Establishing DNA Combing to Investigate DNA Replication Stress *in vitro*

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Declaration

I declare that this thesis is my own work and has not been submitted in part, or as a whole, for the award of a higher degree of qualification at this University or elsewhere.

James Tollitt

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Abbreviations

DNA	Deoxyribonucleic Acid
CDK	Cyclin Dependent Kinase
Ap4A	Diadenosine tetraphosphate
APC/C	Anaphase Promoting Complex
ARS	Autonomously Replicating Sequences
ATP	Adenosine Triphospate
ATR	Ataxia Telangiectasia and Rad3-Related Protein
BCA	Bicinchoninic Acid
CAK	CDK Activating Complex
CDC6	Cell Division Cycle 6
CHK1	Checkpoint Kinase 1
Ciz1	Cip1-Interacting Zinc Finger Protein
CKI	Cyclin Dependent Kinase Inhibitor
CldU	Chlorodeoxyuridine
CMG	CDC45-MCM2-7-GINS Complex
CTP	Cytidine Triphosphate
DAPI	4',6-Diamidino-2-Phenylindole
dATP	DeoxyATP
dCTP	DeoxyCTP
DDK	Dbf4-Dependent Kinase
DDR	DNA Damage Response
dGTP	DeoxyGTP
DMEM	Dulbecco's Modified Eagle Medium
DPBS	Dulbecco's PBS
dsDNA	Double Stranded DNA
dUTP	DeoxyUTP
E. coli	Escherichia coli
E1	Ubiquitin-Activating Enzyme
E2	Ubiquitin Conjugating Enzyme
E3	Ubiquitin Protein Ligase
EDTA	Ethylenediaminetetraacetic Acid
EdU	Ethynyl Deoxyuridine
FBS	Fetal Bovine Serum
G1 Phase	Gap 1 Phase
G2 Phase	Gap 2 Phase
GST	Glutathione S-Transferase
GTP	Guanosine Triphosphate
HBS	HEPES Buffered Saline
IdU	Iododeoxyuridine
IOD	Inter Origin Distance
M phase	Mitosis
MCM	Mini-Chromosome Maintenance Proteins
MEF	Mouse Embryonic Fibroblast

ORC	Origin Recognition Complex
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PFA	Paraformaldehyde
PIP	PCNA-Interacting Peptide
preIC	Pre-Initiation Complex
preRC	Pre-Replication Complex
Rb	Retinoblastoma
RFC	Replication Factor C
RPA	Replication Protein A
S phase	Synthesis Phase
S. cerevisiae	Saccharomyces cerevisiae
SCF	Skp, Cullin, F-box Containing Complex
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS Polyacrylamide Gel Electrophoresis
ssDNA	Single Stranded DNA
TBS	Tris Buffered Saline
TGS	Tris Glycine SDS Solution
tRNA	Transfer RNA
UTP	Uridine Triphosphate

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Abstract

During the mitotic mammalian cell cycle cells faithfully replicate their DNA utilizing multiple DNA replication sites known as origins of replication. DNA is replicated to provide each daughter cell a complete copy of the genome. Replication proceeds bi-directionally from a minority of potential origins licensed for replication by a variety of replication factor proteins. Replication is catalysed by processive replication enzymes known as DNA polymerases and is limited to the synthesis phase (S phase) of the cell cycle. Changes in the timing of replication, origin usage and replication rate are indicative of DNA replication stress, a proposed hallmark of cancer that causes genome instability. Cell cycle progression is largely controlled by the activity of cyclin dependent kinases (CDKs) and their cyclin binding partners. Here using an *in vitro* cell-free DNA replication system we analyse the interplay between Ciz1 and cyclin A/CDK2 in regulation of the initiation phase of DNA replication. This demonstrates that Ciz1 modulates and enhances the activity of cyclin A-CDK2 in cell free DNA replication assays and that Ciz1 increases the permissive CDK range that can promote DNA replication.

Next the inhibitory effect of Ap4A in cell free DNA replication assays is studied. These data suggest that Ap4A inhibits initiation by reducing loading of the replicative helicase MCM2-7 and the DNA polymerase sliding clamp PCNA. These data suggest that Ap4A can inhibit the firing of replication origins through disruption of replication complex assembly. Finally, DNA combing is established to measure replication parameters. Here we find that the replication fork progresses at 1.3kbp/min in mouse fibroblast cells, consistent with other studies, and quantify replication fork stalling by replication inhibitor aphidicolin. These data demonstrate the potential for cell free DNA replication assays to be combined with DNA combing to dissect replication parameters and characterise DNA replication stress in future studies.

Chapter 1: Introduction

1.1 Introduction

Multicellular organisms have evolved multiple overlapping pathways to restrict cell division, unless the correct environmental signals are present for growth, or repair of tissues. These pathways restrict cellular proliferation in the absence of sustained mitogenic signalling. In response to mitogenic signalling cells exit a quiescent state, known as G_0, and enter the cell cycle (Duronio & Xiong, 2013). Multiple layers of regulation maintain the fidelity of this process and therefore control of cellular proliferation. Cancer can be defined as an uncontrolled cellular proliferation and arises from multiple mutations that deregulate the cell cycle and DNA repair pathways leading to a mutant phenotype and uncontrolled proliferative state (Hanahan & Weinberg, 2000). Understanding how cancer arises has important implications for its diagnosis, treatment and prevention.

Cancer is a diverse group of diseases that can be loosely described by the deregulation of the cell cycle resulting in the uncontrolled, unrestricted proliferation of cells leading to tumour formation resulting in a potentially fatal condition. As life expectancies have increased cancer prevalence has also, this has resulted in approximately 8,200,000 cancer deaths worldwide, with a predicted 70% increase over the next 20 years (WHO, 2015). Unsurprisingly perturbations in each stage of the cell cycle are linked to cancer causing dysregulation, emphasising the importance of understanding the regulatory networks that control cellular division.

As cancer is not a single disease, it has to be described through hallmarks originally described by Hanahan & Weinberg (2000), the original hallmarks were: sustained growth signalling, evasion of anti-growth signalling, resistance to apoptosis, metastasis, cells becoming immortalised and angiogenesis. These hallmarks were updated by Hanahan & Weinberg (2011) to include: immune evasion, loss of genome stability, inflammation and altering cells energy metabolism. These hallmarks provide a framework to study cancer to increase treatment and diagnostic options, another common feature of cancers that has been proposed to be a hallmark of cancer is DNA replication stress (Macheret & Halazonetis, 2015).

Replication stress is a feature of cancerous cells. Replication stress is loosely defined as the slowing or stalling of replication forks (Macheret & Halazonetis, 2015). Replication stress causes unwinding of DNA causing coating in protein RPA through decoupling of replicative helicases and polymerases (Ciccia & Elledge, 2010). RPA activates a DNA damage response (DDR) causing cells to halt the cell cycle. This allows forks to be restarted, or if damage persists, forks collapse and are repaired (Zeman & Cimprich, 2014). Activation of the DDR

provides a barrier to tumourigenesis which if bypassed, for example by inactivation of tumour suppressor gene p53, can result in incomplete replication, genome instability and carcinogenesis (Bartkova et al., 2006).

Replication stress is induced by multiple mechanisms associated with increased cellular proliferation. Increased oncogenic signalling mediated by the mutation of proto-oncogenes into oncogenes induces constitutive activation of transcriptional networks that control cellular proliferation (Bos, 1989; Nevins, 2001) and increased expression of the cyclin proteins, which activate cell cycle promoting cyclin dependent kinases (CDKs) (Keck et al., 2007; Musgrove et al., 2011). Both increased oncogenic transcription and increased cyclin-CDK activity DNA replication stress, which leads to genome instability further promoting the progression of tumorigenesis (Gaillard et al., 2015).

This project aims to develop techniques that enable investigation of DNA replication stress responses to altered CDK activity that is common in cancer. The following sections will explore the regulatory mechanisms that control cell cycle progression and timely DNA replication. An overview of this process is described and leads into a description of common mechanisms that induce DNA replication stress in cancer biology. A better understanding of how these events are regulated have important implications for genome stability and prevention of tumourigenesis *in vivo*.

To gain a better understanding of how deregulated cyclin expression can contribute to DNA replication stress, an *in vitro* DNA replication assay is used. Using an *in vitro* DNA replication system that can precisely titrate cyclin A-CDK2 revealed that there is a narrow permissive activity range that can promote DNA replication (Copeland et al., 2010; Coverley et al.). Using this approach, the activating concentration of CDK activity can be established and the effects of both low and high CDK activity on replication could be monitored *in vitro*. This approach is expanded here to assess how Ciz1 (Reviewed in Section 3.1.2) can enhance, and expand the permissive range of cyclin A/CDK2 to promote initiation of replication in a mammalian *in vitro* DNA replication system. This has implications for how increased cyclin-CDK activity could contribute to DNA replication stress (Section 1.7).

In addition, data is presented that suggests that a novel dinucleotide molecule Ap4A (reviewed in Section 3.1.3) can block initiation of DNA replication in a mammalian *in vitro* replication system. Here the inhibitory activity is studied at the level of chromatin association of pre-replication complex (preRC) assembly and replisome markers. This approach clearly identifies potential for Ap4A to regulate initiation of DNA replication and is consistent with the

proposed secondary messenger function in response to genotoxic stress including interstrand crosslinks.

Finally, DNA combing enables high resolution visualisation and quantification of DNA replication. DNA combing enables the visualisation of nascent DNA replication forks by immunofluorescence microscopy. Here we aim to develop and establish this methodology to visualise active DNA replication forks in combination with *in vitro* DNA replication assays. This methodology is used to visualise replication fork progression and monitor fork stalling in mouse fibroblasts (Chapter 4). This provides a framework for detailed future analysis of DNA replication stress.

1.2 Regulation of the Cell Cycle by Cyclin Dependent Kinases

The cell cycle is the co-ordinated series of events a cell undergoes to divide. During this process, a cell must ensure that its DNA is replicated accurately and precisely. Across 23 chromosome pairs, human cells contain around 3 billion base pairs of DNA (genome, 2010). Precise regulation of the cell cycle contributes to genomic stability. The eukaryotic cell cycle is divided into 4 phases: G1, S, G2 and mitotic (M) phases (Figure 1.1). Separation of the cell cycle into these phases ensures faithful unidirectional progression. Failure to precisely replicate DNA can result in mismatches and mutations, which can cause abnormal cell function and can lead to tumorigenesis and cancer.

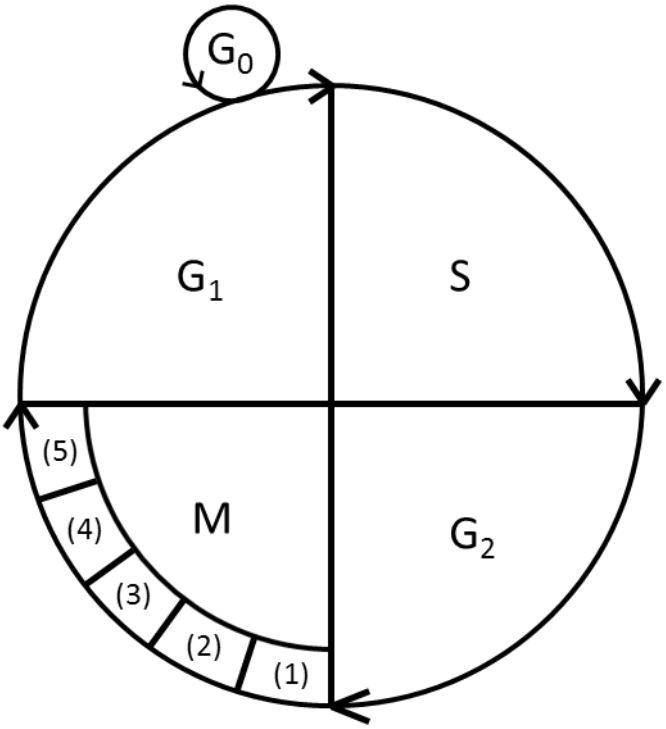


*Figure 1.1-**The Eukaryotic Cell Cycle**. Divided into 4 sections: G_1, S phase, G_2 and mitosis. In G_1 cells prepare for DNA replication, in S phase DNA is replicated, in G_2 cells prepare for the events of mitosis. Mitosis is where chromosome segregate and cells divide, mitosis is split into 5 stages: prophase, prometaphase, metaphase, anaphase and telophase. Some cells can exit the cell cycle prior to S phase entering quiescence. This is known as G_0. (Malumbres & Barbacid, 2009; Sullivan, 2007)*

G1 phase occurs after mitosis in cycling cells, although some cells exit the cell cycle. Cells recover from division and begin preparation for DNA replication. Many cells can exit the cell cycle at the G_1 phase entering G_0 phase (Duronio & Xiong, 2013). Some permanently differentiated cells enter quiescence and never leave G_0 until they are required to replicate once more. Cultured mammalian cells will enter G_0 in response to nutrient starvation for example by serum depletion or contact inhibition (Coverley et al., 2002). During S phase the entire genome is replicated in preparation for segregation during mitosis, a process that occurs at multiple sites across the genome, the replication of the genome occurs in roughly half an hour in yeast and 9.5 hours in mammalian cells (Brewer et al., 1984; Chagin et al., 2016). During G2 phase cells prepare for M phase, DNA is checked for mismatches and

abnormalities and the mitotic machinery is built. In M phase chromosomes segregate and cells divide. (Schafer, 1998)

Cyclin dependent kinases (CDKs) phosphorylate downstream targets driving the unidirectional progression through the cell cycle. CDKs are a family of serine/threonine kinases, without dimerization with a cyclin protein they are inactive. Mammalian genomes contain approximately 20 CDKs, some have roles in cell cycle progression and some have roles in transcriptional regulation (Malumbres, 2014). CDK activity is regulated by dimerization with transiently expressed proteins called cyclins , first discovered in sea urchin eggs in 1983 (Evans et al. 1983). Cyclins are expressed in a temporally regulated manner; there are many cyclins that have roles in cell cycle control, gene expression and transcription (Malumbres, 2014). Cyclin binding alone is insufficient for activation of CDKs and requires phosphorylation of the regulatory threonine 160 conserved in CDK1, 2, 4 and 6 mediated by the CDK activating kinase (CAK) complex of cyclin H, CDK 7 and MAT1. (Lolli & Johnson, 2005).

There are approximately 30 identified cyclin proteins in the mammalian genome, some having roles in cell cycle progression and some in transcriptional regulation (Malumbres, 2014). Cyclins A, B, D and E have major roles in the cell cycle and display considerable degeneracy in their activity yet are selective in binding of CDK partners (Lee & Sicinski, 2006). Cyclin D complexes with CDK 4/6 early during G1 in response to ERK/MAPK signalling from extracellular growth signals (Cheng et al., 1998). Cyclin E complexes with CDK2 promoting initiation of DNA synthesis i.e. the G1/S transition, cyclin A regulates S phase binding to both CDK2 and CDK1 (Donjerkovic & Scott, 2000). Cyclin B regulates mitosis complexed with CDK1. (Lim & Kaldis, 2013). Figure 1.2 shows a schematic for the temporal regulation of cyclin levels during the cell cycle.

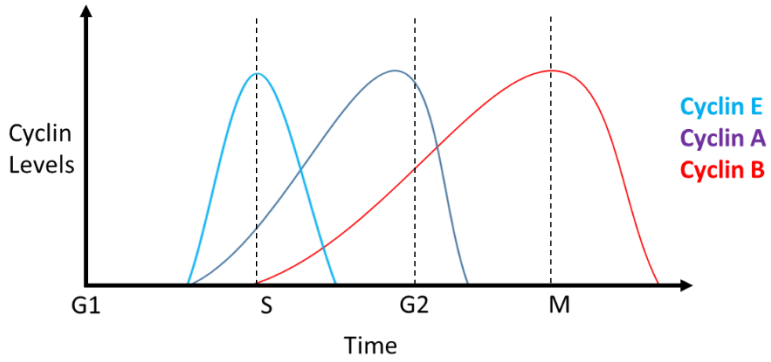


*Figure 1.2-**The Temporal Control of Cyclin Levels:** The changes in levels of cyclins E (blue), A (purple), and B (red) during one round of the eukaryotic cell cycle, dashed lines represent barriers between cell cycle phases. (Hochegger et al., 2008)*

Precise regulation of cyclin/CDK activity controls progression though the cell cycle and contributes to genome stability. Cyclin/CDK activity is controlled by multiple mechanisms that ensure activity is calibrated to cell cycle phase. A further level of regulation of CDK activity are CDK inhibitor (CDKI) proteins (Yoon et al., 2012). Two CDKIs that are important for regulating G1 and restraining entry to S phase are $p21^{Cip1/Waf1}$ and $p27^{Kip1}$. p27 and p21 bind to and inhibit cyclin E/CDK2 and cyclin A/CDK2 preventing its kinase activity (Abukhdeir & Park, 2008). Additionally, p21 protein levels modulates CDK2 activity in cells exiting mitosis promoting longer G1 phase when present at high protein levels and shorter G1 phases at low protein levels (Spencer et al., 2013).

A further layer of cyclin regulation and therefore CDK activity is ubiquitin-mediated proteolysis. Briefly, proteins can be targeted for proteolysis in proteasomes through the addition of multiple chains of the small peptide ubiquitin onto lysine residues through the activity of ubiquitin ligase enzymes. Ubiquitination is a multistep process driven by the activity of ubiquitin ligase enzymes. First, ubiquitin-activating enzymes (E1) are loaded with ubiquitin coupled to ATP hydrolysis, secondly ubiquitin is transferred to a ubiquitin conjugating enzyme (E2), finally the ubiquitin protein ligase (E3) brings together target proteins and E2-Ubiquitin complexes catalysing the transfer of a ubiquitin moiety to a lysine side chain of the target. This is repeated for poly-ubiquitination adding further ubiquitin moieties to the bound ubiquitin moiety targeting proteins for destruction in the proteasome. (Berndsen & Wolberger, 2014)

Selection of target proteins for degradation is driven by the E3 ligase enzymes. Ubiquitin mediated control of the cell cycle is driven mainly by two families of E3 ligases, the activities

of which differ temporally: These are the anaphase promoting complex (APC/C) and the Skp, cullin, F-box containing complex (SCF complex), both complex with different proteins modifying ubiquitination targets. The APC/C is activated during anaphase of mitosis until G_1 and the SCF is active during the G_1/S transition till the onset of mitosis. (Teixeira & Reed, 2013)

During anaphase the APC/C E3 ligase is activated. Principal targets of APC/C include cyclin B and geminin. Proteolytic destruction of these proteins coupled with nuclear membrane dissolution mediated dilution creates the low kinase environment that allows for licensing proteins such as ORC1 and CDC6 to bind, as well as removing the CDT1 inhibitory protein geminin. The activity of the APC/C creates the environment that allows daughter cells to prepare for DNA replication again. (Rizzardi & Cook, 2012; Teixeira & Reed, 2013).

