdt1 could be a consequence of PCNA dependent degradation in response to DNA damage (Higa et al., 2003). Re-replication has recently been shown to activate the DNA damage induced PCNA dependent degradation of Cdt1 preventing origin licensing after DNA damage (Hall et al., 2008).

However, Hall and colleagues (2008) showed enhanced PCNA dependent Cdt1 degradation after detecting substantial re-replication and robust ATR-Chk1 checkpoint activation. In contrast we detected decreased levels of Cdt1 within the first S-phase after geminin depletion in the absence of measurable re-replication and DNA damage checkpoint activation. Therefore it seems more likely that Cdt1 proteolysis is increased upon geminin depletion as response to unbound Cdt1, being not in the inhibitory and protective complex with geminin as suggested by Ballabeni and colleagues (2004). Thus the steric hindrance is not provided by geminin allowing Cdt1 degradation, causing the decline in protein levels. Moreover geminin depletion was shown to reduce Cdt1 mRNA levels in *Drosophila* SD2 cells (Mihaylov et al., 2002) indicating that transcriptional down-regulation could also account for the Cdt1 decrease upon geminin depletion.

We postulate that in geminin depleted cells the low Cdt1 levels during the first S-phase are the reason why re-replication cannot occur before the levels build up during G2 phase. This suggests that the key role of geminin is to prevent re-licensing and re-replication in G2 phase (Figure VI. 1).

In contrast, when Cdt1 proteolysis is suppressed by MLN4924, immediate origin re-firing and continuous incorporation of BrdU takes place resulting in significant re-replication within S-phase (Klotz-Noack et al., 2012; Lin et al., 2010; Milhollen et al., 2011; Soucy et al., 2009). The data provided so far suggests that the levels of Cdt1 determine when re-replication can start and that the efficient proteolysis of Cdt1 prevents substantial re-replication in S-phase when geminin is depleted.

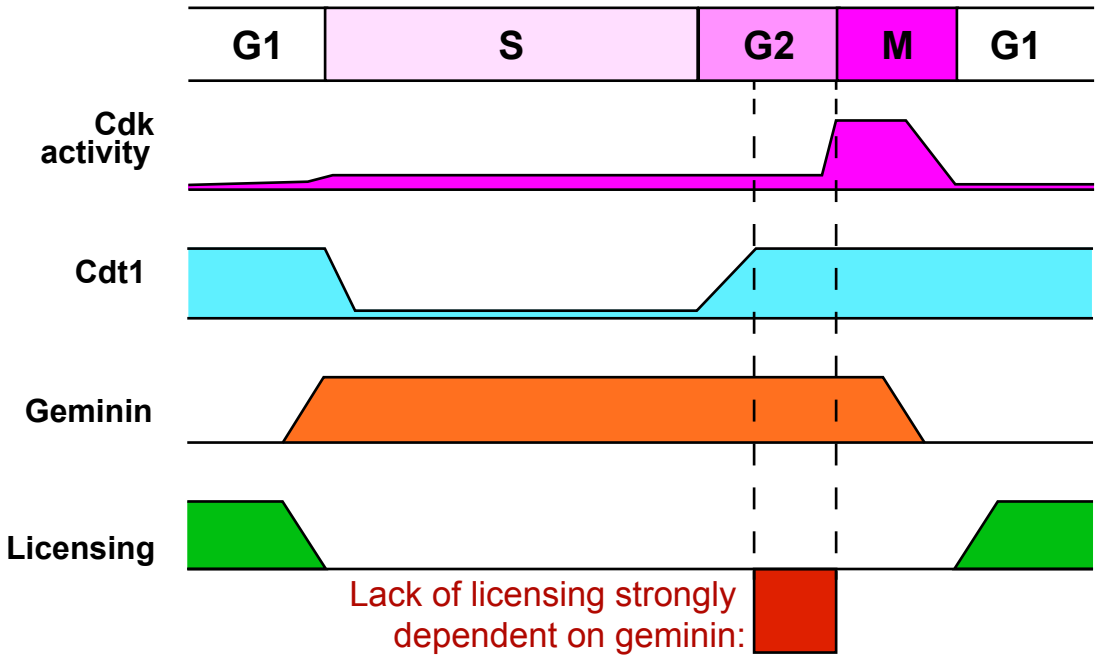


Figure VI. 1. Cartoon of licensing control by Cdt1 and geminin. Activity levels of Cdks, Cdt1 and geminin are shown throughout the cell cycle. This allows licensing to take place only during late mitosis and G1. In the absence of geminin, rising Cdt1 levels in G2 can promote re-licensing of replicated DNA, and in the presence of Cdk activity, can cause re-replication of DNA.

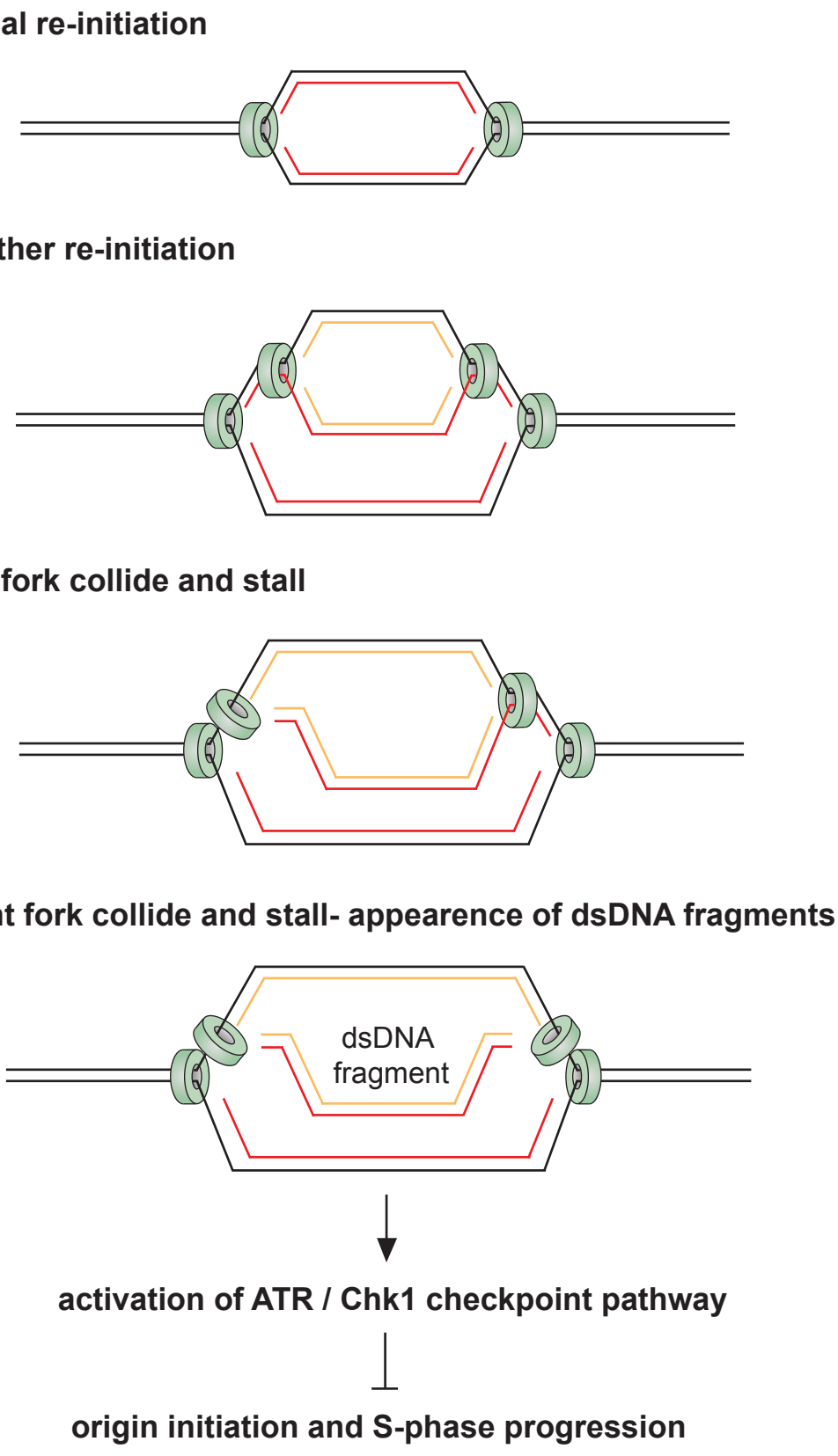
VI. 1. 3. The role of re-replication in activation of cell cycle checkpoints

Although geminin depletion does not cause major detectable problems in the first S-phase, 20 – 40% of geminin depleted cells were delayed or blocked in very late S-phase or G2. This appears to be due to activation of checkpoint kinases as the G2 arrest was associated with phosphorylation of p53 and was abolished by treating cells with checkpoint inhibitors such as caffeine or the Chk1 inhibitor UCN-01.

Although it seems likely that activation of the G2/M checkpoint caused by geminin depletion is a consequence of a small amount of re-replication, we could find no evidence of significant amounts of re-replication having occurred at this stage. Similar results were reported by Liu et al, who showed that Cdt1 over-expression in a number of cell lines caused checkpoint activation in the absence of detectable re-replication (Liu et al., 2007). It is not clear why a small amount of re-replication should cause checkpoint activation.

One mechanism by which re-replication induces checkpoint activation is a head-to-tail collision of replication forks after chasing one another along the same DNA template (Davidson et al., 2006). Under those circumstances the rear fork can run into the front fork causing fork stalling and checkpoint activation. If both forks in a replication bubble undergo this sort of collision, small double stranded DNA fragments would be generated (Figure VI. 2). In order for a head-to-tail collision to occur, re-licensing and re-initiation must occur rapidly after a first initiation event and multiple rounds of re-replication are required. This seems incompatible with the undetectable levels of re-replication occurring in the first S phase after geminin depletion. However, head-to-tail fork collisions could explain the rapid inhibition of DNA replication occurring upon over-expression of Cdt1. Consistent with Davidson *et al*. moderate levels of Cdt1 such as obtained by MLN4924 treatment caused re-replication of DNA while high levels caused inhibition of replication.

Figure VI. 2.



modified from Davidson *et al.*, 2006

Figure VI. 2. Head-to-tail fork collision model to activte cell cycle checkpoints in response to Cdt1 overexpression. A section of DNA with a replication origin is shown. The replication forks are indicated by green rings. **A.** Reinitiation at the replication origin forms a replication bubble. **B.** Further reinitiation events form a second replication bubble within the first bubble with the rear forks chasing the front fork. **C.** The left rear fork collides with the front fork. **D.** The right forks undergo head-to-tail fork collision releasing double stranded DNA fragments. Fork collision and the resulting fork stalling cause ATR / Chk1 checkpoint activation which inhibit further initiation and S-phase progression.

In both studies phosphorylation of the Chk1 checkpoint kinase was observed, suggesting that head-to-tail fork collision took place after massive over-expression of Cdt1 thereby activating Chk1 kinase and consequently leading to replication inhibition and cell cycle arrest. The time frame of this project did not allow further investigation of Cdt1 over-expression to provide evidence for head-to-tail fork collision by checking the appearance of DNA fragments.

Liu and colleagues found that uncontrolled DNA unwinding by MCM2-7 protein upon deregulation of the licensing machinery results in accumulation of ssDNA and ATR checkpoint activation. In geminin depleted cells re-loading of MCM2-7 proteins can occur upon Cdt1 increase in late S-phase or G2 and could therefore result in uncontrolled DNA unwinding and the generation of DNA lesions together with activation of the ATR checkpoint pathway. If this is the case, why should re-loading of the MCM2-7 complex and re-initiation of DNA replication lead to uncontrolled DNA unwinding? One possibility is an incomplete assembly of the replisome, for example as a consequence of defective DNA polymerase activity (Walter and Newport, 2000) or a lack of other replisome components causing enhanced replication forks stalling (Byun et al., 2005).

Consistent with this interpretation, it has been shown in yeast that re-replicating forks may be particularly prone to collapse due to generation of DNA lesions produced by the re-replication itself (Green et al., 2010; Green and Li, 2005). However, it is not clear why re-replication would cause DNA damage that is not generated during 'normal' replication thus activating checkpoints.

