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DNA mediated chromatin pull-down: a novel method for the study of chromatin replication

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the degree of Doctor of Philosophy

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Abbreviations

ACN	acetonitrile
APS	ammonium persulfate
ATP	adenosine-5'-triphosphate
BER	base excision repair
bp	base pair(s)
5'-BMA	5-bromo-5'-azido-2', 5'-dideoxyuridine
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
ChIP	chromatin immunoprecipitation
CL	cytoplasmic lysis buffer
C-terminus	carboxy terminus
DABCO	1, 4-diazabicyclo [2.2.2] octane
DAPI	4', 6-diamidino-2-phenylindole
DDR	DNA damage response
Dm-ChP	DNA mediated chromatin pull-down
DMEM	dulbeco's modified eagle media
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
dNTP	deoxyribonucleotide-5'-triphosphate
DSB	double-strand breaks
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EdU	5-ethynyl-2'-deoxyuridine
FASP	filter aided sample preparation
FBS	fetal bovine serum
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
<i>g</i>	gravity
GFP	green fluorescent protein
HR	homologous recombination

HRP	horseradish peroxidase
IgG	immunoglobulin G
IMS	industrial methylated spirit
IP	immunoprecipitation
IR	ionising radiation
kb	kilobase pair(s)
kDa	kilodaltons
M	molar, (moles/liter)
Milli-Q water	ultrapure water purified on a Milli pore Q system
mRNA	messenger RNA
NP-40	Nonidet-P40
N-terminus	amino terminus
NHEJ	non-homologous end joining
NER	nucleotide excision repair
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pI	isoelectric point
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
RNAse	ribonuclease
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
Ser	serine
SILAC	stable isotope labelling by amino acids in cell culture
ssDNA	single-stranded DNA
TAE	Tris acetate EDTA
TEMED	N, N, N, N'-tetramethylethylenediamine
TFA	trifluoroacetic acid
Thr	threonine
Tris	Tris (hydroxymethyl) aminomethane
Triton X-100	t-octylphenoxypolyethoxyethanol
Tween-20	polyoxyethylene-sorbitan monolaurate
Tyr	tyrosine
UV	ultraviolet

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**I dedicate this thesis to my best friend and
husband Maciej Kliszczyk**

Abstract

The duplication of DNA is possibly the most critical process for the faithful transmission of the chromosomes from the parental cell to the two daughter cells. At the molecular level, multiple events necessary for DNA replication, DNA damage recognition and histone deposition occur at the proximity of the replication fork. An important field of investigation is to determine the identity of the proteins that are either stably or transiently associated with the replication forks during normal DNA replication or when the DNA template is damaged.

To achieve this goal we have developed an innovative approach, described as DNA mediated chromatin pull-down (Dm-ChP), that allows for specific recovery of newly assembled chromatin by capturing the newly synthesised DNA and to analyse the protein component associated with it.

The Dm-ChP procedure has been thoroughly validated in its specificity and captured chromatin bound proteins can be analysed by both immunoblotting and large scale proteomic approaches. We have confirmed that proteins known to be either constitutively or transiently associated with chromatin during DNA duplication and DNA repair can be captured with newly synthesised DNA. Proteomic analysis of the Dm-ChP captured material indicates that several hundred different proteins can be identified with sensitive mass spectrometry approaches.

Furthermore, we have used this technique to begin investigating relationship between chromatin protein composition and the temporal programme of DNA synthesis. Using a quantitative mass spectrometry approach together with a synchronisation procedure we provided preliminary evidence that different relative amounts of replication proteins can be found at DNA replicating at different times during S phase

Finally, using the Dm-ChP methodology in combination with pulse and chase experiments we defined the dynamics of replication proteins binding with newly synthesised and mature chromatin. Replication proteins such as PCNA, Mcm7, RPA and Fen-1 were enriched at the nascent DNA and after replication forks moved away they are not retrieved on mature chromatin. On the contrary, scaffold attachment protein such as lamin B1 or NONO, a DNA and RNA

binding protein, involved in several nuclear processes including pre-mRNA splicing and non homologous end joining repair of double-stranded DNA breaks were absent on nascent DNA and recruited to chromatin after passage of replication machinery.

Our studies establish DNA mediated chromatin pull-down as a powerful technique that can be applied to investigate many aspects of DNA metabolism.

Chapter 1 Introduction

1.1 Cell division and the cell cycle

The cell cycle is the sequence of events that includes chromosome duplication and their distribution into new daughter cells. It is a fundamental process required for the development of all living organisms. The eukaryotic cell cycle is divided into four stages: S phase, M phase and two gap phases G_1 and G_2 (Figure 1.1). Cells can also enter G_0 phase that is quiescent or resting state occurring outside of the cell cycle (Morgan, 2007).

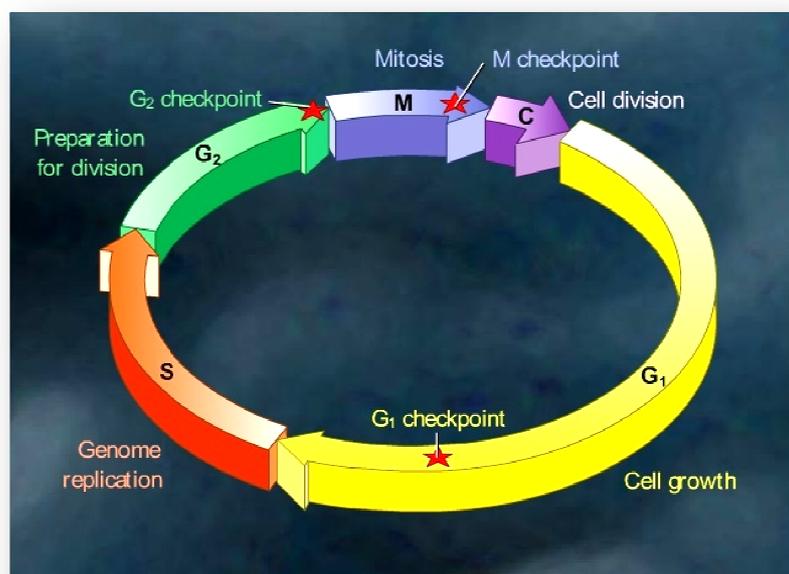


Figure 1.1 Scheme of eukaryotic cell cycle. The cell cycle consists of four distinct phases: G_1 , S, G_2 and M. The transition from one phase of the cell cycle to the next is controlled by cycle-dependent kinases. Checkpoints are represented by stars. G_0 phase is not shown. Picture taken from <http://www.1lec.com/Genetics/Cell Cycle/Control of Cell Cycle.swf>

The G_1 phase occurs before DNA synthesis and during that phase cells grow and synthesise proteins necessary for DNA replication. During S phase DNA is duplicated to ensure proper DNA content and cell division later in M phase. In G_2 , cells continue to grow and synthesise proteins essential for execution of M phase. This includes production of microtubules and membrane components. During mitosis, DNA condensation and chromosome separation occurs, followed by cell division, a process known as cytokinesis (Morgan, 2007). DNA replication and cell division must be achieved with extreme

precision to avoid abnormalities. Many mechanisms have evolved to maintain cell cycle integrity, and their loss may induce cell death or pathological states, such as cancer.

1.2 Molecular mechanism of the cell cycle progression

1.2.1 Cyclin-dependent kinases and cyclins

The cell cycle is regulated through the family of enzymes called the Cdks (Cyclin-dependent kinases). Activity of these serine/threonine protein kinases is required for efficient progression of the cell cycle. Cdks modify different cellular substrates regulating their cell cycle functions (reviewed in (Suryadinata et al., 2010)). Each phase possesses specific Cdk complexes that stimulate progression of that phase.

Cyclins were first characterised by Richard Timothy Hunt during the studies of the cell cycle of sea urchins (Evans et al., 1983). Cyclins can be divided into four classes: cyclin A, B, D and E (Satyanarayana and Kaldis, 2009). Cyclin levels oscillate throughout the cell cycle with cyclin E having the sharpest and cyclin D the broadest window of protein expression (Figure 1.2).

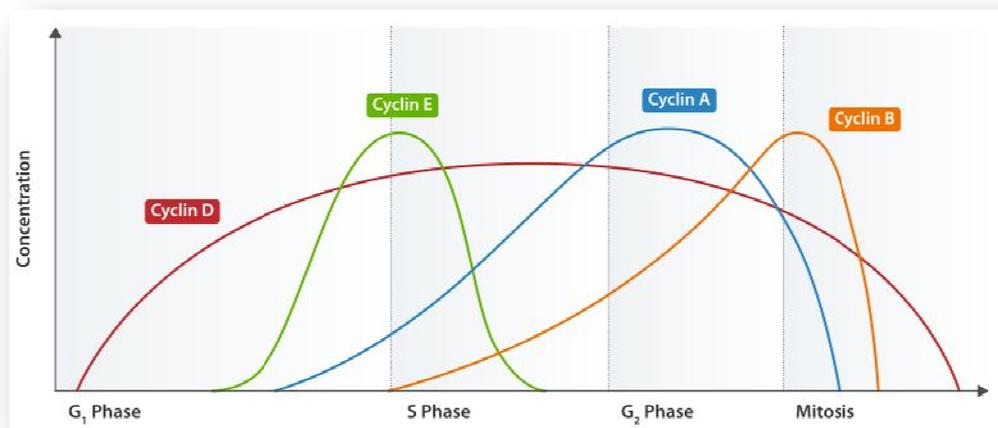


Figure 1.2 Level of cyclins throughout the stages of the cell cycle. Picture taken from http://upload.wikimedia.org/wikipedia/commons/c/ce/Cyclin_Expression.svg

Cyclin A, D and E are targeted to the nucleus, while most of the cyclin B is found in the cytoplasm (Satyanarayana and Kaldis, 2009). All cyclins are broadly expressed in various types of tissue, indicating that their functions are required for proliferation of cells with different origin (Sherr, 1993).

Cdks associate with different cyclins and *vice versa* to form a complex in which the Cdk shows the catalytic activity, however cyclins are mandatory for activation of Cdks. Activity of Cdk/cyclin complexes changes during the cell cycle and corresponds with important events in each phase of the cell cycle. Broadly, Cdk4/cyclin D regulates G₁ phase, Cdk2/cyclin E is active at the G₁/S transition, Cdk2/cyclin A is essential for S phase entry, Cdk1/cyclin A and Cdk1/cyclin B complexes play a role during the transition from G₂ to M phase (Brown et al., 1999; Humphrey and Brooks, 2005).

1.2.2 Regulation of cyclin-dependent kinases activity

Cdks activity is precisely regulated by different mechanisms to ensure proper execution of the cell cycle. One of them is controlled by cyclins access (Figure 1.2) and other by post-translational modification of Cdks.

As mentioned in the previous section, levels of cyclins change to allow for temporal activation of particular Cdk at different time during the cell cycle. Cyclins availability is regulated at transcriptional and post-transcriptional levels (Mazumder et al., 2007). For example, studies of cyclin E in human cells demonstrated that no cyclin E mRNA is present in serum-starved fibroblasts (G₀ phase), as represented on the Figure 1.2. However, levels of cyclin E mRNA appeared in middle-G₁ when cells processed to S phase (Koff et al., 1991). Consistently, overexpression of cyclin E leads to shorter G₁ phase and G₁/S phase transition (Almasan et al., 1995). The levels of cyclin E are regulated by either transcription factor E2F or by proteasomal degradation (Mazumder et al., 2007). Free cyclin E can be phosphorylated by GSK3 complex (glycogen synthase kinase-3), resulting in rapid ubiquitin-dependent proteolysis (Clurman et al., 1996; Mazumder et al., 2007). In contrast, binding to its Cdk partner Cdk2 leads to change in cyclin E conformation, thereby stabilising cyclin E and preventing proteasome degradation (Clurman et al., 1996). Cyclin E can be also degraded after formation of Cdk2/cyclin E complex, through autophosphorylation at Ser380. This automodification of Cdk2 results in cyclin E release and disassembly of Cdk2/cyclin E complex and subsequent destruction of cyclin E by the SCF^{Cdc4} (Skp1-Cullin-F-box) ubiquitin ligase (Reed, 2006; Won and Reed, 1996).

In addition to cyclin-dependent regulation of Cdk activity, Cdk complexes, such as mitotic Cdk, require post-translational modification at the activating (Thr160) and inhibitory (Thr14) sites by CAK (cyclin activating kinase) and Wee1 kinase, respectively (reviewed in (Sanchez and Dynlacht, 2005)). For full activation of Cdk1/cyclin B complex phosphorylation of inhibitory site Thr14 has to be removed. This is catalysed by a member of the Cdc25 family of phosphatases, Cdc25A. Upon Cdk1 activation, APC (anaphase promoting complex) is stimulated and activates mitosis.

Cdk activity can be also regulated by the group of proteins known as CKIs (Cdk inhibitors) (reviewed in (Besson et al., 2008)). There are two families of CKIs: the Ink4 (inhibitors of Cdk4) and the Cip/Kip (cyclin-dependent kinase inhibitory protein/kinase inhibitory protein) (Sherr and Roberts, 1999). The Ink4 family includes p15^{Ink4b}, p16^{Ink4a}, p18^{Ink4c} and p19^{Ink4d} proteins that regulate progression of G₁ by disrupting the interactions between D-type cyclins and Cdk4 and Cdk6 kinases. The p21^{Cip1}, p27^{Kip1} and p57^{Kip2} are the members of Cip/Kip family that interact with both cyclins (cyclins A, B and E) and Cdks (Cdk1 and Cdk2). The Cip/Kip family inhibits activity of Cdk complexes in all phases of the cell cycle (reviewed in (Besson et al., 2008)). The Cip/Kip inhibitors can also indirectly inhibit activity of CAK, thereby averting phosphorylation of Cdks (Kato et al., 1994; Polyak et al., 1994). Overexpression of CKIs leads to inhibition of cell proliferation and G₁ arrest (Chen et al., 1995; Hirai et al., 1995).

1.3 DNA damage response and cell cycle checkpoints

DNA damage response (DDR) is an entangled network of checkpoint proteins that cooperate to restore DNA integrity (Giglia-Mari et al., 2011). DDR components can be divided into three main classes: DNA damage sensors, signal mediators and effectors (Figure 1.3) (reviewed in (Ciccio and Elledge, 2010)). The DDR pathway is heavily controlled by protein-protein interactions and post-translational modifications such as phosphorylation and SUMOylation (reviewed in (Bergink and Jentsch, 2009)). In the event of DNA damage, DNA damage sensor proteins quickly accumulate at the DNA damage sites. Activity of these proteins at DNA lesions attracts DNA damage signal mediators that further

enhances the response (Harper and Elledge, 2007). Finally, effector proteins are activated leading to the cell cycle arrest and DNA repair or programmed cell death (apoptosis) (reviewed in (Zhou and Elledge, 2000)). The cell cycle can be stopped by specific mechanisms termed the cell cycle checkpoints (described in following subsections), to allow efficient DNA repair. However, the DNA damage response can also activate apoptosis to remove cells that were unable to efficiently repair their DNA (Figure 1.3).

Two families of sensor proteins play a central role in DNA damage response: the PIKKs family (phosphatidylinositol 3-kinase like protein kinases) that includes the DNA-PK (DNA-dependent protein kinase), ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia related) (reviewed in (Harper and Elledge, 2007)). The second DNA damage sensor family are PARPs (poly(ADP)ribose polymerases) (reviewed in (Ciccio and Elledge, 2010; Harper and Elledge, 2007)). The ATM and DNA-PK kinases are activated in response to DNA double strand breaks (DSB), while PARP proteins are recruited to DNA single strand breaks (SSB) where they facilitate SSB repair through base excision repair (BER) (reviewed in (Ciccio and Elledge, 2010)). By contrast, ATR kinase forms a complex with ATRIP (ATR-interacting protein) and regulates both firing of replication origins and repair of stalled replication forks in response to DNA damage (reviewed in (Cimprich and Cortez, 2008)).

The sensors regulate activity and functions of mediator proteins that are efficiently recruited to the sites of DNA damage. Mediators include proteins such as Mdc1 (mediator of DNA damage checkpoint protein 1), BRCA1 (breast cancer type susceptibility protein 1) and 53BP1 (53 binding protein 1), MRN complex (Mre11, Rad50 and Nbs1) and Claspin (reviewed in (Ciccio and Elledge, 2010; Harper and Elledge, 2007)). Many of the mediator proteins contain BRCA 1 C-terminus (BRCT) domains that facilitate interaction with phospho-proteins and are important for DNA damage signaling (reviewed in (Harper and Elledge, 2007)). Finally, mediator proteins activate effectors such as Chk1 (checkpoint 1) and Chk2 (checkpoint 2) kinases (reviewed in (Ciccio and Elledge, 2010; Harper and Elledge, 2007)). Phosphorylation of Chk1 and Chk2 by ATR and ATM, respectively, activates phosphorylation cascade leading to cell cycle arrest through inactivation of Cdk by Cdc25. Moreover, effector proteins stimulate assembly and activity of DNA repair complexes or induce

apoptosis through regulation of nuclear guardian protein p53 (reviewed in (Ciccia and Elledge, 2010)).

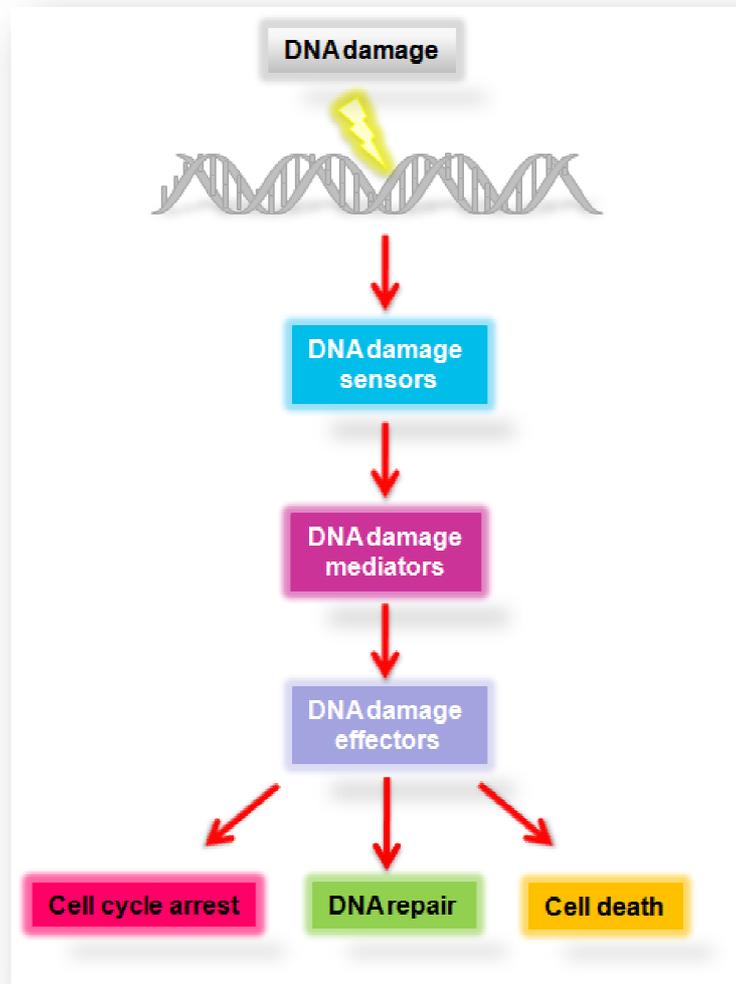


Figure 1.3 DNA damage response (DDR) pathways. After DNA damage sensor proteins recognise the lesions and recruit mediator proteins. DDR cascade amplifies the signal by recruitment of many effector proteins, thereby facilitates initiation of cell cycle arrest, DNA repair or programmed cell death.

1.3.1 Cell cycle checkpoints

Essential for cell survival and proliferation is the ability of cells to maintain genomic integrity. Any aberrations in DNA replication and maintenance can induce cell death or cancer as a result of deleterious mutations. To prevent the occurrence of different mistakes, a cell's progress through the cell cycle is monitored by specific pathways called checkpoints that are present in G₁, S, G₂ and M phases (Figure 1.1) (Lodish et al., 2000; Shackelford Rodney E et al.,

1999). Additionally, in the event of DNA damage, checkpoints delay the cell cycle progression and provide essential time for repair before DNA replication and mitosis (Kaufmann and Paules, 1996).

1.3.1.1 G₁ phase checkpoint

The G₁ checkpoint delays entry into S phase if cells contain damaged DNA (reviewed in (Zhou and Elledge, 2000)). This mechanism protects and prevents replication of a damaged template (Shackelford Rodney E et al., 1999). Activation of G₁ checkpoint is dependent on the two master regulators: the ATM and ATR (Harper and Elledge, 2007). Upon DNA damage, ATM and ATR kinases are activated through autophosphorylation and control functions of downstream proteins by their post-translational modifications (Zhou and Elledge, 2000). The tumour suppressor protein (p53) is phosphorylated at Ser15 and Ser20 by ATM and ATR or by the ATM/ATR target kinases Chk2 and Chk1 (Canman, 1998). In unstressed cells, p53 interacts with the ubiquitin ligase MDM2 (murine double minute 2 protein), thereby it is constantly targeted for degradation (Chehab et al., 1999). Phosphorylation of p53 leads to its accumulation as modified p53 can be no longer a substrate of MDM2 (Chehab et al., 1999). In addition, after DNA damage, MDM2 is also modified by the ATM/ATR kinases and targeted for proteasomal degradation, leading to enhancement of p53 protein release and accumulation (Maya et al., 2001). Activation of p53 leads to expression of target genes, including p21, the cyclin-dependent kinase inhibitor 1 (Kastan and Bartek, 2004). p21-mediated inactivation of Cdk2/cyclin E stalls progression of G₁ phase (Costanzo, 2003). Moreover, a parallel Chk1/Chk2-Cdc25A-dependent pathway exists and amplifies signal for the cell cycle arrest through deactivation of Cdk2/cyclin E complex (Lukas et al., 2004). Whether the initial arrest is induced by the ATM-Chk2-Cdc25A pathway or the ATR-Chk1-Cdc25A pathway, this rapid response is followed by the p53-mediated maintenance of G₁/S arrest (Bartek and Lukas, 2001).

1.3.1.2 S phase checkpoint

The S phase checkpoint network can be induced by damage encountered by replication forks or by unrepaired DNA that evaded G₁ checkpoint (Bartek et al.,

2004; Osborn et al., 2002). It is crucial to protect replication fork integrity as collapsed forks are highly toxic (reviewed in (Bartek et al., 2004)). Upon DNA damage, the S phase checkpoint is activated via at least two parallel branches, ATM- and ATR-dependent (Falck et al., 2002). In the first pathway, ATR-Chk1 and ATM-Chk2 phosphorylate Cdc25A and target it for ubiquitin-dependent degradation. The SCF ubiquitin ligase β TrCP modifies Cdc25A, which is then hydrolysed (Busino et al., 2003). Destabilisation of Cdc25A leaves the Cdk2/cyclin E and Cdk2/cyclin A complexes inactive, thereby preventing Cdc45 loading onto origins and completion of DNA synthesis (Falck et al., 2001; Moyer et al., 2006). In addition, in the absence of Cdc45, the DNA polymerase δ cannot be loaded onto DNA, thereby inhibiting DNA synthesis (reviewed in (Bell and Dutta, 2002)).

In the second pathway, mobilisation of a number of DNA damage response proteins, such as ATM, BRCA1, Nbs1 (Nijmegen breakage syndrome 1) and Smc1 (structural maintenance of chromosomes 1) occurs (Falck et al., 2002; Kitagawa et al., 2004; Olson et al., 2007; Yazdi et al., 2002). ATM phosphorylates BRCA1 (Ser1387, Ser1423, Ser1524), Nbs1 (Ser343, Ser966) and Smc1 (Ser957, Ser966) (Kastan and Bartek, 2004; Kim, 2003; Scully and Livingston, 2000; Xu et al., 2002). Nbs1 has been shown to be a part of the Mre11-Rad50 complex, important for recombination repair of DNA double-strand breaks (Abraham, 2001). Depending on the phosphorylation state of Nbs1, ATM phosphorylates one of the components of the cohesin complex, Smc1 and this phosphorylation is also required for the S phase checkpoint (Xu et al., 2002; Yazdi et al., 2002). In addition, ATM-dependent phosphorylation of BRCA1 is essential for efficient checkpoint and DNA repair after DNA damage (Scully and Livingston, 2000).

1.3.1.3 G₂/M checkpoint

The G₂ checkpoint prevents cells from entering mitosis with unrepaired or not fully replicated DNA (Xu et al., 2002). As previously described, ATM-Chk2 and ATR-Chk1 branches lead to degradation of Cdc25C phosphatase in response to genotoxic stress (Capasso et al., 2002; Falck et al., 2001). Cdc25C phosphorylation at Ser216 creates a binding site for 14-3-3 proteins. This

interaction targets Cdc25C to the cytoplasm and mediates its subsequent degradation (Mailand et al., 2000). Loss of Cdc25C activity impedes the full activation of the mitosis-specific Cdk1/cyclin B complex (reviewed in (Sancar et al., 2004)) and prevents activation of Cdk1 through dephosphorylation of the Thr14 and Tyr15 by Cdc25A. This results in inactivation of Cdk1/cyclin B complex and blockage of entry into mitosis (Boddy and Russell, 1999; Peng, 1997). Additionally, phosphorylation of p53 by ATR-Chk1 and ATM-Chk2 pathways increases expression of p21 leading to inhibition of Cdk1/cyclin B complex (Bunz et al., 1998).

1.3.1.4 Spindle assembly checkpoint

The SAC (Spinde assembly checkpoint) is necessary for proper attachment of microtubules to chromosomes. This checkpoint pathway is conserved from yeast to human and takes place in early mitosis. The SAC ensures the fidelity of chromosome segregation in mitosis and defects in this pathway lead to chromosome mis-segregation and aneuploidy (Vallee et al., 2006). The SAC components in budding yeast include Aurora B kinase, Bub1 (budding uninhibited by benzimidazole), Mps1 (monopolar spindle 1), Bub3, Mad1 (mitotic arrest deficient) and BubR1 that localise to kinetochores during prometaphase (reviewed in (Przewloka and Glover, 2009)). The SAC checkpoint monitors proper kinetochore-microtubule attachment to chromosomes and spindle tension (reviewed in (Musacchio and Salmon, 2007)). In response to abnormal kinetochore-microtubule attachment, Mps1 is phosphorylated by Bub1 in a Bub3-dependent manner leading to formation of the MCC (mitotic checkpoint complex) (Hardwick and Murray, 1995; Hardwick et al., 1996). The MCC complex contains Mad2, BubR1/Mad3, Bub3 and Cdc20 (reviewed in (Musacchio and Salmon, 2007)). The Cdc20/Mad2 sub-complex binds to BubR1 and inhibits E3 ubiquitin ligase activity of the APC/C (anaphase promoting complex/cyclosome), thereby preventing degradation of cyclin B and Pds1/Securin (precocious dissociation of sisters) (McGuinness et al., 2009; Peters, 2006). When correct microtubule-kinetochore attachment and spindle tension is restored, activated APC/C complex removes residual sister chromatin cohesion at kinetochores through Securin degradation, thereby promoting mitosis entry (reviewed in (Nezi and Musacchio, 2009)).

1.4 DNA synthesis

1.4.1 Overview

DNA replication, repair and the cell cycle control are the most important cellular processes necessary to transmit genetic information from the parental cell to the two daughter cells (Jiang et al., 1999a). DNA replication is a tightly coordinated process and recruits different replication proteins to ensure that one genome equivalent is completely duplicated once, and only once, per cell cycle (Boye et al., 2000; Lei and Tye, 2001). In addition, studies of DNA synthesis in different organisms revealed that most of the replication factors are highly conserved from yeast to human (Cvetic and Walter, 2005; Semple and Duncker, 2004).

1.4.2 Discovery of DNA

Deoxyribonucleic acid (DNA) is a carrier of genetic information found in all living organisms (except some viruses) (Sinden, 1994). It was first isolated and analysed in 1869 by the Swiss physician Friedrich Miescher during leukocyte extraction (Miescher, 1871). He characterised the novel molecule with large numbers of phosphorous groups and named it “nuclein” (Dahm, 2008; Miescher, 1871).

In 1919, another biochemist Phoebus Levene performed partial hydrolysis of yeast nucleic acid and identified major components of DNA: adenine, guanine, thymine, cytosine, deoxyribose and a phosphate group (Levene, 1919). He demonstrated that identical units, that he called nucleotides, were formed by the various components linked together through the phosphate groups. Levene proposed "tetranucleotide hypothesis" saying that DNA was made up of equal amount of bases repeated in a fixed order (i.e., G-C-T-A-G-C-T-A) (Levene, 1910).

This hypothesis was disproved by Erwin Chargaff in the late 1940s. Chargaff postulated new base pairing rules, known as Chargaff's rules. According to Chargaff the amount of adenine base is always equal to thymine residues and the amount of cytosine equals guanine (Chargaff et al., 1952). Therefore, DNA strands must be complementary and one strand contains

information about the sequence of the other strand. Chargaff also postulated that two sets of hydrogen bonds are formed between the bases. Adenine always pairs with thymine by two hydrogen bonds and cytosine always interact with guanine by three hydrogen bonds (Chargaff et al., 1951).

A few years later, the chemical structure of DNA was elucidated by Watson and Crick (Watson and Crick, 1953). X-ray diffraction analysis showed that DNA is a linear, un-branched polymer of four nucleotides connected through phosphodiester bonds. The DNA helix is stabilised by hydrogen interactions between the bases from both strands, and a negatively charged sugar-phosphate backbone provides high water solubility. DNA strands are aligned in an anti-parallel manner. One end called 3'-end contains a terminal hydroxyl group and the other 5'-end possesses a phosphate group. Watson and Crick postulated a three-dimensional model of DNA in which two right-handed DNA strands are twisted around the same axis.

1.4.3 Features of DNA synthesis in prokaryotes and eukaryotes

DNA synthesis is a semi-conservative process in which each DNA strand serves as a template for replication reaction. Double-stranded DNA has to be melted to allow polymerase access to the ssDNA template. DNA duplication is coordinated by complex of proteins that are organised into a structure known as the replication fork (Langston et al., 2009). Replication forks are bidirectional “V” shape-like structures where nucleotide incorporation occurs (Voet and Voet, 1995). Because DNA polymerases can read the nucleotide sequence only from 3' to 5' direction (Champe et al., 2008), one of the strands is replicated continuously as the leading strand, meanwhile the other lagging strand is replicated through short intermediates known as Okazaki fragments (Nelson et al., 2000). To start DNA synthesis of the leading and lagging strands, DNA primase needs to add short RNA primers that will then be extended by replicative polymerases. These primers are later removed and any resulting gaps are filled by polymerase I and DNA fragments are ligated by DNA ligase I.

DNA replication in both prokaryotes and eukaryotes is bidirectional (Abdurashidova et al., 2000; Diffley, 1996; Tasheva and Roufa, 1994). Unlike in

prokaryotic cells, where the genome has only a single origin of replication (Mott and Berger, 2007), the genome of eukaryotic cells contains an estimated 30 000 replication origins (Arias and Walter, 2007; Francis et al., 2009; Tudzarova et al., 2010). Prokaryotic DNA synthesis is carried out by a single polymerase III, whereas eukaryotic DNA is duplicated by polymerase ϵ on the leading strand and polymerases δ and α on the lagging strand (Burgers, 2009; Fang et al., 1999). Prokaryotic DNA is not packed as extensively as it is observed in eukaryotes, however it forms structures called the nucleoid, whereas eukaryotic DNA is organised into chromatin (Voet and Voet, 1995). Another difference is the shape of DNA, which in prokaryotes is circular, whereas eukaryotic DNA is linear (Jackson, 2005). Moreover, the rate of DNA synthesis in prokaryotic cells is relatively faster (100 kb/minute) compared to eukaryotic DNA synthesis (0.5-5 kb/minute) (Herrick and Bensimon, 2008; Kornberg and Baker, 1992).

1.5 Prokaryotic DNA replication

1.5.1 Bacterial DNA synthesis

Escherichia coli has provided the greatest understanding of DNA metabolism and replication (McGlynn and Lloyd, 2002). *E. coli* genome contains about four million base pairs, 10^3 times less than the human genome (Champe et al., 2008). *oriC* is a single, unique origin (Champe et al., 2008; Marsh and Worcel, 1977; Ozaki and Katayama, 2009) that has approximately 250 base pairs in length (Kaguni, 1997; Mott and Berger, 2007; Stansfield et al., 1996). *oriC* contains two distinct elements at its ends, three repeats of thirteen base pairs and four repeats of nine base pairs namely 13-mers or 9-mers, respectively (Kaguni, 1997; Mott and Berger, 2007; Stansfield et al., 1996). 13-mers contains AT-rich clusters and plays a role as DNA unwinding element (DUE), whereas 9-mers also called DnaA boxes binds DnaA initiator protein (Fuller et al., 1984; Speck and Messer, 2001).

Bacterial DNA replication is initiated when DnaA protein binds specifically to the DnaA boxes (Kaguni, 1997; Mott and Berger, 2007). Only DnaA-ATP complex is active during initiation (Messer, 2002; Sekimizu et al., 1987) and ATP-dependent melting of DNA strands leads to separation and formation of ssDNA (Champe et al., 2008). In the next step, the DUE region is

unwound and DnaB helicase is loaded (Figure 1.4). As DnaB protein has a low affinity to bind ssDNA covered with single-stranded DNA binding protein (SSB), it needs to be loaded with the help of initiator protein DnaA and helicase loader DnaC (Messer, 2002). DnaC binds ATP and stabilises the DnaB-DnaC complex (Wahle et al., 1989), but to activate DnaB helicase ATP-dependent release of the DnaC loader has to occur (Bárcena et al., 2001; Messer, 2002; Wahle et al., 1989). The two hexamers of DnaB assemble onto the replication origin and mediate expansion of ssDNA region, thus formation of replication forks occurs (Fang et al., 1999).

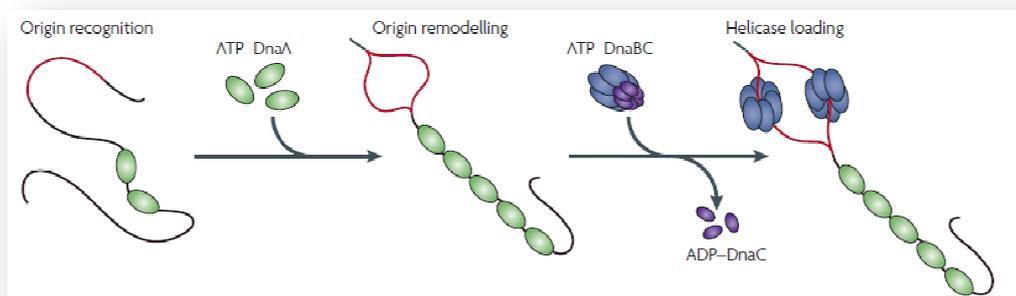


Figure 1.4 Initiation of bacterial DNA replication. *DnaA* protein (green), associates with high-affinity to specific DNA binding sites throughout the cell cycle. During initiation of DNA replication, additional molecules of ATP-DnaA bind to the origin, oligomerise into a large nucleoprotein complex and melt the DNA-unwinding element (red). In the next step, *DnaB* helicase (blue) is loaded onto DNA by the helicase loader *DnaC* (purple). Picture taken from (Mott and Berger, 2007).

DnaG primase requires approximately a 100 nucleotide bubble to initiate synthesis of RNA primers on both lagging and leading strands (Messer, 2002). In the next step, DNA polymerase III holoenzymes are loaded onto each template, thus initiating DNA replication (Fang et al., 1999). The RNA primers are subsequently removed from the lagging strand by RNase H endonuclease and the synthesised gap DNA is ligated by DNA ligase. DNA replication is terminated in the region opposite to the initiation site and occurs when replication forks reach specific sequences called termination sites (Mulugu et al., 2001). The terminus sequence (Ter) contains multiple sites that bind terminus utilisation protein (Tus). This complex arrests replication forks in a polar fashion. Depending on the terminator orientation, such replication blockage stops replication forks coming

from one direction, whereas the converging fork approaching from the opposite direction is not stalled (Bussiere and Bastia, 1999).

1.5.2 Regulation of bacterial origin firing

Escherichia coli evolved different mechanisms that limit origin firing. These include origin sequestration and DnaA titration (Messer, 2002). In both mechanisms, DnaA and *oriC* are targets of regulatory control (Mott and Berger, 2007). After initiation of DNA replication, *oriC* chromosome region and DnaA initiator are transiently inactivated by direct contact with the assembled replisome (Boye et al., 2000; Gille et al., 1991; Margolin and Bernander, 2004). A second mechanism that prevents re-replication involves titration of DnaA protein (Margolin and Bernander, 2004; Roth and Messer, 1998). *E. coli* chromosome contains five regions called *datA* (DnaA titration), which have high affinity for DnaA binding (Kitagawa et al., 1998). *DatA* loci control excess to DnaA protein, by reducing the levels of free DnaA below this, required to stimulate further rounds of DNA replication (Kitagawa et al., 1998; Margolin and Bernander, 2004; Mott and Berger, 2007).

1.6 Initiation of eukaryotic DNA replication

Eukaryotic DNA replication is divided into two major steps: the initiation of DNA synthesis and the elongation reaction. Firstly, ORC complex binds to the origins (Nishitani and Lygerou, 2004) and facilitates recruitment of two other proteins Cdc6 and Cdt1 (Cvetic and Walter, 2005). When these replication factors are loaded onto chromatin the last component of pre-replicative complex (pre-RC), the Mcm2-7 helicase is recruited (Evrin et al., 2009; Tsakraklides and Bell, 2010). The formation of the pre-RC during G₁ phase of the cell cycle also known as licensing reaction is essential for efficient initiation of DNA replication (Figure 1.5) (Lei and Tye, 2001; Thommes and Hubscher, 1990).

Initiation of DNA synthesis takes place during G₁/S phase transition for early origins and throughout S phase for middle and late origins (Cvetic and Walter, 2005; Lei and Tye, 2001). Thus, not all origins are activated simultaneously (Lei and Tye, 2001; Santocanale and Diffley, 1996; Walter and Newport, 1997). Two S phase promoting kinases Cdc7/Dbf4 and Cdk2 (Jares

and Blow, 2000) mediate firing of replication origins (Walter and Newport, 2000). The Cdc7/Dbf4 kinase phosphorylates Mcm2 and other members of the MCM helicase at the N-terminal serine/threonine cluster, thus leading to attraction of Cdc45 protein onto origin (Masai et al., 2006; Sclafani and Holzen, 2007).

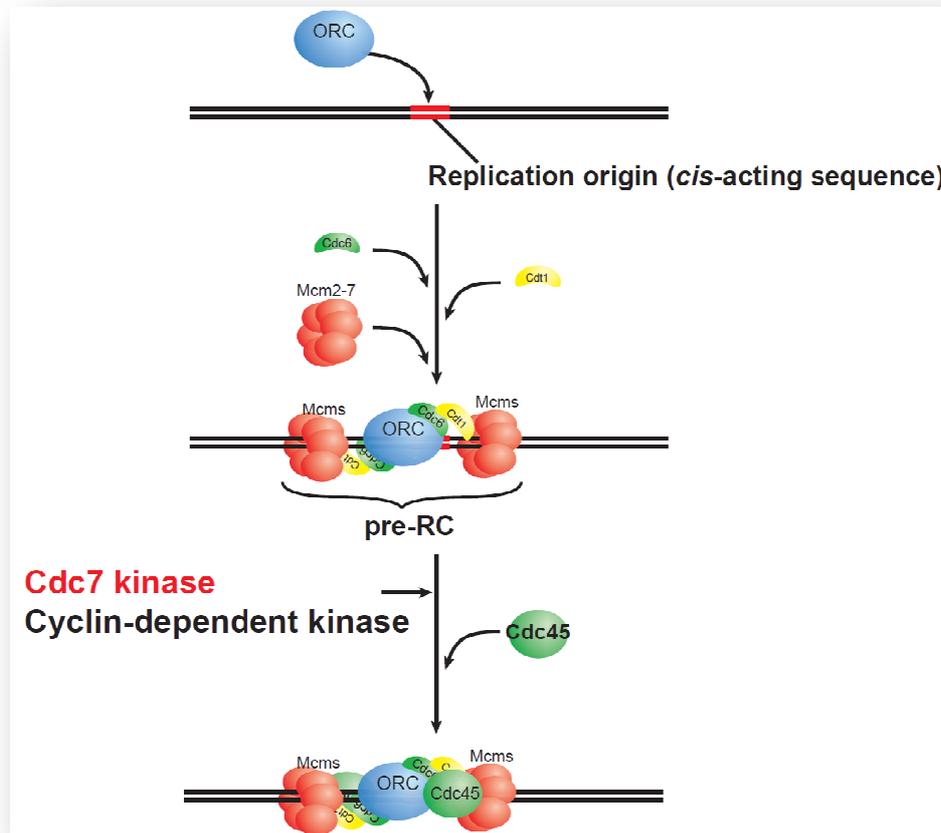


Figure 1.5 Model of pre-RC formation and initiation of DNA synthesis. There are two separate steps to initiate origin firing. First step involves the assembly of pre-replicative complexes by sequential loading of ORC, Cdc6, Cdt1 and the MCM helicase to the origin. In the second step, origins are activated by Cdc7 and Cdk2-dependent phosphorylation of MCM complex and the recruitment of Cdc45, thereby formation of pre-initiation complex (pre-IC). Picture taken from (Sawa and Masai, 2008).

1.6.1 Proteins involved in formation of pre-replication complexes

1.6.1.1 Origin specification

1.6.1.1.1 DNA sequences

Replication origins in *Saccharomyces cerevisiae* contain 150 bp ARS (autonomous replication sequence) elements (Raghuraman et al., 2001). These

sequences mediate interactions with replication protein such as ORC (Gilbert, 2001b). Budding yeast genome possesses 200-400 ARS elements and most of them serve as a potential replication origin (Brewer and Fangman, 1987; Huberman et al., 1988). All ARS sequences share three functional modules: an essential 11 bp ACS (ARS consensus sequences) and three additional B sub-domains B1, B2 and B3 (Newlon and Theis, 1993; Rowley et al., 1995). Beside the conserved ACS core, ARS elements contain additional less conserved 17 bp EACS (extended ACS sequences) at both 5' and 3' sites of the ACS (Chang et al., 2011). The B sub-domains are located 3' to the T-rich strand of the ACS and any mutation of the B1 region abolishes the ARS activity (Rao and Stillman, 1995). The ACS together with B1 element play an important role in origin function as ORC complex binding sequence (Bell and Stillman, 1992). The B2 element is believed to mediate pre-replication complex formation through interaction with replication protein such as Mcm2-7p helicase and Cdc6p. Additionally, role of B2 element as a DNA unwinding element has been also proposed (Wilmes and Bell, 2002). The B3 element is bound by ABF1 (replication factor ARS-binding factor 1) (Rao et al., 1994).

1.6.1.1.2 The Origin Recognition Complex (ORC)

In contrast to yeast, higher eukaryotes do not possess a specific sequence that determines position of the replication origins or such has not been identified yet (Chang et al., 2011). Heterohexameric initiator complex ORC arrives first at eukaryotic replication origins, thereby marking the physical sites of replication origins (Bell and Dutta, 2002; Bell and Stillman, 1992; Lau et al., 2007; Thome et al., 2000). The six subunits of ORC (Orc1-Orc6) are evolutionary conserved and bind to the DNA in an ATP-dependent manner (Bell and Stillman, 1992; Speck et al., 2005). ORCs are the foundation for assembly of pre-replication complex (Bell and Stillman, 1992; Makise et al., 2003). The largest subunit of recognition complex, Orc1 together with Orc5 form active ATPase (Klemm et al., 1997; Makise et al., 2003), but only Orc1 ATPase is crucial for DNA binding and assembly of pre-RC in budding yeast (Speck et al., 2005). Both Orc1 and Orc5 contain consensus nucleotide-binding sequences (Klemm et al., 1997; Loo et al., 1995). Orc1 contains two motifs referred to as Walker A and B, contrary to Orc5 which contains only Walker B (Koonin, 1993; Makise et al., 2003). Walker

A motif, but not Walker B, binds ATP and with help of the latter hydrolyses ATP (Klemm and Bell, 2001). Additionally, Orc1 ATPase activity was shown to be blocked or reduced after establishment of ORC-DNA complex at replication origin (Klemm et al., 1997; Klemm and Bell, 2001). In contrast to Orc2-Orc6 levels which appear constant throughout the cell cycle, Orc1 is present in G₁ phase, but destabilised during S phase (DePamphilis, 2003; Semple and Duncker, 2004; Tatsumi et al., 2003).

1.6.1.1.3 Origin selection in higher eukaryotes

Identification of replication initiation sites has been widely studied to understand how genome duplication is coordinated. Many different systems and methodological approaches are used to map DNA replication initiation sites in higher eukaryotes. The majority of origins were identified by mapping of ORC and MCM binding sites *in vivo* by ChIP. Moreover, purification of nascent DNA, microarray hybridisation or chromatin fibers and large scale sequencing were also used to unravel origin firing events (reviewed in (Méchali, 2010)). However, current approaches suffer from various difficulties; therefore novel concepts are needed to find a unified model of initiation of DNA replication in higher eukaryotes.

Chromatin immunoprecipitation of ORC, MCM and analysis of nascent DNA sequences in higher eukaryotes identified more than a few hundred origins per genome (Cadoret et al., 2008). Moreover, only a subset of assembled pre-RCs is activated while the rest remain silent contributing to the overall origin map. Early labelled-fragment hybridisation (ELFH) in hamster cells showed that the origin firing decision is already made in G₁ phase (Wu and Gilbert, 1996). Moreover, chromatin fibers analysis revealed that the majority of replication origins is activated in clusters and these are conserved between cell cycles (Takebayashi et al., 2001). However, unlike in yeast, the consensus origin sequence has not been found in higher eukaryotes (Méchali, 2010). Additionally, no major histone modifications has been currently implicated in regulation of replication origins in human and *Drosophila*, suggesting that origins in higher eukaryotes may be independent of DNA sequence and histone marks (Martin et al., 2011). Possibly the lack of autonomous origin sequence in higher eukaryotes may be a consequence of genome heterogeneity where presence of euchromatin

and heterochromatin affects origin regulation (Hamlin et al., 2008). Indeed, ChIP experiments in *Drosophila* demonstrated that ORC binding sites co-localise with euchromatin (Karnani et al., 2010; MacAlpine et al., 2004). Moreover, detailed analysis of *Drosophila* chromosome 4 revealed that this heterochromatic chromosome possesses small number of origins and these fire later in S phase (Cayrou et al., 2011). On the contrary, a large body of data suggests that origins are closely associated with transcriptionally active regions of chromatin. For instance, high throughput sequencing and microarray experiments in mouse and human cells detected origins downstream of transcription initiation sites (Lucas et al., 2007; Martin et al., 2011; Sequeira-Mendes et al., 2009). ORC binding sites overlap with RNA polymerase II and transcription regulatory factor binding regions (RFBR) (Karnani et al., 2010; MacAlpine et al., 2004). Consistently, analysis of short nascent strands in human cells revealed positive correlation between localisation of origins and transcription regulatory elements such as c-Jun and c-Fos (Cadoret et al., 2008). These data suggest that chromatin structure regulates origin localisation in higher eukaryotes and these are more likely to be found within more relaxed and active euchromatin. Additionally, the majority of mouse and human origins are found within close proximity of GC-rich regions and CpG islands and these are also associated with euchromatin (Aladjem et al., 1995; Cadoret et al., 2008; Sequeira-Mendes et al., 2009).

It is still unclear how these factors influence selection and usage of the origins. The identification of a true number of origins may be necessary to decode the principles of origin regulation. High-throughput sequencing of newly synthesized DNA has become a leading method for mapping and characterisation of replication origins. However, these techniques suffer several limitations such as usage of an amplification step, poor recovery of nascent DNA fragments or large set of microarrays needed to sufficiently cover the genome. On the contrary, protein-based methods such as mapping of ORC and MCM DNA binding sites *in vivo* are not specific because of high cellular levels and multiple roles of ORC and MCM beside DNA replication (Prasanth et al., 2004). Additionally, from the large pool of assembled pre-RCs only a subset is activated and the rest remain dormant origins. Identification of the conformation properties or specific mutations that lead these proteins to the replication initiation sites could be an alternative method to characterise replication origins in higher

eukaryotes. Genome-wide mapping of DNA replication initiation sites is necessary to understand if the position of replication origin has an important role for cell survival and development.

1.6.1.2 The Cdt1 protein

In eukaryotic cells, Cdt1 (Ccd10-dependent transcript 1) is responsible for licensing and directly interacts with the Mcm2-7 complex (Khayrutdinov et al., 2009). Furthermore, Cdt1 interaction with Dbf4, the regulatory subunit of Cdc7 kinase, promotes recruitment of Cdc45 and Mcm2-7 onto chromatin (Ballabeni et al., 2009). Additionally, gel shift and DNA helicase assays show that Cdt1 stimulates helicase activity of Mcm2-7 complex, suggesting that it may promote DNA unwinding (You and Masai, 2008).

The Cdt1 protein is a member of the nucleotide-dependent loading factors and it is conserved from yeast to human (Nishitani et al., 2001). Cdt1 levels are tightly controlled during the cell cycle. Cdt1 is only present during early G₁ phase and is quickly degraded as cells enter S phase (Bell and Dutta, 2002; Liu et al., 2004; Nishitani et al., 2001; Semple and Duncker, 2004). Regulation of Cdt1 is mediated by two different mechanisms: SCF^{Skp2} - and Geminin-dependent (Li et al., 2003; Liu et al., 2004; Wohlschlegel et al., 2000; Xouri et al., 2007). Cdt1 degradation by two different ubiquitin ligases SCF^{Skp2} and DDB1-Cul4 has been shown *in vivo* and *in vitro* (Li et al., 2003) (see section 1.6.2). Tight control of Cdt1 levels limits its functions in initiation of DNA replication, abolishes Cdt1-MCM helicase interaction later in the cell cycle and prevents re-replication of DNA (McGarry and Kirschner, 1998; Xouri et al., 2007).

1.6.1.3 The Cdc6 protein

Cdc6 is another member of the nucleotide-dependent loading factors (Perkins and Diffley, 1998; Semple and Duncker, 2004). Cdc6 contains conserved nucleotide binding/ATPase domains (Bell and Dutta, 2002) and plays a role as an ATP-dependent MCM protein loader (Mendez and Stillman, 2000; Saha et al., 1998; Weinreich et al., 1999). ORC, Cdt1 and Cdc6 form a complex that clamps MCM helicase around DNA (Oehlmann et al., 2004). In mammalian cells, Cdc6 is crucial for DNA replication and immunodepletion of Cdc6 inhibits initiation of DNA replication in HeLa cells (Yan et al., 1998).

In contrast to Cdt1, human Cdc6 protein level is relatively stable throughout the cell cycle and lower levels of Cdc6 are observed in G₀ and mitosis (Coverley et al., 2000; Mendez and Stillman, 2000; Williams et al., 1997). However, similarly to Cdt1, as cells enter S phase, Cdc6 functions are terminated by Cdk2-mediated phosphorylation and transport of the protein to the cytoplasm (Cook et al., 2002; Furstenthal et al., 2001; Saha et al., 1998). In addition, Cdc6 can be phosphorylated by both Cdk2/cyclin A and Cdk2/cyclin E *in vitro* (Jiang et al., 1999b; Petersen et al., 1999). Overexpression of Cdc6 in G₂ phase human cells inhibits Cdk1/cyclin B and blocks mitosis entry, suggesting that increased levels of Cdc6 are toxic for cells prior to mitosis (Clay-Farrace et al., 2003).

1.6.1.4 The minichromosome maintenance (Mcm2-7) proteins

The MCM complex belongs to the large family of the AAA⁺ ATPases and it is the core component of the replicative helicase (Costa and Onset, 2008; Forsburg, 2004; Iyer et al., 2004; Semple and Duncker, 2004). MCM family members are well conserved and all eukaryotes possess six MCM genes (Bell and Dutta, 2002; Costa and Onset, 2008; Kearsey and Labib, 1998). All six genes are essential in both budding and fission yeast (Sclafani and Holzen, 2007). In *Xenopus* egg extracts MCM is required for efficient initiation of DNA replication (Chong et al., 1995; Kubota et al., 1997; Madine et al., 1995). In budding yeast, cellular localisation of Mcm2-7 complex is tightly regulated during the cell cycle (Nguyen et al., 2000). MCM proteins co-localise in the nucleus in G₁ and S phases, but are excluded from nucleus in G₂ and M phases through activity of Cdks (Bell and Dutta, 2002). Only two MCM members (Mcm2 and Mcm3) possess nuclear localisation signals (NLSs) indicating that MCM hexamer has to be formed prior to nuclear localisation (Bell and Dutta, 2002).

The archaeal MCM proteins can be functionally divided into three domains: N-terminal, AAA⁺ and C-terminal (Figure 1.6). An N-terminal portion that retains helicase activity contains Z-finger motif and it is important for DNA binding and formation of MCM hexamer. An AAA⁺ catalytic domain located in the centre of the protein is required for ATPase and DNA unwinding activities. Moreover, the AAA⁺ domain can be divided into three motifs: Walker A and B needed for ATP binding and hydrolysis and β - α - β insert essential for DNA duplex unwinding (Sakakibara et al., 2009). The third domain located at the C-terminus contains a

helix-turn-helix (HTH) motif of unknown function (Costa and Onset, 2008; Jenkinson and Chong, 2006; Sakakibara et al., 2009). Studies of the MCM proteins from *Methanothermobacter thermautotrophicus* and *Sulfolobus solfataricus* showed that C-terminal deletions had increased ATPase and helicase activities, suggesting an inhibitory role of the C-terminal domain in DNA unwinding (Barry et al., 2007; Jenkinson and Chong, 2006).

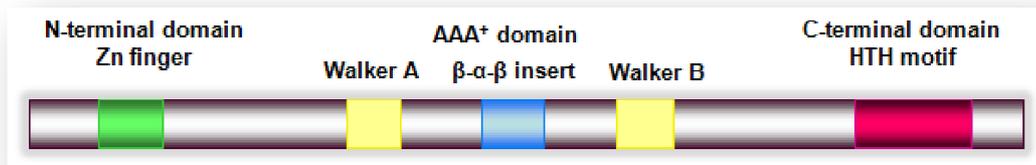


Figure 1.6 Domains organisation and structural motifs of the MCM helicase. MCM protein contains three main domains: N-terminal with zinc-finger motif, catalytic AAA⁺ ATPase domain containing Walker A and B motifs and conserved β-α-β insert, and the HTH C-terminal domain.

Although the MCM proteins exist as a heterohexameric Mcm2/3/4/5/6/7 structure, sub-complexes such as Mcm2/4/6/7, Mcm4/6/7 and Mcm3/5 were identified *in vivo* and *in vitro* (Lee and Hurwitz, 2000; Poplawski et al., 2001). It has been demonstrated that all subunits of MCM complex contain an ATPase motif, but only the Mcm4/6/7 sub-complex possesses helicase activity (Lee and Hurwitz, 2001; You et al., 2003). Furthermore, the DNA helicase activity of the Mcm4/6/7 heterotrimer is abolished by the interactions with either Mcm2, -3 or -5 (Ishimi et al., 1998). Additionally, an ATP-dependent gate was identified in the Mcm2-7 complex between Mcm2 and Mcm5 subunits (reviewed in (Bochman and Schwacha, 2009)) (Figure 1.7). Introduction of point mutations either in Mcm2 (Mcm2RA) or Mcm5 (Mcm5KA) prevents closing of the Mcm2/5 gate, perturbs communication between subunits and blocks helicase activity. Additionally, mutation of Mcm3 and Mcm6 Walker A motifs results in increased ATP hydrolysis but weak DNA unwinding activity (reviewed in (Bochman and Schwacha, 2009)).

Four hypothetical models have been proposed for DNA unwinding process: the steric-exclusion model, the ploughshare model, the rotary pump model and dsDNA pump model (Figure 1.8) (Bochman and Schwacha, 2009; Laskey and Madine, 2003; Sakakibara et al., 2009).

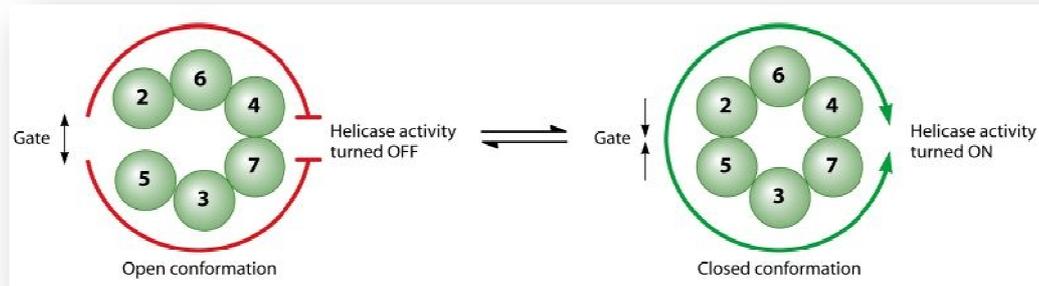


Figure 1.7 Role of Mcm2/5 gate in MCM complex. In physiological conditions gate between Mcm2/5 stays in the balance between open and closed conformations. ATP binding is required to activate Mcm2/5 gate (closed state), thus stimulating DNA unwinding by MCM complex. In contrast, open conformation does not possess helicase activity but may facilitate loading of Mcm2-7 complex onto chromatin. Picture taken from (Bochman and Schwacha, 2009).

In the steric-exclusion model the helicase encircles ssDNA with high affinity, translocates ahead one strand of DNA and excludes the complementary strand from the core of the Mcm2-7 that promotes strands separation and DNA unwinding (Bochman and Schwacha, 2009; Takahashi et al., 2005). The ploughshare scheme is related to the steric-exclusion model. This mechanism postulates that helicase moves along duplex DNA as a single hexamer (Takahashi et al., 2005). Mcm2-7 wraps dsDNA, melts DNA, moves away from the origin by helical rotation and separates the DNA duplex using rigid wedge or pin (it can be part of helicase, associated protein or protein domain) (Bochman and Schwacha, 2009; Sakakibara et al., 2009). The rotary pump model involves direct DNA rotation and loading of multiple helicases onto the replication origin. After translocation at a distance from the replication origin, helicases rotate intervening DNA in the opposite direction through the axis of its ring structure, causing unwinding of the origin (Bochman and Schwacha, 2009). In the dsDNA pump model two Mcm2-7 helicases accumulate on chromatin on both sides of replication fork forming head-to-head complex. dsDNA is pumped toward the origin and extruded as a ssDNA through channels in the helicases (Bochman and Schwacha, 2009; Laskey and Madine, 2003).

In addition to MCM roles in initiation and elongation stages of DNA replication (Barry et al., 2007; Nitani et al., 2008; Shechter et al., 2004), MCM complex is believed to be involved in DNA damage response (Cortez et al.,

2004; Forsburg, 2008; Snyder et al., 2005) and transcription regulation (Forsburg, 2004; Snyder et al., 2005).

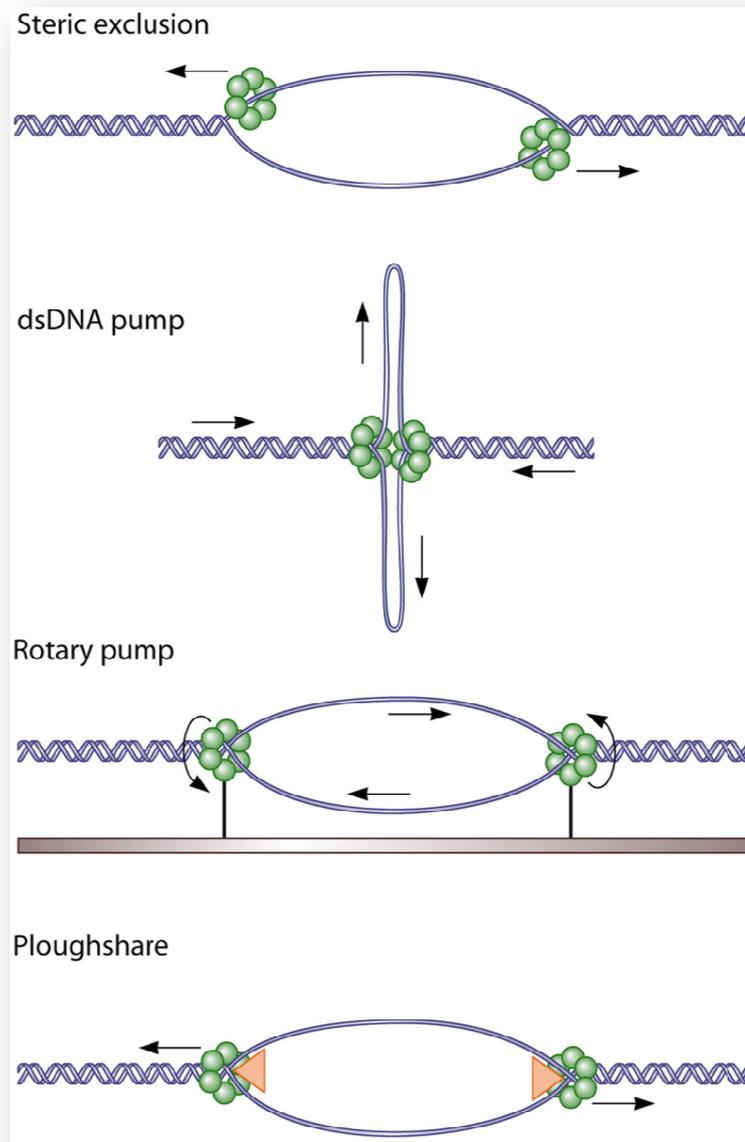


Figure 1.8 Theoretical models of DNA unwinding by MCM helicase. In the steric-exclusion model helicase encircles ssDNA, translocates ahead one strand of DNA and excludes the complementary strand what promotes DNA unwinding. In the ploughshare scheme helicase moves along duplex DNA as a single hexamer and sterically separates the DNA duplex using rigid wedge or pin. The rotary pump model suggests directly DNA rotation and multiple helicases loading on a both sides of replication forks. Helicases rotate intervening DNA in opposite directions through the axis of its ring structure, causing unwinding of the DNA. The dsDNA pump model involves formation of head-to-head conformation between two helicases that pump dsDNA and extruded it later as an ssDNA. Picture taken from (Bochman and Schwacha, 2009).