From the start of mitosis through to metaphase APC/C pairs with co-activator CDC20, APC/C^{CDC20} promotes anaphase by targeting proteins such as securin for destruction (Nakayama & Nakayama, 2006; Sivakumar et al., 2015). During late mitosis and early G1 APC/C interacts with CDH1, during this time it targets proteins for degradation. APC/C^{cdh1} targets mitotic cyclin B for degradation, creating the low kinase activity required for G_1, but also preventing accumulation of cyclins during G_1 that could trigger untimely DNA replication (Li & Zhang, 2009). In G_1, accumulating cyclin E/CDK2 and cyclin A/CDK2 inactivates APC/C^{cdh1} by CDH1 phosphorylation. This is a critical step in the irreversibility of the G_1/S transition as CDH1 degrades skp2 preventing its accumulation. However, phosphorylation of CDH1 by cyclin E/CDK2 and cyclin A/CDK2 promotes degradation via SCF^{skp2} mediated poly ubiquitination. This CDK2 mediated switch of active E3 ligase proteins is one of the major events mediating the G1/S transition, powering the oscillation between cell division and DNA replication. SCF^{skp2} activation triggers the proteolytic destruction of CKI such as CKI p27, which inhibit cyclin E/CDK2 activity preventing onset of S phase. After destruction of CDH1, cyclin E/CDK2 becomes activated phosphorylating pRB promoting more cyclin E expression, creating the positive feedback loop that commits cells to S phase. (Giacinti & Giordano, 2006; Rizzardi & Cook, 2012; Teixeira & Reed, 2013)

1.3 Replication Origin Usage

Compared to mammalian cells bacteria have small genomes, *E. coli* strain K-12 has a genome organised into a single 4.6 million base pair circular chromosome encoding around 4,000 genes (Blattner et al., 1997). Usually, *E. coli* can replicate their DNA from a single location on the DNA known as an origin of replication termed *OriC*. *OriC* is a sequence defined region that

is bound by protein DnaA which recruits further proteins that allow unwinding of DNA and loading and recruitment of DNA primase, the DNA polymerase III holoenzyme and other replication factors (Kaguni, 2011; Robinson & Van Oijen, 2013). DNA replication now proceeds bi-directionally from *OriC* until replication is complete when linked daughter chromosomes are separated by topoisomerase IV in a process known as decatenation (Drlica & Zhao, 1997). Duplication and separation of the *E. coli* genome takes roughly an hour, when exposed to the right nutrients *E. coli* can initiate multiple rounds of DNA replication concurrently from replicated *OriC* to allow a rapid doubling time of 20 minutes (Fossum et al., 2007).

In Eukaryotic systems, multiple linear chromosomes and large genome size require more sophisticated mechanisms to ensure timely replication of the genome (Yekezare et al., 2013). Eukaryotes replicate their genomes through a complex yet flexible replication programme. The budding yeast *Saccharomyces cerevisiae* has a genome approximately 3 times larger than that of *E. coli* as well as having a much slower replication fork speed (Ranghuraman et al., 2001). In budding yeasts, replication initiation is determined by a specific sequence of DNA, these consensus sequences vary across species, but DNA replication begins at so called autonomously replicating sequences (ARS) (Liachko & Dunham, 2014). The Saccharomyces cerevisiae genome contains approximately 400 origin sites (Nieduszynski et al., 2006), although they are not all used in every round of replication and some origins are used more frequently than others (Muller et al., 2014). Use of multiple replication origins in *S. cerevisiae* allow genome replication to occur in approximately 30 minutes (Brewer et al.,1984).

Mammalian DNA replication origins are more complex, mammalian genomes do not contain ARS, instead relying on epigenetic mechanisms to determine origin location. However, GC rich sequence elements such as CpG islands are common features (Delgado et al., 1998). Between 30,000 and 50,000 origins fire in human DNA replication (Leonard & Méchali, 2013). Flexibility of mammalian origin usage is high as less than half of the same origins fire in sequential S phases (Tuduri et al., 2010). Approximately 10% of origins fire in each cell during replication, dormant origins are licensed ready to be used in response to DNA damage or replicative stress (McIntosh & Blow, 2012). Suppression of these dormant origins is required to confer resistance to DNA fork stalling agents that induce replication stress such as hydroxyurea (Ge et al., 2007). Origin usage varies between cells, between tissues and between developmental stages, certain changes in the replication programme are observed in cancerous cells (Amiel et al., 1998). Replication origins fire asynchronously across the whole genome and can be divided into early and late firing replication origins. Origin sites appear to be of high density in early firing replicons and lower for late acting replicons (Cayrou et al., 2011).

In budding yeast, *S. cerevisiae* firing of an origin at one ARS inhibits the firing of neighbouring origins (Brewer & Fangman, 1993) the same is true of mammalian cells (Lebofsky et al., 2006). During mammalian S phase, approximately 5,000 replication foci fire to copy DNA at sites located close within 3D space through formation of DNA loop structures (Chagin et al., 2016). The timing and density of origin usage, coupled with speed of replication fork progression can be collectively known as the DNA replication programme. The replication programme ensures that the entire eukaryotic genome is replicated in the short window of S phase. This replication programme is adapted in response to replication stress, to ensure complete genome replication (Ge et al., 2007). Replication stress can be measured by changes in origin usage and replication fork velocity changes, one technique commonly used for this is DNA combing (reviewed in Section 4.1).

1.4 Regulation of DNA Replication Initiation

DNA replication occurs in the S phase of the cell cycle, the G1/S transition is a tightly controlled component of the cell cycle. Broadly, DNA replication is composed of three stages: initiation, elongation and termination. During initiation sites along the DNA are prepared to begin replication, these sites are known as origins, the number of replication origins vary across species; from one in some bacteria such as *E. coli,* to 400 in the budding yeast *S. cerevisiae* to up to 50,000 in the human genome (Leonard and Méchali, 2013; Nieduszynski et al., 2006). During elongation, replicative polymerases proceed bi-directionally from origins replicating DNA in a semi conservative manner.

In metazoans, the firing of a replication origin is a tightly controlled multi-stepped process. It can be split into 2 phases: replication licensing and replication firing. During replication licensing origins are loaded sequentially with a variety of proteins forming a pre-replication complex (preRC). Origin licensing occurs from anaphase to G1 of the cell cycle ensuring that DNA replication occurs only once per cell cycle. First, origins are bound by the origin recognition complex (ORC) (Shackleton and Peltier, 1992). The ORC is a heterohexamer built of subunits named ORC 1, 2, 3, 4 5 and 6. The core ORC is made up of ORC2-5 with ORC1 and ORC6 binding less strongly. ORC1 binding is an early event in replication regulation (Dhar et al., 2001). The ORC then recruits CDC6 and CDT1. The complex of ORC 1-6, CDC6 and CDT1 is the PreRC. The PreRC then allows recruitment of the heterohexamer minichromosome maintenance complex (MCM) composed of 6 subunits MCM2-7, which promotes recruitment of a second MCM2-7 forming a double hexamer of MCM2-7 on chromatin during replication licensing (Evrin et al., 2009; Fragkos et al., 2015; Ticau et al., 2015). Figure 1.3 shows the

events that occur during replication licensing. Prevention of re-replication is largely driven by increased CDK activity (Section 1.5) and ubiquitin mediated proteolysis (Guarino et al., 2011; Laman et al., 2001; Méndez et al., 2002; Petersen et al., 1999).

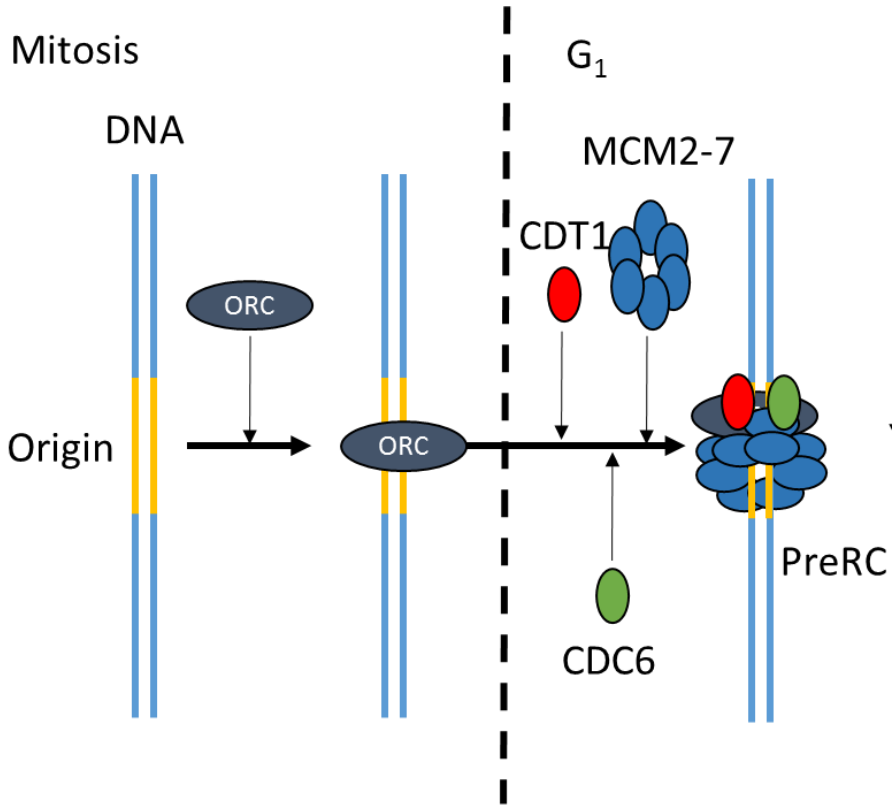


*Figure 1.3-**Loading of Pre RC onto a Replciation Origin:** The events that occur during DNA replication origin licensing. ORC recognises and binds to origins recruiting CDC6 and CDT1. Two MCM hexamers are recruited forming an inactive pre-RC. (Fragkos et al., 2015)*

Replication licensing and replication origin firing are temporally separated into two distinct phases. This is largely driven by CDK activity, after mitogen stimulation of the cell, two kinase proteins, cyclin E/CDK2 and DDK, promotes recruitment of CDC45-MCM2-7-GINS complex (CMG). The CMG allows separation of the MCM2-7 hexamers activating their helicase activity (Takeda & Dutta, 2005). Activation of CMG requires the activity of MCM10 which binds with a high efficiency during S phase and a lower efficiency during G_1 (Douglas & Diffley, 2016). The replicative polymerases are now recruited to these pre-initiation complexes (PreICs), pol α synthesises short strands of RNA that primes DNA replication of the leading and lagging strand by proccessivity factor PCNA bound pol ε and pol δ respectively (Kelman, 1997). However,

there is emerging evidence that pol δ is required for correct balance of leading and lagging strand synthesis and may have an increased role in leading strand synthesis during replication stress (Yeeles et al., 2016).

Interestingly, a minimal framework for eukaryotic DNA replication has been recapitulated *in vitro* using purified yeast replication proteins. This revealed that the basic requirement for budding yeast DNA replication initiation, involving 16 replication factors, and cyclin A/CDK2 and DDK phosphorylation (Yeeles et al., 2015). To achieve replication rates similar to those observed *in vivo* this framework was added to include replisome protein Mrc1 and Csm3/Tof1 which appeared to stabilise Mrc1 activity (Yeeles et al., 2016).

1.5 Role of Cyclin/CDKs in Control of Re-replication

It quickly became apparent that CDK activity was required for the initiation of eukaryotic DNA replication. The activity of CDKs also works to prevent relicensing of origins after DNA replication has begun, an important mechanism to prevent DNA re-replication.

The ORC binds DNA independently of CDK activity, however its protein levels are regulated indirectly through the activity of CDKs. ORC1 protein is constitutively expressed, and changes in ORC1 levels are regulated by ubiquitin mediated proteolysis. (Tatsumi et al., 2003). ORC1 is targeted for degradation after the onset of S phase by the activity of SCF^{Skp2}, the N terminus of ORC1 is phosphorylated in multiple sites by cyclin A/CDK2. This phosphorylation leads to an increase in nuclear export of ORC1 and targets it for poly-ubiquitination by SCF^{Skp2} (Laman et al., 2001; Méndez et al., 2002). The S phase cyclin A/CDK2 complex prevents the loading of a functional PreRC providing a mechanism to prevent re-replication.

Unlike ORC1, CDC6 activity in mammalian cells is not regulated by ubiquitin mediated proteolysis directly following S phase, but through cellular localisation. Prior to DNA replication initiation CDKs phosphorylate CDC6 to block its targeted destruction by APC/C^{cdh1}, allowing replication licensing during early G1 (Mailand & Diffley, 2005). During G_1 phase CDC6 accumulate in the nucleus, allowing licensing of DNA (Saha et al., 1998). After the onset of S phase when cyclin A levels have accumulated the N terminus of CDC6 becomes phosphorylated by cyclin A/CDK2 resulting in the nuclear export of CDC6 (Petersen et al., 1999). CDK2 phosphorylation again prevents relicensing of DNA replication origins safe guarding DNA from re-replication. CDC6 and CDT1 overexpression has been found in a number of human cancers including ovarian and lung cancers (Deng et al., 2016; Karakaidos et al., 2004).

CDT1 regulation is primarily driven by the activity of geminin. Geminin transcription is regulated by E2F transcription factors which are released upon phosphorylation of pRb (Yoshida & Inoue, 2004). Geminin is ubiquitinated through the activity of APC^{CDH1} during mitosis, APC^{Cdh1} becomes inactivated after S phase has begun, allowing geminin levels to recover during S phase. Geminin binds to and sequesters CDT1 after S phase preventing relicensing of origins (Rizzardi & Cook, 2012). CDT1 activity is also regulated by the activity of cyclin A/CDK2. Cyclin A/CDK2phosphorylates CDT1, making it a target for poly-ubiquitination by SCF^{Skp2} resulting in degradation of CDT1 in the proteasome. (Liu et al., 2004). Additionally, CDT1 contains 2 variant PCNA-interacting peptide (PIP) motifs, once bound to processivity factor PCNA CDT1 becomes a target for degradation helping to prevent re-licensing and re-replication (Guarino et al., 2011).

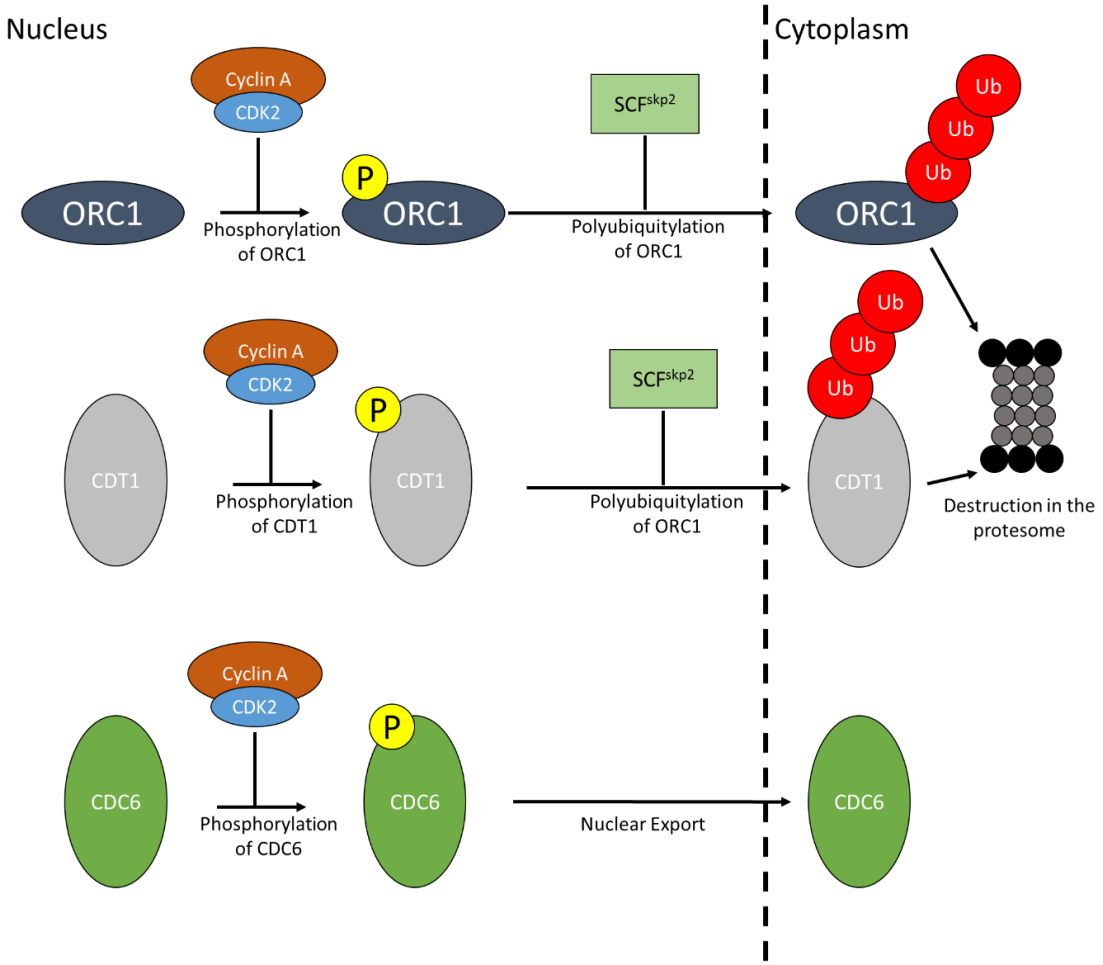


*Figure 1.4-**How CDK Phosphorylation Prevents Re-replication:** The activity of CDK2 after the onset of S phase promotes the inhibition of the activities of the PreRC proteins CDC6, CDT1 and ORC1. CDK activity prevents replication relicensing after DNA replication. (Guarino et al., 2011; Laman et al., 2001; Méndez et al., 2002; Petersen et al., 1999)*

Consistent regulation of replication fork proteins displays a pattern in which low kinase activities directly following mitosis through the activity of the APC/C result in an environment

that facilitates the loading of replication factors (Rizzardi & Cook, 2012). Directly after the onset of S phase a high kinase activity driven by the accumulation of cyclin A and E and the formation of cyclin A/CDK2 and cyclin E/CDK2 heterodimers results in the targeted destruction and re-localisation of early replication factors; figure 1.4 displays ways increased CDK2 activity regulates PreRC proteins.

1.6 CDK Quantitative model of DNA Replication

The cell cycle of mice fibroblasts can be completed using only the activity of CDK1 to complete cell division (Santamaria et al. 2007) suggesting that the various CDK activities display functional redundancy. One simple model for the cell cycle suggests that kinase level is the key requirement for unidirectional progression through the cell cycle (Figure 1.5)(Hochegger et al., 2008).