An alternative possibility is that replisomes performing re-replication are essentially normal and have the same probability of stalling as forks performing normal replication. However, it is likely that after geminin depletion, re-licensed origins on replicated DNA may be very sparse. If these forks stall, then they would be less likely to be rescued by initiation from nearby dormant origins than in case of the normal first-

round replication (Blow et al., 2011; Ge and Blow, 2010; Ge et al., 2007; Woodward et al., 2006). A lack of dormant origins under replicative stress would result in activation of Chk1 checkpoint kinase (Ge and Blow, 2010), which is consistent with ATR and the Fanconi Anaemia pathway being activated when geminin is lost (Liu et al., 2007; Zhu and Dutta, 2006).

VI. 1. 4. The G2/M checkpoint mediates an 'all or nothing' response

I have shown that, paradoxically, activation of the G2/M checkpoint in response to loss of geminin actually promotes DNA re-replication. Only after being delayed in G2 do geminin depleted cells undergo re-replication. This is likely because the PCNA-dependent Cdt1 degradation pathway is no longer active in G2 and Cdt1 levels can start to accumulate. G2 is therefore the critical stage of the cell cycle where the presence of geminin is most important for preventing re-replication (Figure VI. 1). By holding cells at this stage, the G2/M checkpoint allows the amount of re-replication to increase, thereby amplifying the low levels of re-replication that may have initially been caused by geminin depletion. This could represent a protective mechanism to prevent small amounts of re-replication from accumulating in cells, by making re-replication an 'all or nothing' response. Consistent with this idea, it was not possible to grow out colonies from cells that had already undergone re-replication. Therefore even modest amounts of re-replication ultimately led to cell death.

When the G2/M checkpoint was inhibited by Caffeine, cells were not arrested in G2 and progressed through mitosis into G1 without indications of substantial re-replication. Interestingly, progression through G1 and S-phase was delayed in Caffeine treated and geminin depleted cells compared to control cells. One explanation is that a small degree of re-replication took place, causing activation of the G1/S checkpoint and G1/S delay. Alternatively, the low Cdt1 levels caused by geminin depletion could have

caused defects in licensing of replication origins, which would lead to impairments of DNA replication (Ballabeni et al., 2004).

Since Caffeine is known to inhibit ATM and ATR, either of these kinases could be responsible for the G2 delay in geminin depleted cells. Treatment with the ATM inhibitor KU55933 as well as depletion of p53 showed no impact on the levels of re-replication or the ability of geminin depleted cells to progress through mitosis, suggesting that ATR kinase activity was responsible for the observed G2 delay. Moreover treatment of geminin depleted cells with the Chk1 inhibitor UCN-01 gave identical results to caffeine treatment.

Our conclusions are consistent with several previous studies showing that inhibition of checkpoint signalling or DNA damage response pathways in geminin-defective cells leads to decreased levels of re-replication (Lin and Dutta, 2007; Melixetian et al., 2004; Zhu et al., 2004; Zhu and Dutta, 2006). These previous reports assumed that reduced levels of re-replication were due to increased apoptosis when DNA damage and checkpoint pathways were inhibited, but this could equally well be explained by reducing the period of time cells spend in G2, as we show here.

Consistent with our interpretation, re-replication induced by Cdt1 over-expression is enhanced by inhibition of checkpoint kinases, since Cdt1-induced re-replication occurs directly within S phase (no G2 delay is required) and checkpoint kinases suppress re-initiation of re-licensed origins (Davidson et al., 2006; Lee et al., 2007a; Li and Blow, 2005; Liu et al., 2007; Vaziri et al., 2003).

Taken together our data suggest that re-replication can be induced by de-regulation of geminin and Cdt1 and that depending on the cell cycle state, different checkpoint pathways play a role in preventing or enhancing re-replication.

A key example is the p53 checkpoint pathway, which seems to be activated in geminin depleted cells but plays little role in preventing re-replication (Melixetian et al., 2004; Zhu et al., 2004). In contrast, re-replication induced by over-expression of Cdt1, Cdc6 and Cyclin A in p53-negative H1299 cells is inhibited by over-expression of p53

or p21 by inducing G1/S phase arrest (Vaziri et al., 2003). Recent studies showed that re-replication is induced by depletion of Forkhead box (FOX) proteins in human cells (Lo et al., 2010). FOX proteins are transcription factors that play a role in embryonic development, cell cycle progression and cell survival (Carlsson and Mahlapuu, 2002; Hannenhalli and Kaestner, 2009; van der Horst and Burgering, 2007). When FOX1 is depleted in HCT116 (p53-/-, p21-/-) cells re-replicate and apoptosis increases dramatically compared to wild-type cells, which arrest in G1/S-phase. Similarly re-replication is enhanced when FOX1 and p53 are co-depleted in wild-type cells (Lo et al., 2012). It seems that the p53 checkpoint pathway plays an essential role in preventing initiation and S-phase progression when re-replication is induced in early S-phase but is less important when re-replication is induced in G2 phase of the cell cycle.

PART VI. 2. GENOMIC CONSEQUENCES OF CHROMOSOMAL RE-REPLICATION

To gain insight into genomic consequences of geminin induced re-replication of the human genome, the DNA content of cells that had undergone significant re-replication was analysed via Comparative Genomic Hybridisation and Solexa deep sequencing. There are two different ways that re-replication could occur within the genome (Figure VI. 3). Origins could either re-fire reproducibly at preferential sites within the human genome causing detectable amplifications or re-initiate randomly across the genome resulting in no detectable copy number changes.

The initial 8x60K CGH microarray provided results that indicated re-replication of DNA may occur at preferential regions of the genome in geminin depleted cells. However, a second 4x44K CGH microarray and Deep sequencing revealed no amplification of specific regions across the whole human genome. The different results from both arrays are likely to be explained by technical problems with the first array. Although the Cy3 and Cy5 labelling quality was well in the range requested by the manufacturer, the quality plots clearly showed a very poor correlation of Cy5 signal intensity between technical replicates. Correspondence with Agilent technologies revealed that Cy5 has been shown to be sensitive to ozone degradation and levels as low as 10 µg/ m^3 can compromise microarray results. Recently an updated version of the protocol includes stabilisation steps and an ozone-barrier slide cover to prevent degradation. Most likely ozone degradation could contribute to the bad quality of the array. Due to the bad quality, invalid data points were filtered thus removing up to 40% of the data, which may generate the misleading results. However, the bad array quality raised sufficient doubts to immediately start reproducing the findings with a second array and deep sequencing.

Figure VI. 3.

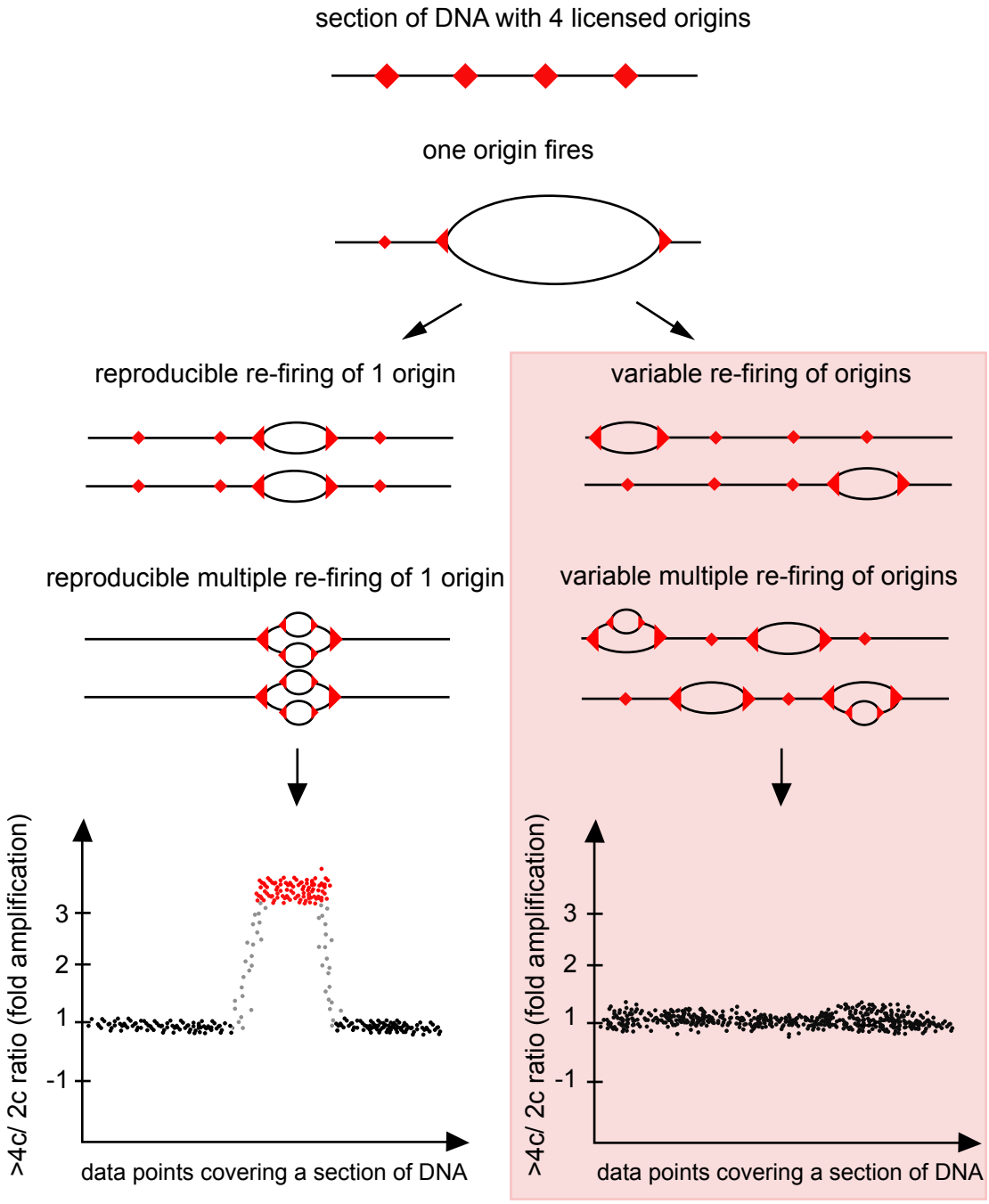


Figure VI. 3. Refire option of replication origins in response to geminin depletion. Shown is a section of DNA with 4 licensed replication origins (A). One origin fires and the section is replicated in S-phase (B). When rereplication is induced in G2 by geminin depletion two possibilities come in mind how refiring of origins takes place. The right panel illustrates how variable re-firing of origins or multiple variable re-firing would be visualised on CGH or deep sequencing analysis. When all origins have the same probability to re-fire, no regions would appear amplified when DNA of a pool of cells is analysed. On the right panel the reproducible re-firing of one preferential origin or multiple re-firing of one origin is presented. This scenario would show ampliefied regions of the genome if a pool of cells is analysed. The results presented in this work suggest that origins re-fire stochastically without preference for certain regins (light red box)

Deep Sequencing and the 4x 44K microarray did not provide any evidence for preferential amplification of any specific DNA sequences (Klotz-Noack et al., 2012). This is in marked contrast to studies in *S. cerevisiae* (Green et al., 2010; Green et al., 2006) and *Drosophila* (Ding and MacAlpine, 2010) showing a preferred amplification of specific DNA regions after re-replication had been induced by de-regulation of the licensing system.

Green and colleagues designed an assay where re-firing of a specific origin could be achieved in G2/M phase in *S. cerevisiae*. They showed that non-allelic homologous recombination between repetitive elements took place leading to re-replication induced gene amplification (RRIGA) in close proximity to the re-fired origin (Green et al., 2010). We cannot conclude that similar processes did not take place in our U2OS experiments but we can clearly say that they do not occur in the proximity of preferred origins (e.g. early or late origins), as this would result in amplified regions within the human genome which we did not observe by high resolution Solexa sequencing. It is currently unclear why our results differ from this mentioned report. It is hard to draw parallels between our results in human U2OS cells and results obtained in *S. cerevisiae*, since the mechanisms that suppress licensing in yeast are different from those in animal cells, and yeast even lacks geminin.