1.6.2 Regulation of licensing

To prevent re-licensing of origins after initiation of DNA replication, pre-initiation complex is inactivated and transformed into post-replicative complex. There are several pathways to control this phenomenon (reviewed in (Truong and Wu, 2011)).

First, assembly of pre-RC occurs only during M/G₁ phase transition, therefore pre-RCs are inactive during S phase and new pre-RCs cannot be formed until mitosis is completed (Li et al., 2004). After DNA replication, proteins involved in initiation of DNA replication are disassociated from the origins, inactivated or targeted for proteasomal degradation (reviewed in (Blow and Dutta, 2005)). As ORC, Cdc6 and Cdt1 are necessary for MCM complex loading, the levels of these proteins diminish (Takisawa et al., 2000; Tsakraklides and Bell, 2010). Cdc6 phosphorylation by Cdk2 changes the sub-cellular localisation of the protein, which is transported from the nucleus to the cytoplasm, thereby prohibiting re-replication of DNA (Cook et al., 2002; Furstenthal et al., 2001; Saha et al., 1998). It has also been reported that during S phase the Orc1, the largest subunit of ORC complex, undergoes phosphorylation by Cdk1/cyclin A which results in loss of Orc1 affinity to DNA, thus inhibiting formation of active ORC complexes (Li et al., 2004).

The regulation of Cdt1 protein is mediated by two different mechanisms: through the SCF^{Skp2} ubiquitination pathway and high levels of geminin, the main Cdt1 inhibitor (Li et al., 2003; Liu et al., 2004; Wohlschlegel et al., 2000; Xouri et al., 2007). Cdt1 is degraded by two different ubiquitin ligases SCF^{Skp2} and DDB1-Cul4 *in vivo* and *in vitro* (Li et al., 2003). The second mechanism, sufficient to block re-replication, involves activity of geminin. Geminin is absent in G₁ and M phases and accumulates exclusively during S and G₂ phases (McGarry and Kirschner, 1998). These fluctuations limit Cdt1 actions and abolish Cdt1 interaction with Mcm2-7 helicase, thus preventing re-replication of DNA (McGarry and Kirschner, 1998; Xouri et al., 2007). Recent studies revealed that Cdt1 forms two complexes with geminin: inhibitory (no Cdt1 activity) and permissive (partial Cdt1 activity) (De Marco et al., 2009; Lutzmann et al., 2006). Differences between these complexes lie in the number of geminin and Cdt1 molecules involved in their formation. The inhibitory complex possesses three

geminins and single Cdt1 molecule, whereas in permissive conformation two geminins interact with Cdt1. The latter complex allows chromatin association of MCM, thus origin licensing even in the presence of geminin (De Marco et al., 2009).

Re-initiation of DNA synthesis can be also inhibited through regulation of Cdk1 and Cdk2 activities (reviewed in (Kelly and Brown, 2000). Cdc20 and Cdh1, the subunits of APC/C, mediate degradation of cyclin A/B and geminin (Machida and Dutta, 2007). In addition, the Emi1 (early mitotic inhibitor 1) inactivates the APC/C complex at G₁/S transition leading to accumulation of cyclin A and geminin (Moshe et al., 2011). High levels of geminin and Cdk2/cyclin A prevent pre-RC assembly by inhibition of Cdt1 activity (Xouri et al., 2007) and degradation of Cdc6, respectively (Cook et al., 2002).

1.6.3 Proteins involved in activation of licensed origins

1.6.3.1 The Cdc7 and Cdk2 kinases

Cdk2 and Cdc7 (Cell division cycle 7) kinases are the two enzymes essential for initiation of DNA synthesis (Sawa and Masai, 2008; Walter, 2000). Cdc7 was first characterised in budding yeast as a serine/threonine kinase that promotes DNA replication by activating eukaryotic replication origins (Hereford and Hartwell, 1974; Sawa and Masai, 2008). Genetic and biochemical studies in different species propose a crucial role of Cdc7 in replication of DNA. *Cdc7* gene is essential for cell viability in budding and fission yeast (Masai et al., 1995). Similarly, mice lacking *Cdc7* gene are not viable. Cdc7 depletion in conditionally targeted mouse embryonic stem (ES) cells leads to activation of the S phase checkpoint and eventual p53-dependent cell death (Kim et al., 2002).

Cdc7 is essential for origin firing, but not for ongoing replication fork activity (Bousset and Diffley, 1998; Donaldson et al., 1998). Cdc7 kinase activity is regulated in a cell cycle-dependent manner (Masai and Arai, 2002; Masai et al., 1999). Cdc7 forms two different complexes with Dbf4 (Dumbbell former 4) also known as activator of S phase kinase or Drf1 (Dbf4-related factor 1) (Montagnoli et al., 2002), but the roles of these regulatory subunits are not fully understood. Studies in *Xenopus* showed that Cdc7/Drf1 complex is required for initiation of DNA replication in embryonic cells, whereas Cdc7/Dbf4 plays an

essential role in DNA synthesis in somatic cells (Takahashi and Walter, 2005). Additionally, a second activator protein Drf1 appears to be also involved in both S and M phase progression (Yoshizawa-Sugata et al., 2005). Activity of Cdc7 is strictly regulated upon binding of the regulatory subunits and thus, is dependent on their levels (Montagnoli et al., 2002). Additionally, Cdc7 plays a role in checkpoint response induced by replication stress (Costanzo, 2003; Heffernan et al., 2007). The major Cdc7 substrates are MCM proteins such as Mcm2, Mcm4 and Mcm6 (Sheu and Stillman, 2010). Phosphorylation of Mcm2 by Cdc7 stimulates DNA unwinding and initiates DNA replication (Sclafani, 2000). Cdc7 phosphorylates Mcm2 among others at Ser40, Ser53 and Ser108 (Cho et al., 2006; Montagnoli et al., 2006). Interestingly, phosphorylation of the Mcm2 at Ser40 and Ser26 by Cdc7 appears after sequential phosphorylation of Ser41 and Ser27 by Cdk2 (Cho et al., 2006). These Mcm2 phospho-sites at Ser40 and Ser53 are sensitive biomarkers of Cdc7 activity *in vivo* (Montagnoli et al., 2008; Natoni et al., 2011).

It has been reported that Cdc7 interacts with other proteins such as Claspin (Kim et al., 2007; Lei et al., 1997), Cdt1 (Ballabeni et al., 2009), Cdk2 (Grishina and Lattes, 2005) and CAF-1 (Chromatin assembly factor 1) (Gerard et al., 2006). *In vitro* studies suggested that budding yeast polymerase α is a substrate of Cdc7 kinase (Weinreich and Stillman, 1999). Studies from *Xenopus* illustrated that Cdc7/Drf1 forms a stable complex with Scc2/Scc4 and that it is essential for Scc2/Scc4 recruitment to chromatin. From this one may hypothesise that loading of Scc2/Scc4 onto chromatin may be dependent on the kinase activity of Cdc7 (Takahashi et al., 2008).

Cdk2 is a second kinase critical for initiation of DNA synthesis (Bell and Dutta, 2002). Studies in many systems demonstrated an essential role of Cdk2 in DNA replication. In *Xenopus* egg extracts, immunodepletion of Cdk2 or inhibition of its activity leads to accumulation of cells in S phase (Fang and Newport, 1991). However, knockout of *Cdk2* in mice reveals that *Cdk2* is not an essential gene in this system (Berthet et al., 2003). It has been reported that human Cdk2 is physically associated with chromatin and interacts with several replication proteins such as ORC complex, Cdc6 (Bell and Dutta, 2002), Mcm3 and Mcm4 (Chi et al., 2008). In budding yeast, the single Cdk phosphorylates Sld2 and Sld3 and this is essential for initiation of DNA synthesis. These proteins

are the two major substrates of Cdk that once phosphorylated mediate interaction between Cdc45 and GINS (Tanaka et al., 2007).

1.6.3.2 The Cdc45 protein

The Cdc45 (Cell division cycle 45) is a nuclear initiation factor, essential for assembly of the pre-initiation complex onto chromatin (Jares and Blow, 2000; Masai et al., 2006; Pacek and Walter, 2004). Cdc45 interacts with proteins at the replication forks such as ORC, RPA, MCM helicase or DNA polymerase ϵ (Bell and Dutta, 2002). Cdc45 localises to the nucleus where its levels are relatively constant throughout the cell cycle (Owens et al., 1997). Recruitment of Cdc45 onto chromatin is mediated by its association with Cdt1 and Cdc7 (Ballabeni et al., 2009). Moreover, Cdc45 is required for the recruitment of DNA polymerase α onto DNA, thereby initiating replication origin firing (Zou and Stillman, 2000). Chromatin association of Cdc45 strongly depends on Cdk activity and correlates with initiation of DNA replication (Walter and Newport, 2000). S phase progression releases Cdc45 from chromatin, thereby preventing re-initiation of DNA replication (Bell and Dutta, 2002).

1.6.3.3 The GINS complex

The GINS (go-ichi-ni-san) is a complex composed of four subunits: Sld5, Psf1, Psf2 and Psf3 (Chang et al., 2007; MacNeill, 2010). All four subunits of the complex are highly conserved in all eukaryotes and present at constant levels throughout the cell cycle (MacNeill, 2010; Pospiech et al., 2010). The GINS complex has 1:1:1:1 stoichiometry and a horseshoe shape (De Falco et al., 2007). The GINS is an essential component of the replisome that moves with the replication forks and it is required during both the initiation and elongation stages of DNA synthesis (Boskovic et al., 2007; Chang et al., 2007; Ilves et al., 2010). Additionally, GINS complex interacts with DNA polymerase α to facilitate synthesis of primers on the lagging strand in budding yeast (Gambus et al., 2009). In *Xenopus*, similarly to PCNA, GINS complex interacts and stimulates activity of the DNA polymerases ϵ and α . However, electron microscopy (EM) experiments excluded that GINS acts as the DNA clamp (De Falco et al., 2007; Kamada et al., 2007; Shikata et al., 2006). The GINS complex binds preferentially to single-stranded DNA and it is involved in recruitment of RPA to

ssDNA (Labib and Hodgson, 2007; Pospiech et al., 2010). Time-resolved ChIP-chip analysis showed that the GINS complex is present at replication origins and migrates with replication forks in both directions as S phase progresses (Sekedat et al., 2010). Together with Cdc45 and MCM helicase, GINS forms complex called CMG where the GINS mediates stable interactions between Mcm2-7 and Cdc45 (Choi et al., 2007; Gambus et al., 2009). The CMG complex from *Drosophila melanogaster* embryo extracts has an ATP-dependent helicase activity and it is essential for fork progression (Kamada et al., 2007). Furthermore, CMG complex is a central subunit of replisome progression complex (RPC) (Aparicio et al., 2006; Gambus et al., 2006). Budding yeast RPC contains many proteins, such as Mrc1, Tof1, Csm3 (required for replication fork stability), chromatin-associated protein Ctf4 (involved in sister chromatid cohesion), histone chaperone FACT, Topoisomerase 1 and Mcm10 (Gambus et al., 2006).

1.6.3.4 The Mcm10 protein

The Mcm10 is the conserved nuclear replication factor required for efficient initiation of DNA synthesis (Nishitani and Lygerou, 2004). *Mcm10* gene was first characterised in the same genetic screen as *Mcm2-7*, but shows no sequence conservation with the other members of the MCM family. Two-hybrid assay indicated that physical interaction between Mcm10 and at least five members of the Mcm2-7 helicase occurs, suggesting that functional and physical relationship between these proteins is important for initiation of DNA replication (Homesley et al., 2000; Tye, 1999). In addition, Mcm10 interactions with ORC complex and subunits of DNA polymerase ϵ and δ have been also demonstrated (Kawasaki et al., 2000; Ricke and Bielsky, 2004). Studies in fission yeast have shown that Mcm10 interacts with Cdc7/Dbf4 kinase and stimulates effective phosphorylation of MCM helicase by Cdc7/Dbf4 *in vitro* (Lee et al., 2003). Loss of *Mcm10* in budding yeast impedes initiation of DNA replication and cell cycle progression, suggesting its role in both initiation and elongation stages of DNA synthesis (Ricke and Bielsky, 2004). Furthermore, depletion of *Mcm10* in *Xenopus* and budding yeast prevents loading of replication factors such as Cdc45 and RPA onto DNA and promotes dissociation of Mcm2-7 complex from the chromatin (Homesley et al., 2000; Wohlschlegel et al., 2002). Moreover, recent

studies demonstrated that Mcm10 physically interacts with RecQ4 helicase (potential Sld2 homologue) and promotes its association with Mcm2-7 and GINS during initiation of DNA replication (Xu et al., 2009).

1.7 The elongation reaction

Unwinding of DNA strands triggers recruitment of the single-stranded DNA-binding protein RPA (Sclafani and Holzen, 2007). Furthermore, polymerases α , δ and ϵ together with the GINS complex are loaded onto DNA in an ATP-dependent manner (Masai et al., 2005; Nishitani and Lygerou, 2002; Waga and Stillman, 1998). Polymerase α synthesises short RNA primers on both DNA strands (Albertson et al., 2009; Bermudez et al., 2011). RNA primers are

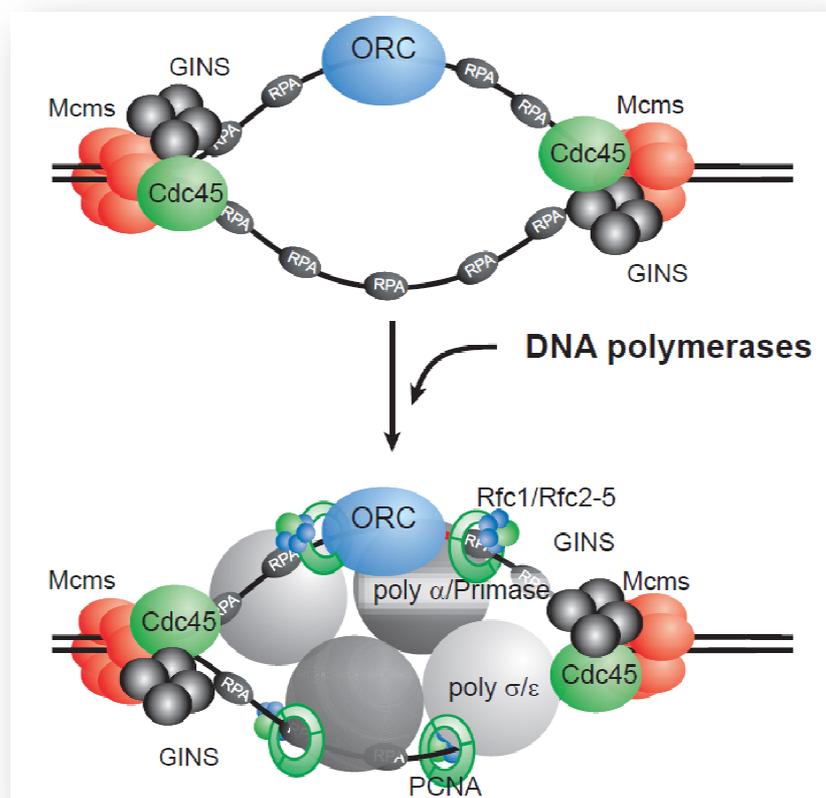


Figure 1.9 Model of replication fork formation and elongation of DNA replication. After assembly of pre-initiation complex, phosphorylated MCM recruits essential DNA replication factors such as Cdc45 and GINS. Chromatin association of PCNA and DNA polymerases α , δ and ϵ generates active replication forks. Picture taken from (Sawa and Masai, 2008).

subsequently extended (Burgers, 2009) and serve as a template recognised by the PCNA-RFC complex (Bermudez et al., 2011). From that point DNA synthesis is

continued by polymerase δ and polymerase α on the lagging strand and polymerase ϵ on the leading strand (Figure 1.9) (Burgers, 2009; Kunkel and Burgers, 2008; Waga and Stillman, 1998).

1.7.1 Proteins involved in elongation of DNA synthesis

Different factors were identified to be involved in the elongation step of DNA synthesis in eukaryotes. These are listed in Table 1.1. Several proteins that were used in this study as replication markers are described in some detail. These factors were used to characterise changes in proteins associated with newly synthesised DNA at different times during S phase (see Chapter 5, section 5.2). We also described DNA polymerases α , δ and ϵ the major enzymes involved in DNA replication and incorporation of nucleotide analogues such as 5-bromo-2'-deoxyuridine (BrdU) or 5-ethynyl-2'-deoxyuridine (EdU).

1.7.1.1 Replication protein A

The RPA (Replication protein A) is the most abundant single-stranded binding protein (SSB) in mammalian cells (Iftode et al., 1999; Prakash et al., 2011) and it is essential for stabilisation of single-stranded DNA (Fanning et al., 2006; Iftode et al., 1999). RPA was first isolated from human cells as a crucial component of SV40 DNA replication system (Fairman and Stillman, 1988). RPA exists as a heterotrimeric complex composed of subunits p70, p34, and p14 (Prakash et al., 2011; Wold, 1997). All three RPA subunits are essential in *Saccharomyces cerevisiae* (Brill and Stillman, 1991). Binding of the RPA to ssDNA is dependent on Cdc45 and precedes recruitment of polymerase α and polymerase δ onto DNA (Kenny et al., 1989; Walter and Newport, 2000). In addition, RPA stimulates DNA unwinding by MCM helicase through stabilisation of ssDNA, thus preventing DNA re-annealing or degradation (Waga and Stillman, 1998). RPA is phosphorylated in a cell cycle-dependent manner. During DNA replication, the p34 subunit of RPA is modified by Cdks at the N-terminal sites Ser23 and Ser29 (Dutta and Stillman, 1992).

In addition to its role in DNA replication, RPA has been implicated in major DNA repair pathways, including nucleotide excision repair (NER) (De Laat et al., 1998; Sancar et al., 2004), base excision repair (BER) (DeMott et al.,

1998) and homologous recombination (HR), where it binds to ssDNA generated during these processes (Stauffer and Chazin, 2004) (all pathways reviewed in (Oakley and Patrick, 2010)). Furthermore, RPA undergoes hyperphosphorylation in S and G₂ phases in response to DNA damage (Zou et al., 2006). Nine phosphorylation sites have been identified: Ser4, Ser8, Ser11, Ser12, Ser13, Thr21, Ser23, Ser29 and Ser33 (Nuss et al., 2005). Moreover, RPA interacts with nucleosome remodelling complex FACT (VanDemark et al., 2006), DNA polymerase α , DNA repair factor XPA, p53 (reviewed in (Fanning et al., 2006)) and different DNA damage proteins such as Rad51, Rad5 (Hays et al., 1998; Park et al., 1996; Sugiyama and Kowalczykowski, 2002) and ATRIP (Zou and Elledge, 2003).

1.7.1.2 PCNA and RFC complex

The PCNA (Proliferating cell nuclear antigen) plays a pivotal role in both the DNA duplication and DNA repair (Essers et al., 2005; Moldovan et al., 2007). PCNA contains well conserved domains found only in DNA sliding clamps (Moldovan et al., 2007; Naryzhny, 2008). At the C-terminus, PCNA possesses a hydrophobic pocket that contains a protein-protein interaction domain (Moldovan et al., 2007; Naryzhny, 2008). Most of the PCNA partners contain PCNA binding motif namely PIP box (consensus sequence QXX(L/M/I)XX(F/Y)(F/Y)) (Warbrick, 2000). Proteins like CAF-1, DNA polymerase β or the p50 subunit of DNA polymerase δ interact with PCNA through the PIP-related sequence QLXLF (Dalrymple et al., 2001; Miller et al., 2010; Naryzhny, 2008). Additionally, PCNA is involved in DNA repair pathways and its interactions with the DNA repair proteins such as Fen-1 (Flap endonuclease-1) and Msh-2 (mutS homologue 2) have been described (Maga and Hubscher, 2003).

This ring-shaped homotrimeric structure of PCNA encircles DNA and can slide spontaneously along DNA molecule in both directions (Moldovan et al., 2007). PCNA has been shown to travel with the replication forks and tether DNA polymerases to DNA (Moldovan et al., 2007; Prosperi, 1997). PCNA interaction with double-stranded DNA results in enhanced processivity of DNA polymerases ϵ and δ (Essers et al., 2005; Majka and Burgers, 2004) as replication of both leading and lagging strands is PCNA-dependent (Trakselis and Bell, 2004).

Additionally, PCNA coordinates efficient movement of replication forks and prevents dissociation of polymerases from the DNA template (Bowman et al., 2004; Maga and Hubscher, 2003).

Both RFC complex (Replication factor C) and ATP are essential for PCNA chromatin association, however, ATP hydrolysis is dispensable for the RFC and PCNA interaction (Mossi and Hubscher, 1998). RFC is a chaperone-like, arc-shaped complex composed of five subunits and possesses DNA binding and ATPase activities (Majka and Burgers, 2004). RFC complex binds to the PCNA ring and through ATP hydrolysis deposits the clamp onto DNA (Bowman et al., 2004). Interestingly, RFC-PCNA complex binds preferentially to primed DNA at its 3'-ends, suggesting that these may be primary binding sites of the RFC-PCNA complex (Moldovan et al., 2007).

1.7.1.3 DNA polymerases

DNA polymerases are the main effectors of the DNA replication process. At least 19 different eukaryotic polymerases have been discovered (Hubscher et al., 2002), including the five main DNA polymerases classified by Greek letters: α , β , γ , δ , and ϵ (Thommes and Hubscher, 1990). DNA polymerase β is mainly responsible for DNA repair and recombination (Podlutzky et al., 2001), while DNA polymerase γ is involved in mitochondrial DNA synthesis and repair (Thommes and Hubscher, 1990). Because polymerase β and γ are not involved in the replication of bulk nuclear DNA, they will not be described in this section. The process of DNA replication is jointly supported by the three polymerases α , δ , and ϵ (Bermudez et al., 2011; Burgers, 2009).

DNA polymerase α is the only polymerase that has a unique ability to start DNA synthesis *de novo* (Burgers, 2009; Waga and Stillman, 1998). Polymerase α is a heterotetrameric enzyme consists of four subunits: Pol1, Pol12, Pri1 and Pri2. All subunits of polymerase α possess different functions. The largest Pol1 subunit (p140) harbours DNA polymerase activity, whereas Pri1 (p48) catalyses the RNA primers synthesis (Garg and Burgers, 2005; Santocanale et al., 1993; Waga and Stillman, 1998). Both Pri2 (p58) and Pol12 (p79) subunits are involved in stabilisation and regulation of the holoenzyme (Garg and Burgers, 2005). DNA primase initiates DNA replication by synthesis of 10 nucleotides

long RNA primer on both DNA strands (Albertson et al., 2009; Bermudez et al., 2011). RNA primers are subsequently extended for another 20 nucleotides by polymerase α (Burgers, 2009). As mentioned before, this short RNA-DNA hybrid is efficiently recognised by PCNA-RFC complex. Upon the binding of PCNA-RFC and establishment of PCNA tethering, DNA polymerase α is displaced from the primer (Bermudez et al., 2011). From that point, DNA synthesis is continued by either polymerase δ or polymerase ϵ on the lagging or leading strand, respectively (Burgers, 2009; Kunkel and Burgers, 2008; Waga and Stillman, 1998).

DNA polymerase ϵ is the first polymerase loaded onto DNA after assembly of the pre-initiation complex (Bermudez et al., 2011; Garg and Burgers, 2005). It has also been proposed that polymerase ϵ is a component of the pre-replication complex and may interact with proteins required for initiation of DNA replication (Bermudez et al., 2011). Similarly to DNA polymerase α , DNA polymerase ϵ contains four subunits Pol2, Dpb2, Dpb3, and Dpb4 (Garg and Burgers, 2005). Budding and fission yeast gene of polymerase ϵ is essential for cell viability (Feng et al., 2003). Zlotkin and colleagues showed that polymerase ϵ is required for *in vivo* DNA synthesis, but not for SV40 (simian virus 40) DNA replication *in vitro*. This indicates that polymerase ϵ is the leading strand enzyme (Zlotkin et al., 1996). In budding yeast, analysis of polymerase ϵ mutation revealed that polymerase ϵ executes replication of the leading but not lagging strand (Pursell et al., 2007). Polymerase ϵ possesses 3'-5' exonuclease activity that allows proof-reading of errors arising during DNA replication (Essers et al., 2005; Majka and Burgers, 2004).

DNA polymerase δ is the second major polymerase at the replication fork, and it is involved in the lagging strand synthesis (Burgers, 2009; Stillman, 2008). This enzyme comprises of four subunits Pol2, Pol31, Pol32 and Cdm1 (Garg and Burgers, 2005) and it is the most conserved polymerase between eukaryotes (Hubscher et al., 2002). *In vitro* studies in SV40 DNA replication system demonstrated that polymerase δ interacts with replicating DNA and together with polymerase α is sufficient to duplicate leading and lagging strands (Nick McElhinny et al., 2008). Together with Fen-1 and PCNA it is involved in maturation of Okazaki fragments (Burgers, 2009; Jin et al., 2003). DNA

polymerase δ is required for several processes beside DNA synthesis (Xie et al., 2005). DNA polymerase δ participates in the telomere addition *in vivo* (Diede and Gottschling, 1999) and DNA repair mechanisms (Burgers, 1998). Polymerase δ possesses 3'-5' exonuclease activity that allows proof-reading of errors (Kunkel and Burgers, 2008; Pavlov et al., 2006) and similarly to polymerase ϵ , processivity of polymerase δ is increased by the presence of PCNA (Essers et al., 2005; Majka and Burgers, 2004).

1.7.1.4 Flap structure-specific endonuclease 1

Fen-1 is a member of the XPG/RAD2 family of endonucleases with 5'-flap endonuclease and 5'-3' exonuclease activities (Lieber, 1997). Fen-1 is involved in both DNA synthesis and DNA repair (Nikolova et al., 2009). It has been reported that Fen-1 removes RNA primers attached to the 5'-end of each Okazaki fragment (Waga and Stillman, 1998) and together with PCNA and RPA stimulates Okazaki fragment maturation (Zheng and Shen, 2011). Mammalian Fen-1 interacts with cyclin A, Cdk1 and Cdk2 (Henneke et al., 2003). Activity of Fen-1 is promoted by physical interaction with PCNA (Frank et al., 2001), but recent studies demonstrated that RFC also stimulates activity of Fen-1 independent of ATP hydrolysis (Cho et al., 2009). Additionally, Fen-1 is required for a long-patch base excision repair (Kim et al., 1998) and restart of stalled replication forks (Nikolova et al., 2009).

Name	Functions	References
Cdc5 Polo-like kinase (cell division cycle 5)	through interaction with Dbf4 could regulate the function of Cdc7/Dbf4 kinase	(Chen and Weinreich, 2010).
Cdc14p (cell division cycle 14 protein phosphatase)	dephosphorylates replication factors Sld2 and Dpb2 in budding yeast	(Bloom and Cross, 2007); (reviewed in (Mocciaro and Schiebel, 2010)).
Ctf4 (Chromosome transmission fidelity 4)	recruits polymerase α to DNA via interaction with Mcm10	(Zhu et al., 2007).

Dna2 endonuclease	removes RNA primer and cleaves long RPA-bound flap structures on the lagging strand	(Kao et al., 2004; Kim et al., 2006).
DNA ligase	ligates the Okazaki fragments on the lagging strand	(Pascal et al., 2004).
GEMC-1 (geminin coiled-coil containing protein 1)	interacts with TopBP1 and Cdc45, its phosphorylation by Cdk2 promotes initiation of DNA synthesis and loading of Cdc45	(Balestrini et al., 2010).
Mcm8	recruits RPA p34 and facilitates the processivity of DNA polymerases	(Maiorano et al., 2005).
Noc3 (Nucleolar complex-associated protein 3)	interacts with ORC and MCM helicase; promotes association of Cdc6 and Mcm2 with chromatin in budding yeast	(Zhang et al., 2002b).
RecQ1 (ATP-dependent DNA helicase Q1)	may play a role in modulation of replication forks activity and rate; plays role in DNA unwinding and promotes PCNA loading onto chromatin	(Thangavel et al., 2010).
RecQ4 (ATP-dependent DNA helicase Q4)	interacts with the MCM helicase through Mcm10, plays a role in DNA unwinding and promotes RPA and PCNA loading onto chromatin; potential orthologue of Sld2 in human	(Thangavel et al., 2010; Xu et al., 2009); reviewed in (Bachrati and Hickson, 2008).
RFC (Replication factor C)	PCNA clamp loader; stimulates activity of Fen-1; facilitates polymerases switch	(Cho et al., 2009; Yao et al., 2003).
RNase H2 (Ribonuclease H2)	removes RNA primers from lagging strand and single ribonucleotides integrated in a DNA; interacts with PCNA	(Bubeck et al., 2011).
Sld2 (Synthetically lethal with Dpb11-2)	Cdk substrate; promotes loading of TopBP1 and Cdc45 in budding yeast	(Pospiech et al., 2010; Sclafani and Holzen, 2007).

Sld3 (Synthetically lethal with Dpb11-3)	Cdk substrate; promotes loading of TopBP1; required for Cdc45-Mcm2-7 interaction in budding yeast	(Pospiech et al., 2010; Sclafani and Holzen, 2007).
Pob3 (DNA polymerase α binding protein 3)	interacts with DNA polymerase α and facilitates DNA accessibility. Subunit of FACT complex in budding yeast	(Formosa et al., 2001; Ransom et al., 2010).
Top1 (DNA topoisomerase I)	involved in control and modification of DNA topology; mediates a transient single-strand break in the phosphodiester backbone at the replication forks	(Austin and Marsh, 1998).
Top2 (DNA topoisomerase II)	involved in control and modification of DNA topology during synthesis; catalyses formation of double-strand breaks in DNA	(Austin and Marsh, 1998).
Treslin	together with TopBP1 promotes loading of Cdc45, phosphorylated by Cdk2	(Kumagai et al., 2010).

Table 1.1 Other proteins involved in eukaryotic DNA synthesis.

1.7.2 Regulation at stalled replication forks

Replication stress can be induced by different natural impediments such as: alternative DNA structures (e.g. left-handed Z-DNA), changes in the expression of components necessary for the DNA synthesis, protein-DNA interactions or collisions between replication and transcription machineries (reviewed in (Branzei and Foiani, 2010; Mirkin and Mirkin, 2007)). Replication stress can also be induced artificially (reviewed in (Burhans and Weinberger, 2007)) by drugs that can cause depletion of dNTP supply (Koç et al., 2004), inhibition of polymerase activity (Krokan et al., 1981), inhibition of the topoisomerase I or the topoisomerase II α activities (Allen et al., 2011). To ensure proper DNA duplication cells have evolved different pathways to preserve genome integrity, such as S phase checkpoint (see section 1.3.3), proofreading of DNA template during DNA synthesis and DNA repair mechanisms (Allen et al., 2011).

Stalled replication forks can be repaired by different pathways (Allen et al., 2011; Michel et al., 2004). Upon replication stress, the activity of DNA

polymerase is inhibited, however *in vitro* studies in *Xenopus* oocytes confirmed that Mcm2-7 helicase continues to unwind DNA strands leading to production of long ssDNA tracks (Byun et al., 2005). The mechanism of DNA helicase inhibition after replication stress is still unclear, however, high-density oligonucleotide array and co-immunoprecipitation analyses in budding yeast suggest that direct interaction of Mcm2-7 with fork pausing complex (FPC) (Mrc1/Tof1/Csm3) may stabilise the replisome and prevent further unwinding of dsDNA (Katou et al., 2003). Accumulation of ssDNA occurs at blocked replication forks and ssDNA is immediately covered by RPA (Zou and Elledge, 2003). Excess of ssDNA and accumulation of RPA lead to activation of the S phase checkpoint (Shiotani and Zou, 2009). Interestingly, analysis of *Xenopus* oocytes extracts containing reduced levels of RPA showed that checkpoint activation and phosphorylation of Chk1 is independent on RPA hyperloading onto stalled replication forks, suggesting that additional mechanisms are involved in complete activation of S phase checkpoint (Recolin et al., 2011).

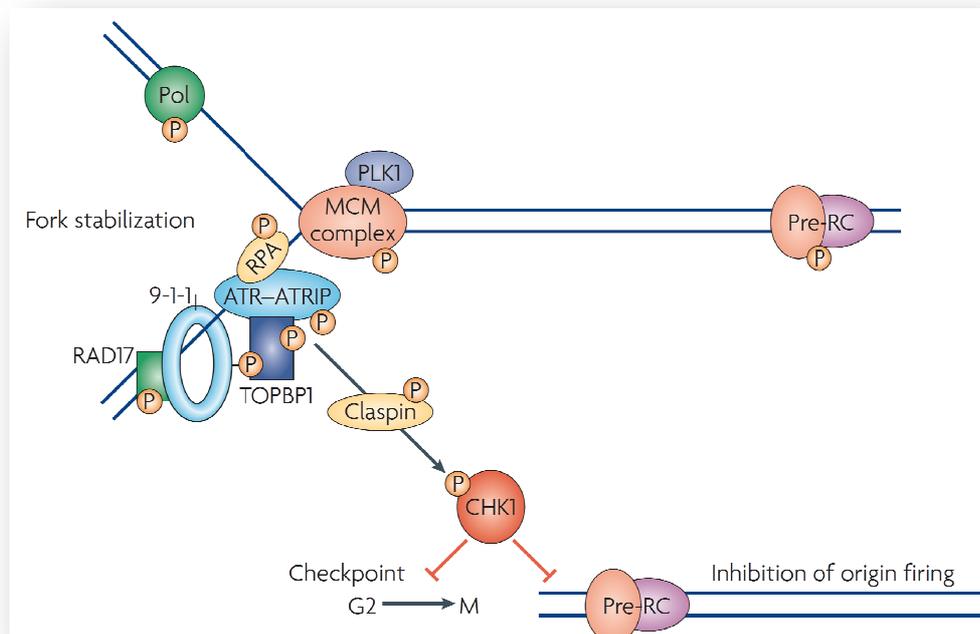


Figure 1.10 Stabilisation of stalled replication forks. The ATR kinase plays a major role in replication fork stabilisation. Upon replication stress, ATR promotes cascade phosphorylation of protein complexes loaded onto the replication forks. Additionally, phosphorylation of Mcm2 helicase by ATR recruits Polo-like kinase 1 (PLK-1) and facilitates the completion of DNA synthesis. Phosphorylated substrates are indicated on the picture with letter P. Picture taken from (Cimprich and Cortez, 2008).

Once stalled, replication forks are stabilised to prevent their collapse and replisome dissociation (Errico and Costanzo, 2010) (Figure 1.10). The maintenance of the stalled replication forks and activation of the checkpoint mechanism is mediated by the FPC complex (Errico and Costanzo, 2010; Yoshizawa-Sugata and Masai, 2007). The FPC is composed of Tim1 (Timeless protein homologue 1), Tipin (Timeless-interacting protein) and Claspin (Errico and Costanzo, 2010). RNAi analysis in human cells indicated that both Tipin and Timeless are essential for Chk1 phosphorylation upon genotoxic stress (Kemp et al., 2010). Moreover, Tipin-RPA interaction stabilises the FPC complex and facilitates Claspin-mediated Chk1 phosphorylation at Ser317 and Ser345 by ATR. This leads to activation of ATR/Chk1 response pathway (Branzei and Foiani, 2010; Kemp et al., 2010). *In vitro* binding assays with purified ATR, ATRIP and naked or RPA-coated biotinylated oligomers revealed that RPA-coated ssDNA is able to recruit ATRIP or ATR/ATRIP complex to the DNA damage sites, but it is not required for ATR association with ssDNA (Zou and Elledge, 2003). Additionally, RNAi-mediated knockdown of RPA confirmed that RPA is essential for ATR/ATRIP foci formation after DNA damage (Ball et al., 2005). Zou and colleagues also illustrated that phosphorylation of the Rad17-RFC2-5 clamp loader by ATR/ATRIP is dependent on RPA *in vitro* (Zou and Elledge, 2003). In budding yeast and human cells Rad9-Hus1-Rad1 and Rad17-RFC2-5 complexes co-localise at the site of DNA damage (Kondo et al., 2001; Zou et al., 2002). Electron microscopy analysis of 9-1-1 and Rad17-RFC2-5 complexes in insect cells demonstrated that phosphorylated Rad17-RFC2-5 stimulates loading of Rad9-Hus1-Rad1 (9-1-1) heterotrimer onto 5'-DNA template junctions (Bermudez, 2003). Additionally, recent evidences obtained by immunodepletion of TopBP1 and polymerase α from NIB-250 chromatin extract in *Xenopus* confirmed that TopBP1 and polymerase α directly mediate the assembly of 9-1-1 complex onto stalled replication forks *in vitro* (Yan and Michael, 2009).

Studies in both human cells and DT40 mutants of *Rad9*^{-/-}, and *Rad17*^{-/-} confirmed that phosphorylation of Rad9 at Ser387 is sufficient for Chk1 activation. Additionally, Rad9 phosphorylation is required for interaction with TopBP1 through BRCT domains I-II (Delacroix et al., 2007). Further analysis of *Xenopus* TopBP1 mutants lacking BRCT I-II repeats showed that interaction

between Rad9 and TopBP1 is essential for further recognition of TopBP1 by ATR/ATRIP, and thus for full activation of ATR kinase (Delacroix et al., 2007; Lee et al., 2007). Once activated, ATR phosphorylates Mcm2 at Ser108 (Yoo et al., 2004), thus facilitating recruitment of PLK1 (Polo-like kinase 1) as indicated by immunodepletion of *Xenopus* PLK1 homologue Pxi1 (Trenz et al., 2008) (Figure 1.10). In the event of stalled replication forks, PLK1 promotes firing of adjacent DNA replication origins by recruitment of Cdc45 and DNA polymerases onto chromatin (reviewed in (Cimprich and Cortez, 2008)).

Stalled replication forks must restart to continue the DNA synthesis. A number of different DNA proteins are involved in this process including DNA helicases such as BLM (Bloom syndrome) (Bachrati and Hickson, 2008), WRN (Werner syndrome), FANCM (Fanconi-anaemia complementation group M) (Luke-Glaser et al., 2010), HLTF (helicase like transcription factor) (Unk et al., 2010) and SMARCAL1 (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A-like protein 1) (Driscoll and Cimprich, 2009). Most of them are recruited near blocked replisome machinery by RPA (reviewed in (Cimprich and Cortez, 2008)). Terminally arrested replication forks can collapse and DNA synthesis in this region can be rescued and completed by activation of nearby dormant origins (Errico and Costanzo, 2010). The dormant origins do not normally fire during unchallenged DNA synthesis (Ge et al., 2007).

In the recent years techniques such as chromosome fibre and DNA combing become leading approaches to study fork stalling, speed and movement of individual replication forks and firing of new origins. Additionally, combination of DNA fibre with FISH (fluorescence *in-situ* hybridisation) allows the dynamics of replication fork at specific genome locus to be studied. However, these methods cannot distinguish defects in fork stabilisation and detect specific DNA structures associated with DNA damage such as Holliday junctions. Moreover, usage of nucleotide derivatives to label DNA can affect replication forks progression and create artifacts during analysis when two different labelling fluorophores are used (Petermann and Helleday, 2010).

In vitro cell-free systems such as *Drosophila* embryos and *Xenopus* oocytes allows for the study of proteins associated with DNA damage sites and their potential role in DNA damage response and replication stress. Recently, the

Helleday group described CldU-based method for labelling of ssDNA to analyse the role of Rad51 in the restart of stalled replication forks (Petermann et al., 2010). However, different parameters must be taken under consideration during this procedure such as denaturation of dsDNA and sensitivity of antibody-based purification. Nevertheless, site-specific analysis of stalled replication fork has not been performed so far. Dm-ChP methodology developed and described in this study allows purifying proteins that are associated with active or stalled replication forks in mammalian cells *in vivo*. This approach can be also used to study post-translational modification of different proteins upon replication stress. Additionally, Dm-ChP permits investigation of time-dependent dynamics of proteins associated with chromatin under replication stress conditions.

1.8 Termination of DNA replication

1.8.1 Termination by converging replication forks

Termination of replication in eukaryotic cell is not well understood. Linear DNA of eukaryotic cell contains thousands of different origin sites (Arias and Walter, 2007; Francis et al., 2009)), thus replication forks meet and terminate randomly in the regions between activated origins (Codlin and Dalgaard, 2003; Fachinetti et al., 2010).

In budding yeast, termination of DNA synthesis occurs non-specifically within a 4.3 kb region located between two origins (Fachinetti et al., 2010). ChIP-chip analysis in fission and budding yeast, confirmed the presence of 5 kb termination regions (TERs) where the two replication forks converge (Codlin and Dalgaard, 2003; Fachinetti et al., 2010). These termination regions were present between both the early and late replicated origins. In budding yeast, replication forks can terminate at specific replication fork barriers (RFBs) that mediate termination by arresting one of the replication forks (Fachinetti et al., 2010). Topoisomerase II is involved in termination of DNA replication *in vitro* and *in vivo* (Baxter and Diffley, 2008). Topoisomerase II function in this process is to remove DNA catenates that were created during DNA replication, thus allowing subsequent chromosome segregation (Field-Berry and DePamphilis, 1989; Lucas et al., 2001).

1.8.2 Termination at telomeres

Eukaryotic chromosome ends are protected by telomeres (Jackson, 2005; Vega et al., 2003). Vertebrate telomeres are unique DNA sequences containing tandem TTAGGG repeats at the ends of the chromosome (reviewed in (Blackburn, 2005; Jackson, 2005)). Synthesis of telomere caps during chromosome duplication is stimulated by the telomerase, thus preventing chromosome shortening and degradation (Greider and Blackburn, 1989; Jackson, 2005). Most human cells have a finite replication capacity known as a Hayflick limit (Hayflick, 1965) and it is the consequence of telomeres shortening with each cell division due to the lack of telomerase activity in somatic cells (Harley et al., 1990). Chromosome shortening was observed in aging cells, confirming contribution of telomerase activity to aging and senescence (Jackson, 2005).

Precise termination mechanism of the DNA replication at telomeres is well understood. Telomeres are added at the 3'-end of eukaryotic chromosomes by telomerase. Telomerase is RNA-dependent DNA polymerase composed of catalytic subunit containing reverse transcriptase domain (TERT) and RNA molecule (TERC) that serves as a template for telomeric DNA synthesis. Using RNA primers telomerase binds to the 3'-end of the chromosome and synthesises a new telomere using the RNA template. After elongation of single telomere sequence, telomerase translocates to initiate synthesis of new TTAGGG repeat (reviewed in (Autexier and Lue, 2006)).

1.9 Temporal regulation of DNA replication

All eukaryotic cells duplicate their genome during S phase of the cell cycle (Gilbert, 2010). The location of all replication origins and firing time have been well defined in budding yeast (Raghuraman et al., 2001). *Saccharomyces cerevisiae* genome consists of 16 chromosomes that are duplicated within 25-30 minutes of S phase (Yabuki et al., 2002). Replication origins in budding yeast are fired in a temporally coordinated manner (Kim and Huberman, 2001) and it has been estimated that approximately 40 kb of DNA is replicated from a single replication origin (replicon) (Donaldson, 2005). Isotopic-labelling of newly synthesised DNA in budding yeast allowed characterisation of all replication origins (Donaldson, 2005; Raghuraman et al., 2001). Additionally, DNA

microarray analysis demonstrated that most of the replication origins are firing in the middle of the S phase and their activation occurs in the clusters of adjacent origins. Moreover, base composition of budding yeast genome showed that in contrast to human cells, there is no relationship between GC-rich regions and early replication timing. Furthermore, origins placed in vicinity of telomeres are firing later than centromeric origins (Raghuraman et al., 2001).

Temporal hierarchy of origin firing in humans is established during G₁ phase of the cell cycle (Raghuraman et al., 1997). Replication origins are firing, as clusters of replication domains, in a time-dependent manner to duplicate specific chromosome segments (Costantini and Bernardi, 2008). Early replicating origins are spread randomly within the nucleus, whereby late replicating origins are localised at the nuclear periphery (Dimitrova and Gilbert, 1999; Masai et al., 2010). The temporal regulation of DNA synthesis correlates with chromatin dynamics, chromosome architecture and gene expression (Pliss and Malyavantham, 2009). Chromosomes are divided into regions that contain either Gimesa dark chromosome bands (G bands) or Gimesa light chromosome bands (R bands) (Gilbert, 2002). G bands are highly homogeneous in AT content, while R bands are enriched in GC content (Holmquist et al., 1982). GC-rich regions that contain more accessible euchromatin and highly expressed genes tend to replicate early, while AT-rich regions containing genetically inactive heterochromatin and infrequently transcribed genes are generally replicated in late S phase (reviewed in (Grewal and Jia, 2007); (Göndör and Ohlsson, 2009)) (see section 1.10.1). Several studies on human (White et al., 2004; Woodfine et al., 2005; Woodfine et al., 2004), mouse (Farkash-Amar et al., 2008; Hiratani et al., 2008) or *Drosophila* models (MacAlpine et al., 2004; Schübeler et al., 2002; Schwaiger et al., 2009) have revealed that early replication is correlated with chromatin structure, transcription activation, gene density and GC content. Details of particular experiments determining replication timing in different organisms are presented in Table 1.2.

Epigenetic marks also control the temporal order of replication. Microarray hybridisation of genomic DNA isolated from S phase arrested HeLa cells revealed that acetylation of histones is a characteristic mark for transcriptionally active genes, while deacetylation and tri-methylation of H3K9 is generally associated with silenced chromatin (Göndör and Ohlsson, 2009) (see section

1.10.6). Additionally, study of human genome using ENCODE microarrays showed that tri-methylation of histone H3 at Lys27 was associated with late replicating chromatin (ENCODE Project Consortium et al., 2007). On the other hand, early replicated regions analysed by anti-BrdU immunoprecipitation, followed by microarray hybridisation were associated with histone acetylation of histone H4 at Lys16 and transcription sites in *Drosophila* (Schwaiger et al., 2009).

Organism	Cell line	Analytical method	Genome coverage	Note	References
<i>Drosophila</i>	Kc (derived from dissociated embryo)	FACS sorting	Expressed sequences at 20.5 kb resolution	40% of the genes showed strong correlation between early replication and transcription	(Schübeler et al., 2002)
			Chromosome 2L	Early replicated genes co-localise with RNA polymerase II binding sites and gene density	(MacAlpine et al., 2004)
			Whole genome	Early replication regions correlate with transcription activation and acetylation of H3K16	(Schwaiger et al., 2009)
Mouse	L1210 (lymphocytic leukemia cells)	'baby-machine' sorting	Whole genome	Relationship between early replication regions, transcription and GC content	(Farkash-Amar et al., 2008)
	Embryonic stem cells and Neural precursor cells	FACS sorting		Early replicated genes are enriched with GC content	(Hiratani et al., 2008)
Human	HFL-1 (Primary lung fibroblast)	FACS sorting	Chromosome 22	Relationship between early replication regions and transcriptional activity	(White et al., 2004)
	CO202 (male lymphoblastoids)	FACS sorting (gene copy number analysed)	Chromosome 6	Relationship between early replication regions, GC content and active transcription	(Woodfine et al., 2005)

HeLa cells	cell-cycle synchronisation (thymidine-aphidicolin)	Chromosome 21 and 22	Higher gene density and GC content are associated with early replicated DNA	(Jeon et al., 2005)
7 different Neuroblastoma cell lines	FACS sorting (copy number analysed)	Whole genome	Chromosome breakpoints preferentially occur within early replicating regions	(Janoueix-Lerosey et al., 2005)
HeLa cells	cell-cycle synchronisation (thymidine-aphidicolin)	ENCODE (1% of genome)	Relationship between early replication and transcriptional activity	(Karnani et al., 2007)
HCT116 (colorectal cancer cells) p53 ^{-/-} and p53 ^{+/+}	FACS sorting	25,000 cDNA microarray	Cell-cycle and apoptosis related genes are replicated early in p53 ^{-/-} cells	(Watanabe et al., 2007)
Erythroid, mesenchymal and Embryonic stem cells	FACS sorting (gene copy number analysed)	Whole genome	Early replicating regions localise near highly expressed genes while late-firing origins are associated with regions lacking active genes	(Desprat et al., 2009)
Various human cell lines	FACS sorting	Whole genome	Replication timing correlates with chromatin accessibility	(Hansen et al., 2010)
HeLa cells	FACS sorting	Whole genome	Chromatin compaction is negatively correlated with replication timing	(Chen et al., 2010)

Table 1.2 Determination of replication timing for various organisms using genome-wide analysis. Table adapted from (Farkash-Amar et al., 2008; Masai et al., 2010).

Additionally, the temporal regulation of genome duplication changes during development. Active X chromosome is duplicated early in S phase and is less condensed than inactive X chromosome as indicated by Schwaiger and colleagues (reviewed in (Pope et al., 2009); (Schwaiger et al., 2009)). On the contrary, study on female mouse embryonic fibroblasts using immuno-RNA FISH and ChIP analyses showed that inactive X chromosome contains tri-

methylated H3K9, a mark for a transcriptionally silenced chromatin that is consistent with late replicating pattern (Heard et al., 2001).

Genome-wide and high-throughput approaches provide a significant contribution into understanding of temporal regulation of the mammalian genome. However, these techniques need to be improved in order to clarify the several aspects of replication timing that still remain unexplored. Firstly, resolution of synchronisation procedures is limited by the speed of the mammalian replication forks that move at average 1-2 kb/minute, thus labelling times need to be reduced to improve technique resolution. Moreover, optimisation of purification of replication regions is necessary to increase resolution and sensitivity of sequencing methods. As different sets of replication origins are fired in various cells, recent methods provide an average origin choice in a cell population, the single cell analysis would be appropriate. Understanding how replication timing varies in the single cell and how it is regulated during cell development is crucial to determine mechanism of DNA replication. Methods such as fluorescence *in situ* hybridization (FISH) or DNA fibres could be used to determine temporal regulation in single cell, thus addressing the issue of replication-timing heterogeneity.

Alternatively, a method such as Dm-ChIP due to its specificity and high resolution could provide a powerful tool to investigate how replication timing affects chromatin deposition and DNA component associated with replication origins at different times during S phase.

1.10 Structure and organisation of the chromatin

Each human cell contains 1.7 meters of DNA that is packed into a nucleus of 5 μm in diameter (Ho and Crabtree, 2010; Mario-Ramirez et al., 2005). In mammalian cells DNA is organised in a highly complex structure that contains DNA and DNA binding proteins called chromatin. Chromatin has several levels of organisation (reviewed in (Fedorova and Zink, 2008)) and this packaging is necessary for regulation of DNA accessibility during nuclear processes, such as DNA replication, transcription and DNA repair (Scharf et al., 2009a).

1.10.1 Euchromatin and heterochromatin

The chromatin packaging depends on the stage of the cell cycle and it is most condensed in metaphase and less condensed in interphase (reviewed in (Grigoryev et al., 2006)). Interphase cell DNA is organised into two morphologically different forms: euchromatin and heterochromatin (reviewed in (Fedorova and Zink, 2008)).

Heterochromatin was first described by Emil Heitz based on his work with the moss model (Heitz, 1928). He noticed that moss nucleus contains regions which remain condensed throughout the cell cycle (Dillon, 2004; Straub, 2003). Heitz distinguished euchromatin ('true chromatin') as a less packed form of DNA from the heterochromatin ('other chromatin') which is highly and constitutively condensed (reviewed in (Grewal and Jia, 2007; Grigoryev et al., 2006)). Further findings demonstrated that heterochromatin contains infrequently transcribed genes and it is also less accessible for the replication and transcription machineries, whereas euchromatin is more accessible and contains genes that are highly expressed (reviewed in (Grewal and Jia, 2007)).

Heterochromatin contributes to important biological functions, such as chromosome segregation (reviewed in (Tamaru, 2010)) and maintaining of genomic stability (reviewed in (Cann and Dellaire, 2011)). Heterochromatin protein 1 (HP1 α) is a multifunctional protein involved in heterochromatin formation, gene silencing and heterochromatic gene expression (Grewal and Jia, 2007). However, recent studies revealed that HP1 γ plays an additional role in regulation of euchromatin genes (Kwon and Workman, 2011). Heterochromatin can be found in the regions occupied by lamin proteins, which are involved in formation of nuclear membrane structures (reviewed in (Olins et al., 2010)). Lamins provide heterochromatin with molecular docking sites at the nuclear periphery (Shumaker et al., 2006). Consistently, lamin B is observed to localise to late replicating DNA and heterochromatin regions (Kennedy et al., 2000). Additionally, euchromatin and heterochromatin can be distinguished by nucleosome modifications. Heterochromatin is typically associated with hypoacetylation and histone H3 tri-methylation at Lys9 (H3K9), whereas euchromatin is associated with acetylated histone H3 and H4 and tri-methylated histone H3 at Lys4 (reviewed in (Tamaru, 2010)).

1.10.2 The nucleosome organisation

Nucleosomes are the basic, repeating blocks that pack DNA into higher-order structures (Woodcock and Ghosh, 2010). The nucleosome core consists of 147 base pairs of double-stranded DNA that wraps around a histone octamer. The histone octamer is composed of two copies of each core histones H3 and H4 (central tetramer) and side flanking dimers of histones H2A-H2B (reviewed in (Groth et al., 2007b), (Arents and Moudrianakis, 1993)). A fifth histone H1 associates with linker DNA and serves as an anchor between DNA and nucleosome, thereby mediating DNA compaction (Figure 1.11) (reviewed in (Delage and Dashwood, 2008; Marino-Ramirez et al., 2005; Ramakrishnan, 1997)).

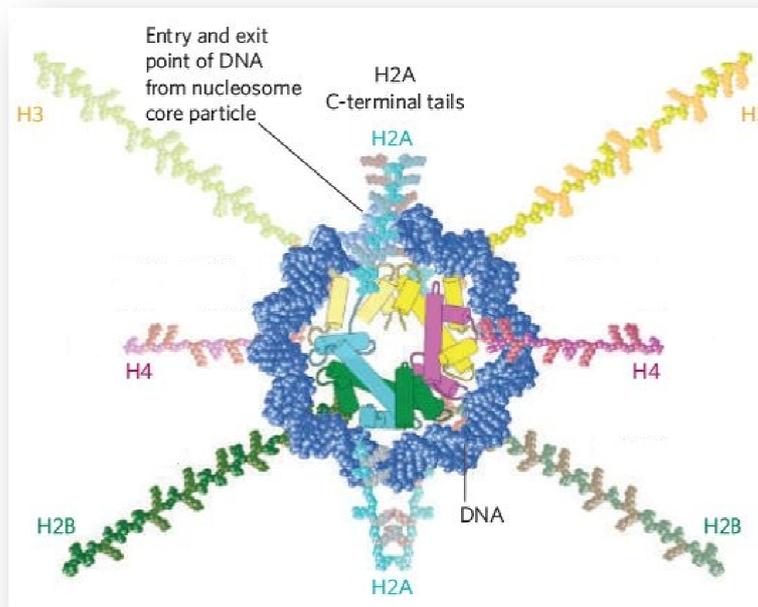


Figure 1.11 Organisation of the nucleosome. The single nucleosome contains histone octamer (two molecules of each histones: H2A (light blue), H2B (green), H3 (yellow) and H4 (pink)) that wraps 147 base pairs of double-stranded DNA (dark blue). Picture adapted from (Down et al., 2007).

The 2.8 Å resolution structure of the nucleosome core revealed that all core histones in the nucleosome interact with double-stranded DNA (Luger et al., 1997). In a single nucleosome, DNA interacts non-covalently with the histone core in 14 different regions (Luger, 2006). These specific regions are the target for chromatin remodelling factors that catalyse exchange of histone variants.

Nevertheless, the contact regions of histone H3 and H2A are both located in central and ends of the nucleosome core. In the absence of DNA, the histone octamer dissociates into H3-H4 tetramer and two H2A-H2B dimers, suggesting that presence of the DNA helix stabilises formation of the histone octamer (reviewed in (Szerlong and Hansen, 2011)). Consistently, at high ionic strength conditions, the intact nucleosome core can be formed even in the absence of DNA or cross-linking agents (reviewed in (Ramakrishnan, 1997)).

1.10.3 Higher-order DNA structures

DNA of the eukaryotic cell is packed according to a chromatin folding system (Grigoryev et al., 2006). The primary level of chromatin compaction is determined by 10 nm fibre structures of nucleosomes, also called "string-of-beads" (reviewed in (Li and Reinberg, 2011)). In the presence of high ionic strength, nucleosomes are further coiled into 30 nm chromatin fibres (reviewed in (Woodcock and Ghosh, 2010)).

This superhelical structure of chromatin is present in both interphase and metaphase chromosomes (reviewed in (Szerlong and Hansen, 2011)). 30 nm fibre condensation depends on interactions between the N-terminal tail of histone H4 and the acidic region of the H2A-H2B dimer (reviewed in (Marino-Ramirez et al., 2005)). Microscopy studies proposed three distinct models for the 30 nm chromatin fibre, the two-start helical ribbon (Zig-zag) model, the two-start crossed-linker model and the one-start solenoid model (Figure 1.12) (reviewed in (Li and Reinberg, 2011; Marino-Ramirez et al., 2005)).

In the one-start helix/solenoid model, the adjacent nucleosome units are positioned in a hand-to-hand orientation and connected by a bent DNA linker (reviewed in (Li and Reinberg, 2011; Marino-Ramirez et al., 2005)). The one-start solenoid superhelix is formed by the superhelical patch around an inner cavity with 6-8 nucleosomes per turn and 11 nm pitch (reviewed in (Szerlong and Hansen, 2011)). In this structure, neighbouring nucleosomes interact with each other, which is not observed in two-start helix conformation (Figure 1.12 a) (Kruithof et al., 2009).

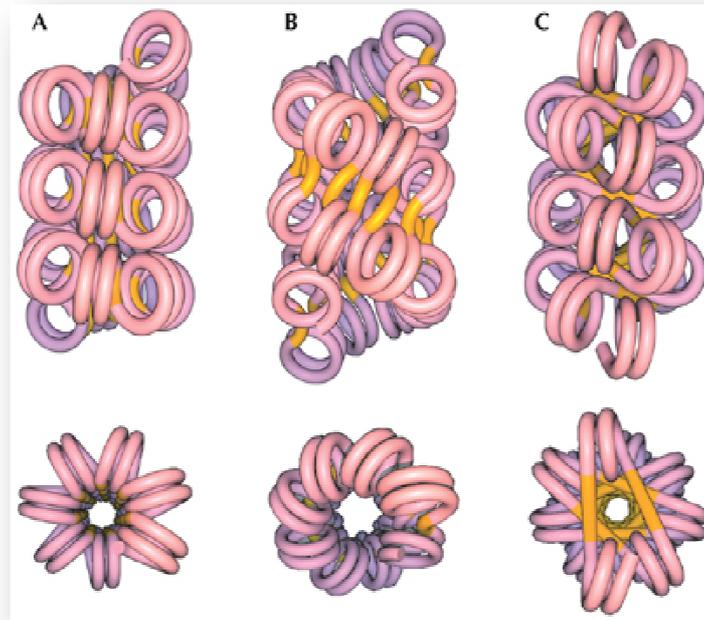


Figure 1.12 *Three models of the 30 nm chromatin fibre. a) The one-start solenoid model b) The two-start helical ribbon (Zig-zag) model c) The two-start crossed-linker model. Nucleosome is pink and linker DNA is yellow. Picture taken from (Wu et al., 2007).*

The two-start helix model is based on the conformation in which repeating units of nucleosome are oriented on the opposite sides of the fibre and connected by a straight DNA linker (reviewed in (Li and Reinberg, 2011; Marino-Ramirez et al., 2005)). In the Zig-zag arrangement nucleosome units are assembled across each other. In addition, two stacks of nucleosomes are rotated by about 70° with respect to each other (Robinson and Rhodes, 2006). The orientation of Zig-zag ribbon is directed parallel to the long axis of the fibre (Figure 1.12 b) (reviewed in (Szerlong and Hansen, 2011)).

The two-start crossed-linker model was originally proposed by Richmond and colleagues (Robinson and Rhodes, 2006). This model is based on the crystal structure of four-nucleosome cores lacking the linker histone. In this model, nucleosome units form twisted ribbon with a diameter of 25 nm and compaction of 5-6 nucleosome per 11 nm (Li and Reinberg, 2011). The linker DNA was located perpendicular to the long axis of the fibre and the two stacks of nucleosomes are connected together by two DNA helical turns since they do not interact with each other directly (Figure 1.12 c) (reviewed in (Szerlong and Hansen, 2011); (Robinson and Rhodes, 2006)).

1.10.4 Canonical histones

Histones are the main architectural proteins involved in chromatin packaging (see section 1.10.2-1.10.3). All four core histones H2A, H2B, H3 and H4 together with linker histone H1 are known as canonical histones (Marzluff et al., 2008). In higher eukaryotes canonical histones are encoded by a cluster of genes. These genes are highly expressed during S phase and their transcription is tightly associated with DNA synthesis (Marzluff et al., 2008; Talbert and Henikoff, 2010). During S phase, approximately 10^8 molecules of each histone core are synthesised and mRNA levels are rapidly decreased when genome duplication is finished. There are three replication-dependent histone gene loci in human cells: the major HIST1 that is located on chromosome 1 and two smaller HIST2 and HIST3 that are located on chromosome 6 (Marzluff et al., 2002). All H1 histone genes are located in a large HIST1 cluster, whereas all core histone genes are clustered together in all three loci (Albig and Doenecke, 1997). The HIST1 locus contains genes encoding 49 core histone genes and 6 histone H1 genes. The HIST2 locus contains one of each H2B, H3 and H4 genes and 3 of H2A genes, while HIST3 cluster contains 3 genes for each H2A, H2B and H3 histones (Marzluff et al., 2002). All 14 histone H4 genes encode the same protein, while 11 histone H3 genes encode 3 different histone H3 proteins (H3, H3.1 and H3.2). Histones H2A and H2B genes encode 10-12 different H2A and H2B proteins (Koessler et al., 2003; Marzluff et al., 2002).