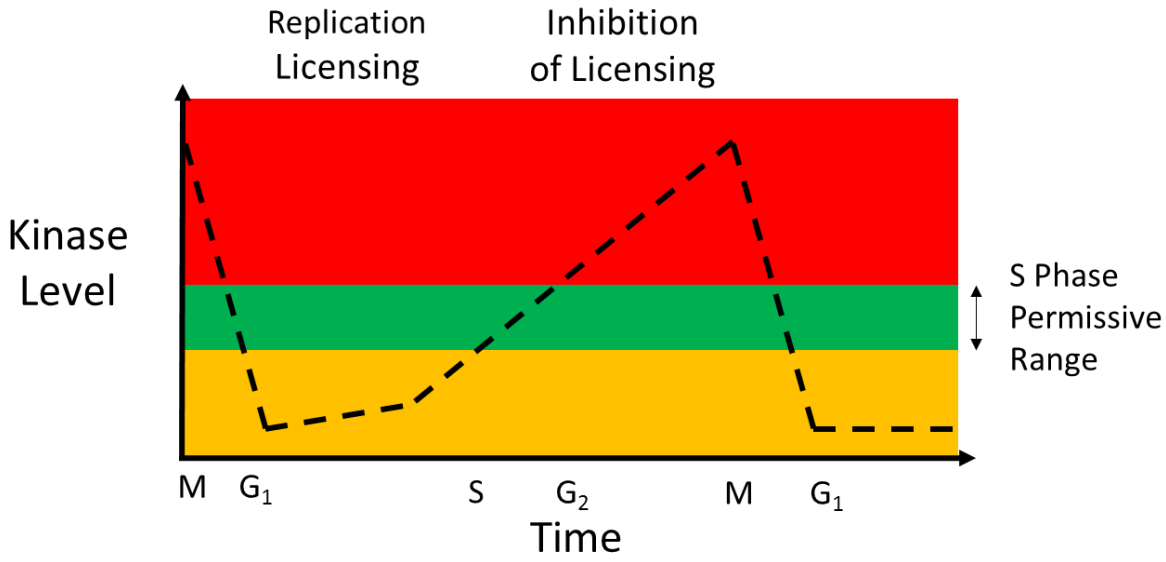


*Figure 1.5-**The Quantitative Model of DNA Replication:** The increase of kinase activity that drives the eukaryotic cell, showing the thresholds required to initiate S phase and mitosis and the small window of kinase activity that allows DNA replication to occur driving the unidirectional progression of the cell cycle. (Coudreuse & Nurse, 2010; Hochegger et al., 2008; Spencer et al., 2013)*

The quantitative model accurately models intracellular CDK activity in both yeast and mammalian model systems. In fission yeast *Schizosaccharomyces pombe* the mitotic cell cycle can be controlled by oscillating the activity of a fusion protein of a yeast cyclin/CDK, revealing the kinase activity and two kinase thresholds at S phase and M phase to be the minimal framework for the mitotic cell cycle (Coudreuse & Nurse, 2010).

The mechanisms described earlier on how CDK activity prevents DNA replication re-licensing after the onset of S phase creates the upper limit of the kinase level threshold in which DNA replication can initiate. This combined with lower kinase limit creates a permissive range of kinase concentrations in which DNA replication can occur (Figure 1.5). During anaphase of mitosis the APC/C^{CDC20} becomes activated resulting in the targeting of cyclins for degradation (Teixeira & Reed, 2013). This results in a reduction of kinase activity within the cell and enables protein phosphatases to reduce protein phosphorylation, thereby resetting the cell cycle (Wurzenberger & Gerlich, 2011). In this low kinase environment, licensing of replication origins can be mediated allowing S phase to occur.

In G1 phase cyclin CDK activity is maintained at a low level and accumulates throughout the cell cycle peaking in metaphase in early mitosis (Spencer et al., 2013). Within the cell cycle, there are two kinase thresholds that must be met for cell cycle progression: The S phase threshold and the M phase threshold. As discussed in section 1.5, S phase does not occur at low kinase levels that allow replication licensing, the activity of cyclin E/CDK2 and DDK is required to transition from replication licensing to the replication origin firing following loading of the CMG, PCNA and replicative polymerases (Takeda & Dutta, 2005). Intracellular kinase levels can be visualised using fluorescent sensors that are exported from the nucleus when phosphorylated by CDK2, when coupled with measuring S phase entry through degradation of fluorescent CDT1 this was elegantly used to demonstrate that S phase entry does not occur until after an increase in kinase levels. This defines a low kinase S phase threshold (Figure 1.5) (Spencer et al., 2013).

The high kinase threshold required for mitotic entry can be seen in the classical cell fusion experiments by Rao and Johnson (1970). Briefly, DNA replication can be initiated by fusion of G_1 and S phase phase cells but not when fusing G_2 and S phase cells. The high kinase activity promotes inhibition of replication licensing factors, such as CDC6, the ORC through degradation and re-localisation (Laman et al., 2001; Méndez et al., 2002; Petersen et al., 1999). Additionally, in budding yeast, at higher kinase levels ORC1 is phosphorylated by CDK activity, preventing CDT1 recruitment and inhibiting replication licensing (Chen & Bell, 2011).

Further additions can be made to the quantitative model if the inhibitory effect of cyclin A-CDK2 activity that prevents re-replication at high CDK activity is considered. The bifurcated response to cyclin A-CDK2 was shown by Coverley et al. (2002) in which an *in vitro* replication system is used to show that mammalian nuclei can be induced to replicate in a G_1 extract supplemented with cyclin A/CDK2 but only in a tight concentration window, this window

represents the permissive range (Figure 1.5). inhibition of S phase entry at low and high kinase levels, and the presence of an S phase kinase threshold creates a permissive range of kinase activity in which replication origins are licensed and can be initiated surrounded by a lower S phase kinase threshold and an upper inhibitory S phase kinase threshold.

1.7 Mechanisms that Promote DNA Replication Stress

Mutations cause deregulation of the cell cycle partially through altering cyclin regulation. It is important to study the genes that affect this in the search for diagnostic and therapeutic tools. It is also important to study the mechanisms by which mutations cause cancer development and progression. As cancer progresses genes regulating cell cycle control mutate. There are two broad types of genes that mutate in cancer: proto-oncogenes and tumour suppressor genes. Proto-oncogenes mutate into oncogenes causing an increase in cell proliferation signals (Croce, 2008). An example of this is Ras, which is a signalling protein that can become constitutively activated by loss of an intrinsic GTPase activity, this increased cell signalling leads to DNA replication stress (Maya-Mendoza et al., 2015). Tumour suppressors, however, usually act to restrain cell growth but when mutated lose this function (Sun & Yang, 2010). Many of the oncogenes and tumour suppressor genes identified interfere with CDK activity disrupting regulation of the cell cycle. For example, constitutive activation of mitogen signalling leads to cell cycle dysregulation. One prominent example of this is the ERK pathway, Activation of this pathway results in cell proliferative signals being activated including the expression of cyclin D1 (Filmus et al., 1994).

Replication stress can be described as the stalling or slowing of replication forks causing a change in the normal cellular replication programme. It is an early event in cancer progression. Changes in origin usage and replication fork speed can be measure directly by visualisation of stretched fibres of DNA in a technique called DNA combing (Reviewed in section 4.1). Replication stress is common in cancer development that it has even been proposed to be included as a hallmark of cancer itself (Macheret & Halazonetis, 2015). As an important early event in cancer progression it provides a target for the development of diagnostics and therapeutics.

One major source of replication stress in cancer is the activation of oncogenes that induce de-regulation of the cell cycle. Mutations in signalling pathways that induce constitutive activation such as Ras, overexpression of G1 cyclin D or cyclin E or overexpression of the apoptosis inhibitor Bcl2 have been shown to induce DNA replication stress (Maya-Mendoza et al., 2015; Jones et al., 2013;Xie et al., 2014). Oncogene activation causes replication stress

within the genome. Replication stress causes activation of the DNA damage response arresting the cell cycle until DNA can be successfully repaired or cells targeted for apoptosis; this provides a barrier to tumorigenesis. Replication stress induces genome instability, further mutations in DDR genes such as p53 result in tumorigenesis and cancer progression (Gaillard et al., 2015).

In response to replication stress cells activate the DNA damage response. Replication stress causes uncoupling of replicative polymerases and helicases resulting in large stretches of ssDNA (Byun et al., 2005). ssDNA becomes coated with RPA (an ssDNA binding protein), that facilitates the recruitment of the serine/threonine kinase, ataxia telangiectasia and Rad3 related protein (ATR) (Ciccia & Elledge, 2010) ATR phosphorylates various proteins, a noteworthy target of ATR is checkpoint kinase 1 (Chk1) (Cimprich & Cortez, 2008). Chk1 has many functions, a notable one is the phosphorylation of Cdc25. Cdc25 proteins are phosphatases that remove inhibitory phosphorylations from CDKs resulting in their activation. Chk1 activation causes inhibition of Cdc25 proteins resulting in the inhibition of CDKs and arrest of the cell cycle. Cell cycle arrest gives cells time to repair damage done to DNA or if necessary enter apoptosis. (Cimprich & Cortez, 2008). Some cancer cells under replicative stress rely on the activity of DDR proteins to survive. ATR and CHK1 have been shown to be required for cancer cell survival. Inhibition of ATR and CHK1 causes large regions of ssDNA to be exposed and replication forks to collapse triggering cell death. The activity of ATR and CHK1 is required to stabilise DNA with RPA and prevent replication fork collapse (Sanjiv et al., 2016). This may provide a novel therapeutic target by inducing unresolvable replication stress in cancer cells through inhibition of the DDR.

Due to the importance of CDKs in both the initiation of DNA synthesis through firing of replication forks and prevention of re-replication, it is unsurprising that dysregulation of CDK activity leads to abnormal DNA replication and is often dysregulated in cancers. Deregulation of CDKs through a diverse range of mechanisms can induce DNA replication stress: overexpression of cyclins upregulating CDK activity, changes in cyclin localisation, removal of inhibitory phosphorylations. Evidence from multiple cell lines clearly demonstrate that mislocalisation or overexpression of cyclin subunits can induce DNA replication stress and genomic instability. Cyclin D1 overexpression is found in a variety of cancers including breast and lung cancer (Gilett et al., 1994; Gautschi et al., 2007). Cyclin D overexpression is also implicated in the development of tumour resistance to radiotherapy (Shimura et al., 2010). Cyclin D1 overexpression has been shown to increase double-strand breaks that occur in HeLa cells. Cyclin D1 overexpression has been shown through DNA fibre assays to reduce the

velocity of replication forks, although this appears to be independent of CDK4 activity (Shimura et al., 2013).

Regulation of Cyclin D1 localisation is also key to cell cycle control. Cyclin D is localised in the nucleus during G1 phase and is exported to the cytoplasm during S-phase (Baldin et al., 1993). However, if nuclear localisation of cyclin D persists during S phase re-replication of DNA occurs at higher frequency, a feature of replicative stress. This appears to be through stabilisation of the PreRC protein CDT1 allowing origins to fire after replication (Aggarwal et al., 2007; Vaites et al., 2011). Cancers have been found with cyclin D mutations that prevent nuclear export of cyclin D after DNA replication initiation (Benzemo et al., 2006). This shows disregulation of the localisation of Cyclin D/CDK 4 during S phase promotes replication stress inducing genome instability which could accelerate carcinogenesis.

Cyclin E overexpression is implicated with cancers such as ovarian cancer (Courjal et al., 1996). U2OS cells induced to over express cyclin E activate a DDR detectable through p53 activation and CHK1 phosphorylation (Bartkova et al., 2005). DNA combing was used to show that cyclin E overexpression caused an increase in replication fork stalling indicating an induction of replication stress in cells (Bartkova et al., 2006). Cells overexpressing cyclin E have increased numbers of replication origins firing, with reduced replication fork velocity. Transcriptional regulation appears to have a role in cyclin E induced replication stress. Inhibition of transcription elongation in cells overexpressing cyclin E partially restores replication fork rate closer to wild type cells (Jones et al. 2013). Cyclin E overexpression forces replication with insufficient nucleotide levels through premature activation of the RB-E2F pathway, slow fork rates can be rescued by supplementation of an exogenous supply of nucleotides. (Bester et al., 2011) Deregulation of cyclin E induces replicative stress, implicating CDK activity once more. Overexpression of CMG component CDC45 causes replication stress, observed by increased origin firing and reduced DNA replication fork speed (Köhler et al., 2016).

Cyclin A overexpression has been shown to induce phosphorylation of the histone γH2AX in a variety of mammalian cell lines in a CDK2 dependent manner (Tane & Chibazakura, 2009). γH2AX is a key component of the DDR and indicator of replication stress produced in response to DSBs. Furthermore, cells that are deficient in NEK8 show double strand break (DSB) formation. NEK8 is involved in the cellular response to DSBs by limiting the activity of cyclin A/CDK2 (Choi et al., 2013). Control of CDK regulation is once more implicated in replicative stress and genome instability.

Further evidence that CDK activity can induce replication stress comes from studies that hyperactivate CDK activity. CDK is inhibited by inhibitory phosphorylations mediated by various kinases including Wee1 kinase and PKMYT1 kinase (Abukhdeir et al., 2008). Mutation of these sites to produce a deregulated CDK mutant induced genome instability and abhorrent mitosis. Furthermore, genetic silencing of the modified form of CDK2 in heterozygous cell lines resulted in reduction in the DDR. Fibre assays showed that loss of inhibitory phosphorylation caused a change in the replication programme that results in reduction in replication fork speed and increase in origin firing (Hughes et al., 2013). Once again showing that reduction in the control of the regulation of CDK activity results in replication stress and genome instability.

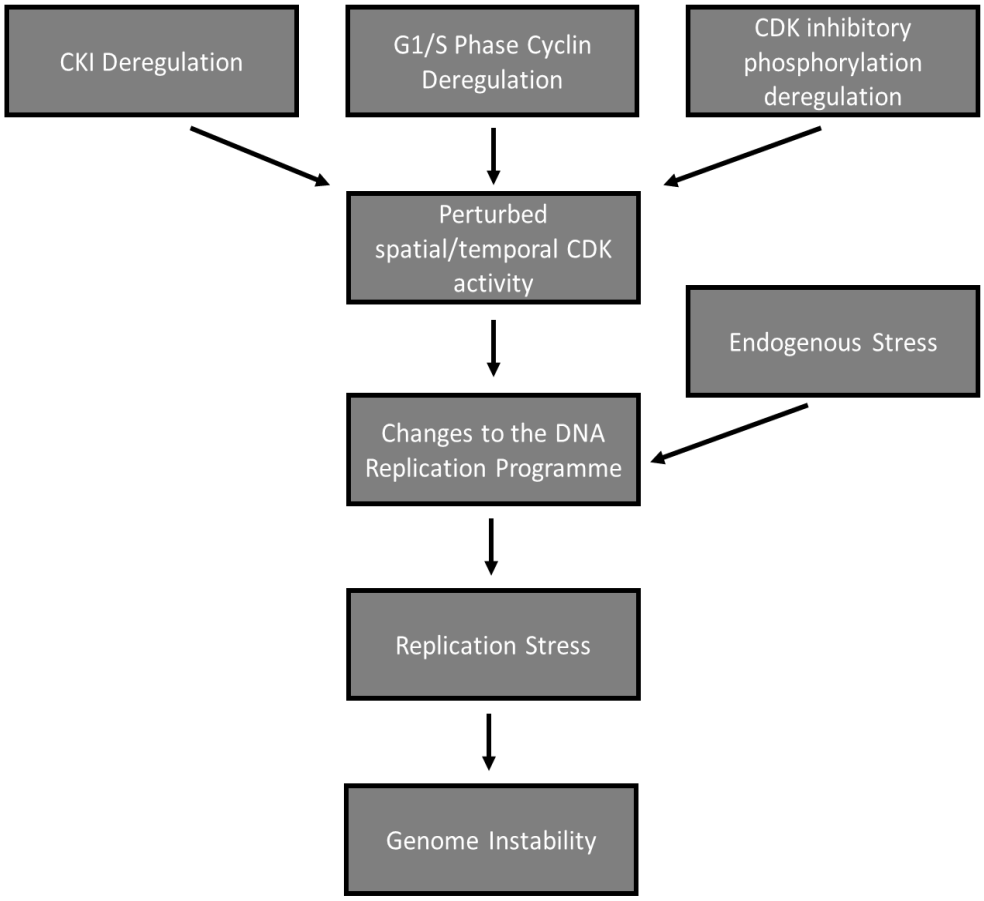


*Figure 1.6-**A Model for Inducing Replication Stress:** A pathway by which modifications to CDK regulation can induce genomic instability through inducing of replication stress. (Bester et al., 2011; Hughes et al., 2013; Quereda et al., 2016)*

The activity of G1 CKIs is also important in maintaining replication stability. As discussed earlier p27 and p21 inhibit the activity of CDK2, CDK 4 and CDK6 during G1, preventing the phosphorylation of RB, release of E2F transcription factors, inhibiting the G1/S transition. Mice have been generated that lack the activity of p27 and p21 and express a CDK4 form resistant to inhibition, cells displayed phenotypes typical of increased replication stress. This replication stress could be resolved by inhibition of CDK 4 and 6. (Quereda et al., 2016) This displays the importance of correct regulation of CDKs during G1 phase to ensure correct timely replication.

Consistently, changes in CDK spatial or temporal regulation, causes changes in the replication programme of DNA, causing replication stress and leading to genome instability (Figure 1.6) (Zeman & Cimprich, 2014). Critically, phenotypes associated with CDK activity dysregulation are found in a variety of cancers (Benzemo et al., 2006; Courjal et al., 1996). CDK disregulation induced replication stress is important to study in the context of cancer progression and tumorigenesis.

1.8 Aims

Here we will establish techniques for studying DNA replication, focusing on CDK activity, replication initiation and replication stress. Here *in vitro* mammalian DNA experiments will be used to investigate CDK activity during the events of DNA replication initiation. *In vitro* experiments will also be used to investigate the effect of Ciz1, a cyclin interacting oncogene (Reviewed in Section 3.1.3), on CDK mediated initiation. Additionally, *in vitro* experiments will be used to investigate how loading of replication licensing factors MCM2, CDC6, and PCNA is affected by Ap4A (Reviewed in 3.1.2), an alarmone produced upon DNA damage.

A powerful technique used frequently to measure replication parameters to quantify replication stress is DNA combing. Here, this technique will be established with the aim of using it to measure if abnormal Ciz1 levels has a role in promoting replication stress. Finally, work will be made towards combining in vitro replication experiments with DNA combing to establish a toolkit for further measuring replication parameters in vitro.

Chapter 2: Materials and Methods

2.1 Cell Culture

NIH3T3 and HeLa cells were cultured using Dulbecco's modified eagle medium (1 g/l glucose, with pyruvate and GlutaMAX I™) (DMEM) (GIBCO) supplemented with 1 % v/v penicillin-streptomycin-glutamine (100 X) (GIBCO) and 10 % (v/v) foetal bovine serum (FBS) (labtech). Cells were grown in 15 cm diameter dishes in 30 ml of media. Cells were incubated in a ThermoFisher Scientific Heracell^{TM} 150i, at 37 °C in 5 % CO_2.