It is more surprising that our results differ from those results obtained by depletion of geminin in *Drosophila*, which preferentially showed re-replication of heterochromatin. Although we detected a small-scale (≈10%) increase in the re-replication around some centromeres (chromosomes 4, 7, 10, 18 and 19) and telomeres (chromosomes 6, 10, 11, 12, 19 and 21), the increase was small and other known heterochromatic regions such as the p arms of chromosome 13, 14, 15, 21, and 22 were not elevated. In addition to a differently used cell type, the *Drosophila* study used asynchronous cells (Ding et al (2010), and reached higher re-replication levels (~8C DNA content), though it is not obvious why these features would be critical for causing preferential re-replication of specific regions.

We had initially expected re-replicating DNA to be enriched in sequences normally replicated early in S-phase, as these would be the first to replicate again following progression through mitosis and into the next cell cycle. However, a recent study in mouse cells revealed that G2 phase chromatin lacks any determinants of replication timing established during G1 phase of the cell cycle (Lu et al., 2010). When a complete genome re-duplication was induced in G2 phase cells, either by incubation in *Xenopus* egg extracts or following transient Cdk inhibition, replication did not follow any defined temporal sequence. Lu *et al.* used CGH analysis to compare the ratio of 'normal' early versus late S-phase chromatin, which in a normal S-phase showed clear features of gain and loss according to early and late firing origins. During re-duplication of the genome starting from early G2 versus late S-phase chromatin clearly showed a ratio of approximately one, indicating that origins fire randomly if re-replication starts in G2 after timing information is lost. Taking this into consideration it would be expected that if geminin depletion induced re-replication predominantly from a G2 state, re-replication would occur without any defined temporal sequence. Therefore all chromosomal domains have an equal probability of being re-replicated and DNA from a pool of >4C cells might not show preferential regions of re-replication.

As suggested by a previous report (Lin et al., 2010), it could be predicted that this would not be the case for re-replication induced by Cdt1 over-expression or MLN4924 treatment. When re-licensing of origins and consequently re-replication is induced in S-phase the timing pattern is present and replication starts at early replicating origins. Consequently re-replication would probably also start at early replicating origins as those would be re-licensed immediately by Cdt1 mediated MCM2-7 re-loading. Consistent with this idea, Vaziri *et al.* (2003) showed that re-replication of human cells induced by over-expression of Cdt1 and Cdc6 generated heavy-heavy DNA that hybridised preferentially to early replicating regions on metaphase chromosome spreads (Vaziri et al., 2003). This is in contrast to the data presented in this thesis, but can be explained by the different experimental setup. Cdt1 stabilisation has been shown to induce re-replication in S-phase (Klotz-Noack et al., 2012; Lin et al.,

2010; Milhollen et al., 2011; Soucy et al., 2009) while geminin depletion induces re-replication from G2 (Klotz-Noack et al., 2012). These two pathways are likely to be different in terms of re-replication origins. It could well be that Solexa sequencing of MLN4924 treated cells would reveal that re-replication starts at early replication origins, simply because Cdt1 is active in S-phase and can facilitate the immediate re-licensing and re-firing of origins according to the replication timing programm.

Since MLN4924 is in trial as an anti-cancer agent, it would be interesting to know if different ways of inducing re-replication can be used to exploit different sensitivities between normal and cancer cells.

References

Aggarwal, P., M.D. Lessie, D.I. Lin, L. Pontano, A.B. Gladden, B. Nuskey, A. Goradia, M.A. Wasik, A.J. Klein-Szanto, A.K. Rustgi, C.H. Bassing, and J.A. Diehl. 2007. Nuclear accumulation of cyclin D1 during S phase inhibits Cul4-dependent Cdt1 proteolysis and triggers p53-dependent DNA rereplication. *Genes Dev*. 21:2908-2922.

Aggarwal, P., L.P. Vaites, J.K. Kim, H. Mellert, B. Gurung, H. Nakagawa, M. Herlyn, X. Hua, A.K. Rustgi, S.B. McMahon, and J.A. Diehl. 2010. Nuclear cyclin D1/CDK4 kinase regulates CUL4 expression and triggers neoplastic growth via activation of the PRMT5 methyltransferase. *Cancer Cell*. 18:329-340.

Ahn, J., M. Urist, and C. Prives. 2004. The Chk2 protein kinase. *DNA Repair (Amst)*. 3:1039-1047.

Ahn, J.Y., J.K. Schwarz, H. Piwnica-Worms, and C.E. Canman. 2000. Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation. *Cancer Research*. 60:5934-5936.

Albertson, D.G. 2006. Gene amplification in cancer. *Trends Genet*.

Aparicio, O.M., D.M. Weinstein, and S.P. Bell. 1997. Components and dynamics of DNA replication complexes in S- cerevisiae: Redistribution of MCM proteins and Cdc45p during S phase. *Cell*. 91:59-69.

Archambault, V., A.E. Ikui, B.J. Drapkin, and F.R. Cross. 2005. Disruption of mechanisms that prevent rereplication triggers a DNA damage response. *Mol. Cell Biol.* 25:6707-6721.

Arentson, E., P. Faloon, J. Seo, E. Moon, J.M. Studts, D.H. Fremont, and K. Choi. 2002. Oncogenic potential of the DNA replication licensing protein CDT1. *Oncogene*. 21:1150-1158.

Arias, E.E., and J.C. Walter. 2005. Replication-dependent destruction of Cdt1 limits DNA replication to a single round per cell cycle in Xenopus egg extracts. *Genes Dev.* 19:114-126.

Arias, E.E., and J.C. Walter. 2006. PCNA functions as a molecular platform to trigger Cdt1 destruction and prevent re-replication. *Nat. Cell Biol.* 8:84-90.

Arias, E.E., and J.C. Walter. 2007. Strength in numbers: preventing rereplication via multiple mechanisms in eukaryotic cells. *Genes & Development*. 21:497-518.

Avery, O.T., C.M. Macleod, and M. McCarty. 1944. Studies on the Chemical Nature of the Substance Inducing Transformation of Pneumococcal Types : Induction of Transformation by a Desoxyribonucleic Acid Fraction Isolated from Pneumococcus Type Iii. *J Exp Med*. 79:137-158.

Bakkenist, C.J., and M.B. Kastan. 2003. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature*. 421:499-506.

Ballabeni, A., M. Melixetian, R. Zamponi, L. Masiero, F. Marinoni, and K. Helin. 2004. Human geminin promotes pre-RC formation and DNA replication by stabilizing CDT1 in mitosis. *EMBO J.* 23:3122-3132.

Bartek, J., and J. Lukas. 2003. Chk1 and Chk2 kinases in checkpoint control and cancer. *Cancer Cell*. 3:421-429.

Bell, S.P., J. Mitchell, J. Leber, R. Kobayashi, and B. Stillman. 1995. The multidomain structure of Orc1p reveals similarity to regulators of DNA replication and transcriptional silencing. *Cell*. 83:563-568.

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

Bielinsky, A.K., and S.A. Gerbi. 1999. Chromosomal ARS1 has a single leading strand start site. *Molecular Cell*. 3:477-486.

Blow, J.J. 1993. Preventing re-replication of DNA in a single cell cycle: evidence for a replication licensing factor. *J Cell Biol*. 122:993-1002.

Blow, J.J., and A. Dutta. 2005. Preventing re-replication of chromosomal DNA. *Nature Reviews Molecular and Cellular Biology*. 6:476-486.

Blow, J.J., and X.Q. Ge. 2009. A model for DNA replication showing how dormant origins safeguard against replication fork failure. *EMBO Reports*. 10:406-412.

Blow, J.J., X.Q. Ge, and D.A. Jackson. 2011. How dormant origins promote complete genome replication. *Trends in Biochemical Sciences*. 36:405-414.

Blow, J.J., and P.J. Gillespie. 2008a. Replication licensing and cancer--a fatal entanglement? *Nat Rev Cancer*. 8:799-806.

Blow, J.J., and P.J. Gillespie. 2008b. Replication licensing and cancer--a fatal entanglement? *Nature Reviews Cancer*. 8:799-806.

Blow, J.J., P.J. Gillespie, D. Francis, and D.A. Jackson. 2001. Replication Origins in Xenopus Egg Extract Are 5–15 Kilobases Apart and Are Activated in Clusters That Fire at Different Times. *Journal of Cell Biology*. 152:15-25.

Blow, J.J., and R.A. Laskey. 1988. A role for the nuclear envelope in controlling DNA replication within the cell cycle. *Nature*. 332:546-548.

Boveri, T. 1902. Ueber mehrpolige Mitosen als Mittel zur Analyse des Zellkern. *Verhandlungen der physikalisch-medizinischen Gesellschaft zu Wuerzburg*. 35:67-90.

Bowers, J.L., J.C. Randell, S. Chen, and S.P. Bell. 2004. ATP hydrolysis by ORC catalyzes reiterative Mcm2-7 assembly at a defined origin of replication. *Molecular Cell*. 16:967-978.

Brauchle, M., K. Baumer, and P. Gonczy. 2003. Differential activation of the DNA replication checkpoint contributes to asynchrony of cell division in C. elegans embryos. *Current Biology*. 13:819-827.

Brewer, B.J., and W.L. Fangman. 1987. The localization of replication origins on ARS plasmids in S. cerevisiae. . *Cell*. 51:463-471.

Broek, D., R. Bartlett, K. Crawford, and P. Nurse. 1991. Involvement of p34cdc2 in establishing the dependency of S phase on mitosis. *Nature*. 349:388-393.

Brown, E.J., and D. Baltimore. 2000. ATR disruption leads to chromosomal fragmentation and early embryonic lethality. *Genes & Development*. 14:397-402.

Brown, E.J., and D. Baltimore. 2003. Essential and dispensible roles of ATR in cell cycle arrest and genome maintenance. *Genes & Development*. 17:615-628.

Burkhart, R., D. Schulte, D. Hu, C. Musahl, F. Gohring, and R. Knippers. 1995. Interactions of human nuclear proteins P1Mcm3 and P1Cdc46. *European Journal of Biochemistry*. 228:431-438.

Byun, T.S., M. Pacek, M.C. Yee, J.C. Walter, and K.A. Cimprich. 2005. Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. *Genes Dev*. 19:1040-1052.

Carlsson, P., and M. Mahlapuu. 2002. Forkhead transcription factors: key players in development and metabolism. *Developmental biology*. 250:1-23.

Carney, J.P., R.S. Maser, H. Olivares, E.M. Davis, M. Le Beau, J.R. Yates, 3rd, L. Hays, W.F. Morgan, and J.H. Petrini. 1998. The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: linkage of double-strand break repair to the cellular DNA damage response. *Cell*. 93:477-486.

Chandrasekaran, S., T.X. Tan, J.R. Hall, and J.G. Cook. 2011. Stress-stimulated mitogen-activated protein kinases control the stability and activity of the Cdt1 DNA replication licensing factor. *Mol Cell Biol*. 31:4405-4416.

Chesnokov, I., D. Remus, and M. Botchan. 2001. Functional analysis of mutant and wild-type Drosophila origin recognition complex. *Proc Natl Acad Sci U S A*. 98:11997-12002.

Chong, J.P., H.M. Mahbubani, C.Y. Khoo, and J.J. Blow. 1995. Purification of an mcm-containing complex as a component of the DNA replication licensing system. *Nature*. 375:418-421.

Chong, J.P., P. Thommes, and J.J. Blow. 1996. The role of MCM/P1 proteins in the licensing of DNA replication. *Trends in Biochemical Sciences*. 21:102-106.

Clay-Farrace, L., C. Pelizon, D. Santamaria, J. Pines, and R.A. Laskey. 2003. Human replication protein Cdc6 prevents mitosis through a checkpoint mechanism that implicates Chk1. *EMBO Journal*. 22:704-712.

Cocker, J.H., S. Piatti, C. Santocanale, K. Nasmyth, and J.F. Diffley. 1996. An essential role for the Cdc6 protein in forming the pre-replicative complexes of budding yeast. *Nature*. 379:180-182.

Coleman, T.R., P.B. Carpenter, and W.G. Dunphy. 1996. The Xenopus Cdc6 protein is essential for the initiation of a single round of DNA replication in cell-free extracts. *Cell*. 87:53-63.

Cortez, D., G. Glick, and S.J. Elledge. 2004. Minichromosome maintenance proteins are direct targets of the ATM and ATR checkpoint kinases. *Proceedings of the National Academy of Sciences of the U.S.A.* 101:10078-10083.