All core histones share characteristic structural features termed the “histone fold” motif. The histone fold is a hydrophobic structure found on the N-terminal of each histone and consists of a core of three α -helices: a long α -helix flanked by the two short α -helices. These heterodimerise forming a ‘handshake motif’ necessary for interactions of core histones (Alva et al., 2007). Furthermore, histones N-terminus possesses flexible amino acid ‘tail’ domain (NTD), that is subjected to different post-translational modifications (reviewed in (Groth, 2009)). Modifications of histone tails are essential for nucleosome stability and facilitate its assembly and disassembly. Moreover, N-terminal tail of histone H4 interacts with histone H2A to stabilise higher-order chromatin structure (reviewed in (Marino-Ramirez et al., 2005)).

1.10.5 Histone variants

Chromatin architecture is modulated by histone variants, post-translational modifications and histone chaperones (reviewed in (Park and Luger, 2006a)). Assembly of histone variants into the nucleosome occurs independently from DNA replication and leads to chromatin differentiation (reviewed in (Henikoff and Ahmad, 2005)). The canonical histones play a primary function in DNA packaging and gene regulation, while non-canonical variants are involved in a wide range of processes including DNA repair, meiotic recombination, chromosome segregation, transcription initiation and termination, sex chromosome condensation and sperm chromatin packaging (reviewed in (Talbert and Henikoff, 2010)). Histone variants include: histone H2A (H2A.Z, H2A.X, macroH2A, H2ABbd), histone H2B (H2BFWT and hTSH2B) and histone H3 (H3.3, H3.4, H3.5 and CENP-A). There are no sequence variants of histone H4 (reviewed in (Marino-Ramirez et al., 2005)). Functions and localisation of the major histone variants are presented in Table 1.3.

The H2A variant H2A.Z is evolutionary conserved across species (Guillemette et al., 2005). The H2A.Z is essential in mammalian cells, but not in *Saccharomyces cerevisiae* (reviewed in (Henikoff and Ahmad, 2005)). It is recruited to the nucleosome in ATP-dependent manner by chromatin remodelling complex Swr1 in budding yeast (Keogh et al., 2006). The Swr1 complex does not replace individual histones, but replaces the entire H2A-H2B for H2A.Z-H2B dimers. The H2A.Z histone is deposited onto chromatin independently of DNA replication (Jin and Felsenfeld, 2007). In mammalian cells H2A.Z shows heterochromatic distribution and interactions with HP1 α and Inner centromere protein (INCENP) (Fan et al., 2004), and thus plays role in heterochromatin formation and chromosome segregation (reviewed in (Dalmaso et al., 2011), (Guillemette et al., 2005)). Nucleosomes containing H2A.Z variant are necessary for recruitment of RNA polymerase II in both mammalian and budding yeast (Adam et al., 2001; Hardy et al., 2009). Additionally, the H2A.Z variant is believed to be involved in many processes including DNA repair, gene activation and silencing (reviewed in (Talbert and Henikoff, 2010)). Studies in *Drosophila* and *Saccharomyces cerevisiae* also demonstrated that H2A.Z prevents spreading

of silenced heterochromatin (reviewed in (Zlatanova and Thakar, 2008)). The H2A.Z functions are influenced by post-translational modifications in particular

Canonical histone	Histone variant	Localisation and function	References
H2A	H2A.Z	<ul style="list-style-type: none"> - associated with both transcriptionally active and inactive chromatin; - prevents the spreading of silence heterochromatin; - implicates in transcription activation 	(Altaf et al., 2009; Zlatanova and Thakar, 2008).
	H2A.X	<ul style="list-style-type: none"> - phosphorylated upon DNA damage - recruits DNA repair machinery 	(Talbert and Henikoff, 2010; van Attikum and Gasser, 2009).
	H2A.Bbd	<ul style="list-style-type: none"> - associated with transcriptionally active chromatin; - excluded from the inactivated X-chromosome; - reduces nucleosome stability 	(Chadwick and Willard, 2001; Gautier et al., 2004).
	MacroH2A	<ul style="list-style-type: none"> - associated with inactive X-chromosomes - contains non-histone enzymatic active macrodomain 	(Ladurner, 2003; Mietton et al., 2009).
H2B	H2BFWT	<ul style="list-style-type: none"> - associated with telomeric sequence - induces nucleosome instability when associated with somatic-type histone 	(Boulard et al., 2006; Gaucher et al., 2010).
	hTSH2B	<ul style="list-style-type: none"> - enriched in genes for spermatogenesis - induces nucleosome instability when associated with somatic-type histone 	(Hammoud et al., 2009)
H3	H3.3	<ul style="list-style-type: none"> - associated with transcriptionally active chromatin - derepression of genes 	(Ahmad and Henikoff, 2002)
	H3.4	<ul style="list-style-type: none"> - found in primary spermatocytes 	(Witt et al., 1996)
	H3.5	<ul style="list-style-type: none"> - expressed in the seminiferous tubules of human testes - preferentially associated with euchromatin - can replace an essential function of H3.3 in cell growth 	(Schenk et al., 2011).
	CENP-A	<ul style="list-style-type: none"> - localised in centromeric chromatin - essential for assembly and preservation of kinetochores 	(Malik and Henikoff, 2003; Santaguida and Musacchio, 2009).

Table 1.3 The major histone variants.

acetylation (Bruce et al., 2005). In chicken cells nucleosome core particles containing non-acetylated H2A.Z are more stable and in compact conformation than those containing acetylated histone, suggesting that acetylation of H2A.Z is essential for efficient chromatin relaxation (Ishibashi et al., 2009). The H2A.X variant of histone H2A has a similar structure to canonical H2A except for the presence on the C-terminus consensus S-Q-(D/E)-Ø motif, where Ø represents a hydrophobic residue. The Ser139 residue in the consensus motif is rapidly phosphorylated by ATM, ATR and DNA-PK in response to DSBs DNA damage (reviewed in (Henikoff and Ahmad, 2005; Talbert and Henikoff, 2010)). H2A.X phosphorylated at Ser139 is also known as γ H2A.X and this phosphorylation results in the recruitment of DNA repair factors such as those that facilitate non-homologous end joining (NHEJ) or homologous recombination (HR) repair pathways (Morrison and Shen, 2009; van Attikum and Gasser, 2009). Additionally, SQEY motif of mammalian H2A.X is phosphorylated at Tyr142 by the WSTF (Williams–Beuren syndrome transcription factor) and it is pivotal for a DNA damage response. Phosphorylation of Tyr142 may facilitate changes in chromatin structure, thus promoting maintenance of γ -H2A.X. However, the relation between dephosphorylation of Try142 and phosphorylation of Ser139 still remain unclear (Xiao et al., 2009). It has been demonstrated that direct interaction between phosphorylated Ser139 and Mdc1 facilitates accumulation of DNA repair proteins on the sites of damage such as 53BP1, Nsb1, ATM and prevents γ H2A.X dephosphorylation. On the contrary, phosphorylation of Tyr142 masks binding sites of the γ H2A.X, thus prevents Mdc1 binding (Stucki et al., 2005). This inhibitory activity of phosphorylated Tyr142 is abolished by the dephosphorylation of Tyr142 by the EYA1/3 phosphatase upon DNA damage, thus generating and maintaining of γ -H2A.X (reviewed in (Talbert and Henikoff, 2010; Xiao et al., 2009). Beside the role in DNA damage response, the H2A.X variant is also involved in remodelling and inactivation of male mouse sex chromosomes in meiosis (Talbert and Henikoff, 2010).

MacroH2A is another characterised variant of H2A histone that contains an additional non-histone globular ‘macrodomain’ at the C-terminus (Talbert and Henikoff, 2010). This domain plays an important role in nucleosome assembly *in vitro*. Moreover, the macroH2A preferentially pairs with a canonical histone H2A and increases octamer stability (Chakravarthy and Luger, 2006). The

macroH2A variant is found in the inactive regions of mammalian X chromosome and it is required for silencing of the heterochromatin (Chakravarthy and Luger, 2006).

On the contrary, another vertebrate-specific variant of H2A histone, the H2A Barr body-deficient (H2A.Bbd) contains a short C-terminal truncation of the docking domain (Talbert and Henikoff, 2010). The H2A.Bbd shows association with active chromatin and it is excluded from inactive regions of X chromosomes in fibroblasts (Chadwick and Willard, 2001). Study in mammalian cells revealed that H2A.Bbd is exchanged in nucleosome faster than its canonical histone H2A (Gautier et al., 2004). Additionally, the H2A-H2B dimers are preferentially replaced for H2A.Bbd-H2B by nucleosome assembly protein 1-like 1 (NAP1L1) in reconstituted nucleosomes *in vitro* (Okuwaki et al., 2005). Additionally, reconstituted H2A.Bbd nucleosomes are unstable in the absence of DNA and do not undergo SWI/SNF remodelling (Talbert and Henikoff, 2010).

The hTSH2B and H2BFWT are the two-testes-specific variants of the histone H2B (Talbert and Henikoff, 2010). The hTSH2B is enriched in genes for spermatogenesis and induces nucleosome instability when associated with somatic-type histone (Hammoud et al., 2009), whereas H2BFWT, is associated with telomere binding complex in human sperm (Dalmaso et al., 2011). The hTSH2B contains N-terminal domain of S/T amino acids that undergoes phosphorylation. In chicken cells the reconstituted histone octamer containing the hTSH2B variant showed reduced stability compared to nucleosome containing the H2B counterpart, but presence of hTSH2B does not affect nucleosome mobility or nucleosomal DNA topology (Li et al., 2005).

CENP-A was the first homologue of histone H3 that was found to co-purify with nucleosome core particles (Palmer et al., 1987). CENP-A localisation at kinetochore provides structure for spindle microtubules attachment during mitosis and meiosis (Henikoff and Ahmad, 2005). It is an epigenetic marker for centromeric chromatin that mediates proper chromosome segregation (Malik and Henikoff, 2003). CENP-A incorporation into nucleosomes occurs during late telophase to G₁ phase independently of DNA synthesis (Jansen et al., 2007). Several histone chaperones play a role in CENP-A deposition such as Retinoblastoma binding accessory protein 48 (RbAp48; subunit of hCAF-1 chaperone), Nucleophosmin and HJURP (Holliday junction recognition protein)

(reviewed in (Ransom et al., 2010)). Purification of pre-deposited CENP-A complexes revealed that CENP-A specifically interacts with Nucleophosmin, RbAp48 and HJURP (Dunleavy et al., 2009). Nevertheless, RNAi studies showed that HJURP and RbAp48, but not nucleophosmin are essential for localisation of CENP-A at centromeres (Dunleavy et al., 2009; Foltz et al., 2009).

The H3.3 variant is expressed throughout the cell cycle (Loyola and Almouzni, 2007) and unlike the canonical histone H3 can undergo assembly onto the nucleosome in a replication-dependent or independent fashion (Talbert and Henikoff, 2010). The H3.3 is highly structurally similar to histone H3; the difference appears only at four amino acids positions 31, 87, 89 and 90. Residues 87 and 90 play an essential role in histone H3.3 nucleosome incorporation through replication-independent mechanism (Dalmaso et al., 2011). Assembly of H3.3 histone to actively transcribed regions is mediated by histone regulator A complex (HIRA) (Lewis et al., 2010), while H3 counterpart requires chromatin-assembly factor 1 (CAF-1) for deposition onto chromatin during DNA synthesis (Dalmaso et al., 2011). Recent studies demonstrated that proteins such as the death domain associated protein (DAXX) and the α -thalassemia X-linked mental retardation protein (ATRX) alone or in complexes co-localise with heterochromatin and facilitate efficient assembly of newly synthesised nucleosomes containing H3.3 variant onto telomeric chromatin (Lewis et al., 2010). Mutation of histone H3.3 Lys27 results in abnormal heterochromatin formation in the mouse embryo (Santenard et al., 2010).

The H3.1 and H3.2 variants are highly conserved and show 99% identity to their canonical counterpart (Loyola and Almouzni, 2007). The H3.1 and H3.2 are only expressed during S phase and are deposited onto chromatin in replication-dependent pathway by CAF-1 chaperone (Tachiwana et al., 2011). In addition, both H3.1 and H3.2 co-localise with heterochromatin and their nucleosomes deposition is mediated by universal histone chaperones, such as nucleosome assembly protein 1 (Nap-1) (Osakabe et al., 2010) and nuclear autoantigenic sperm protein (sNASP) (Loyola and Almouzni, 2007). Post-translational modifications of H3.2 are associated with gene silencing (tri-methylation of H3.2K27), whereas modifications of H3.1 are specifically enriched during both

gene activation (acetylation of H3.1K14) and silencing (di-methylation of H3.1K9) (Hake et al., 2006).

Recent studies demonstrated that the H3.5 variant preferentially co-localised with euchromatin and is specifically expressed in the seminiferous tubules of human testes. Interestingly, RNAi-mediated depletion of H3.3 results in the cell growth retardation and this can be rescued by H3.5 variant, suggesting potential redundant functions of some histone variants (Schenk et al., 2011).

1.10.6 Histone post-translational modifications

The histones tail is subject to post-translational modifications (PTMs) that directly influence chromatin organisation and dynamics. Histone PTMs provide epigenetic marks that are inherited by daughter cells (Yun et al., 2011). The combination of these marks are known as the ‘histone code’ initially proposed by Allis and Turner (Barth and Imhof, 2010). The ‘histone code’ or ‘epigenetic code’ is an hypothesis predicting that post-translational modifications of the histones tail could serve as a signal platform for specific regulatory proteins, thereby modulating chromatin structure and other downstream cellular processes (Fillingham and Greenblatt, 2008; Strahl and Allis, 2000). Many different histone modifications have been identified and these include acetylation, methylation, ubiquitylation, SUMOylation of lysine and arginine residues, phosphorylation of serines and threonines and ADP-ribosylation of glutamate residues (Gelato and Fischle, 2008; Kouzarides, 2007).

1.10.6.1 Acetylation

Acetylation of lysine and arginine residues is one of the most explored histone tail modifications. Acetylation of lysine amino acids neutralises positive charge of lysines or arginines and leads to destabilisation of nucleosomes (Bannister and Kouzarides, 2011). This modification of histones is a dynamic process mediated by two groups of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). There are two classes of HATs: the type-A and B. Type-A HATs acetylate multiple sites on the N-terminal tail of histones and they are usually associated with large multiprotein complexes. The type-B HATs function in cytoplasm and acetylate newly synthesised free histones before

they are transported and assembled onto chromatin (Bannister and Kouzarides, 2011). All type-B HATs are highly conserved and share sequence homology to budding yeast Hat1 (Kuo and Allis, 1998).

Acetylated histones are recognised by proteins containing HATs bromodomains. The large family of histone modification readers include the double-bromodomain containing proteins TAF1, Spt, Ada, Gcn5 acetyltransferase (SAGA) complex (Gcn5 and Spt7), the triple chromodomain containing chromatin-remodelling complex (RSC) or no bromodomain containing proteins such as HP1 α that non-specifically recognises acetylated histones (Yun et al., 2011).

On the contrary, the HDAC enzymes remove acetylated residues from histones after their assembly onto chromatin. This restores the positive charge of lysine residues and stabilises histone-DNA interactions, and therefore higher-order chromatin structure (Bannister and Kouzarides, 2011).

The acetylation of histones plays an important role in assembly and can be found in most of the transcriptionally active regions of chromatin (Sternier and Berger, 2000). Analysis of radioactively labelled histones revealed that acetylation marks are highly dynamic and exchange rapidly with a half-life of a few minutes (Barth and Imhof, 2010; Chestier and Yaniv, 1979).

Acetylation of the H4K5 and H4K12 by the two-subunit HAT1-RbAp46 enzyme is a characteristic mark present only on newly synthesised histone H4 (Barman et al., 2006). Both marks are important for deposition of newly synthesised histone H4 onto chromatin by CAF-1 (Zhang et al., 2002a). The H4K5 and H4K12 deacetylation occurs 20-60 minutes after assembly of histone H4 onto chromatin (Taddei et al., 1999). Acetylation of H4K16 increases chromatin accessibility and plays a role in transcriptional activation and euchromatin maintenance (Shogren-Knaak et al., 2006). Similarly, acetylation of histone H3 at Lys9 and Lys16 serves as a marker for newly synthesised histone H3 and these are often associated with euchromatin regions (Gelato and Fischle, 2008).

Acetylation of newly synthesised histone H3K56 facilitates its deposition onto newly replicated chromatin and plays a direct role in transcriptional activation and chromatin reassembly following the DNA damage (Chen et al., 2008; Costelloe and Lowndes, 2010; Williams et al., 2008). Acetylated H3K56

decrease binding affinity of the H3-H4 tetramer to DNA, and thus increases DNA unwrapping in budding yeast (Williams et al., 2008). After DNA damage, H3K56 acetylation facilitates nucleosome assembly by stimulation of H3-H4 tetramer chaperones (reviewed in (Zhu and Wani, 2010)). Recent findings suggested that acetylation of Lys56 is induced by DNA damaging agents such as IR, UV, HU or MMS and co-localises with γ H2AX foci after IR in human. Additionally, assembly of acetylated H3K56 at the sites of DNA repair is mediated by CAF-1 and Asf-1 chaperones (Das et al., 2009). Similar studies published by Tjeertes and colleagues demonstrated opposite effect for acetylated H3K56 that was rapidly reduced in response to DNA damage (Tjeertes et al., 2009).

1.10.6.2 Methylation

Methylation is another post-translational modification of histones (Martin and Zhang, 2005). Methylation occurs on the side chain of lysines and arginines. Additionally, modification of lysine comes in three different flavours: mono- di- and tri-methylation (Barth and Imhof, 2010). This covalent modification is the most stable histone modification known and is mediated by histone lysine methyltransferases (HKMTase) such as Su(var)3-9H1 (Suppressor of position effect variation) (Bannister and Kouzarides, 2011; Yun et al., 2011), ASH1L (Absent, small or homeotic disc 1 like) (An et al., 2011), Set1 and Dot1L (Disruptor of telomeric silencing 1 like protein) (Nguyen and Zhang, 2011). Many histone lysine methyltransferases contain highly conserved SET chromodomain consisting of two flanking cysteine-rich regions (Zhang and Reinberg, 2001). On the contrary, Dot1L does not contain SET chromodomain, but instead possesses SAM motif (S-adenosyl-L-methionine) (Min et al., 2003). Histone H3 lysine and arginine residues are methylated by Su(var)3-9H1 and nuclear receptor CARM1 (co-activator associated arginine methyltransferase 1), respectively (Zhang and Reinberg, 2001). PRMT1 (protein arginine N-methyltransferase 1) is a specific histone H4 arginine methyltransferase *in vitro* and *in vivo* (Wang et al., 2001; Zhang and Reinberg, 2001).

Histones methylation is associated with different regions of chromatin. Tri-methylation of histone H3K4 and H3K36 mark transcriptionally active euchromatin and are required for transcriptional initiation and elongation,

respectively (Maze and Nestler, 2011). This is opposite to mono-methylation of H3K27, tri-methylation of H3K9 and H4K20 that appear to localise largely to heterochromatin (Gelato and Fischle, 2008). The H3K9 di-methylation, H3K27 tri-methylation, H4K20 mono-methylation, H3K4 di-methylation are major marks of the inactive X chromosome (Brinkman et al., 2006). Different localisation of individual histone modification marks reflects various functions. Mono-methylation of histone H4K20 is essential for cell-cycle regulation, whereas di-methylation of H4K20 controls DNA damage checkpoints (Wang and Jia, 2009). Furthermore, tri-methylation of H3K9 stimulates binding to HP1 α protein, thereby influences formation of heterochromatin and chromatin compaction (Grewal and Moazed, 2003). All methylation marks are relatively stable, showing a half-life between 0.4-3 days. Additionally, the turnover rate for active modifications such as mono-methylation of H3K4 and H3K36 is higher than for repressive marks such as di-methylation of H3K9 or H3K27 and tri-methylation of H3K9 or H3K27 (Barth and Imhof, 2010).

1.10.6.3 Phosphorylation

Phosphorylation on serine, threonine and tyrosine amino acids mainly associated with transcriptional activation (Barth and Imhof, 2010; Maze and Nestler, 2011). The half-life of histone phosphorylation varies between 30 minutes to 2 hours (Barth and Imhof, 2010). Originally, the phosphorylation marks of histone were associated with chromosome assembly and segregation during mitosis (Johansen and Johansen, 2006). The best characterised phosphorylation site of histone H3 is Ser10 (Maze and Nestler, 2011). The H3S10 phosphorylation promotes subsequent acetylation on Lys9 and Lys16 of histone H3 by the recruitment of associated HATs enzymes (Barth and Imhof, 2010; Maze and Nestler, 2011). This phosphorylation event is facilitated by Aurora B kinase, and it is a characteristic hallmark for chromosome condensation and mitotic progression (Adams et al., 2001; Hirota et al., 2005).

1.10.7 3D organisation of nuclear structure and functions

Different sub-nuclear structures present within the nucleus such as the nuclear membrane, nuclear bodies and chromosome territories (CTs) determine

the three-dimensional organisation of the human genome (Lenser et al., 2010). Genome architecture influences different processes such as gene expression, DNA replication and DNA repair (Cremer et al., 2006). The three-dimensional organisation of the nucleus was first suggested by Carl Rabl and Theodori Boveri (Boveri, 1909). Using light microscopy, they observed that interphase chromosomes are divided into distinct territories. Further studies of nucleus structure using electron microscopy revealed that except mitosis chromosomes are attached to nuclear membrane and arranged into non-overlapping territories (Comings, 1968). However, development of a FISH approach (fluorescence *in situ* hybridization) in a combination with confocal laser scanning microscopy allows direct visualisation of individual chromosome domains (Langer-Safer et al., 1982). Additionally, chromosome conformation capture (3C) technique identified chromatin interactions between two distinct genomic loci located at the same or different chromosomes by PCR-based analysis of cross-lined DNA fragments (Dekker et al., 2002).

Positioning pattern seems to be conserved through evolution indicating functional role of genome self-organisation (Takizawa et al., 2008). FISH analysis revealed that chromosome territories were placed into the nucleus in specific positions. Analysis of human lymphocyte chromosomes 18 and 19 demonstrated their radial position in the human nucleus. Chromosome 18 composed of GC-poor regions showed peripheral localisation, while chromosome 19 containing a high gene-density was associated with interior of the nucleus (Croft et al., 1999). Moreover, further studies of chromosome morphology confirmed that transcriptionally active euchromatin and gene-dense regions are located in the centre of the nucleus, while gene-poor segments and transcriptionally silenced heterochromatin are located at the nuclear periphery (Gilbert, 2001a). Studies of the *β-globin* gene in differentiating mouse erythroid cells (Kosak et al., 2002) and analysis of *GATA-3* and *c-maf* genes during murine T lymphocytes development by FISH demonstrated that inactive genes are located at the nuclear periphery and upon activation these genes relocate toward to the nuclei centre (Hewitt et al., 2004). The work of Osborne and colleagues on mouse chromosome 7 using combination of FISH and 3C analyses provided evidence for coalescence of active genes into functional transcription factories (Osborne et al., 2004). Additionally, analysis of tRNA genes in yeast using

fluorescent oligonucleotide probes indicated that rDNA and tRNA genes located at different chromosomes co-localise to form nucleolar clusters (Thompson et al., 2003). Consistent with these data, immuno-DNA FISH analysis confirmed that rDNA genes in human cells are located in nucleolar organiser regions (NORs) (Dimitrova, 2011). It has been also found that the size of chromosome affects its position in the nucleus. The FISH analysis of q-arm telomeres of human chromosomes revealed that small chromosomes are located centrally in the nuclei, while large chromosomes tend to localise to the nucleus periphery (Sun et al., 2000). Recent improvement of the 3C technique allowed chromosome conformation capture-on-chip (4C) (Simonis et al., 2006) and Hi-C methods (Lieberman-Aiden et al., 2009) to study nuclear organisation in high-throughput manner.

4C technique was used to identify specific chromosome segments that interact with the mouse *β-globin* gene (Simonis et al., 2006). This result emphasises clusters of interactions that were found in a *cis* position on chromosome 7 and located a few megabases away from the *β-globin* locus. Comparison between transcriptionally active fetal liver cells and fetal brain cells that do not express the *β-globin* gene revealed different interaction clusters. In fetal liver, 80% of the interacting DNA segments contained one or more active genes and were located toward the telomere of the chromosome. On the contrary, 74% of the *β-globin* interacting genes in brain cells were localised toward the centromere of chromosome and were transcriptionally silenced. These observations suggested cell-to-cell variability in chromosome organisation and that features of the neighbouring segments may facilitate gene-gene interactions (Simonis et al., 2006).

Most recently developed Hi-C approaches combine proximity-based ligation with massively parallel sequencing and allows for mapping of chromatin interactions across the human genome at a resolution of 1Mb (Lieberman-Aiden et al., 2009). Maps generated using Hi-C were consistent with previous data that gene-rich chromosomes are located at the centre of the nuclei and preferentially interact with each other (Boyle et al., 2001). Additionally, it has been shown that open and closed conformation of the chromatin associate with different spatial compartments in the nucleus (Lieberman-Aiden et al., 2009). Recently, Rajapakse and colleagues used computational approaches to define dynamic

correlation between gene expression and the spatial organisation of chromosomes during *in vitro* differentiation of mouse hematopoietic progenitors (Rajapakse et al., 2009). Analysis of rosette-based chromosomal association matrices showed that chromosome organisation increases during differentiation, opposite to the ordering of gene expression. Therefore, the position of the chromosome is coordinated by gene expression activity.

Electron microscopy and FISH methods provided understanding of genome three-dimensional organisation by characterisation of different genome territories. However, these approaches suffer from various limitations. Electron microscopy allows high resolution but it is laborious and partially destroys 3D structure. Moreover, it cannot be applied to study specific genome loci. On the contrary, light microscopy preserves 3D structure, but due to limited resolution is not suitable to define chromosome conformation at the molecular level (Dekker et al., 2002). A FISH approach visualises only a certain number of DNA loci but involves harsh treatment during sample preparation that may affect chromosome organisation. Additionally, microscopy-based assays do not allow for unbiased analysis of the genome segments that interact with a particular locus (Fraser and Bickmore, 2007).

Development of chromosome conformation capture method and its variants 4C and Hi-C approaches allow for screening of an entire genome and identification of different DNA sequences interacting with each other (Simonis et al., 2006). All 3C-based methods provide only average insight into properties of the chromosome conformation in the cell population (Marti-Renom and Mirny, 2011). Additionally, 3C technique has a very limited throughput and sequence-specific primers have to be used to identify analysed gene. This method cannot be applied to characterise unknown sequences that interact with a locus of interest (Lanctot et al., 2007). To overcome this limitation 4C method based on re-circulisation of the template permits identification of different DNA sequences interacting with specific locus by inverse PCR and high-throughput sequencing (Marti-Renom and Mirny, 2011). Nevertheless, identification of interacting loci using both methods is still limited as only one input sequence can be analysed at the time. An additional Hi-C technology has been successfully used to characterise chromatin interactions across whole genome at 1 Mb resolution.

However, different challenges involving computational data analysis such as high background noise or the difficulties of increasing resolution need to be still improved.

In conclusion, all techniques described in this section are powerful tools that will further allow understanding the relationship between chromosome organisation and genome activity during interphase. Moreover, detailed three-dimensional map of the chromosomes conformation and characterisation of specific chromatin modification proteins and transcription factors facilitating interactions between different loci would provide a better understanding of the genome organisation.

1.11 Chromatin assembly

1.11.1 Dynamics of chromatin assembly *in vivo*

The dynamic nature of the chromatin is a hallmark of chromatin organisation (reviewed in (Kumaran Ileng et al., 2008)). The assembly of the nucleosome occurs by sequential deposition of a histone H3-H4 tetramer and two H2A-H2B heterodimers onto double-stranded DNA. Most of the histones H3 and H4 are incorporated into chromatin during DNA synthesis and stay bound to it, whereas more rapid exchange of histone H2A and H2B have been observed (Kimura and Cook, 2001). The fluorescence recovery after photobleaching (FRAP) experiments in living cells, that utilised histone GFP-H1 demonstrated that the histone H1 linker binds to chromatin with the residence time between 220-250 seconds. It was also observed that it is continuously exchanged in both euchromatin and heterochromatin regions (Misteli et al., 2000). New histones H3-H4 are deposited only onto newly replicated chromatin, whereas new histones H2A-H2B and linker histone H1 are believed to associate with newly and pre-existing chromatin (reviewed in (Groth, 2009)).

1.11.2 Replication-dependent chromatin assembly and maturation

Chromatin assembly is tightly associated with DNA synthesis (Clemente-Ruiz and Prado, 2009). Progression of the replication fork requires disruption of

the parental nucleosome ahead of the replication forks and reassembly of two new nucleosomes after its passage, thereby leading to restoration of proper chromatin conformation (Clemente-Ruiz and Prado, 2009; Groth et al., 2007b). Nucleosome disassembly is facilitated by the movement of replication fork machinery and occurs at one or two nucleosomes ahead of the replication forks (reviewed in (Groth, 2009)). In addition, co-immunoprecipitation and RNAi studies in human cells indicated that the H2A-H2B dimer and H3-H4 tetramer are displaced ahead of the replication fork by histone chaperones FACT and Asf-1 (Groth et al., 2007a; Tan et al., 2006). Electron micrographs of replicating SV40 minichromosomes treated with psoralen showed destabilisation of the nucleosome that leads to formation of approximately 300 bp naked DNA ahead of the forks (Gasser et al., 1996). H2A-H2B dimers and H3-H4 tetramers can be further assembled onto newly synthesised DNA (Figure 1.13) (Groth et al., 2007b). As the excess of histones is toxic for the cells, monitoring of histones supply and their deposition onto newly replicated DNA is an important cellular mechanism. Both pre-existing and *de novo* synthesised histones are distributed between new nucleosome binding sites (Polo and Almouzni, 2006). Different approaches including cell-free models, RNAi-mediated knockdowns, co-immunoprecipitation and reconstitution of nucleosome assembly machinery confirmed an essential role of histone chaperones in histone deposition (Groth et al., 2007a; Ito et al., 1996). Newly synthesised histones H3-H4 dimers are bound by histone chaperone Asf-1, thus preventing tetramer formation. Asf-1 facilitates distribution of *de novo* synthesised H3-H4 dimer, thereby assists in CAF-1 and HIRA-mediated histone assembly (reviewed in (Groth, 2009); (Groth et al., 2007a)). In similar fashion, other histone chaperones, such as Nap-1 meditates *de novo* nucleosome assembly by transporting of newly synthesised histone H2A-H2B dimers into the nucleus and their deposition onto DNA. This was clearly demonstrated by nucleosome assembly on short mononucleosome-sized DNA fragments or x-ray crystallography of budding yeast Nap-1 (Mazurkiewicz et al., 2006; Park and Luger, 2006b). Additionally, FRET (Fluorescence resonance energy transfer) analysis of nucleosome assembly thermodynamics indicated that histone chaperone Nap-1 destabilises non-nucleosome DNA-histone interactions, thus facilitating assembly of the nucleosomes *in vitro* (Andrews et al., 2010). Moreover, studies of salt-dependent assembly and disassembly of the DNA-

labelled nucleosomes by single-pair FRET experiments, revealed stepwise mechanism of nucleosome disassembly (Gansen et al., 2009). These findings demonstrate that H2A-H2B dimer is displaced from the nucleosome before H3-H4 tetramer. Additionally, dissociation of H2A-H2B dimer from the nucleosome is preceded by opening of the H2A-H2B dimer and H3-H4 tetramer interface. Interestingly, nucleosome assembly follows the exact same pathway, but in a reverse direction (Böhm et al., 2011).

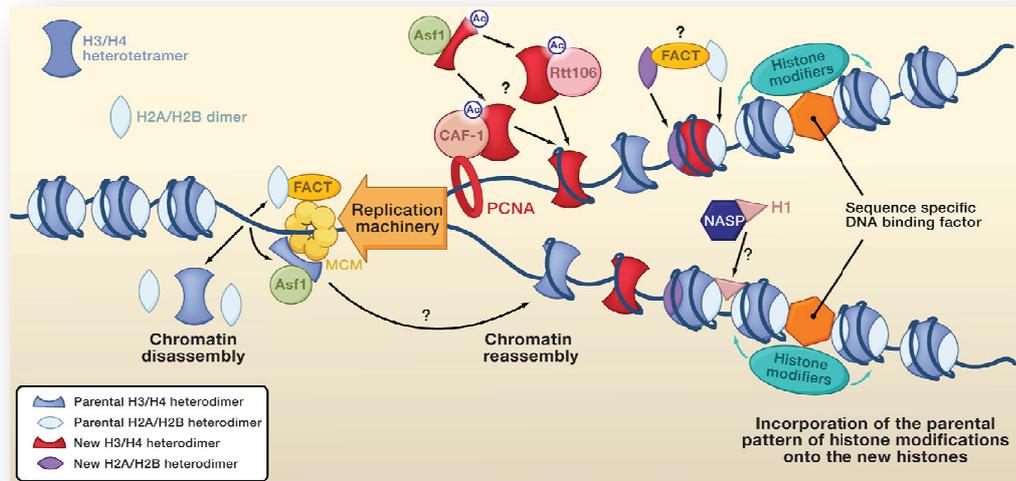


Figure 1.13 Nucleosome disruption and assembly. Replication machinery and histone chaperones cooperate to disrupt pre-existing nucleosomes in front of the replication forks and assembly of new nucleosomes after the passage of replication forks. Picture taken from (Ransom et al., 2010).

Newly assembled nucleosomes contain a 1:1 ratio of histones recycled from the parental strands and *de novo* synthesised. Assembled H3-H4 tetramer is stably associated with nucleosome, while H2A-H2B dimers can be exchanged with free histones H2A and H2B (reviewed in (Ransom et al., 2010)). In addition, *in vitro* analysis of radio- and density-labelled histones revealed that exchange of histones H3 and H4 occurs slower than histones H2A and H2B (Jackson, 1990). The same approach was used to illustrate that newly assembled H3-H4 heterotetramer consists of parental or *de novo* synthesised H3-H4 dimers, but not a mixture of both in the same nucleosome. On the contrary, newly deposited H2A-H2B heterodimer can contain a mixture of new and parental H2A-H2B dimers (Figure 1.13) (Jackson, 1987, 1988; Jackson, 1990). Furthermore, histones acquire a specific pattern of post-translational

modifications that are passed to newly synthesised histones to facilitate chaperone recognition and nucleosome assembly (Barth and Imhof, 2010) (see section 1.10.6). Using S150 chromatin extract in *Drosophila* embryos, Scharf and colleagues demonstrated that mono-methylation of histone H4 at Lys20 is required for efficient deacetylation of histone H4, thus for nucleosome assembly and chromatin maturation (Scharf et al., 2009b). Nucleosome assembly begins rapidly as the replication fork machinery moves leaving behind 250 bp of naked DNA that can immediately wrap around the histone core (Ransom et al., 2010; Sogo et al., 1986).

First, acetylation of H4-H3 tetramer (H4K5/12 and H3K9/16/56; see section 1.10.6.1) mediates CAF-1/Rtt106 assembly and deposition of H3-H4 tetramer onto newly replicated DNA (Gelato and Fischle, 2008; Zhang et al., 2002a). This is followed by two H2A-H2B dimers binding to the complex (reviewed in (Groth, 2009)). The acetylation marks are essential for nucleosome formation, but not for chromatin maturation, thereby deacetylation step occurs 20-60 minutes after assembly to stabilise nucleosome structure (Taddei et al., 1999; Worcel et al., 1978). Recent study of histone modifications dynamics, by selective isotopic labelling of newly synthesised histones (pSILAC), followed by mass spectrometry confirmed that deacetylation of newly synthesised histones occurs within 2 hours after histone deposition in human cells (Scharf et al., 2009a). Lastly, H1 histone linker has to be assembled onto chromatin to facilitate chromatin maturation. Native gel electrophoresis revealed that NASP (nuclear autoantigenic sperm protein) chaperone specifically interacts with histone H1 (Finn et al., 2008). Moreover, nucleosome array experiments determined that NASP mediates binding of H1 histone linker within 30-40 seconds after passage of the replication machinery, thereby facilitating maturation of newly assembled chromatin (Bavykin et al., 1993; Finn et al., 2008).

Genome duplication involves not only synthesis of a new copy of DNA but also maintaining of epigenetic information that facilitates chromatin assembly. These processes are critical for genome stability and for maintaining of cell-type identity (Margueron and Reinberg, 2010). Various approaches such as ChIP-chip and ChIP-seq have been extensively used to identify the distribution of a given DNA interacting protein across the genome (see section 12.1). However, these methods suffer from several limitations such as low resolution and sensitivity.

Recently developed fluorescence techniques such as FRET and its combination with single molecule studies (spFRET) were successfully used to determine dynamics of nucleosome assembly (Andrews et al., 2010; Böhm et al., 2011; Buning and van Noort, 2010). However, the main limitations of the FRET and the spFRET methods are an unspecific incorporation of fluorescent dyes and sensitivity. FRET is limited by incomplete labelling and the time resolution. Magnetic tweezers is another single-molecule technique that allows investigation of nucleosome assembly. Recently, Gupta and colleagues illustrated that chromatin assembly is inhibited by positive superhelical tension in the DNA (Gupta et al., 2009). However, magnetic tweezers in comparison with other single-molecule techniques suffers from manipulation settings. Additionally, the large size of the connected bead can change biological properties of any analysed DNA molecule. Moreover, unspecific binding between DNA and glass surface and time resolution of this approach is limited by so called the grabbing rate.

Mechanism of chromatin reassembly, maturation and duplication of epigenetic marks is still poorly understood due to the lack of techniques that allow recovery of proteins that are associated with newly synthesised DNA. Dm-ChP approach provides a powerful tool to study chromatin assembly and maturation as small fragments of nascent chromatin can be efficiently recovered and proteins associated with newly synthesised or matured DNA can be analysed by both immunoblotting and mass spectrometry. Specificity and flexibility of Dm-ChP, together with the possibility of manipulating the cellular systems, labelling times and conditions of extract preparation will allow investigation of how chromatin remodelling and maturation are linked to the duplication of its basic constituent the DNA.

1.11.2.1 Role of chaperones in nucleosome assembly

Chaperones associate with histones and promote their interactions with DNA and other proteins (Loyola and Almouzni, 2004). They facilitate the nucleosome formation, but are not part of the nucleosome itself (Ransom et al., 2010). Different classes of histone chaperones have been identified by their ability to bind either H3-H4 tetramer or H2A-H2B dimer (Loyola and Almouzni, 2004). The H3-H4 tetramer is recognised by Asf-1 (Anti-silencing function 1), CAF-1 (Chromatin assembly factor 1), HIRA (histone regulatory homologue A)

and Spt6 (Suppressor of Ty 6) (Moshkin et al., 2009; Ransom et al., 2010), while H2A-H2B dimer can be bound by histone chaperones including Nap-1 (Nucleosome assembly protein-1), Nucleolin and Nucleophosmin (De Koning et al., 2007; Loyola and Almouzni, 2004). Both H3-H4 tetramer and H2A-H2B dimer are recognised by FACT complex (Facilitates chromatin transcription) (Das et al., 2010).

Anti-silencing function 1 complex (Asf-1) is highly conserved and one of the major histone H3-H4 chaperones (Moshkin et al., 2009). Asf-1 possesses three helical linkers on the top of the core β sandwich domain with highly conserved acidic patches that mediates interaction with histones (Bao and Shen, 2006). Asf-1 is implicated in chromatin assembly in replication-dependent manner through interaction with CAF-1 and Mcm2-7 helicase (Groth et al., 2007a). Additionally, Asf-1 can act in replication-independent pathway through interaction with HIRA complex (Tagami et al., 2004). Moreover, Asf-1 mediates chromatin assembly and disassembly following DNA repair by binding to the histone acetyltransferase Rtt109 (Chen et al., 2008; English et al., 2006). In budding yeast, Asf-1 directly interacts with DNA damage checkpoint kinase Rad53, suggesting that Asf-1 activity may be important for recovery after DNA damage (Emili et al., 2001).

Nucleosome assembly protein 1 (Nap-1) is a well conserved histone chaperone that interacts with H2A-H2B dimers, but can also bind with similar affinity all histones *in vivo* (Andrews et al., 2008; Krogan et al., 2006). Microscopy studies in mammalian cells demonstrated that localisation of Nap-1 protein is cell cycle-dependent. Nap-1 is predominantly present in cytoplasm throughout the cell cycle and only in S phase localises in to the nucleus (Marheineke and Krude, 1998). Nap-1 mediates *de novo* nucleosome assembly by transporting newly synthesised histone H2A-H2B dimers into the nucleus and their deposition onto the DNA (Mazurkiewicz et al., 2006; Park and Luger, 2006b). Similarly to Asf-1, Nap-1 mediates chromatin assembly by both replication-dependent and independent pathways (Park et al., 2005). Studies in budding yeast demonstrated that Nap-1 is a component of Swr1 complex that specifically exchanges H2A.Z variant (Mizuguchi et al., 2004). Furthermore, Nap-1 catalyses active histone exchange *in vitro* by the reversible removal and

replacement of H2A-H2B dimers with canonical histones or its variants (Park et al., 2005), thereby promoting nucleosome sliding (Park and Luger, 2006b).

Facilitates chromatin transcription complex (FACT) was first identified in budding yeast as an important factor for transcriptional elongation (Orphanides et al., 1998; Winkler and Luger, 2011). Further studies connected this chaperone to nucleosome re-organisation during DNA synthesis and DNA repair (Winkler and Luger, 2011). FACT complex contains two subunits: SSRP-1 (Structure specific recognition protein 1) and hSpt16 (human Suppressor of Ty 16) (Abe et al., 2011). FACT forms a stable complex with H3-H4 tetramers as well as H2A-H2B dimers, thereby promoting their assembly and disassembly onto DNA (Tan et al., 2006; Winkler and Luger, 2011). Releasing of the H2A-H2B dimer and nucleosome destabilisation by FACT complex mediates passage of the RNA polymerase II and allows gene transcription (Birch et al., 2009; Winkler and Luger, 2011). Recently, *Drosophila melanogaster* HP1 γ has been shown to mediate interaction between FACT and RNA polymerase II *in vitro*. Additionally, interaction with HP1 γ leads to recruitment of FACT complex to euchromatin and supports transcriptional activation of euchromatin (Kwon and Workman, 2011). FACT complex is involved in the loading of histone H3.3 variant to pre-existing nucleosomes through association with other histone chaperone HIRA (Nakayama et al., 2007). Additionally, recruitment of CHD1 by FACT complex permits CENP-A assembly onto centromeric chromatin (Okada et al., 2009). In DNA synthesis, FACT was identified as a component of the RPC complex (Gambus et al., 2006). Direct interactions with replication proteins, such as RPA (Fanning et al., 2006), DNA polymerase α (Wittmeyer and Formosa, 1997) and MCM helicase (Tan et al., 2010) indicate that FACT is an important factor for DNA synthesis (Tan et al., 2006). Depletion of FACT subunit SSRP-1 in chicken cells inhibits progression of replication forks and reduces the length of newly replicated DNA tracks, suggesting that FACT complex is involved in the elongation stage of DNA synthesis (Abe et al., 2011). Moreover, in response to DNA damage, FACT mediates exchange of H2A.X variants and when in complex with CK2 (Casein kinase 2) promotes phosphorylation of the tumour suppressor protein p53 at Ser392 (Heo et al., 2008; Keller and Lu, 2002).

The histone chaperone Chromatin assembly factor 1 (CAF-1) mediates *de novo* nucleosome assembly during DNA synthesis (Stillman, 1986; Takami et al., 2007; Verreault, 2000). CAF-1 co-localises to replication foci *in vivo* and interacts preferentially with histones H3 and H4 (Krude, 1995). These interactions are essential for proper assembly of the nucleosomes onto newly synthesised DNA (Takami et al., 2007). CAF-1 is a heterotrimeric protein that consists of three subunits p150, p60 and p48 (Kaufman et al., 1995). Subunits p48 and p60 bind histones H3 and H4 through WD40-repeat domain. The p48 subunit has been characterised as a member of the HAT1 and HDAC1 families in chicken cells (Ahmad et al., 1999). Two larger subunits p150 and p60 are essential for nucleosome assembly in mammalian cells (Kaufman et al., 1995). Additionally, p60 promotes interaction with histone chaperone Asf-1 and p150 mediates binding to PCNA (Nabatiyan et al., 2006) and HP1 α (Quivy et al., 2008). Despite the role of CAF-1 in DNA replication, studies in mammalian cells also linked CAF-1 to chromatin assembly following DNA repair (Hoek et al., 2011). This chaperone is involved in nucleotide excision repair (Gaillard et al., 1996), where it deposits histones onto regions where UV-induced DNA damage was repaired (Green and Almouzni, 2003). Moreover, CAF-1 was shown to interact with BLM (Jiao et al., 2004) and WRN and therefore it is considered to play a role in the maintenance of genome stability (Jiao et al., 2007).

1.11.3 Replication-independent chromatin assembly

Assembly of chromatin can occur outside of the S phase, independently of the DNA synthesis (Avvakumov et al., 2011; Lewis et al., 2010). Deposition of histone variants in a replication-independent manner is mediated by histone chaperones Asf-1, CAF-1 and FACT, but also by unique chaperones such as DAXX, ATRX and HIRA (Lewis et al., 2010; Ray-Gallet et al., 2002). Assembly and disassembly of the nucleosome plays an important role during the repair of damaged DNA or transcription (Avvakumov et al., 2011; Lewis et al., 2010). Deposition of nucleosomes after DNA damage repair is referred to as “access-repair-restore” model (Smerdon, 1991). Upon DNA damage, nucleosomes have to be first disassembled to allow repair machinery access to the DNA damage site and subsequently re-establish after repair (reviewed in (Ransom et al., 2010)). It is still unclear if histones are disassembled or just moved away from

damage sites by histone chaperones (reviewed in (Avvakumov et al., 2011)). It has been suggested that histone eviction may involve activities of the chromatin remodelers such as SWI/SNF, INO80 and RSC (reviewed in (Ransom et al., 2010)). During DSBs repair, RSC and INO80 mediate accessibility of DNA ends for the end processing enzymes, thereby facilitate loading of repair proteins such as Mre11, RPA and Rad51 to the damage sites (van Attikum et al., 2007).

After DNA damage repair, histone variant γ H2A.X is removed from the chromatin by Swr1 complex in budding yeast (reviewed in (Rossetto et al., 2010); (Papamichos-Chronakis et al., 2006)). Histone chaperone FACT mediates further exchange of γ H2A.X-H2B dimers with unmodified versions (Heo et al., 2008). To complete chromatin re-establishment, histone chaperones CAF-1 and Asf-1 mediate assembly of newly synthesised H3.1-H4 dimers similar to nucleosome restoration after DNA replication (reviewed in section 1.11.2 and (Avvakumov et al., 2011)). In addition, acetylation at Lys56 of newly synthesised histone H3 plays an important role in the chromatin re-assembly following DNA damage (Chen et al., 2008; Costelloe and Lowndes, 2010; Das et al., 2009; Williams et al., 2008). It has been reported that CAF-1 is tightly regulated during DSBs repair by the RecQ family of DNA helicases such as the BLM and WRN. The WRN helicase recruits CAF-1 to sites of DNA damage (Jiao et al., 2007), whereas BLM inhibits CAF-1-mediated chromatin restore after DNA repair (reviewed in (Ransom et al., 2010)). Chromatin restoring by both Asf-1 and CAF-1 is essential for deactivation of the checkpoint pathway after DNA repair (Kim and Haber, 2009).

Transcription is another process that requires chromatin disruption. Studies in *Xenopus* egg extracts demonstrated that the presence of nucleosomes at DNA affects transcription *in vitro* (Knezetic and Luse, 1986). The transcription initiation requires assembly of the transcription machinery onto promoters, followed by binding of RNA polymerase II (reviewed in (Williams and Tyler, 2007)). Transcription regulation is mediated through chromatin remodelling and post-translational modification of histones (reviewed in (Li et al., 2007)). In budding yeast, nucleosome loss was detected at the PHO5 gene during active gene transcription (Reinke and Horz, 2004). Disassembly of nucleosomes by histone chaperones such as Asf-1, Nap-1, FACT and HIRA is mediated after PTMs of the histone tail (reviewed in (Avvakumov et al., 2011; Li et al., 2007)).

To disrupt existing nucleosome, chromatin remodelers such as Nap-1, RSC or FACT remove H2A-H2B dimers and this is followed by H3-H4 disassembly by Asf-1. During transcription elongation histones can be displaced by both active RNA polymerase II and FACT complex (reviewed in (Williams and Tyler, 2007)). Recruitment of FACT onto chromatin and deposition of H2A-H2B dimer is facilitated by mono-ubiquitination of histone H2B at Lys123 (Pavri et al., 2006). Ubiquitination of H2BK123 is mediated by Paf1 transcriptional elongation complex (Ng et al., 2003). Paf1 is a multiprotein complex composed of four subunits Ctr9, Cdc73, Leo1, and Rtf1 (Rosonina and Manley, 2005) that together with RNA polymerase II (Shi et al., 1997) and FACT promote transcription elongation in budding yeast (Formosa et al., 2002).

Beside DNA repair and transcription, deposition of histone variants such as H3.3 and CENP-A also occurs independent of DNA synthesis. CENP-A is deposited onto centromeres by HJURP and RbAp48, whereas newly synthesised H3.3 is incorporated at telomeric chromatin or actively transcribed genes by DAXX-ATRX complex and HIRA, respectively (Jansen et al., 2007; Lewis et al., 2010).

1.11.3.1 Chromatin remodelling factors

Different factors have been identified to facilitate chromatin dynamics. These factors called remodelers possess high affinity to nucleosomes (Clapier and Cairns, 2009). Remodelers have conserved ATP binding/hydrolysis domains and utilise energy from ATP breakdown to fulfil their specific biological roles (Khorasanizadeh, 2004) (Figure 1.14). Chromatin remodelers are classified based on their sequence and structure of the ATPase domain. Four families: SWI/SNF, INO80, CHD and ISWI have been distinguished (Ho and Crabtree, 2010).

The SWI/SNF class of remodelers was first identified in budding yeast during screening for genes influencing the mating-type switching (SWI) and sucrose fermentation (SNF) (Carlson et al., 1981; Halliday et al., 2009). Beside the catalytic ATPase domain essential for remodelling and translocation of DNA, SWI/SNF family possesses C-terminal bromodomain composed of four helices. Bromodomain binds to acetylated histones but also to helicase-SANT (HAS) subunit. HAS is a part of N-terminal SNF domain involved in actin and actin-related proteins interactions (Figure 1.14) (Halliday et al., 2009; Tang et al.,

2010). The SWI/SNF remodelers bind to nucleosome with 1:1 stoichiometry (Hargreaves and Crabtree, 2011) and may exchange histone H2A.Z variant (Mizuguchi et al., 2004), but does not assist in global chromatin assembly, suggesting a local nucleosome processing by these remodelers (Hargreaves and Crabtree, 2011). Furthermore, SWI/SNF remodelling activity is dependent on histone linker H1.

The ISWI (Imitation Switch) is the second family of ATP-dependent remodelers. It was first purified from *Drosophila* embryo extracts (Tsukiyama et al., 1995). The ISWI family can be divided into three classes: NURF (Nucleosome remodelling factor), ACF (ATP-dependent chromatin-assembly factor) and CHRAC (Chromatin accessibility complex) (reviewed in (Dirscherl and Krebs, 2004)). NURF assists in both transcription by RNA polymerase I/II and transcriptional repression (Morillon et al., 2003; Zhou et al., 2002). The ACF and CHRAC complexes facilitate the heterochromatin formation and regulation of the nucleosome assembly/spacing (Dirscherl and Krebs, 2004). All members of the ISWI family contain a core catalytic ATPase domain and the unique SLIDE and SANT domains at the C-terminus (Figure 1.14) (Clapier and Cairns, 2009). The SANT domain appears to be involved in transcriptional regulation, while the SANT-related SLIDE motif plays a role in nucleosome recognition (Boyer et al., 2002).

The Chromodomain helicase DNA-binding (CDH) family is a third class of ATP-dependent chromatin-remodelling complexes. CDH contains a characteristic chromodomain tandem at the N-terminus (Marfella and Imbalzano, 2007) (Figure 1.14). There are three subfamilies of CHD, classified according to their similarities in domains structure (Hargreaves and Crabtree, 2011). The first subfamily contains CDH1 and CDH2 proteins that possess C-terminal DNA binding motif (Stokes and Perry, 1995). CHD3 and CHD4 represent a second subfamily that includes the N-terminal PHD zinc-finger-like domain but lack of the DNA binding domain (Woodage et al., 1997). The third subfamily includes the proteins of CHD5-9. This subfamily has additional domains at their C-terminus, such as BRK (Brahma and Kismet), a SANT-like and CR domains (Clapier and Cairns, 2009; Marfella and Imbalzano, 2007). The tandem of chromodomain in CHD family mediates recognition of the modified histone H3 (tri-methylation of H3K4). Additionally, CHD1 participates in histone H3.3

deposition onto chromatin *in vivo* (Konev et al., 2007). CHD3 and CHD4 are part of the large protein complex NURD (nucleosome-remodelling and histone deacetylase) that mediates access to acetylated histone through chromatin remodelling (Xue et al., 1998).

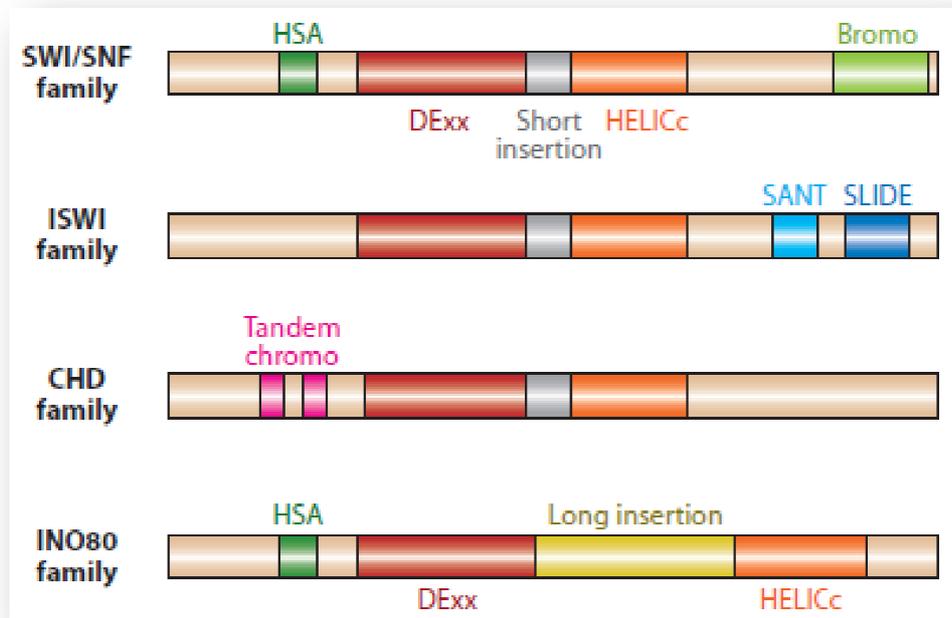


Figure 1.14 Chromatin remodelers. All families contain DExx and HELICc ATPase domains. Each family contains unique domains: Bromo, SANT, SLIDE, Tandem, chromo or long-insertion. Picture taken from (Clapier and Cairns, 2009).

The INO80 family is the last family of SWI-like ATP-dependent chromatin-remodelling complexes. The core catalytic ATPase domain is divided into two fragments by long insertion domain (Hargreaves and Crabtree, 2011) (Figure 1.14). This insert motif binds helicase related RuvB1/2 proteins and this suggests a potential role of INO80 in DNA damage response (Morrison and Shen, 2009). INO80 members remove canonical H2A-H2B dimers and replace them with H2A.Z-H2B variant (Clapier and Cairns, 2009). Furthermore, INO80 complex has a special affinity to bind histone H2A.Z and γ H2A.X, suggesting that it is required for DNA repair or checkpoint responses (Morrison and Shen, 2009).

1.12 Techniques widely used for the study of DNA replication

1.12.1 Evaluating cell proliferation methods

1.12.1.1 [³H] thymidine incorporation

Radioactive [³H] thymidine labelling was first used to visualise *Vicia faba* (English broad bean) chromosomes (Taylor et al., 1957). The labelling procedure involved growing of the roots in the media containing [³H] thymidine in the presence or absence of cell proliferation inhibitors. The roots were subsequently released from [³H] thymidine containing media and allowed to grow further. Finally, DNA fragments were extracted from the roots and incorporation of radioactive [³H] thymidine into cellular DNA was measured using autoradiography or scintillation counters (Taylor et al., 1957). Nevertheless, this technology suffers several limitations, such as the handling and disposal of radioisotopes or time consuming autoradiography detection step that initially lasted for several months (Salic and Mitchison, 2008). Additionally, microscopy images obtained with [³H] thymidine were characterised by the poor resolution and low signal-to-noise ratios (Salic and Mitchison, 2008). Furthermore, incorporation of radioactive [³H] thymidine is not suitable for all cell type models or conditions (Drach et al., 1981).

1.12.1.2 Incorporation of halogenated nucleotides

Halogenated nucleotides such as 5-bromo-2'-deoxyuridine (BrdU), 5-chloro-2'-deoxyuridine (CldU) and 5-iodo-2'-deoxyuridine (IdU) are non-radioactive alternative to [³H] thymidine-based cell proliferation studies (Yokochi and Gilbert, 2001). These permeable, pyrimidine analogues similarly to tritiated thymidine, are incorporated into newly synthesised DNA during DNA replication *in vitro* and *in vivo* (Morstyn et al., 1983). Within the cells, these analogues are phosphorylated and incorporated into DNA via the nucleotide salvage pathway (Kuebbing and Werner, 1975; WlkramasInghe, 1981; Zupanc and Horschke, 1996).

BrdU detection based on quenched fluorochromes was developed by Samuel A. Latt (Latt, 1973) and immediately introduced in flow cytometric

studies (Franceschini, 1974). Further development of specific antibodies raised against the halogenated nucleosides allowed to extend analysis of labelled DNA by immunofluorescence (Gratzner, 1982) and immunohistochemistry (Morstyn et al., 1983). Additionally, fluorophore-conjugated secondary antibodies provide another significant tool to study DNA synthesis (Nagashima and Hoshino, 1985). BrdU can be also applied in high-resolution chromosome combing that can detect parameters, such as density of active origins, speed of replication forks and replicone size (Michalet et al., 1997).

Nevertheless, the major disadvantage of halogenated nucleotides incorporation is requirement to denature the double-stranded DNA to allow epitope exposure for antibody recognition (Gratzner, 1982). Typically harsh conditions for DNA denaturation, such as strong acid/base treatment or extreme temperatures often result in degradation of protein epitopes (Salic and Mitchison, 2008; Zeng et al., 2010).

1.12.1.3 Incorporation of 5-ethynyl-2'-deoxyuridine

Recently, a novel method based on incorporation of 5-ethynyl-2'-deoxyuridine (EdU) into newly synthesised DNA was described (Salic and Mitchison, 2008; Zeng et al., 2010). EdU is a thymidine-like nucleotide and it is incorporated into DNA very efficiently during S phase (Salic and Mitchison, 2008). Detection of EdU is based on a Huisgen-Sharpless 1, 4-cycloaddition reaction also known as Click reaction, which is a copper (I) catalysed transformation between an azide and an alkyne moieties (Moses and Moorhouse, 2007). The EdU contains the alkyne functional group that can be coupled to any azide group containing reagent to form a stable, triazole ring. As the reaction occurs under mild conditions, protein epitopes or DNA secondary structures are not adversely affected (Buck et al., 2008). The major advantages of DNA labelling with EdU is the detection step that does not required DNA denaturation (Salic and Mitchison, 2008).

Originally, EdU methodology was developed for cell proliferation assays such as flow cytometry or fluorescence microscopy, where EdU was coupled to fluorophore azide-containing probe and fluorescence was measured directly (Zeng et al., 2010). Additionally, EdU labelled DNA can be detected by conventional anti-BrdU antibody after EdU coupling to BrdU azide derivative

called 5'-BMA (5-bromo-5'-azido-2',5'-dideoxyuridine) (Cappella et al., 2008). Recent studies demonstrated that the EdU approach can be introduced to analyse components of labelled chromatin (this study, (Kliszczak et al., 2011) and (Sirbu et al., 2011). In this methodology, EdU is linked to a biotin azide analogue and labelled chromatin can be further recovered by conventional pull-down with streptavidin-coated resin, followed by immunoblotting analysis (Kliszczak et al., 2011; Sirbu et al., 2011).

1.12.2 Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) methodology was first developed by Alexander Varshavsky to study histone distribution in *Drosophila* (Solomon et al., 1988; Solomon and Varshavsky, 1985) and quickly revolutionised experimental approaches to study transcription, DNA replication and DNA repair (Orlando et al., 1997). Chromatin immunoprecipitation methodology involves the covalent cross-linking of proteins to DNA strands by cross-linking agents such as formaldehyde, cells lysis and DNA shearing into small fragments. The DNA-protein complex is subsequently immunoprecipitated using an antibody specific to a feature of interest. After capturing, DNA-protein cross-link is reversed, followed by DNA purification and amplification by PCR using primers specific to predicted binding site. ChIP is a widely applied technique that allows for determination of genomic sequences of specific chromosomal proteins *in vivo* (Aparicio et al., 2004). Additionally, ChIP assay was used to characterise genome binding site of mammalian transcription factors (Ren and Dynlacht, 2004) or to detect checkpoint regulatory proteins recruited to sites of DNA damage such as Nbs1, ATM, XRCC4, Tof1 and Mrc1 (Berkovich et al., 2007; Katou et al., 2003). Multiple approaches based on chromatin immunoprecipitation method are available to investigate global DNA-protein interactions across the genome such as DNA microarray hybridisation (ChIP-chip) (Katou et al., 2006; Ren et al., 2000) or massively parallel sequencing (ChIP-seq) (Robertson et al., 2007; Wang et al., 2009). These techniques are widely used, for example to study histone post-translational modifications at genomic scale (reviewed in (Schones and Zhao, 2008)).