Cells were passaged every 2-3 days unless being brought to confluence. Media was discarded from cells and cells were rinsed in 10 ml Dulbecco's PBS (GIBCO), cells were then trypsinsed in 9 ml DPBS supplemented with 1 ml 0.5% trypsin-EDTA (GIBCO) for 1-2 minutes until cells were released from the plates. To protect cells, trypsin was neutralised with an equal volume DMEM and split evenly across the desired number of plates and made up to 30 ml with DMEM. When trypsinising confluent plates the concentration of trypsin EDTA was doubled and incubation was increased to 5 minutes.

2.2 Cell Synchronisation

2.2.1 Synchronisation of NIH3T3 by Contact inhibition and Serum Depletion

NIH3T3 cells were synchronised in G_0 through contact inhibition and nutrient depletion. Briefly, NIH3T3 cells were grown until they reached confluency, medium removed and replaced with fresh growth media and cells incubated for a further 48 hours. Cells were released from G0 by trypsinisation and cells replated at 1 in 4 dilution into fresh growth medium. All subsequent timings for synchronised cell populations refer to hours after release into fresh media. Figure 2.1 shows a schematic for this synchronisation showing the timeline for cells entering S phase.

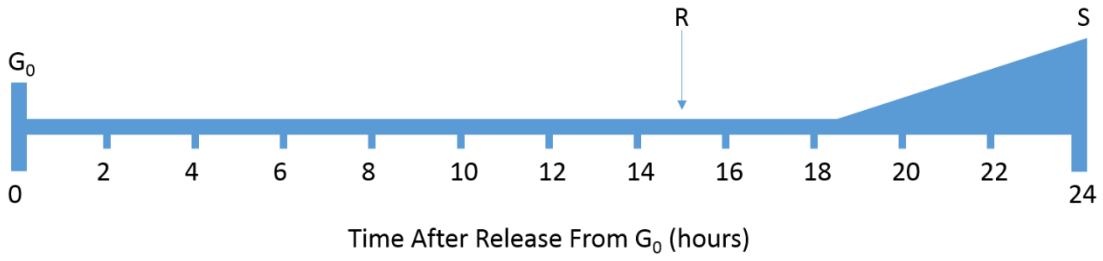


Figure 2.1 ***Cell Synchrony Schematic:*** *Schematic for synchronous release of NIH3T3 cells into S phase from G0 after release from G0, displaying the restriction point R and the cells entering S phase as determined previously for this methodology (Coverley et al., 2002)*

2.2.2 Determination of Percentage S-phase Cells by Ethynyl Deoxyuridine Incorporation

Half an hour before harvesting, cells were pulse labelled with 1 µM EdU (Invitrogen). Cells were fluorescently labelled using the protocol outlined in the click-iT® EdU imaging kits protocol (Invitrogen). Coverslips were mounted in vectashield with DAPI (Vector Laboratories).

Percentage of cells in S phase was calculated using a Zeiss confocal microscope and counting the fraction of DAPI labelled nuclei that had fluorescently labelled replication foci (alexafluor 555). Only nuclei with replication foci that were present throughout the nuclei were scored positive. Percentage of cells in S phase cells was calculated using the equation below.

$$\% \, S \, phase = \frac{Positively \, Labelled \, Nuclei}{Total \, Nuclei}$$

2.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein fractions were analysed by resuspension of cell fraction at a 1 in 4 ratio 4x SDS PAGE loading buffer (200 mM tris HCl pH 6.8, 27.7 mM SDS, 40% (v/v) glycerol, 1 mM DTT) and boiled for 10 minutes. Samples were run on 15 well 4–15% ,mini-PROTEAN® TGX™ precast protein gels (BioRad) in tris glycine SDS solution (TGS) (250 mM tris, 1.92 M glycine , 1% (w/v) SDS) at 200 V for 35 minutes. Gels were analysed by Coomassie staining (2.4) or western blotting (2.5).

2.4 Staining of SDS-PAGE Gels

Samples were run on an SDS-PAGE, gels were stained using GelCode™ blue safe protein stain (ThermoFisher Scientific) using the procedure outlined in its manual.

2.5 Western Blotting

Samples were run on SDS-PAGE. Protein was transferred onto an Amersham Protran 0.45 nitro-cellulose membrane (GE Life Sciences) using a semi dry transfer system. Filter paper, nitrocellulose membrane and gels were all soaked in transfer buffer (750 µM trizma base (SIGMA), 10 µM CAPS (SIGMA), 0.01% (w/v) SDS, 10% (v/v) ethanol (Fisher)). Four layers of filter paper were stacked, followed by the nitrocellulose membrane, gel and then four more layers of filter paper. Protein was transferred at 1 mA/cm^2 of membrane for two hours.

Membranes were placed in 10 ml blocking buffer (1% (w/v) BSA (Biowest), 0.05 M tris, 0.138 M NaCl, 0.0027 M KCl, pH 8.0, 0.1% (v/v) TWEEN 20 (Sigma Aldrich)) for 30 minutes to an hour. Membranes were incubated with primary antibody (table 1) for either two hours at

room temperature or overnight at 4 °C as indicated. Blots were washed four times for five minutes in 5 ml blocking buffer. If primary antibody was HRP conjugated blots were simply washed and developed. If unconjugated primary antibodies were used, blots were incubated with secondary antibody (Table 1) for one hour. Blots were washed four times for 5 minutes in 5 ml washing buffer (0.05 M tris, 0.138 M NaCl, 0.0027 M KCl, pH 8.0, 0.1% (v/v) TWEEN 20) prior to imaging.

Blots were developed using the BIO-RAD ChemiDoc™ MP system. HRP conjugated antibodies were developed using Westar etaC (ηC) (cyanagen).

Antibody Target	Supplier	Code	Dilution	Antibody Species	Conjugate
β-Actin	SIGMA	A-1978	1/5000	Mouse	N/A
Histone H3	Abcam	ab1791	1/10000	Rabbit	N/A
MCM2	BD Biosciences	610700	1/500	Mouse	N/A
CDC6	Santa Cruz Biotechnology	sc-9964	1/500	Mouse	HRP
PCNA	Abcam	ab201673	1/500	Mouse	HRP
Mouse IgG	Sigma Aldrich	A4416	1/5000	Goat	HRP
Rabbit IgG	Abcam	Ab7051	1/5000	Goat	HRP

*Table 2.1-**Antibodies Used for Western Blot Analysis***

2.6 Standardising Protein Loads

Protein loads were standardised using actin or histone H3 levels. 10 µl of sample was ran on SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were probed for their load control and developed. Loading was adjusted across samples to ensure uniform load control band intensity.

Band intensities were quantified using the BIO-RAD image lab software, 1 band was selected to be normalised too. The equation below was used to calculate the volume of each sample that needed to be loaded to standardise protein loads.

$$\boldsymbol{Volume\ of\ Sample\ to\ Load\ in\ Normalised\ Gel}\ (\mu\boldsymbol{l}) = \frac{\boldsymbol{Intensity\ of\ Band\ to\ Normalise\ to}}{\boldsymbol{Intesity\ of\ Sample\ band}} \times \boldsymbol{Volume\ Loaded}\ (\boldsymbol{\mu l})$$

2.7 Preparation of synchronised Cell-free Materials for *in vitro* DNA replication assays

2.7.1 Preparation of synchronised G1 nuclei for cell-free DNA replication assays

To prepare synchronised cells nuclei 5 plates of NIH3T3 cells were synchronise in G_0, and released into 20 plates as described in section 2.2.1. After 17.5 hours, cells were harvested. All further steps were performed and buffers cooled to 4 °C. Media was removed and cells were rinsed in hypotonic buffer (20 mM HEPES (pH 7.8), 0.5 mM $MgCl_2$, 5 mM potassium acetate, 1 mM DTT), buffer removed, then incubated in hypotonic buffer for 5 minutes. Hypotonic buffer was discarded and plates were left at a 45° angle for a further 5 minutes. Excess hypotonic buffer was discarded, cells removed from plates by scraping, and cell slurry pooled. Cells were lysed using a Wheaton 1 mL dounce homogeniser with seven strokes. Nuclei were removed from lysed cells by centrifugation at 6,000 RPM for five minutes at 4^{O}C. The nuclei pellet was resuspended in an equal volume of hypotonic buffer, typically around 200 µl of hypotonic buffer. Resuspended nuclei were flash frozen directly in liquid nitrogen in 10 µl beads and stored in cryovials in liquid nitrogen until use.

2.7.2 Preparation of Synchronised Soluble Extracts for Cell-Free DNA Replication Assays

G1 phase extract was isolated from NIH3T3 cells synchronised as per section 2.2. After release from quiescence, cells were scrape harvested at 15.5 hours, collected in cold 1.5ml eppendorfs and dounce homogenised using 20 strokes, nuclei were removed by centrifugation at 13,300 RPM for 15 minutes at 4°C. Cell extract supernatant was flash frozen in 50 µl beads and stored in cryovials in liquid nitrogen until use.

2.7.3 Preparation of S-phase Extracts for Cell-free DNA Replication Assays

HeLa cells were synchronised in S phase using a double thymidine block. HeLa cells were cultured in 15cm dishes and passaged until 20 plates were produced at 30-40 % confluence. At this point thymidine was added at a 2.5 mM final concentration to growth medium for 24 hours, cells were washed in 10 ml PBS and replaced with fresh media (-thymidine) for 8 hours and a second thymidine incubation (2.5 mM thymidine) for a further 16 hours. Cells were released from thymidine block into fresh media and harvested one hour later. Cells were scrape harvested, collected in cold 1.5 ml eppendorfs and dounce homogenised using 20 strokes, and nuclei were removed by centrifugation at 13,300 RPM for 15 minutes at 4°C. Cell extract supernatant was flash frozen in 50 µl beads and stored in cryovials in liquid nitrogen until use.

2.7.4 Preparation of Polylysine Coated Coverslips

Round 12 mm diameter coverslips (ThermoFisher Scientific) were dipped in a 1 mg/ml solution of poly-l-lysine (Sigma Aldrich), covered to protect from dust and dried overnight. Coverslips were stored in falcon tubes at room temperature until use.

2.7.5 Cell-free Replication Assay

Cell-free replication assays reaction mixtures added 1:9 nuclei:soluble cell extracts supplemented with a premix solution containing an energy regenerating solution, deoxynucleotides, biotin-16-dUTP and ribonucleotides Table 2.2 shows the components of 10x premix solution, Table 2.3 shows the ratios of reagents added to cell cycle specific cell extracts.

Premix	
HEPES pH 7.8	400 mM
$MgCl_2$	70 mM
DTT	1 mM
Phosphocreatine	2 mM
Adenosine triphosphate (ATP)	30 mM
Guanosine triphosphate (GTP)	1 mM
Cytidine triphosphate (CTP)	1 mM
Uridine triphosphate (UTP)	1 mM
DeoxyATP (dATP)	1 mM
DeoxyGTP (dGTP)	1 mM
DeoxyCTP (dCTP)	1 mM

*Table 2.2-**Components of 10x Premix Solution for in vitro Replication Assays***

Cell-free Replication Assay Reaction Mixture	**Ratio added to extract**
10 x premix solution	1:10
0.1 M $MgCl_2$	1:50
10 µg/ml CPK	1:50
Biotin-16-dUTP	1:50

*Table 2.3-**Reagents Added to Cell Cycle Specific Cell Extracts for Cell-free Reactions***

Cell-free DNA replication assays were incubated at 37 °C for half an hour and reactions quenched and fixed in 4 % paraformaldehyde (PFA) for 15 minutes at room temperature. Poly-l-lysine coated coverslips were added to the bottom of 13 mm diameter scintillation

tubes, and 800 µl of 30 % (w/v) sucrose was layered over the coverslips. Fixed nuclei were added to the surface of the sucrose solution without breaking the meniscus. Nuclei were dense enough to spin through the sucrose cushion by centrifugation at 1500 RPM for 10 minutes in a Harrier 18/80 centrifuge. The sucrose solution was removed and coverslips recovered. Coverslips were transferred to a 24 well plate and washed three times for five minutes in PBS. Coverslips were then washed three times for five minutes in antibody buffer (0.02 % (w/v) SDS, 0.1 % (v/v) triton X-100, 0.01 M PBS, 0.138 M NaCl, 0.0027 M KCl, pH 7.4, 1 % (w/v) BSA). Coverslips were transferred to a humidity chamber and incubated for half an hour in 20 µl of a 1 in a 1000 dilution of streptavidin, Alexa Fluor® 555 Conjugate (Thermo Fisher Scientific) in antibody buffer. Coverslips were recovered and washed three times for five minutes in antibody buffer, then three times for five minutes in PBS. Coverslips were mounted on glass slides with Vectashield antifade mounting medium with DAPI (Vectorlabs).

Coverslips were visualised using the 40x and 63x magnification of on a Zeiss 810 laser scanning confocal microscope. The proportion of positively labelled nuclei was counted by the proportion of DAPI stained nuclei with labelled replication foci. The percentage initiation of nuclei was calculated using the calculation below.

$$\%\ Initiation = \frac{Experimental\ Positive\ Nuclei - G1\ Positive\ Nuclei}{Total\ Nuclei - G1\ Positive\ Nuclei} \times 100$$

2.7.6 Western Blot Analysis of Cell-free Replication Reactions

For western blot analysis 50 µl reactions were prepared. For total protein fractions reactions were mixed with 4 x SDS-PAGE loading buffer and boiled for 10 minutes.

For isolation of the chromatin fraction triton X-100 was added to a final concentration of 0.5% (v/v) dissolved in PBS and incubated on ice for 2 minutes. Samples were then centrifuged at 14000 x *g* for 5 minutes. Supernatant and pellet were separated, the supernatant was mixed with SDS-PAGE loading buffer and boiled for 10 minutes representing the soluble protein fraction. The pellet was resuspended in SDS-PAGE loading buffer and boiled for 10 minutes representing the DNA bound protein fraction. Samples could then be analysed through SDS-PAGE and western blot analysis.

2.8 Bacterial Culture

2.8.1 Bacterial Transformation

1 µl of a 50-100 ng/µl plasmid solution was added to 50 µl of competent cell on ice and incubated for 30 minutes, bacteria were heat shocked at 42^{O}C for 1 minute and cooled to 4^{O}C for 5 minutes. Bacteria were resuspended in 300 µl of super optimal broth with catabolite repression (SOC media; 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose; ThermoFisher Scientific) and incubated whilst shaking at 37 °C for 1 hour. 100 µl of cultured SOC media was spread onto agar plates and grown overnight at 37 °C using suitable selection.

Transformed bacteria were stored in 50% glycerol. Colonies were picked from agar plates and inoculated into 5 ml of LB broth. 500 µl of culture bacteria was added to 500 µl of 50% (v/v) glycerol and stored at -80^{O}C.

2.8.2 Plasmid Purification

Transformed *E. coli* were inoculated into 5 ml of LB broth and cultured overnight. Plasmids were purified with the GeneJET plasmid miniprep kit (ThermoFisher Scientific) using the procedure outlined in the manual. Plasmid concentrations were quantified using the ThermoScientific nanodrop 2000c.

2.8.3 Bacterial Culture

All culture material was autoclaved before inoculation with bacteria. For normal growth, bacteria were grown at 37 °C in 2.5 % (w/v) LB broth (tryptone 10 g/l, yeast Extract 5 g/l, sodium chloride 10 g/l) (Melford). Bacterial colonies were cultured on 4 % (w/v) agarose Lb agar (Fisher Scientific). When inducing protein expression, bacteria were grown in auto-induction media (Table 2.4). Auto-induction media contains a low concentration of glucose and a high concentration of lactose. Protein expression begins after glucose is metabolised switching on expression of lacI regulated genes. This system allows slow expression of recombinant protein helping to ensure the expressed protein is soluble. All culture material was supplemented with 100 µg/ml ampicillin (Melford).

Component	Concentration
$(NH_4)_2SO_4$	25 mM
KH_2PO_4	50 mM
Na_2HPO_4	50 mM
Glycerol	54.3 mM
Glucose	2.78 mM
α-Lactose	5.55 mM
$MgSO_4$	1 mM
$FeCl_3$	50 µM
$CaCl_2$	20 µM
$MnCl_2$	10 µM
$ZnSO_4$	10 µM
$CoCl_2$	2 µM
$CuCl_2$	2 µM
$NiCl_2$	2 µM
Na_2MoO_4	2 µM
Na_2SeO_3	2 µM
H_3BO_3	2 µM
Tryptone	1% (w/v)
Yeast Extract	0.5% (w/v)

*Table 2.4-**Components of Bacterial Auto-induction Media***

2.8.4 Protein Expression and Purification

75 ml cultures of BL21 *E. coli* transformed with pGEX-6P-3 vectors containing genes of interest were grown overnight. 75 ml cultures were inoculated into 750 ml auto-induction media (Table 2.4). Bacteria were incubated at 20 °C for at least 20 hours post inoculation.

Bacteria were centrifuged in a Beckman Coulter Avanti J-26 XP centrifuge using a JLA 8.1000 rotor at 4500 RPM for 15 minutes. Pellets were resuspended in 25 ml HEPES buffered saline (HBS) (50 mM HEPES, 135 mM NaCl, 3 mM EDTA, 1 mM DTT, pH 7.8) supplemented with 1 mM PMSF, and cOmplete™, EDTA-free protease inhibitor cocktail (ROCHE). Bacteria were lysed by sonication four times for 15 seconds at 1 minute intervals. Cell lysate was centrifuged in a Beckman Coulter Avanti J-26 XP centrifuge using a JA25.50 rotor at 40,000 *x g* for half an hour at 4 ^{O}C.

0.75 ml glutathione sepharose beads (GE Healthcare Life Sciences) were resuspended in 50 ml of HBS per 750 ml culture for 1 hour. Glutathione sepharose beads were centrifuged at 1000 RPM for 1 minute. Glutathione sepharose beads were resuspended with cell lysate supernatant and left to bind on a mechanical wheel at $4^{O}C$ for one hour. Glutathione sepharose beads were centrifuged at 1000 RPM for 1 minute and washed in 10 ml HBS supplemented with complete protease inhibitors, this was repeated 4 times. Beads were then washed three times by centrifugation and resuspension in 3C buffer (50 mM tris-HCl pH 7.0, 150 mM NaCl, 2 mM DTT). Beads were resuspended in 1.5 ml of 3C buffer, 10 µl of PrecScission 3C protease (GE Healthcare LifeSciences) was added to solution and incubated overnight at $4^{O}C$ on a mechanical wheel. Before addition of 3C protease a sample was taken for SDS-PAGE analysis.

Proteins were eluted using Pierce™ spin columns (ThermoFisher Scientific) using the procedure outlined in the manual. Samples were taken from the beads and elution for SDS-PAGE analysis. Protein samples were flash frozen in liquid nitrogen in 25 µl beads.

Protein concentration was quantified using the procedure outlined in the reducing agent compatible Pierce™ BCA protein assay kit (ThermoFisher Scientific) according to manufacturer's instructions.

2.9 DNA Combing

2.9.1 Preparation of Silanized Cover Slips for DNA Combing

Square 0.5mm thick 22 mm by 22 mm coverslips (Thermo Scientific) were placed in teflon coverslip racks. All washing steps take place in fresh, dust free 250 ml beakers. Coverslips were briefly rinsed in acetone (Sigma-Aldrich) and air-dried. Coverslips were washed in 120 ml 50% (v/v) methanol (Sigma-Aldrich) for 20 minutes in a sonicating water bath. Coverslips were washed in 120ml chloroform (Sigma-Aldrich) for 20 minutes in a sonicating water bath and air-dried.