Cortez, D., S. Guntuku, J. Qin, and S.J. Elledge. 2001. ATR and ATRIP: partners in checkpoint signaling. *Science*. 294:1713-1716.

Coverley, D., C.S. Downes, P. Romanowski, and R.A. Laskey. 1993. Reversible effects of nuclear membrane permeabilization on DNA replication: evidence for a positive licensing factor. *J Cell Biol*. 122:985-992.

Crevel, G., and S. Cotterill. 1991. DNA replication in cell-free extracts from Drosophila melanogaster. *EMBO Journal*. 10:4361-4369.

Cseresnyes, Z., U. Schwarz, and C.M. Green. 2009. Analysis of replication factories in human cells by super-resolution light microscopy. *BMC Cell Biol*. 10:88.

Dahmann, C., J. Diffley, and K. Nasmyth. 1995. S-phase-promoting cyclin-dependent kinases prevent re-replication by inhibiting the transition of replication origins to a pre-replicative state. *Current Biology*. 5:1257-1269.

Davidson, I.F., A. Li, and J.J. Blow. 2006. Deregulated replication licensing causes DNA fragmentation consistent with head-to-tail fork collision. *Molecular Cell*. 24:433-443.

de Klein, A., M. Muijtjens, R. van Os, Y. Verhoeven, B. Smit, A.M. Carr, A.R. Lehmann, and J.H. Hoeijmakers. 2000. Targeted disruption of the cell-cycle checkpoint gene ATR leads to early embryonic lethality in mice. *Curr Biol*. 10:479-482.

De Marco, V., P.J. Gillespie, A. Li, N. Karantzelis, E. Christodoulou, R. Klompmaker, S. van Gerwen, A. Fish, M.V. Petoukhov, M.S. Iliou, Z. Lygerou, R.H. Medema, J.J. Blow, D.I. Svergun, S. Taraviras, and A. Perrakis. 2009. Quaternary structure of the human Cdt1-Geminin complex regulates DNA replication licensing. *Proceedings of the National Academy of Sciences of the United States of America*. 106:19807-19812.

Del Bene, F., K. Tessmar-Raible, and J. Wittbrodt. 2004. Direct interaction of geminin and Six3 in eye development. *Nature*. 427:745-749.

Delacroix, S., J.M. Wagner, M. Kobayashi, K. Yamamoto, and L.M. Karnitz. 2007. The Rad9-Hus1-Rad1 (9-1-1) clamp activates checkpoint signaling via TopBP1. *Genes Dev*. 21:1472-1477.

DePamphilis, M.L., J.J. Blow, S. Ghosh, T. Saha, K. Noguchi, and A. Vassilev. 2006. Regulating the licensing of DNA replication origins in metazoa. *Current Opinion in Cell Biology*. 18:231-239.

Dhar, S.K., L. Delmolino, and A. Dutta. 2001. Architecture of the human origin recognition complex. *Journal of Biological Chemistry*. 276:29067-29071.

Di Micco, R., M. Fumagalli, A. Cicalese, S. Piccinin, P. Gasparini, C. Luise, C. Schurra, M. Garre, N. P.G., A. Bensimon, R. Maestro, P. P.G., and F. Di Fagagna. 2006. Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature*. 444:638-642.

Diffley, J.F. 1996. Once and only once upon a time: specifying and regulating origins of DNA replication in eukaryotic cells. *Genes and Development*. 10:2819-2830.

Dimitrova, D.S., and D.M. Gilbert. 1999. The spatial position and replication timing of chromosomal domains are both established in early G1 phase. *Molecular Cell*. 4:983-993.

Dimitrova, D.S., I.T. Todorov, T. Melendy, and D.M. Gilbert. 1999. Mcm2, but not RPA, is a component of the mammalian early G1-phase prereplication complex. *Journal of Cell Biology*. 146:709-722.

Ding, Q., and D.M. MacAlpine. 2010. Preferential re-replication of Drosophila heterochromatin in the absence of geminin. *PLoS Genetics*. 6:e1001112.

Donnellan, R., and R. Chetty. 1999. Cyclin E in human cancers. *Faseb J*. 13:773-780.

Donovan, S., J. Harwood, L.S. Drury, and J.F. Diffley. 1997. Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. *Proceedings of the National Academy of Sciences of the U.S.A.* 94:5611-5616.

Drury, L.S., G. Perkins, and J.F.X. Diffley. 2000. The cyclin-dependent kinase Cdc28p regulates distinct modes of Cdc6p proteolysis during the budding yeast cell cycle. *Current Biology*. 10:231-240.

Duch, A., G. Palou, Z.O. Jonsson, R. Palou, E. Calvo, J. Wohlschlegel, and D.G. Quintana. 2011. A Dbf4 mutant contributes to bypassing the Rad53-mediated block of origins of replication in response to genotoxic stress. *J Biol Chem*. 286:2486-2491.

Dutta, A., and S.P. Bell. 1997. Initiation of DNA replication in eukaryotic cells. *Annual review of cell and developmental biology*. 13.

Edwards, M.C., A.V. Tutter, C. Cvetic, C.H. Gilbert, T.A. Prokhorova, and J.C. Walter. 2002. MCM2-7 complexes bind chromatin in a distributed pattern surrounding the origin recognition complex in Xenopus egg extracts. *Journal of Biological Chemistry*. 277:33049-33057.

Ekholm-Reed, S., J. Mendez, D. Tedesco, A. Zetterberg, B. Stillman, and S.I. Reed. 2004. Deregulation of cyclin E in human cells interferes with prereplication complex assembly. *Journal of Cell Biology*. 165:789-800.

Elsasser, S., Y. Chi, P. Yang, and J.L. Campbell. 1999. Phosphorylation controls timing of Cdc6p destruction: A biochemical analysis. *Molecular Biology of the Cell*. 10:3263-3277.

Evrin, C., P. Clarke, J. Zech, R. Lurz, J. Sun, S. Uhle, H. Li, B. Stillman, and C. Speck. 2009. A double-hexameric MCM2-7 complex is loaded onto origin DNA during licensing of eukaryotic DNA replication. *Proceedings of the National Academy of Sciences of the United States of America*. 106:20240-20245.

Feng, D., Z. Tu, W. Wu, and C. Liang. 2003. Inhibiting the expression of DNA replication-initiation proteins induces apoptosis in human cancer cells. *Cancer Research*. 63:7356-7364.

Ferenbach, A., A. Li, M. Brito-Martins, and J.J. Blow. 2005. Functional domains of the Xenopus replication licensing factor Cdt1. *Nucl. Acids Res.* 33:316-324.

Feulgen, R., and H. Rossenbeck. 1924. Mikroskopisch-chemischer Nachweis einer Nucleisäure vom Typus der Thymonucleinsäure und die darauf beruhende elektive Färbung von Zellkernen in mikroskopischen Präparaten. *Hoppe Seyler Z Physiol Chem*. 135:203-248.

Fletcher, R.J., B.E. Bishop, R.P. Leon, R.A. Sclafani, C.M. Ogata, and X.S. Chen. 2003. The structure and function of MCM from archaeal M. Thermoautotrophicum. *Nature Structural Biology*. 10:160-167.

Franklin, R.E., and R.G. Gosling. 1953. Molecular configuration in sodium thymonucleate. *Nature*. 171:740-741.

Franz, A., M. Orth, P.A. Pirson, R. Sonneville, J.J. Blow, A. Gartner, O. Stemmann, and T. Hoppe. 2011. CDC-48/p97 coordinates CDT-1 degradation with GINS chromatin dissociation to ensure faithful DNA replication. *Mol Cell*. 44:85-96.

Fujita, M., T. Kiyono, Y. Hayashi, and M. Ishibashi. 1997. In vivo interaction of human MCM heterohexameric complexes with chromatin. Possible involvement of ATP. *J Biol Chem*. 272:10928-10935.

Fujita, M., C. Yamada, H. Goto, N. Yokoyama, K. Kuzushima, M. Inagaki, and T. Tsurumi. 1999. Cell cycle regulation of human CDC6 protein. Intracellular localization, interaction with the human mcm complex, and CDC2 kinase-mediated hyperphosphorylation. *J Biol Chem*. 274:25927-25932.

Gambus, A., G.A. Khoudoli, R.C. Jones, and J.J. Blow. 2011. MCM2-7 form double hexamers at licensed origins in Xenopus egg extract. *Journal of Biological Chemistry*. 286:11855-11864.

Garcia, V., K. Furuya, and A.M. Carr. 2005. Identification and functional analysis of TopBP1 and its homologs *DNA Repair (Amst)*. 4:1227-1239.

Gavin, K.A., M. Hidaka, and B. Stillman. 1995. Conserved initiator proteins in eukaryotes. *Science*. 270:1667-1671.

Ge, X.Q., and J.J. Blow. 2010. Chk1 inhibits replication factory activation but allows dormant origin firing in existing factories. *Journal of Cell biology*. 191:1285-1297.

Ge, X.Q., D.A. Jackson, and J.J. Blow. 2007. Dormant origins licensed by excess Mcm2-7 are required for human cells to survive replicative stress. *Genes & Development*. 21:3331-3341.

Gillespie, P.J., and J.J. Blow. 2010. Clusters, factories and domains: The complex structure of S-phase comes into focus. *Cell Cycle*. 9:3218-3226.

Gillespie, P.J., A. Li, and J.J. Blow. 2001. Reconstitution of licensed replication origins on Xenopus sperm nuclei using purified proteins. *BMC Biochem.* 2:15.

Gonzalez, M.A., K.E. Tachibana, R.A. Laskey, and N. Coleman. 2005. Control of DNA replication and its potential clinical exploitation. *Nat Rev Cancer*. 5:135-141.

Gonzalez, M.A., K.E.K. Tachibana, D.J. Adams, L. van der Weyden, M. Hemberger, N. Coleman, A. Bradley, and R.A. Laskey. 2006. Geminin is essential to prevent endoreduplication and to form pluripotent cells during mammalian development. *Genes & Development*. 20:1880-1884.

Goren, A., and H. Cedar. 2003. Replicating by the clock. *Nat Rev Mol Cell Biol*. 4:25-32.

Gossen, M., D.T. Pak, S.K. Hansen, J.K. Acharya, and M.R. Botchan. 1995. A Drosophila homolog of the yeast origin recognition complex. *Science*. 270:1674-1677.

Grallert, B., and P. Nurse. 1996. The ORC1 homolog orp1 in fission yeast plays a key role in regulating onset of S phase. *Genes Dev*. 10:2644-2654.

Green, B.M., K.J. Finn, and J.J. Li. 2010. Loss of DNA replication control is a potent inducer of gene amplification. *Science*. 329:943-946.

Green, B.M., and J.J. Li. 2005. Loss of rereplication control in Saccharomyces cerevisiae results in extensive DNA damage. *Mol. Biol. Cell*. 16:421-432.

Green, B.M., R.J. Morreale, B. Ozaydin, J.L. Derisi, and J.J. Li. 2006. Genome-wide mapping of DNA synthesis in Saccharomyces cerevisiae reveals that mechanisms preventing reinitiation of DNA replication are not redundant. *Mol. Biol. Cell*. 17:2401-2414.

Hall, J.R., H.O. Lee, B.D. Bunker, E.S. Dorn, G.C. Rogers, R.J. Duronio, and J.G. Cook. 2008. Cdt1 and Cdc6 are destabilized by rereplication-induced DNA damage. *Journal of Biological Chemistry*. 283:25356-25363.

Hanahan, D., and R.A. Weinberg. 2000. The hallmarks of cancer. *Cell*. 100:57-70.

Hanby, A.M. 2005. Aspects of molecular phenotype and its correlations with breast cancer behaviour and taxonomy. *Br J Cancer*. 92:613-617.

Hannenhalli, S., and K.H. Kaestner. 2009. The evolution of Fox genes and their role in development and disease. *Nat Rev Genet*. 10:233-240.

Hara, K., K. Nakayama, and K. Nakayama. 2006. Geminin is essential for the development of preimplantation mouse embryos. *Genes Cells*. 11:1281-1293.

Harland, R.M., and R.A. Laskey. 1980. Regulated Replication of DNA Micro-Injected into Eggs of Xenopus-Laevis. *Cell*. 21:761-771.