The ChIP-chip approach was developed by Bing Ren to monitor genome specific localisation of two transcription factors Gal4 and Ste12 in budding yeast (Ren et al., 2000). The method was further used by Richard Young's group to determine localisation of 106 transcription factors across the budding yeast genome (Lee et al., 2002). The ChIP-chip technique involves combination of chromatin immunoprecipitation and DNA microarray analysis. After immunoprecipitation of chromatin, DNA sequences pulled-down specifically or non-specifically are amplified by LM-PCR (ligation-mediated-polymerase chain reaction) and labelled with different fluorophores. Finally, DNA fragments are hybridised to a single DNA microarray containing ORFs and intergenic regions of budding yeast genome (Ren et al., 2000). Nowadays, DNA microarrays were replaced with high-density DNA tiled arrays (oligonucleotide DNA chip) containing whole budding yeast or human genome. These tiled arrays possess shorter 25 bp oligonucleotides probes compared to 500-1000 bp probes in single microarrays, thus increase the resolution of the assay (Katou et al., 2006). ChIP-chip has quickly become a powerful method for high-resolution study of localisation of chromatin bound proteins such as transcription factors or histone post-translational modifications (reviewed in (Schones and Zhao, 2008)). The ChIP-chip method is not suitable to study mammalian genomes, due to their large size and presence of repetitive sequences (Bernstein et al., 2007; Mikkelsen et al., 2007). Instead of whole human genome analysis, many studies in mammalian cells have focused on specific regions such as promoters or small chromosomes (Bernstein, 2005; Kim et al., 2005; Koch et al., 2007; Roh et al., 2006). Technical limitations of ChIP-chip approach, such as large set of microarrays needed to sufficiently cover the genome, large amounts of DNA used and potential cross-hybridisation that impedes analysis of repeat sequences lead to development of more suitable methods to study human genome such as the ChIP-sequencing (Mikkelsen et al., 2007).

The ChIP-sequencing approach couples chromatin immunoprecipitation with massively parallel sequencing (ChIP-seq) (Mikkelsen et al., 2007). Principles of the ChIP-seq involve several steps. The first, DNA fragments are precipitated according to the standard ChIP protocol. After DNA purification, DNA fragments are blunted and conjugated at both ends with the specific adaptors. These fragments are then immobilised and solid-phase PCR amplifies

precipitated DNA. Finally, sequencing by DNA synthesis is performed and resulting fragments are analysed by comparison with genomic reference sequences (Schmidt et al., 2009; Schones and Zhao, 2008).

In contrast to the ChIP-chip assay, the ChIP-seq has a greater coverage and allows the analysis of DNA-protein interactions in human genome (Mikkelsen et al., 2007; Robertson et al., 2007). Additionally, ChIP-seq does not require a hybridisation step as amplified DNA is directly quantified by the sequencing methods (Morozova and Marra, 2008), thus gives better signal resolution with fewer artefacts than ChIP-chip microarrays approach (reviewed in (Park, 2009; Schones and Zhao, 2008)). The ChIP-seq technique has found many applications such as high-resolution analysis of histone post-translational modifications (Barski et al., 2007a) or nucleosome architecture (Barski et al., 2007b; Schmid and Bucher, 2007) and became a pivotal tool to study gene regulation and epigenetic marks (reviewed in (Park, 2009)).

Both ChIP-chip and ChIP-seq approaches are broadly used to study DNA replication in particular to map replication origins in both budding and fission yeast. The identification of replication sites are based on characterisation of sequences bound by the proteins of the pre-replication complex such as ORC or Mcm2-7 helicase. Nevertheless, due to high background noise during the analysis, to date there is no examples of such analysis in mammalian cells (reviewed in (Gilbert, 2010)).

1.13 Aims of this study

It has long been recognised that the fidelity of DNA replication is crucial for the maintenance of genome stability (Sclafani and Holzen, 2007). More recently it has also been realised that the proteins that coat the DNA, such as histones and transcription factors also carry important information that specifies cell function and identity (Kouzarides, 2007). As the DNA is replicated, histones and DNA binding proteins are displaced from the DNA in front of a replication fork and re-occupy their binding sites after its passage. Since twice as many binding sites exist after DNA duplication, previously resident DNA binding proteins are supplemented from a pool of soluble proteins. Therefore the duplication of DNA imposes a source of stress for the maintenance of the epigenetic information and for the regulation of gene expression (Jasencakova and Groth, 2010).

Molecular mechanisms of chromatin dynamics, such as histone assembly/disassembly and duplication of epigenetic marks are not fully understood. This is due to a lack of biochemical techniques that allow analysis of proteins that are associated with replication forks *in vivo*. We predict that if a technique could be established it would provide a powerful tool to investigate different aspects of chromatin dynamics.

Therefore the main goal of this study was to generate a tool for the analysis of proteins associated with newly synthesised DNA. Using the developed technology we wanted to investigate how cells re-assemble chromatin proteins onto newly replicated DNA after passage of the replication forks and how chromatin maturation is coupled to DNA synthesis. Additionally, we wanted to investigate the relationship between chromatin proteins composition and the temporal programme of DNA replication.

Chapter 2 Materials and methods

2.1 Materials

2.1.1 Chemical reagents

Chemicals used during this study were of analytical grade and purchased from Sigma-Aldrich (Arklow, Ireland), Fisher Scientific (Lecienstershire, UK) and GE Healthcare (Buckinghamshire, UK). EdU (5-ethynyl-2'-deoxyuridine), biotin-TEG azide and 6-carboxyfluorescein-TEG azide were purchased from Berry & Associates (Dexter, USA).

All solutions were prepared using Milli-Q purified water (Millipore, Billerica, USA) and autoclaved (125°C for 15 minutes) prior to use if required. Organic solvents were supplied by Sigma-Aldrich (Arklow, Ireland) or Fisher Scientific (Lecienstershire, UK), unless otherwise stated.

All common reagents and buffers used throughout this study are presented in Table 2.1.

Name	Composition	Notes and references
6 x DNA loading dye	20% sucrose, 100 mM EDTA pH 8.0, 1% SDS, 0.25% bromophenol blue, 0.25% xylene cyanol	For DNA sample loading prior to running agarose gel
Click reaction Mix I	10 mM ascorbic acid, 0.1 mM 6-carboxyfluorescein- TEG azide, 2 mM CuSO ₄ - added in the order indicated	For labelling of S phase cells for flow cytometry and fluorescence microscopy
Click reaction Mix II	10 mM ascorbic acid, 0.1 mM 5'-BMA azide, 2 mM CuSO ₄ - added in the order indicated	For the capturing of labelled chromatin in Dm- ChP using 5'-BMA azide
Click reaction Mix III	10 mM ascorbic acid, 0.1 mM biotin-TEG azide, 2 mM CuSO ₄ - added in the order indicated	For the capturing of labelled chromatin in Dm- ChP using biotin-TEG azide

Coomassie Brilliant Blue R	1 g Coomassie in 50% methanol, 10% acetic acid	For staining protein gels
CL buffer	50 mM HEPES pH 7.8, 150 mM NaCl, 0.5% NP-40, 0.25% Triton X-100, 10% glycerol	For lysis and preparation of soluble fraction of human cells in Dm-ChP
DABCO	100 mM Tris-HCl, pH 8.0, 90% glycerol, 200 mM DABCO	To mount slides for fluorescence microscopy
Destain solution	12% methanol, 7% acetic acid	To destain Coomassie stained gels
Elution buffer	1% SDS, 100 mM NaHCO ₃	To elute purified DNA from the agarose beads
Extraction buffer	5% formic acid, 65% acetonitrile	To extract peptides from the gel
Fixation Solution 1	4% methanol, 1% glycerol	To dry Coomassie and silver stained gels
Fixation Solution 2	50% ethanol, 10% acetic acid	To fix SDS-PAGE for a silver staining
Fixation Solution 3	50% methanol, 1% acetic	To fix SDS-PAGE for a GelCode Blue staining
Freezing medium	10% DMSO in 20% FBS	For freezing down mammalian cells
5 x Laemmli buffer - SDS-PAGE sample buffer	5% SDS, 20% glycerol, 0.004% bromphenol blue, 125 mM Tris-HCl pH 8.0, 10% β-mercaptoethanol	For denaturation and loading of proteins prior to SDS-PAGE
Lysis Buffer	50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100	For cell lysate preparation and analysis by SDS-PAGE
10 x PBS	137 mM NaCl, 2.7 mM KCl, 100 mM Na ₂ HPO ₄ , 2 mM KH ₂ PO ₄	Make up to 1x solution with Sigma tablets
PBS-Tween 20	Phosphate buffered saline with 0.05% Tween 20	To wash nitrocellulose membrane
Ponceau S solution	0.5 g Ponceau S, 5% acetic acid	To stain proteins on the nitrocellulose membrane

RIPA buffer	10 mM Tris-HCl pH 8.0, 140 mM NaCl, 0.1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100	Extraction of chromatin fraction in Dm-ChP protocol
Running buffer	25 mM Tris-HCl, 250 mM glycine, 0.1% SDS	For running SDS-PAGE gels
SDS lysis buffer	1% SDS, 50 mM Tris-HCl pH 6.8	For whole cell lysate preparation and analysis by SDS-PAGE
2 x SSC	0.3 M NaCl, 0.03 M sodium citrate, pH adjust to 7.0 with citric acid	For transfer of DNA from agarose gels to nylon membrane
TE	10 mM Tris-HCl pH 8.0, 1 mM EDTA	To dissolve nucleic acids. To protect DNA or RNA from degradation
TAE	40 mM Tris-acetate pH 8.0, 1 mM EDTA	To run agarose gels
Transfer buffer	72 mM Tris-HCl, 58.5 mM glycine, 15% methanol	For the wet transfer of SDS-PAGE onto nitrocellulose membrane
Trypsin digest buffer	50 mM ammonium bicarbonate, 10% acetonitrile	To digest proteins for mass spectrometry analysis
UA buffer	8 M urea, 10 mM Tris-HCl pH 8.0	To wash Microcon YM-10 column after SDS elution for mass spectrometry analysis
UB buffer	8 M urea, 10 mM Tris-HCl pH 8.5	To wash Microcon YM-10 column after SDS elution for mass spectrometry analysis
Wash buffer	10 mM Tris-HCl pH 8.0, 200 mM NaCl, 0.5 mM DTT	To wash beads after IP and Dm-ChP assays

Table 2.1 Common reagents and buffers.

2.1.2 Molecular biology reagents

DNA size ladders were obtained from New England Biolabs (Hertfordshire, UK). Protein size markers were supplied by New England Biolabs, Bio-Rad (Hercules, USA) or Fermentas (Glen Burnie, USA). Protein A-agarose beads for immunoprecipitation assays were supplied by GeneSpin (Milano, Italy). High capacity Streptavidin-coated agarose beads for DNA mediated chromatin pull-down (Dm-ChP) assays were obtained from Thermo Scientific (Rockford, USA).

Table 2.2 lists the antibodies used in western blotting throughout this study, their working dilutions and source.

Antigen	Species type	Working dilution	Supplier
anti-BrdU (clone B44)	Mouse monoclonal	1/50	Becton Dickinson (Oxford, UK)
anti-BrdU (clone 3D4)	Mouse monoclonal	1/1000	Becton Dickinson (Oxford, UK)
anti-biotin-Peroxidase (Clone BN-34)	Mouse monoclonal	1/8000	Sigma-Aldrich (Arklow, Ireland)
anti-RbAp48 (CAF-1)	Rabbit polyclonal	1/1000	Abcam (Cambridge, UK)
anti-Cdc7	Mouse monoclonal	1/2000	Abcam (Cambridge, UK)
anti-Cdc45	Rat monoclonal	1/50	Gift from Dr. H.P. Nasheuer (Bauerschmidt et al., 2007)
anti-pSer317 Chk1	Rabbit polyclonal	1/2000	Cell Signalling (Danvers, USA)
anti-Chk1	Mouse monoclonal	1/2000	Santa Cruz Biotechnology (Santa Cruz, USA)
anti-Claspin	Rabbit polyclonal	1/2000	Abcam (Cambridge, UK)
anti-FACT (SSRP-1)	Mouse monoclonal	1/1000	Abcam (Cambridge, UK)

anti-GFP	Mouse monoclonal	1/2000	Roche (Mannheim, Germany)
anti-Fen-1	Rabbit polyclonal	1/2500	Abcam (Cambridge, UK)
anti-Histone H2A	Rabbit polyclonal	1/2000	Upstate (Billerica, USA)
anti-Histone H3	Rabbit polyclonal	1/8000	Abcam (Cambridge, UK)
anti-Histone H4	Rabbit polyclonal	1/2000	Abcam (Cambridge, UK)
anti-Lamin B1	Rabbit polyclonal	1/1000	Abcam (Cambridge, UK)
anti-Mcm2	Rabbit polyclonal	1/2000	Abcam (Cambridge, UK)
anti-Mcm7	Mouse monoclonal	1/1000	Thermo Scientific (Rockford, USA)
anti-Msh2	Mouse monoclonal	1/250	Calbiochem (Darmstadt, Germany)
anti-p54nrb (NONO)	Rabbit polyclonal	1/2000	Abcam (Cambridge, UK)
anti-Nucleolin	Sheep polyclonal	1/1000	Gift from Prof. Brian McStay (NUIG)
anti-Nucleophosmin	Rabbit polyclonal	1/2000	Abcam (Cambridge, UK)
anti-PCNA	Mouse monoclonal	1/1000	Santa Cruz Biotechnology (Santa Cruz, USA)
anti-RPA	Rabbit polyclonal	1/2000	Abcam (Cambridge, UK)
anti-Smc1	Rabbit polyclonal	1/2000	Bethyl (Montgomery, USA)
anti-Smc3	Goat polyclonal	1/2000	Santa Cruz Biotechnology (Santa Cruz, USA)

Table 2.2 Primary antibodies used in this study.

Antigen	Species type	Working dilution	Supplier
FITC (fluorescein isothiocyanate)-conjugated anti-mouse whole antibody	Goat polyclonal	1/32	Sigma-Aldrich (Arklow, Ireland)
HRP (horseradish peroxidase)-conjugated anti-mouse whole antibody	Sheep polyclonal	1/10 000	GE Healthcare (Buckinghamshire, UK)
HRP (horseradish peroxidase)-conjugated anti-rabbit whole antibody	Donkey polyclonal	1/10 000	GE Healthcare (Buckinghamshire, UK)
HRP (horseradish peroxidase)-conjugated anti-goat whole antibody	Donkey polyclonal	1/10 000	Abcam (Cambridge, UK)
HRP (horseradish peroxidase)-conjugated anti-rat whole antibody	Donkey polyclonal	1/10 000	Jackson Labs (Bar Harbor, USA)

Table 2.3 Secondary antibodies used in this study.

2.1.3 Tissue culture cell line and reagents

2.1.3.1 Cell line

HeLa CCL-2 cells (Epitheloid carcinoma, cervix, human) were obtained from The American Type Culture Collection (Middlesex, UK). The cell line was negative when tested for mycoplasma contamination using MycoTrace mycoplasma PCR detection kit according to the manufactures' instructions (PAA Laboratories Ltd, Yeovil, UK). The cell stock was maintained in culture for a maximum of four months and then replaced from a frozen sample. The cell concentrations were determined with an automated cell counter Countess™ (Invitrogen, Carlsbad, USA).

Table 2.4 shows drugs used in this study for pharmacological treatment of HeLa cells.

Drug	Concentration	Application	Source
Hydroxyurea	10 mM	To arrest replication forks	Sigma-Aldrich (Arklow, Ireland)
Thymidine	2 mM	To synchronise the cells in G ₁ /S phase	

Table 2.4 Drugs used in this study.

2.1.3.2 Cell culture reagents

Cell culture reagents were obtained from Sigma-Aldrich (Arklow, Ireland) and sterile plastic-ware from Sarstedt AG (Nümbrecht, Germany). HeLa cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with heat inactivated 10% fetal bovine serum, 1 x non-essential amino acids, 2 mM L-glutamine and 1% penicillin-streptomycin (all from Sigma-Aldrich, Arklow, Ireland).

Cell culture procedures were carried out in an S@feFlow 1.2 hood (Bioair EuroClone Division, Italy). Disposable sterile plastic-ware were used for all cell culture protocols. Surfaces were sprayed with 70% IMS solution prior to carrying out cell culture procedures. The cells were cultured in a Steri-Cycle CO₂, HEPA Class 100 incubator (Thermo Scientific, Rockford, USA) at 37°C and 5% CO₂.

2.1.4 Computer programmes

For classification analysis of proteins identified by mass spectrometry analysis, *UniProt* (Universal Protein Resource) protein Knowledgebase (<http://www.uniprot.org>), Genome browser *Ensembl* (<http://www.ensembl.org>) and *GeneCards* (<http://www.genecards.org>) online databases were used.

Immunofluorescence was performed using an Olympus BX-51 microscope with 60 x (NA 1.4) or 100 x (NA 1.35) and driven by OpenLab software (version 5, Improvision, Emeryville, USA).

Flow cytometry analysis was carried out using Cell Quest (version 3.3, Becton Dickinson, Oxford, UK) or BD FACS Diva Software (version 6.1.2, Becton Dickinson, Oxford, UK).

2.2 Nucleic acid methods

2.2.1 Preparation of genomic DNA

To prepare genomic DNA, 1.7×10^7 HeLa cells were harvested and chromatin fraction was prepared in RIPA buffer (see Table 2.1; see section 2.3.7.1). The chromatin was subsequently treated with 0.1 mg/ml RNase A at 37°C for 30 minutes, followed by 2 hours incubation at 45°C with 0.1 mg/ml Proteinase K (both from Sigma-Aldrich, Arklow, Ireland). DNA was extracted by adding an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) to the lysate, followed by mixing and centrifugation for 2 minutes at 16 100 x g at 4°C. The aqueous phase was transferred to a new tube. To precipitate the DNA, 1/20 volume of 3 M sodium acetate pH 5.2 together with at least 2 volumes of 100% ice-cold ethanol were added to the aqueous phase. Precipitated DNA was recovered by centrifugation at 16 100 x g for 20 minutes at 4°C. DNA was air dried for 15 minutes, re-suspended in 50 µl of 1x TE buffer (see Table 2.1) and store at 4°C or -20°C for a long-term storage.

2.2.2 Agarose gel electrophoresis

Generally 0.8% or 1.5% agarose gels were prepared using electrophoresis grade agarose (Sigma-Aldrich, Arklow, Ireland) in 1 x TAE buffer containing 0.1 µl/ml SYBR Safe DNA gel stain (Invitrogen, Carlsbad, USA). Gels were run in 1 x TAE buffer for 40-60 min at 110V in the Mini Horizontal Gel Units (Medical Supply Co. Ltd., Dublin, Ireland). After electrophoresis, gels were analysed using a Multi Image Light Cabinet (ChemiImager 5500, Alpha Innotech, Medical Supply Co. Ltd., Dublin, Ireland) and the images were captured with a digital camera.

2.2.3 DNA transfer and detection

DNA was purified as previously described in section 2.2.1 and was then separated on an agarose gel. DNA was nicked by treatment with 0.25 M HCl for 20 minutes at room temperature on a platform shaker and then denatured in 0.5 M NaOH/1.5 M NaCl for an additional 20 minutes. DNA was transferred by capillary transfer onto positively charged nylon membrane (Hydron-N, GE

Healthcare, Buckinghamshire, UK) in 2 x SSC transfer buffer (see Table 2.1). To permanently attach the DNA to the membrane, DNA was irradiated with UV 300 J/cm² using a UV Cross-linker (Hoefer UVC500, GE Healthcare, Buckinghamshire, UK); alternatively the membrane was incubated in an Economy incubator (Weiss-Gallenkamp, Loughborough, UK) at 70°C for an hour. After incubation, the membrane was washed in Milli-Q water and incubated in blocking solution (5% not-fat, dry milk in 0.05% (v/v) Tween 20 in PBS) for an hour. The DNA was probed by overnight incubation with anti-BrdU antibody (see Table 2.2) and detection step was carried out as for western blot analysis (see section 2.3.5).

2.2.4 Polymerase Chain Reaction (PCR)

Polymerase chain reaction was performed using Sigma Taq polymerase. PCR experiments were carried out on a TGradient (Biometra, Göttingen, Germany). Table 2.5 gives an example of the PCR conditions and programme used. Prior to PCR reaction, DNA recovered by Dm-ChP assay (see section 2.3.8) was purified using PCR purification Clean-up kit (Sigma-Aldrich, Arklow, Ireland). DNA concentration was determined using Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Rockford, USA) and 100 ng of DNA was used for each PCR reaction.

SigmaTaq Polymerase		
Reagent concentrations	buffer (10x)	1x, supplemented with 2.5 mM MgCl ₂
	Primers	0.25 µM
	dNTP's	200 µM
	Enzyme	0.2 µl (5U/µl)
PCR steps	'hot start'	94°C – 2 minutes
	Denaturation	94°C – 30 seconds
	Annealing	60°C – 30 seconds
	Extension	72°C – 2 minutes
	final extension	72°C – 10 minutes
No. of cycles		50

Table 2.5 Example of typical PCR reaction conditions.

2.3 Protein methods

2.3.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The Mini-Protean[®] Cell System Bio-Rad (Hercules, USA) was used for mini gels (10 x 10 cm). Wide gels (10 x 20 cm) were carried in the Vertical Maxi 2 Gel Device, Medical Supply Co. Ltd. (Dublin, Ireland). Standard gels (mini) were generally run at 25 mA for about 60-90 minutes in 1 x running buffer (see Table 2.1). Wide gels were run at 55-70 mA for 2-3 hours depending on gel percentage. Standard SDS-PAGE gels were prepared according to Table 2.6. 30% acrylamide: bisacrylamide (37:5:1) stock was obtained from Sigma-Aldrich (Arklow, Ireland).

	10% gel	12% gel
Resolving Gel Mix	375 mM Tris-HCl pH 8.8	375 mM Tris-HCl pH 8.8
	10% acrylamide/bis	12% acrylamide/bis
	0.1% SDS	0.1% SDS
	0.05% APS	0.05% APS
	0.05% TEMED	0.05% TEMED
Stacking Gel Mix	125 mM Tris-HCl pH 6.8	
	4% acrylamide/bis	
	0.1% SDS	
	0.05% APS	
	0.1% TEMED	

Table 2.6 Preparation of SDS-PAGE gels for western blotting procedure.

2.3.2 Protein sample preparation

Protein samples for western blotting were generated by lysing HeLa cells for 10 minutes on ice bath in lysis buffer of choice (supplemented with protease and phosphatase inhibitors) depending on the experiment performed. Lysis buffers used are described in Table 2.1. After lysis, DNA and cell membranes were removed by centrifugation at 16 100 x g for 10 minutes at 4°C. Protein

concentration in the supernatant was determined by Bradford Protein assay (see section 2.3.3.1) or BCA Protein assay (see section 2.3.3.2). Normally 20-25 μg of cell lysate per well was loaded onto a gel. Each protein sample was boiled at 95°C for 3 minutes in 1 x *Laemmli* sample buffer (see Table 2.1). The samples were either stored at -20°C or loaded directly onto the gel.

2.3.3 Methods to determine protein concentration

2.3.3.1 Bradford Protein assay

To determine protein concentration in samples, the Bradford assay was used (Bradford, 1976). Bovine serum albumin (BSA) was used to prepare a standard curve and the appropriate lysis buffer was used as a blank. Briefly, 1 μl of lysate was diluted in 0.5 ml of Bradford solution obtained from Sigma-Aldrich (Arklow, Ireland) and brought up to 1 ml using Milli-Q water. Samples were transferred to the plastic cuvettes and an absorbance at 595 nm was measured by a spectrophotometer (Eppendorf, Hamburg, Germany). The protein concentration was calculated based on a BSA standard curve, in which absorbance was plotted vs. varying concentrations of the BSA protein.

2.3.3.2 BCA Protein assay

For a protein samples containing high concentration of detergents (e.g SDS) the BCA Protein kit (Thermo Scientific, Rockford, USA) was used to determine proteins concentration. According to the manufacturers' instructions, BCA Protein assay was performed in 96-well multiple well plate. A series of BSA protein dilutions were used to create standard curve. The appropriate lysis buffer solution was used as a blank. Briefly, an aliquot of 10 μl of each sample, standard and blank sample were diluted in 200 μl of BCA working reagent (50:1 solution A: solution B). The plate was incubated at 37°C for 30 minutes and after cooling down the absorbance was measured at 562 nm using the Wallac 1420 VICTOR³ multilabel plate reader (Perkin Elmer, Massachusetts, USA). Using the BSA standard curve the protein concentration in each sample was calculated.

2.3.4 SDS-PAGE staining methods

2.3.4.1 Coomassie Blue staining

Proteins in the gel were visualised by incubation with Coomassie Blue dye solution (see Table 2.1) for an hour on a platform shaker at room temperature. Gels were then transferred into the destain solution (see Table 2.1), placed on a shaker and rinsed several times with fresh destain solution until the excess dye was removed. Gel images were taken using HP Scanjet G 2410 scanner and further dried after treatment with a fixation solution 1 (see Table 2.1) using the Hoefer Slab Gel Dryer SE1160 (Amersham Biosciences, Piscataway, USA) for 90 minutes at 70°C.

2.3.4.2 GelCode Blue staining

After SDS-PAGE electrophoresis gels were rinsed three times with Milli-Q water for 5 minutes and fixed in fixing solution 3 (see Table 2.1) for 15 minutes on a platform shaker at room temperature. To visualise proteins, GelCode Blue stain reagent (Thermo Scientific, Rockford, USA) was added and gels were incubated with shaking for an hour at room temperature. To remove the excess of dye, gels were rinsed several times with fresh Milli-Q water. Images of gels were taken as described in section 2.3.4.1.

2.3.4.3 Silver staining

To visualise protein bands on gel for further characterisation by mass spectrometry, the ProteoSilver Plus kit (Sigma-Aldrich, Arklow, Ireland) was used. After electrophoresis, the gel was placed into a clean tray with 50 ml of the fixing solution 2 (see Table 2.1) and incubated overnight at room temperature. The following day, the gel was rinsed in sequence with 30% (v/v) ethanol in Milli-Q water, followed by Milli-Q water wash for 10 minutes and further exposed to sensitizer solution for 10 minutes. The gel was rinsed twice with Milli-Q water leaving each wash for 10 minutes. The ProteoSilver Silver solution was added to equilibrate the gel for 10 minutes and washed away for 1 minute with Milli-Q water. Proteins were detected using the ProteoSilver Developing solution for 2-5 minutes to produce the desired staining intensity. Developing reaction was stopped by adding the ProteoSilver Stop solution to the mixture for

5 minutes. The stained gel was stored in Milli-Q water at 4°C and later dried as described in section 2.3.4.1.

2.3.5 Western blotting

For immunoblotting, proteins were separated by SDS-PAGE and then transferred onto nitrocellulose membrane (Whatman GmbH, Dassel, Germany). This was carried out using a wet transfer system for 90 minutes at 250 mA in 1 x transfer buffer (see Table 2.1) either at 4°C or room temperature. For mini gels, the Mini Trans-Blot Cell transferred system was used. Wide gels were transferred using the Trans-Blot Cell (both from Bio-Rad, Hercules, UK). The gel and membrane were placed between sponge pads and filter papers (Whatman GmbH, Dassel, Germany). Air bubbles that formed between the gel and the membrane were removed. Prior to transfer, the transfer cassettes were assembled with the nitrocellulose membrane on the cathode side and the gel on the anode side. Two filter papers were placed on each side of the membrane and gel and a sponge was then placed outside the filter papers. The transfer apparatus was assembled; an ice-pack was placed in the tank and the apparatus was filled with 1 x transfer buffer (see Table 2.1). The quality of protein transfer was confirmed either by the presence of the pre-stained protein marker on the membrane or by staining with Ponceau S solution for 5 minutes (Sigma-Aldrich, Arklow, Ireland) (see Table 2.1). The Ponceau S solution was removed from the membrane by rocking in 0.05% (v/v) Tween 20 in PBS washing solution (see Table 2.1) for 10 minutes. To prevent non-specific binding of either the primary or secondary antibodies, the membrane was incubated in blocking solution (5% not-fat, dry milk in 0.05% (v/v) Tween 20 in PBS) at room temperature for an hour under agitation and then washed once in 0.05% (v/v) Tween 20 in PBS to remove the excess of milk. Meanwhile, the primary antibody was prepared in 5% not-fat milk or 1-5 % (w/v) BSA in PBS at the recommended dilution (see Table 2.2). The membrane was incubated with the primary antibody overnight at 4°C on the rolling mixer (SRT9, Stuart Scientific, Stone, UK). The following day, the membrane was washed three times in 0.05% (v/v) Tween 20 in PBS while agitating for 10 minutes to remove the residual primary antibody. After rinsing, membrane was incubated for an hour at room temperature with secondary

antibody conjugated to HRP (see Table 2.3). Before detection with enhanced chemiluminescence, the membrane was washed three times in 0.05% (v/v) Tween 20 in PBS. The membrane was placed in a clean tray and incubated for 1 minute with the Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK) or Immobilon Western-Chemiluminescent HRP substrate detection system (Millipore, Billerica, USA). The membrane was placed protein side up in the cassette with an autoradiograph film (Konica Minolta & Graphic, USA). Films were exposed initially for 2 minutes and then re-exposed for the optimal time as needed. Films were developed and fixed by passing it through a developing machine (CP 1000, AGFA, Brentford, UK) or by using solutions in the trays. If necessary the membrane was stripped with the Restore Western Blot Stripping buffer (Thermo Scientific, Rockford, USA) for 30 minutes at 37°C and incubated with primary antibody again, as previously described.

2.3.6 Dot Blot procedure

For a spotting sample assay, the Bio-Dot Apparatus (Bio-Rad, Hercules, USA) was used. A sheet of nitrocellulose membrane was pre-wet in PBS and then clamped between gasket and 96-well sample template. After assembly of the apparatus, 100 µl of each sample was slowly filtered through the membrane using vacuum pressure. As soon as the buffer solution dried out the vacuum was disconnected, the dried membrane was placed into a washing tray and rinsed in PBS. Protein signal was detected as described in previous section 2.3.5.

2.3.7 Chromatin immunoprecipitation

2.3.7.1 Chromatin preparation

To cross-link proteins to DNA, typically, 1.7×10^7 HeLa cells were incubated for 10 minutes at 4°C with an aqueous solution of 1% (v/v) formaldehyde. To quench un-reacted formaldehyde, 0.125 M glycine was added and cells were incubated additionally for 10 minutes at 4°C. Cells were then washed three times with PBS and lysed in 1.2 ml of CL buffer containing protease inhibitors (Protease inhibitor cocktail III, Fisher Scientific, Leciensershire, UK) by incubating at 4°C with end-over-end mixing for 10 minutes. This was followed by centrifugation at 160 x g for 5 minutes.

Supernatant was collected and labelled as a soluble fraction. The residual pellet was then washed in 1.2 ml of wash buffer (see Table 2.1) supplemented with protease inhibitor cocktail for 10 minutes at 4°C by end-over-end mixing and centrifuged again. The pellet was then re-suspended in 1.2 ml of RIPA buffer (see Table 2.1) containing a protease inhibitor cocktail. To shear chromatin, lysate was sonicated on ice at 40% amplitude for six rounds of 10 seconds with 2 minutes interval between rounds using a Digital Sonifier (Branson, London, UK). The extract was clarified by centrifugation at 16 100 x g for 10 minutes at 4°C. Protein concentration was determined using either Bradford or BCA protein assays (see sections 2.3.3.1 and 2.3.3.2).

2.3.7.2 Immunoprecipitation

For immunoprecipitation experiments, the chromatin fraction previously extracted (see section 2.3.7.1) was pre-cleaned with 50 µl of wet Protein A-agarose beads on a rotating mixer (Stuart Scientific, Stone, UK) to remove proteins that non-specifically bind to the beads. Meanwhile, 50 µl of wet pre-washed Protein A-agarose resin was re-suspended in 1 ml of RIPA buffer containing 2 µg of appropriate antibody and incubated for 2 hours at 4°C. After antibody binding and three washes with wash buffer (see Table 2.1), beads were used for immunoprecipitation. Typically, 1.5 mg of lysate was incubated for 2 hours at 4°C with antibodies previously bound to the Protein A-agarose beads. After binding, the resin was washed three times with 1 ml of wash buffer (see Table 2.1), centrifuged at 160 x g for 3 minutes at 4°C and transferred to a fresh tube. To reverse protein-DNA cross-linking, beads were incubated for 6 hours at 65°C. Immuno-complexes were recovered by adding 1 x *Laemmli* sample buffer (see Table 2.1), followed by boiling for 3 minutes at 95°C.

2.3.8 Immunoprecipitation of BrdU labelled, purified DNA

Chromatin fraction from HeLa cells labelled with 30 µM BrdU (Sigma-Aldrich, Arklow, Ireland) for 24 hours was prepared as described in section 2.3.7.1. Later, DNA was purified from the chromatin fraction (see section 2.2.1). Purified DNA was re-suspended in RIPA buffer and heat denatured at 95°C for 5 minutes, followed by rapid cooling on ice. 4% of the supernatant was taken and

then used as control input sample. DNA solution was incubated at 4°C by end-over-end mixing with anti-BrdU (see Table 2.2) or control IgG antibodies, that were previously pre-bound to the Protein A-agarose resin (see section 2.3.7.2). After immunoprecipitation, a sample of supernatant was taken and kept as an unbound fraction. The beads were then washed three times with 1 ml of wash buffer (see Table 2.1) and centrifuged at 160 x g for 3 minutes at 4°C. The purified DNA was eluted from beads using IP elution buffer (see Table 2.1) and either electrophoresed in a 1.5% agarose gel and transferred onto nylon membrane (see section 2.2.3) or used as a template for PCR reaction (see section 2.2.4).

2.3.9 Immunoprecipitation of BrdU labelled chromatin

HeLa cells were labelled with 10 µM BrdU for an hour, collected and incubated in 2M HCl supplemented with 1% (v/v) Triton-X 100 for 10 minutes to denature DNA before chromatin preparation (see section 2.3.7.1). Protein content was quantified by BCA Protein assay (see section 2.3.3.2) and 25 µg of the supernatant was saved as the input for immunoblotting.

To remove non-specific binding of proteins to the beads, pre-cleaning of chromatin fraction was achieved by incubation with 50 µl of Protein A-agarose beads on a rotating mixer (Stuart Scientific, Stone, UK). Typically, 1.5 mg of lysate was incubated for 2 hours at 4°C with anti-BrdU or control IgG mouse antibodies previously bound to the Protein A-agarose beads (see section 2.3.7.2). After binding, beads were washed three times with 1 ml of wash buffer (see Table 2.1) and centrifuged at 160 x g for 3 minutes at 4°C. To reverse protein-DNA cross-linking, the resin was incubated for 6 hours at 65°C or 5 minutes at 95°C. Immuno-complexes were recovered by adding 1 x *Laemmli* sample buffer (see Table 2.1), followed by boiling for 3 minutes at 95°C.

2.3.10 Immunoprecipitation of EdU labelled, naked DNA using 5'-BMA azide

HeLa cells were grown in the presence of 5 µM EdU for 24 hours, fixed in formaldehyde and permeabilised with 0.1% (v/v) Triton X-100 in PBS for 10 minutes on ice. The Click reaction was performed in PBS for 30 minutes at room

temperature in the dark by adding the Click reaction Mix II to the cells (see Table 2.1), followed by the addition of 10 volumes of 1% (w/v) BSA, 0.5% (v/v) Tween 20 in PBS and incubated for a further 10 minutes. After three washes in PBS, the chromatin fraction was extracted as described in section 2.3.7.1 and DNA was purified as previously described in section 2.2.1. 4% of the supernatant was taken as the input sample for agarose gel electrophoresis. Purified labelled DNA was incubated at 4°C by end-over-end mixing with anti-BrdU or control IgG antibodies (see Table 2.2) previously pre-bound to the Protein A-agarose resin (see section 2.3.7.2). After immunoprecipitation, the unbound sample was saved. Beads were washed three times with 1 ml of wash buffer (see Table 2.1) and centrifuged at 160 x g for 3 minutes at 4°C. Purified DNA was eluted from the beads using IP elution buffer (see Table 2.1). DNA was then electrophoresed in a 1.5% agarose gel, stained with 0.1µl/ml of SYBR Safe DNA gel stain (Invitrogen, Carlsbad, USA) and later transferred onto nylon membrane (see section 2.2.3).

2.3.11 Immunoprecipitation of EdU labelled chromatin using 5'-BMA azide

HeLa cells were labelled with 10 µM EdU for an hour and the chromatin fraction was extracted as described in section 2.3.7.1 and 2.3.10. Protein content was quantified using the BCA Protein assay (see section 2.3.3.2) and 25 µg of the supernatant was saved as the input for immunoblotting. Chromatin fraction was pre-cleared with 50 µl of wet Protein A-agarose beads on a rotating mixer (Stuart Scientific, Stone, UK) to remove non-specific binding of proteins to the beads. Typically, 1.5 mg of lysate was incubated for 2 hours at 4°C with anti-BrdU or control IgG antibodies previously pre-bound to the Protein A-agarose beads (see section 2.3.7.2). After binding, beads were washed three times with 1 ml of wash buffer (see Table 2.1) and centrifuged at 160 x g for 3 minutes at 4°C. To reverse protein-DNA cross-linking, resin was incubated for 6 hours at 65°C or for 5 minutes at 95°C. Immuno-complexes were recovered by adding 1 x *Laemmli* sample buffer (see Table 2.1), followed by boiling for 3 minutes at 95°C.

2.3.12 DNA mediated chromatin pull-down of EdU labelled chromatin using biotin-TEG azide

HeLa cells were pulsed with 10 μ M EdU before the Click reaction was performed in the presence of the Click reaction Mix III (see Table 2.1). The chromatin fraction was extracted as described in section 2.3.7.1. Protein content was quantified using BCA protein assay (see section 2.3.3.2) and 25 μ l of the supernatant was saved as the input for western blotting. Typically 1-2 mg of extract was used for the pull-down step with 50 μ l of wet streptavidin-coated resin. Before use, streptavidin-coated beads were washed twice with 1 ml of wash buffer (see Table 2.1), equilibrated in RIPA buffer (see Table 2.1) and blocked overnight at 4°C with 0.5 mg/ml BSA and 0.4 mg/ml pre-sheared salmon sperm DNA (both from Sigma-Aldrich, Arklow, Ireland). Chromatin extracts were incubated for 2 hours or overnight at 4°C with pre-blocked streptavidin-coated beads. After binding, unbound material was collected and resin was washed three times with 1 ml of wash buffer (see Table 2.1) and centrifuged at 160 x g for 3 minutes at 4°C. To reverse protein-DNA cross-linking samples and elute proteins from streptavidin-coated beads, samples were incubated for 5 minutes at 95°C in 1 x *Laemmli* sample buffer and loaded onto protein SDS-PAGE gel.

2.4 Mass spectrometry methods

2.4.1 Filter aided sample preparation (FASP) method

Dm-ChP was performed as described in 2.3.12 and captured proteins were prepared for proteomic analysis using FASP method (Wisniewski et al., 2009). Streptavidin eluted material was transferred to a Microcon YM-10 column (Millipore, Billerica, USA) and the volume was reduced to 30 μ l by centrifugation at 16 100 x g at room temperature. To remove detergent, the column was washed three times in sequence with 200 μ l of UA and UB buffers (see Table 2.1) and centrifuged at 16 100 x g for 30 minutes at room temperature. To carboxyamidomethylate thiol residues, proteins were incubated for 20 minutes at room temperature in the dark with 100 μ l of 50 mM iodoacetamide (IAA). Filter units were washed again three times with UB buffer and

centrifuged at 16 100 x *g* for 30 minutes at room temperature. Proteins were digested overnight at 37°C with trypsin digest buffer (see Table 2.1) containing 5 ng/ml of trypsin. Digested peptides were eluted by centrifugation at 16 100 x *g* for 30 minutes and filter units were additionally washed twice in 100 µl of 500 mM NaCl. All fractions were combined, peptides were acidified using 100% TFA and then desalted using ZipTip columns (Millipore, Billerica, USA, see section 2.4.2).

2.4.2 Peptides purification using ZipTip

ZipTip (C₁₈) desalting column (Millipore, Billerica, USA) was placed in a 1.5 ml Eppendorf tube and the resin was activated using 50 µl of 100% methanol. The column was centrifuged at 400 x *g* for about 5-10 seconds and the level of supernatant was monitored to prevent drying of the resin. After two washes with 100% methanol, the column was washed four times using 50 µl of 0.1% (v/v) TFA, 70% (v/v) acetonitrile in water and centrifuged at 400 x *g* for about 5-10 seconds. After the third wash, the Eppendorf tube was changed for a new one and the column was washed four times with 0.1% (v/v) TFA. The digested peptides (see section 2.4.1) were loaded onto the column and centrifuged at 400 x *g* for 5-10 seconds. After peptide binding, the column was again washed four times with 0.1% (v/v) TFA and transferred to a new tube. Peptides were eluted using 200 µl of 70% (v/v) acetonitrile in water by centrifugation, dried in a MiVac vacuum centrifuge (Barnstead, GeneVac, Suffolk, UK) and analysed by mass spectrometry.

2.4.3 Mass spectrometry sample preparation and protein identification after FASP method

Samples for mass spectrometry analysis were prepared according to the protocol for Filter Aided Sample Preparation method (see sections 2.4.1 and 2.4.2). Peptide samples were analysed with a nanoelectrospray enabled Agilent 6510 Q-TOF mass spectrometer interfaced with an enrichment column (Michrom Bioresources Inc, Cap TrapTM 500 nl volume) and separation column (Agilent 150 mm length, 73 µm diameter Zorbax 300SB-C18 5 µm particles) and driven by Agilent Technologies 1200 series nano/capillary LC system. The system was

controlled by MassHunter Workstation Data Acquisition (ver B.02.00. Build 1128.5, Patches 1, 2, 3, Agilent Technologies). Peptides were loaded onto the enrichment column at 7 $\mu\text{l}/\text{min}$ for 3 minutes in 1% (v/v) acetonitrile (ACN) and 0.1% (v/v) formic acid with the trapping column set to enrichment using the capillary pump. At 3.1 minutes an inbuilt switching valve was set to separation, and peptides were eluted directly into the Q-TOF during a 60 minutes gradient (1–60% ACN) at 0.4 $\mu\text{l}/\text{min}$ using the nano pump. ACN was then increased to 99% at 65 minutes and maintained at this concentration until 75 minutes. At 75.1 minutes, gradients were set to initial conditions and re-equilibrated for 20 minutes. The Q-TOF was run in positive ion mode using source settings as suggested by Agilent and with MS scans from 360–2000 m/z at 2 scans/s and with MS/MS scans set from 60–2400 m/z at 1 scan/s. Top 3 precursor ions were selected for auto MS/MS at an absolute threshold of 500 counts and a relative threshold of 0.01%, with maximum of three precursors *per* cycle, and active exclusion set at 2 spectra and released after 1 minute. Precursor ion charge-state selection and preference were set to 2, 3 and >3 precursors, respectively and selection was by charge then abundance. Q-TOF collision energy settings were adjusted for a slope of 3.6 volts (V) per 100 Daltons with an offset of minus 4.8V. Resulting MS/MS spectra were opened in MassHunter Workstation Qualitative Analysis (ver B.02.02, Build 2.0.197.0, Patches 3 Agilent Technologies) and MS/MS compounds exported as *Mascot generic files* which were then searched using the Global Proteome Machine search engine using both XTandem! and XHunter! algorithms. The search results are stored on the GPM database (www.thegpm.org, index search numbers GPM32100033377 and GPM20100004555, respectively).

2.5 Cell biology methods

2.5.1 Tissue culture techniques

2.5.1.1 Cryopreservation

Cells were removed from flasks by treatment with 1 x trypsin in PBS for 3 minutes. The cells were centrifuged at 160 x g for 5 minutes, counted and re-suspended at $1.5 \times 10^6/\text{ml}$ in cold freezing medium (see Table 2.1). The cell

suspension was transferred to pre-labelled 1.5 ml cryovials (Sarstedt AG, Nümbrecht, Germany) in 1 ml aliquots. To maintain cell membrane integrity, the cells were slowly cooled at a rate of approximately 1°C/minute by placing the cryovials in a Cryo 1°C Freezing Container-Mr Frosty (Nalgene[®], Sigma-Aldrich, Arklow, Ireland) containing 250 ml of 100% isopropanol and stored at -80°C.

2.5.1.2 Resuscitation

HeLa cells were resuscitated by rapid thawing of the cell at 37°C. 1 ml of pre-warmed medium was placed in a 15 ml sterile tube, and the cell suspension was added and incubated at 37°C for 1 minute. 2 ml of DMEM culture medium was then added, followed by incubation at 37°C for 1 minute. 15 ml of pre-warmed DMEM culture medium was placed in a 75 cm² flask, and the cell suspension was then added to the flask. The cells were incubated at 37°C and 5% CO₂. The culture medium was changed the following day.

2.5.1.3 Cell cycle synchronisation using double thymidine block

HeLa cells were grown in DMEM culture medium to subconfluent density. The following day, the cells were exposed to 2 mM thymidine for 18 hours at 37°C. After the first block, thymidine was removed by rinsing the cells three times with PBS and the culture was released into fresh medium for 9 hours at 37°C. After the indicated time, cells were blocked for a second time with 2 mM thymidine for a further 17 hours at 37°C. Treatment was terminated by washing cells with PBS three times and releasing synchronised cells into fresh medium to progress throughout the cell cycle. The cells were further collected at different time points and cell synchrony was monitored by flow cytometry of propidium iodide-stained cells.

2.5.1.4 Stable isotope labelling with amino acids in cell culture (SILAC) method

HeLa cells were grown in three sets of SILAC media (all purchased from Dundee Cell Products, Dundee, UK). Medium labelled medium contains ¹³C labelled arginine and deuterium labelled lysine amino acids (R6K4) and heavy

labelled medium contains ^{13}C and ^{15}N labelled arginine and ^{13}C and ^{15}N labelled lysine (R10K8). Control cells were grown in SILAC medium that contained unlabelled arginine and lysine amino acids (R0K0). All media were supplemented with filtered FCS (Dundee Cell Products, Dundee, UK). The cells were cultured for six cell divisions in labelled and unlabelled media to metabolically incorporate heavy amino acids into synthesised proteins. Growth media were replaced with fresh media every three days over a period of a week.

2.5.2 Detection of DNA synthesis by fluorescence microscopy

2.5.2.1 BrdU labelling

To detect DNA synthesis by immunofluorescence, cells growing on round coverslips (\O 13 mm, VWR International, Dublin, Ireland) were incubated with 30 μM BrdU (Sigma-Aldrich, Arklow, Ireland) for 15 minutes. After labelling, cells were rinsed twice with PBS and fixed with 70% cold ethanol for 30 minutes at room temperature. Ethanol excess was removed by rinsing the coverslips three times with PBS. At this point the coverslips could be stored at 4°C prior to immunostaining. The cells were incubated in 2 M HCl for 30 minutes at 37°C to fragment and denature DNA of the labelled cells. The cells were then washed three times with PBS and non-specific antibody binding sites were blocked with 1% (w/v) BSA in PBS for 30 minutes at room temperature. Cells were incubated with anti-BrdU antibody (see Table 2.2) for 30 minutes at 37°C . After three washes in PBS, a secondary anti-FITC conjugated antibody (see Table 2.3) was applied and coverslips were incubated in the dark for 30 minutes. DNA was counterstained with 1 $\mu\text{g/ml}$ of DAPI (Sigma-Aldrich, Arklow, Ireland), and the Superfrost slides (Menzer-Glasser-Fisher, Dublin, Ireland) were mounted in DABCO solution (see Table 2.1). The image analysis was carried out using an Olympus BX51 microscope with 60 x (NA 1.4) or 100 x objective (NA 1.35). Images were analysed using OpenLab software (version 5, Improvision, Emeryville, USA).

2.5.2.2 EdU labelling

For detection of DNA synthesis by fluorescence microscopy, cells growing on round coverslips (\O 13 mm, VWR International, Dublin, Ireland) were

incubated with 10 μ M EdU for 30 minutes at 37°C and fixed with an aqueous solution of 1% (v/v) formaldehyde for 10 minutes at room temperature. After PBS wash, cells were permeabilised with 0.3% (v/v) Triton X-100 in cold PBS for 15 minutes on ice and subsequently washed three times with 1% (w/v) BSA in PBS. Non-specific sites on the slide were blocked with 1% (w/v) BSA in PBS for 30 minutes at room temperature and coverslips were rinsed three times with PBS. The Click reaction was performed in PBS for 30 minutes at room temperature in the dark by adding to the cells Click reaction Mix I (see Table 2.1). After staining, cells were washed three times with 1% (w/v) BSA, 0.5% (v/v) Tween 20 in PBS. Nuclei was counterstained with 1 μ g/ml of DAPI (Sigma-Aldrich, Arklow, Ireland), and after three further washes, coverslips were mounted onto Superfrost slides (Menzer-Glasser-Fisher, Dublin, Ireland) in DABCO solution (see Table 2.1). Images were taken and analysed as previously described in section 2.5.2.1.

2.5.3 Flow cytometry analysis

2.5.3.1 Analysis of DNA content and DNA synthesis using propidium iodide and BrdU

2 x 10⁶ HeLa cells were incubated with 30 μ M BrdU for 30 minutes, re-suspended in 5 ml of PBS and fixed by drop wise addition of pre-chilled water solution of 70% (v/v) ethanol and stored at 4°C prior to flow cytometry analysis. The following day, cells were washed in pre-warmed DMEM culture medium to remove precipitated salt and DNA was then denatured using 2 M HCl supplemented with 0.7% (v/v) Triton X-100 in PBS for 30 minutes at room temperature. Next, cells were washed twice with PBS and incubated in the dark with primary anti-BrdU antibody for 30 minutes (see Table 2.2), washed again twice with PBS, followed by incubation for 20 minutes with secondary anti-FITC antibody (see Table 2.3). Before analysis, cells were re-suspended in 1 x PBS supplemented with 100 μ g/ml RNase A and 40 μ g/ml propidium iodide (BD Pharmingen, San Diego, USA). After incubation at room temperature for 20 minutes, optionally overnight in the dark, cells were analysed using a FACS Calibur (Becton Dickinson, San Jose, USA) and Cell Quest software (version 3.3, Becton Dickinson, Oxford, UK).

2.5.3.2 Analysis of DNA content and DNA synthesis using 7-AAD and 5'-BMA

2 x 10⁶ HeLa cells were labelled with 10 µM EdU for 30 minutes at 37°C. After incubation, cells were collected and fixed in an aqueous solution of 70% (v/v) ice-cold ethanol overnight at 4°C. The cells were then washed with 1% (w/v) BSA in PBS, followed by an additional wash in PBS. The Click reaction was performed in PBS for 30 minutes at room temperature in the dark by adding to the cells Click reaction Mix II (see Table 2.1), followed by addition of 10 volumes 1% (w/v) BSA, 0.5% (v/v) Tween 20 in PBS and incubated for a further 10 minutes. Later, cells were washed twice with PBS and incubated in the dark with primary anti-BrdU antibody for 30 minutes (see Table 2.2), washed again twice with PBS followed by incubation for 20 minutes with secondary anti-FITC antibody (see Table 2.3). Finally, after three washes with PBS, samples were re-suspended in 1 x PBS supplemented with 100 µg/ml RNase A and 40 µg/ml propidium iodide (BD Pharmingen, San Diego, USA) and incubated for an hour in the dark. Analysis was carried out using a FACS Canto (Becton Dickinson, San Jose, USA) and data were processed using BD FACS Diva software (version 6.1.2, Becton, Dickinson, Oxford, UK).

2.5.3.3 Analysis of DNA content and DNA synthesis using 7-AAD and 6-carboxyfluorescein-TEG azide

To analyse the cell cycle profile using 6-carboxyfluorescein-TEG azide cells were processed as described previously in section 2.5.3.2. Click reaction was performed with the Click Reaction Mix I (see Table 2.1) and after three washes with PBS, the cells were re-suspended in 1 ml of PBS containing 1.25 µg of 7-AAD (Invitrogen, Carlsbad, USA) as a DNA intercalator and incubated for an hour in the dark. Analysis was carried out as previously using a FACS Canto and BD FACS Diva software.

Chapter 3 Development of DNA mediated chromatin pull-down technique

3.1 Introduction

Chromatin immunoprecipitation (ChIP) methodology has revolutionised experimental approaches to study transcription, replication and DNA repair (Orlando et al., 1997). It allows the association occurring *in vivo* between defined proteins at specified loci to be investigated. The traditional ChIP method was modified and described here as DNA mediated chromatin pull-down (Dm-ChP) (see section 2.3.12). The Dm-ChP procedure involves several steps: protein to DNA cross-linking using formaldehyde, DNA shearing, protein pull-down, cross-link reversal and protein detection. Each of these steps was optimised individually as outlined in the following sections.

3.2 Preliminary steps for development of Dm-ChP method

3.2.1 Optimisation of the cross-link step

Cross-linking is required to stabilise DNA and protein interactions before subsequent analysis. The aim of cross-linking is to fix the protein of interest to its chromatin binding site. Proteins like histones are generally tightly associated with DNA and may not require cross-linking, but other DNA binding proteins have a weaker affinity and need to be cross-linked to avoid their dissociation from the chromatin during lysis. The cross-linking agent should possess several unique properties: a suitable cross-linking distance, controllable kinetics of cross-linking and ease of reversibility. Formaldehyde reversibly cross-links primary amino groups in proteins forming a CH₂ methylene cross-link bridge (Kiernan, 2000). It is one of the shortest cross-linkers known (Klockenbusch and Kast, 2010). The distance between cross-linked groups is in the proximity of 2.3-2.7 Å, suggesting an interaction at the range of van der Waals radii (Zeng et al., 2006). The formaldehyde cross-link can be reversed by a high temperature treatment (Orlando et al., 1997). Time of cross-linking is a critical parameter and excessive cross-linking can interfere with cell breakage during lysis, affect fragmentation

and solubilisation of the DNA during sonication. Therefore it is necessary to perform a time course experiment to optimise cross-linking conditions.

To optimise protein-DNA cross-linking, cells were treated with 1% (v/v) formaldehyde (see section 2.3.7), harvested at different times and analysed by western blotting using anti-PCNA antibody. Immunoreactive bands corresponding to the PCNA were detected in all samples analysed. Additionally, after fixation, the cross-linked form of the PCNA was detected in the chromatin fraction as an additional slower migrating band (Figure 3.1; lanes 2-6).

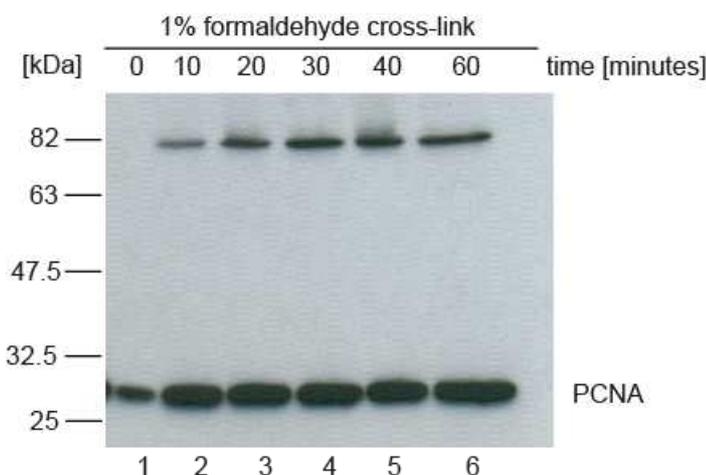


Figure 3.1 *Optimisation of protein-DNA cross-linking step.* Cells were treated with 1% (v/v) formaldehyde for different times, harvested and analysed by western blotting using anti-PCNA antibody.

The apparent molecular weight of the cross-linked form of PCNA corresponds to the homotrimeric form of protein. This form was not present in the control, non cross-linked cells (Figure 3.1; lane 1). The intensity of the band did not increase much across a defined time window, suggesting that 10-20 minutes fixation was possibly sufficient to generate the protein-DNA and protein-protein cross-links (Figure 3.1; lanes 2-6).

3.2.2 Preparation of chromatin enriched fraction

To eliminate cytoplasmic proteins that are not normally present in the nucleus but may interfere with subsequent analysis of chromatin and to further enrich for proteins associated with DNA, a cell fractionation was performed. We investigated whether the cross-linking step could affect the fractionation process.

We hypothesised that if a protein interacts loosely with DNA it should be enriched in the chromatin fraction after formaldehyde cross-linking.

To assess this, HeLa cells were either mock treated or incubated for 10 or 30 minutes at 4°C with 1% (v/v) formaldehyde. Cells were subsequently lysed in isotonic, low stringent CL buffer to extract soluble proteins. The residual pellet containing nuclei was resuspended in RIPA buffer to prepare the chromatin enriched fraction (see section 2.3.7.1). Proteins were separated on SDS-PAGE and analysed by western blotting using antibodies recognising the indicated proteins (Figure 3.2). Claspin, Cdc7, Mcm2, Cdc45 and RPA were chosen as examples of nuclear and chromatin associated proteins.

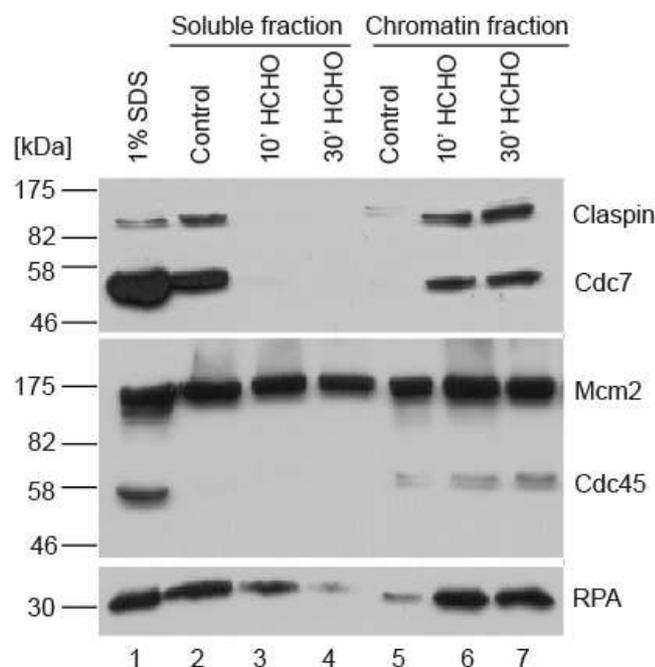


Figure 3.2 Preparation of chromatin enriched fraction. The soluble fraction was extracted using CL buffer, followed by chromatin preparation in RIPA buffer. Proteins were separated on SDS-PAGE and analysed by western blotting using antibodies recognising the indicated proteins.

We could detect all of the replication proteins analysed in the positive control extract prepared in 1% SDS. In the absence of cross-linking Claspin, Cdc7 and RPA were mostly extracted with CL buffer (Figure 3.2; lane 2). As predicted, following cross-linking these same proteins are recovered in the insoluble fraction (Figure 3.2; lane 6 and 7). On the contrary, Cdc45 was only detected in the insoluble fraction both in absence and presence of cross-linking. This suggests that in absence of cross-linking, Cdc45 is more tightly bound to the

chromatin and CL buffer is not stringent enough to release Cdc45 into the soluble fraction. Constant level of the Mcm2 throughout samples indicates that not all cellular proteins are cross-linking to DNA (Figure 3.2; lanes 1-7). We conclude that all of the replication proteins analysed can be easily retained in the insoluble fraction after cross-linking to DNA and that the cross-linking step helps to stabilise protein-DNA interactions.

3.2.3 Optimisation of sonication step

In the standard chromatin immunoprecipitation protocols the approximate size of DNA fragments ranges between 100-500 base pair in length. DNA fragments of this length ensure good resolution during analysis as they cover one to three nucleosomes. Our goal was to find the experimental conditions to generate DNA fragments of similar size.

To achieve this, HeLa cells were incubated for 10 minutes at 4°C with 1% (v/v) formaldehyde. The chromatin fraction was prepared in RIPA buffer and sonicated on ice for different periods of time from 0 to 70 seconds. Between each round of 10 seconds sonication, samples were allowed to cool down on ice for 2 minutes (see section 2.3.7.1). DNA was isolated, purified with phenol:chloroform: isoamyl alcohol extraction and separated on an agarose gel (see sections 2.2.1 and 2.2.2).

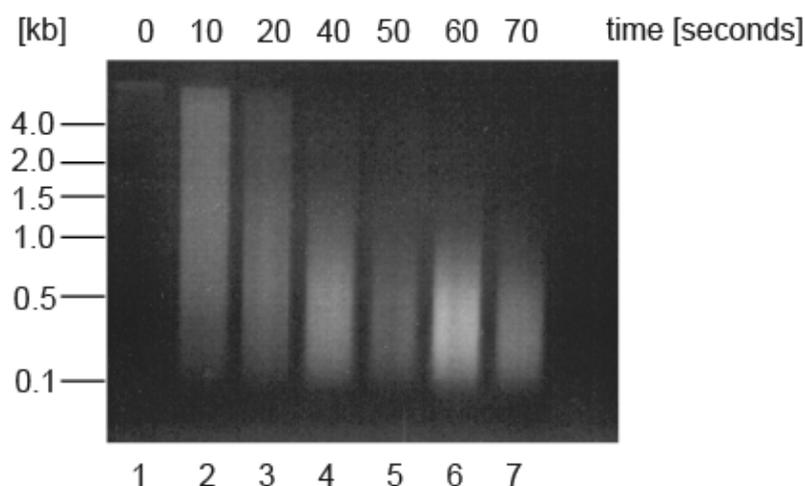


Figure 3.3 DNA shearing by sonication. Chromatin was sheared by sonication at 40% amplitude for up to 70 seconds. DNA fragments were isolated and after purification separated on an agarose gel.