Piranha solution was prepared by mixing concentrated sulphuric acid (99% w/v) (Sigma-Aldrich) and hydrogen peroxide (35% w/v) (Sigma-Aldrich) at a 7:3 ratio, adding hydrogen peroxide first. Coverslips were washed in 120 ml of piranha solution in a water bath filled from a warm tap for 20 minutes.

Coverslips were sequentially washed in 120 ml distilled water then chloroform and repeated until viscous acid was visibly removed from the coverslips. Coverslips were placed in 120 ml heptane (Sigma-Aldrich) with a 1:1000 dilution of (7-octen-1-yl) trimethoxysilane (Sigma-Aldrich) and left overnight in a desiccator. Coverslips were sequentially washed in 120 ml heptane, distilled water, and then chloroform for 5 minutes in a sonicating water bath. Coverslips were left to dry then stored individually in 50 ml falcon tubes.

2.9.2 Pulse Labelling of Cells for DNA Combing

Pulse labelling was performed on NIH 3T3 cells grown to approximately 70 % confluence in 15 ml plates. Cells were labelled with iodo-deoxyuridine (IdU) (Sigma-Aldrich), thymidine (Sigma-Aldrich) and chlorodeoxyuridine (CldU) (Sigma-Aldrich). Cells were pulsed labelled with 25 µM IdU supplemented DMEM (section 2.1) with for 20 minutes. Cells were washed with 10 ml DPBS then pulse labelled with 30 ml complete DMEM containing 2.5 µM thymidine for 20 minutes. Cells were washed with 10 ml DPBS then pulse labelled with 30 ml DMEM containing 250 µM CldU. Cells were washed with 10 ml DPBS then incubated for an hour in 30 ml DMEM containing 50µM thymidine. Timings of labelling varied between experiments as indicated in Figure legends.

2.9.3 Purifying Genomic DNA from Pulse Labelled Cells

Labelled cells were trypsinised, neutralised with 10ml of DMEM and centrifuged at 500 *x g* for 5 minutes, pellets were resuspended in 10 ml of PBS. Cells were counted, centrifuged at 500 *x g* for 5 minutes then resuspended. Where possible, cell number was adjusted to $1x10^7$ cells/ml, if cell number too low cells were resuspended in 330 µl of PBS.

Cells were warmed to 42 °C. 330 µl of warmed cell suspension was mixed with 195 µl of 42 °C melted low melting point agarose (Thermo Scientific) (1 % w/v dissolved in PBS). 100 µl cell agarose mixture was added to plug moulds from CHEF genomic DNA plug kit (BIO-RAD) and set at 4 *°C* for 20 minutes. Plugs were added individually to 50 ml falcon tubes containing 250 µl of 2mg/ml proteinase K in proteinase K buffer from CHEF genomic DNA plug kit (BIO-RAD). Plugs were incubated overnight at 42^OC.

Plugs were washed 4 times at room temperature in TNE 50 (10 mM tris-HCl, pH 7.5, 20 mM NaCl, 50 mM EDTA) for 1 hour, 1 mM PMSF was included on the third wash. Plugs were rinsed in 2 ml of MES-E (50mM MES, 1mM EDTA, pH 5.7) at room temperature for 30 minutes. Plugs were incubated in 1.5 ml of MES-E at 65 °C for half an hour then cooled to 42 °C. 100 µl of a 2% (v/v) beta agarase I (NEB) in MES-E was added to plugs, which were digested overnight at 42 °C releasing DNA into solution.

2.9.4 DNA Combing

DNA solutions were cooled to room temperature. DNA solutions were poured into milled (25x25x4 mm) slots in 100 % teflon blocks. Silanized coverslips were lowered into DNA solution and DNA was allowed to bind for 10 minutes. Silanized coverslips were removed from wells at a constant speed of 300 µm/sec using a KSV NIMA small dip coater (Biolin Scientific).

Coverslips were tested for their ability to stretch single DNA molecules. Coverslips were combed, glued to slides then mounted directly with 0.1 µM YOYO-1 iodide (ThermoFisher Scientfic) in ProLong® diamond antifade mountant (ThermoFisher Scientific).

For probing labelled DNA coverslips were glued to slides and baked at 60 °C for 1 hour. DNA was denatured with 0.5 M NaOH for 30 minutes. Slides were washed three times for three minutes in PBS-T (0.01 M phosphate buffered saline, 0.138 M NaCl, 0.0027 M KCl, pH 7.4, 0.1% (v/v) Tween20), filtered through 0.2 µm filters. Coverslips were blocked with 200 µm of 1% (w/v) BSA PBS-T (filtered through 0.2 um filters).

Primary antibodies were used to label IdU raised in mouse, CldU raised in rat and ssDNA raised in mouse. Anti-IdU primary antibody was detected with a single Alexa Fluor® 633 conjugated anti mouse IgG secondary antibody raised in goat. Anti-CldU primary antibody was detected with an Alexa Fluor® 488 conjugated anti-rat IgG antibody raised in chicken and an Alexa Fluor® 488 anti-chicken IgY antibody raised in goat, to intensify the signal. anti ssDNA primary antibody was detected using an Alexa Fluor® 568 conjugated anti-mouse IgG raised in rabbit and Alexa Fluor® 568 anti-rabbit IgG raised in goat, to intensify the signal (Table 2.5).

Coverslips were probed with 50 µl primary antibodies for IdU (1/20) and CldU (1/250) (Table 2.5) dissolved in 1% BSA PBS-T in a humidity chamber for either 2 hours at room temperate or overnight at 4 °C. Slides were washed three times for three minutes in PBS-T. Coverslips were probed with 50 µl of 1/50 Alexa Fluor® 488 and 1/50 Alexa Fluor® 633 conjugated secondary antibodies (Table 2.5) dissolved in 1% BSA PBS-T in a humidity chamber for 30 minutes. Coverslips were washed three times for three minutes in PBS-T.

Coverslips were probed with 50 µl of a 1/50 dilution of the primary antibody for ssDNA (Table 2.5) dissolved in 1% BSA PBS-T in a humidity chamber for either 2 hours at room temperate or overnight at 4 °C. Slides were washed three times for three minutes in PBS-T. Coverslips were probed with 50 µm of Alexa Fluor® 568 conjugated secondary antibodies (Table 2.5) dissolved in 1% BSA PBS-T in a humidity chamber for 30 minutes. Coverslips were mounted with ProLong® diamond antifade mountant (Thermo Fisher Scientific) and sealed with clear nail varnish. DNA was imaged using a Zeiss confocal microscope using the 63 x objective lens.

Antibody Target	Supplier	Code	Dilution	Antibody Species	Conjugate
IdU	BD Biosciences	347580	1/20	Mouse	N/A
CldU	BioRad	OBT0030	1/250	Rat	N/A
ssDNA	Merck Millipore	MAB3034	1/50	Mouse	N/A
Rat IgG	Thermo Fisher Scientific	A-21470	1/50	Chicken	Alexa Fluor® 488
Chicken IgY	Thermo Fisher Scientific	A-11039	1/50	Goat	Alexa Fluor® 488
Mouse IgG	Thermo Fisher Scientific	A-21050	1/50	Goat	Alexa Fluor® 633
Mouse IgG	Thermo Fisher Scientific	A-11061	1/50	Rabbit	Alexa Fluor® 568
Rabbit IgG	Thermo Fisher Scientific	A-11036	1/50	Goat	Alexa Fluor® 568

Table 2.5-Antibodies used for Detecting Labelled Combed DNA

Chapter 3: *In vitro* analysis of the role of Ciz1 and Ap4A in regulation of the initiation phase of DNA replication

3.1 Introduction

3.1.1 Eukaryotic *in vitro* Replication Assays

Initiation of DNA replication at the G_1/S transition is a tightly controlled process. Genetic and biochemical dissection of this complex process has provided great insight into the regulatory events that facilitate precise control of replication origin firing. Eukaryotic DNA replication has been studied extensively in yeast and findings have largely been found to be conserved in Animalia. Techniques have been developed in which DNA replication can be reconstituted *in vitro.* These *in vitro* replication experiments have been important for investigating the events surrounding the G_1/S transition.

Early experiments using budding yeast *S. cerevisiae,* and fission yeast *S. pombe* as model organisms to identify both yeast and human CDK and identify their role in cell cycle progression. Cdc2 in fission yeast, and cdc28 in budding yeast were identified to be the key drivers entering the cell cycle and mitosis (Hartwell, 1971; Nurse & Bissett, 1981). Complementation was then used to show that the cdc2 and cdc28 genes were functionally homologous to each other, and identified the mammalian homologue CDK1 (Lee & Nurse, 1987). *S. cerevisiae* was also used also used to identify the ORC proteins, CDC6, MCM proteins, CDC45 (Bell & Stillman, 1992; Piatti et al., 1995; Yan et al., 1991; Zou et al., 1997) . More recently budding yeast DNA replication has been recapitulated *in vitro.* This revealed a minimum framework for DNA replication initiation and replication factors required to increase fork rate to those observed *in vivo* (Yeeles et al., 2015; Yeeles et al., 2016)

Originally cell-free replication experiments were perfomed using cell extracts obtained from the eggs of *Xenopus* species. *Xenopus* eggs are transcriptionally inactive during their early development. Because of this, *Xenopus* eggs contain many copies of the proteins and replication factors required to initiate DNA synthesis. These extracts can stimulate replication of *Xenopus* sperm DNA which can then be used to investigate a variety of factors including DNA replication timing and protein loading (Gillespie et al., 2012). *Xenopus* cell-free DNA replication systems are used as a model for higher eukaryotes, cell-free experiments were used to show that mitosis is inhibited until DNA replication is completed and showed that yeast replication licensing factor homologs ORC proteins, MCM proteins and CDC6 are required for higher eukaryotic DNA replication (Dasso et al., 1990; Kubota et al., 1995; Romanowski et al., 1996).

Mammalian cell-free replication protocols were developed to investigate the events of the G1/S transition in the mammalian cell cycle. An early *in vitro* replication experiment used to investigate mammalian DNA replication was the SV40 virus. This virus contains one replication origin and uses host cell proteins to replicate, providing a useful model for DNA replication in eukaryotes. Human and monkey cell extracts were used to initiate replication (Huwitz et al., 1990; Waga & Stillman, 1994). SV40 in vitro replication experiments were used to show that single strand binding protein RPA was required for DNA synthesis. They were also used to show the function of polymerase and primase enzymes on lagging strand synthesis. Furthermore, SV40 *in vitro* experiments were used to investigate the role of PCNA in DNA synthesis (Prelich & Stillman, 1988; Tsurimoto et al., 1990; Nasheuer et al., 1992).

More recently, techniques have been developed in which nuclei can be isolated from mammalian cells that can support replication initiation. These are known as replication competent nuclei. A variety of different techniques exist to generate these replication competent nuclei. These include isolating nuclei after release from G_0 back into the cell cycle and chemical arrest of cells at late G_1 using metal ion chelator mimosine (Krude, 2006).

The number of nuclei in S phase can be measured by the incorporation of modified nucleotides such a biotin-16-dUTP. Simply, a biotin moiety is added to a uridine nucleotide, this can be detected utilizing the interaction between biotin and streptavidin (Coverley et al., 2002). Streptavidin conjugated antibodies will bind to this nucleotide providing a frame work for the immunofluorescent labelling of S phase nuclei. When counterstained with DAPI the proportion of nuclei in S phase can be calculated.

In mouse fibroblasts, quiescence is used to synchronise cells and cytosolic extracts and nuclei produced at defined points within G1 phase. Using this approach nuclei are isolated from cells after re-entry to the cell cycle following contact inhibition and serum starvation. Nuclei isolated after the restriction point but before cells begin to enter S phase are licensed for DNA replication and are termed 'replication competent' (Coverley et al., 2002). For NIH3T3 cells this is approximately 17.5 hours after release from G_0. Nuclei isolated in this way have been used to investigate how G1/S phase cyclins A and E function during replication initiation and how cyclin A/CDK2 serves to prevent re-replication.

In vitro replication of replication competent mammalian nuclei provides a valuable tool for investigating the events surrounding the G_1/S transition and how replication factors and replication inhibitors act. This Chapter will use cell-free DNA replication assays to establish the protocols and procedures to test the requirements for proteins for the initiation of DNA

replication. In addition, we will use this methodology to test the activity of an alarmone (Bochner et al., 1984) Ap4A to inhibit the initiation phase of DNA replication and monitor replication complex assembly in cell-free replication assays.

3.1.2 Ciz1

Cip1 interacting zinc finger protein (Ciz1) is an 898 amino acid, 95 kDa protein. Ciz1 was shown to contain an MH3 domain, three zinc finger motifs and two glutamine rich domains (Mitsui et al., 1999). It has apparent roles in several cancers including prostate, colon and lung cancer (Higgins et al., 2012; Liu et al., 2014; Wang et al., 2014). Ciz1 was first identified by Mitsui et al. (1999) using a modified yeast two hybrid system in which recombinant cyclin E/p21Cip1/waf 1 was used as bait. Ciz1 cDNA was isolated, cloned and identified as a cip1 interacting protein. Warder and Keherly (2003) confirmed that Ciz1 was a nuclear localised protein. They used a selection and amplification binding assay to identify a consensus sequence to which small nucleotide chains were used to immunoprecipitate with Ciz1. Ciz1 was found to bind the consensus sequence ARYSR(0–2)YYA.

Ciz1 was thought to have a role in cell cycle control and oncogenesis due to its ability to bind cyclin E interacting proteins. Using cell-free replication experiments it was discovered that recombinant Ciz1 could contribute to initiation of replication competent nuclei when supplemented into G1 extract containing recombinant cyclin A/CDK2. Ciz1 nuclear localisation and interactions with cyclins led to investigation as to its role during DNA replication. Ciz1 has been shown to have a role co-ordinating the activity of cyclin A/CDK2 and Cyclin E during the early events preceding replication initiation (Copeland et al., 2010).

Ciz1 has been proposed to be used in the diagnostics of certain types of cancer. A splice-variant version of CIZ1 has been shown to be detectable in small volumes of blood that is associated with lung cancers, the test has been shown to be highly sensitive, non-intrusive and selective. Furthermore, it was demonstrated that reducing the levels of this form of Ciz1 reduced the growth of tumours, solidifying a direct link between Ciz1 and cancer indicating that Ciz1 may act as an oncogene (Higgins et al., 2012)

Intriguingly Ciz1 has not only been shown to have an oncogenic role but it has also been implicated as a tumour suppressor. Nishibe et al. (2013) developed mouse embryonic fibroblasts (MEFs) Ciz1 knockouts that displayed increased sensitivity to hydroxyurea mediated replication stress and developed leukaemia in response to retroviral infection. It is

not unheard of for proteins to act as both tumour suppressor and an oncogene for example p21 can function as both through both inhibiting cell growth and apoptosis (Roninson, 2002).

Ciz1 could act as a kinase sensor, linking together spatial and temporal control of DNA replication. Ciz1 has hypophosphorylated and hyperphosphorylated states. In its hyperphosphorylated state, it is phosphorylated at threonine 144, 192 and 293. When hyperphosphorylated Ciz1 does not interact with cyclin A/CDK2 or promote DNA replication initiation (Copeland et al., 2015). This suggests a link between DNA initiation, cellular kinase levels and Ciz1. The effects of Ciz1 levels and phosphorylation states on mammalian replication should be studied at the DNA replication level to investigate if changes alter the replication programme.

3.1.3 Diadenosine 5 , 5-P1,P4-tetraphosphate (Ap4A)

Diadenosine 5 , 5-P1,P4-tetraphosphate (Ap4A) is a small alarmone molecule with structural similarity to ATP (Pojoga et al., 2004). Ap4A appears to have a role in the cell cycle in a wide range of organisms ranging from prokaryotes to eukaroytes (Bambara et al., 1985; Nishimura et al., 1997). Ap4A is found in cells progressing through the cell cycle, but not in cells that have exited the cell cycle (Rapaport & Zamecnik, 1976). Basal levels of Ap4A are provided as a by-product of transfer RNA (tRNA) synthesis by aminoacyl-tRNA synthetases (Brevet et al., 1989).

Ap4A could have an inhibitory role in replication initiation in response to DNA lesions, Ap4A is synthesised in cells that have been exposed to a variety of types of DNA damage and *in vitro* experiments have indicated that Ap_4A can inhibit replication initiation at cellular levels. (Marriott et al., 2015).

Ap4A metabolism is also implicated in certain cancers. The nudix hydrolase enzyme NUDT2 is responsible for the degradation of cellular Ap4A. Marriott et al. (2016) increased Ap4a levels by disturbing the NUDT2 gene. When compared with wild type cells, a distinct change in gene expression profile was observed, including a reduction in the expression of many cancer promoting genes. Whether this is down to increased Ap4A levels or a pleiotropic effect of NUDT2 is yet to be seen. However, it provides potential evidence that Ap4A is used by cells to control growth in response to DNA damage.

In this Chapter, we will establish *in vitro* replication assays from post quiescent NIH3T3 nuclei and use them to investigate Ap4A's effect on replisome assembly, to further investigate the

role of Ap4A in restraint of the cell cycle at levels produced in cells in response to DNA damage.

3.1.4 Aims

To investigate the role of Ciz1 and Ap4A in the regulation of the initiation phase of DNA replication. We will use a mammalian cell-free DNA replication system. This chapter outlines the principles for preparation of synchronised cell extracts and nuclei to reconstitute DNA replication *in vitro*. Initially, the synchronisation and quality control of reagents will be performed. To ensure the quality of reagents, cell-free DNA replication assays will be performed in S-phase extract to ensure that materials are competent to initiate. This will be followed by reconstitution of initiation by use of recombinant cyclin A-CDK2.

The *in vitro* DNA replication system will then be used to analyse the concentration dependence of cyclin A-CDK2 for initiation of DNA replication, together with Ciz1 that has previously been shown to enhance cyclin A-CDK2 function. Analysis of the role of Ciz1 and how it modulates Cyclin A/CDK2 activity will be assessed during *in vitro* DNA replication studies.

Finally, the role of Ap4A in the inhibition of DNA replication will be determined using cell-free DNA replication assays. We will analyse replication protein complex assembly in reactions including Ap4A to gain greater insight into the mechanism by which Ap4A inhibits initiation of DNA replication.

3.2 Generation of Materials for Cell-free Replication Experiments

3.2.1 Quiescence Release of NIH3T3 Cells

To ensure NIH3T3 cells could be released from quiescence into S phase to a large enough level to be used in cell-free experiments the post-quiescence release technique was tested. Cells were released as they would be in cell-free experiments, grown onto coverslips, then pulse labelled with EdU. The percentage of nuclei in S phase was scored by incorporation of EdU. Figure 3.1 shows a typical result using this type of release.