Hartwell, L.H., R.K. Mortimer, J. Culotti, and M. Culotti. 1973. Genetic Control of the Cell Division Cycle in Yeast: V. Genetic Analysis of cdc Mutants. *Genetics*. 74:267-286.

Harvey, K.J., and J. Newport. 2003. CpG methylation of DNA restricts prereplication complex assembly in Xenopus egg extracts. *Molecular and Cellular Biology*. 23:6769-6779.

Hemerly, A.S., S.G. Prasanth, K. Siddiqui, and B. Stillman. 2009. Orc1 controls centriole and centrosome copy number in human cells. *Science*. 323:789-793.

Hennessy, K.M., C.D. Clark, and D. Botstein. 1990. Subcellular-Localization of Yeast Cdc46 Varies with the Cell-Cycle. *Genes & Development*. 4:2252-2263.

Hershey, A.D., and M. Chase. 1952. Independent functions of viral protein and nucleic acid in growth of bacteriophage. *J Gen Physiol*. 36:39-56.

Higa, L.A., I.S. Mihaylov, D.P. Banks, J. Zheng, and H. Zhang. 2003. Radiation-mediated proteolysis of CDT1 by CUL4-ROC1 and CSN complexes constitutes a new checkpoint. *Nature Cell Biology*. 5:1008-1015.

Hodgson, B., A. Li, S. Tada, and J.J. Blow. 2002. Geminin becomes activated as an inhibitor of Cdt1/RLF-B following nuclear import. *Current Biology*. 12:678-683.

Hofmann, J.F., and D. Beach. 1994. cdt1 is an essential target of the Cdc10/Sct1 transcription factor: requirement for DNA replication and inhibition of mitosis. *Embo J*. 13:425-434.

Hook, S.S., J.J. Lin, and A. Dutta. 2007. Mechanisms to control rereplication and implications for cancer. *Curr Opin Cell Biol*. 19:663-671.

Hua, X.H., and J. Newport. 1998. Identification of a preinitiation step in DNA replication that is independent of origin recognition complex and cdc6, but dependent on cdk2. *Journal of Cell Biology*. 140:271-281.

Huberman, J.A., K.A. Spotila, and S.M. Nawotka. 1987. The in vivo replication origin of the yeast 2 micron plasmid. *Cell*. 51:473-481.

Hyrien, O., and M. Mechali. 1992. Plasmid replication in Xenopus eggs and egg extracts: a 2D gel electrophoretic analysis. *Nucleic Acids Res*. 20:1463-1469.

Ibarra, A., E. Schwob, and J. Mendez. 2008. Excess MCM proteins protect human cells from replicative stress by licensing backup origins of replication. *Proc Natl Acad Sci U S A*. 105:8956-8961.

Ishii, T., Y. Shiomi, T. Takami, Y. Murakami, N. Ohnishi, and H. Nishitani. 2010. Proliferating cell nuclear antigen-dependent rapid recruitment of Cdt1 and CRL4Cdt2 at DNA-damaged sites after UV irradiation in HeLa cells. *J Biol Chem*. 285:41999-42000.

Ishimi, Y. 1997. A DNA helicase activity is associated with an MCM4, -6, and -7 protein complex. *Journal of Biological Chemistry*. 272:24508-24513.

Jackson, D.A., and A. Pombo. 1998. Replicon clusters are stable units of chromosome structure: evidence that nuclear organization contributes to the efficient activation and propagation of S phase in human cells. *J Cell Biol*. 140:1285-1295.

Jallepalli, P.V., G.W. Brown, M. MuziFalconi, D. Tien, and T.J. Kelly. 1997. Regulation of the replication initiator protein p65(cdc18) by CDK phosphorylation. *Genes & Development*. 11:2767-2779.

Jia, L.J., H. Li, and Y. Sun. 2011. Induction of p21-Dependent Senescence by an NAE Inhibitor, MLN4924, as a Mechanism of Growth Suppression. *Neoplasia*. 13:561-569.

Jiang, W., N.J. Wells, and T. Hunter. 1999. Multistep regulation of DNA replication by Cdk phosphorylation of HsCdc6. *Proc Natl Acad Sci U S A*. 96:6193-6198.

Jin, J., E.E. Arias, J. Chen, J.W. Harper, and J.C. Walter. 2006a. A family of diverse Cul4-Ddb1-interacting proteins includes Cdt2, which is required for S phase destruction of the replication factor Cdt1. *Mol Cell*. 23:709-721.

Jin, J.P., E.E. Arias, J. Chen, J.W. Harper, and J.C. Walter. 2006b. A family of diverse Cul4-Ddb1-interacting proteins includes Cdt2, which is required for S phase destruction of the replication factor Cdt1. *Molecular Cell*. 23:709-721.

Karnani, N., and A. Dutta. 2011. The effect of the intra-S-phase checkpoint on origins of replication in human cells. *Genes Dev*. 25:621-633.

Kawabata, T., S.W. Luebben, S. Yamaguchi, I. Ilves, I. Matise, T. Buske, M.R. Botchan, and N. Shima. 2011. Stalled fork rescue via dormant replication origins in unchallenged S phase promotes proper chromosome segregation and tumor suppression. *Mol Cell*. 41:543-553.

Kelly, T.J., and G.W. Brown. 2000. Regulation of chromosome replication. *Annu Rev Biochem*. 69:829-880.

Kerr, J.F., A.H. Wyllie, and A.R. Currie. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*. 26:239-257.

Kim, J., H. Feng, and E.T. Kipreos. 2007. C. elegans CUL-4 prevents rereplication by promoting the nuclear export of CDC-6 via a CKI-1-dependent pathway. *Current Biology*. 17:966-972.

Kisielewska, J., and J.J. Blow. 2012. Dynamic interactions of high Cdt1 and geminin levels regulate S phase in early Xenopus embryos. *Development*. 139:63-74.

Klotz-Noack, K., and J.J. Blow. 2011. A role for dormant origins in tumor suppression. *Molecular Cell*. 41:495-496.

Klotz-Noack, K., D. McIntosh, N. Schurch, N. Pratt, and J.J. Blow. 2012. Re-replication induced by geminin depletion occurs from G2 and is enhanced by checkpoint activation. *J Cell Sci*.

Koonin, E.V. 1993. A common set of conserved motifs in a vast variety of putative nucleic acid-dependent ATPases including MCM proteins involved in the initiation of eukaryotic DNA replication. *Nucleic Acids Res*. 21:2541-2547.

Kornberg, A., I. Lieberman, and E.S. Simms. 1955. Enzymatic synthesis and properties of 5-phosphoribosylpyrophosphate. *J Biol Chem*. 215:389-402.

Kreitz, S., M. Ritzi, M. Baack, and R. Knippers. 2001. The human origin recognition complex protein 1 dissociates from chromatin during S phase in HeLa cells. *J Biol Chem*. 276:6337-6342.

Krude, T., C. Musahl, R.A. Laskey, and R. Knippers. 1996. Human replication proteins hCdc21, hCdc46 and P1Mcm3 bind chromatin uniformly before S-phase and are displaced locally during DNA replication. *Journal of Cell Science*. 109:309-318.

Kubota, Y., S. Mimura, S. Nishimoto, T. Masuda, H. Nojima, and H. Takisawa. 1997. Licensing of DNA replication by a multi-protein complex of MCM/P1 proteins in Xenopus eggs. *EMBO Journal*. 16:3320-3331.

Kubota, Y., S. Mimura, S.I. Nishimoto, H. Takisawa, and H. Nojima. 1995. Identification of the Yeast Mcm3-Related Protein as a Component of Xenopus DNA-Replication Licensing Factor. *Cell*. 81:601-609.

Kulartz, M., and R. Knippers. 2004. The replicative regulator protein geminin on chromatin in the HeLa cell cycle. *J Biol Chem*. 279:41686-41694.

Kumagai, A., and W.G. Dunphy. 2000. Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in Xenopus egg extracts. *Mol Cell*. 6:839-849.

Kumagai, A., J. Lee, H.Y. Yoo, and W.G. Dunphy. 2006. TopBP1 activates the ATR-ATRIP complex. *Cell*. 124:943-955.

Kundu, L.R., Y. Kumata, N. Kakusho, S. Watanabe, A. Furukohri, S. Waga, M. Seki, H. Masai, T. Enomoto, and S. Tada. 2010. Deregulated Cdc6 inhibits DNA replication and suppresses Cdc7-mediated phosphorylation of Mcm2-7 complex. *Nucleic Acids Res*. 38:5409-5418.

Labib, K., and G. De Piccoli. 2011. Surviving chromosome replication: the many roles of the S-phase checkpoint pathway. *Philos Trans R Soc Lond B Biol Sci*. 366:3554-3561.

Labib, K., J.F.X. Diffley, and S.E. Kearsey. 1999. G1-phase and B-type cyclins exclude the DNA-replication factor Mcm4 from the nucleus. *Nature Cell Biology*. 1:415-422.

Labib, K., J.A. Tercero, and J.F.X. Diffley. 2000. Uninterrupted MCM2-7 function required for DNA replication fork progression. *Science*. 288:1643-1647.

Landis, G., R. Kelley, A.C. Spradling, and J. Tower. 1997. The k43 gene, required for chorion gene amplification and diploid cell chromosome replication, encodes the Drosophila homolog of yeast origin recognition complex subunit 2. *Proc Natl Acad Sci U S A*. 94:3888-3892.

Larner, J.M., H. Lee, and J.L. Hamlin. 1994. Radiation effects on DNA synthesis in a defined chromosomal replicon. *Mol Cell Biol*. 14:1901-1908.

Laskey, R. 2005. The Croonian Lecture 2001 - Hunting the antisocial cancer cell: MCM proteins and their exploitation. *Philosophical Transactions of the Royal Society B-Biological Sciences*. 360:1119-1132.

Laskey, R.A. 1985. Chromosome replication in early development of Xenopus laevis. *J Embryol Exp Morphol*. 89 Suppl:285-296.

Lau, E., T. Tsuji, L. Guo, S.H. Lu, and W. Jiang. 2007. The role of pre-replicative complex (pre-RC) components in oncogenesis. *Faseb J*. 21:3786-3794.

Lavin, M.F., and Y. Shiloh. 1997. The genetic defect in ataxia-telangiectasia. *Annu Rev Immunol*. 15:177-202.

Leatherwood, J., A. Lopez-Girona, and P. Russell. 1996. Interaction of Cdc2 and Cdc18 with a fission yeast ORC2-like protein. *Nature*. 379:360-363.

Lee, A.Y., E. Liu, and X. Wu. 2007a. The Mre11/Rad50/Nbs1 complex plays an important role in the prevention of DNA rereplication in mammalian cells. *Journal of Biological Chemistry*. 282:32243-32255.

Lee, C., B. Hong, J.M. Choi, Y. Kim, S. Watanabe, Y. Ishimi, T. Enomoto, S. Tada, and Y. Cho. 2004. Structural basis for inhibition of the replication licensing factor Cdt1 by geminin. *Nature*. 430:913-917.

Lee, J., A. Kumagai, and W.G. Dunphy. 2007b. The Rad9-Hus1-Rad1 checkpoint clamp regulates interaction of TopBP1 with ATR. *J Biol Chem*. 282:28036-28044.

Lee, J.H., and T.T. Paull. 2005. ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. *Science*. 308:551-554.

Lei, M., Y. Kawasaki, and B.K. Tye. 1996. Physical interactions among Mcm proteins and effects of Mcm dosage on DNA replication in Saccharomyces cerevisiae. *Molecular and Cellular Biology*. 16:5081-5090.

Lengauer, C., K.W. Kinzler, and B. Vogelstein. 1998. Genetic instabilities in human cancers. *Nature*. 396:643-649.

Leno, G.H., C.S. Downes, and R.A. Laskey. 1992. The nuclear membrane prevents replication of human G2 nuclei but not G1 nuclei in Xenopus egg extract. *Cell*. 69:151-158.

Li, A., and J.J. Blow. 2004. Non-proteolytic inactivation of geminin requires CDK-dependent ubiquitination. *Nat. Cell Biol.* 6:260-267.