Untreated DNA (Figure 3.3; lane 1) migrates slowly as a single band at the top of the gel, whereas during sonication DNA was sheared in a time-dependent manner and runs as a smear. Sixty seconds of sonication was sufficient to generate DNA fragments with the desired size of approximately 300-500 base pairs in length (Figure 3.3; lane 6).

3.2.4 Protein detection after DNA-protein cross-link reversal

To permit the recovery and analysis of proteins, the protein-DNA and protein-protein cross-linking must be reversed. This step includes treating the sample at high temperatures, but this has a potential to cause protein degradation. To investigate the effect of cross-linking reversal, chromatin fractions from cross-linked and mock treated cells (see section 2.3.7.1) were incubated at 65°C over a time course of 0-6 hours. PCNA was used as a marker to assess the cross-linking reversal.

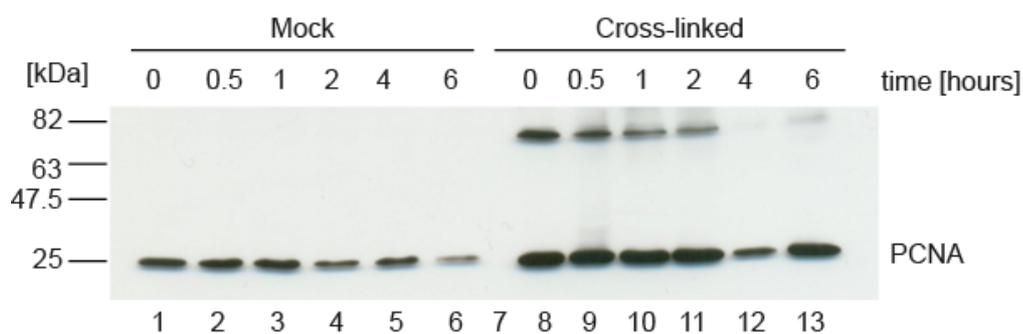


Figure 3.4 Detection of PCNA after reversal of cross-linking. Chromatin fractions from cross-linked and mock treated cells were incubated at 65°C for up to 6 hours. Proteins were separated on SDS-PAGE and analysed by western blotting using anti-PCNA antibody.

Western blotting analysis verified that we could detect PCNA in all of the samples analysed. PCNA from mock treated cells migrates as a single band, but after exposure to cross-linking agent, we could detect a slower migrating band, corresponding to the homotrimeric form of the protein. This band disappeared in a time-dependent manner (Figure 3.4; lanes 9-13). Incubation for 4-6 hours at 65°C was enough to reverse most of the formaldehyde cross-linking (Figure 3.4; lanes 12 and 13). The 6 hour time point was used in most of the future experiments. Alternatively, to reverse the action of formaldehyde samples can be incubated in 1 x *Laemmli* sample buffer (see Table 2.1) for 5 minutes at 95°C.

3.3 Chromatin pull-down of replication proteins

3.3.1 Immunoprecipitation of Mcm2

As a proof of concept that we can pull-down known replication proteins that interact with DNA under these experimental conditions, we performed an immunoprecipitation experiment using an antibody against Mcm2. Briefly, HeLa cells were either mock treated or cross-linked for 10 minutes with 1% (v/v) formaldehyde. The chromatin fractions from cross-linked and mock treated cells were prepared in RIPA buffer as previously described (see section 2.3.7.1) and subsequently incubated with control IgG or anti-Mcm2 antibodies for 2 hours at 4°C (see section 2.3.7.2). After immunoprecipitation, protein-DNA cross-linking was reversed by incubation for 6 hours at 65°C. Immuno-complexes were recovered by boiling for 3 minutes at 95°C in 1 x *Laemmli* sample buffer (see Table 2.1) and analysed by immunoblotting.

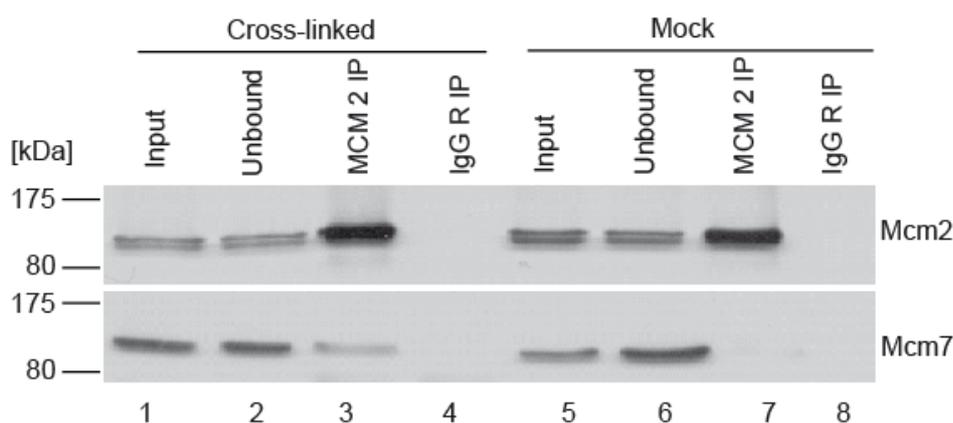


Figure 3.5 Immunoprecipitation of Mcm2 protein. Chromatin fractions from cross-linked and mock treated cells were incubated for 2 hours at 4°C with control IgG rabbit or anti-Mcm2 antibodies. After IP we reversed cross-link and analysed the input material (Input), material that did not bind to the anti-Mcm2 or IgG antibodies (Unbound) and material eluted from the beads (Mcm2 IP or IgG R IP) by western blotting using the indicated antibodies.

Mcm2 protein was successfully detected in the input and unbound samples from both cross-linked and mock treated cells (Figure 3.5, top panel; lanes 1, 2 and 5, 6). A similar amount of Mcm2 protein was immunoprecipitated in mock treated and cross-linked cells (Figure 3.5, top panel; lanes 3 and 7), but not when a control IgG rabbit was used (Figure 3.5, top panel; lanes 4 and 8).

Additionally, we looked at interactions between Mcm2 and other MCM proteins. Under the experimental conditions used, Mcm7, a known subunit of the MCM helicase complex, was detected in the input and unbound material from both cross-linked and mock treated cells (Figure 3.5, bottom panel; lanes 1 and 2). Using the anti-Mcm2 antibody we could co-immunoprecipitate Mcm7 from cross-linked cells (Figure 3.5, bottom panel; lane 3), but in mock treated cells this interaction was weaker, possibly because the MCM complex is disrupted during cell lysate preparation (Figure 3.5, bottom panel; lane 7). This result suggests that cross-linking stabilises the MCM helicase throughout the various stages of the experiment.

3.4 Immunoprecipitation of BrdU labelled DNA

The aim of my project was to capture newly synthesised DNA and analyse the protein components associated with it. To achieve this we first used 5-bromo-2'-deoxyuridine (BrdU) to label nascent DNA, followed by immunoprecipitation of chromatin with an anti-BrdU antibody (see Table 2.1).

Halogenated nucleosides, such as BrdU have been exploited as an alternative to [³H] thymidine-based cell proliferation studies (Yokochi and Gilbert, 2001). This permeable analogue is widely used to detect cellular DNA synthesis in a variety of organisms in both cell-based assays or *in vivo* models (Morstyn et al., 1983). Upon phosphorylation, BrdU is incorporated into the nascent DNA by the DNA polymerases (Kuebbing and Werner, 1975; Wlkramaslnghe, 1981; Zupanc and Horschke, 1996). The labelled DNA is then detected by using specific antibodies raised against halogenated nucleosides (Gratzner, 1982).

3.4.1 Detection of BrdU incorporation in HeLa cells

To detect DNA synthesis, HeLa cells were grown on coverslips and labelled for 30 minutes with 30 μ M BrdU (see section 2.5.2.1). Replication foci were detected by immunofluorescence using an anti-BrdU antibody coupled to a secondary FITC-fluorescein fluorophore (Figure 3.6, green fluorescence; middle panel). To visualise the nuclei, DNA was stained using DAPI (see Table 2.1)

(Figure 3.6, blue fluorescence; left panel). The right panel shows overlaid images (Figure 3.6; right panel).

We observed a fraction of cells positive for BrdU staining and this corresponded to actively replicating cells (Figure 3.6). Moreover, dispersed patterns of replication foci throughout the nucleus were observed in cells that incorporated BrdU (Figure 3.6, green fluorescence; middle panel). No green fluorescence was detected in non S phase, control cells (Figure 3.6, green fluorescence; right panel).

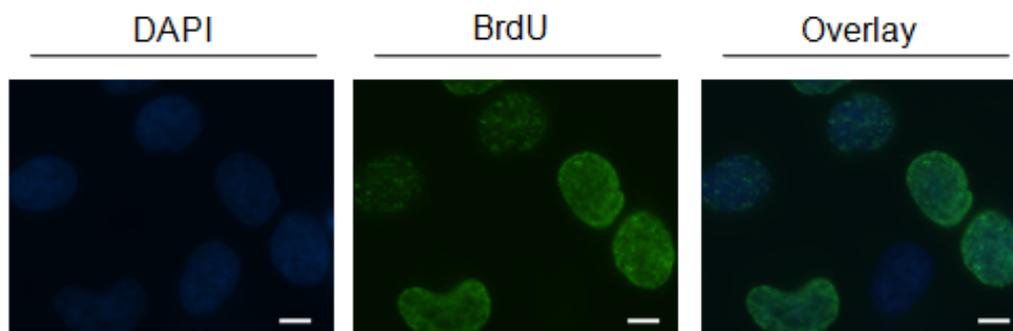


Figure 3.6 Immunofluorescence analysis of BrdU stained cells. Non-synchronised HeLa cells were grown on coverslips and incubated with BrdU for 30 minutes, followed by anti-BrdU antibody detection. DNA was visualised using DAPI. Left panel shows DNA nuclear staining (blue fluorescence), middle panel shows BrdU labelled DNA (green fluorescence) and right panel shows overlaid images (Overlay). (Scale bar 10µm).

We also used flow cytometry to further assess the incorporation of BrdU into nascent DNA (see section 2.5.3.1). Cells were labelled for 30 minutes with 30 µM BrdU, fixed and stained using anti-BrdU and anti-FITC antibodies (Figure 3.7). DNA content was determined by staining DNA with propidium iodide (PI). A characteristic DNA content histogram for an asynchronous population of HeLa cells is presented (Figure 3.7; left panel). Cells in G₁, G₂ and M phases have a 2n and 4n DNA content, respectively, with S phase cells lying in between these two DNA content markers (Figure 3.7; left panel). To specifically detect BrdU positive cells, DNA content was plotted against FITC fluorescence using scatter plots. We detected background levels of fluorescence in mock treated cells (Figure 3.7, middle panel), whereas the population of cells that incorporated BrdU showed a characteristic fluorescence pattern known as “horseshoe” (Figure 3.7, right panel). The number of cells in S phase was

expressed as a percentage of total cells analysed (Figure 3.7. right panel). Microscopy and flow cytometry analysis were routinely used to monitor DNA synthesis in HeLa cells.

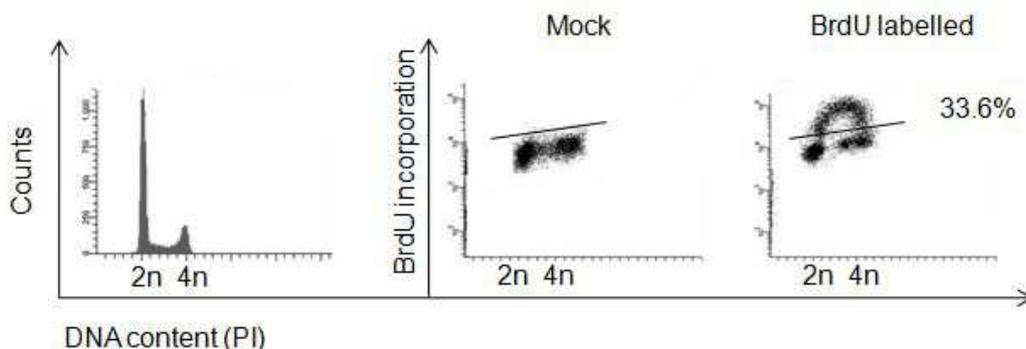


Figure 3.7 DNA content and BrdU incorporation using flow cytometry. HeLa cells were labelled for 30 minutes with 10 μ M BrdU and later analysed by flow cytometry for its incorporation. DNA content was measured using the PI. The percentage of BrdU positive cells is indicated in the figure.

3.4.2 Immunoprecipitation of BrdU labelled, naked DNA

To assess if BrdU labelled DNA can be immunoprecipitated using an anti-BrdU antibody, HeLa cells were incubated with 30 μ M BrdU for 24 hours, the chromatin fraction was prepared in RIPA buffer as described previously (see section 2.3.7.1) and DNA was purified with phenol: chloroform: isoamyl alcohol extraction and ethanol precipitation (see section 2.2.1). To allow epitope exposure for antibody recognition, DNA was denatured by heat shock and used in immunoprecipitation using either anti-BrdU or control IgG antibodies (see section 2.3.8). After the immunoprecipitation, the captured DNA was eluted from the beads in elution buffer (see Table 2.1). To detect if DNA was present in the immunoprecipitated samples, we used a PCR-based assay that relies on amplification of DNA fragments using primers specific to a 300 bp actin fragment (see section 2.2.4). Amplified DNA was subsequently separated by electrophoresis on a 1.5% agarose gel.

Gel electrophoresis demonstrated that DNA containing the target 300 bp actin sequence was present in all samples from input, unbound and immunoprecipitated material (Figure 3.8, lanes 1-7). The labelled DNA was captured with anti-BrdU antibody (Figure 3.8, lane 4), but also with control IgG

antibody (Figure 3.8, lane 7). Therefore we concluded that non-specific capturing of DNA occurred during immunoprecipitation of BrdU labelled DNA.

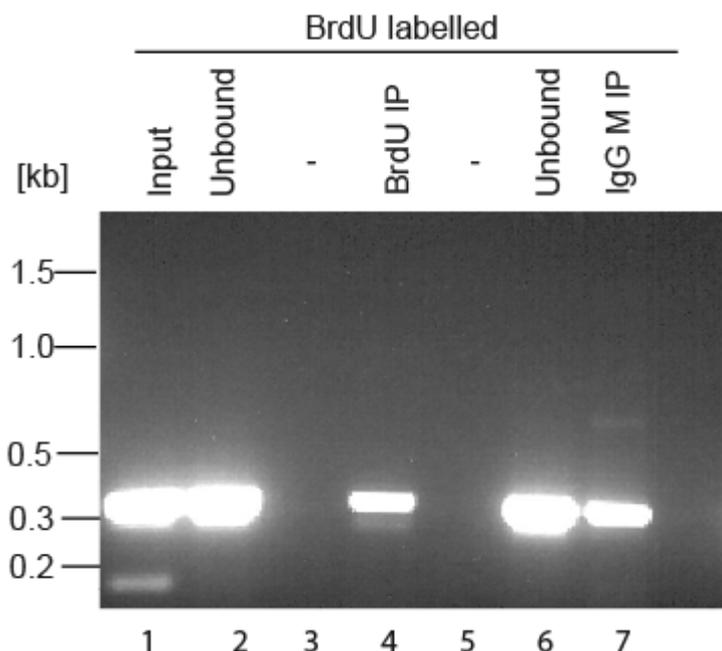


Figure 3.8 Detection of anti-BrdU immunoprecipitated DNA by PCR. After IP, purified DNA from input material (Input), material that did not bind to the anti-BrdU or IgG mouse antibodies (Unbound) and material eluted from the beads (BrdU IP or IgG M IP) was amplified by PCR reaction using primers specific for a 300 bp actin fragment and then separated onto 1.5% agarose gel.

To minimise non-specific binding of DNA, Protein A-agarose beads were blocked overnight using 0.4 mg/ml pre-sheared salmon sperm DNA and the pre-blocked resin was subsequently used in the immunoprecipitation experiment. After elution, captured DNA and salmon sperm DNA either on their own (Figure 3.9; lanes 2 and 3) or as a mixture (Figure 3.9; lane 1) were used in the PCR reaction with the same actin primers. Similarly, the 300 bp actin fragment was amplified in all samples. We observed that pre-sheared salmon sperm DNA used for Protein A-agarose beads blocking was also a template in the PCR reaction (Figure 3.9; lane 3). Due to the above experimental limitations we switched to an alternative method for detection of naked, labelled DNA after immunoprecipitation procedure.

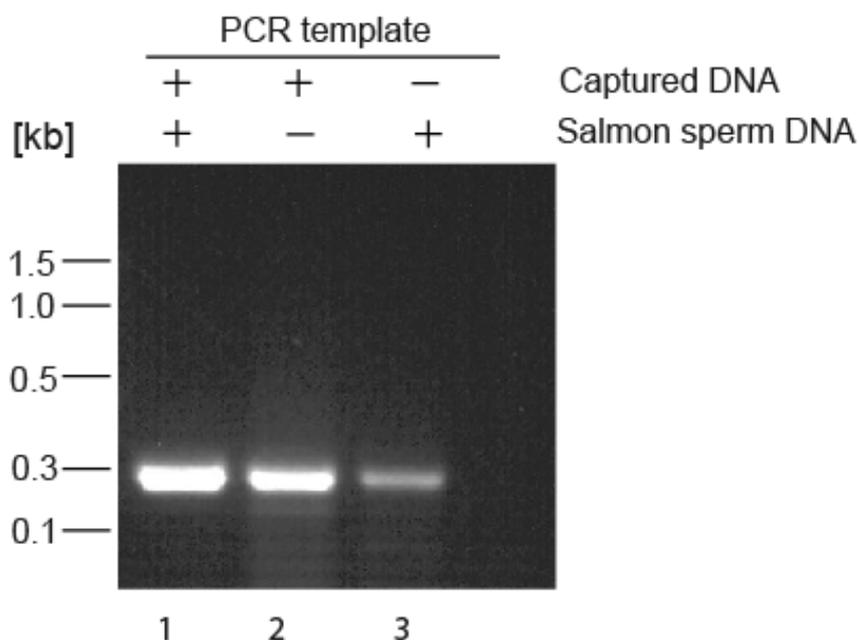


Figure 3.9 Amplification of an actin fragment by PCR. Immunoprecipitation of labelled DNA was performed using anti-BrdU antibody conjugated to Protein A-agarose beads either pre-blocked with pre-sheared salmon sperm DNA or not. Eluted DNA and salmon sperm DNA either in the mixture or alone were used as a template for PCR reaction using primers specific for a 300 bp actin fragment and then separated onto 1.5% agarose gel.

We abandoned this PCR-based assay in favour of a Southwestern blotting-based assay. DNA labelling and immunoprecipitation were performed as described above. After capture, the DNA was eluted from the beads in elution buffer (see Table 2.1), resolved by electrophoresis on a 1.5% agarose gel and transferred by capillarity onto a nylon membrane (see section 2.2.3). The membrane was subsequently blocked and probed with the anti-BrdU antibody (see Table 2.1) similar to western blotting. Gel electrophoresis and SYBR Safe DNA gel staining showed that DNA was present in the input and unbound samples from both BrdU labelled and mock treated cells. However, we observed a significantly lower amount of DNA isolated from mock treated cells. We could not detect immunoprecipitated DNA in the pulled-down samples (Figure 3.10 a). This might be due to a low amount of pulled-down DNA fragments which are below the limit of detection for this technique. After the transfer onto the nylon membrane and the probing with anti-BrdU antibody, BrdU labelled DNA was detected in the input and unbound samples from BrdU labelled cells, but not in mock treated cells (Figure 3.10 b; lanes 1, 2 and 6, 7). Importantly, labelled DNA fragments were recovered when the immunoprecipitation was performed with the

chromatin was used as starting material for the immunoprecipitation. HeLa cells were either mock treated or labelled with 30 μ M BrdU for an hour. After formaldehyde fixation, cells were harvested and DNA was denatured using 2M HCl supplemented with 1% (v/v) Triton-X 100 (see section 2.3.9). The chromatin fractions were prepared in RIPA buffer (see section 2.3.7.1). As a last step, labelled DNA was immunoprecipitated using anti-BrdU or IgG antibodies and protein complexes were analysed by western blotting (see section 2.3.9). We hypothesised that using this approach we should be able to detect proteins closely associated with the DNA molecule such as histones, and for this reason histone H2A was used as a probe.

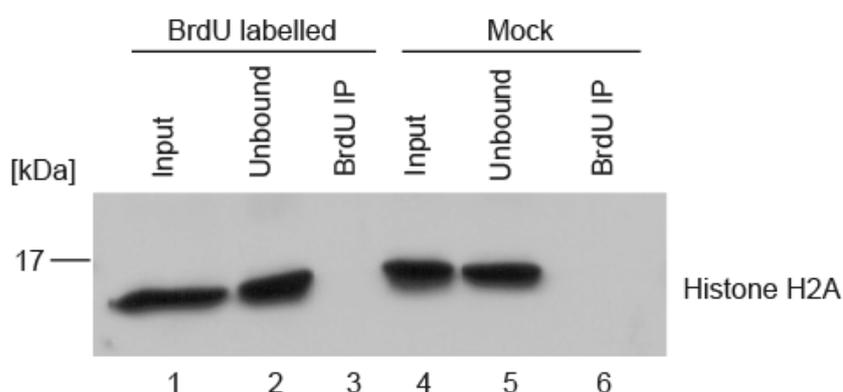


Figure 3.11 Immunoprecipitation of newly synthesised chromatin using anti-BrdU antibody. DNA from chromatin fraction was denatured with 2M HCl, followed by immunoprecipitation of labelled DNA using anti-BrdU antibody. Input material (Input), material that did not bind to anti-BrdU antibody (Unbound) and material eluted from the beads (BrdU IP) were analysed by western blotting using histone H2A antibody.

After immunoblotting, histone H2A was detected in the input and unbound fractions from either BrdU labelled or mock treated cells (Figure 3.11; lanes 1, 2 and 4, 5). Unfortunately, histone H2A was not recovered in the immunoprecipitation using anti-BrdU antibody neither in the BrdU labelled or mock treated samples, suggesting that we failed to pull-down BrdU labelled chromatin using this approach (Figure 3.11; compare lanes 3 to 6).

Taking these data together we concluded that labelled, naked DNA, but not labelled DNA from the chromatin fraction, can be immunoprecipitated under our experimental conditions. This may reflect either failure of the DNA denaturation step or the inability of the anti-BrdU antibody to recognise DNA under such conditions.

3.5 DNA mediated chromatin pull-down methodology

Due to unsuccessful immunoprecipitation of BrdU labelled DNA from the chromatin fraction we decided to explore a different nucleotide derivative called 5-ethynyl-2'-deoxyuridine (EdU). EdU is a thymidine-like nucleotide that is incorporated into DNA during S phase of the cell cycle very efficiently. Detection of EdU is based on a Huisgen-Sharpless 1, 4 cycloaddition reaction (also known as Click reaction), which is a copper (I) catalysed transformation between an azide and an alkyne moieties (Moses and Moorhouse, 2007).

The EdU contains the alkyne functional group which can be covalently coupled to an azide-containing molecule such as a fluorochrome, biotin or 5'-BMA (5-bromo-5'-azido-2',5'-dideoxyuridine) to form a stable, triazole ring (Cappella et al., 2008). As this reaction occurs under mild conditions and it does not requires the denaturation of double-stranded DNA proteins are not adversely affected (Buck et al., 2008) (Figure 3.12).

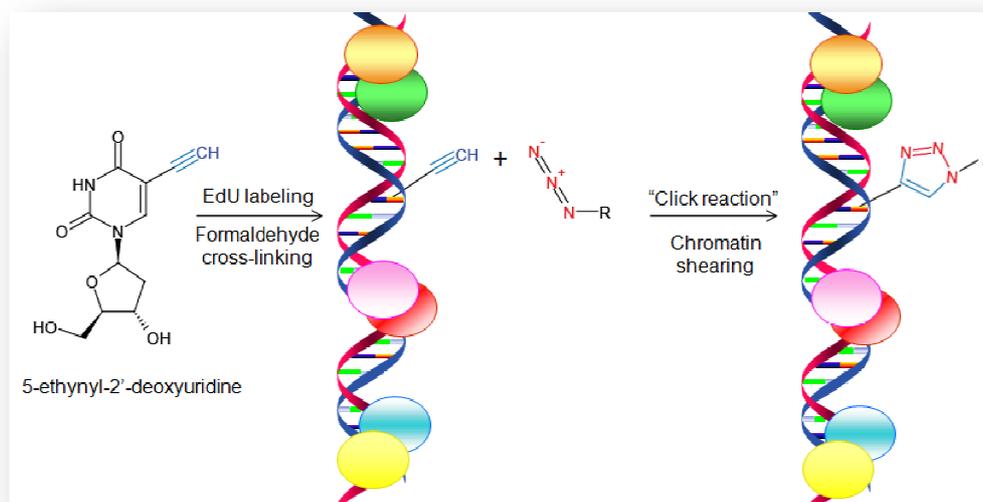


Figure 3.12 General strategy for tagging of newly synthesised chromatin with EdU. 5-ethynyl-2'-deoxyuridine is used to label newly replicating DNA. Next, azide-containing reagent is selectively linked to the reactive alkyl group of EdU containing DNA through Click reaction.

3.5.1 Detection of EdU incorporation in HeLa cells

To confirm that EdU can be used as an alternative method for DNA labelling, HeLa cells were grown on coverslips and pulsed for 30 minutes with 10 μ M of EdU. Cells were fixed with formaldehyde and permeabilised. Upon

Click reaction the EdU incorporated into the DNA was linked to a 6-carboxyfluorescein-TEG azide molecule (see section 2.5.2.2). Coverslips were then washed with PBS and nuclei were stained with DAPI (Figure 3.13, blue fluorescence; left panel).

As before (see section 3.4.1) we observed a fraction of cells positive for EdU staining and this corresponded to actively replicating cells (Figure 3.13). The characteristic patterns of replication foci dispersed throughout the nucleus were observed in cells that incorporated EdU in early S phase, whereas replication foci patterns typical for cells in middle/late S phase shown perinuclear and nucleolar staining (Anachkova et al., 2005; Dimitrova and Gilbert, 1999) (Figure 3.13, green fluorescence; middle panel).

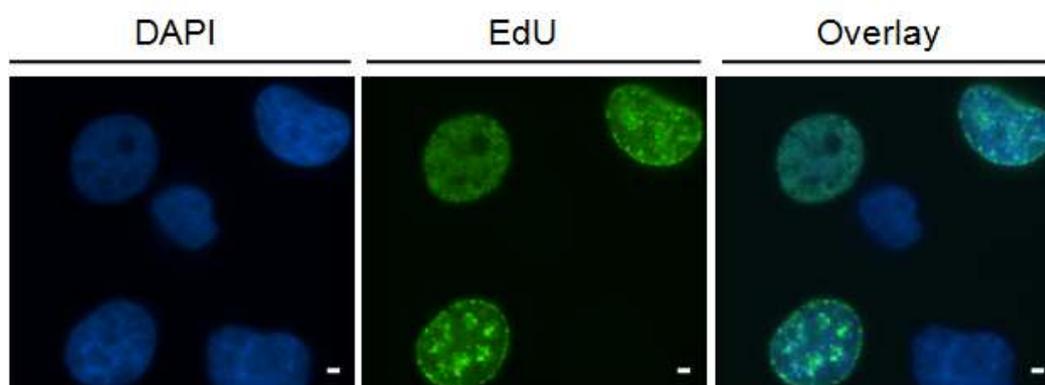


Figure 3.13 Analysis of DNA synthesis using EdU and 6-carboxyfluorescein-TEG azide. HeLa cells grown on coverslips were incubated with EdU for 30 minutes and afterwards EdU was conjugated to 6-carboxyfluorescein-TEG azide by Click reaction. DNA was visualised using DAPI. Left panel shows DNA nuclear staining (blue fluorescence), middle panel shows replication foci of EdU labelled DNA (green fluorescence) and right panel shows overlaid images (Overlay). (Scale bar 10 μ m).

We also used flow cytometry to assess incorporation of EdU into nascent DNA. HeLa cells were incubated with 10 μ M EdU for 30 minutes and fixed with ice-cold 70% (v/v) ethanol (see section 2.5.3.3). Upon Click reaction the EdU was coupled to 6-carboxyfluorescein-TEG azide and DNA was stained with 7-amino-actinomycin D (7-AAD).

A characteristic DNA content histogram for an asynchronous population of HeLa cells is presented by plotting cell count against 7-AAD content (Figure 3.14, left panel). To specifically detect S phase EdU positive cells, 7-AAD

content was plotted against 6-carboxyfluorescein-TEG azide fluorescence using scatter plots. We detected background levels of fluorescence in mock treated cells (Figure 3.14, middle panel), whereas the population of cells that incorporated EdU showed a characteristic pattern known as “horseshoe” (Figure 3.14, right panel). The number of cells in S phase was expressed as a percentage of total cells analysed (Figure 3.14, right panel).

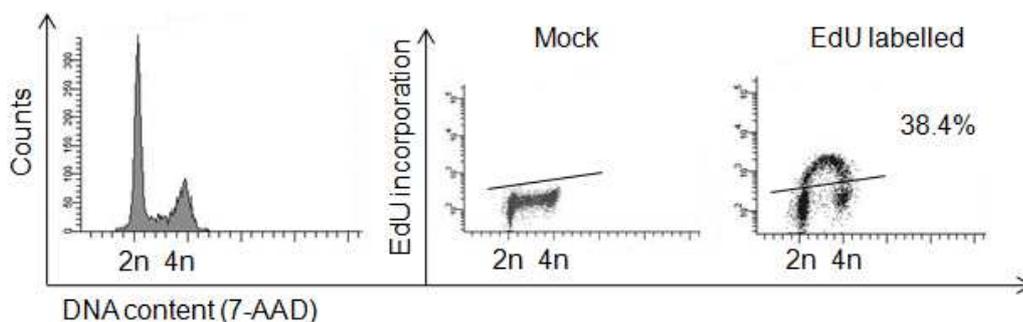


Figure 3.14 Detection of DNA content and EdU incorporation using flow cytometry. HeLa cells were incubated with 10 μ M EdU for 30 minutes and EdU incorporation into the DNA was detected by flow cytometry after Click reaction with 6-carboxyfluorescein-TEG azide. DNA content was measured using the DNA intercalator 7-AAD. The percentage of EdU positive cells is indicated in the figure.

Microscopy and flow cytometry analysis confirmed that DNA labelling using EdU was detected *in vivo*. EdU was successfully incorporated into newly replicated DNA undergoing semi-conserved synthesis and could be detected by covalent linkage to a fluorochrome through a specific Click chemistry reaction. We compared EdU and BrdU protocols and observed that data obtained are identical and in addition EdU approach was faster and did not required denaturation of DNA.

3.6 Capturing of EdU labelled DNA from chromatin enriched fraction

To address the question whether EdU labelled DNA can be recovered from the chromatin fraction, two approaches were investigated. Firstly, EdU labelled DNA was covalently linked to BrdU azide probe called 5-bromo-5'-azido-2',5'-dideoxyuridine (5'-BMA) through Click reaction (Cappella et al., 2008) and chromatin was captured using an anti-BrdU antibody (Figure 3.15). The second

approach involves the covalent linkage of biotin-TEG azide. Recovery of newly replicated chromatin was achieved by streptavidin-coated resin (Figure 3.18).

3.6.1 Immunoprecipitation of EdU labelled, naked DNA using 5'-BMA azide

First we investigated if EdU labelled, naked DNA can be coupled to the 5'-BMA molecule and further immunoprecipitated using anti-BrdU antibody (Figure 3.15). To assess that, HeLa cells were either mock treated or incubated for 24 hours with 5 μ M EdU and proteins were cross-linked to DNA with formaldehyde. Cells were permeabilised and the EdU was coupled to a 5'-BMA molecule through Click chemistry reaction (Figure 3.15). Cells were lysed in isotonic buffer, followed by nuclei extraction in RIPA buffer and DNA shearing by sonication (see section 2.3.7.1). DNA molecules were further purified as previously described (see section 2.2.1).

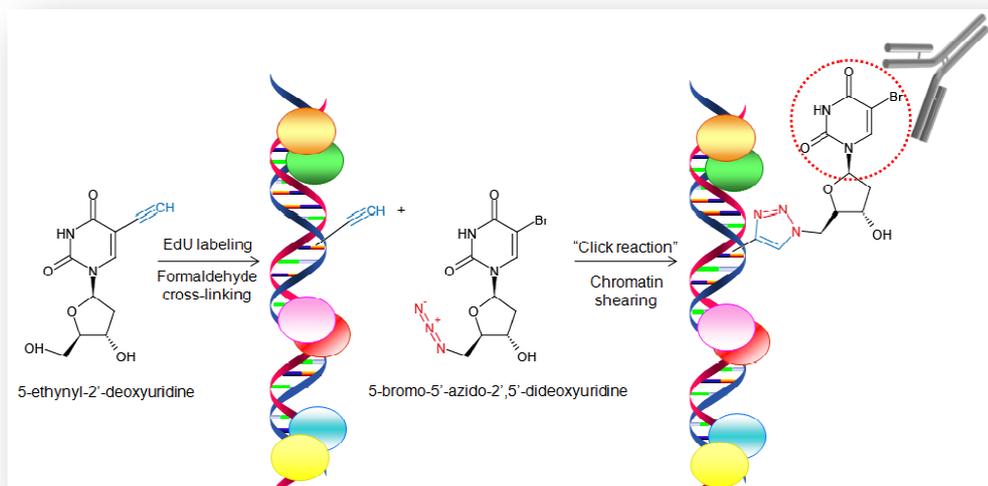


Figure 3.15 Strategy for tagging and capturing of newly synthesised chromatin using 5'-BMA azide. EdU is used to label newly replicating DNA. After protein DNA-cross-linking, 5'-BMA azide is selectively linked to the reactive alkyl group of EdU containing DNA. After DNA shearing small fragments of chromatin are captured using anti-BrdU antibody.

Finally, labelled DNA was immunoprecipitated using anti-BrdU or IgG antibodies pre-bound to Protein A-agarose beads (see section 2.3.10). DNA was eluted from the resin by incubation in elution buffer (see Table 2.1). DNA was separated on a 1.5 % agarose gel (Figure 3.16 a), subsequently transferred onto

nylon membrane and probed with anti-BrdU antibody (see section 2.2.3) (Figure 3.16 b). After staining the agarose gel with SYBR Safe we detected DNA in the input and unbound samples from both EdU labelled and mock treated cells.

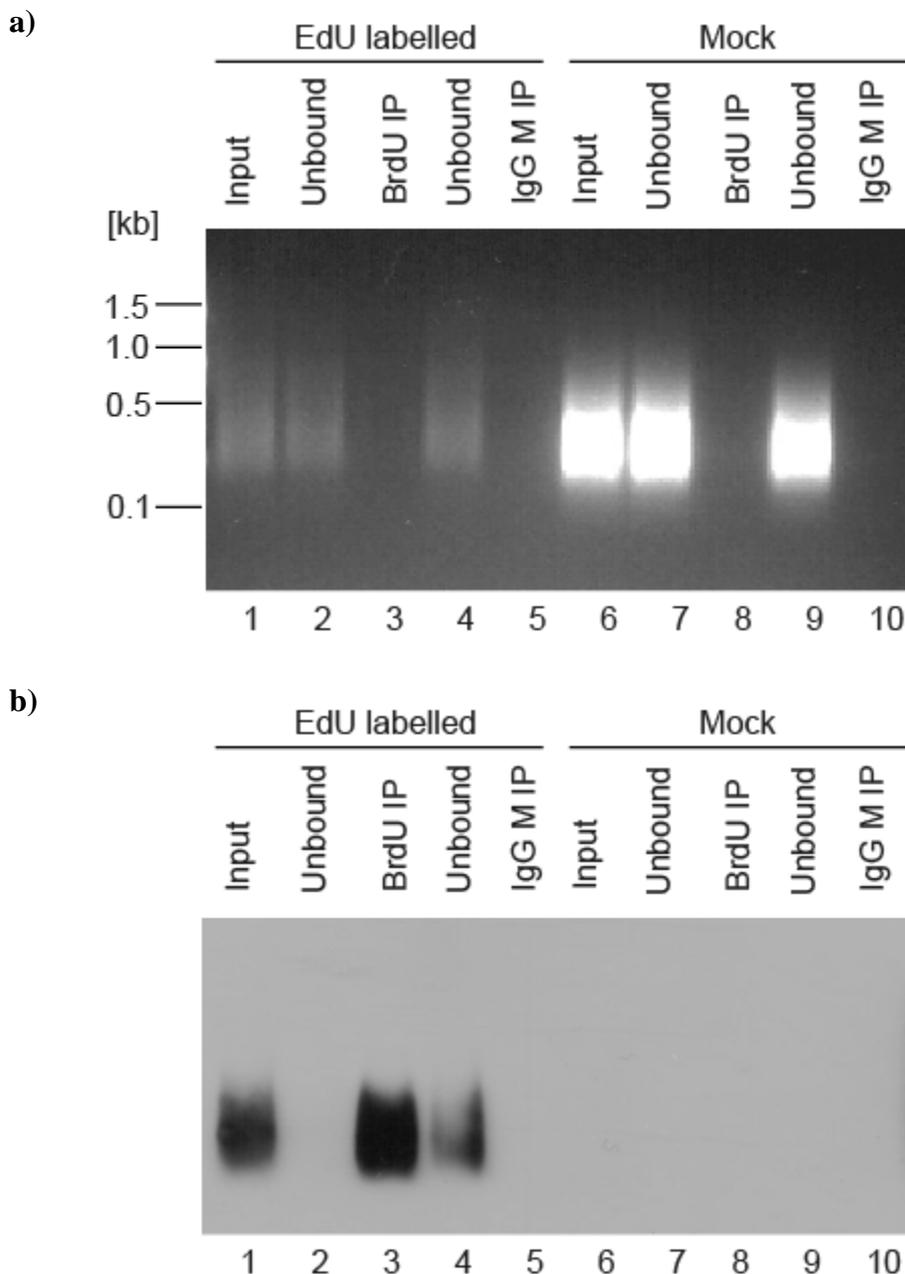


Figure 3.16 Immunoprecipitation of EdU labelled, naked DNA. DNA prepared from either EdU labelled or mock treated cells was purified from the input material (Input), material that did not bind to the anti-BrdU or IgG antibodies (Unbound) and material eluted from the beads (BrdU IP or IgG M IP) **a)** DNA fragments were electrophoresed on agarose gel and visualised by SYBR Safe **b)** DNA was then transferred onto nylon membrane and EdU labelled DNA tagged with 5'-BMA was detected using anti-BrdU antibody.

We observed a significantly lower amount of DNA isolated from EdU labelled cells. We could not detect DNA in immunoprecipitated samples (Figure 3.16 a). This again could be due to the low amount of recovered DNA and the detection limit of SYBR Safe. After transferring the DNA from the gel onto the nylon membrane and blotting with anti-BrdU antibody, EdU labelled DNA linked to 5'-BMA was detected in the input sample from EdU labelled (Figure 3.16 a; lane 1), but not from mock treated cells (Figure 3.16 b; lanes 6 and 7). We observed that labelled DNA was efficiently depleted from unbound sample probably due to the low amount of labelled DNA in the starting material (Figure 3.16 b; lane 2). Labelled DNA fragments were recovered with anti-BrdU antibody only when the cells previously incorporated EdU (Figure 3.16 b; lane 3), but not in control IgG (Figure 3.16 b; lane 5) or mock treated cells immunoprecipitation (Figure 3.16 b; lanes 8 and 10). Since we observed unlabelled DNA in the unbound sample (Figure 3.16; compare lanes 2 a and b) it is likely that 24 hours labelling with 5 μ M EdU was not sufficient to label all DNA. Alternatively, it is possible EdU labelled DNA was in excess to 5'-BMA azide during Click reaction. From these experiments we concluded that combination of EdU and 5'-BMA allows naked, labelled DNA immunoprecipitation using anti-BrdU antibody.

3.6.2 Immunoprecipitation of EdU labelled DNA from chromatin fraction using 5'-BMA azide

To test whether EdU labelled 5'-BMA tagged newly synthesised chromatin can be purified, HeLa cells were either mock treated or incubated for an hour with 10 μ M EdU. Proteins were subsequently cross-linked to DNA using formaldehyde, permeabilised and the 5'-BMA azide was linked to EdU molecule through the Click reaction. Cells were lysed in CL buffer, followed by nuclei extraction in RIPA buffer (see Table 2.1 and section 2.3.7.1). DNA was sheared by sonication and finally, immunoprecipitations were performed using anti-BrdU or IgG antibodies previously bound to Protein A-agarose beads (see section 2.3.11). Beads were eluted by incubation in 1 x *Laemmli* sample buffer at 95°C, thus obtaining DNA and protein containing fractions that were subsequently analysed by western blotting using anti-histone H3 antibody.

We found that histone H3 was present in the input and unbound samples from both EdU labelled or mock treated cells (Figure 3.17; compare lanes 1, 2 to 6, 7). Histone H3 was not captured in the immunoprecipitation performed with chromatin prepared from mock treated cells (Figure 3.17; lanes 3 and 5). Unfortunately, histone H3 was non-specifically immunoprecipitated from chromatin fractions by either anti-BrdU or control IgG antibodies (Figure 3.17; lanes 8 and 10).

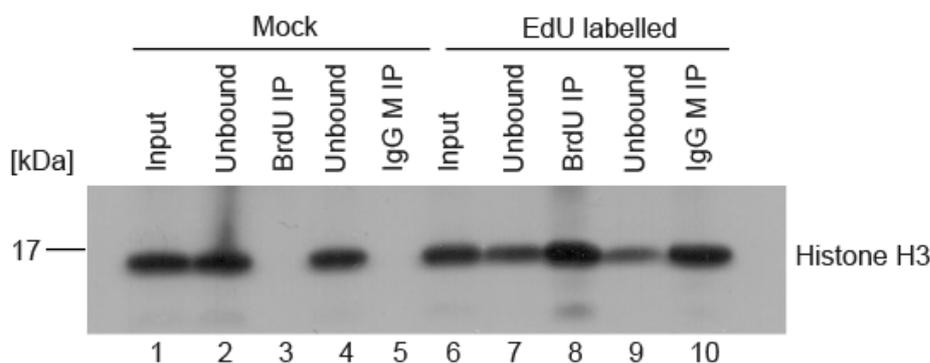


Figure 3.17 Immunoprecipitation of EdU labelled 5'-BMA tagged DNA from chromatin using anti-BrdU antibody. Chromatin fractions were prepared either from EdU labelled or mock treated cells. EdU labelled DNA was linked to 5'-BMA azide and immunoprecipitated using anti-BrdU or control IgG antibodies. The input material (Input), material that did not bind to anti-BrdU or IgG antibodies (Unbound) and material eluted from Protein A-agarose beads (BrdU IP or IgG M IP) were analysed by western blotting using anti-histone H3 antibody.

The EdU-5'-BMA-based approach was initially promising, but under our experimental conditions the non-specific binding of labelled DNA to Protein A-agarose beads was most likely the reason for the lack of specificity. Therefore we concluded that this method may not be suitable for studying protein content associated with newly synthesised DNA.

3.6.3 Capture of EdU labelled, naked DNA using biotin-TEG azide

Due to the technical problems described in the previous section we decided to use an alternative tagging molecule, biotin-TEG azide and capturing beads such as streptavidin-coated resin (Figure 3.18). To assess the feasibility and specificity of DNA mediated chromatin pull-down, logarithmically growing cells were either mock treated or incubated with EdU for 24 hours prior to collection.

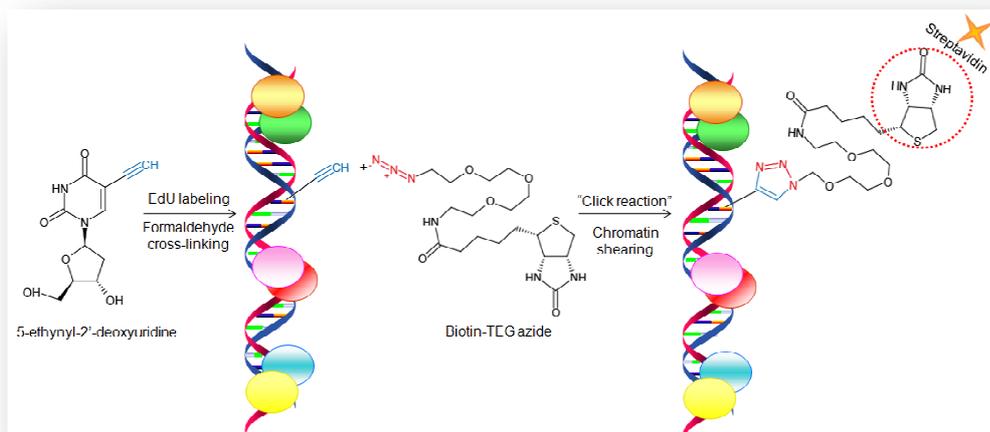


Figure 3.18 Schematic principles for tagging and capturing of newly synthesised chromatin using biotin-TEG azide. EdU is used to label newly replicating DNA. After protein DNA-cross-linking, biotin-TEG azide is selectively linked to the reactive alkyl group of EdU containing DNA. After DNA shearing small fragments of chromatin are captured using streptavidin-coated beads.

Chromatin fractions were prepared in RIPA buffer (see section 2.3.7.1) and DNA molecules were purified as previously described (see section 2.2.1). Finally, EdU labelled, biotin-TEG azide tagged DNA was captured using streptavidin-coated beads. DNA was eluted from the resin by incubation in elution buffer (see Table 2.1), resolved on a 1.5 % agarose gel and stained with SYBR Safe DNA gel stain (see section 2.2.2).

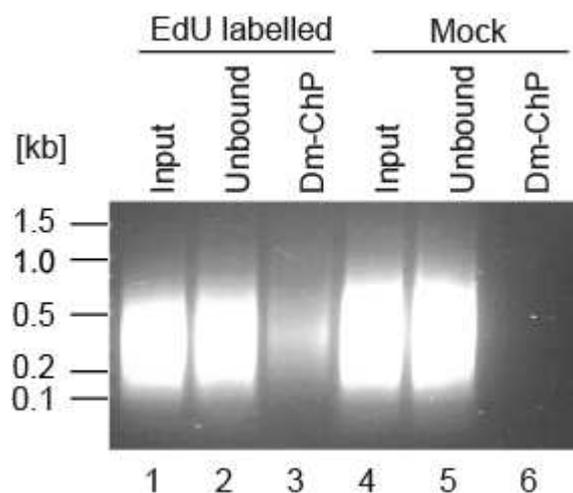


Figure 3.19 Pull-down of EdU labelled, naked DNA using biotin-TEG azide. Dm-ChP assay was performed from either EdU labelled or mock treated cells. Purified DNA fragments from the input material (Input), material that did not bind to streptavidin-coated beads (Unbound) and material eluted from streptavidin-coated beads (Dm-ChP) were separated on an agarose gel.

After staining of the gel, we detected DNA in the input and unbound samples from both EdU labelled and mock treated cells (Figure 3.19, compare lanes 1, 2 and 4, 5). We could also detect recovered DNA in pulled-down sample only when the cells were previously incubated with EdU (Figure 3.19, lane 3), but not in the mock treated cell pull-downs (Figure 3.19; lane 6). This is the first time that we observed a strong signal in the recovered sample using SYBR Safe, suggesting that biotin tagging and pull-down is more efficient than previous approaches.

To confirm the presence of biotinylated residues on this DNA, the recovered material was subsequently analysed by dot blot (see section 2.3.6). DNA from either mock treated or EdU labelled cells from the input or pulled-down material was spotted onto nitrocellulose membrane and analysed using anti-biotin HRP conjugated antibody (see Table 2.2). Biotinylated residues were detected only in the input and pulled-down samples from the cells previously labelled with EdU. No biotinylated DNA was present in the input and pulled-down samples from the mock treated cells (Figure 3.20).

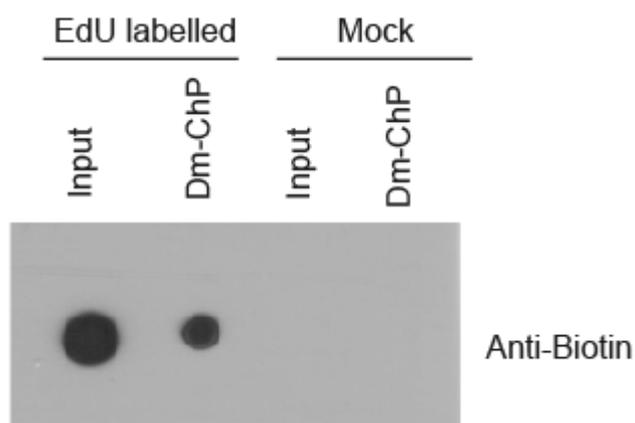


Figure 3.20 Detection of biotin conjugated to EdU in purified DNA. Purified DNA from extract (Input) and biotin pull-down (Dm-ChP) from either EdU labelled or mock treated cells was spotted onto nitrocellulose membrane and blotted with anti-biotin antibody.

Together these data suggest that the capture of purified DNA is dependent on EdU labelling and it is not due to non-specific binding to streptavidin-coated beads or aggregation during the pull-down step.

3.6.4 Capture of EdU labelled DNA from chromatin fraction

To test whether EdU labelled, biotin-TEG azide tagged chromatin can be captured using streptavidin-coated beads, HeLa cells were mock treated or incubated with 10 μ M EdU for an hour. After EdU labelling and formaldehyde protein-DNA cross-linking, cells were permeabilised and upon Click reaction the EdU incorporated into the DNA was linked to a biotin-TEG azide. Cells were lysed in CL buffer, followed by re-suspension of the nuclei in RIPA buffer and DNA fragmentation by sonication (see section 2.3.7.1). Finally, labelled DNA together with bound proteins was recovered using pre-blocked streptavidin-coated beads. Chromatin fragments were then eluted from the resin by incubation in 1 x *Laemmli* sample buffer at 95°C, a step that also reverses the formaldehyde cross-link, thus obtaining DNA and protein containing fractions (see section 2.3.12). Proteins present in the chromatin fractions from the input, unbound and streptavidin captured material were analysed by western blotting using anti-histone H3 antibody.

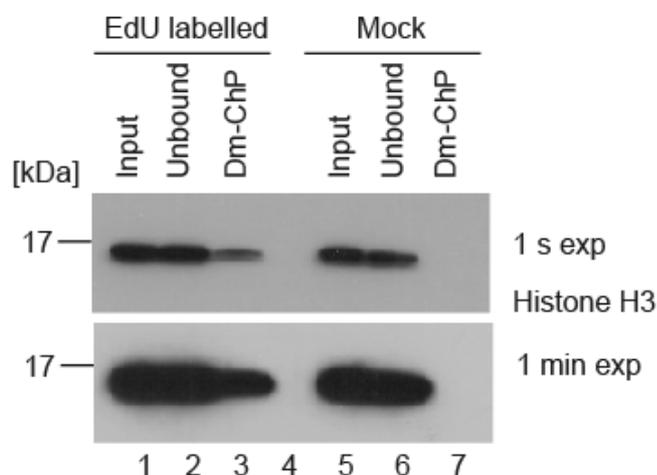


Figure 3.21 Dm-ChP analysis of EdU labelled chromatin. Dm-ChP assay was performed from either EdU labelled or mock treated cells and material from the input (Input), material that did not bind to streptavidin-coated beads (Unbound) or material eluted from streptavidin-coated beads (Dm-ChP) were analysed by western blotting using anti-histone H3 antibody. Two exposures of the same film are shown.

This analysis revealed that histone H3 was detected in the input and unbound fractions from both EdU labelled and mock treated cells (Figure 3.21; compare lanes 1, 2 and 5, 6). Histone H3 was pulled-down in EdU labelled, but

not detected in the mock treated cells (Figure 3.21; lanes 3 and 7) even after a prolonged exposure (Figure 3.21, bottom panel; lane 7). Thus, unlike in the 5'-BMA approach, using biotin tagging and streptavidin-coated beads the capturing of labelled chromatin was specific (Figure 3.21; lane 3).

Chapter 4 Characterisation of DNA mediated chromatin pull-down technology

4.1 Introduction

The DNA mediated chromatin pull-down methodology relies on incorporation of a dNTP analogue such as EdU, by active replication forks, into newly synthesised DNA. EdU labelled DNA can be linked to a biotin-TEG azide molecule allowing it to be affinity purified using streptavidin-coated resin. In Chapter 3 we provided evidences that the Dm-ChP methodology has been established and in principle can be applied to the capturing of proteins associated with EdU labelled DNA. In this chapter we further validate Dm-ChP technique aiming to assess its sensitivity and specificity.

4.2 Requirements for Dm-ChP

To pull-down labelled chromatin using streptavidin-coated beads, EdU tagged DNA needs to be covalently linked to a biotin derivative. This reaction is catalysed by copper (II) sulphate that needs to be *in situ* reduced to copper (I) by a reducing agent such as sodium ascorbate (Salic and Mitchison, 2008). The reaction requires an alkyne moiety (EdU labelling molecule), an azide group (biotin tag molecule) and copper (I) as a catalyst to be present in the mixture. To assess if all the reagents necessary for Click reaction are required for the binding of the labelled chromatin to the streptavidin-coated beads, HeLa cells were either mock treated or incubated with 10 μ M EdU for an hour. After EdU labelling and formaldehyde protein-DNA cross-linking, cells were permeabilised. The Click reaction was subsequently performed in the absence or presence of each particular component: EdU, biotin-TEG azide or copper (II) sulphate. The chromatin fraction was further extracted in RIPA buffer and DNA was fragmented by sonication. EdU labelled DNA fragments together with bound proteins were recovered using pre-blocked streptavidin-coated resin and eluted in 1 x *Laemmli* sample buffer at 95°C (see section 2.3.12). As previously described, histone H3 was used as a probe to detect chromatin.

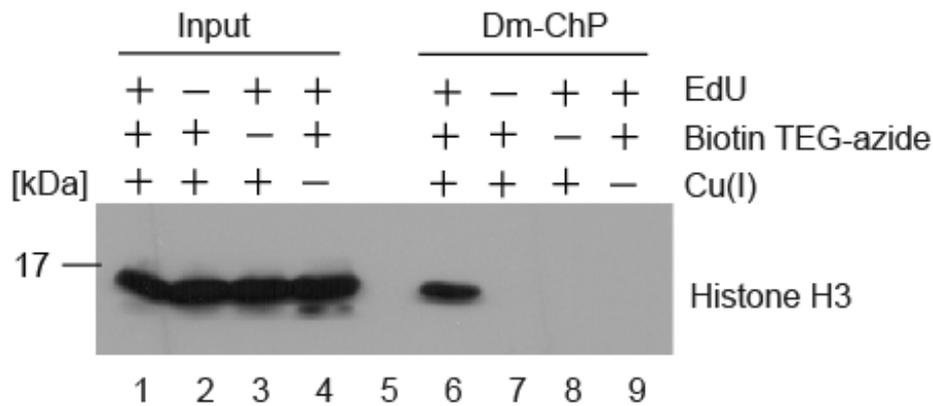


Figure 4.1 Requirements for DNA mediated chromatin pull-down procedure. *Dm-ChP* was performed in the presence (+) or absence (-) of the indicated component of the Click reaction. Material from the input (Input) and material eluted from streptavidin-coated beads (*Dm-ChP*) were analysed by western blotting using anti-histone H3 antibody.

Immunoblotting analysis demonstrated that histone H3 was present in the input from all samples (Figure 4.1; lanes 1-4). Histone H3 was only recovered in samples where all reagents necessary for the Click reaction were present (Figure 4.1; lane 6). The omission of EdU, biotin-TEG azide or copper (II) sulphate prevented chromatin capture as assessed by a lack of histone H3 in the pulled-down samples (Figure 4.1; lanes 7-9). These data indicate that pull-down of labelled chromatin is Click reaction-dependent.

4.3 Specificity of *Dm-ChP*

Using a non-synchronised cell population and short EdU labelling times, only a fraction of cells are in S phase and of these cells only a small proportion of cellular DNA is labelled with EdU. Therefore a chromatin extract prepared from these cells contains both labelled and unlabelled DNA.

Because of this, we asked the question if *Dm-ChP* specifically captures EdU labelled chromatin from a mixture of labelled and unlabelled chromatin. To assess this, we mixed extracts prepared from HeLa cells that along with normal histones also express a functional GFP-histone H3 fusion (Figure 4.2, green fluorescence; middle panel) with extracts from unmodified HeLa cells, either labelled or unlabelled EdU. Briefly, unmodified HeLa cells or HeLa cells expressing H3-GFP were either mock treated or incubated with 10 μ M EdU for an hour. Chromatin fractions were prepared as previously described (see section

2.3.7.1). An equal amount of extracts prepared from both cell lines was combined and labelled chromatin was recovered using pre-blocked streptavidin-coated beads (see section 2.3.12).

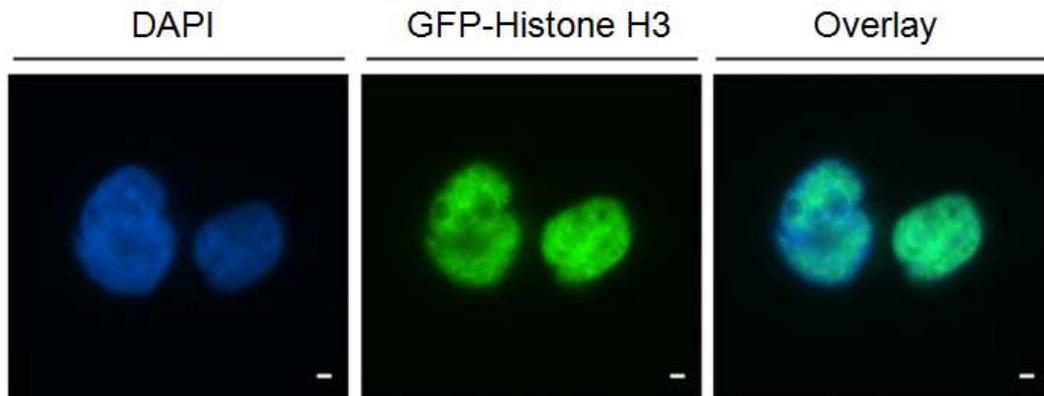


Figure 4.2 *Stable expression of GFP-histone H3 in HeLa cell line.* HeLa cells expressing GFP-histone H3 were visualised by fluorescence microscopy. Images show nuclear DNA stained with DAPI (blue fluorescence, left panel), GFP-histone H3 (green fluorescence, middle panel) and overlaid images (Overlay, right panel). (Scale bar 10 μm).

As expected after immunoblotting we found that histone H3 and histone H4 were present in all input samples. We also detected GFP-histone H3 in input samples prepared from cells expressing the fusion protein (Figure 4.3; lanes 1-6). We could specifically capture GFP-histone H3 only when HeLa expressing GFP-histone H3 were labelled with EdU (Figure 4.3, top panel; lanes 9, 10 and 12). Unmodified histone H3 and histone H4 were present in all EdU treated samples (Figure 4.3, middle and bottom panels, respectively; lanes 8-12). Chromatin was not recovered from mock treated cells (Figure 4.3; lane 13).

Importantly, GFP-histone H3 was not detected when extracts from EdU labelled HeLa cells were mixed with extracts of unlabelled chromatin that was marked with GFP-histone H3, indicating that intermolecular aggregation between biotinylated and untagged chromatin fragments does not occur (Figure 4.3, top panel; lane 11). Together these results support the notion that chromatin can be specifically pulled-down by EdU labelling and biotin tagging of nascent DNA.

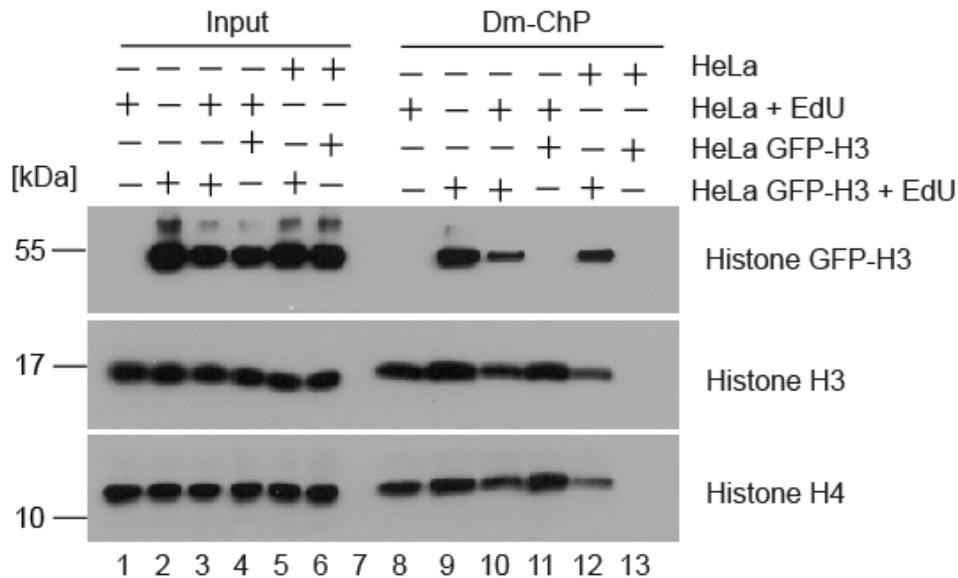


Figure 4.3 Specificity of Dm-ChP technology. Dm-ChP was performed from cells labelled (+) or not labelled (-) with EdU either expressing GFP-Histone H3 fusion protein (HeLa GFP-H3) or parental HeLa cells (HeLa). The input (Input) and pulled-down material (Dm-ChP) were analysed by western blotting using anti-GFP (top panel), anti-histone H3 (middle panel) or anti-histone H4 (bottom panel) antibodies.

4.4 Sensitivity of Dm-ChP

The DNA mediated chromatin pull-down methodology was devised with the idea of capturing and analysing proteins present at the replication forks. Eukaryotic replication forks progress at a velocity of 0.74-2.3 kb/minute (Herrick and Bensimon, 2008) and the DNA fragments obtained during the sonication step are approximately 300 bp in length. Therefore, the length of the EdU pulse is a critical parameter if the aim is to capture chromatin in close proximity to replication machinery.