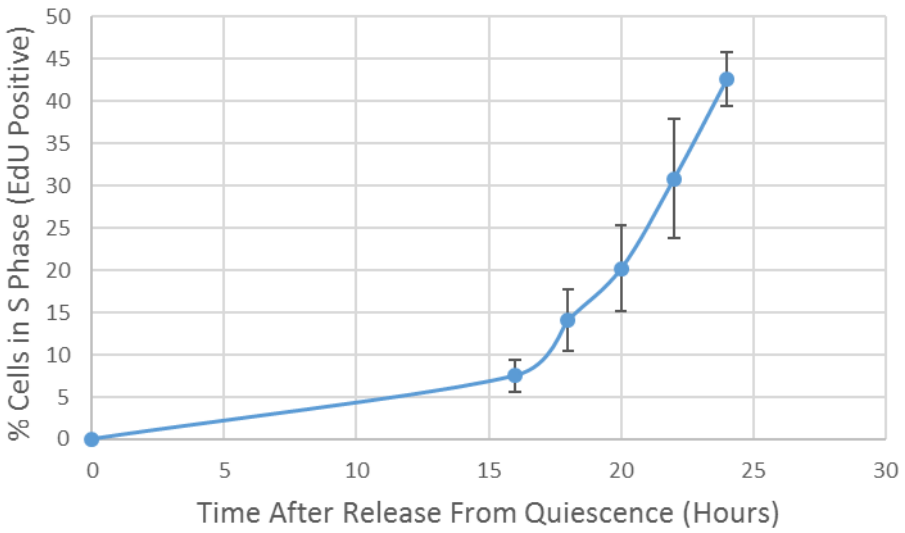


*Figure 3.1-**Quiescence Release of NIH3T3 Cells:** Release of NIH3T3 cells from quiescence into S phase, showing the increase in mean number of cells entering S phase between 16 and 24 hours (n=3) after release from G_0. At each time point >100 nuclei were scored and percentage EdU positive cells are shown.*

Quiescence release experiments showed an increase in the number of cells entering S phase between 16 hours and 24 hours after release from G_0. This showed the degree by which cells could be expected to enter S phase. This demonstrated that the quiescence release technique was effective, meaning it could be used for generation of replication competent nuclei. Having established the cell cycle kinetics for 3T3 cells facilitates determination of the correct time point for harvesting late G1 nuclei. Kinetics may still slightly vary due to temperature changes, changes of serum and changes in time at confluence. Consequently, nuclei used experimentally are first batch tested to ensure the quality of reagents prior to use.

In subsequent experiments, late G1 nuclei are harvested at around 17-17.5 hours where typically contains 10-15% of cells are in S-phase. That is monitored as a control in all experiments to establish the baseline of initiating nuclei, but there is potential to stimulate

this population of nuclei into S-phase by addition of S-phase cell extracts or addition or recombinant cyclin A-CDK2 (Coverley et al., 2002; Krude et al., 1997).

In addition, G1 extracts were produced by harvesting cells at 15-15.5 hours post-release from quiescence. 15 hours is approximately the restriction point of NIH 3T3 cells released in this manner (Coverley et al, 2002) This population of cell has approximately 5% of cells in S-phase providing a cytosolic extract that is low in CDK activity, and does not stimulate high amounts of replication initiation (Copeland et al., 2010). This extract will be used as the negative control extract in subsequent replication assays (Coverley et al., 2002; Rahman et al., 2007; Copeland et al., 2010).

3.2.2 Generation of nuclei and extracts for Cell-free DNA replication assays

Having established that cells can be synchronised (Section 3.2.1) cell cycle specific extracts and replication competent nuclei could be isolated for use in *in vitro* replication experiments. Three components needed to be isolated from cells: Replication competent nuclei, harvested approximately 17 hours after release; mid G_1 cell extract (15hrs post release) (Figure 3.2) and S phase cell extract produced in HeLa cells (section 2.7.3 from materials and methods). Originally three batches of nuclei were isolated from NIH3T3 cells, each batch was generated from synchronising five plates of NIH3T3 cells and releasing them into 20 plates. Nuclei were then isolated 17.5 hours after release from G_0.

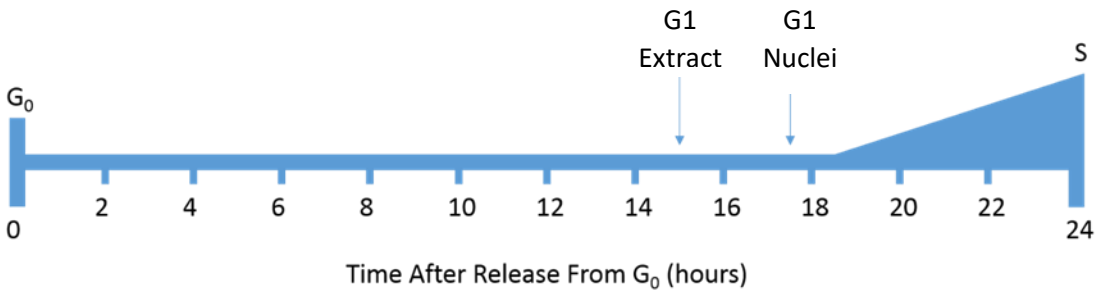
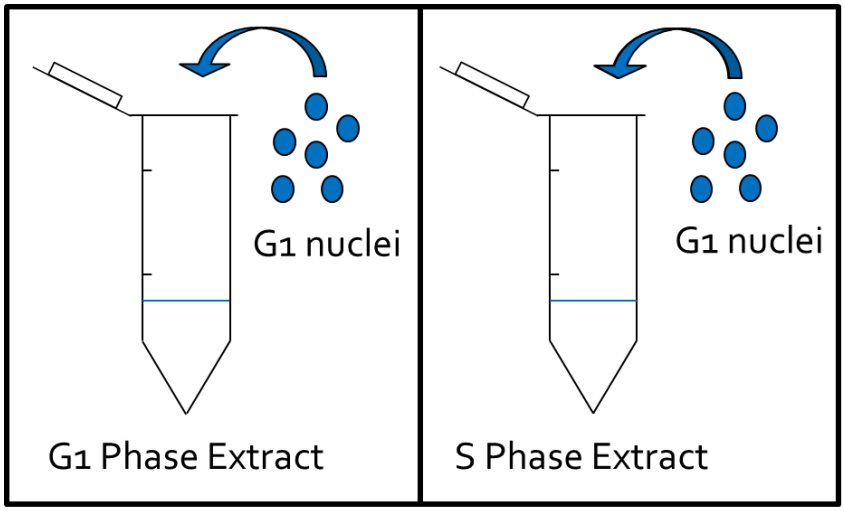
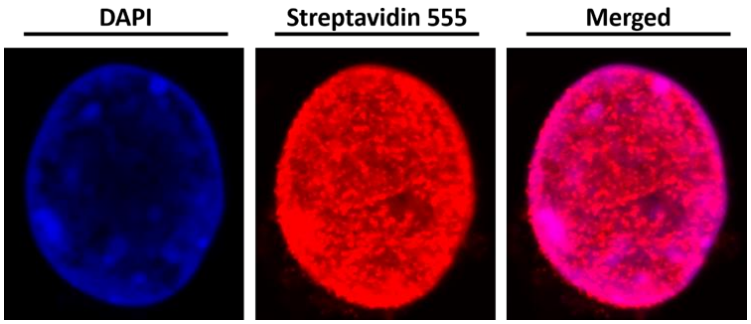


*Figure 3.2-**Timepoints for Harvesting Cell Cycle Specific Extracts and Nuclei:** Schematic displaying the times at which cell-free components were harvested from NIH3T3 cells after release from G0, G1 nuclei were harvested around 17.5 hours (N) and G1 extracts at around 15 hours (R).*

A



B



C

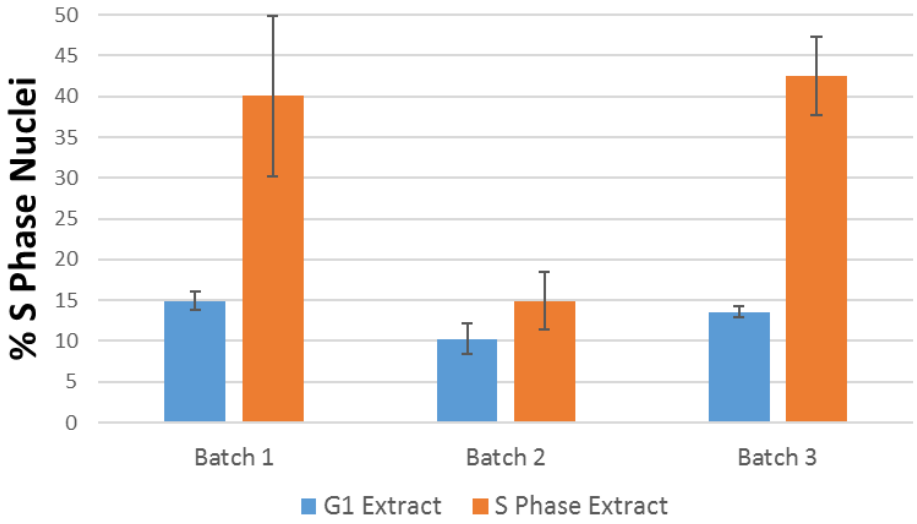


Figure 3.3 – ***Testing of synchronised populations of nuclei for replication competence*** *A) Schematic for reaction mixtures in in vitro replication experiments used to test the replication competence of batches of NIH3T3 nuclei harvested 17.5 hours after release from quiescence. B) A confocal image of a positively biotin 16 dUTP labelled S phase nucleus C) Testing of three batches of nuclei harvested from NIH3T3 cells 17.5 hours after release from G_0. Nuclei were incubated with G1 and S phase extracts in in vitro reactions and the proportion of replicating nuclei in S phase was counted. Data shows mean ± Standard Deviation, n=3*

Each batch of nuclei was tested against G1 and S phase extracts that had previously been shown to initiate (S phase extract) and not initiate (G_1 extract) DNA replication in replication competent nuclei from NIH3T3 Cells. Simply, cell-free reactions were set up where nuclei were added to G_1 extracts and S phase extracts (Figure 3.3a), and S-phase nuclei determined by incorporation of biotin-16-dUTP into nascent DNA during DNA replication. The percentage of nuclei with labelled DNA replication foci were scored to determine which nuclei batches were replication competent. (Figure 3.3c)

Figure 3.3b shows the results from the testing of replication competent nuclei. Batches 1 and 3 were shown to respond as expected to G_1 and S phase extracts, the amount of positively labelled nuclei increased by a factor of 2.6 and 3.1 between reactions in G_1 and S phase extract respectively, with a low number of nuclei replicating in G_1 extract. Batch 2 showed effectively no increase in the proportion of replicating nuclei in response to S phase extract compared to G_1 extract. This batch was not used in further experiments, any nuclei used in future experiments were tested in this manner, only nuclei that increased replication by at least a factor of 2 were used. These experiments confirmed that replication competent nuclei had been generated, now replication competent cell cycle specific extracts needed to be generated and tested.

Occasionally, like in nuclei batch 2 (Figure 3.3c), nuclei did not initiate in response to a HeLa S phase extract. This could be due to differences during the synchronisation process. This could be from differences in the degree of confluence during the initial media change, differences in the length of time at confluence, quickness of the nuclei harvesting process or simple differences between batches of cells. Hence, all nuclei were quality controlled before use experimentally. Titration experiments below were performed using multiple batches of nuclei, using percentage initiation (2.7.4) to compare results across nuclei batches.

3.3 Cyclin A/CDK2, Ciz1-N471 and Replication Initiation

The initiation phase of eukaryotic DNA replication requires the activity of multiple kinases including DDK and CDK2 (Yeeles et al., 2015). In mouse fibroblasts, the consensus theory is that both cyclin E/CDK2 activity and cyclin A/CDK2 activity are redundant in the activation of DNA replication in cycling fibroblasts (Geng et al., 2003; Geng et al., 2007; Kalaszczynska et al., 2009). However, in nuclei isolated during G_1 phase from post- quiescent cells, cyclin E/CDK2 does not promote initiation of DNA replication (Copeland et al., 2010; Coverley et al., 2002). Interestingly, the biochemical reconstitution of DNA replication initiation in *S. cerevisiae* proteins also use human cyclin A/CDK2 to initiate DNA replication (Yeeles et al., 2015).

3.3.1 Cyclin A/CDK2 Initiates DNA Replication in vitro

Cyclin A/CDK2 is the cyclin/CDK complex primarily used for the regulation of S phase, cyclin A/CDK2 can also initiate DNA replication (Coverley et al., 2002). To investigate the role of cyclin A/CDK2 replication initiation *in vitro* replication assays were used. *In vitro* replication reactions were set up as shown in Figure 3.4. Three types of reaction were prepared, nuclei were added to a G1 extract, an S phase extract and further G1 reactions were prepared where G1 extract was supplemented with three concentrations of cyclin A CDK2: 0.01 ng/µl, 0.1 ng/µl and 1 ng/µl. This was to investigate the extent to which different concentrations of recombinant cyclin A CDK2 could affect replication initiation. Figure 3.4b shows the proportion of nuclei that entered S phase upon addition of varying concentrations of cyclin A/CDK2 reaction.

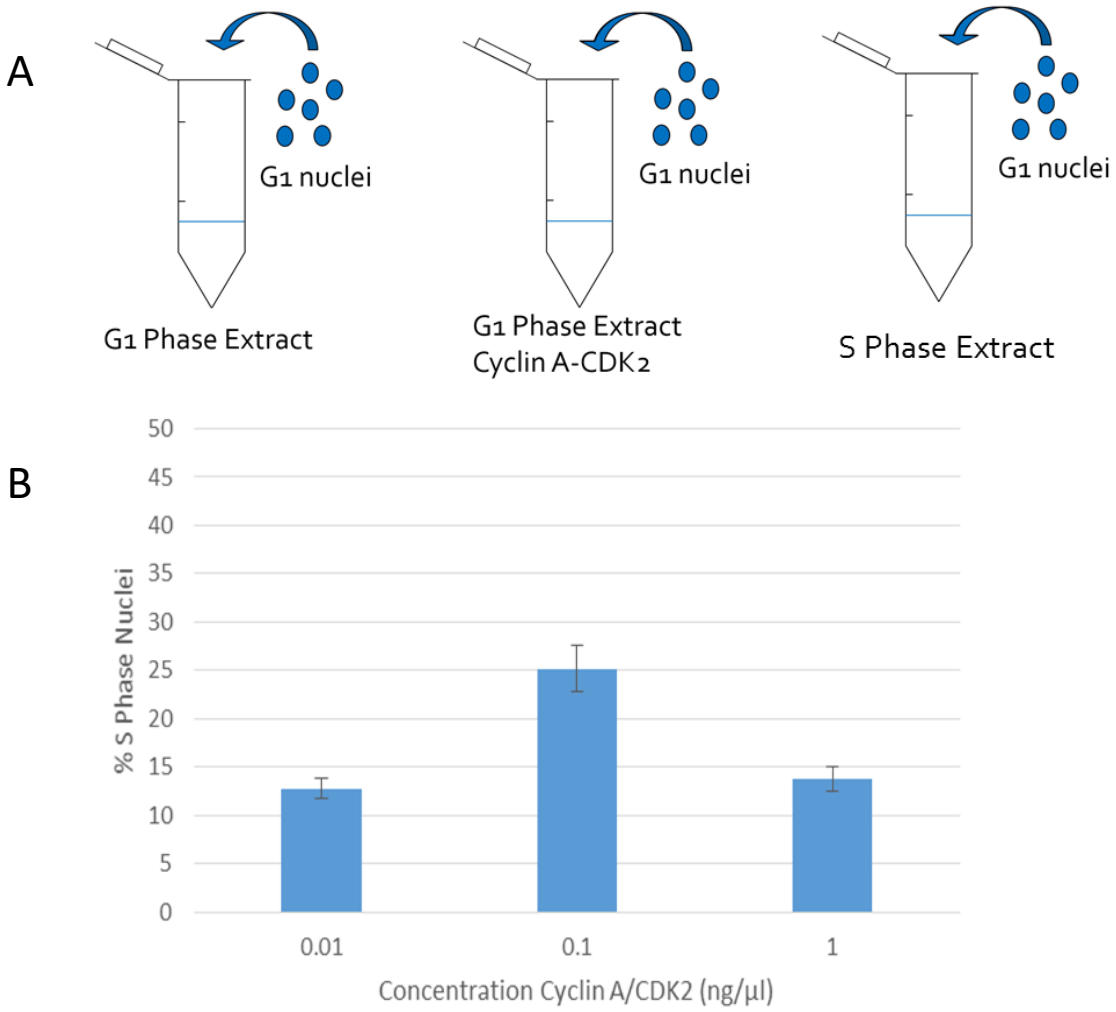


Figure 3.4- ***Cyclin A/CDK2 Promotes Replication Initiation*** *A) A schematic for the cell-free reactions performed in this section B) The effects of a varying concentration of cyclin A/CDK2 on the mean (N=3) initiation of replication competent nuclei ± standard deviation.*

These results demonstrate that G_1 extract supplemented with 0.1 ng/µl cyclin A/CDK2 can cause a small increase in the number of replication competent nuclei. Hela S phase extract stimulates a mean initiation of 46.3% of the nuclei used in these experiments. Whereas, G1 extract stimulated a mean initiation of 13.7% of the nuclei used in these experiments. Cyclin A/CDK2 can promote DNA replication initiation, but not to the extent of a HeLa S phase extract. Addition of 0.1 ng/µl Cyclin A/CDK2 yielded a 2-fold increase in S phase population. To investigate further the activity of cyclin A/CDK2 a range of concentrations of cyclin A/CDK2 around 0.1 ng/µl was used (Figure 3.5).

Reactions were set up as before (Figure 3.4) titrating recombinant cyclin A/CDK2 as before using an increased number of cyclin A/CDK2 concentration in the range between 0.01 and 1 ng/µl. These reactions showed that the optimal replication initiating activity of cyclin A/CDK2 in G_1 extract peaked tightly at 0.1 ng/µl with some increased initiation between 0.08 and 0.12

ng/µl. Resuming to slightly above base levels of replication at concentrations either side of these concentration. This reveals that there is a biphasic concentration dependent activation and inhibition of DNA replication. This creates a narrow window of CDK2 activity to promote initiation. This inhibitory effect of CDK2 has been associated with the dissociation of replication proteins (Chen & Bell, 2011).