Li, A., and J.J. Blow. 2005. Cdt1 downregulation by proteolysis and geminin inhibition prevents DNA re-replication in Xenopus. *EMBO J.* 24:395-404.

Li, J.J., and I. Herskowitz. 1993. Isolation of ORC6, a component of the yeast origin recognition complex by a one-hybrid system. *Science*. 262:1870-1874.

Li, X., Q. Zhao, R. Liao, P. Sun, and X. Wu. 2003. The SCF(Skp2) ubiquitin ligase complex interacts with the human replication licensing factor Cdt1 and regulates Cdt1 degradation. *Journal of Biological Chemistry*. 278:30854-30858.

Lin, J.J., and A. Dutta. 2007. ATR pathway is the primary pathway for activating G2/M checkpoint induction after re-replication. *Journal of Biological Chemistry*. 282:30357-30362.

Lin, J.J., M.A. Milhollen, P.G. Smith, U. Narayanan, and A. Dutta. 2010. NEDD8-targeting drug MLN4924 elicits DNA rereplication by stabilizing Cdt1 in S phase, triggering checkpoint activation, apoptosis, and senescence in cancer cells. *Cancer Research*. 70:10310-10320.

Liu, E., A.Y. Lee, T. Chiba, E. Olson, P. Sun, and X. Wu. 2007. The ATR-mediated S phase checkpoint prevents rereplication in mammalian cells when licensing control is disrupted. *Journal of Cell Biology*. 179:643-657.

Liu, E., X. Li, F. Yan, Q. Zhao, and X. Wu. 2004. Cyclin-dependent kinases phosphorylate human Cdt1 and induce its degradation. *Journal of Biological Chemistry*. 279:17283-17288.

Liu, P., D.M. Slater, M. Lenburg, K. Nevis, J.G. Cook, and C. Vaziri. 2009. Replication licensing promotes cyclin D1 expression and G1 progression in untransformed human cells. *Cell Cycle*. 8:125-136.

Liu, Q., S. Guntuku, X.S. Cui, S. Matsuoka, D. Cortez, K. Tamai, G. Luo, S. Carattini-Rivera, F. DeMayo, A. Bradley, L.A. Donehower, and S.J. Elledge. 2000. Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. *Genes Dev.* 14:1448-1459.

Lo, P.K., J.S. Lee, X. Liang, L. Han, T. Mori, M.J. Fackler, H. Sadik, P. Argani, T.K. Pandita, and S. Sukumar. 2010. Epigenetic inactivation of the potential tumor suppressor gene FOXF1 in breast cancer. *Cancer Res*. 70:6047-6058.

Lo, P.K., S.J. Lee, and S. Sukumar. 2012. The p53-p21WAF1 checkpoint pathway plays a protective role in preventing DNA rereplication induced by abrogation of FOX1 function. *Cellular Signalling*. 24:316-324.

Loeb, K.R., H. Kostner, E. Firpo, T. Norwood, D.T. K, B.E. Clurman, and J.M. Roberts. 2005. A mouse model for cyclin E-dependent genetic instability and tumorigenesis. *Cancer Cell*. 8:35-47.

Lopez-Mosqueda, J., N.L. Maas, Z.O. Jonsson, L.G. DeFazio-Eli, J. Wohlschlegel, and D.P. Toczyski. 2010. Damage-induced phosphorylation of Sld3 is important to block late origin firing. *Nature*. 467:479-U133.

Lovejoy, C.A., K. Lock, A. Yenamandra, and D. Cortez. 2006. DDB1 maintains genome integrety through regulation of Cdt1. *Mol Cell Biol*. 26.

Lu, J., F. Li, C.S. Murphy, M.W. Davidson, and D.M. Gilbert. 2010. G2 phase chromatin lacks determinants of replication timing. *Journal of Cell Biology*. 189:967-980.

Lukas, C., J. Bartkova, L. Latella, J. Falck, N. Mailand, T. Schroeder, M. Sehested, J. Lukas, and J. Bartek. 2001. DNA damage-activated kinase Chk2 is independent of proliferation or differentiation yet correlates with tissue biology. *Cancer Research*. 61:4990-4993.

Luo, L., X. Yang, Y. Takihara, H. Knoetgen, and M. Kessel. 2004. The cell-cycle regulator geminin inhibits Hox function through direct and polycomb-mediated interactions. *Nature*. 427:749-753.

Lutzmann, M., D. Maiorano, and M. Mechali. 2006. A Cdt1-geminin complex licenses chromatin for DNA replication and prevents rereplication during S phase in Xenopus. *Embo J*. 25:5764-5774.

Machida, Y.J., and A. Dutta. 2007. The APC/C inhibitor, Emi1, is essential for prevention of rereplication. *Genes & Development*. 21:184-194.

Madine, M.A., C.Y. Khoo, A.D. Mills, C. Musahl, and R.A. Laskey. 1995. The nuclear envelope prevents reinitiation of replication by regulating the binding of MCM3 to chromatin in Xenopus egg extracts. *Current Biology*. 5:1270-1279.

Madine, M.A., M. Swietlik, C. Pelizon, P. Romanowski, A.D. Mills, and R.A. Laskey. 2000. The roles of the MCM, ORC, and Cdc6 proteins in determining the replication competence of chromatin in quiescent cells. *Journal of structural biology*. 129:198-210.

Mahbubani, H.M., J.P. Chong, S. Chevalier, P. Thömmes, and J.J. Blow. 1997. Cell cycle regulation of the replication licensing system: involvement of a Cdk-dependent inhibitor. *Journal of Cell Biology*. 136:125-135.

Mahbubani, H.M., T. Paull, J.K. Elder, and J.J. Blow. 1992. DNA replication initiates at multiple sites on plasmid DNA in Xenopus egg extracts. *Nucleic acid research*. 20:1457-1462.

Mailand, N., J. Falck, C. Lukas, R.G. Syljuasen, and M. Welcker. 2000. Rapid de-struction of human Cdc25A in response to DNA damage. *Science*. 288:1425-1429.

Maine, G.T., P. Sinha, and B.K. Tye. 1984. Mutants of S. cerevisiae defective in the maintenance of minichromosomes. *Genetics*. 106:365-385.

Maiorano, D., L. Krasinska, M. Lutzmann, and M. Mechali. 2005. Recombinant Cdt1 induces rereplication of G2 nuclei in Xenopus egg extracts. *Curr. Biol.* 15:146-153.

Maiorano, D., J. Moreau, and M. Mechali. 2000. XCDT1 is required for the assembly of pre-replicative complexes in Xenopus laevis. *Nature*. 404:622-625.

Majka, J., A. Niedziela-Majka, and P.M. Burgers. 2006. The checkpoint clamp activates Mec1 kinase during initiation of the DNA damage checkpoint. *Mol Cell*. 24.

Malkin, D., F.P. Li, L.C. Strong, J.F. Fraumeni, Jr., C.E. Nelson, D.H. Kim, J. Kassel, M.A. Gryka, F.Z. Bischoff, M.A. Tainsky, and et al. 1990. Germ line p53

mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science*. 250:1233-1238.

Mamely, I., M.A. van Vugt, V.A. Smits, J.I. Semple, B. Lemmens, A. Perrakis, R.H. Medema, and R. Freire. 2006. Polo-like kinase-1 controls proteasome-dependent degradation of Claspin during checkpoint recovery. *Curr Biol*. 16:1950-1955.

Matsuoka, S., M. Huang, and S.J. Elledge. 1998. Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. *Science*. 282:1892-1897.

May, N.R., M. Thomer, K.F. Murnen, and B.R. Calvi. 2005. Levels of the origin-binding protein double parked and its inhibitor geminin increase in response to replication stress. *J Cell Sci*. 118:4207-4217.

Maya-Mendoza, A., P. Olivares-Chauvet, A. Shaw, and D.A. Jackson. 2010. S Phase Progression in Human Cells Is Dictated by the Genetic Continuity of DNA Foci. *PLoS Genetics*. 6.

McGarry, T.J., and M.W. Kirschner. 1998. Geminin, an inhibitor of DNA replication, is degraded during mitosis. *Cell*. 93:1043-1053.

Melchionna, R., X.B. Chen, A. Blasina, and C.H. McGowan. 2000. Threonine 68 is required for radiation-induced phosphorylation and activation of Cds1. *Nature Cell Biology*. 2:762-765.

Melixetian, M., A. Ballabeni, L. Masiero, P. Gasparini, R. Zamponi, J. Bartek, J. Lukas, and K. Helin. 2004. Loss of Geminin induces rereplication in the presence of functional p53. *J. Cell Biol.* 165:473-482.

Mendez, J., and B. Stillman. 2000. Chromatin association of human origin recognition complex, Cdc6, and minichromosome maintenance proteins during the cell cycle: Assembly of prereplication complexes in late mitosis. *Molecular and Cellular Biology*. 20:8602-8612.

Mendez, J., X.H. Zou-Yang, S.Y. Kim, M. Hidaka, W.P. Tansey, and B. Stillman. 2002. Human origin recognition complex large subunit is degraded by ubiquitin-mediated proteolysis after initiation of DNA replication. *Molecular Cell*. 9:481-491.

Meselson, M., and F.W. Stahl. 1958. The Replication of DNA in Escherichia Coli. *Proc Natl Acad Sci U S A*. 44:671-682.

Miescher, F. 1871. Ueber die chemische Zusammensetzung der Eiterzellen. *Medizinische Untersuchung* 4:441-460.

Mihaylov, I.S., T. Kondo, L. Jones, S. Ryzhikov, J. Tanaka, J. Zheng, L.A. Higa, N. Minamino, L. Cooley, and H. Zhang. 2002. Control of DNA replication and chromosome ploidy by geminin and cyclin A. *Mol. Cell. Biol.* 22:1868-1880.

Milhollen, M.A., U. Narayanan, T.A. Soucy, P.O. Veiby, P.G. Smith, and B. Amidon. 2011. Inhibition of NEDD8-Activating Enzyme Induces Rereplication and Apoptosis in Human Tumor Cells Consistent with Deregulating CDT1 Turnover. *Cancer Research*. 71:3042-3051.

Moyer, S.E., P.W. Lewis, and M.R. Botchan. 2006. Isolation of the Cdc45/Mcm2-7/GINS (CMG) complex, a candidate for the eukaryotic DNA replication fork helicase. *Proc Natl Acad Sci U S A*. 103:10236-10241.

Mullis, K.B., and F.A. Faloona. 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol*. 155:335-350.

Murakami, H., S.K. Yanow, D. Griffiths, M. Nakanishi, and P. Nurse. 2002. Maintenance of replication forks and the S-phase checkpoint by Cdc18p and Orp1p. *Nature Cell Biology*. 4:384-388.

Musahl, C., H.P. Holthoff, R. Lesch, and R. Knippers. 1998. Stability of the replicative Mcm3 protein in proliferating and differentiating human cells. *Exp Cell Res*. 241:260-264.

Myllykangas, S., and S. Knuutila. 2006. Manifestation, mechanisms and mysteries of gene amplifications. *Cancer Lett*. 232:79-89.

Nakada, D., Y. Hirano, and K. Sugimoto. 2004. Requirement of the Mre11 complex and exonuclease 1 for activation of the Mec1 signaling pathway. *Mol Cell Biol*. 24:10016-10025.

Neuwald, A.F., L. Aravind, J.L. Spouge, and E.V. Koonin. 1999. AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res*. 9:27-43.

Nevis, K., M. Cordeiro-Stone, and J.G. Cook. 2009. Origin licensing and p53 status regulate Cdk2 activity during G(1).
Nevis KR, Cordeiro-Stone M, Cook JG. *Cell Cycle*. 8:1952-1963.

Nguyen, V.Q., C. Co, and J.J. Li. 2001. Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms. *Nature*. 411:1068-1073.

Nishitani, H., Z. Lygerou, and T. Nishimoto. 2004. Proteolysis of DNA replication licensing factor Cdt1 in S-phase is performed independently of geminin through its N-terminal region. *Journal of Biological Chemistry*. 279:30807-30816.

Nishitani, H., Z. Lygerou, T. Nishimoto, and P. Nurse. 2000. The Cdt1 protein is required to license DNA for replication in fission yeast. *Nature*. 404:625-628.

Nishitani, H., and P. Nurse. 1995. p65cdc18 plays a major role controlling the initiation of DNA replication in fission yeast. *Cell*. 83:397-405.