First we decided to test the minimum time required to detect EdU incorporation into nascent DNA. To assess this, HeLa cells were incubated with 10 μ M of EdU for different periods of time (1 minute to 1 hour), harvested and fixed for cell cycle analysis (see section 2.5.3.2). Afterwards, EdU was coupled to 5'-BMA azide through the Click reaction and further stained using anti-BrdU and anti-FITC antibodies. DNA content was determined by staining DNA with PI. To specifically detect EdU positive cells PI content was plotted against FITC fluorescence using scatter plots (Figure 4.4, bottom panel). We detected

background levels of fluorescence in mock treated cells (Figure 4.4, bottom panel), whereas the population of cells that incorporated EdU showed a characteristic fluorescence pattern known as “horseshoe”. Fluorescence from EdU labelled cells increases linearly with the time of EdU incubation (Figure 4.4, bottom panel).

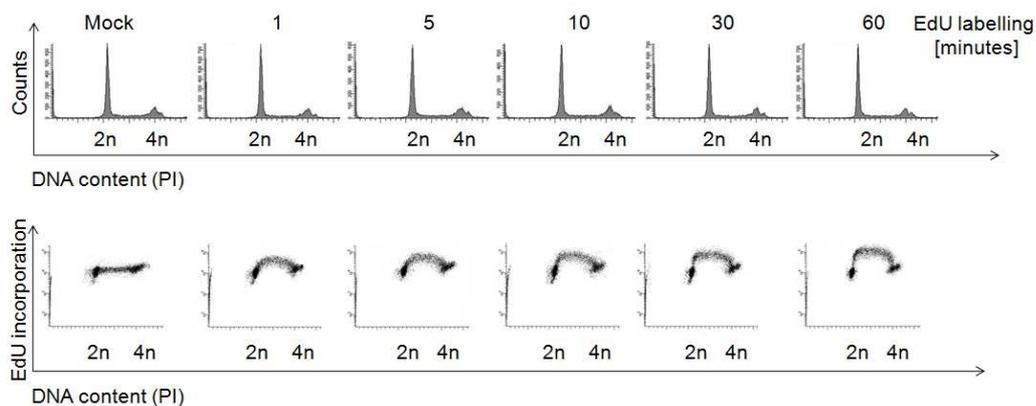


Figure 4.4 Cell cycle analysis of DNA content and EdU incorporation. HeLa cells were labelled with 10 μ M EdU for the indicated times and EdU incorporation was detected by flow cytometry analysis after Click reaction with 5'-BMA azide molecule. DNA content was measured using the PI staining.

To investigate the minimum time of EdU labelling required to detect pulled-down proteins associated with nascent, labelled DNA such as histones, logarithmically growing HeLa cells were mock treated or labelled with 10 μ M of EdU for different periods of time (10 minutes to 2 hours), harvested and permeabilised. The chromatin fraction was extracted in RIPA buffer, followed by sonication to shear genomic DNA (see section 2.3.7.1). Upon Click reaction EdU was coupled to biotin-TEG azide and recovered using pre-blocked streptavidin-coated beads (see section 2.3.12). Eluted material was analysed either by western blotting (Figure 4.5 a) or by SDS-PAGE stained with CodeBlue Gel Stain Reagent (see section 2.3.4.2; Figure 4.5 b).

As previously described, histone H3 was used as a probe to detect labelled chromatin. Histone H3 was detected in the input from all of the samples analysed. Furthermore, using the Dm-ChP strategy we were able to capture histone H3 associated with DNA that was synthesised within 10 minutes of labelling. The amount of pulled-down histone H3 appears to increase in a time-

dependent manner and after 30 minutes it levels out, suggesting a positive correlation between the amount of labelled chromatin and recovered histone within a certain range (Figure 4.5 a).

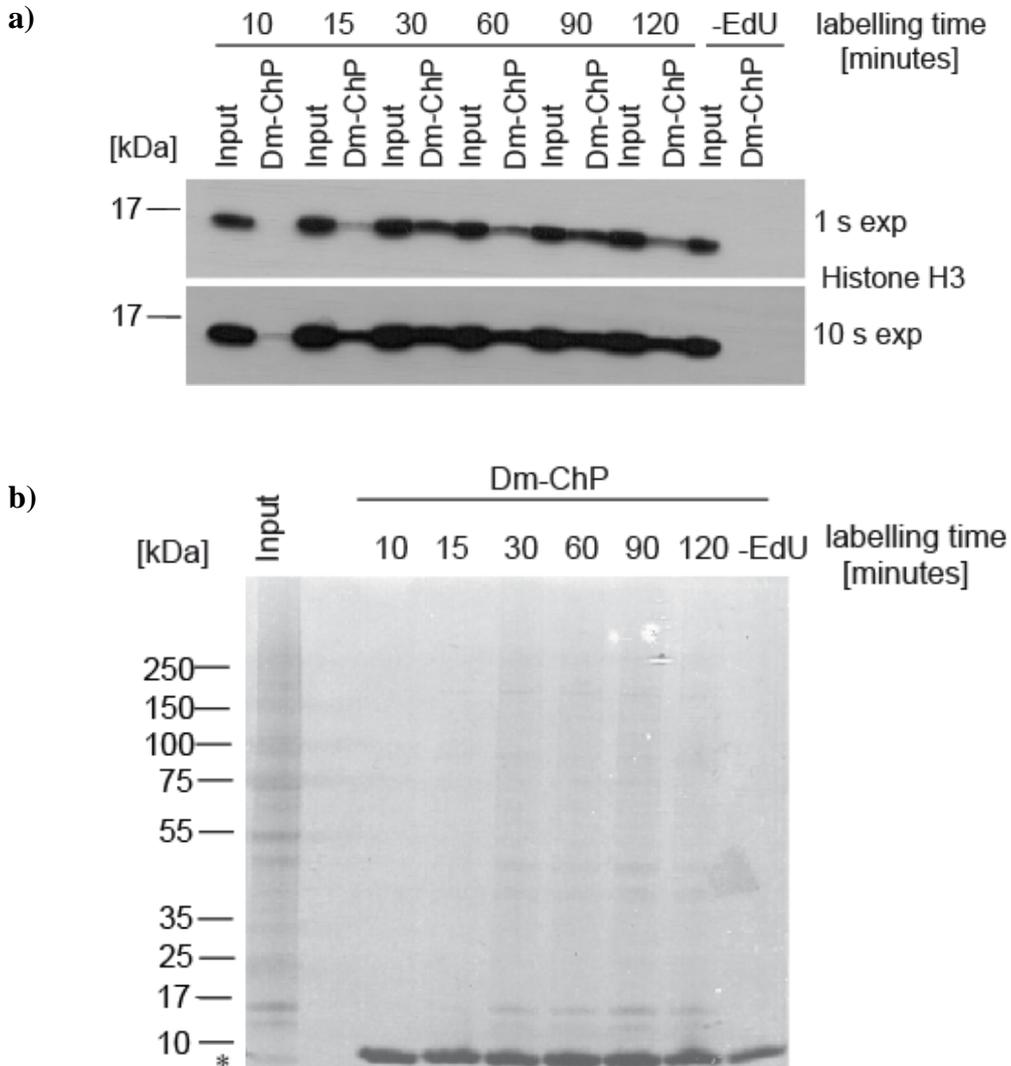


Figure 4.5 Detection of histone H3 and other proteins upon different EdU labelling times. HeLa cells were either mock treated or incubated with 10 μ M EdU for the different times before harvesting and processed by Dm-ChP (a) The input material (Input) and material eluted from streptavidin-coated beads (Dm-ChP) were analysed by western blotting using an anti-histone H3 antibody (b) or by staining the SDS-PAGE with CodeBlue Gel Stain Reagent. Streptavidin (*) band is indicated.

Additionally, SDS-PAGE followed by CodeBlue staining further confirmed our observation. In addition, to the predominant streptavidin band present in all pulled-down samples, we observed an increase in the intensity of histone derived bands that corresponded to time of EdU pulse. Moreover, we

detected a number of non-histone bands that co-purified with newly synthesised DNA (Figure 4.5 b).

4.5 Linearity and resolution of Dm-ChP

To investigate the relationship between the amount of EdU labelled chromatin in the starting material and the amount of recovered proteins in the pull-down step, HeLa cells were either mock treated or incubated with 10 μ M EdU for an hour. If there is a positive correlation between these two parameters, we expected to pull-down increasing amount of proteins with the increasing amount of EdU labelled DNA. After harvesting, cells were permeabilised and EdU was coupled to biotin-TEG azide upon Click reaction. Chromatin fractions were extracted in RIPA buffer and DNA was fragmented by sonication (see section 2.3.7.1). The EdU labelled extract was combined with mock treated extract at different ratios before the pull-down step in such a way that 1 mg of protein extract was used in each sample. EdU labelled DNA fragments together with bound proteins were then recovered using pre-blocked streptavidin-coated beads (see section 2.3.12). Pulled-down material was analysed by western blotting using anti-PCNA antibody (Figure 4.6).

Western blotting analysis showed that we recovered PCNA in EdU labelled, but not in mock treated cells. A positive correlation between the amount of EdU labelled chromatin and pulled-down PCNA was observed (Figure 4.6). These data suggest that Dm-ChP procedure is not only specific, but it can be potentially semi-quantitative.

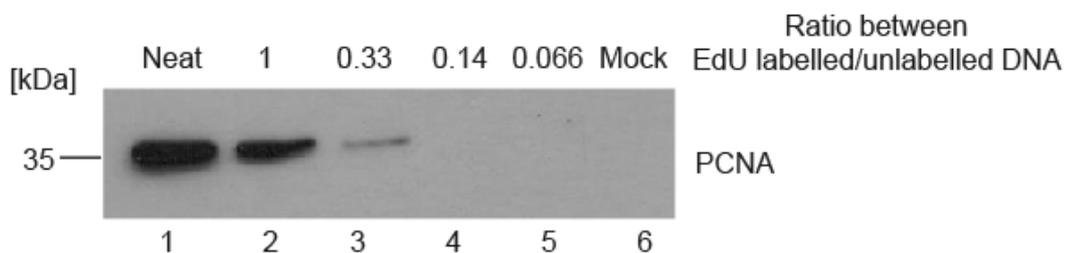


Figure 4.6 Linearity of Dm-ChP procedure. HeLa cells were mock treated or incubated with 10 μ M EdU for an hour. EdU labelled chromatin was diluted with mock treated chromatin as indicated. Streptavidin recovered material was analysed by western blotting using anti-PCNA antibody.

To assess the efficiency of the EdU labelled DNA capture we performed dot blot analysis of the input and unbound material from previous experiments. Different concentrations of extract (3 μ g-12 μ g) were spotted onto nitrocellulose membrane and blotted with anti-biotin HRP conjugated antibody (see Table 2.2 and section 2.3.6).

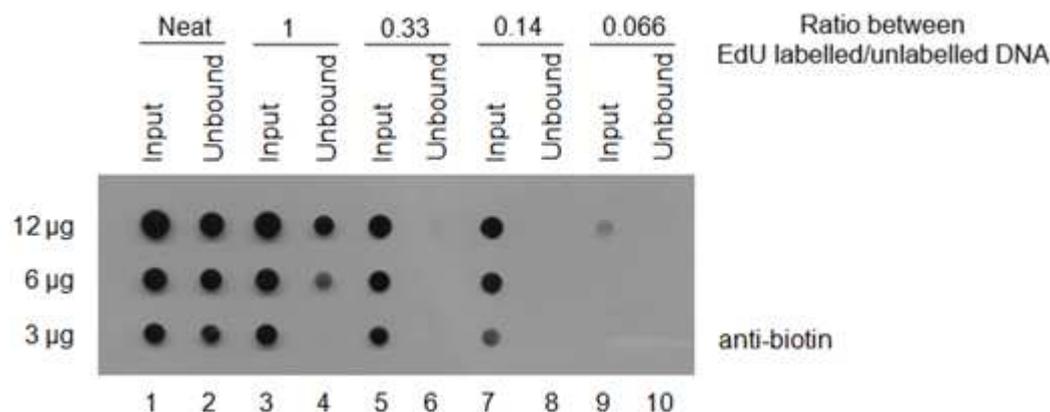


Figure 4.7 Dot blot analysis of EdU labelled chromatin. Different concentrations of chromatin from the input material (Input) and material that did not bind to the streptavidin-coated beads (Unbound) were spotted onto nitrocellulose membrane and blotted with anti-biotin antibody.

Biotin-tagged DNA was detected in all starting material analysed. Additionally, the intensity of the signal produced by the anti-biotin HRP conjugated antibody correlated with the amount of labelled chromatin spotted onto the membrane. We observed that by using neat or 1:1 diluted extracts, we saturated the streptavidin-coated beads as labelled chromatin was still detected in unbound material (Figure 4.7; lanes 2 and 4). EdU labelled chromatin was fully depleted from extract containing 25% or less of labelled DNA (Figure 4.7; lane 6, 8 and 10) indicating that resin saturation occurs when 25-50% of labelled DNA is used during pull-down step.

4.6 Saturation of streptavidin-coated resin during Dm-ChP

Next we decided to investigate whether saturation of streptavidin-coated beads occurs during Dm-ChP procedure. Moreover, we titrated the streptavidin and biotin-TEG azide to test how recovery of proteins correlates with the amount of these reagents. To address these questions, HeLa cells were mock treated or incubated with 10 μ M EdU for an hour. After harvesting, cells were

permeabilised and EdU was coupled to 50 μ l, 100 μ l or 200 μ l of biotin-TEG azide through the Click reaction. The chromatin fraction was extracted in RIPA buffer, followed by DNA shearing (see section 2.3.7.1). Labelled chromatin fragments were captured using 50 μ l, 100 μ l or 150 μ l of pre-blocked streptavidin-coated beads and further separated onto SDS-PAGE (see section 2.3.12). To visualise proteins the gel was stained with GelCode Blue Stain Reagent (see section 2.3.4.2; Figure 4.8).

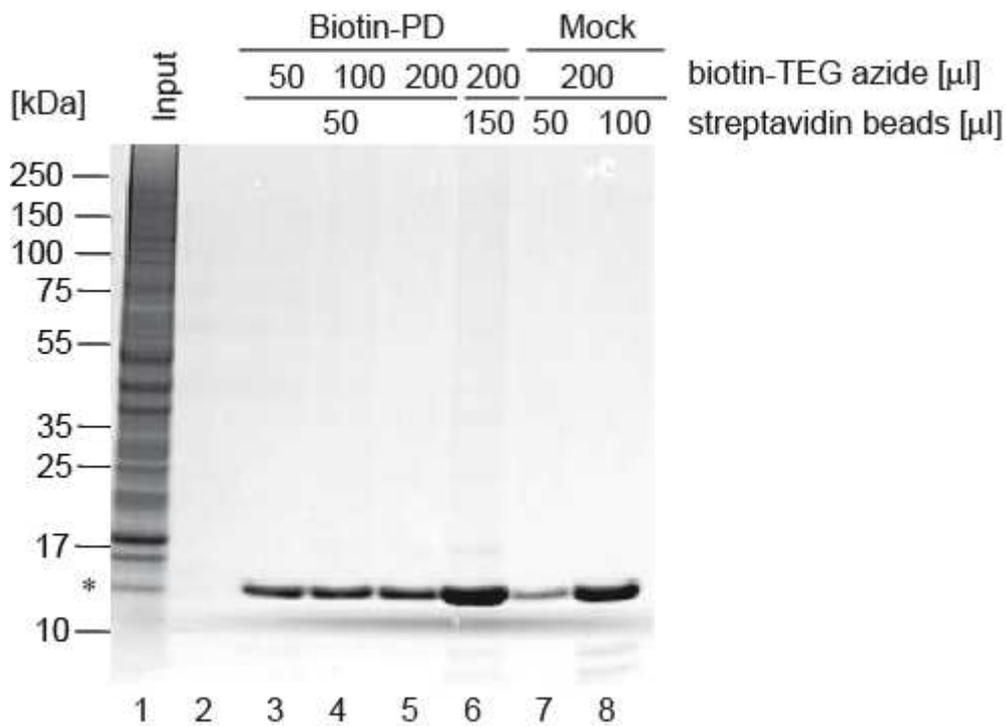


Figure 4.8 Relationship between amount of streptavidin-coated beads, biotin-TEG azide and captured material. Dm-ChP was performed using different amounts of streptavidin-coated beads and biotin-TEG azide in pull-down and labelling steps, respectively. The input material (Input) and captured material (Dm-ChP) from EdU labelled or mock treated cells were separated onto SDS-PAGE and stained with CodeBlue Gel Stain Reagent. Streptavidin (*) band is indicated.

We could detect the streptavidin moiety as a single band in the lanes where recovered material was loaded, but not in the input sample (Figure 4.8; compare lanes 1 and 3-8). The intensity of the streptavidin band correlated with the amount of beads used (Figure 4.8; lanes 3-8). In addition to streptavidin, we could also detect proteins that were captured with labelled chromatin. Proteins were not detected when chromatin was prepared from mock treated cells (Figure 4.8; lanes 7 and 8). By assessing the intensity of CodeBlue gel staining, we

observed that proteins recovery correlated with increasing amount of streptavidin-coated beads (Figure 4.8; lane 6), but not with the increasing amount of biotin-TEG azide used (Figure 4.8; lanes 3-5). This suggests that all EdU labelled DNA was tagged with the biotin molecule and streptavidin-coated beads may be a limiting factor in Dm-ChP.

To confirm that amount of streptavidin-coated beads is a limiting factor for Dm-ChP, we performed immunoblotting analysis of protein complexes captured using different concentrations of streptavidin-coated beads. HeLa cells were either mock treated or labelled for an hour with 10 μ M EdU. Dm-ChP procedure was subsequently performed as previously described (see section 2.3.12). Constant amount of lysate was incubated with increasing amounts of streptavidin-coated resin and the input and streptavidin captured material were then analysed by western blotting using anti-PCNA and anti-histone H3 antibodies.

PCNA and histone H3 were detected in the input from either EdU labelled and mock treated cells (Figure 4.9; compare lanes 1 and 2). Additionally, PCNA and histone H3 were specifically recovered only when cells were treated with EdU (Figure 4.9; lanes 4-6). Proteins were not observed when chromatin was pulled-down from mock treated cells (Figure 4.9, lane 3).

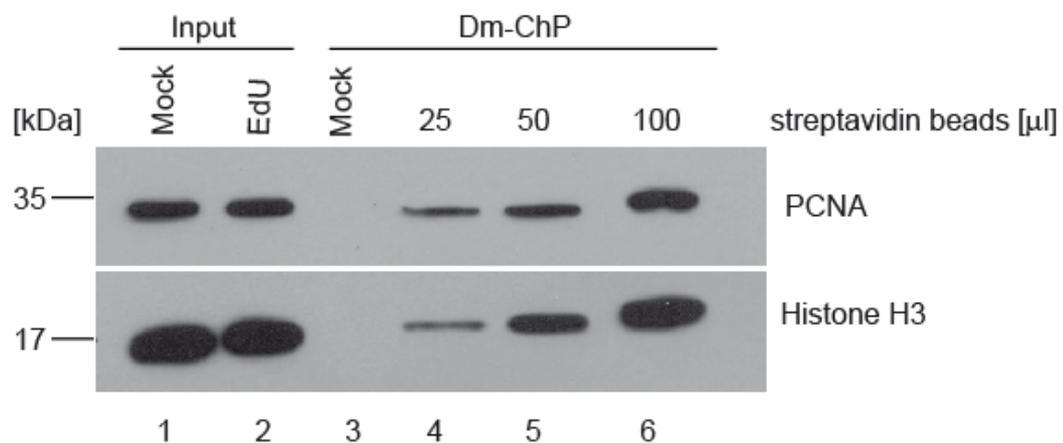


Figure 4.9 Titration of streptavidin-coated beads. HeLa cells were either mock treated or labelled with EdU. Dm-ChP assay was performed and DNA-protein complexes were recovered using either 25 μ l, 50 μ l or 100 μ l of streptavidin-coated beads. The input material (Input) and streptavidin recovered material (Dm-ChP) were analysed by western blotting using an indicated antibodies.

Similar to the previous experiment, the levels of recovered PCNA and histone H3 increased with the amount of streptavidin-coated beads used (Figure 4.9; lanes 4-6). This data strongly suggests that under these experimental conditions and time used for DNA labelling, streptavidin-coated beads become saturated when 25 or 50 μl and possibly 100 μl of streptavidin-coated beads are used. Thus, titration experiment suggests that the amount of streptavidin-coated beads is a limiting factor for the quantitative capture of biotinylated chromatin.

4.7 Conclusions

Dm-ChP approach was developed and validated in its sensitivity and feasibility. Brief summary of the major Dm-ChP steps are presented in Table 4.1. We confirmed that chromatin can be specifically pulled-down by EdU labelling and biotin tagging of nascent DNA.

Optimised conditions for Dm-ChP	
Number of cells used	Optimisation and validation of Dm-ChP: - 2 x 10 ⁶ cells for mass spectrometry analysis - 2 x 10 ⁷ cells for biological studies
EdU labelling	10 μM EdU for short pulses and 5 μM for 24 hours labelling
Formaldehyde cross-linking	10 minutes at 4°C
Chromatin enriched fraction	1.2 ml of RIPA buffer supplemented with protease inhibitor cocktail
Chromatin shearing	10 seconds sonication at 40% amplitude repeated six times
Click reaction	30 minutes at RT
Amount of biotin-azide used for 2 x 10⁷ cells	100 μl of 10 mM stock
Amount of pre-blocked Streptavidin beads	100 μl of bed volume
Pull-down step	Overnight at 4°C
Reverse of DNA-protein cross-link	5 minutes at 95°C

Table 4.1 Summary of major steps of Dm-ChP approach.

4.8 Proteomic analysis of labelled chromatin recovered after Dm-ChP

4.8.1 Introduction

Histones are the most abundant proteins associated with chromatin, but many different low abundance proteins bind to DNA. We aimed to identify novel non-histone proteins that are associated with EdU labelled chromatin by combining Dm-ChP and mass spectrometry approaches. Moreover, we wanted to assess compatibility of the Dm-ChP method with proteomic analysis. Additionally, we decided to further validate the Dm-ChP method and confirm that pulled-down material contains proteins known to be present at active replication forks and can be specifically captured by Dm-ChP. We hypothesised that using Dm-ChP we will purify several hundred proteins that specifically associate with nascent DNA. We also aimed to identify new components of the replisome that could be subsequently characterised. We strongly believe that such knowledge could help to understand DNA replication and shed light onto different biological mechanisms involved in DNA synthesis and chromatin maturation.

Mass spectrometry is a powerful tool used to identify and quantify unknown proteins present in a sample. Briefly, mass spectrometry separates peptides according to their mass to charge ratios (Walther and Mann, 2010). Peptide sequences can be identified by mass spectrometry and this information can be further used to specifically search a variety of online databases to determine protein identity (Beavis, 2006). Initial attempts at mass spectrometry analysis of the streptavidin eluted material were performed by Brendan Harhen at NCBES at NUI Galway, using an Agilent Q-TOF in MS/MS mode. A schematic diagram of sample preparation is presented in Figure 4.12. For quantitative proteomic analysis based on SILAC (Stable isotope labelling with amino acids in cell culture) samples were analysed in collaboration with Dundee Cell Product Laboratory Ltd. (Dundee, UK).

4.8.2 Detection of chromatin associated proteins after Dm-ChP

To characterise proteins associated with newly replicated chromatin, EdU labelling was performed for an hour and samples were processed according to the Dm-ChP protocol (see section 2.3.12). Recovered proteins were separated on SDS-PAGE and analysed by silver staining (see section 2.3.4.3).

Using SDS-PAGE staining we observed the predominant streptavidin band in both EdU labelled and mock treated cells pull-down (Figure 4.10; lanes 1 and 2). Additionally, in the sample pulsed with EdU, we detected histone derived bands together with a great number of bands that co-purified with newly synthesised labelled DNA fragments (Figure 4.10; lane 1). Although silver staining is not quantitative, the lack of bands in the sample from mock treated cells further indicates that capturing of the proteins is specific due to EdU labelling (Figure 4.10; lane 2).

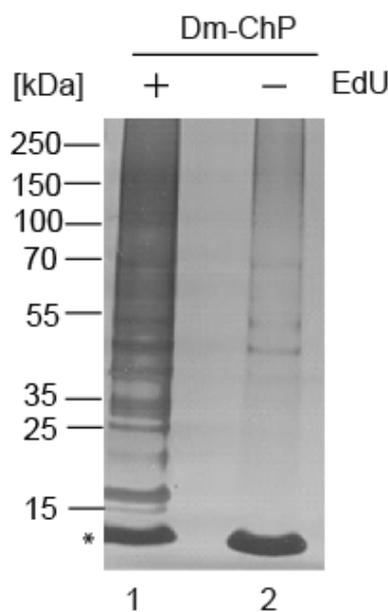


Figure 4.10 Detection of non-histone proteins in pulled-down material after Dm-ChP. Dm-ChP pulled-down material (Dm-ChP) performed from EdU labelled (+) or mock treated (-) cells was separated on SDS-PAGE and stained with silver. Streptavidin (*) band is indicated.

4.8.3 Preparation of protein complexes for mass spectrometry analysis using FASP method

To identify the proteins associated with labelled chromatin we performed proteomic analysis of Dm-ChP captured material. After the Dm-ChP procedure, recovered proteins were eluted from the beads in 1% SDS elution buffer (see Table 2.1) at 95°C. Eluted material was subsequently prepared for trypsin digestion of proteins by adapting the FASP method (Wisniewski et al., 2009) (see section 2.4.1; Figure 4.11). Briefly, eluted material was applied onto a Microcon YM-10 column (Millipore, Billerica, USA) and concentrated by centrifugation. To remove the detergent, the column was washed with UA and UB buffers in sequence (see Table 2.1), followed by protein reduction with dithiothreitol and alkylation using iodoacetamide. Proteins were digested overnight at 37°C with trypsin (Sigma-Aldrich, Arklow, Ireland) and purified peptides were eluted from the filter unit, acidified and further purified on the ZipTip desalting columns (see section 2.4.2).

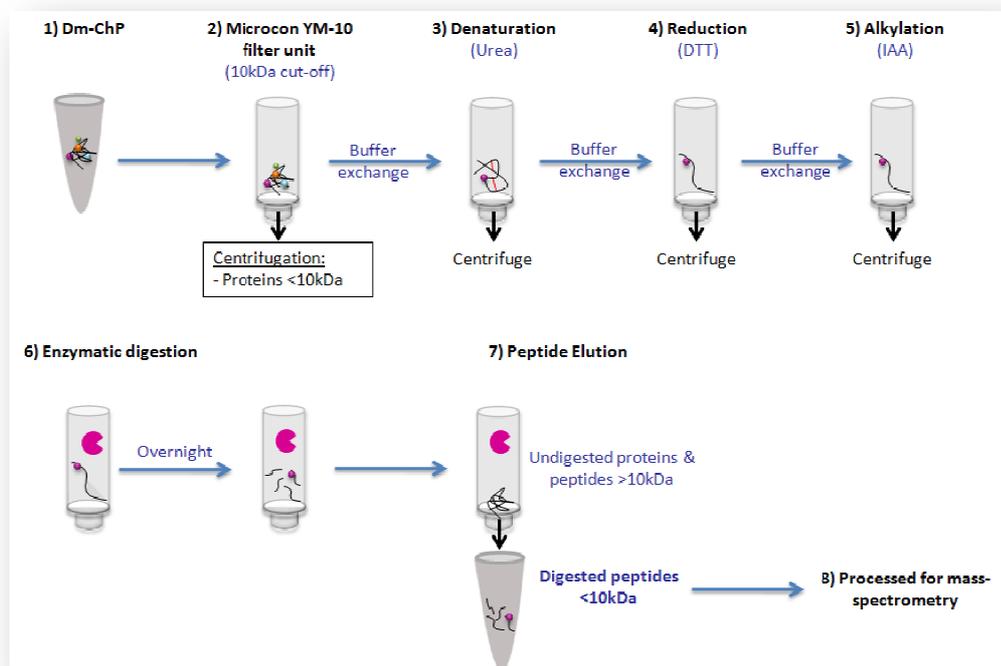


Figure 4.11 Filter-aided sample preparation (FASP) for MS-based proteomic analysis. FASP method consists of six simple steps. Eluted material recovered after Dm-ChP was applied onto ultrafiltration devices. SDS was further removed by exchange with urea buffer followed by reduction and alkylation steps. As a last step, proteins are digested overnight with digestion enzyme like trypsin and collected as a filtrate.

The ZipTip desalting column was activated by washing with methanol, followed by washes with 0.1% (v/v) TFA and 70% (v/v) acetonitrile. Peptides were bound to the resin and eluted with 70% (v/v) acetonitrile. Eluted peptides were dried in a MiVac vacuum centrifuge (Barnstead, GeneVac, Suffolk, UK) and analysed by mass spectrometry (see section 2.4.3).

4.8.4 Functional classification of Dm-ChP proteome

Pure peptides were analysed by mass spectrometry using an Agilent Q-TOF in MS/MS mode (see section 2.4.3). The generated list of proteins was exported as a GPM (Global Proteome Machine) generic file. To obtain the name and function of each protein *UniProt* (<http://www.uniprot.org>) and *GeneCards* (<http://www.genecards.org>) databases were used (Consortium, 2011; Rebhan et al., 1998). 277 proteins were recognised and subsequently manually classified into 29 different classes (Figure 4.13; see Appendix 2). As expected, proteins known to interact with nucleic acids like histones or proteins involved in chromosome function like histone modification factors and chaperones (Retinoblastoma binding protein, CAF-1, nucleophosmin, FACT complex) were identified. Chromosomal proteins like Smc1, Smc3 were also pulled-down with EdU labelled chromatin fragments.

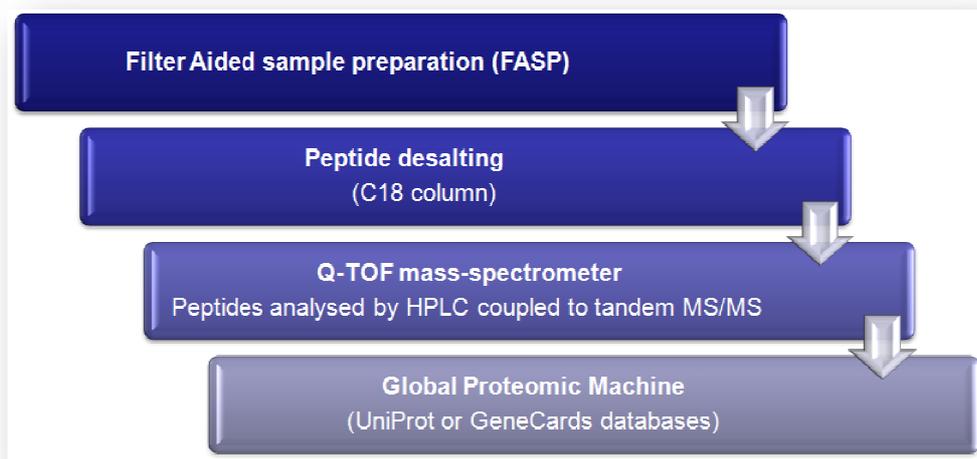


Figure 4.12 Scheme of the procedure used for protein identification. Eluted proteins were prepared for a tryptic digestion using FASP methodology, followed by desalting of peptides. Purified peptides were subsequently analysed using Agilent Q-TOF in MS/MS mode. Protein list was generated using Global Proteomic Machine and proteins were characterised using UniProt and GeneCards databases.

DNA replication proteins like MCM helicase and PCNA or DNA repair proteins including DNA helicases Ku80, Ku70 and PARP were also present in captured material. Additionally, we identified a large number of ribosomal proteins. These proteins are highly positively charged and may show adventitious binding to chromosomes. For this reason they were previously defined as chromosome hijacker proteins (Ohta et al., 2010). Contamination in the form of structural and cytoskeletal proteins (e.g. actin, vimentin) were also found in streptavidin captured material. The full list of the proteins identified with the relevant information is reported in Table 2 of Appendix 2.

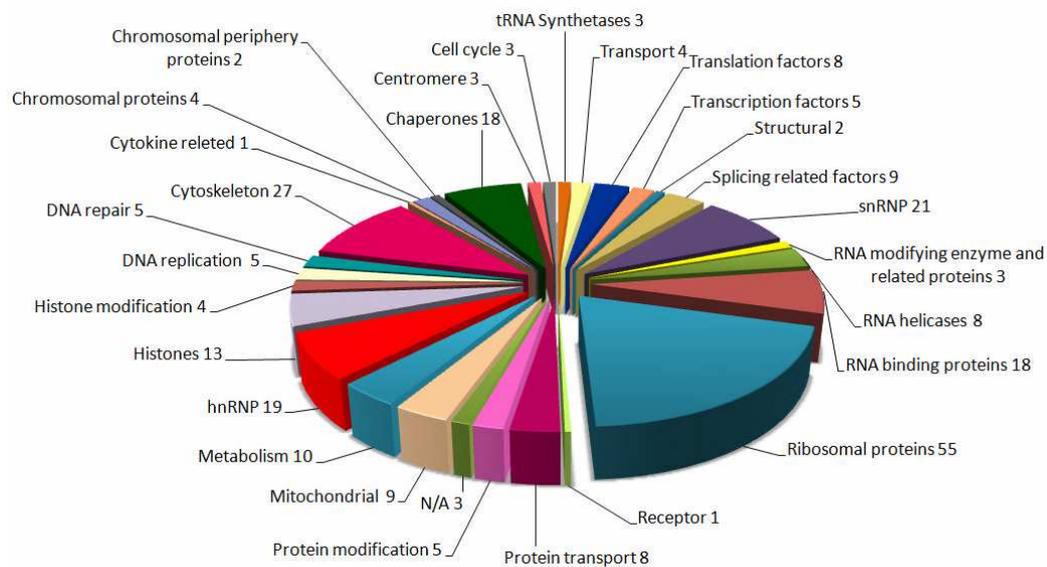


Figure 4.13 Functional classification of Dm-ChP proteome. 277 proteins identified were classified into 29 different classes using information from UniProt (Universal Protein Resource) protein Knowledgebase and GeneCards databases. Number of proteins in each class is indicated.

4.8.5 Identification of non-histone proteins associated with EdU labelled chromatin by western blotting

To confirm mass spectrometry results, Dm-ChP was performed from HeLa cells either mock treated or labelled with 10 μ M of EdU for an hour (see section 2.3.12). Material eluted from the streptavidin-coated beads was analysed by western blotting (Figure 4.14). A range of available antibodies against chromatin associated proteins were used as probes.

We could detect all proteins analysed in the input samples from either EdU labelled or mock treated cells (Figure 4.14; lanes 1 and 2). We were also able to

detect the presence of non-histone proteins such as Smc1 and Smc3, involved in sister chromatid cohesion, Mcm7 and PCNA, key components of the DNA replication machinery, and the nuclear chaperone nucleophosmin, specifically in the pulled-down material from EdU labelled cells (Figure 4.14; lane 4). These proteins were absent in the pull-down from mock treated cells (Figure 4.14; lane 5). This result indicates that the Dm-ChP procedure can be used to analyse proteins associated with newly synthesised chromatin by both immunoblotting and mass spectrometry.

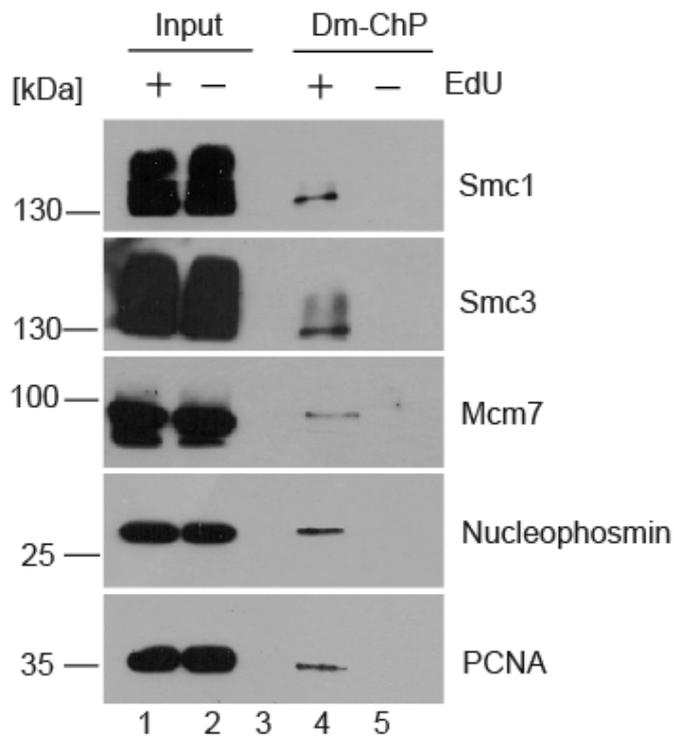


Figure 4.14 Detection of non-histone proteins in pulled-down material after Dm-ChP. Dm-ChP pulled-down material (Dm-ChP) performed from EdU labelled (+) or mock treated (-) cells was analysed together with the input material (Input) by western blotting using antibodies recognising the indicated proteins.

4.8.6 Conclusions

Mass spectrometry and immunoblotting results confirmed that the Dm-ChP procedure can be used to analyse proteins associated with newly synthesised chromatin. Mass spectrometry approach revealed a large number of contaminants (cytoskeletal and structural proteins) that were captured with nascent DNA. Different cellular fractionation method such as French press or Dounce

homogenisation could be tested to exclude the membrane and cytoplasmic proteins from the final nuclear fraction thus, increase the sensitivity of the Dm-ChP reaction. Moreover, to verify whether ribosomal proteins bind non-specifically to DNA during extract preparation, an additional pre-cleaning step with an excess of internal unlabelled DNA fragments could be performed prior to Dm-ChP. As proteins specifically associated with chromatin are cross-linked to DNA, only proteins that non-specifically bind to the DNA during extract preparation will be removed using this step and subsequently excluded from the Dm-ChP captured material. Additionally, several high stringent wash buffers such as those containing high salt or detergent could be used to remove non-specific binders. Furthermore, immunoblotting analysis of the ribosomal proteins could also be helpful to understand the nature of their interactions with EdU labelled DNA.

Chapter 5 Assessment of protein dynamics during DNA synthesis and chromatin maturation

5.1 Introduction

In previous chapters we demonstrated that Dm-ChP is specific and can be used to analyse proteins associated with labelled chromatin. In this chapter we provide evidence that the combination of Dm-ChP, quantitative mass spectrometry and immunoblotting approaches allows studying changes occurring in the protein-DNA complexes at different times during S phase of the cell cycle.

5.2 Dm-ChP studies of DNA replicating at different times during S phase

5.2.1 Introduction and SILAC methodology

After the development and initial analysis of the DNA mediated chromatin pull-down we started to address the qualitative and quantitative differences in the protein content associated with early and late replicating chromatin. To assess this we decided to apply highly sensitive and quantitative approaches for protein characterisation.

As different genomic regions are replicated during early and late S phase, we hypothesised that different amount of known and novel proteins may be associated with DNA replicating at different stages of S phase. As more replication origins is fired in early S phase compared to late S phase, we speculated that replication factors involved in DNA synthesis will be enriched with euchromatin. Furthermore, late replicating heterochromatin should be then associated with the proteins involved in heterochromatin assembly such as HP1 α , chromatin remodelling factors or cohesin and condensin complexes.

SILAC (Stable isotope labelling with amino acids in cell culture) is a powerful approach characterised by high accuracy and used to detect small changes in protein abundance among different samples (Ong et al., 2002). This method relies on incorporation of non-radioactively labelled amino acids into mammalian proteins *in vivo*. By culturing cells in media supplemented with

normal and heavy isotopes of amino acids, proteins can be differentially labelled during their synthesis. After several cell cycles, each amino acid will be replaced by its isotope labelled analogue. Since there is no chemical difference between labelled and “light” amino acids, cell behavior is identical in labelled and regular culture. However, proteins prepared from the “light” and “heavy” media can be easily distinguished due to small differences in their masses using mass spectrometry (Figure 5.1) (Zhang and Neubert, 2009).

We decided to take an advantage of SILAC methodology and combine it with Dm-ChP procedure. This approach will allow us to directly compare and quantify proteins present in the various samples.

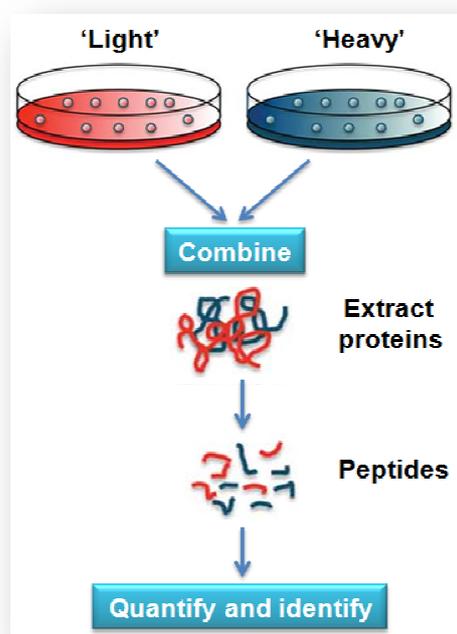


Figure 5.1 Cartoon representation of SILAC methodology. Cells are cultured in “light” and “heavy” SILAC medium, respectively to differentially label proteins. After a number of cell divisions, each amino acid was replaced by its isotope labelled analogue. After harvesting cells were combined together and proteins were isolated, digested and subjected to mass spectrometry analysis. Picture adapted from (Gingras et al., 2007).

5.2.2 Proteome analysis of early and late replicating chromatin using SILAC approach

To elucidate the molecular correlation between chromatin features and the temporal programme of DNA replication, Dm-ChP technology was combined

with cell cycle synchronisation and quantitative proteomic analysis based on SILAC. Briefly, isotope pre-labelled HeLa cells (see section 2.5.1.4) were synchronised at the G₁/S border using a double thymidine block (see section 2.5.1.3). After arrest, cells were released for 2 and 6 hours into a synchronous S phase to analyse early and middle/late replicating chromatin, respectively. Cells were labelled with 10 μM of EdU for 30 minutes before collection and processed according with the Dm-ChP protocol as described previously (see section 2.3.12). After Dm-ChP, beads were mixed together and eluted in 1% SDS. The early S phase cell population was labelled with isotope “medium” media, while the middle/late S phase cell population was labelled with “heavy” media. Mock treated cells were grown on “light” media (see section 2.5.1.4). Cell synchrony and EdU incorporation was monitored by flow cytometry and fluorescence microscopy, respectively.

DNA content was determined by staining DNA with 7-AAD. A characteristic DNA content histogram for an asynchronous population of HeLa cells is presented (Figure 5.2; top panel).

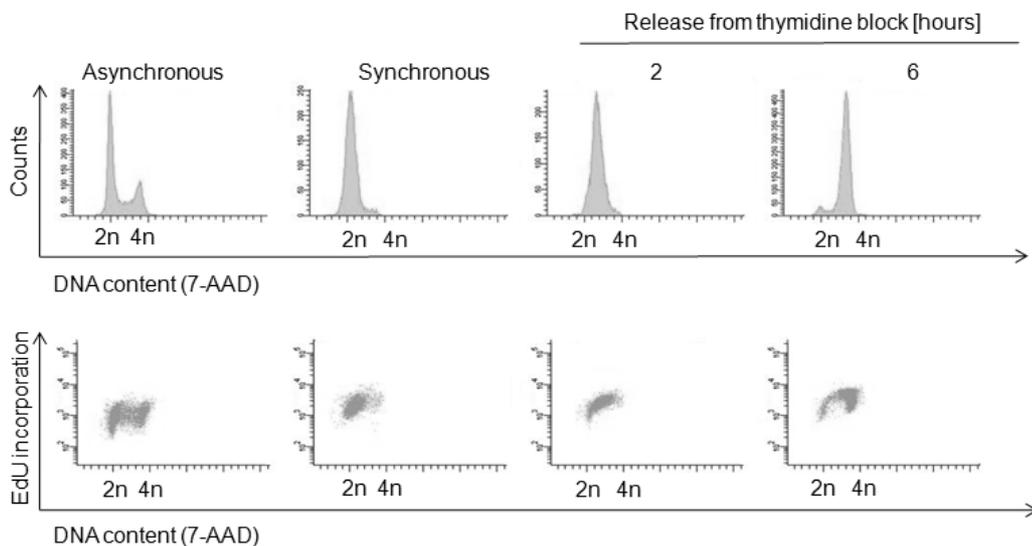


Figure 5.2 DNA content of metabolically labelled cells released from a thymidine block for 0h, 2h or 6h. HeLa cells arrested at the G₁/S transition were released from the cell cycle block and allowed to progress into a synchronous S phase. DNA content of logarithmically growing cell population (Asynchronous, mock treated) and cells collected at the 0, 2 and 6 hour post-release was analysed by flow cytometry.

Cells in G_1 phase and at G_2/M border have a $2n$ and $4n$ DNA content, respectively, with S phase cells lying in between these two DNA content markers (Figure 5.2; top panel). Cells arrested with double thymidine showed accumulation at the G_1/S boundary, confirming a successful synchronisation procedure. Cells re-entered S phase after 2 hours post-release (Figure 5.2, top panel), whereas 6 hours after block, cells reached middle/late S phase (Figure 5.2, top panel). To specifically detect EdU positive cells, 7-AAD content was plotted against 6-carboxyfluorescein-TEG azide fluorescence using scatter plots. We detected background levels of fluorescence in mock treated, asynchronous cells (Figure 5.2, bottom panel). Only cells labelled with EdU (synchronous, 2 and 6 hours post-release) showed increased levels of fluorescence (Figure 5.2, bottom panel).

To further demonstrate that cells were synchronised and labelled with EdU we performed fluorescence microscopy. Briefly, synchronised HeLa cells were grown on coverslips and labelled for 30 minutes with $10 \mu\text{M}$ of EdU before collection at 2 hours and 6 hours post-release from double thymidine block (see section 2.5.1.3). Cells were fixed with formaldehyde and permeabilised. Upon Click reaction the EdU incorporated into the DNA was linked to a 6-carboxyfluorescein-TEG azide molecule (see section 2.5.2.2). Coverslips were then washed with PBS and nuclei were stained with DAPI (Figure 5.3, blue fluorescence; left panel).

We verified that over 90 % of the cells were labelled with EdU and EdU signal was exclusively nuclear (Figure 5.3). Moreover, classical patterns of early and middle/late replicating chromatin were observed (Dimitrova and Gilbert, 1999; Frum et al., 2009); cells taken at 2 hour post-release were all EdU positive and showed replication foci distributed homogenously throughout the nucleus as a dispersed, punctate pattern (Figure 5.3, top panel; green fluorescence). Six hours after release, replication foci are mostly located at the periphery of the nucleus and around the nucleolus, confirming that cells were enriched for middle/late S phase population (Figure 5.3, bottom panel; green fluorescence). Together these experiments demonstrated that cells were labelled with EdU, synchronised and efficiently released into S phase generating cell populations either in early or middle/late stages of S phase.

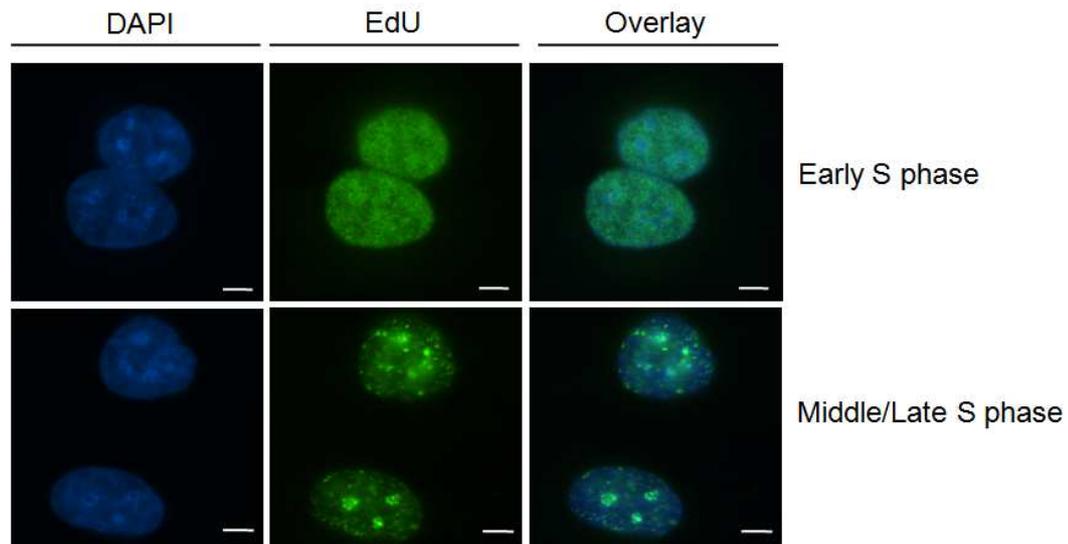


Figure 5.3 DNA replication foci identified by EdU incorporation in cells released into S phase. *HeLa* cells grown on coverslips were arrested at the G_1/S transition released from the cell cycle block and allowed to progress into a synchronous S phase. EdU labelling was performed for 30 minutes and afterwards EdU was conjugated to 6-carboxyfluorescein-TEG azide by Click reaction. DNA was visualised using DAPI. Left panels show DNA nuclear staining (blue fluorescence), middle panels show replication foci characteristic for an early and middle/late S phase (green fluorescence; top and bottom panels, respectively) and right panels show overlaid images (Overlay). (Scale bar 10 μm).

Finally, proteins from either EdU labelled or mock treated cells from early (2 hours post-release) and middle/late (6 hours post-release) replicating cells were recovered by Dm-ChP (see section 2.3.12). 10% of eluted material after Dm-ChP was analysed next to 1 μg of input sample on SDS-PAGE, followed by silver staining (see section 2.3.4.3; Figure 5.4). The rest of the material was analysed using a quantitative mass spectrometry approach based on SILAC labelling (Dundee Cell Product Ltd., Dundee, UK).

Gel staining showed that the eluted material contained a sufficient amount of proteins for MS analysis (Figure 5.4). In the mass spectrometry analysis, 315 proteins were identified and characterised by parameters such as unique peptide number, signal intensity or sequence coverage. Intensities of the medium and heavy labelled peptides to light labelled ones express medium/light (M/L), heavy/light (H/L) and heavy/medium (H/M) ratios. MaxQuant algorithm was used for protein quantification determined as the median value of multiple peptides (Cox and Mann, 2008).

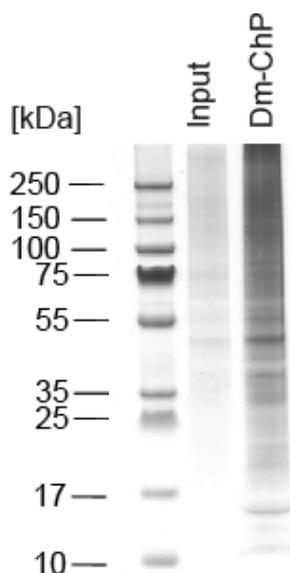


Figure 5.4 Detection of proteins recovered with EdU labelled chromatin after Dm-ChP. Dm-ChP pulled-down material (Dm-ChP) performed from EdU labelled cells together with the input material was separated on SDS-PAGE and stained with silver.

Studying ratios between heavy, medium and light labelled peptides we monitored for small changes between analysed conditions. Therefore, groups of the proteins with high M/L and H/L ratios (values >1) represent specific interactions with EdU labelled DNA and proteins with M/L and H/L ratios less than 1 were considered as contaminants. Similarly, proteins with high H/M ratio (values >1) were more abundant in the sample labelled with heavy amino acids.

Table 3 in Appendix 3 shows 241 proteins identified with at least 2 unique peptides. We also detected 4 uncharacterised proteins such as C14orf166, C7orf13, C20orf174 (Zinc-finger protein 831) and C12orf112 (T-complex protein 1). These proteins were excluded from the final list as they were either identified with low confidence (one unique peptide) or were classified as experimental contaminants according to the M/L and H/L ratios.

The average normalised signal intensity of the particular peptide was calculated using MaxQuant software. MaxQuant uses internal calibration method to normalise peptide ratios. In this method, the median logarithmic value of the SILAC ratios was obtained by its comparison with a group of predicted contaminants whose median logarithmic values were set as zero (Cox and Mann, 2008). The graphical representation of mass spectrometry data can be obtained by plotting $\log_2(H/M)$ SILAC ratio for all proteins identified on the y axis and

ordinal number for each proteins on the x axis (Figure 5.5). One hundred and sixteen proteins with high heavy/medium ratio (between 1.85-0.9) were identified as enriched in middle/late S phase compared to early S phase, whereas 125 proteins with a lower ratio (between 0.8-0.09) indicated association with early synthesised chromatin (see Appendix 3).

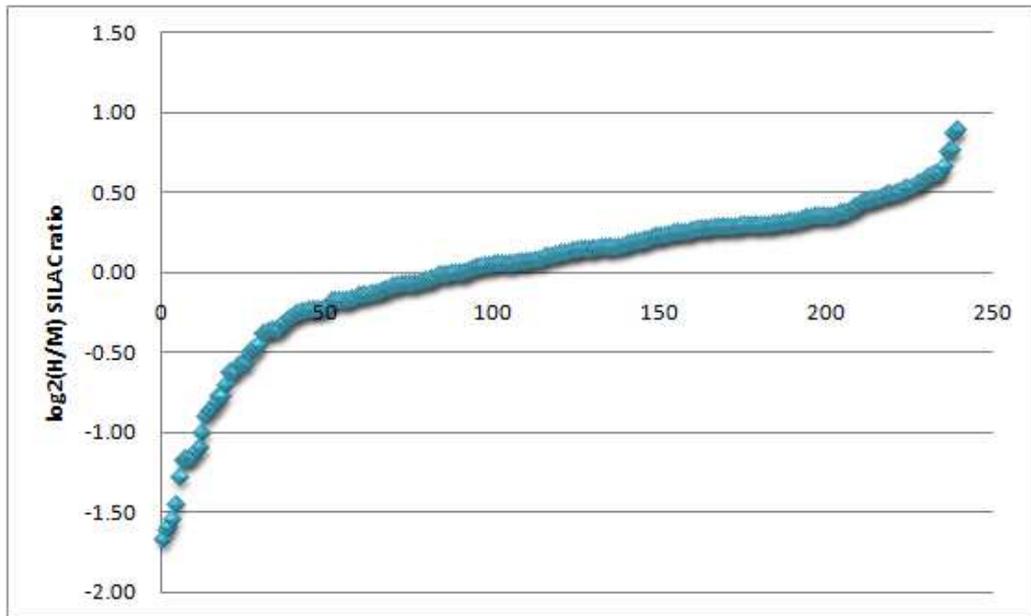


Figure 5.5 Graphical representation of SILAC data.

Proteins such as histone chaperones FACT and CAF-1 showed preferential association with chromatin synthesised after 2 hours post-release from thymidine block. In addition, several factors involved in DNA synthesis, such as Replication factor C, Flap endonuclease 1, Topoisomerases 1 and 2, Mcm7, PCNA, Smc1 and Smc3 were enriched in the fraction associated with early replicating DNA. On the contrary, proteins involved in chromatin organisation such as scaffold attachment factor B2 (SAFB2) and lamin A and B1 were more abundant with the middle/late replicating DNA. Furthermore, proteins involved in nucleosome assembly such as nucleophosmin and nucleolin were also found in pull-down of late chromatin. Intriguingly, we observed that ribosomal proteins were enriched in the 6 hours compared to 2 hours time point (see Appendix 3).

To confirm SILAC results, we performed similar experiment where Dm-ChP material was analysed by mass spectrometry (data not shown). We have identified 363 proteins with minimum 2 unique peptides and these included 195

unique proteins compared to the first experiment. However, analysis of heavy, medium and light ratios indicated that most of the proteins (only 3 proteins had M/L ratio values >1 and 26 proteins have H/L ratio values >1) were enriched in the control sample, suggesting that we pulled-down proteins non-specifically. It is rather unlikely, that proteins such as Lamin B1 or condensin that we have previously observed as enriched with late replicating chromatin would be now more abundant in control unlabelled cells. We do not have an explanation for this discrepancy but it is possible that MS analysis was not performed properly in the second experiment or despite that the proteins were cross-linked to DNA some complexes could exchange among labelled and unlabelled variants. The observed differences between both SILAC analyses could be related to different experimental approaches. Unlike in the first experiment where separate pulled-down samples were mixed after Dm-ChP, we combined cells from different time points (2 and 6 hours post-release from thymidine block) and then processed by Dm-ChP and mass spectrometry.

5.2.3 Characterisation of proteins associated with DNA synthesised at different time during S phase

To confirm the findings from quantitative SILAC analysis and extend our examination to regions of the genome replicating very late in S phase, HeLa cells were released from the G₁/S boundary and labelled with 10 μ M EdU for 15 minutes before collection at the 2 hours (early S phase), 6 hours (middle S phase) and 8 hours (late S phase) periods. Samples were processed by Dm-ChP (see section 2.3.12) and analysed by flow cytometry and fluorescence microscopy (Figure 5.6 a, b).

As previously described, flow cytometry and fluorescence microscopy were used to assess synchronisation and DNA labelling. DNA content was determined by staining DNA with 7-AAD. Flow cytometry analysis confirmed that cells released into S phase and harvested at 2, 6 and 8 hours post-release were labelled with EdU and enriched in early, middle and late S phase, respectively (Figure 5.6 a). Moreover, fluorescence microscopy analysis confirmed previous observations of cell synchrony and showed a characteristic

pattern for early, middle and late replicating chromatin in particular time points (Dimitrova and Gilbert, 1999; Frum et al., 2009) (Figure 5.6 b).

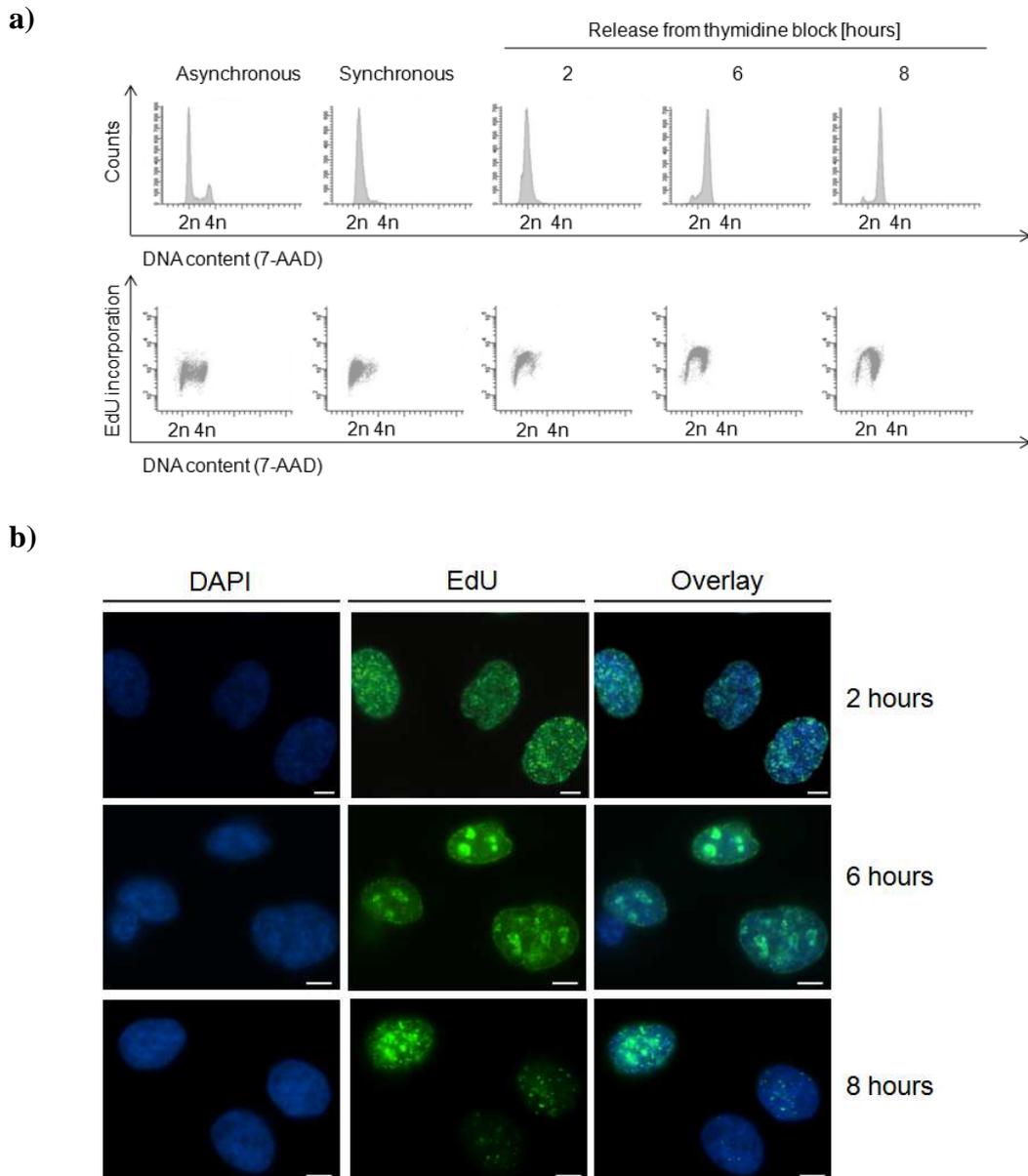


Figure 5.6 Analysis of chromatin replicating at early, middle and late stages of the S phase. HeLa cells arrested at the G_1/S transition were released from the cell cycle block and allowed to progress into a synchronous S phase **a)** DNA content and EdU incorporation of logarithmically growing cell population (Asynchronous, mock treated) and cells collected at different times post-release were analysed **b)** Representative images of DNA replication foci identified by EdU incorporation in cells harvested at the indicated times post- release into S phase. (Scale bar 10 μm).

We scored EdU positive cells and quantified number of cells showing patterns consistent with early, middle and late S phase at each time point.