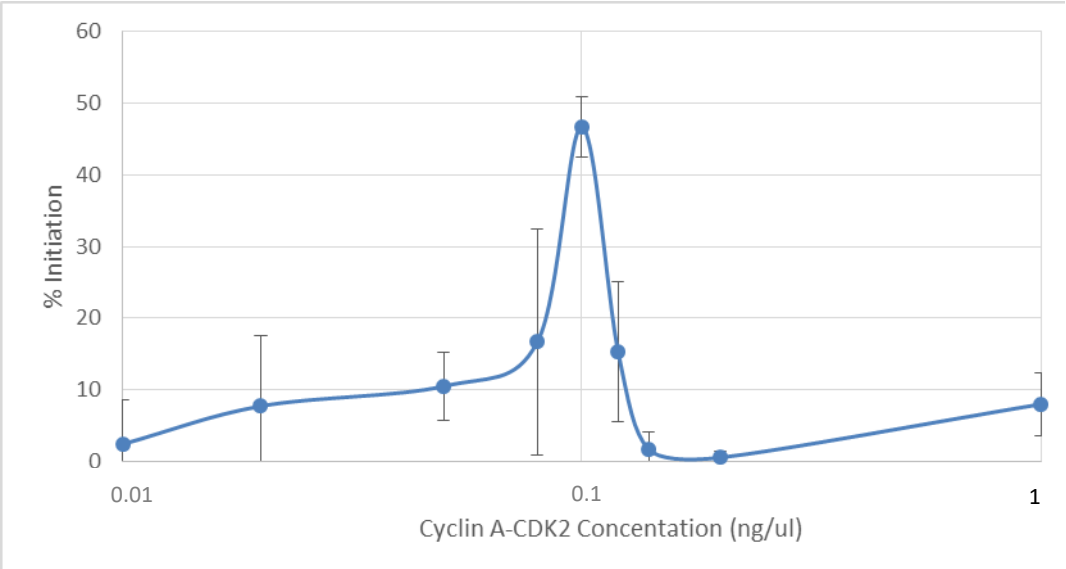


*Figure 3.5-**Cyclin A/CDK2 Replication Initiation Titration**: The effects of various levels of cyclin A/CDK2 supplemented G1 extracts ability to initiate DNA replication in a mammalian in vitro replication assay. Data has been normalised using G1 extract and S phase extract initiating values. Data is presented as the percentage of nuclei that were initiated out of the total that can be initiated. Data shows mean ± Standard Deviation, n=3*

This indicated that cyclin A/CDK2 only initiates replication at a specific concentration that defines the activating threshold for CDK activity to promote initiation of DNA replication. Furthermore it also identifies the inhibitory concentration of CDK activity to inhibit initiation of DNA replication. These data are consistent with the quantitative model of CDK activity (Section 1.6). Low and high kinase concentration inhibit DNA replication initiation to ensure DNA only replicates at permissible kinase levels during S phase.

3.3.2 Purification of Ciz1-N471

This study uses the DNA replication active Ciz1 N471 construct that retains full activity in DNA replication assays but which lacks the C-terminal anchor domain, termed Ciz1 N-471. This Ciz1 truncation can interact with cyclin E, cyclin A and the replication licensing protein Cdc6 (Copeland et al., 2010; Copeland et al., 2015). In order to investigate the effects of addition of recombinant Ciz1-N471 on the initiation process, GST-Ciz1-N471 was purified by immobilisation on glutathione sepharose followed by cleavage with 3C protease to release recombinant Ciz1-N471 (Figure 3.6).

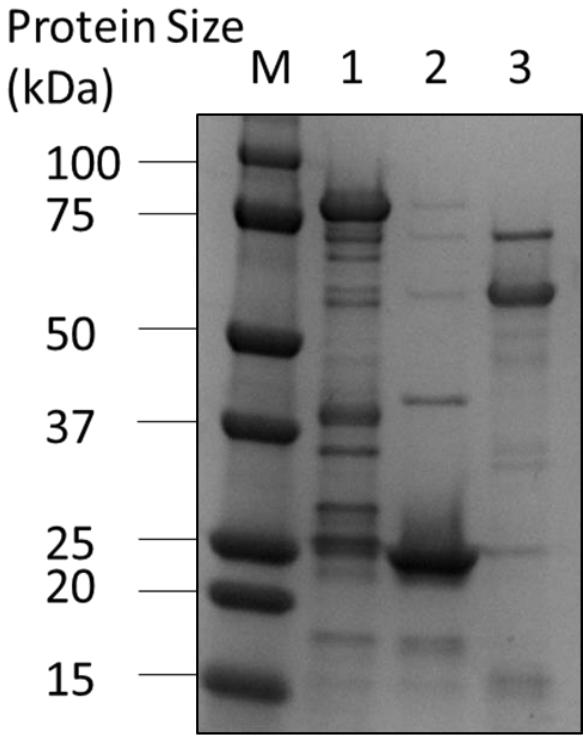


*Figure 3.6 -**Purification of Ciz1-N471 Using a GST Tag:** Lane 1 shows the purified recombinant protein prior to cleavage of the GST tag. Lane 2 shows the GST tag after cleavage by 3C protease. Lane 3 shows the eluted protein after proteolytic clevage and separation from glutathione sepharose beads.*

GST-Ciz1-N471 was successfully expressed and bound to glutathione sepharose. Lane 1 shows GST-Ciz1-N471 with an approximate molecular weight of approximately 75kDa. This protein was digested overnight with 3C prescission protease that released soluble Ciz1-N471, and residual GST was retained on the glutathione sepharose beads. Soluble Ciz1-N471 protein (lane 3) was found to contain a band at approximately 70 kDa that was previously identified as Hsp70 by mass spectrometry (Copeland, unpublished observation). The presence of this protein may be related to the predicted native disorder of N-terminus Ciz1. This is a common feature of Ciz1 expression in *E. coli* but does not affect Ciz1 function in *in vitro* assays (Coverley et al., 2005; Copeland 2010; Copeland 2015).

3.3.3 Ciz1-N471 Co-operates with Cyclin A/CDK2 During Replication Initiation

To investigate the effect of recombinant Ciz1-N471 on recombinant cyclin A/CDK2 initiation activity more cell-free reactions were prepared. Ciz1 promotes the activity of cyclin A/CDK2 in *in vitro* DNA replication assays by increasing localisation of cyclin A/CDK2 to chromatin (Copeland et al., 2010). The preliminary investigations were initiated to test the effect of Ciz1 on cyclin A/CDK2 activity *in vitro* (Figure 3.7). As seen previously, addition of Ciz1 to optimal concentrations of cyclin A/CDK2 (determined in Section 3.3.1) increased the proportion of nuclei that initiated consistent with its co-operative effect (Figure 3.7). Ciz1-N471 was titrated at increasing concentrations between 0.02 nM and 10 nM into G_1 cell-free reactions supplemented with 0.1 ng/µl cyclin A/CDK2 (Figure 3.8). Figure 3.7 illustrates the co-operation between Ciz1-N471 and cyclin A/CDK2 during replication initiation. In reactions where Ciz1 was added to G_1 extract with no cyclin A/CDK2, no increase in replicating nuclei was observed.

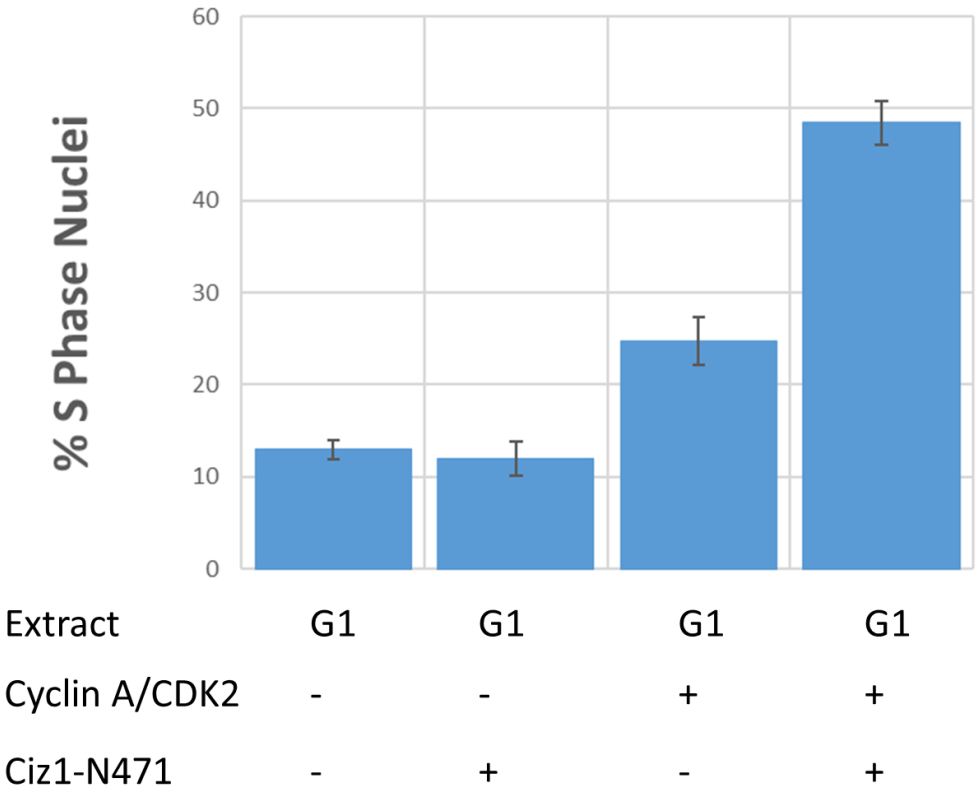


Figure 3.7- ***Ciz1-N471 Co-operates with Cyclin A/CDK2****: The proportion of nuclei that initiated DNA replication in in vitro replication assays when reacted in G1 extracts supplemented with 0.1 ng/µl cyclin A/CDK2 and 0.1 nM Ciz1-N471 as indicated. S phase extract induced a mean initiation of 47.2% in the nuclei used in these experiments. Data is mean ± Standard Deviation. n=3*

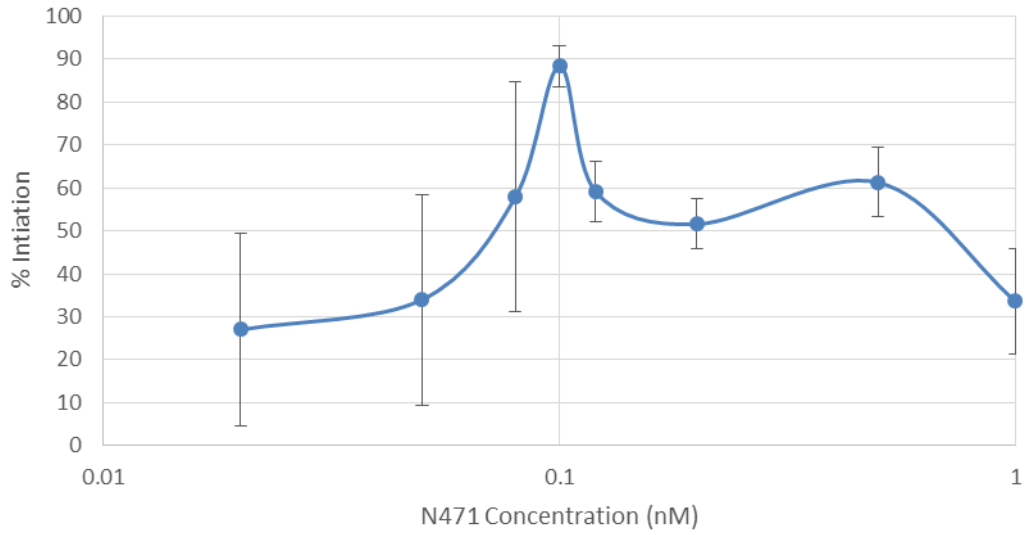


Figure 3.8- ***Ciz1-N471 Activity is Concentration Dependent.*** *The effects of various levels of Ciz1-N471 on G1 extract supplemented with 0.1 ng/µl cyclin A/CDKs ability to initiate DNA replication in a mammalian in vitro replication assay Data has been normalised using G1 extract and S phase extract initiating values. Data is presented as the percentage of nuclei that were initiated out of the total that can be initiated. N=3 ± Standard Deviation*

These results show that Ciz1-N471 is not sufficient to initiate replication alone, but at 0.1 nM Ciz1-N471 promotes cyclin A/CDK2 replication initiation, increasing the replicative capability to similar levels as an S phase extract. This is shown by an average initiation of 89% when both Ciz1-N471 and cyclin a/CDK2 are present at optimal levels (Figure 3.8). Ciz1 contributes to promoting the activity of cyclin A/CDK2 with respect to initiation of DNA replication. This was previously related to the ability of Ciz1 to localise cyclin A/CDk2 to chromatin (Copeland et al., 2010) or potentially by localising to sites proximal to putative origins of replication (Copeland 2015).

3.3.4 Ciz1-N471 Broadens the Concentrations at which Cyclin A/CDK2 Promotes Replication initiation

To investigate whether Ciz1 influenced the effect of cyclin A/CDK2 activity on initiation of replication we have used G_1 nuclei and G_1 extract supplemented with 0.1 nM Ciz1-N471. The activity of cyclin A/CDK2 was assessed between 0.08 ng/µl and 1 ng/µl when co incubated with optimal concentration of Ciz1-N471. Figure 3.9 shows the results of this on the proportion of replicating nuclei with and without Ciz1-N471.

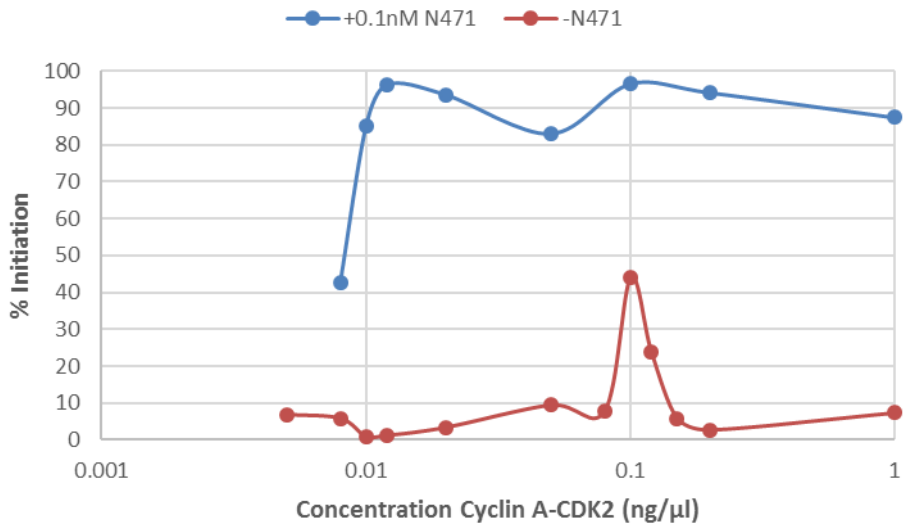


*Figure 3.9- **Ciz1-N471 Expands the Range of Concentrations Cyclin A/CDK2 Initiated Replication**: The effect of G1 extracts supplemented with a range of concentrations of cyclin A/CDK2 on replication initiation with and without 0.1nM Ciz1-N471. Data has been normalised using G1 extract and S phase extract initiating values. Data is presented as the percentage of nuclei that were initiated out of the total that can be initiated. Data displayed as mean (N=2).*

Ciz1-N471 not only seems to contribute to activation of DNA replication through the addition of cyclin A/CDK2 but also appears to broaden the range of concentrations at which cyclin A/CDK2 can contribute to the initiation of DNA replication over a 10-fold range either side. This agonistic and antagonistic interaction with cyclin A/CDK2 activity may play an important role in Ciz1s role in cell cycle control as well as its role as an oncogene. This may be important for its function observed in a number of cancers, as these perturbations may cause untimely S phase entry, which as discussed earlier may induce replicative stress (Pauzaite, et al., 2016).

3.4 Ap4As Role in Replication Initiation

3.4.1 Ap4A inhibits initiation of DNA replication in S Phase cytosolic Extracts

Ap4A is induced in response to interstrand crosslinking agents that are commonly used in cancer therapy. Cells that are exposed to sub-lethal concentrations of mitomycin C induce Ap4A production, with intracellular concentrations reaching up to 20 µM. It has previously been demonstrated that 25 µM Ap_4A had an inhibitory effect on replication initiation in a cell-free system (Marriott et al., 2015). Here, the effect of addition of Ap4A on DNA replication complex assembly will be determined in cell-free DNA replication assays. First the effect of Ap4A was tested using a G1 extract, and an S phase extract in the presence and absence of 25µM Ap4A (Figure 3.10).

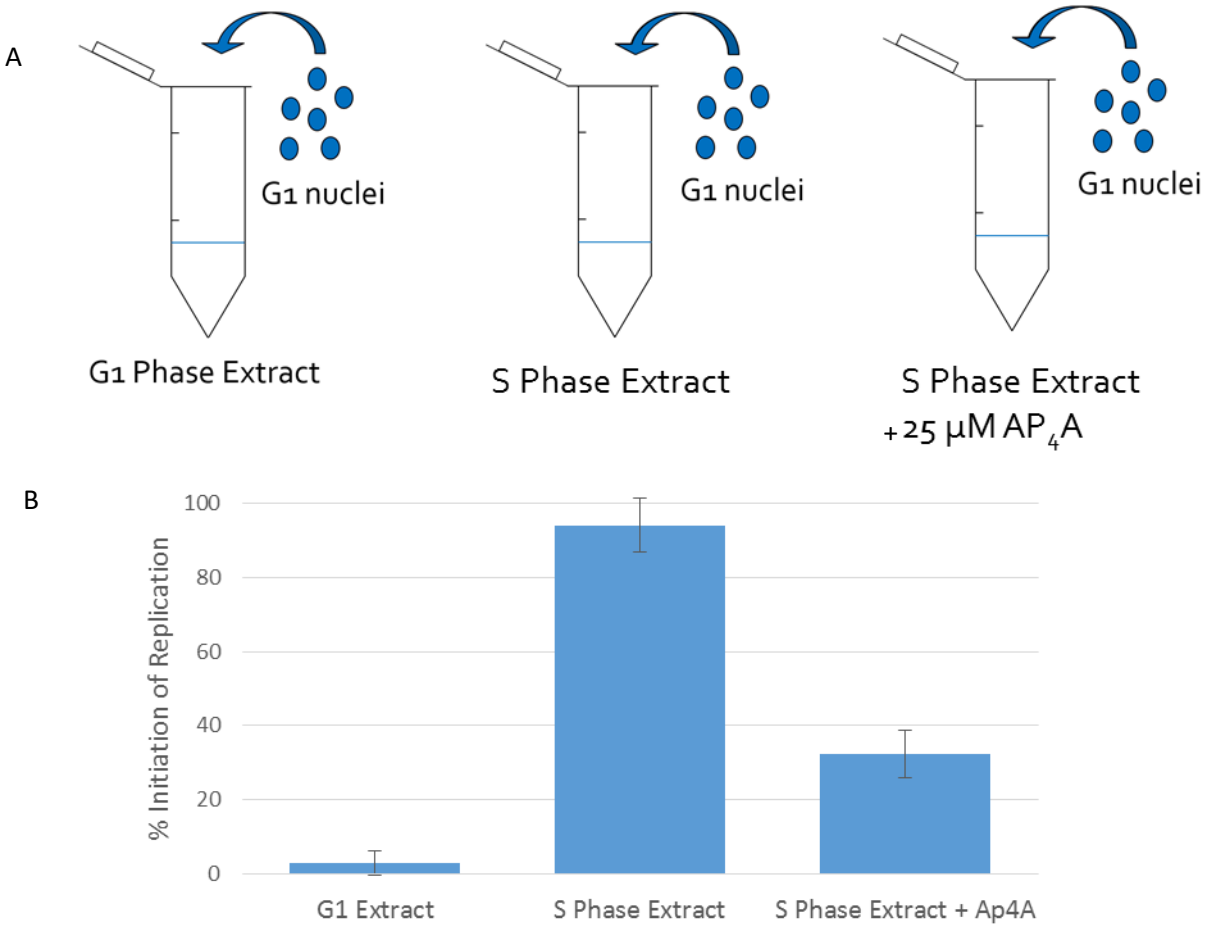


Figure 3.10- ***Ap4A Inhibits DNA Replication in vitro:*** *A) Schematic of in vitro replication reactions. B) Proportion of initiated nuclei for three reactions: G_1 extracts and nuclei (left) provided the baseline, G_1 nuclei and S phase extract (middle), G_1 nuclei and S phase extract with 24 µM Ap4A. data shows mean ± standard deviation, n=3..*

Replication initiation was reduced by approximately 65% by addition of Ap4A (Figure 3.10). To further investigate the mechanism by which Ap4A prevents the replication initiation of replication competent G_1 nuclei by a HeLa S phase extract the effects on loading of replication licensing and replication factors onto DNA should be investigated.