Nishitani, H., N. Sugimoto, V. Roukos, Y. Nakanishi, M. Saijo, C. Obuse, T. Tsurimoto, K.I. Nakayama, K. Nakayama, M. Fujita, Z. Lygerou, and T. Nishimoto. 2006. Two E3 ubiquitin ligases, SCF-Skp2 and DDB1-Cul4, target human Cdt1 for proteolysis. *EMBO Journal*. 25:1126-1136.

Nishitani, H., S. Taraviras, Z. Lygerou, and T. Nishimoto. 2001. The human licensing factor for DNA replication Cdt1 accumulates in G1 and is destabilized after initiation of S-phase. *J. Biol. Chem.* 276:44905-44411.

Nyberg, K.A., R.J. Michelson, C.W. Putnam, and T.A. Weinert. 2002. Toward maintaining the genome: DNA damage and replication checkpoints. *Annu Rev Genet*. 36:617-656.

O'Keefe, R.T., S.C. Henderson, and D.L. Spector. 1992. Dynamic organization of DNA replication in mammalian cell nuclei: spatially and temporally defined replication of chromosome-specific alpha-satellite DNA sequences. *J Cell Biol*. 116:1095-1110.

Oda, E., R. Ohki, H. Murasawa, J. Nemoto, T. Shibue, T. Yamashita, T. Tokino, T. Taniguchi, and N. Tanaka. 2000. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science*. 288:1053-1058.

Ode, K.L., K. Fujimoto, Y. Kubota, and H. Takisawa. 2011. Inter-origin cooperativity of geminin action establishes an all-or-none switch for replication origin licensing. *Genes Cells*. 16:380-396.

Oehlmann, M., A.J. Score, and J.J. Blow. 2004. The role of Cdc6 in ensuring complete genome licensing and S phase checkpoint activation. *Journal of Cell Biology*. 165:181-190.

Ohtsubo, M., and J. Roberts. 1993. Cyclin dependent regulation of G1 in mammalian fibroblasts. *Science*. 259:1908-1912.

Ohtsubo, M., A.M. Theodoras, J. Schumacher, J.M. Roberts, and M. Pagano. 1995. Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. *Mol Cell Biol*. 15:2612-2624.

Pacek, M., A.V. Tutter, Y. Kubota, H. Takisawa, and J.C. Walter. 2006. Localization of MCM2-7, Cdc45, and GINS to the site of DNA unwinding during eukaryotic DNA replication. *Mol Cell*. 21:581-587.

Pefani, D.E., M. Dimaki, M. Spella, N. Karantzelis, E. Mitsiki, C. Kyrousi, I.E. Symeonidou, A. Perrakis, S. Taraviras, and Z. Lygerou. 2011. Idas, a Novel Phylogenetically Conserved Geminin-related Protein, Binds to Geminin and Is Required for Cell Cycle Progression. *Journal of Biological Chemistry*. 286:23234-23246.

Peng, C.Y., P.R. Graves, R.S. Thoma, Z. Wu, A.S. Shaw, and H. Piwnica-Worms. 1997. Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. *Science*. 277:1501-1505.

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

Peschiaroli, A., N.V. Dorrello, D. Guardavaccaro, M. Venere, T. Halazonetis, N.E. Sherman, and M. Pagano. 2006. SCFbetaTrCP-mediated degradation of Claspin regulates recovery from the DNA replication checkpoint response. *Mol Cell*. 23:319-329.

Pession, A., and R. Tonelli. 2005. The MYCN oncogene as a specific and selective drug target for peripheral and central nervous system tumors. *Curr Cancer Drug Targets*. 5:273-283.

Petersen, B.O., C. Wagener, F. Marinoni, E.R. Kramer, M. Melixetian, E. Lazzerini Denchi, C. Gieffers, C. Matteucci, J.M. Peters, and K. Helin. 2000. Cell cycle- and cell growth-regulated proteolysis of mammalian CDC6 is dependent on APC-CDH1. *Genes Dev*. 14:2330-2343.

Petersen, P., D.M. Chou, Z. You, T. Hunter, J.C. Walter, and G. Walter. 2006. Protein phosphatase 2A antagonizes ATM and ATR in a Cdk2- and Cdc7-independent DNA damage checkpoint. *Mol. Cell. Biol.* 26.

Pruitt, S.C., K.J. Bailey, and A. Freeland. 2007. Reduced Mcm2 expression results in severe stem/progenitor cell deficiency and cancer. *Stem Cells*. 25:3121-3132.

Quinn, L.M., A. Herr, T.J. McGarry, and H. Richardson. 2001. The Drosophila Geminin homolog: roles for Geminin in limiting DNA replication, in anaphase and in neurogenesis. *Genes & Development*. 15:2741-2754.

Raman, M., C.G. Havens, J.C. Walter, and J.W. Harper. 2011. A genome-wide screen identifies p97 as an essential regulator of DNA damage-dependent CDT1 destruction. *Mol Cell*. 44:72-84.

Randell, J.C., J.L. Bowers, H.K. Rodriguez, and S.P. Bell. 2006. Sequential ATP hydrolysis by Cdc6 and ORC directs loading of the Mcm2-7 helicase. *Mol Cell*. 21:29-39.

Rao, P.N., and R.T. Johnson. 1970. Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis. *Nature*. 225:159-164.

Remus, D., F. Beuron, G. Tolun, J.D. Griffith, E.P. Morris, and J.F. Diffley. 2009. Concerted loading of Mcm2-7 double hexamers around DNA during DNA replication origin licensing. *Cell*. 139:719-730.

Resnitzky, D., M. Gossen, H. Bujard, and S.I. Reed. 1994. Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system. *Mol Cell Biol*. 14:1669-1679.

Ritzi, M., M. Baack, C. Musahl, P. Romanowski, R.A. Laskey, and R. Knippers. 1998. Human minichromosome maintenance proteins and human origin recognition complex 2 protein on chromatin. *Journal of Biological Chemistry*. 273:24543-24549.

Rogakou, E.P., D.R. Pilch, A.H. Orr, V.S. Ivanova, and W.M. Bonner. 1998. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem*. 273:5858-5868.

Romanowski, P., M.A. Madine, A. Rowles, J.J. Blow, and R.A. Laskey. 1996. The Xenopus origin recognition complex is essential for DNA replication and MCM binding to chromatin. *Current Biology*. 6:1416-1425.

Romanowski, P., J. Marr, M.A. Madine, A. Rowles, J.J. Blow, J. Gautier, and R.A. Laskey. 2000. Interaction of Xenopus Cdc2 x cyclin A1 with the origin recognition complex. *J Biol Chem*. 275:4239-4243.

Roukos, V., A. Kinkhabwala, J. Colombelli, P. Kotsantis, S. Taraviras, H. Nishitani, E. Stelzer, P. Bastiaens, and Z. Lygerou. 2011. Dynamic recruitment of licensing factor Cdt1 to sites of DNA damage. *J Cell Sci*. 124:422-434.

Rowles, A., J.P. Chong, L. Brown, M. Howell, G.I. Evan, and J.J. Blow. 1996. Interaction between the origin recognition complex and the replication licensing system in Xenopus. *Cell*. 87:287-296.

Rowles, A., S. Tada, and J.J. Blow. 1999. Changes in association of the Xenopus origin recognition complex with chromatin on licensing of replication origins. *Journal of Cell Science*. 112:2011-2018.

Saha, P., J.J. Chen, K.C. Thome, S.J. Lawlis, Z.H. Hou, M. Hendricks, J.D. Parvin, and A. Dutta. 1998. Human CDC6/Cdc18 associates with Orc1 and cyclin-cdk and is selectively eliminated from the nucleus at the onset of S phase. *Molecular and Cellular Biology*. 18:2758-2767.

Sakahira, H., M. Enari, and S. Nagata. 1998. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature*. 391:96-99.

Sanchez, Y., C. Wong, R.S. Thoma, R. Richman, R.Q. Wu, H. PiwnicaWorms, and S.J. Elledge. 1997. Conservation of the Chk1 checkpoint pathway in mammals: Linkage of DNA damage to Cdk regulation through Cdc25. *Science*. 277:1497-1501.

Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A*. 74:5463-5467.

Sansam, C.L., J.L. Shepard, K. Lai, A. Ianari, P.S. Danielian, A. Amsterdam, N. Hopkins, and J.A. Lees. 2006. DTL/CDT2 is essential for both CDT1 regulation and the early G2/M checkpoint. *Genes & Development*. 20:3117-3129.

Santocanale, C., and J.F. Diffley. 1998. A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication. *Nature*. 395:615-618.

Sato, M., T. Gotow, Z. You, Y. Komamura-Kohno, Y. Uchiyama, N. Yabuta, H. Nojima, and Y. Ishimi. 2000. Electron microscopic observation and single-stranded DNA binding activity of the Mcm4,6,7 complex. *J Mol Biol*. 300:421-431.

Savitsky, K., A. Bar-Shira, S. Gilad, G. Rotman, and Y. Ziv. 1995. A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science*. 268:1749-1753.

Saxena, S., P. Yuan, S.K. Dhar, T. Senga, D. Takeda, H. Robinson, S. Kornbluth, K. Swaminathan, and A. Dutta. 2004. A dimerized coiled-coil domain and an adjoining part of geminin interact with two sites on Cdt1 for replication inhibition. *Molecular Cell*. 15:245-258.

Senga, T., U. Sivaprasad, W. Zhu, J.H. Park, E.E. Arias, J.C. Walter, and A. Dutta. 2006. PCNA is a cofactor for Cdt1 degradation by CUL4/DDB1-mediated N-terminal ubiquitination. *Journal of Biological Chemistry*. 281:6246-6252.

Seo, S., A. Herr, J.W. Lim, G.A. Richardson, H. Richardson, and K.L. Kroll. 2005. Geminin regulates neuronal differentiation by antagonizing Brg1 activity. *Genes Dev*. 19:1723-1734.

Sherr, C.J. 1996. Cancer Cell Cycles. *Science*. 274:1672-1676.

Shima, N., A. Alcaraz, I. Liachko, T.R. Buske, C.A. Andrews, R.J. Munroe, S.A. Hartford, B.K. Tye, and J.C. Schimenti. 2007. A viable allele of Mcm4 causes chromosome instability and mammary adenocarcinomas in mice. *Nature Genetics*. 39:93-98.

Shiomi, Y., A. Hayashi, T. Ishii, K. Shinmyozu, J.I. Nakayama, K. Sugasawa, and H. Nishitani. 2012. Two different RFC proteins, Ctf18 and RFC1 separately control PCNA-CRL4Cdt2-mediated Cdt1 proteolysis during S phase and following UV-irradiation. *Mol Cell Biol*.

Shirahige, K., Y. Hori, K. Shiraishi, M. Yamashita, K. Takahashi, C. Obuse, T. Tsurimoto, and H. Yoshikawa. 1998. Regulation of DNA-replication origins during cell-cycle progression. *Nature*. 395:618-621.

Shreeram, S., A. Sparks, D.P. Lane, and J.J. Blow. 2002. Cell type-specific responses of human cells to inhibition of replication licensing. *Oncogene*. 21:6624-6632.

Sogo, J.M., M. Lopes, and M. Foiani. 2002. Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. *Science*. 297:599-602.

Sonneville, R., M. Querenet, A. Craig, A. Gartner, and J.J. Blow. 2012. The dynamics of replication licensing in live Ceanorhabditis elegans embryos. *J Cell Biol*. 196:233-246.

Soucy, T.A., P.G. Smith, M.A. Milhollen, A.J. Berger, J.M. Gavin, S. Adhikari, J.E. Brownell, K.E. Burke, D.P. Cardin, S. Critchley, C.A. Cullis, A. Doucette, J.J. Garnsey, J.L. Gaulin, R.E. Gershman, A.R. Lublinsky, A. McDonald, H. Mizutani, U. Narayanan, E.J. Olhava, S. Peluso, M. Rezaei, M.D. Sintchak, T. Talreja, M.P. Thomas, T. Traore, S. Vyskocil, G.S. Weatherhead, J. Yu, J. Zhang, L.R. Dick, C.F. Claiborne, M. Rolfe, J.B. Bolen, and S.P. Langston. 2009. An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. *Nature*. 458:732-736.