Approximately 300 cells per slide were scored (Table 5.1). We observed that at the 2 and 6 hour time points 95% of cells were EdU positive and possess the classical pattern for early and middle/late replicating chromatin, respectively (Dimitrova and Gilbert, 1999). At the 8 hour time point only 40% of cells incorporated EdU and from those only 63% were scored as cells with a late S phase replication pattern (Table 5.1).

All these experiments demonstrated that cells were synchronised in S phase and successfully labelled with EdU upon release in to S phase. Additionally, cells harvested at the 2, 6 and 8 hour post-release corresponded to population of cells in early, middle and late S phase, respectively.

	EdU positive			EdU negative
	Early	Middle	Late	
2h	95.6	1.8	-	2.7
6h	27.3	63.6	3.4	5.7
8h	7.4	7.0	25.1	60.5

Table 5.1 Quantification of cells showing EdU incorporation pattern consistent with early, middle and late S phase. Cells were synchronised by double thymidine block, released into S phase and labelled with 10 μ M EdU before collection at the 2, 6 and 8 hour post-release. 300 cells were scored in each time point using fluorescence microscopy.

Finally, Dm-ChP captured material prepared as described at the beginning of this section was analysed by immunoblotting (Figure 5.7). Different replication proteins, previously identified by mass spectrometry analysis, were used as probes to validate their association with chromatin at different stages of S phase. We could observe all proteins analysed in the input samples. Proteins were not recovered in Dm-ChP from mock treated cells (Figure 5.7; lanes 11-13). We observed that many replication proteins, such as Mcm7, PCNA FACT, Msh2 and Fen-1 were specifically enriched in the pull-downs from 2 and 6 hour time points (Figure 5.7; lanes 8 and 9) and their capture was significantly reduced at 8 hour post-release (Figure 5.7; lane 10). The binding of lamin B1, nucleolin and NONO (non-POU-domain-containing, octamer-binding protein) was more efficient in chromatin replicating in late S phase (Figure 5.7; lanes 8 and 9). As observed by SILAC data and previous studies these proteins are associated with

transcriptionally inactive genes, that are replicated late in S phase (Olins et al., 2010; Shav-Tal and Zipori, 2002). Moreover, levels of histone H4, histone chaperone CAF-1 and Smc1 remained fairly constant (Figure 5.7; lanes 7-9). These data show that Dm-ChP can be applied to investigate protein complexes and their association with early-, middle- and late-replicating chromatin.

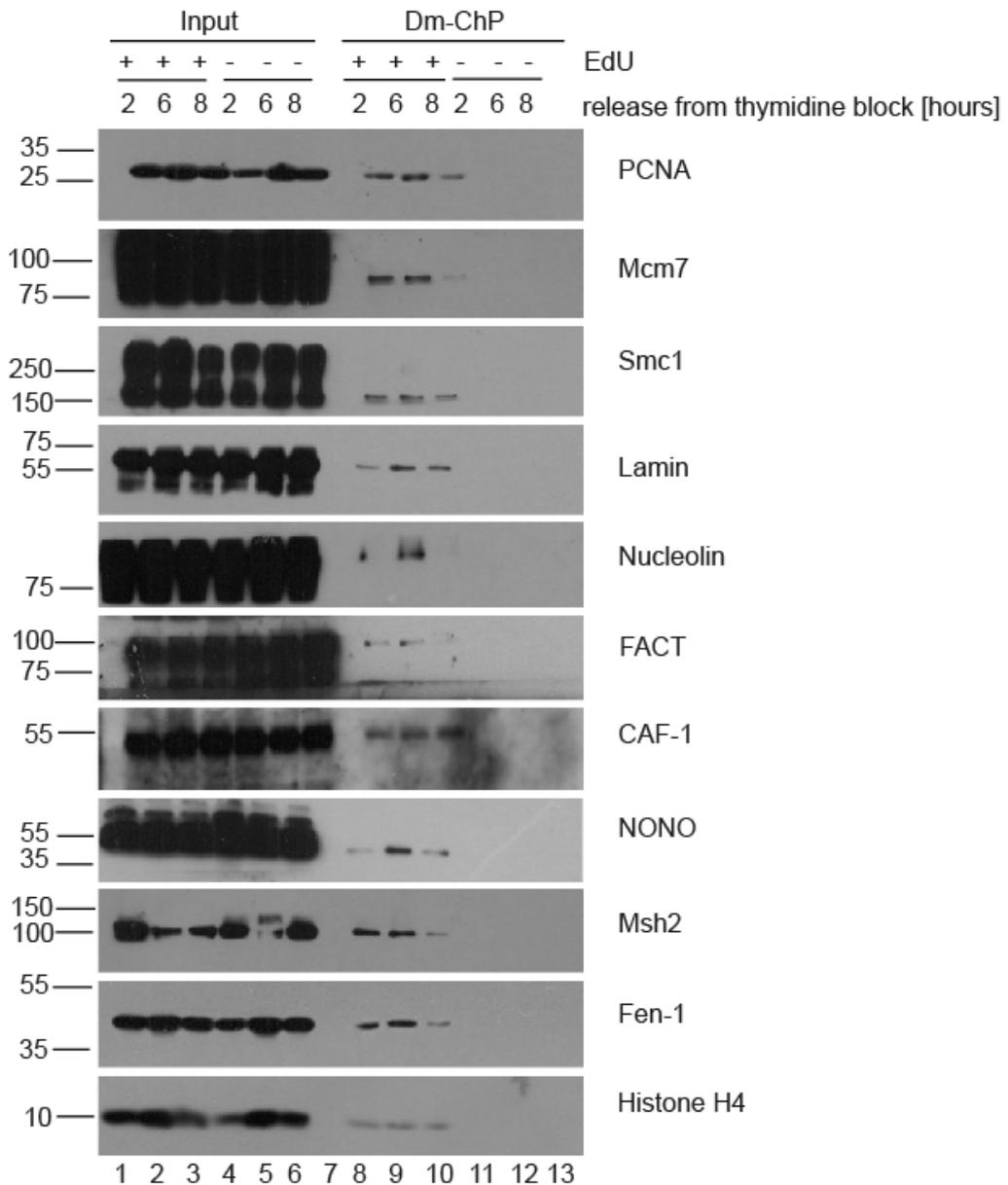


Figure 5.7 Proteins associated with chromatin replicating in early, middle and late S phase. HeLa cells were synchronised at G₁/S boarder using double thymidine block and released into S phase for 2, 6 and 8 hours. Before collection cells were either mock treated (-) or labelled with EdU (+) for 15 minutes. After Dm-ChP, the input material (Input) and streptavidin captured material (Dm-ChP) were analysed by western blotting using an indicated antibodies.

5.2.4 Conclusions

Using the Dm-ChP method with a combination of proteomic techniques, we provided preliminary evidence that DNA replicating at different times during S phase can be found together with different relative amount of proteins involved in DNA replication. At present we speculate that this observation is possibly consistent with the idea that in mammalian cells in late S phase, replication forks move at a faster rate (Frum et al., 2009; Herrick and Bensimon, 2008), thus the ratio between EdU labelled DNA in close proximity to the replication machinery and EdU labelled DNA away from it and not cross-linkable to replication factors is lower. Nevertheless, to perform statistic analysis and to be able to properly interpret the SILAC data it is necessary to repeat the mass spectrometry analysis. Moreover, the careful quantification of the levels of any given protein in different samples is a key technical hurdle for the use of this technique in future studies. To be able to compare samples prepared under different experimental conditions it is necessary to perform Dm-ChP using the constant amount of EdU labelled DNA instead of constant protein concentration as was performed so far.

Because immunoblotting analysis did not fully recapitulate mass spectrometry data it is essential to use more sensitive and quantifiable approaches such as infrared fluorescence imaging systems. An alternative approach would be to carefully titrate pulled-down material prior to loading onto SDS-PAGE thus allowing for signal quantification. Additionally, to confirm that we indeed pull-down euchromatin or heterochromatin, the known chromatin markers, such as histone modifications or presence of specific transcription factors could be analysed. Early replicated chromatin can be detected by the presence of histone modifications such as acetylation of histone H4 at Lys5 and Lys12 or acetylation of histone H3 at Lys9 and Lys16. On the contrary, trimethylation of histone H3 at Lys9 is characteristic mark for late replicating heterochromatin. Additionally, early and late replicating chromatin is marked with different transcription factors such as intermediary factor TIF α and transcriptional repressor protein YY1, respectively.

5.3 Dm-ChP studies of protein dynamics during chromatin maturation

5.3.1 Introduction

In the previous section, we demonstrated that a combination of Dm-ChP with mass spectrometry approaches can be used to study protein enrichment at replicating DNA at different time points during S phase. Then we sought to determine the identity of proteins stably or transiently associated with active or stalled replication forks. To achieve this, we combined cell synchronisation procedure with pulse-chase methodology.

We predicted that replisome factors will be associated with newly synthesised DNA and displaced from the chromatin as replication forks move away from the site of nucleotide incorporation. Conversely, proteins involved in chromatin maturation such as lamin B1, condensin or chromatin remodelling factors should not be associated with nascent DNA and will be recruited to DNA after passage of replication machinery as chromatin matures.

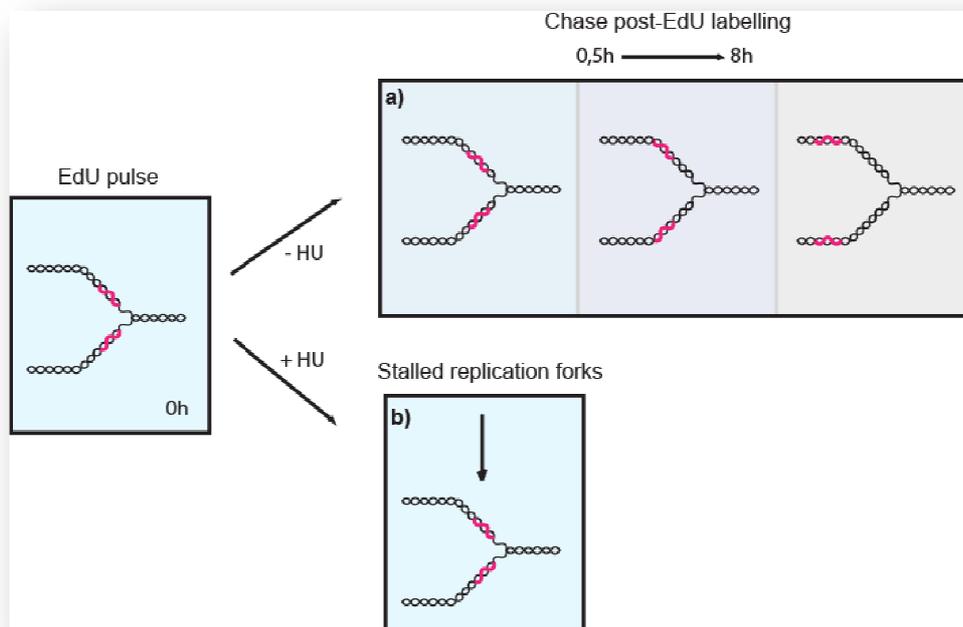


Figure 5.8 Graphical representation of EdU pulse-chase experiments. During labelling period EdU is incorporated into nascent DNA **a)** After EdU labelling and during chase replication forks move away from the labelled DNA in time-dependent manner **b)** In the presence of hydroxyurea replication forks are stalled in close proximity to the EdU labelled DNA.

5.3.2 Proteins associated with active replication forks

To investigate protein assembly and disassembly from a nascent DNA, HeLa cells were synchronised at the G₁/S boundary using double thymidine block and released into early S phase for 105 minutes, followed by labelling for 15 minutes with 10 μM EdU. Afterwards, cells were released into fresh medium without EdU, collected at different time points and processed by the Dm-ChP protocol as previously described (Figure 5.8 a; see section 2.3.12). Cell synchrony and EdU incorporation were monitored by flow cytometry and fluorescence microscopy, respectively.

DNA content was determined by staining DNA with 7-AAD. Similarly as before, flow cytometry analysis confirmed cell synchrony at the G₁/S boundary (Figure 5.8). We observed that 2 hours post-release from thymidine block cells re-entered S phase (early replication stage) (Figure 5.9). During the chase procedure, cells progress through S phase and at 6 hours post-chase, cells

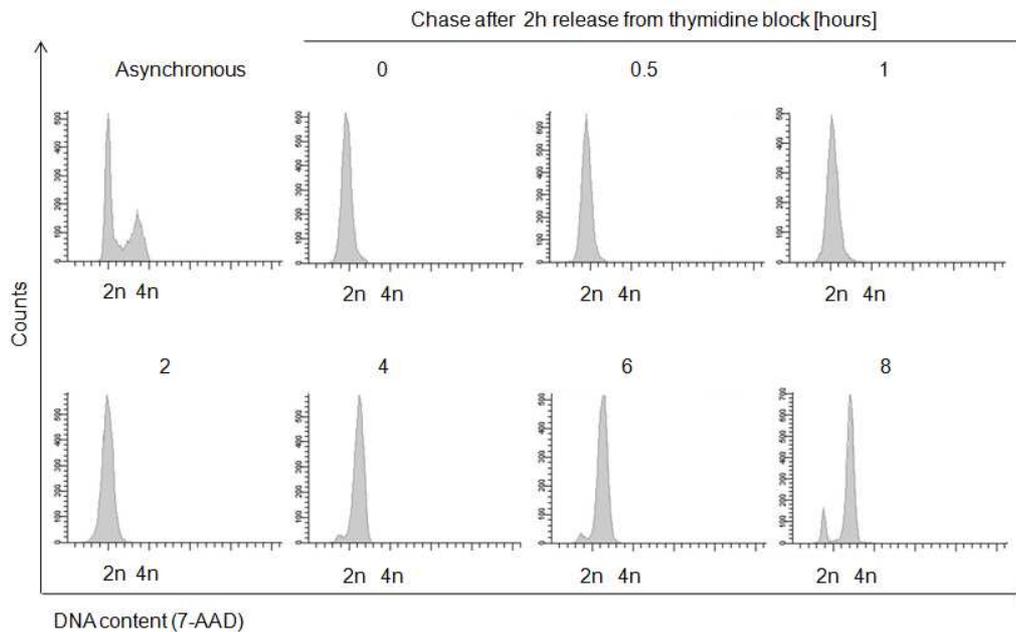


Figure 5.9 DNA content analysis of early replicating cells pulsed and chased into synchronous S phase. Cells were synchronised by double thymidine block, released into early S phase and labelled with EdU for 15 minutes. Cells were subsequently chased into fresh medium for different periods of time. DNA content of logarithmically growing cell population (Asynchronous) and cells collected at indicated times post-chase (0-8 hours) were analysed.

reached middle S phase (Figure 5.9), whereas at the 8 hour time point cells finished S phase and accumulated at the G₂/M border. A small portion of G₁ cells was observed at 8 hours post-release (Figure 5.9).

Fluorescence microscopy analysis further confirmed that cells were synchronised and were EdU positive. We could observe characteristic patterns of early replicating chromatin and its maturation. Cells taken 0-4 hours post-chase were EdU positive and showed early S phase punctate patterns of replication foci (Figure 5.10, bottom panel; green fluorescence). Six hours after chase, we can observe early events of chromatin condensation (Figure 5.10, bottom panel; green fluorescence). At the 8 hour time point some cells reached G₂/M border and EdU labelled DNA could be observed in condensed form.

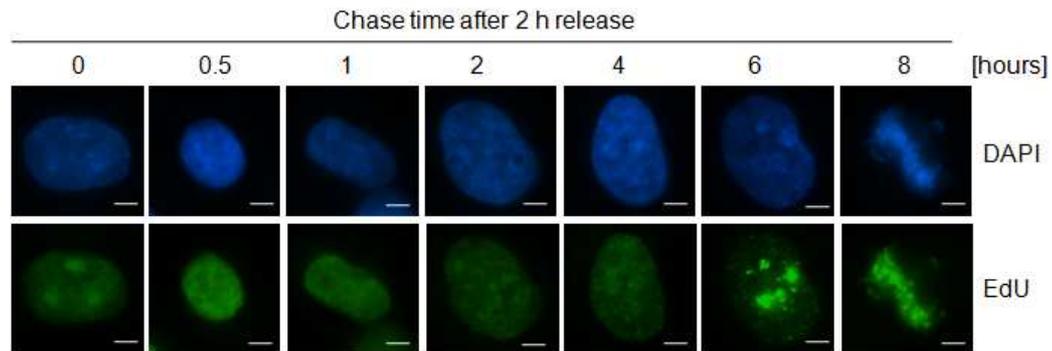


Figure 5.10 Analysis of EdU foci in pulse-chase experiment. *HeLa cells arrested at the G₁/S transition were released from the cell cycle block and after EdU labelling chromatin was incubated in EdU free media. Representative images of EdU incorporation sites were collected and show cells harvested at the indicated times post-EdU pulse. (Scale bar 10 μ m).*

Finally, Dm-ChP was performed as described at the beginning of this section and the captured material was analysed by western blotting (Figure 5.11; see section 2.3.12). As representative samples we analysed input material taken at the 0 and 8 hour time point. We could detect all replication proteins analysed in the input samples. No proteins were recovered from mock treated cells (Figure 5.11; lane 4). Replication proteins such as PCNA, Mcm7, Cdc45, RPA, Fen-1 and FACT were enriched at newly synthesised DNA (Figure 5.11; lanes 5 and 6). With time, association of these proteins with labelled DNA was significantly reduced, suggesting that these replication factors are not associated with mature chromatin (Figure 5.11; lanes 7-11). The amount of Smc1 recovered was constant at early

time points (Figure 5.11; lanes 5-9) when the cell population was in S phase, but at the 6 and 8 hour post-chase levels of Smc1 decreased (Figure 5.11; lanes 10 and 11), suggesting that cohesin complex is displaced from chromatin as cells

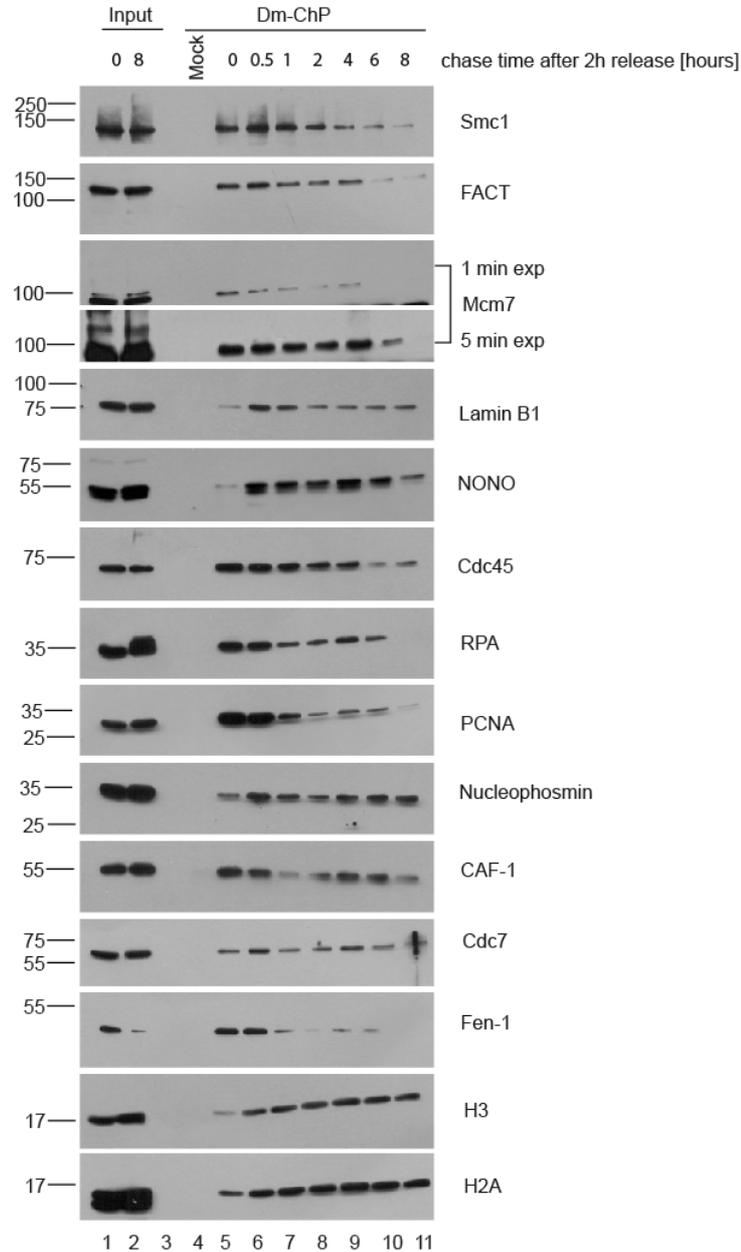


Figure 5.11 Protein dynamics during chromatin maturation. *HeLa* cells were synchronised at the G_1/S boarder using double thymidine block, released into S phase for 2 hours and cells were either mock treated or labelled with EdU for 15 minutes. Cells were subsequently chased into fresh medium for different periods of time before harvesting. Dm-ChP was performed and the input (Input) and recovered material (Dm-ChP) were analysed by western blotting using indicated antibodies.

exit S phase. The pull-down of the histone chaperone nucleophosmin as well as Cdc7 remained fairly constant throughout the analysis (Figure 5.11; lanes 5-11). The binding of NONO and lamin B1 was more efficient in the late time points as these proteins show a mark preference only for a matured DNA (Figure 5.11; lanes 6-10), consistently with our previous data. Intriguingly, capturing of histone H3 and H2A was delayed at the early time points (Figure 5.11; lane 5) and remained constant at later times (Figure 5.11; lanes 6-11), suggesting that reassembly of chromatin may not occur immediately after passage of the replication forks.

5.3.3 Conclusions

Immunoblotting analysis of nascent versus mature chromatin revealed that replication factors are indeed displaced from the DNA after passage of replication forks, suggesting that these may not be involved in chromatin maturation. However, we could detect most of the replication proteins such as Cdc45 and PCNA at matured chromatin which is consistent with their role in processing of Okazaki fragments. Moreover, we were able to detect weak signal from other replication proteins at the mature chromatin but this could be due to ongoing EdU incorporation after removal of media containing the nucleotide derivative. In the future, chase media should be supplemented with an excess of thymidine to ensure complete inhibition of EdU incorporation. Additionally, a more specific set of antibodies could be used to monitor chromatin maturation and nucleosome assembly after passage of replication machinery. We could analyse histone modifications such as mono-methylation of H4 at Lys20 or deacetylation of histone H4 at Lys5 or Lys12, chromatin factors, including HP1 α or histone modification enzymes Suv39h1 (histone methyltransferase) and HDAC1 (histone deacetylase 1) to study chromatin maturation. Moreover, proteomic analysis of proteins associated with nascent and matured chromatin should be performed to provide novel insights into the all components involved in chromatin duplication and maturation.

5.4 Dm-ChP studies of protein dynamics during chromatin maturation after DNA damage

5.4.1 Introduction

HU inhibits deoxyribonucleotide reductase and it is a common reagent used for a S phase synchronisation. Immediately, after HU treatment S phase checkpoint is activated and as a result replication forks are stabilised. However, when cells are exposed to HU for a longer period of time, double-strand breaks (DSBs) are induced (Petermann et al., 2010).

As we were interested in the effects of replication stress on the replisome we decided to perform similar experiments as described above and analyse chromatin maturation under replication stress conditions. We hypothesised that components of the replisome such as PCNA or Mcm7 will be associated with nascent DNA prior to addition of HU. However, we wondered whether different proteins may be recruited or displaced from the nascent DNA upon replication stress. We expected that HU treatment should stabilise replication forks to prevent their collapse, thus levels of many replication proteins should not change during the time of the experiment. We also predicted that after prolonged exposure to HU we may be able to observe destabilisation of the replisome machinery.

5.4.2 Proteins associated with stalled replication forks

To investigate proteins assembly and disassembly from a nascent DNA when replication fork are stalled, synchronised and EdU labelled HeLa cells were released from double thymidine block into early S phase for 105 minutes, labelled with EdU for 15 minutes and released into fresh medium without EdU in the presence of 10 mM HU prior to performing Dm-ChP (Figure 5.8 b). Cell cycle progression and checkpoint activation were monitored by flow cytometry and western blotting, respectively. EdU labelling was examined by fluorescence microscopy.

DNA content was determined by staining DNA with 7-AAD. As previously demonstrated, flow cytometry analysis confirmed synchronisation of

cell after double thymidine block (synchronous). In addition, we observed that at the 2 hour time point cell had just re-entered S phase and initiated DNA synthesis (0 hour time point) (Figure 5.12 a). Subsequently, HU was added to the medium and cells were collected at different times from 0.5 to 8 hours. We observed that HU treatment inhibited DNA synthesis; therefore cells were arrested in S phase and did not progress through the cell cycle as DNA profile did not change (Figure 5.12 a).

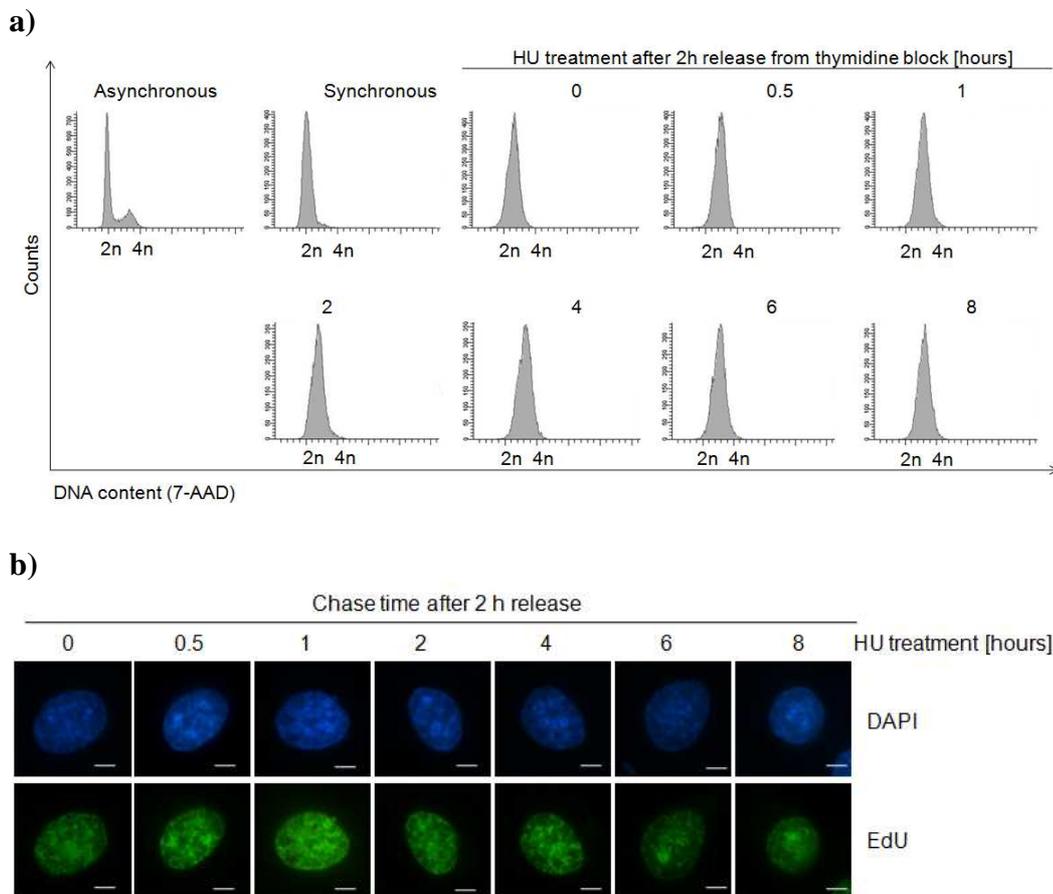


Figure 5.12 Analysis of DNA content and EdU foci in pulse-chase experiment in the presence of HU. HeLa cells arrested at the G_1/S transition were released from the cell cycle block and after EdU labelling chromatin was incubated in EdU free but HU containing medium **a)** DNA content of logarithmically growing cell population (Asynchronous) and cells collected at different times after EdU labelling (0-8 hours) **b)** Representative images of EdU incorporation sites were collected and show cells harvested at the indicated times post-EdU pulse. (Scale bar 10 μm).

Fluorescence microscopy analysis was consistent with flow cytometry results. We could observe replication foci in all cells analysed. The cell foci were

identical throughout the experiment and characteristic for early S phase, either before or after HU treatment, confirming S phase arrest (Figure 5.12 b).

To assess if replication checkpoint was activated, we measured phosphorylation of Chk1 at Ser317. We could detect activation of S phase checkpoint after half an hour post-HU treatment that lasted till the end of the experiment. Chk1 Ser317 phosphorylation slightly increased in time-dependent manner, showing that cells were efficiently arrested by HU (Figure 5.13).

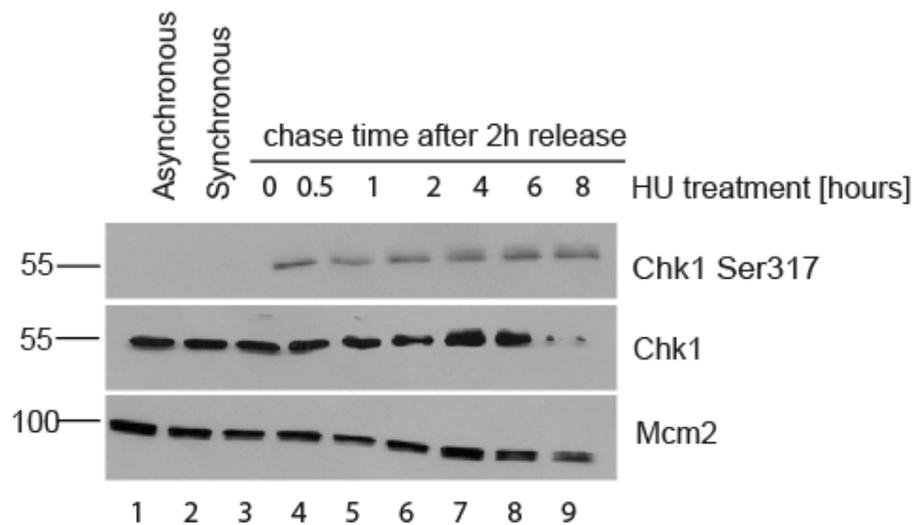


Figure 5.13 Analysis of S phase checkpoint activation. *HeLa cells were synchronised in S phase using double thymidine block and released for 2 hours into early S phase and further chased for different periods of time into HU medium. After collecting, cells were lysed in 1% SDS lysis buffer and analysed by western blotting using indicated antibodies. Mcm2 and Chk1 were used as loading controls.*

Finally, Dm-ChP captured material prepared as described at the beginning of the section was analysed by immunoblotting (see section 2.3.12). Different antibodies against replication proteins were used as probes. All proteins were present in the input samples taken at the 0 and 8 hour time points. Proteins were not recovered from mock treated cells (Figure 5.14; lane 4). We observed that levels of most proteins such as Smc1, Smc3, Mcm7, PCNA, CAF-1, nucleophosmin, lamin B1 and histone H2A are fairly constant throughout the time of the experiment (Figure 5.14; lanes 5-11). The level of replication proteins such as Cdc45 and RPA decreased in time-dependent manner (Figure 5.14; lanes 5-11).

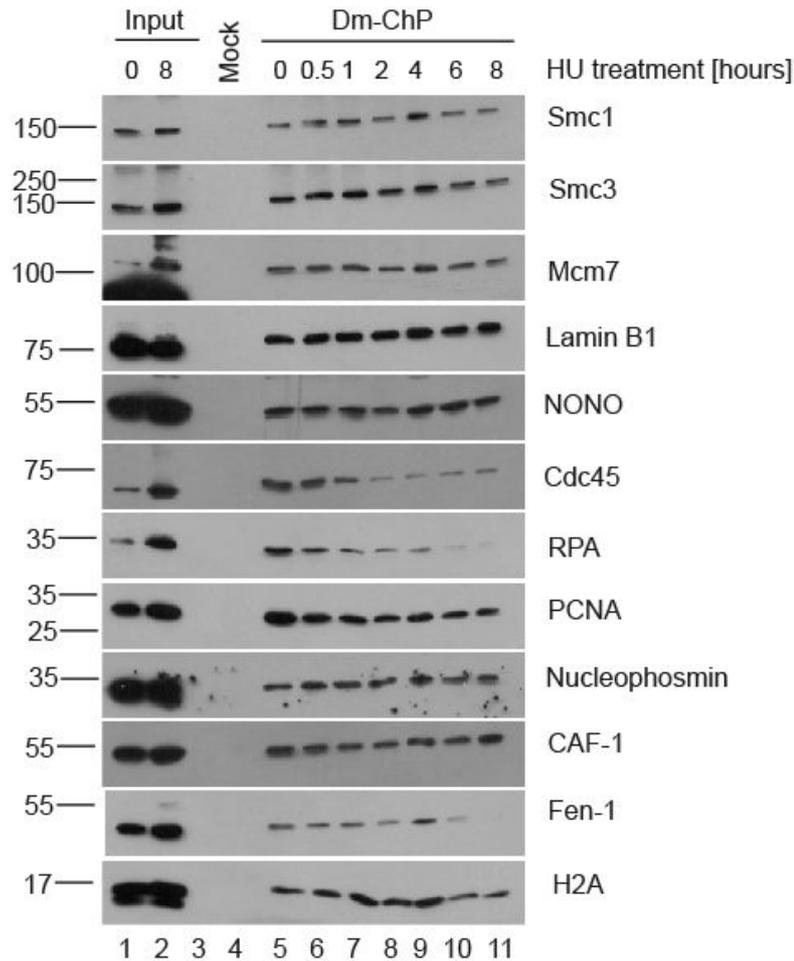


Figure 5.14 Protein dynamics during chromatin maturation after replication forks stall. HeLa cells were synchronised at the G₁/S boarder using double thymidine block and released into S phase for 2 hours. Cells were either mock treated or labelled with EdU for 15 minutes and subsequently chased into HU medium for different periods of time before harvesting. Dm-ChP was performed and the input material (Input) and recovered material (Dm-ChP) were analysed by western blotting using indicated antibodies.

5.4.3 Conclusions

As we expected, the immunoblotting analysis of Dm-ChP pulled-down material after replication stress showed replication forks arrest and replisome stabilisation. Levels of PCNA, Mcm7 or Smc1 were unaffected upon HU treatment. On the contrary, proteins such as Cdc45 and RPA were displaced from labelled DNA in a time-dependent manner. This suggests that Dm-ChP is able to detect early events of replisome stabilisation and disassembly. This could be due to recruitment of DNA repair proteins to stalled replication forks. To confirm this hypothesis, we could analyse chromatin association of proteins such as γ H2A.X,

Rad51, MRN complex and Ku70/80 that are known to be recruited to DNA damage sites. This analysis could provide novel information about spatial and temporal regulation of different DNA damage repair proteins at stalled or collapsed replication forks. Additionally, histone marks could be used to study arrested replication forks in two different approaches. Firstly, in response to replication blockage we should not detect histone modifications, such as trimethylation of histone H3 at Lys9 that are associated with chromatin maturation and assembly as this process should be inhibited in HU treated cells. Secondly, histone modifications at the site of replication block could be also analysed. However, to properly interpret such Dm-ChP experiments, pull-downs should be performed using identical amounts of EdU labelled DNA for each time point as previously discussed.

5.5 Preliminary attempt to capture the replisome

Our previous experiments showed that 1 minute pulse with EdU is sufficient to detect labelled DNA by flow cytometry analysis (see Figure 4.4 in section 4.4). Additionally, using Dm-ChP in combination with western blotting we were able to specifically detect histone H3 in the pulled-down material from asynchronous cell populations using EdU pulses as short as 10 minutes (see Figure 4.5 a in section 4.4). Under these conditions replication forks have synthesised approximately 20 kb of nascent DNA. As DNA fragments generated during sonication step are approximately 300 bp then in average every 66th recovered fragment should contain a replication fork. Therefore to increase the chances of capturing the replisome we hypothesised that the labelling time could be dramatically reduced since the cell population is synchronised in S phase.

To test whether replication proteins can be captured at or within close proximity to replication forks, HeLa cells were synchronised by double thymidine block, released into early S phase for 2 hours and labelled with EdU for 2 and 5 minutes prior to collection (see section 2.5.1.3). Additionally, after EdU labelling other cells were also incubated in EdU free, but HU containing medium for 30 minutes before harvesting. Cell synchrony and HU-induced stalling of replication forks were monitored by flow cytometry and western blotting, respectively.

As previously demonstrated, flow cytometry analysis confirmed synchronisation of cells that after thymidine block showed accumulation at the G₁/S boundary (synchronous).

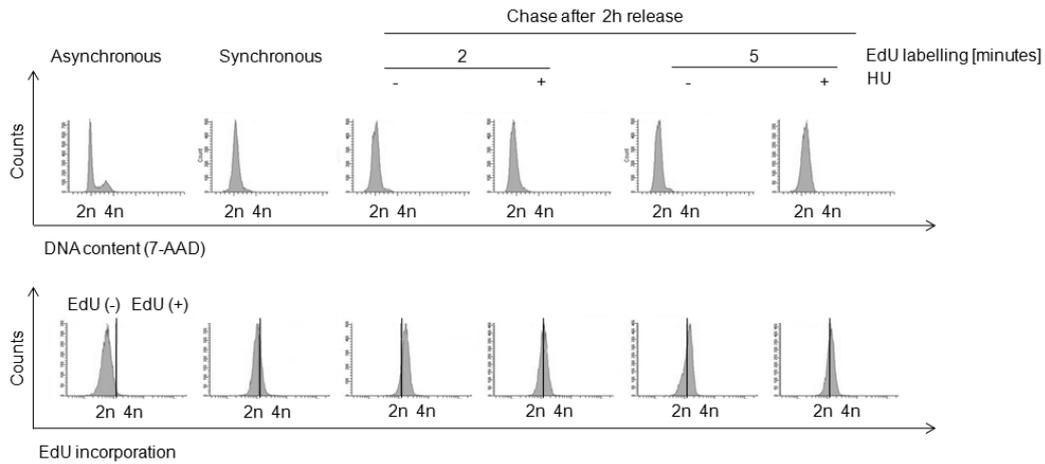


Figure 5.15 DNA content and EdU incorporation analyses of cells labelled with EdU for 2 and 5 minutes. HeLa cells were synchronised by double thymidine block, released into early S phase and labelled with EdU for 2 and 5 minutes prior collection. Additionally, after EdU labelling different set of cells was chased into 10 mM HU medium for 30 minutes. DNA content and EdU incorporation were analysed by flow cytometry.

To detect EdU incorporation, cell counts were plotted against 6-carboxyfluorescein-TEG azide fluorescence using histogram plots (Figure 5.15; bottom panel). We detected background levels of fluorescence in mock treated, asynchronous cells (Figure 5.15, bottom panel). Only cells labelled with EdU for 2 and 5 minutes showed increased levels of fluorescence and had similar cell cycle profiles as G₁/S arrested cells (Figure 5.15, bottom panel). Additionally, after HU treatment we observed similar DNA profiles and levels of EdU incorporation compared to mock treated cells, suggesting that DNA synthesis was inhibited efficiently (Figure 5.15, top panel).

To further confirm replication fork stalling and checkpoint activation after HU treatment, we analysed Chk1 Ser317 phosphorylation by western blotting (Figure 5.16). Chk1 was present in all samples analysed, however, phosphorylation of Chk1 Ser317 was only observed after HU treatment (Figure 5.16; lanes 4 and 6).

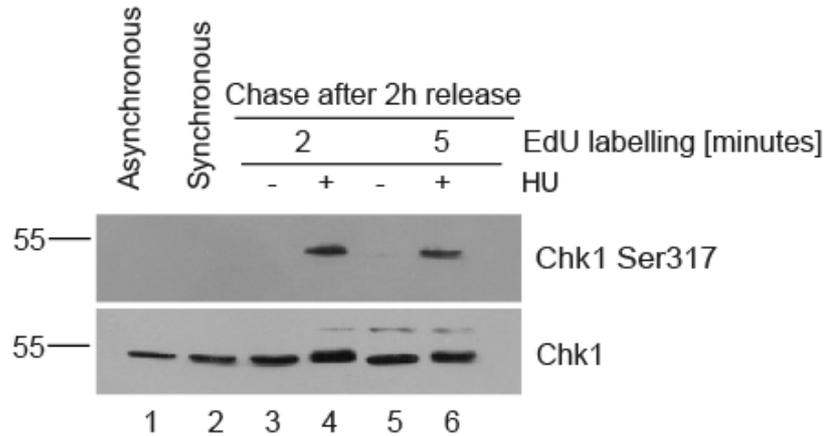


Figure 5.16 Detection of Chk1 phosphorylation in HU treated cells. *HeLa cells were synchronised by double thymidine block, released into early S phase and labelled with EdU for 2 and 5 minutes prior collection. Additionally, after EdU labelling different set of cells was chased into 10 mM HU medium for 30 minutes. After harvesting cells were lysed in 1% SDS lysis buffer and analysed by western blotting using indicated antibodies.*

The same samples were analysed by the Dm-ChP procedure (see section 2.3.12) using antibodies against different replication proteins (Figure 5.17). We could detect a time-dependent increase of Cdc45 and PCNA levels after as short as 2 and 5 minutes following EdU labelling. This suggests that we are possibly capturing proteins closely associated with the replication machinery (Figure 5.17; lanes 7 and 9). With the labelled chromatin that was synthesised within 5 minutes we could also recover the FACT complex, Cdc7 and RPA (Figure 5.17; lanes 7 and 9), suggesting that either these proteins associate with DNA after replication fork passage or their amount is below detection limit in the 2 minute EdU pulse sample. Interestingly, histone H3 was not recovered after the 2 and 5 minute EdU pulses. This is consistent with our previous observations that there could be a delay in histone loading onto newly replicated chromatin (Figure 5.17; lane 10).

After HU treatment, we could detect all proteins but not histone H3 in both samples labelled for 2 and 5 minutes. Histone H3 was only recovered after 5 minutes of EdU pulse followed by HU (Figure 5.17; lane 10). This suggests that these proteins associate with chromatin in response to HU-induced replication forks stalling or that EdU incorporation was not fully inhibited by HU treatment (Figure 5.17; lanes 8 and 10). Moreover, we observed a faster migrating form of RPA only in samples exposed to HU. This could possibly correspond to the

phosphorylated form of RPA although this would require further confirmation (Figure 5.17; lanes 8 and 10).

This short time pulse experiment revealed that Dm-ChP can be a good tool to study proteins that are part of the replisome and proteins that associate with stalled replication forks.

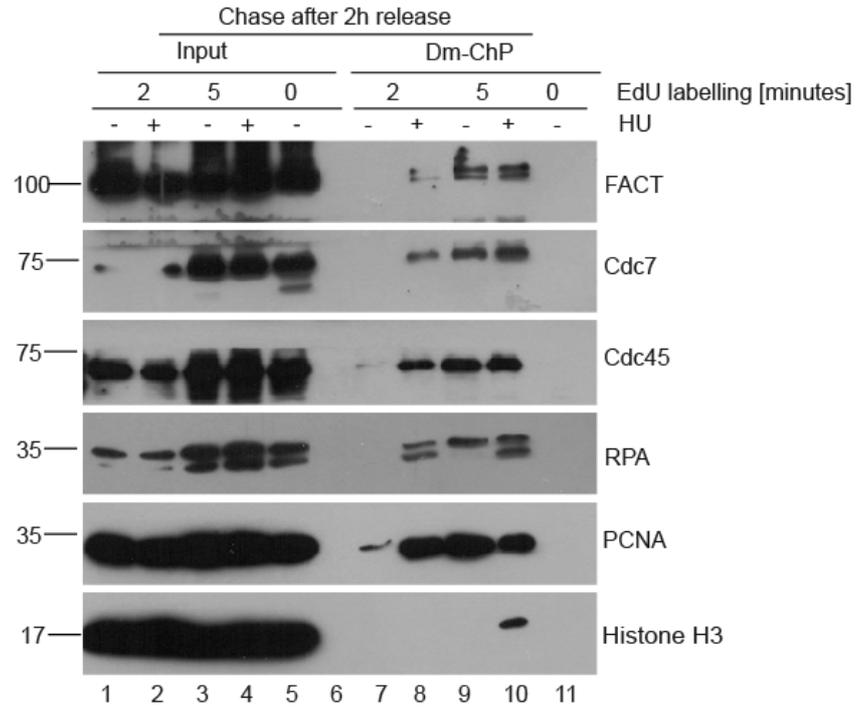


Figure 5.17 Dm-ChP analysis of proteins in close proximity to the replication forks. HeLa cells were synchronised by double thymidine block, released into early S phase and labelled with EdU for 2 and 5 minutes prior collection. Additionally, after EdU labelling different set of cells was chased into 10 mM HU medium for 30 minutes. Dm-ChP procedure was performed and the input material (Input) and captured material (Dm-ChP) were analysed by western blotting using indicated antibodies.

Chapter 6 Discussion

In this study we have developed an innovative methodology referred to as DNA mediated chromatin pull-down (Dm-ChP). The Dm-ChP allows specific recovery of the newly synthesised chromatin and analysis of the protein components associated with it. This novel technique relies on incorporation of a thymidine analogue EdU by active replication forks, into nascent DNA. This analogue is further coupled to a biotin-TEG azide tagging molecule through the Click reaction allowing nascent chromatin to be affinity purified using streptavidin-coated resin (Kliszczak et al., 2011).

6.1 Development of DNA mediated chromatin pull-down technique

To investigate proteins associated with newly synthesised DNA we have based our approach on the chromatin immunoprecipitation protocol. We have addressed each individual step, including formaldehyde protein-DNA cross-linking, DNA shearing and DNA-protein cross-linking reversal (see section 3.2). Under these optimised experimental conditions we were able to capture known replication proteins such as Mcm2. We could also detect a known subunit of the MCM helicase complex, Mcm7, that co-immunoprecipitated with Mcm2 (Figure 3.5). These data indicate that after several steps during lysate preparation such as formaldehyde cross-linking and cross-link reversal, chromatin bound proteins are largely unaffected and can be further analysed by immunoblotting.

To identify proteins associated with newly replicating chromatin, we explored three different strategies. These approaches were based on tagging of nascent DNA with distinct molecules such as BrdU, 5'-BMA and biotin.

The first strategy involved BrdU labelling of nascent DNA, followed by immunoprecipitation with an anti-BrdU antibody. Using this approach we were able to capture purified, labelled DNA (Figure 3.10). However, we failed to immunoprecipitate nascent DNA from the chromatin fraction as assessed by a lack of histone H2A recovery in anti-BrdU pull-downs (Figure 3.11). To allow epitope exposure for antibody recognition, the double-stranded DNA needs to be denatured, therefore we initially believed that this failure was due to inefficient

exposure of the BrdU moiety (Gratzner, 1982). We tested several DNA denaturation conditions such as different pH ranges (2M HCl, 0.1M NaOH), high temperature (heat-shock) or 4M urea (Ageno et al., 1969). However, we were not able to purify BrdU labelled chromatin in any of the conditions analysed (data not shown). We suspect that the denaturation step was critical for chromatin immunoprecipitation from cell lysate. This might cause protein degradation that could further affect the ability to detect recovered proteins by immunoblotting. Moreover, we cannot exclude the other potential factors such as lower accessibility of labelled DNA when immunoprecipitated from the chromatin fraction.

Interestingly, a similar approach has recently been described where anti-BrdU antibody was used to capture 5-chloro-2'-deoxyuridine (CldU) labelled ssDNA to study the role of Rad51 in the restart of HU stalled replication forks (Petermann et al., 2010). The affinity of the BrdU antibody towards the CldU analogue is higher than for BrdU, suggesting that it may be easier to capture CldU labelled DNA than BrdU labelled DNA. These data suggest that capturing of the labelled chromatin based on this BrdU tagging approach is possible but the denaturation step needs to be further improved and optimised.

Due to unsuccessful pull-down of BrdU labelled DNA from the chromatin fraction, we decided to explore a labelling and tagging strategy based on a different nucleotide derivative called EdU. The major advantage of DNA labelling with EdU is the detection step which allows access to the incorporated thymidine analogue without DNA denaturation (Salic and Mitchison, 2008). Using this approach, EdU labelled DNA was covalently conjugated to a BrdU probe called 5'-BMA through the Click reaction (Cappella et al., 2008). Next chromatin was captured using an identical approach as described above for BrdU. Under the experimental conditions used we could specifically recover purified, labelled DNA fragments (Figure 3.16). Moreover, we were able to pull-down labelled chromatin from cell lysate. Unfortunately, recovery of labelled DNA from the chromatin fraction was not specific (Figure 3.17). To minimise non-specific binding of labelled chromatin to the Protein A-agarose beads we performed a pre-cleaning of the lysate. However, this step did not prevent non-specific capturing of labelled chromatin as histone H3 was again detected in a

control immunoprecipitation sample (data not shown). The EdU-5'-BMA-based approach was initially promising but under our experimental conditions it may not be suitable for studying proteins associated with newly synthesised chromatin. Pre-blocking of the Protein A-agarose beads before the pull-down step could prevent the non-specific binding of labelled chromatin to the resin although this was not investigated.

6.2 Validation of Dm-ChP technique

As a third strategy, we investigated a different tagging molecule, biotin-TEG azide, to capture nascent DNA, followed by streptavidin pull-down. This method excludes the use of antibodies that may later interfere with detection of the recovered proteins. Additionally, since the affinity of streptavidin to biotin is higher than the affinity between BrdU and anti-BrdU antibody (Diamandis and Christopoulos, 1991), we hypothesised that this approach could be more efficient at pulling-down labelled chromatin. In a similar approach to the second strategy (EdU-5'-BMA-based approach), newly replicated chromatin was labelled with EdU. Through the Click reaction EdU labelled DNA was subsequently conjugated to biotin moiety and recovered using streptavidin-coated beads. This method was referred to as DNA mediated chromatin pull-down (Dm-ChP). Using the Dm-ChP approach the capturing of labelled chromatin was specific as histone H3 was recovered only in the pull-down from the EdU labelled cells (Figure 3.21).

The sensitivity of the Dm-ChP methodology depends on several factors including the time period of EdU labelling. We have determined that a 1 minute pulse with EdU was sufficient to detect labelled DNA by flow cytometry (Figure 4.4). Additionally, using Dm-ChP in combination with western blotting approach we were able to specifically capture histone H3 with the labelled DNA that was synthesised within 10 minutes (Figure 4.5 a). These data indicate that DNA labelling with EdU is robust and very efficient. In addition, specific recovery of histone H3 suggests that the Dm-ChP could be applied to study the protein components of different processes that involve DNA synthesis.

To further characterise this novel Dm-ChP approach we performed several control experiments to validate its specificity. We demonstrated that omission of

each of the components required for the Click reaction prevented capturing of the labelled chromatin (Figure 4.1). Additionally, we showed that Dm-ChP specifically captures EdU labelled chromatin from a mixture of labelled and unlabelled DNA. We verified that intermolecular aggregation/precipitation between biotinylated and untagged chromatin fragments does not occur as showed by lack of GFP-histone H3 signal in pulled-down samples when extracts from EdU labelled HeLa cells were mixed with extracts of unlabelled chromatin that was marked with GFP-histone H3. This clearly demonstrates that the Dm-ChP is specific and that the capturing of chromatin is due to EdU-biotin tagging of the nascent DNA (Figure 4.3).

We performed titration experiments which revealed that one of the limiting factors for capturing of the biotinylated chromatin is the amount of streptavidin-coated beads used in the pull-down step. Levels of PCNA and histone H3 that were recovered with labelled chromatin after the Dm-ChP increased with the amount of resin used (Figure 4.9). Moreover, when different amounts of labelled chromatin were presented in the starting material we observed a positive correlation between the amount of EdU labelled DNA and PCNA recovered in the pull-down (Figure 4.6). These data demonstrate that the Dm-ChP methodology meets the important requirements of every assay, because it is specific and potentially semi-quantitative.

Finally, we were able to confirm our hypothesis that the efficiency of chromatin capture is dependent on the amount of labelled DNA and the amount of streptavidin-coated resin used in the pull-down step. In titration experiments we varied the amount of the biotinylated DNA in the input sample and found that labelled DNA can be fully depleted at particular ratios of labelled DNA to streptavidin-coated beads. This indicates that all biotin-tagged chromatin is accessible and can be successfully recovered (Figure 4.7).

When a constant amount of streptavidin-coated beads were used in pull-downs and increasing amounts of biotin-TEG azide were used to tag labelled chromatin we did not enrich protein capture (Figure 4.8). This result indicates that there was no more available EdU alkyne moiety that could react with biotin azide to increase the efficiency of recovered chromatin fragments. This also strengthens the possibility that all EdU incorporated into nascent DNA was tagged with biotin. We cannot exclude the possibility that un-reacted EdU is left

in DNA and cannot be tagged with biotin moieties due to steric hindrance making some of the labelled DNA unavailable for recovery. However, the average GC-content of an 100 kb fragment of the mammalian genome ranges from 35-60% depending upon the genomic location (Romiguier et al., 2010). Therefore a 300 bp DNA fragment obtained under our experimental conditions is estimated to contain between 60 and 98 thymidine residues which can be replaced with EdU molecules during DNA synthesis, therefore it is probable that each DNA fragment should contain at least one biotin residue after the conjugation reaction. A single biotin moiety is sufficient to capture a labelled DNA fragment as shown by Syvänen and colleagues (Syvänen et al., 1988). Taken together these data suggest that streptavidin-coated beads are the limiting factor for the quantitative capture of biotinylated chromatin. Careful quantification of the amount of biotin labelled DNA would be also essential to address this question in future work.

After thorough characterisation and validation of the Dm-ChP procedure, we used this technique in combination with mass spectrometry to analyse proteins associated with nascent chromatin (see section 4.8). Using the Global Proteome Machine search engine we have identified 326 proteins using XTandem! and XHunter! algorithms. We recognised 277 proteins with a $\log(e) \leq -3$, where the $\log(e)$ corresponds to the base-10 log of the expectation that any particular protein assignment was made at random (E-value) (Appendix 2; Table 2). Proteins were classified manually into 29 different classes using information from *UniProt* protein database (Figure 4.13). We identified proteins known to be either constitutively associated with chromatin (histones, cohesin), or transiently during DNA duplication (DNA helicase, topoisomerases, PCNA, MCM), DNA repair (Ku70/80, DNA-PK) or histone chaperones (CAF-1, nucleolin, nucleophosmin) that can be captured with newly replicated DNA (Appendix 2; Table 2). Similar to other proteomic studies where the composition of chromosomes were examined (Ohta et al., 2010), we recovered ribosomal proteins using Dm-ChP. The majority of the ribosomal proteins have high pI values and may bind adventitiously to DNA possibly during extract preparation. Therefore, these proteins have been defined as chromosome hijacker proteins (Ohta et al., 2010). Additionally, we also recovered proteins involved in mRNA processing (transcription and splicing factors). It has recently been confirmed

that DNA replication and transcription processes are closely associated (Bermejo et al., 2011). During transcription hybrids between DNA and an mRNA product is formed and creates R-loop like structures (Bermejo et al., 2009; Bermejo et al., 2011; Gomez-Gonzalez et al., 2009; Zlatanova and van Holde, 1992). It is possible that the newly synthesised and labelled DNA is transcribed immediately after passage of the replication fork, thus transcription factors that are bound to this chromatin become cross-linked to DNA and recovered during the process of Dm-ChP pull-down.

6.3 Dm-ChP studies of DNA replicating at different times during S phase

The Dm-ChP approach in combination with highly quantitative mass spectrometry analysis was used to investigate proteins associated with replicating chromatin at different times during S phase. The proteins identified were listed according to an increasing heavy/medium normalised ratio (H/M), reflecting their relative abundance in the early and middle/late synthesised DNA, respectively. Most of the proteins present at the replication forks such as PCNA, Mcm7, Replication factor C, Fen-1 and Topoisomerases 1 and 2 were enriched in the early replicating chromatin (Appendix 3; Table 3). This data is consistent with the evidence that the average rate of mammalian replication forks is not constant throughout S phase (Housman and Huberman, 1975). The replication forks in early stages of S phase are 2-3 times slower when compared to those firing in late S phase (Herrick and Bensimon, 2008), thus the probability of capturing the replisome at this time point is higher than in late S phase. On the contrary, we observed that the lamin B1, involved in formation of the nuclear envelope, was enriched in the late replicating chromatin. This is consistent with the evidence that lamin marks transcriptionally silenced genes (heterochromatin) that are replicated later in S phase (Olins et al., 2010). We also detected increased levels of ribosomal proteins associated with late replicating chromatin, a time when replication of rDNA occurs, suggesting that ribosomal DNA transcription and ribosome assembly are closely coupled (Boisvert et al., 2007). As the Dm-ChP procedure involves *in vivo* protein-DNA cross-linking we suggest that ribosomal proteins may interact *in vivo* with the chromatin in a localised manner.

Using semi-quantitative immunoblotting analysis we were able to confirm some of the SILAC data. We distinguished two categories of proteins. In the first group we found proteins that either completely or partially confirmed the SILAC results, while the second category included proteins that were not consistent with this analysis. Similarly to SILAC data, the binding of lamin B1 (H/M ratio 1.05), and NONO (H/M ratio 1.15) to chromatin was more efficient in cells replicating in late S phase (Figure 5.7). Proteins that were identified, and were consistent with mass spectrometry data, included replication factors: PCNA (H/M ratio 0.58), Mcm7 (H/M ratio 0.55), FACT (H/M ratio 0.46) and Fen-1 (H/M ratio 0.64). These proteins were enriched in the early S phase (2 hour post-release) and reduced at the 6 hour time point when analysed by SILAC, whereas such small difference has not been observed using immunoblotting (Figure 5.7). However, we did detect decreased chromatin binding of these proteins in late S phase (8 hour post-release). In contrast to SILAC data, where proteins such as histone H4 (H/M ratio 0.92), histone chaperone CAF-1 (H/M ratio 0.50) and Smc1 (H/M ratio 0.31) showed a marked preference for early replicating chromatin, the immunoblotting revealed constant levels of these proteins throughout the analysis (Figure 5.7). The differences observed between immunoblotting and SILAC could be related to the low dynamic range of chemiluminescence immunoblotting and slightly different experimental design such as a 15 minutes shorter EdU labelling pulse in immunoblotting analysis. It may be possible to resolve this problem by using quantitative immunoblotting such as the Odyssey infrared fluorescence imaging system as was performed by Sirbu and colleagues (Sirbu et al., 2011).

6.4 Dm-ChP studies of protein dynamics during chromatin maturation

Next, we sought to define chromatin bound proteins that are stably or transiently associated with active replication forks. To assess this we used a combination of the Dm-ChP and pulse-chase approaches. Using immunoblotting we monitored maturation of the chromatin that was labelled with EdU in early S phase. Replication proteins such as PCNA, Mcm7, Cdc45, RPA and FACT were abundant at newly synthesised DNA (section 5.5; Figure 5.11). As chromatin

was maturing, interaction of these proteins with nascent DNA was reduced, suggesting that these replication factors are not associated with mature chromatin (Figure 5.11). During our analysis we also observed that level of proteins such as lamin B1 and nucleolin was fairly low on nascent DNA and increased on matured chromatin, indicating that these proteins are loaded onto the DNA after passage of replication machinery. Intriguingly, the observation that lower levels of histone H3 and H2A are associated with nascent DNA compared to mature chromatin, suggest that there may be a delay in the loading of histones onto newly synthesised chromatin.

PCNA not only travels with the replication forks and tethers DNA polymerases to a DNA template (Moldovan et al., 2007; Prosperi, 1997) but also marks nascent chromatin for the histone chaperone CAF-1 (Shibahara and Stillman, 1999). Association of PCNA with chromatin after DNA synthesis was demonstrated in *Xenopus* where PCNA promotes Cdt1 ubiquitination, thus preventing re-replication (Arias and Walter, 2006). Moreover, the presence of PCNA on matured chromatin supports the role of PCNA in the process of maturation of Okazaki fragments. Additionally, PCNA interacts with Fen-1 to remove RNA primers and subsequently with Lig1 (DNA ligase 1) to ligate the nick between fragments of nascent DNA (Ayyagari et al., 2003). The decreased levels of PCNA observed during our analysis might be a result of its displacement from synthesised Okazaki fragments. Similarly, we observed in our analysis that Fen-1 is associated with nascent DNA and it is gradually displaced from maturing chromatin, which is consistent with its role in maturation of the lagging strand (Ayyagari et al., 2003). Cdc45 is another chromatin bound protein analysed that was more abundant with the nascent DNA and its levels were reduced on mature chromatin. Studies in budding yeast are consistent with our observations and suggest the role of Cdc45 in maturation of Okazaki fragments (Reid et al., 1999). Furthermore, it has been shown that S phase progression releases Cdc45 from chromatin, thereby preventing re-initiation of DNA replication (Bell and Dutta, 2002). Pulse-chase analysis revealed that MCM complex similarly to replication proteins mentioned above was preferentially associated with newly replicated chromatin and its association with the DNA was reduced as chromatin was maturing. This is consistent with different studies presented in the literature. MCM complex is required for DNA unwinding and it

is constitutively displaced from DNA as cells progress through S phase (Dimitrova et al., 1999). Recent studies in *Xenopus* egg extracts, confirmed that Mcm2-7 helicase is associated with chromatin after passage of the replication forks and is disassembled from the chromatin by MCM-BP (MCM-binding protein) at the end of S phase (Nishiyama et al., 2011). Additionally, MCM/FACT complex interacts with Cdc45 (Tan et al., 2010) that is consistent with the similar behavior of these proteins after passage of replication machinery presented in our analysis. We observed low levels of Smc1 at late time points during pulse-chase experiments and this is consistent with its role in sister chromatid cohesion. Smc1 is the component of the cohesin complex that is loaded onto chromatin during G₁ phase by Scc2/Scc4 heterodimer (Ciosk et al., 2000). While cells progress to M phase the cohesin ring is dissolved, thereby facilitating chromosome separation (Cuylen and Haering, 2011).

Association of Cdc7 with chromatin has not been well characterised. Cdc7/Dbf4-dependent phosphorylation of Mcm2 is essential for initiation of DNA synthesis (Sheu and Stillman, 2006), but there is no direct evidence supporting whether Cdc7 physically interacts with chromatin or if it travels with replication forks. To our knowledge this is the first report demonstrating that Cdc7 not only binds to newly replicating DNA but is also associated with mature chromatin, suggesting possible novel role of Cdc7 in chromatin maturation.

The single-stranded DNA binding protein RPA showed preferential binding to the nascent DNA and its levels decreased during chromatin maturation. The presence of RPA on mature chromatin was rather surprising as after replication fork passage it is not expected that there should be any single-stranded DNA that RPA could bind to. From our analysis we also cannot rule out that the observed chromatin association of RPA after replication fork passage is due to its binding to dsDNA, its roles in the processing of Okazaki fragments (Bae et al., 2001) or its interactions with other proteins, such as FACT (VanDemark et al., 2006). These results may also suggest that RPA could have some unidentified roles in chromatin maturation.

6.5 Dm-ChP studies of protein dynamics during chromatin maturation upon replication stress

The Dm-ChP approach was also used to analyse the protein component associated with stalled replication forks. Hydroxyurea inhibits deoxyribonucleotide reductase leading to depletion of deoxyribonucleotides, activation of S phase checkpoint and stabilisation of the replication forks (Petermann et al., 2010). After the EdU pulse, labelled chromatin was allowed to mature in the presence of HU. We observed that HU treatment resulted in stable chromatin association of replication proteins such as PCNA, Smc1, Smc3, Mcm7 or Fen-1, compared to untreated samples (compare Figure 5.11 and 5.14). Levels of proteins such as lamin B1 or NONO that were not present on the nascent DNA and arrived later after passage of active replication forks remained constant after replication forks stall. Intriguingly, chromatin bound proteins such as Cdc45 and RPA were gradually unloaded from the chromatin under both replication stress and physiological conditions.

These data indicate that after inhibition of DNA synthesis, replication proteins that were loaded onto newly synthesised DNA remain associated with this DNA. This is consistent with the notion that replisome stabilisation is required for replication fork restart and completion of DNA synthesis after block removal (Lucca et al., 2003; Segurado and Tercero, 2009). Experiments in budding yeast, showed that proteins such as Cdc45 and MCM helicase are retained at stalled forks after HU treatment (Calzada et al., 2005). Moreover, dissociation of Mcm2-7 complex from stalled replication forks prevents recovery of DNA replication in budding yeast (Katou et al., 2003; Labib et al., 2000). Consistently, we observed stable levels of Mcm7; however levels of Cdc45 decreased after replication fork stalling. This discrepancy may be a result of interspecies differences between yeast and human cells or represents an early event in replisome disassembly. Even though RPA accumulates at single-stranded DNA after fork pausing in both human and budding yeast (reviewed in (Cimprich and Cortez, 2008)), we observed a time-dependent decrease of this protein, suggesting that RPA is unloaded from stalled replication forks at later time points, possibly allowing recruitment of DNA repair and replication fork

stabilising proteins. To test this hypothesis we could monitor levels of Rad51 that displaces RPA from single-stranded DNA to allow replication fork restart through recombination (Stauffer and Chazin, 2004).

6.6 Overall conclusions and future perspectives

The primary goal of the Dm-ChP technology was to purify proteins at or within close proximity to the replication fork. We decided to investigate whether this approach is sensitive enough to capture the replisome. We have already validated that EdU incorporation can be detected by flow cytometry after a 1 minute EdU pulse (Figure 4.4). To maximise the chances for replisome recovery we decided to combine cell synchronisation that allows us to increase the fraction of cells in S phase with short EdU labelling pulses. Additionally, we blocked progression of the replication forks by HU treatment. We provided preliminary evidences that replication proteins such as PCNA, Cdc45, RPA, Cdc7 and FACT can be captured with labelled chromatin synthesised within 2-5 minutes (Figure 5.17). Intriguingly, after HU addition we could observe increased amount of all the proteins analysed, suggesting that EdU incorporation was not immediately inhibited by HU treatment (Figure 5.17). Although, we do not fully understand all of our observations, these preliminary data indicate that the Dm-ChP is highly sensitive and may be suitable to capture proteins at the replication fork.

While completing this study, Bianca M. Sirbu and co-workers reported the development of a new method named iPOND (isolation of proteins on nascent DNA). This technique was used to study protein dynamics at active and stalled replication forks (Sirbu et al., 2011). Similarly to Dm-ChP, iPOND relies on EdU labelling of nascent DNA and biotin-mediated capturing of chromatin using streptavidin beads. The authors use a biotin tag molecule that is photocleavable and allows biotinylated chromatin to be eluted without denaturation conditions. This advantageous elution method increases the purity of captured material and is more compatible with proteomic approaches (Kim et al., 2009) when compared to Dm-ChP. The remaining differences between both methods include experimental variations such as extract preparation, cross-linking, Click reaction conditions and number and type of cell line used. In the iPOND method proteins are cross-linked to DNA for 20 minutes at room temperature and the Click

reaction is performed for 1-2 hours (Sirbu et al., 2011). The Dm-ChP approach relies on 10 minutes cross-linking at 4°C and the Click reaction is performed for 30 minutes. Both cross-linking and the Click reaction were optimised during the development of Dm-ChP and optimal times were used to prevent excessive cross-linking or non-specific labelling of chromatin (Kliszczak et al., 2011). Moreover, the Dm-ChP approach utilises cell fractionation and preparation of the chromatin enriched fraction in RIPA buffer, while whole cell lysate prepared in 1% SDS lysis buffer is used to capture chromatin in iPOND method. The cellular fractionation approach allows for elimination of cytoplasmic proteins that are not normally present in the nucleus but could interfere with any subsequent analysis of the chromatin fraction. Depending on the experiment and EdU labelling pulse typically 2×10^6 to 2×10^7 HeLa cells were used in Dm-ChP and approximately 1.5×10^8 HEK 293T cells in the iPOND method. The higher amount of cells may have been used to allow for efficient capture of chromatin using very short EdU pulses and we are not sure if Sirbu and co-workers tested lower amount of cells (Sirbu et al., 2011).

In summary, we have established a methodology that allows the investigation of proteins that are associated with newly replicated DNA by the EdU labelling and biotin tagging of newly synthesised DNA. As the chromatin bound proteins appear to be largely unaffected by the chemical conditions required for biotin conjugation, formaldehyde cross-linking and cross-link reversal they can be further analysed with simple immunological techniques or in large scale proteomic studies. The specificity and flexibility of this technique, together with the possibility of manipulating the cellular systems, labelling times and conditions of extract preparation, make the Dm-ChP a potential leading method not only for the study of the relationships between chromatin proteins and the temporal regulation of DNA synthesis, but more generally in studies on chromatin maturation and for investigating how these processes are linked to the duplication of its basic constituent the DNA.

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APPENDIX 1

Table 1 Oligonucleotides used for PCR-based amplification

Primer name	Sequence (5'-3')	Use
Actin 1217-1541-FW	GCGTCATGGTCGGTATG	For amplification of captured DNA
Actin 1217-1541-RV	CGGTGGTCCTGCCGGAGG	

FW - forward primer, RV - reverse primer.

APPENDIX 2

Table 2 List of proteins identified by proteomics analysis in the Dm-ChP pulled-down material using either XHunter! or XTandem! algorithms with a log(e) score \leq -3.

UniProt KB	Entrez Gene	pI	Mr (kDa)	Description	Localisation	Function
Q8IZL8	27043	4.38	124.9	Proline-, glutamic acid- and leucine-rich protein 1	Nuclear	Transcription factors
O75607	10360	4.55	19.3	Nucleophosmin	Nuclear	Chaperones
P19338	4691	4.6	76.6	Nucleolin	Chromosome	Chromosomal periphery proteins
Q99733	4676	4.6	42.8	Nucleosome assembly protein 1-like 4	Nuclear	Histone modification factors
P62258	7531	4.63	29.2	14-3-3 protein epsilon	Cytosol	Chaperones
P06748	4869	4.64	32.6	Nucleolar phosphoprotein B23, numatrin	Chromosome	Chromosomal periphery proteins
P19105	10627	4.65	19.8	Myosin, light chain 12A	Cytosol	Cytoskeleton proteins
Q14974	3837	4.68	97.1	Karyopherin (importin) β -1	Nuclear	Protein transport
D6RF23	2771	4.72	11.2	Guanine nucleotide binding protein (G protein)	N/A	N/A
Q09028	5928	4.74	47.6	Retinoblastoma binding protein 4	Nuclear	Histone modification factors
Q9BUF5	84617	4.77	49.8	Tubulin, β -6	Cytosol	Cytoskeleton proteins
P07437	203068	4.78	49.6	Tubulin β chain (Tubulin β -5 chain)	Cytosol	Cytoskeleton proteins
A6NE09	388524	4.78	32.9	Ribosomal protein SA pseudogene 58	Ribosome	Ribosomal proteins
P68371	10383	4.79	49.8	Tubulin, β -2C	Cytosol	Cytoskeleton proteins
P08865	3921	4.79	32.8	40S ribosomal protein SA; Laminin receptor 1	Ribosome	Ribosomal proteins
O00410	3843	4.8	125.5	Importin 5, Importin-b N	Nuclear	Protein transport
Q92973	3842	4.83	102.3	Transportin-1; Importin β -2	Nuclear	Protein transport
Q15029	9343	4.84	109.4	U5 small nuclear ribonucleoprotein component	Nuclear	snRNP
Q1KMD3	221092	4.85	85.1	Heterogeneous nuclear ribonucleoprotein U-like protein 2	Nuclear	hnRNP
P68366	7277	4.93	49.9	Tubulin, α 4a	Cytosol	Cytoskeleton proteins
O60812	343069	4.93	32.1	Heterogeneous nuclear ribonucleoprotein C-like 1	Nuclear	hnRNP

P68363	10376	4.94	50.1	Tubulin, α 1b	Cytosol	Cytoskeleton proteins
P07910	3183	4.95	33.6	Heterogeneous nuclear ribonucleoprotein C (C1/C2)	Nuclear	hnRNP
Q15084	10130	4.95	48.1	Protein disulfide isomerase family A, member 6	Cytosol	Chaperones
P08238	3326	4.96	83.2	Heat shock protein 90kDa α , class B member 1	Cytosol	Chaperones
P12270	7175	4.97	267.1	Nucleoprotein TPR	Chromosome	Centromere proteins
Q86U42	8106	5.04	32.7	Poly(A) binding protein, nuclear 1	Nuclear	RNA modifying enzyme and related proteins
P08670	7431	5.05	53.6	Vimentin	Cytosol	Cytoskeleton proteins
P11021	3309	5.06	72.3	Heat shock 70kDa protein 5(glucose-regulated protein)	Cytosol	Chaperones
P07900	3320	5.07	98.1	Heat shock protein 90kDa α , class A member 2	Cytosol	Chaperones
Q6IS14	143244	5.07	16.8	Eukaryotic translation initiation factor 5A-like 1	Nuclear	RNA binding proteins
P20700	4001	5.11	66.4	Lamin B1	Nuclear	Structural
Q15393	23450	5.13	135.5	Splicing factor 3b, subunit 3	Nuclear	Splicing related factors
Q12905	3608	5.18	43	Interleukin enhancer binding factor 2	Nuclear	RNA binding proteins
Q6IBN1	3190	5.19	51	Heterogeneous nuclear ribonucleoprotein K	Nuclear	hnRNP
Q13813	6709	5.22	284.4	Spectrin, α , non-erythrocytic 1 (α -fodrin)	Cytosol	Cytoskeleton proteins
P68032	70	5.23	42	α -actin, cardiac muscle 1	Cytosol	Cytoskeleton proteins
Q13185	11335	5.23	20.8	Chromobox homologue 3 (HP1 γ homologue, <i>Drosophila</i>)	Chromosome	Chromosomal proteins
P52292	3838	5.25	57.8	Karyopherin α -2	Nuclear	Protein transport
Q9NQX3	10243	5.25	79.7	Gephyrin	Membrane proteins	Cytoskeleton proteins
P06576	506	5.26	56.5	ATP synthase, H ⁺ transporting, mitochondrial F1 complex	Cytosol	Mitochondrial proteins
O43707	81	5.27	104.8	α -actinin 4	Cytosol	Cytoskeleton proteins
O43432	8672	5.27	176.5	Eukaryotic translation initiation factor 4 γ -3	Nuclear	Translation factors
Q04637	1981	5.27	175.4	Eukaryotic translation initiation factor 4 γ -1	Nuclear	Translation factors
Q14566	4175	5.28	92.8	Minichromosome maintenance complex component 6	Chromosome	DNA replication proteins
P60709	60	5.29	41.7	β -actin	Cytosol	Cytoskeleton proteins
Q5JYR4	6185	5.3	18.2	Ribophorin II	Membrane proteins	Protein modification
P63261	71	5.31	41.8	γ -actin 1	Cytosol	Cytoskeleton proteins
P11142	3312	5.37	70.9	Heat shock 70kDa protein 8	Cytosol	Chaperones
P60842	1973	5.32	46.1	Eukaryotic translation initiation factor 4A1, DEAD-like Q motif	Nuclear	RNA helicases
Q16401	5711	5.34	56.2	Proteasome (prosome, macropain) 26S subunit	Nuclear	Chaperones
Q13283	10146	5.36	52.1	GTPase activating protein (SH3 domain) binding protein 1	Nuclear	RNA binding proteins

P52597	3185	5.37	45.6	Heterogeneous nuclear ribonucleoprotein F	Nuclear	hnRNP
Q08043	89	5.37	103.2	α -actinin-3	Cytosol	Cytoskeleton proteins
Q96CG1	2107	5.4	45.4	Eukaryotic translation termination factor 1	Cytosol	Translation factors
P58107	83481	5.43	552.8	Epiplakin, epidermal antigen	Cytosol	Cytoskeleton proteins
Q13838	7919	5.44	49	Spliceosome RNA helicase BAT1, RNA helicase DEAD Q motif	Nuclear	RNA helicases
P48643	22948	5.44	59.6	T-complex protein 1 subunit epsilon; TCP-1-epsilon	Cytosol	Chaperones
O00148	10212	5.46	49.1	ATP-dependent RNA helicase DDX39	Nuclear	RNA helicases
Q59EJ3	3303	5.47	70	Heat shock 70, Hsp 70	Cytosol	Chaperones
O75369	2317	5.47	278	β -Filamin B	Cytosol	Cytoskeleton proteins
Q00610	1213	5.48	191.5	Clathrin, heavy chain 1 (Hc)	Cytosol	Transport
P35579	4627	5.5	226.4	Myosin, heavy chain 9	Cytosol	Cytoskeleton proteins
P55060	1434	5.51	110.3	Exportin/Importin Cse1-like	Nuclear	Protein transport
Q99829	8904	5.52	59	Copine	Cytosol	Transport
Q13263	10155	5.52	88.5	Transcription intermediary factor 1- β	Nuclear	Transcription factors
P25205	4172	5.53	90.9	Minichromosome maintenance complex component 3	Chromosome	DNA replication proteins
P50990	10694	5.53	59.4	T-complex protein 1 subunit theta	Cytosol	Chaperones
P13010	7520	5.55	82.7	ATP-dependent DNA helicase II 80 kDa subunit	Chromosome	DNA repair proteins
Q6PDF7	3895	5.56	149.5	Kinectin 1	Membrane proteins	Protein transport
P35232	5245	5.57	29.8	Prohibitin	Cytosol	Cell-cycle proteins
Q96IE3	5339	5.59	517.7	Plectin	Cytosol	Cytoskeleton proteins
Q07065	10970	5.63	66	Cytoskeleton-associated protein 4	Cytosol	Cytoskeleton proteins
Q14980	4175	5.64	238.7	Nuclear mitotic apparatus protein 1	Chromosome	Chromosomal proteins
P61224	5908	5.65	20.8	Ras-related protein Rap-1b	Cytosol	Cytoskeleton proteins
Q96C61	2316	5.69	279.8	α -Filamin A	Cytosol	Cytoskeleton proteins
P60228	3646	5.71	52.2	Eukaryotic translation initiation factor 3, subunit E	Cytosol	Translation factors
P53618	1315	5.72	107.1	Coatmer protein complex, subunit β -1, HEAT	Cytosol	Protein transport
O75643	23020	5.73	244.4	Small nuclear ribonucleoprotein (U5)	Nuclear	snRNP
Q9Y490	7094	5.77	269.6	Talin 1, Vinculin/catenin	Cytosol	Cytoskeleton proteins
Q8NF37	79888	5.77	59.1	Lysophosphatidylcholine acyltransferase 1	Membrane proteins	Metabolism
O15371	8664	5.79	63.9	Eukaryotic translation initiation factor 3, subunit D	Cytosol	Translation factors
P17987	6950	5.8	60.3	Small nucleolar RNA, H/ACA box 29	Nuclear	snRNP
Q9PIN9	3376	5.82	144.4	Isoleucyl-tRNA synthetase	Nuclear	tRNA Synthetases
Q2F832	6741	5.85	21.4	Sjogren syndrome antigen B	Nuclear	snRNP
P43243	9782	5.87	94.6	Matrin-3	Nuclear	RNA binding proteins

P38646	3313	5.87	73.6	Heat shock 70kDa protein 9 (mortalin)	Cytosol	Chaperones
Q9NZM1	26509	5.87	233.3	Myoferlin (Fer-1-like protein 3)	Membrane proteins	Transport
Q8TBZ0	256309	5.88	96.7	Coiled-coil domain containing protein 110	Nuclear	N/A
P31943	3187	5.89	49.2	Heterogeneous nuclear ribonucleoprotein H1	Nuclear	hnRNP
P55795	3188	5.89	49.2	Heterogeneous nuclear ribonucleoprotein H2	Nuclear	hnRNP
P15311	7430	5.94	69.4	Ezrin; p81; Cytovillin; Villin-2	Cytosol	Cytoskeleton proteins
P04843	6184	5.96	68.5	Ribophorin I	Membrane proteins	Protein modification
P04792	3315	5.98	22.8	Heat shock 27kDa protein 1	Cytosol	Chaperones
P49327	2194	6.01	273.3	Fatty acid synthase	Cytosol	Mitochondrial proteins
P78371	10576	6.01	57.5	T-complex protein 1 subunit β	Cytosol	Chaperones
P29692	1936	6.02	71.2	Elongation factor 1- δ (EF-1- δ)	Cytosol	Transcription factors
P27708	790	6.02	242.8	CAD protein	Nuclear	Metabolism
Q9Y265	8607	6.03	50.2	RuvB-like 1	Chromosome	Chromosomal proteins
O95766	221960	6.06	55.8	UPF0550 protein C7orf28	-	-
Q7Z4Q5	3192	6.07	86.8	Heterogeneous nuclear ribonucleoprotein U	Nuclear	hnRNP
P33993	4176	6.08	81.3	Minichromosome maintenance complex component 7	Nuclear	DNA replication proteins
P26038	4478	6.08	67.8	Moesin	Cytosol	Cytoskeleton proteins
P39656	1650	6.09	50.8	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit	Membrane proteins	Protein modification
Q9UMS4	27339	6.14	55.1	Pre-mRNA-processing factor 19	Nuclear	RNA modifying enzyme and related proteins
Q15019	4735	6.15	41.5	Septin 2	N/A	Cell-cycle proteins
P12956	2547	6.23	69.8	X-ray repair cross-complementing protein 6	Chromosome	DNA repair proteins
P40227	908	6.23	58	T-complex protein 1 subunit zeta	Cytosol	Chaperones
P31942	3189	6.37	36.9	Heterogeneous nuclear ribonucleoprotein H3	Nuclear	hnRNP
P33991	4173	6.28	96.5	Minichromosome maintenance complex component 4	Chromosome	DNA replication proteins
Q14152	8661	6.38	166.5	Eukaryotic translation initiation factor 3, subunit A	Cytosol	Translation factors
P13639	1938	6.41	95.3	Eukaryotic translation elongation factor 2	Cytosol	Translation factors
Q08211	1660	6.41	140.9	ATP-dependent RNA helicase A	Nuclear	RNA helicases
Q08945	6749	6.45	81	Structure specific recognition protein 1 FACT	Nuclear	Histone modification factors
Q5SZY0	10576	6.46	57.9	Chaperonin containing TCP1, subunit γ -3	Cytosol	Chaperones
Q99729	3182	6.49	35.9	Heterogeneous nuclear ribonucleoprotein A/B	Nuclear	hnRNP
Q5VU81	9898	6.49	103.9	Ubiquitin associated protein 2-like I	N/A	Protein modification
P02248	6233	6.56	8.6	Ubiquitin-60S ribosomal protein L40	Ribosome	Ribosomal proteins
P62988	7314	6.56	8.6	Ubiquitin	N/A	Protein modification

P02545	4000	6.57	74.1	Lamin A/C	Nuclear	Structural
Q15365	5093	6.66	37.5	Poly(rC) binding protein 1, KH type 2	Nuclear	RNA binding proteins
P47897	5859	6.71	87.7	Glutaminyl-tRNA synthetase	Nuclear	tRNA Synthetases
O00571	1654	6.73	73.2	ATP-dependent RNA helicase DDX3X	Nuclear	RNA helicases
Q7KZF4	27044	6.74	101.9	Staphylococcal nuclease domain-containing protein 1	Nuclear	Transcription factors
Q9Y3I0	51493	6.77	55.2	UPF0027 protein C22orf28		
B4DL41	5591	6.81	465.1	DNA-dependent protein kinase catalytic subunit	Chromosome	DNA repair proteins
P25398	6206	6.81	14.5	40 S ribosomal protein S12	Ribosome	Ribosomal proteins
Q92945	8570	6.84	73.1	Far upstream element-binding protein 2	Nuclear	hnRNP
P53396	47	6.95	120.8	ATP citrate lyase	Cytosol	Metabolism
P62826	5901	7.01	24.4	RAN, member RAS oncogene family	Nuclear	Transcription factors
P06733	2023	7.02	47.1	α -enolase N	Cytosol	Metabolism
B1AMX0	29085	7.03	13.7	Phosphohistidine phosphatase 1	Cytosol	Metabolism
P31040	6389	7.06	72.6	Succinate dehydrogenase [ubiquinone] flavoprotein subunit	Cytosol	Mitochondrial proteins
O43143	1665	7.12	90.9	Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15	Nuclear	RNA helicases
Q5TCG3	10492	7.17	62.6	Heterogeneous nuclear ribonucleoprotein Q	Nuclear	hnRNP
Q96AE4	8880	7.18	67.5	Far upstream element-binding protein 1	Nuclear	Splicing related factors
Q9Y285	2193	7.31	57.5	Phenylalanyl-tRNA synthetase, α subunit	Nuclear	tRNA Synthetases
P29401	7086	7.58	67.8	Transketolase N	Cytosol	Metabolism
Q2F840	1937	7.59	56.1	Eukaryotic translation elongation factor γ -1	Nuclear	Translation factors
P63244	10399	7.6	35.1	Guanine nucleotide-binding protein subunit β -2-like 1	Ribosome	Ribosomal proteins
Q14103	3184	7.61	38.4	eterogeneous nuclear ribonucleoprotein D	Nuclear	hnRNP
O15260	6836	7.64	30.4	Surfeit 4	Membrane proteins	Protein transport
A8K220	5478	7.69	18	Peptidylprolyl isomerase A (cyclophilin A)	Cytosol	Chaperones
P14174	4282	7.72	12.5	Macrophage migration inhibitory factor	Cytosol	Cytokine related
Q5VYJ4	414153	7.84	10.7	Putative small nuclear ribonucleoprotein polypeptide E-like protein 1	Nuclear	snRNP
P14618	5315	7.96	65.9	Pyruvate kinase, muscle	Cytosol	Metabolism
P23528	1072	8.22	18.5	Cofilin 1 (non-muscle)	Cytosol	Cytoskeleton proteins
Q8WTY5	7155	8.22	182.5	DNA topoisomerase 2- β	Chromosome	Chromosomal proteins
Q9BV64	10236	8.23	71.2	Heterogeneous nuclear ribonucleoprotein R	Nuclear	hnRNP
Q9Y6C9	23788	8.25	33.3	Mitochondrial carrier homologue 2	Cytosol	Mitochondrial proteins
Q06830	5052	8.27	22.1	Peroxisredoxin 1	Cytosol	Metabolism
P27695	328	8.32	35.5	APEX nuclease (multifunctional DNA repair enzyme) 1	Chromosome	DNA repair proteins
Q5JXL7	989	8.35	44.8	Septin-7 (CDC10 protein homologue)	N/A	Cell-cycle proteins

P07737	5216	8.44	15	Profilin 1	Cytosol	Cytoskeleton proteins
P14866	3191	8.46	64.1	Heterogeneous nuclear ribonucleoprotein L	Nuclear	hnRNP
B4DXP5	5094	8.5	33.5	Poly(rC) binding protein 2	Nuclear	RNA binding proteins
C9JFV5	3609	8.56	82.7	Interleukin enhancer binding factor 3	Nuclear	RNA binding proteins
P04406	2597	8.57	36	Glyceraldehyde-3-phosphate dehydrogenase	Cytosol	Metabolism
Q07666	10657	8.73	48.2	KH domain containing, RNA binding, signal transduction associated 1	Nuclear	RNA binding proteins
Q5XXA6	55107	8.76	114	Anoctamin-1	Membrane proteins	Transport
O43809	11051	8.85	26.2	Nudix (nucleoside diphosphate linked moiety X)-type motif 21	Nuclear	RNA modifying enzyme and related proteins
A8MWD9	100130932	8.93	8.5	Small nuclear ribonucleoprotein G-like protein	Nuclear	snRNP
Q8TCM5	4670	8.94	73.6	Heterogeneous nuclear ribonucleoprotein M	Nuclear	hnRNP
P22626	3181	8.97	37.4	Heterogeneous nuclear ribonucleoprotein A2/B1	Nuclear	hnRNP
P62308	6637	8.98	8.5	Small nuclear ribonucleoprotein G	Nuclear	snRNP
P09874	142	8.99	113	Poly [ADP-ribose] polymerase 1 (PARP)	Chromosome	DNA repair proteins
O00425	10643	8.99	63.7	Insulin-like growth factor 2 mRNA-binding protein 3	Nuclear	RNA binding proteins
Q15233	4841	9.01	54.2	Non-POU domain containing, octamer-binding protein	Chromosome	DNA replication proteins
Q9P258	55920	9.02	56	Protein RCC2 (Telophase disk protein)	Chromosome	Centromere proteins
Q9Y2X3	51602	9.03	59.5	Nucleolar protein 58	Nuclear	snRNP
P17844	1655	9.06	69.1	Probable ATP-dependent RNA helicase DDX5	Nuclear	RNA helicases
P51991	220988	9.1	39.6	Heterogeneous nuclear ribonucleoprotein A3	Nuclear	hnRNP
Q05639	1917	9.11	50.4	Eukaryotic translation elongation factor 1 α -2	Nuclear	Metabolism
Q15785	10953	9.12	34.5	Mitochondrial import receptor subunit TOM34	Cytosol	Mitochondrial proteins
P25705	498	9.16	59.7	ATP synthase subunit α , mitochondrial	Cytosol	Mitochondrial proteins
P40939	3030	9.16	82.9	Trifunctional enzyme subunit α	Cytosol	Mitochondrial proteins
Q9BUQ0	5725	9.21	59.6	Polypyrimidine tract binding protein 1	Nuclear	RNA binding proteins
P35268	6146	9.21	14.8	60 S ribosomal protein L22	Ribosome	Ribosomal proteins
Q9BVP2	26354	9.23	62	Small nucleolar RNA, C/D box 19B	Nuclear	snRNP
O00567	10528	9.24	66	Small nucleolar RNA, C/D box 86	Nuclear	snRNP
Q9P1N8	7112	9.26	41.8	Thymopoietin	N/A	N/A
P0C7M2	3178	9.27	34.2	Heterogeneous nuclear ribonucleoprotein A1-like 3	Nuclear	hnRNP
Q13310	8761	9.31	70.7	Poly(A) binding protein, cytoplasmic 4	Cytosol	RNA binding proteins
Q9NR30	9188	9.32	87.3	Nucleolar RNA helicase 2	Nuclear	RNA helicases
Q96FN5	113220	9.33	56.6	Kinesin family member 12	Chromosome	Centromere proteins

Q96EY4	55319	9.33	23.8	UPF0534 protein C4orf43	-	-
B4DW28	6224	9.4	16	40S ribosomal protein S20	Ribosome	Ribosomal proteins
P23284	5479	9.42	23.7	Peptidylprolyl isomerase B (cyclophilin B)	Cytosol	Chaperones
Q8NCF7	5250	9.43	39.9	Solute carrier family 25 member 3	Cytosol	Mitochondrial proteins
P23246	6421	9.45	76.1	Splicing factor, proline- and glutamine-rich	Nuclear	Splicing related factors
P30050	6136	9.48	17.8	60 S ribosomal protein L12	Ribosome	Ribosomal proteins
Q15717	1994	9.5	39	ELAV-like protein 1	Nuclear	RNA binding proteins
P11940	26986	9.52	70.6	Poly(A) binding protein, cytoplasmic 1	Cytosol	RNA binding proteins
Q71UM5	51065	9.57	9.5	40 S ribosomal protein S27-like	Ribosome	Ribosomal proteins
Q8NEJ9	25983	9.57	35.9	Neuroguidin, EIF4E binding protein	Nuclear	RNA binding proteins
Q5QPM2	221913	9.63	30.3	RNA-binding protein Raly	Nuclear	RNA binding proteins
P62913	6135	9.64	20.2	60 S ribosomal protein L11	Ribosome	Ribosomal proteins
P23396	6188	9.68	26.7	40 S ribosomal protein S3	Ribosome	Ribosomal proteins
P05141	292	9.71	32.8	ADP/ATP translocase 2	Cytosol	Mitochondrial proteins
P46782	6193	9.73	22.9	40 S ribosomal protein S5	Ribosome	Ribosomal proteins
P46777	6125	9.73	34.3	Small nucleolar RNA, C/D box 21	Nuclear	snRNP
P61247	6189	9.75	29.9	Small nucleolar RNA, C/D box 73A	Nuclear	snRNP
A8MYX5	6156	9.76	12.9	Ribosomal protein L30	Ribosome	Ribosomal proteins
O75367	9555	9.8	39.6	H2A histone family, member Y	Chromosome	Histones
P08708	6218	9.85	15.5	40 S ribosomal protein S17	Ribosome	Ribosomal proteins
P62906	4736	9.94	24.8	60 S ribosomal protein L10a	Ribosome	Ribosomal proteins
P08621	6625	9.94	51.5	U1 small nuclear ribonucleoprotein	Nuclear	snRNP
P32969	6133	9.96	21.8	60 S ribosomal protein L9	Ribosome	Ribosomal proteins
Q9Y383	51631	10.02	46.5	Putative RNA-binding protein Luc7-like 2	Nuclear	RNA binding proteins
P37108	6727	10.04	14.6	Signal recognition particle 14 kDa protein	Cytosol	Receptor proteins
P38159	27316	10.06	42.3	Heterogeneous nuclear ribonucleoprotein G	Nuclear	hnRNP
P62263	6208	10.07	16.3	40 S ribosomal protein S14	Ribosome	Ribosomal proteins
P62081	6201	10.09	22.1	40 S ribosomal protein S7	Ribosome	Ribosomal proteins
Q9BRL6	10929	10.09	12.7	Serine/arginine-rich splicing factor 8	Nuclear	Splicing related factors
P27635	6134	10.11	24.6	Small nucleolar RNA, H/ACA box 70	Nuclear	snRNP
P62851	6230	10.12	13.7	40 S ribosomal protein S25	Ribosome	Ribosomal proteins
P62244	6210	10.14	14.8	40 S ribosomal protein S15a	Ribosome	Ribosomal proteins
Q6N037	9584	10.14	58.6	RNA binding motif protein 39	Nuclear	RNA binding proteins
P46783	6204	10.15	18.9	40 S ribosomal protein S10	Ribosome	Ribosomal proteins
P62701	6191	10.16	29.6	40 S ribosomal protein S4, X-linked	Ribosome	Ribosomal proteins
P62861	2197	10.17	14.4	40S ribosomal protein S30	Ribosome	Ribosomal proteins
P18621	6139	10.18	21.4	60 S ribosomal protein L17	Ribosome	Ribosomal proteins
P62249	6217	10.21	16.4	40 S ribosomal protein S16	Ribosome	Ribosomal proteins
P27816	4134	10.22	50.9	Microtubule-associated protein 4	Cytosol	Cytoskeleton proteins
P15880	6187	10.25	31.3	40 S ribosomal protein S2	Ribosome	Ribosomal proteins
P42766	11224	10.3	10.6	60 S ribosomal protein L35	Ribosome	Ribosomal proteins
P62807	3017	10.31	13.9	Histone cluster 1, H2bc	Chromosome	Histones
P33778	3018	10.31	13.9	Histone cluster 1, Histone core D	Chromosome	Histones

P62280	6205	10.31	18.4	40S ribosomal protein S11	Ribosome	Ribosomal proteins
P62241	6202	10.32	24.2	Small nucleolar RNA, C/D box 55	Nuclear	snRNP
P62318	6634	10.33	18	Small nuclear ribonucleoprotein D3 polypeptide 18kDa	Nuclear	snRNP
O75494	10772	10.33	22.2	Serine/arginine-rich splicing factor 10	Nuclear	Splicing related factor
P62750	6147	10.44	17.7	Small nucleolar RNA, C/D box 4A, Ribosomal L25/23	Nuclear	snRNP
P61513	6168	10.44	10.3	60 S ribosomal protein L37a	Ribosome	Ribosomal proteins
P46778	6144	10.49	18.6	60 S ribosomal protein L21	Ribosome	Ribosomal proteins
P62266	6228	10.5	15.8	40 S ribosomal protein S23	Ribosome	Ribosomal proteins
P62829	9349	10.51	14.9	60 S ribosomal protein L23	Ribosome	Ribosomal proteins
P62277	6207	10.53	17.2	40 S ribosomal protein S13	Ribosome	Ribosomal proteins
P62899	6160	10.54	14.5	60 S ribosomal protein L31	Ribosome	Ribosomal proteins
P61254	6154	10.55	17.2	60 S ribosomal protein L26	Ribosome	Ribosomal proteins
P61353	6155	10.56	15.8	60 S ribosomal protein L27	Ribosome	Ribosomal proteins
Q02878	6128	10.58	32.7	60S ribosomal protein L6	Ribosome	Ribosomal proteins
P62424	6130	10.61	30	Small nucleolar RNA, C/D box 24	Nuclear	snRNP
P18124	6129	10.66	29.2	60 S ribosomal protein L7	Ribosome	Ribosomal proteins
P46781	6203	10.66	22.6	40 S ribosomal protein S9	Ribosome	Ribosomal proteins
Q02543	6142	10.72	20.7	60 S ribosomal protein L18a	Ribosome	Ribosomal proteins
Q92522	8974	10.76	22.5	H1 histone family, member X	Chromosome	Histones
Q92522	8971	10.76	22.5	Histone H1x	Chromosome	Histones
P62847	6229	10.79	15.4	40 S ribosomal protein S24	Ribosome	Ribosomal proteins
P46779	6158	10.84	19.1	60 S ribosomal protein L28	Ribosome	Ribosomal proteins
P62753	6194	10.85	28.7	40 S ribosomal protein S6	Ribosome	Ribosomal proteins
Q9BTM1	55766	10.9	14	H2A histone	Chromosome	Histones
P20671	3013	10.9	14.1	Histone H2A type 1-D; Histone H2A/g; Histone H2A.3	Chromosome	Histones
P16401	3009	10.91	22.6	Histone cluster 1, H1b	Chromosome	Histones
P16403	3006	10.94	21.4	Histone cluster 1, H1c , Histone H5	Chromosome	Histones
P50914	9045	10.94	23.4	60 S ribosomal protein L14	Ribosome	Ribosomal proteins
P40429	23521	10.94	23.6	Small nucleolar RNA, C/D box 32A	Nuclear	snRNP
P62269	6222	10.99	17.7	40S ribosomal protein S18	Ribosome	Ribosomal proteins
P46776	6157	11	16.6	60 S ribosomal protein L27a	Ribosome	Ribosomal proteins
P62854	6231	11.01	13	40 S ribosomal protein S26	Ribosome	Ribosomal proteins
P16402	3007	11.02	22.3	Histone cluster 1, H1d, Histone H5	Chromosome	Histones
P10412	3008	11.03	21.9	Histone H1.4; Histone H1b	Chromosome	Histones
P62917	6132	11.04	28	60 S ribosomal protein L8	Ribosome	Ribosomal proteins
P04908	3012	11.05	14.1	Histone cluster 1, H2ab	Chromosome	Histones
P36578	6124	11.07	47.7	60 S ribosomal protein L4	Ribosome	Ribosomal proteins
P68431	8350	11.13	15.4	Histone cluster 1, H3a	Chromosome	Histones
P63162	6638	11.2	24.6	Small nuclear ribonucleoprotein-associated protein N	Nuclear	snRNP
P62995	6434	11.25	33.6	Transformer-2 protein homologue β	Nuclear	Splicing related factors
P83731	6152	11.26	17.8	60 S ribosomal protein L24	Ribosome	Ribosomal proteins
P62805	554313	11.36	11.4	Histone cluster 4, H4	Chromosome	Histones
P49207	6164	11.48	13.3	60 S ribosomal protein L34	Ribosome	Ribosomal proteins
P84098	6143	11.48	23.5	60 S ribosomal protein L19	Ribosome	Ribosomal proteins
P62314	6632	11.56	13.3	Small nuclear ribonucleoprotein D1	Nuclear	snRNP
P84103	6428	11.64	19.3	Serine/arginine-rich splicing factor 3	Nuclear	Splicing related factors
P26373	6137	11.65	24.2	60 S ribosomal protein L13	Ribosome	Ribosomal proteins
P47914	6159	11.66	17.7	60 S ribosomal protein L29	Ribosome	Ribosomal proteins
Q07020	6141	11.73	21.6	60 S ribosomal protein L18	Ribosome	Ribosomal proteins
Q16629	6432	11.83	27.4	Serine/arginine-rich splicing factor 7	Nuclear	Splicing related factors

Q9Y3Y2	26097	11.9	26.4	Uncharacterized protein C1orf77	Nuclear	Histone modification factors
Q9UQ35	23524	11.9	299.4	Serine/arginine repetitive matrix protein 2	Nuclear	Splicing related factors

APPENDIX 3

Table 3 List of proteins identified by quantitative proteomic analysis in the Dm-ChP pulled-down material collected either at the 2 or 6 hour after release from a thymidine block.

The proteins identified are listed according to increasing heavy/medium normalised ratio, reflecting their relative abundance in the early versus late replicating DNA. For further details see supplemented Table 4 (Kliszczak et al., 2011).

UniProt KB	Entrez Gene	Mr (kDa)	Description	Ratio H/M	Ratio M/L	Ratio H/L
Q96PK6	10432	69.5	RNA-binding protein 14	0.09	6.33	0.75
Q14683	8243	143.2	Structural maintenance of chromosomes protein 1A	0.31	2.49	0.51
P35249	5984	39.7	Replication factor C subunit 4	0.33	2.30	0.53
Q9UQE7	9126	141.5	Structural maintenance of chromosomes protein 3	0.33	2.18	0.73
P61956	6613	10.8	Small ubiquitin-related modifier 2	0.34	1.75	0.60
Q13263	10155	88.5	Transcription intermediary factor 1- β	0.36	1.90	0.60
Q13185	11335	20.8	Chromobox protein homologue 3	0.41	2.28	0.72
O60264	8467	121.9	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5	0.44	1.71	0.82
P78347	2969	112.4	General transcription factor II-I	0.44	1.50	0.49
P11388	7153	182.7	DNA topoisomerase 2- α	0.45	1.26	0.68
Q9UIG0	9031	170.9	Tyrosine-protein kinase BAZ1B	0.45	1.67	0.87
Q08945	6749	81.07	FACT complex subunit SSRP1	0.46	1.33	0.58
Q9Y5B9	11198	119.9	FACT complex subunit SPT16	0.47	1.87	0.99
Q09028	5928	47.6	Histone-binding protein RBBP4, Chromatin assembly factor 1 subunit C	0.50	1.75	0.94
P35659	7913	48.02	Protein DEK	0.53	1.68	0.92
P33993	4176	81.3	Minichromosome maintenance complex component 7	0.55	1.34	0.58
Q16401	5711	56.19	26S proteasome non-ATPase regulatory subunit 5	0.56	1.35	0.57
P11387	7150	99.07	DNA topoisomerase 1	0.57	1.58	0.95
P0CG48	7316	77.03	Polyubiquitin-C	0.58	0.88	0.51
P12004	5111	28.77	Proliferating cell nuclear antigen	0.58	1.86	1.12
P46063	5965	73.46	ATP-dependent DNA helicase Q1	0.61	0.90	0.62
P39748	2237	42.59	Flap structure-specific endonuclease 1	0.64	1.56	0.95
Q9Y2W1	9967	108.66	Thyroid hormone receptor-associated protein 3	0.65	0.88	0.62
Q9Y265	8607	50.23	RuvB-like 1	0.67	1.51	1.12
Q9Y230	10856	51.16	RuvB-like 2	0.67	1.48	1.04
P52292	3838	57.86	Importin subunit α -2	0.67	0.91	0.67
P78527	5591	469.08	DNA-dependent protein kinase catalytic subunit	0.70	1.22	0.81
P31689	3301	44.87	DnaJ homologue (Hsp40) subfamily A member 1	0.71	0.81	0.70
P62316	6633	13.53	Small nuclear ribonucleoprotein Sm D2	0.72	1.72	1.20
P09874	142	113.08	Poly (ADP-ribose) polymerase 1	0.73	0.97	0.85
Q6FI13	8337	14.09	Histone H2A type 2-A	0.76	0.85	0.59
Q86UP2	3895	156.27	Kinectin	0.77	0.90	0.67

Q15393	23450	135.58	Splicing factor 3B subunit 3	0.77	1.29	0.96
Q562R1	345651	42.003	β -actin-like protein 2	0.77	1.14	0.83
Q01081	7307	27.87	Splicing factor U2AF 35 kDa subunit	0.78	1.67	1.00
P68032	70	42.02	α -actin, cardiac muscle 1	0.78	1.10	0.88
P09429	3146	24.89	High mobility group protein B1	0.80	1.36	0.88
Q01130	6427	25.47	Splicing factor, arginine/serine-rich 2	0.80	1.44	1.17
P08670	7431	53.65	Vimentin	0.82	0.76	0.59
P57053	54145	13.92	Histone H2B type F-S	0.82	0.86	0.72
P67809	4904	41.91	Nuclease-sensitive element-binding protein 1	0.83	0.68	0.61
P0C0S5	3015	13.55	Histone H2A.Z	0.83	1.60	1.17
O95347	10592	135.65	Structural maintenance of chromosomes protein 2	0.84	0.39	0.33
Q16778	8349	13.92	Histone H2B type 2-E	0.84	0.90	0.65
P18754	1104	48.14	RCC1-I	0.85	1.43	1.13
Q08J23	54888	86.47	tRNA (cytosine-5)-methyltransferase NSUN2	0.85	0.96	0.76
Q13838	7919	50.68	Spliceosome RNA helicase BAT1	0.85	1.05	1.09
P46940	8826	189.25	Ras GTPase-activating-like protein IQGAP1	0.85	0.65	0.53
Q92945	8570	73.11	Far upstream element-binding protein 2	0.85	0.96	0.83
P42166	7112	75.49	Lamina-associated polypeptide 2, isoform α	0.85	0.92	0.71
P62826	5901	26.41	Putative uncharacterized protein RAN	0.86	1.29	1.07
P26368	11338	53.5	Splicing factor U2AF 65 kDa subunit	0.88	1.64	1.09
P62995	6434	33.631	Splicing factor, arginine/serine-rich 10	0.88	0.90	0.87
Q9Y383	51631	46.513	Putative RNA-binding protein Luc7-like 2	0.88	0.98	0.79
P58107	83481	555.61	Epiplakin	0.88	0.73	0.60
P16401	3009	22.58	Histone H1.5	0.88	1.07	0.85
P11142	3312	70.89	Heat shock cognate 71 kDa protein	0.88	1.28	1.24
Q92973	3842	102.35	Transportin-1	0.89	0.76	0.63
P13010	7520	82.70	ATP-dependent DNA helicase 2 subunit 2	0.89	0.91	0.94
Q71DI3	333932	15.39	Histone H3.2	0.90	0.84	0.65
Q14974	3837	97.17	Importin subunit β -1	0.90	0.87	0.56
P30153	5518	65.31	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A α isoform	0.90	0.80	0.79
P08107	3303	63.92	Heat shock 70 kDa protein 1	0.90	0.93	0.96
P62081	6201	22.13	40S ribosomal protein S7	0.91	0.93	0.85
Q08211	1660	140.96	ATP-dependent RNA helicase A	0.91	1.07	1.14
P46013	4288	358.69	Antigen KI-67	0.91	0.54	0.58
P35579	4627	226.53	Myosin-9	0.92	0.76	0.71
P62805	8359	11.37	Histone H4	0.92	0.75	0.59
Q15149	5339	516.47	Plectin-1	0.93	0.75	0.59
Q9UBF2	26958	97.62	Coatomer subunit γ -2	0.94	1.14	0.62
P06748	4869	32.57	Nucleophosmin	0.94	1.31	1.21
P35637	2521	53.376	RNA-binding protein FUS	0.94	0.75	0.76
O00571	1654	73.24	ATP-dependent RNA helicase DDX3X	0.94	0.78	0.80
O75367	9555	39.62	Core histone macro-H2A.1	0.95	1.27	1.29
P22626	3181	37.43	Heterogeneous nuclear ribonucleoproteins A2/B1	0.95	0.78	1.15
P16403	3006	21.36	Histone H1.2	0.95	0.59	0.56
P52597	3185	45.67	Heterogeneous nuclear ribonucleoprotein F	0.95	0.91	0.92
O75494	10772	31.3	FUS-interacting serine-arginine-rich protein 1	0.95	1.13	1.16
Q92841	10521	80.27	Probable ATP-dependent RNA helicase DDX17	0.96	0.77	0.80
P17844	1655	69.15	Probable ATP-dependent RNA helicase DDX5	0.96	0.62	0.63
Q08170	6429	56.68	Splicing factor, arginine/serine-rich 4	0.96	0.72	0.74
P42677	6232	9.46	40S ribosomal protein S27	0.97	0.84	0.81
P19338	4691	76.61	Nucleolin	0.97	1.33	1.36
P84103	6428	19.33	Splicing factor, arginine/serine-rich 3	0.98	1.05	0.86
O95373	10527	119.5	Importin-7	0.98	0.78	0.83
Q86U42	8106	32.75	Polyadenylate-binding protein 2	0.98	0.72	0.93
P43243	9782	99.96	Putative uncharacterized protein MATR3	0.99	0.98	0.97
P07910	3183	32.34	Heterogeneous nuclear ribonucleoprotein C1/C2	0.99	1.22	1.15

P12268	3615	55.80	Inosine-5'-monophosphate dehydrogenase 2	0.99	0.68	0.70
P1295	2547	69.84	ATP-dependent DNA helicase 2 subunit 1	1.0	0.98	1.01
P00558	5230	44.61	Phosphoglycerate kinase 1	1.0	0.87	0.80
Q12905	3608	43.06	Interleukin enhancer-binding factor 2	1.0	1.18	1.21
P14866	3191	64.13	Heterogeneous nuclear ribonucleoprotein L	1.01	0.93	0.94
P08107	3303	70.05	Heat shock 70 kDa protein 1	1.01	1.53	1.67
Q07955	6426	27.74	Splicing factor, arginine/serine-rich 1	1.02	0.99	0.97
P61978	3190	48.56	Heterogeneous nuclear ribonucleoprotein K	1.02	1.32	1.26
Q12906	3609	95.81	Interleukin enhancer-binding factor 3	1.02	0.91	0.98
P62263	6208	16.27	40S ribosomal protein S14	1.03	1.19	0.92
Q07666	10657	48.23	KH domain-containing, RNA-binding, signal transduction-associated protein 1	1.03	0.98	1.04
P52272	4670	77.52	Heterogeneous nuclear ribonucleoprotein M	1.03	0.97	0.96
P47914	6159	17.78	Ribosomal protein L29	1.03	0.88	0.89
Q13151	10949	30.84	Heterogeneous nuclear ribonucleoprotein A0	1.03	1.03	0.91
P23528	1072	18.50	Cofilin-1	1.03	1.37	0.93
P26599	5725	59.63	Polypyrimidine tract binding protein 1	1.03	1.12	1.24
P20700	4001	66.41	Lamin-B1	1.03	1.14	1.30
P62241	6202	25.19	40S ribosomal protein S8	1.03	1.08	1.09
Q00341	3069	141.4	Vigilin	1.04	0.66	0.62
P07437	203068	49.67	β -Tubulin chain	1.04	0.78	0.80
P49368	7203	60.53	T-complex protein 1 subunit γ	1.04	0.64	0.83
Q96AG4	55379	34.93	Leucine-rich repeat-containing protein 59	1.05	0.83	0.94
P02545	4000	74.14	Lamin-A/C	1.05	1.26	1.31
Q14103	3184	38.43	Heterogeneous nuclear ribonucleoprotein D0	1.05	1.13	1.33
P08865	3921	33.31	40S ribosomal protein SA	1.05	1.00	0.96
Q5SSJ5	50809	61.20	Heterochromatin protein 1-binding protein 3	1.05	0.76	0.85
P31942	3189	36.93	Heterogeneous nuclear ribonucleoprotein H3	1.05	0.79	0.69
P09651	3178	38.85	Heterogeneous nuclear ribonucleoprotein A1	1.07	1.14	1.16
P38919	9775	46.87	Eukaryotic initiation factor 4A-III	1.07	0.65	0.59
Q04637	1981	175.5	Eukaryotic translation initiation factor 4 γ -1	1.07	0.61	0.63
Q96AE4	8880	68.90	Putative uncharacterized protein FUBP1	1.07	1.04	1.18
P61313	6138	24.17	Ribosomal protein L15	1.07	0.92	1.08
P46379	7917	122.3	Large proline-rich protein BAT3	1.08	0.72	0.69
Q15365	5093	37.49	Poly(rC)-binding protein 1	1.08	0.75	0.94
O00231	5717	47.53	26S proteasome non-ATPase regulatory subunit 11	1.08	0.95	1.11
Q9Y262	51386	70.90	Eukaryotic translation initiation factor 3, subunit E	1.09	0.58	0.89
P31948	10963	62.66	Stress-induced-phosphoprotein 1	1.10	0.76	0.97
Q16629	6432	27.36	Splicing factor, arginine/serine-rich 7	1.10	0.73	0.82
P83731	6152	19.57	60S ribosomal protein L24	1.10	0.94	0.84
P31943	3187	51.23	Heterogeneous nuclear ribonucleoprotein H	1.10	1.11	1.23
P63241	1984	20.17	Eukaryotic translation initiation factor 5A-1	1.10	0.98	0.82
Q01844	2130	69.05	RNA-binding protein EWS	1.10	0.87	1.04
P63244	10399	37.89	Guanine nucleotide-binding protein subunit β -2-like 1	1.11	0.73	0.85
Q13148	23435	44.99	TAR DNA-binding protein 43	1.11	1.01	1.17
O43390	10236	70.94	Heterogeneous nuclear ribonucleoprotein R	1.11	0.94	1.17
P46778	6144	18.56	60S ribosomal protein L21	1.11	1.00	1.05
P39023	6122	46.11	60S ribosomal protein L3	1.11	0.83	0.96
P62249	6217	16.45	40S ribosomal protein S16	1.11	0.73	0.91
P46777	6125	34.36	60S ribosomal protein L5	1.11	1.05	1.27
P18077	6165	12.54	60S ribosomal protein L35a	1.11	1.00	0.93
P62244	6210	14.84	40S ribosomal protein S15a	1.12	1.10	0.95
Q99729	3182	35.97	Heterogeneous nuclear ribonucleoprotein A/B	1.12	1.19	1.33
P68371	10383	49.83	Tubulin β -2C chain	1.13	0.67	0.91
P62851	6230	13.74	40S ribosomal protein S25	1.13	1.14	1.13
Q7KZF4	27044	102	Staphylococcal nuclease domain-containing protein 1	1.13	0.64	0.80
O14979	9987	46.44	Heterogeneous nuclear ribonucleoprotein D-like	1.14	1.08	1.11

O60841	9669	138.8	Eukaryotic translation initiation factor 5B	1.14	0.69	0.74
P29401	7086	68.74	Transketolase	1.15	0.86	0.99
P38159	27316	42.33	Heterogeneous nuclear ribonucleoprotein G	1.15	1.21	1.43
Q15717	1994	38.99	ELAV-like protein 1	1.16	0.75	0.75
P60866	6224	16.01	40S ribosomal protein S20	1.16	1.21	1.10
P25398	6206	14.53	40S ribosomal protein S12	1.17	1.06	1.15
Q13283	10146	52.16	Ras GTPase-activating protein-binding protein 1	1.17	0.81	0.85
P35241	5962	71.05	Radixin isoform b	1.17	0.78	0.86
P07900	3320	98.16	Heat shock protein HSP 90- α	1.17	0.89	1.00
P62266	6228	15.81	40S ribosomal protein S23	1.17	0.87	0.99
P23246	6421	76.15	Splicing factor, proline- and glutamine-rich	1.18	1.07	1.20
P23396	6188	26.69	40S ribosomal protein S3	1.18	0.88	1.00
P08621	6625	51.56	U1 small nuclear ribonucleoprotein 70 kDa	1.18	0.81	1.31
P62701	6191	29.60	40S ribosomal protein S4, X isoform	1.18	0.93	0.96
P61247	6189	29.95	40S ribosomal protein S3a	1.19	1.04	1.08
P18621	6139	21.39	60S ribosomal protein L17	1.19	1.03	1.10
P27635	6134	26.59	60 S ribosomal protein L10	1.19	0.79	0.80
P62753	6194	28.68	40S ribosomal protein S6	1.20	0.93	0.94
Q06830	5052	22.11	Peroxisredoxin-1	1.20	1.06	1.06
P62424	6130	29.99	60S ribosomal protein L7a	1.20	0.97	1.08
P60228	3646	52.22	Eukaryotic translation initiation factor 3 subunit E	1.21	0.63	0.72
-	648000	32.42	Ribosomal protein L7 pseudogene 23	1.21	0.67	1.08
Q00839	3192	90.58	Heterogeneous nuclear ribonucleoprotein U	1.21	1.25	1.45
P46783	6204	18.89	40S ribosomal protein S10	1.21	0.75	0.85
P51991	220988	39.59	Heterogeneous nuclear ribonucleoprotein A3	1.21	0.98	1.01
P08238	3326	83.26	Heat shock protein HSP 90- β	1.21	0.85	1.09
P62906	4736	25.03	60S ribosomal protein L10a	1.21	0.81	0.99
P55884	8662	99.04	Eukaryotic translation initiation factor 3 subunit B	1.21	0.69	1.19
P05388	6175	34.27	60S acidic ribosomal protein P0	1.22	1.22	1.09
P15880	6187	31.32	40S ribosomal protein S2	1.22	0.94	1.09
P46782	6193	22.88	40S ribosomal protein S5	1.22	0.94	1.03
Q8WZ42	7273	3829.8	Titin	1.22	1.08	1.39
P62750	6147	22.69	60S ribosomal protein L23a	1.22	0.80	1.10
Q15233	4841	54.23	Non-POU domain-containing octamer-binding protein	1.22	0.80	1.14
Q13427	5478	24.38	Peptidyl-prolyl cis-trans isomerase A	1.22	0.38	0.72
P46776	6157	16.56	60S ribosomal protein L27a	1.22	0.96	1.00
P31943	10492	62.66	Heterogeneous nuclear ribonucleoprotein Q	1.23	0.85	1.05
-	6219	15.92	Ribosomal protein S17 pseudogene 2	1.23	1.02	1.00
P46781	6203	22.59	40S ribosomal protein S9	1.23	0.91	1.05
P11021	3309	72.42	78 kDa glucose-regulated protein	1.23	0.51	0.45
P40429	23521	23.58	60S ribosomal protein L13a	1.23	0.78	1.00
P62277	6207	17.22	40S ribosomal protein S13	1.23	0.89	0.90
Q1KMD3	221092	85.10	Heterogeneous nuclear ribonucleoprotein U	1.24	0.86	0.86
P21333	2316	280.7	Filamin-A	1.24	0.67	0.82
-	-	40.41	Putative uncharacterized protein DKFZp686P03159	1.24	0.75	0.90
P62913	6135	20.25	60S ribosomal protein L11	1.24	0.98	0.99
Q8NC51	26135	44.97	Plasminogen activator inhibitor 1 RNA-binding protein	1.25	0.79	1.08
P46779	6158	15.75	60S ribosomal protein L28	1.25	0.82	0.96
P30050	6136	17.82	60S ribosomal protein L12	1.26	1.11	1.10
P62917	6132	28.03	60S ribosomal protein L8	1.26	0.91	1.05
P63104	7534	27.75	14-3-3 protein ζ/δ	1.26	1.00	0.96
P13639	1938	95.34	Elongation factor 2	1.26	0.55	0.71
P55010	1983	49.22	Eukaryotic translation initiation factor 5	1.27	0.69	0.89
P49327	2194	273.4	Fatty acid synthase	1.27	0.64	0.75
P11940	26986	70.67	Polyadenylate-binding protein 1	1.27	0.45	0.62
P32969	6133	21.86	60S ribosomal protein L9	1.27	0.97	1.06
P07195	3945	36.64	L-lactate dehydrogenase B chain	1.28	0.73	0.81

Q02878	6128	32.87	60S ribosomal protein L6	1.28	0.92	1.21
P62280	6205	18.43	40S ribosomal protein S11	1.28	0.99	1.00
Q13310	8761	72.39	Polyadenylate-binding protein 4	1.28	0.54	0.81
P62829	9349	14.87	60S ribosomal protein L23	1.29	1.02	1.26
Q07020	6141	21.63	60S ribosomal protein L18	1.29	1.13	1.36
P50914	9045	23.79	60S ribosomal protein L14	1.31	0.69	1.16
P26373	6137	24.26	60S ribosomal protein L13	1.32	0.94	1.09
P50991	10575	57.95	T-complex protein 1, δ subunit	1.33	0.60	1.24
Q14152	8661	166.57	Eukaryotic translation initiation factor 3 subunit A	1.35	0.68	0.86
P62899	6160	14.63	60S ribosomal protein L31	1.36	0.74	0.79
P6317	6169	8.22	60S ribosomal protein L38	1.36	1.03	1.34
Q02543	6142	20.76	60S ribosomal protein L18a	1.37	0.88	1.02
Q7L1Q6	9689	51.28	Basic leucine zipper and W2 domain-containing protein 1	1.37	0.66	0.93
P26641	1937	56.15	Elongation factor γ -1	1.37	0.62	0.91
O43707	81	104.9	α -actinin-4	1.38	0.48	0.65
P29692	1936	71.41	Elongation factor δ -1	1.39	0.83	1.03
Q14151	9667	107.5	Scaffold attachment factor B2	1.40	1.20	1.71
P10809	3329	61.05	60 kDa heat shock protein, mitochondrial	1.40	0.80	1.18
P24534	1933	24.76	Elongation factor 1- β	1.40	0.45	0.60
P42766	11224	14.55	60S ribosomal protein L35	1.41	0.62	0.90
P60842	1973	46.15	Eukaryotic initiation factor 4A-I	1.41	0.51	0.84
Q9UQ80	5036	43.79	Proliferation-associated protein 2G4	1.43	0.62	1.06
O0030	8665	39.15	Eukaryotic translation initiation factor 3 subunit F	1.43	0.82	1.19
P84098	6143	23.47	60S ribosomal protein L19	1.44	1.05	1.13
P36578	6124	47.70	60S ribosomal protein L4	1.45	0.88	1.25
P06733	2023	47.17	α -enolase	1.47	0.75	1.13
P61353	6155	15.79	60S ribosomal protein L27	1.47	1.01	1.35
P18206	7414	123.8	Vinculin	1.48	0.80	0.81
P61254	6154	17.26	60S ribosomal protein L26	1.50	0.78	0.82
P62269	6222	17.72	40S ribosomal protein S18	1.52	0.90	1.16
O43175	26227	56.65	D-3-phosphoglycerate dehydrogenase	1.53	0.49	0.85
P68104	1915	50.14	Elongation factor 1- α -1	1.53	0.70	1.05
P62841	6209	17.04	40S ribosomal protein S15	1.54	0.80	1.07
P62847	6229	15.42	40S ribosomal protein S24	1.58	0.67	1.05
P00338	3939	36.69	L-lactate dehydrogenase A chain	1.68	0.70	1.03
P04406	2597	36.05	Glyceraldehyde-3-phosphate dehydrogenase	1.70	0.61	1.01
P04075	226	45.26	Fructose-bisphosphate aldolase A	1.82	0.46	0.91
P14618	5315	65.93	Pyruvate kinase isozymes M1/M2	1.85	0.45	0.87

APPENDIX 4

Public presentations, posters and publications arising from thesis work

1. Poster presentations

Kliszczak, A.E. and Santocanale, C. *Investigating molecular interactions of DNA replication proteins* (A poster presented at the NCBES Science Research Day, National University of Ireland Galway, 7th January 2010, Galway, Ireland).

Kliszczak, A.E., Harhen, B., Rainey, M.D., Santocanale, C. *DNA mediated chromatin pull-down: a novel method for chromatin analysis* (A poster presented at the Keystone Symposia, DNA Replication and Recombination, 26th February-4th March 2011, Keystone, Colorado, USA).

2. Public presentations

Kliszczak, A.E. and Santocanale, C. *DNA mediated chromatin pull-down: a novel method for the analysis of newly synthesised chromatin* (A seminar presented at the Waters Postgraduate Prize in Mass Spectrometry for Proteomics and Biopharmaceuticals symposium 27th January 2011, Dublin, Ireland; finalist of the Waters Postgraduate competition).

3. Publications

Kliszczak, A.E., Rainey, M.D., Harhen, B., Boisvert, F.M., Santocanale, C. (2011) *DNA mediated chromatin pull-down for the study of chromatin replication* *Sci Rep* 1, 95.