Spruck, C.H., K.A. Won, and S.I. Reed. 1999. Deregulated cyclin E induces chromosome instability. *Nature*. 401:297-300.

Stewart, G.S., R.S. Maser, T. Stankovic, D.A. Bressan, M.I. Kaplan, N.G.J. Jaspers, A. Raams, P.J. Byrd, J.H.J. Petrini, and A.M.R. Taylor. 1999. The DNA double-strand break repair gene hMRE11 is mutated in individuals with an ataxia-telangiectasia-like disorder. *Cell*. 99:577-587.

Stucki, M., and S.P. Jackson. 2006. gamma H2AX and MDC1: Anchoring the DNA-damage-response machinery to broken chromosomes. *DNA Repair*. 5:534-543.

Sugimoto, N., I. Kitabayashi, S. Osano, Y. Tatsumi, T. Yugawa, M. Narisawa-Saito, A. Matsukage, T. Kiyono, and M. Fujita. 2008. Identification of novel human Cdt1-binding proteins by a proteomics approach: proteolytic regulation by APC/CCdh1. *Molecular Biology of the Cell*. 19:1007-1021.

Sugimoto, N., Y. Tatsumi, T. Tsurumi, A. Matsukage, T. Kiyono, H. Nishitani, and M. Fujita. 2004. Cdt1 phosphorylation by cyclin A-dependent kinases negatively regulates its function without affecting geminin binding. *Journal of Biological Chemistry*. 279:19691-19697.

Sutton, W.S. 1902. On the morphology of the chromosome group in Brachystola magna. *Biol. Bull*. 4:24-39.

Tachibana, K.E., M.A. Gonzalez, and N. Coleman. 2005a. Cell-cycle-dependent regulation of DNA replication and its relevance to cancer pathology. *J Pathol*. 205:123-129.

Tachibana, K.E., M.A. Gonzalez, G. Guarguaglini, E.A. Nigg, and R.A. Laskey. 2005b. Depletion of licensing inhibitor geminin causes centromer overdublication and mitotic defects. *Embo Rep.* 6:1052-1057.

Tachibana, K.E., M.A. Gonzalez, G. Guarguaglini, E.A. Nigg, and R.A. Laskey. 2005c. Depletion of licensing inhibitor geminin causes centrosome overduplication and mitotic defects. *EMBO Rep*. 6:1052-1057.

Tada, S., A. Li, D. Maiorano, M. Mechali, and J.J. Blow. 2001. Repression of origin assembly in metaphase depends on inhibition of RLF-B/Cdt1 by geminin. *Nature Cell Biology*. 3:107-113.

Takai, H., K. Tominaga, N. Motoyama, Y.A. Minamishima, H. Nagahama, T. Tsukiyama, K. Ikeda, K. Nakayama, N. Nakanishi, and K. Nakayama. 2000.

Aberrant cell cycle checkpoint function and early embryonic death in Chk1(-/-) mice. *Genes & Development*. 14:1439-1447.

Takara, T.J., and S.P. Bell. 2011. Multiple Cdt1 molecules act at each origin to load replication-competent Mcm2–7 helicases. *EMBO Journal*. 30:4885-4896.

Takeda, D.Y., J.D. Parvin, and A. Dutta. 2005. Degradation of Cdt1 during S phase is Skp2-independent and is required for efficient progression of mammalian cells through S phase. *J Biol Chem*. 280:23416-23423.

Tanaka, S., and J.F. Diffley. 2002. Interdependent nuclear accumulation of budding yeast Cdt1 and Mcm2-7 during G1 phase. *Nature Cell Biology*. 4:198-207.

Tanny, R.E., D.M. MacAlpine, H.G. Blitzblau, and S.P. Bell. 2006. Genome-wide analysis of re-replication reveals inhibitory controls that target multiple stages of replication initiation. *Mol Biol Cell*. 17:2415-2423.

Tenca, P., D. Brotherton, A. Montagnoli, S. Rainoldi, C. Albanese, and C. Santocanale. 2007. Cdc7 is an active kinase in human cancer cells undergoing replication stress. *J Biol Chem*. 282:208-215.

Thepaut, M., D. Maiorano, J.F. Guichou, M.T. Auge, C. Dumas, M. Mechali, and A. Padilla. 2004. Crystal structure of the coiled-coil dimerization motif of geminin: structural and functional insights on DNA replication regulation. *Journal of Molecular Biology*. 342:275-287.

Thomer, M., N.R. May, B.D. Aggarwal, G. Kwok, and B.R. Calvi. 2004. Drosophila double-parked is sufficient to induce re-replication during development and is regulated by cyclin E/CDK2. *Development*. 131:4807-4818.

Thommes, P., Y. Kubota, H. Takisawa, and J.J. Blow. 1997. The RLF-M component of the replication licensing system forms complexes containing all six MCM/P1 polypeptides. *EMBO Journal*. 16:3312-3319.

Thomson, A., P. Gillispie, and J. Blow. 2010. Replication factory activation can be decoupled from the replication timing program by modulating Cdk levels. *Journal of Cell Biology*. 188:209-221.

Todorov, I.T., B.A. Werness, H.Q. Wang, N. Buddharajul, P.D. Todorova, H.K. Slocum, J.S. Brooks, and J.A. Huberman. 1998. HsMcm2/BM28: a novel proliferation marker for human tumours and normal tissues. *Lab. Invest.* 78:73-78.

Uhlmann, F., F. Lottspeich, and K. Nasmyth. 1999. Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. *Nature*. 400:37-42.

van der Horst, A., and B.M.T. Burgering. 2007. Stressing the role of FoxO proteins in lifespan and disease. *Nature Reviews Molecular Cell Biology*. 8:440-450.

Varon, R., C. Vissinga, M. Platzer, K.M. Cerosaletti, K.H. Chrzanowska, K. Saar, G. Beckmann, E. Seemanova, P.R. Cooper, N.J. Nowak, M. Stumm, C.M.R. Weemaes, R.A. Gatti, R.K. Wilson, M. Digweed, A. Rosenthal, K. Sperling, P. Concannon, and A. Reis. 1998. Nibrin, a novel DNA double-strand break repair protein, is mutated in Nijmegen breakage syndrome. *Cell*. 93:467-476.

Vas, A., W. Mok, and J. Leatherwood. 2001. Control of DNA rereplication via Cdc2 phosphorylation sites in the origin recognition complex. *Mol Cell Biol*. 21:5767-5777.

Vaziri, C., S. Saxena, Y. Jeon, C. Lee, K. Murata, Y. Machida, N. Wagle, D.S. Hwang, and A. Dutta. 2003. A p53-dependent checkpoint pathway prevents rereplication. *Mol. Cell*. 11:997-1008.

Vogt, N., S.H. Lefevre, F. Apiou, A.M. Dutrillaux, A. Cor, P. Leuraud, M.F. Poupon, B. Dutrillaux, M. Debatisse, and B. Malfoy. 2004. Molecular structure of double-minute chromosomes bearing amplified copies of the epidermal growth factor receptor gene in gliomas. *Proc Natl Acad Sci U S A*. 101:11368-11373.

Walter, J., and J. Newport. 2000. Initiation of eukaryotic DNA replication: origin unwinding and sequential chromatin association of Cdc45, RPA, and DNA polymerase alpha. *Mol Cell*. 5:617-627.

Watson, J.D., and F.H. Crick. 1953. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature*. 171:737-738.

Weinreich, M., and B. Stillman. 1999a. Cdc7p-Dbf4p kinase binds to chromatin during S phase and is regulated by both the APC and the RAD53 checkpoint pathway. *Embo J*. 18:5334-5346.

Weinreich, M., and B. Stillman. 1999b. Cdc7p–Dbf4p kinase binds to chromatin during S phase and is regulated by both the APC and the RAD53 checkpoint pathway. *Embo J*. 18:5334-5346.

Whittaker, A.J., I. Royzman, and T.L. Orr-Weaver. 2000. Drosophila Double parked: a conserved, essential replication protein that colocalizes with the origin recognition complex and links DNA replication with mitosis and the down-regulation of S phase transcripts. *Genes & Development*. 14:1765-1776.

Williams, G.H., P. Romanowski, L. Morris, M. Madine, A.D. Mills, K. Stoeber, J. Marr, R.A. Laskey, and N. Coleman. 1998. Improved cervical smear assessment using antibodies against proteins that regulate DNA replication. *Proceedings of the National Academy of Sciences of the United States of America*. 95:14932-14937.

Williams, G.H., and K. Stoeber. 2007. Cell cycle markers in clinical oncology. *Curr Opin Cell Biol*. 19:672-679.

Wohlschlegel, J.A., B.T. Dwyer, S.K. Dhar, C. Cvetic, J.C. Walter, and A. Dutta. 2000. Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. *Science*. 290:2309-2312.

Woodward, A.M., T. Gohler, M.G. Luciani, M. Oehlmann, X. Ge, A. Gartner, D.A. Jackson, and J.J. Blow. 2006. Excess Mcm2-7 license dormant origins of replication that can be used under conditions of replicative stress. *Journal of Cell Biology*. 173:673-683.

Xouri, G., Z. Lygerou, H. Nishitani, V. Pachnis, P. Nurse, and S. Taraviras. 2004. Cdt1 and geminin are down-regulated upon cell cycle exit and are over-expressed in cancer-derived cell lines. *Eur. J. Biochem.* 271:3368-3378.

Yanagi, K., T. Mizuno, T. Tsuyama, S. Tada, Y. Iida, A. Sugimoto, T. Eki, T. Enomoto, and F. Hanaoka. 2005. Caenorhabditis elegans geminin homologue participates in cell cycle regulation and germ line development. *J. Biol. Chem.* 280:19689-19694.

Yanagi, K., T. Mizuno, Z. You, and F. Hanaoka. 2002. Mouse geminin inhibits not only Cdt1-MCM6 interactions but also a novel intrinsic Cdt1 DNA binding activity. *Journal of Biological Chemistry*. 277:40871-40880.

Yanow, S.K., D.A. Gold, H.Y. Yoo, and W.G. Dunphy. 2003. Xenopus Drf1, a regulator of Cdc7, displays checkpoint-dependent accumulation on chromatin during an S-phase arrest. *J Biol Chem*. 278:41083-41092.

Yim, H., and R. Erikson. 2010. Cdc6, a mitotic substrate of polo-like kinase 1, regulates chromosome segragation mediated by Cdk1 and separase. *PNAS*. 107:19742-19747.

Yoshida, K., and I. Inoue. 2004. Regulation of Geminin and Cdt1 expression by E2F transcription factors. *Oncogene*. 23:3802-3812.

Yoshida, K., H. Takisawa, and Y. Kubota. 2005. Intrinsic nuclear import activity of geminin is essential to prevent re-initiation of DNA replication in Xenopus eggs. *Genes Cells*. 10:63-73.

Zegerman, P., and J.F. Diffley. 2010. Checkpoint-dependent inhibition of DNA replication initiation by Sld3 and Dbf4 phosphorylation. *Nature*. 467:474-478.

Zhong, W., H. Feng, F.E. Santiago, and E.T. Kipreos. 2003. CUL-4 ubiquitin ligase maintains genome stability by restraining DNA-replication licensing. *Nature*. 423:885-889.

Zhu, W., Y. Chen, and A. Dutta. 2004. Rereplication by depletion of geminin is seen regardless of p53 status and activates a G2/M checkpoint. *Mol. Cell. Biol.* 24:7140-7150.

Zhu, W., and M.L. DePamphilis. 2009. Selective killing of cancer cells by suppression of geminin activity. *Cancer Research*. 69:4870-4877.

Zhu, W., and A. Dutta. 2006. An ATR- and BRCA1-mediated Fanconi anemia pathway is required for activating the G2/M checkpoint and DNA damage repair upon rereplication. *Molecular and Cellular Biology*. 26:4601-4611.

Zink, D. 2006. The temporal program of DNA replication: new insights into old questions. *Chromosoma*. 115:273-287.

Zou, L., and S.J. Elledge. 2003. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science*. 300:1542-1548.

Zou, L., D. Liu, and S.J. Elledge. 2003. Replication protein A-mediated recruitment and activation of Rad17 complexes. *Proceedings of the National Academy of Sciences of the United States of America*. 100:13827-13832.