3.4.2 Ap4A Prevents S Phase Extract Prevents Loading of Replication Complex Proteins

To better understand the molecular mechanism by which Ap4A inhibits initiation, western blotting was used to visualise the chromatin bound fraction in parallel cell free DNA replication assays (Figure 3.11). To investigate the effect of Ap4A on DNA replication, the pre-RC CDC6 and MCM2 were analysed. This was supported by analysis of the loading of the clamp PCNA that is a marker of replisome formation and active DNA replication activity. Reactions were performed as in Figure 3.10, the DNA bound protein fraction of the *in vitro* reactions was isolated and probed for replication factors by Western Blot. Figure 3.11 shows the effects of 25 µM Ap4A on binding of replication proteins to DNA.

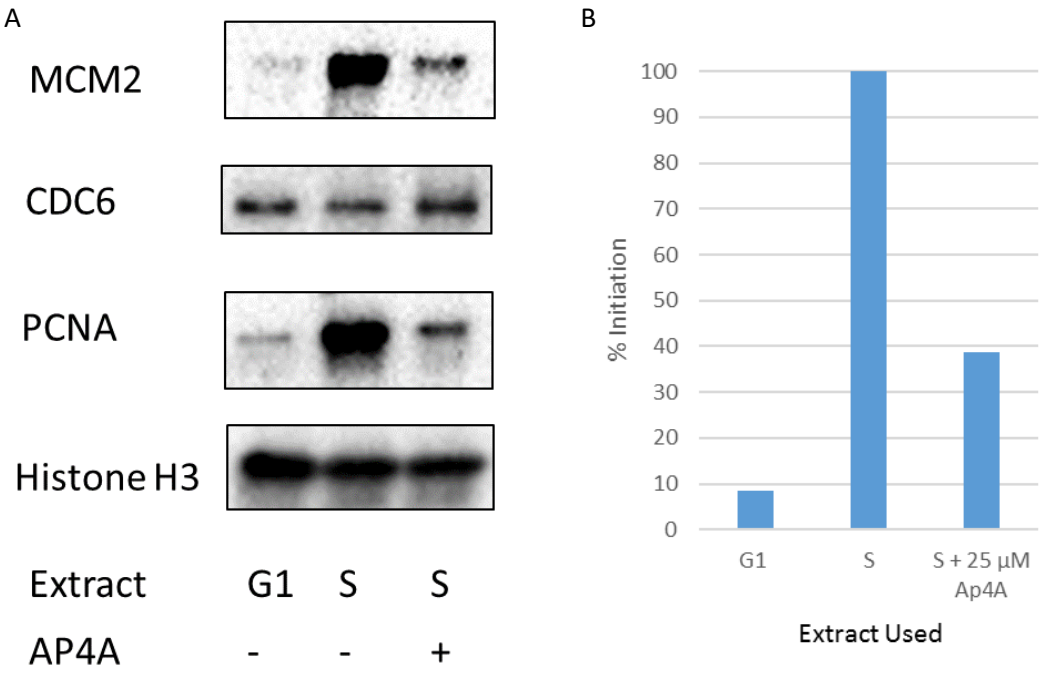


Figure 3.11- ***The Effects of Ap4A on Loading of Replication Protein Assembly:*** *In vitro replication reactions were prepared as in Figure 3.10a. A) The chromatin fraction was isolated Western blotted and probed for replication factors. B) The percentage initiation of the nuclei used in A.*

Data presented in Figure 3.11 is representative of multiple datasets and initiation factors are consistent with data from Figure 3.10. These results show that loading of early replication

factor CDC6 seemed unaffected by Ap4A. MCM2 is loaded onto DNA later during the replication licensing process (Section 1.3), its binding to DNA is impaired when Ap4A is included in an S phase extract in an *in vitro* replication assay. MCM2 binding is reduced but not to the level in reactions in a G_1 extract. PCNA levels were similarly affected by Ap4A as MCM2, levels were reduced but not to the levels of a G_1 extract reaction. PCNA associates with chromatin during active DNA replication and its levels correlate with the number of active replication foci (Chagin et al., 2016). Both reduced MCM2 and PCNA binding correlate with the low numbers of replicating nuclei in control reactions and reactions containing Ap4A. These data suggest that Ap4A replication initiation inhibition appears to be partially mediated by inhibition of licensing that usually occurs when an S phase extract is added to replication competent nuclei preventing loading of CMG preventing activation of the DNA helicase MCM2-7 and recruitment of the replicative DNA polymerases. Further analysis of this process is required to fully identify the mechanism by which Ap4A blocks initiation of DNA replication. However, these data strongly support the data presented by Marriott et al., (2015) demonstrating that Ap4A blocks the initiation phase of DNA replication.

These results have shown that mammalian *in vitro* replication assays can be a useful tool for investigating replication. The effects of replication proteins on replication initiation can be seen at fine detail as well allowing analysis of binding of DNA replication factors important during the G1/S transition.

3.5 Chapter Discussion

Here *in vitro* replication experiments have been established as a technique for measuring replication parameters. Notably: replication initiation and replisome assembly. 0.1 nM Ciz1-N471 has been shown to broaden and amplify the replication initiation activity of cyclin A/CDK2 (Figure 3.9) marking it as an interesting candidate to study in regards to CDK activity deregulation. This capacity to modify nuclei's response to cyclin A/CDK2 may link Ciz1 to the quantitative model of CDK activity (Section 1.6).

Modified expression of Ciz1 is a common feature in a number of cancers including lung, prostate and colon cancer (Higgins et al., 2012; Liu et al., 2015; Wang et al., 2014; Pauzaite et al., 2016). However, precisely why cancer cell lines require high Ciz1 levels for tumourigenesis is poorly understood. Genetic depletion of a variant of Ciz1 in mice xenograft models restrains the growth of tumours (Higgins et al., 2012), indicating Ciz1 has some role in either directly promoting tumour growth or facilitating the conditions that allow tumours to survive. These data suggests that increased Ciz1 levels may increase the permissive range of CDK2 activity for activation of DNA replication initiation. Perhaps, cancer cells require higher Ciz1 levels to replicate in the dysregulated CDK environment found in many replication stressed or cancerous cells (Section 1.7). A proposed model for this is displayed in Figure 3.12 initiation outside the normally permissive zone mediated through Ciz1 may help certain cancerous cells continue replicating in a modified kinase background, this may also contribute to a replicative stress environment which could further tumour developments.

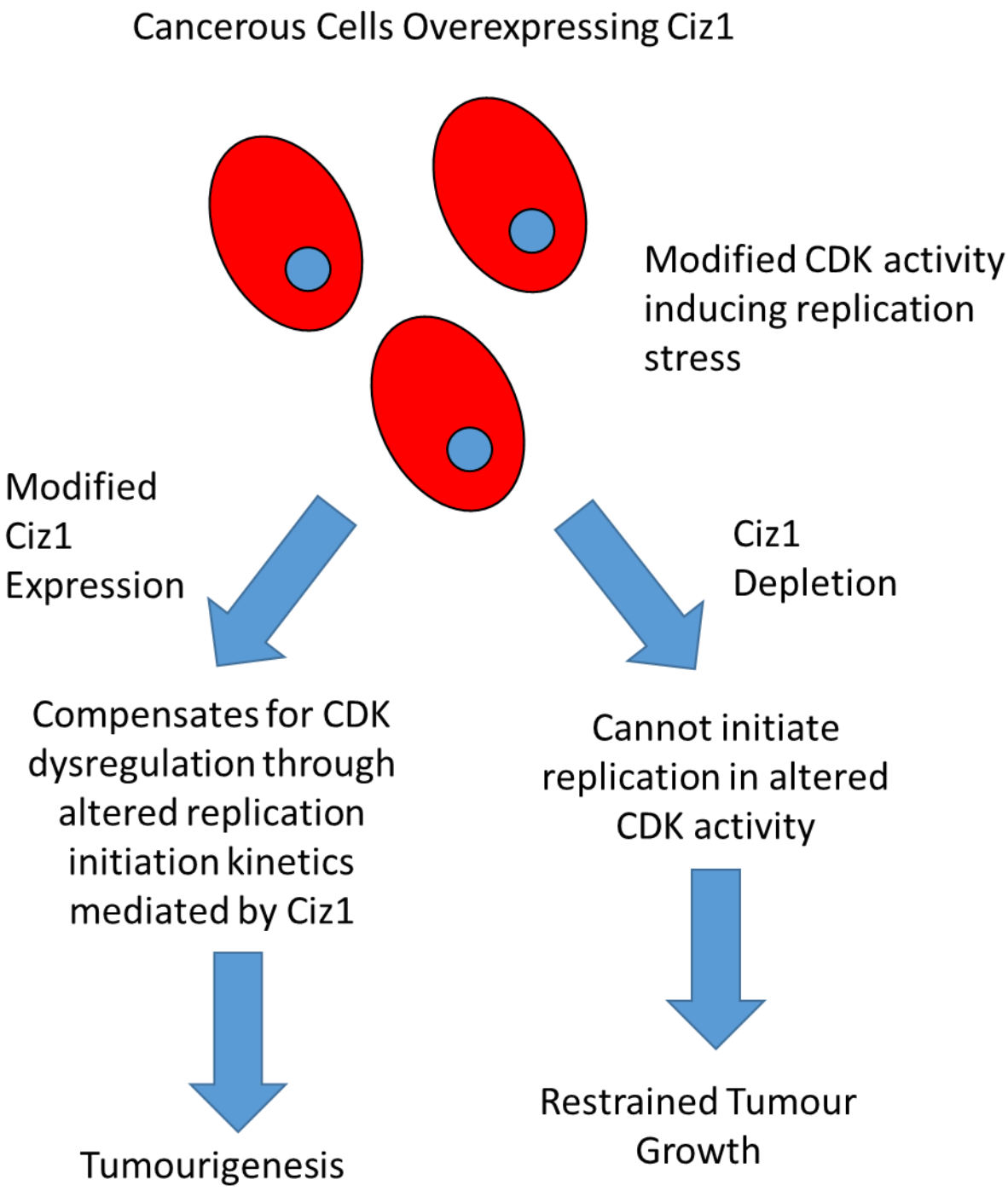


*Figure 3.12-**A Proposed Model for How Ciz1 Could Promote Tumourigenesis:** A model that could explain how Ciz1 is required for tumour development. In this model, Ciz1 allows cancer cells to replicate despite the modified kinase background, resulting in further growth in a replication stress environment.*

Data presented here (Figure 3.11) demonstrated that Ap4A inhibits the initiation phase of DNA replication and corroborate previous results (Marriott et al., 2015). Here for the first time Ap4A has been shown to reduce the amount DNA binding of replication factors PCNA and MCM2 to DNA when supplemented in an S phase extract. Ap4A does not affect CDC6 loading but it reduces Mcm2 and PCNA loading (Figure 3.11). This could indicate that Ap4A does not affect the replication licensing stage of DNA replication, but rather inhibits the loading of the CMG, preventing DNA unwinding by MCM2-7, loading of the replicative polymerases and DNA initiation. In future, a detailed molecular analysis of the effect of Ap4A on replication fork progression could be measured by DNA combing. This approach could investigate active replication on single molecules of DNA could provide further insight into how Ap4A affects DNA replication. Ap4A has long been proposed to regulate initiation of DNA replication, perhaps through the inhibition of DNA pol α (Baxi et al., 1994). The mechanism by which Ap4A restrains loading of MCM2 and PCNA is yet to be fully understood but will be vital in understanding its role in cell cycle restraint in response to DNA damage. Nevertheless, Ap4A has been shown to prevent initiation of DNA replication by blocking replication complex loading on chromatin.

Chapter 4: Investigating Mammalian DNA Replication by DNA Combing

4.1 Introduction

4.1.1 DNA Combing

Visualisation of DNA replication at high resolution is possible with DNA combing. This technique enables visualisation of DNA replication to quantitatively determine fork rate, origin usage, inter-origin distance (IOD), and fork asymmetry. This is achieved through incorporation of modified nucleotides within nascent DNA fibres through pulse labelling of cycling cells. Naked DNA is uniformly stretched onto a hydrophobic surface (silanized coverslip) through the action of a receding air-water meniscus. DNA can then be fluorescently labelled to determine kinetic parameters. This powerful technique can be used to study a variety of factors than can measure origin firing and replication kinetics at a high resolution, importantly this technique can be used to quantify replicative stress through measurements in changes in fork rate and increased fork stalling. Figure 4.1 illustrates how DNA fibres are uniformly stretched onto the surface of a coverslip, adapted from Labit et al. (2008).

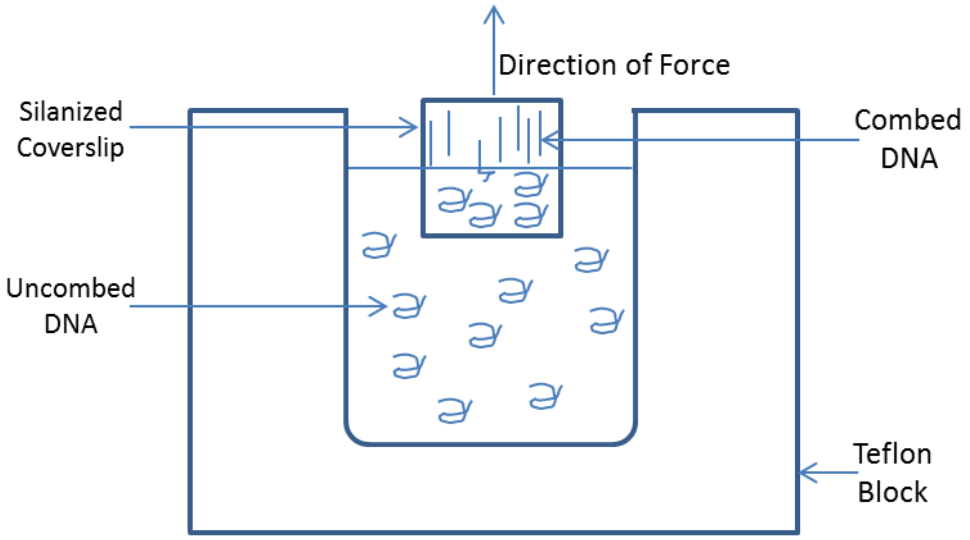


*Figure 4.1- **Molecular DNA Combing:** A pH buffered solution of DNA is prepared, DNA is bound to a hydrophobic silanized coverslip which is then removed at a constant speed. The force applied by the receding air/water meniscus uniformly stretches DNA fibres onto the surface of the coverslip. (Labit et al., 2007)*

This technique was first developed by Bensimon et al. (1994) and greatly increased the reliability, reproducibility and the scope of fibre analysis by allowing longer molecules to be stretched to a constant 2 kbp:1µm ratio. Due to the inability to perfectly synchronise mammalian cells into S phase two different modified nucleotides must be used to investigate origin firing. Two commonly used nucleotides are 5-Iodo-2-deoxyuridine (IdU) and 5-Chloro-2-deoxyuridine (CldU) which can be fluorescently labelled after on slide denaturation of DNA to expose epitopes. An antibody sandwiching approach can then be used to intensify labelling, aiding visualisation. (Bianco et al.,2012)

Replication tracks that are duel labelled with both nucleotides can be considered progressing replication forks. Replication origins that fire shortly before or during the first pulse labelling can also be visualised. Figure 4.2 shows how origins appear depending on when they fire.

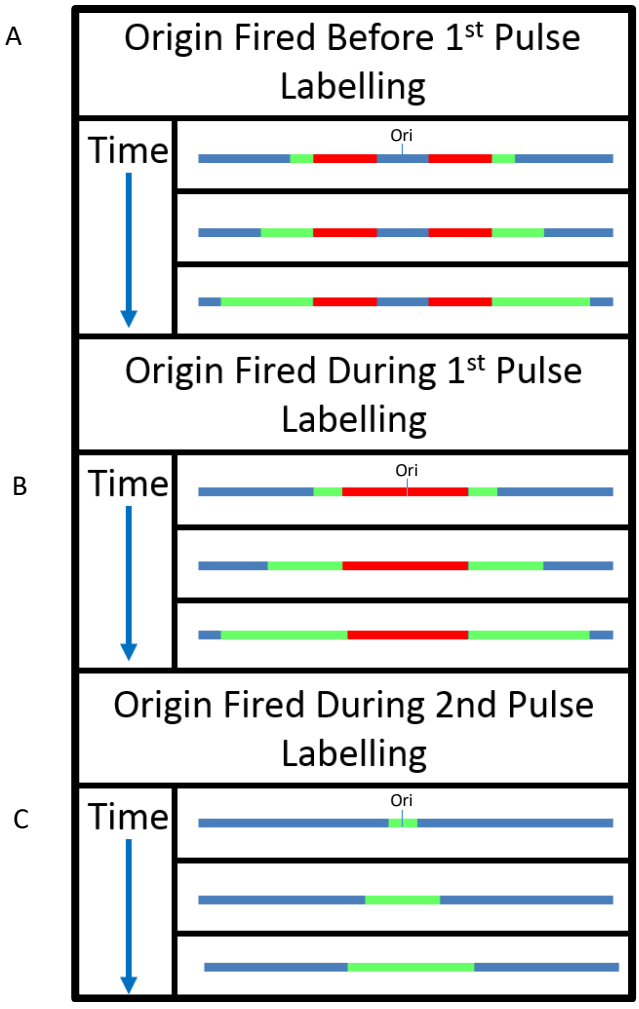


*Figure 4.2-**Molecular DNA Combing Fibre Schematic.** How origins appear depending on the time in which they fire in respect to labelling. A) Origin fires shortly before labelling, producing symmetrical duel labelled replication tracks separated by an unlabelled region. B) Origin fires during the first labelling period producing a single labelled region flanked by the other. C) Origin fires during the second labelling producing only a single labelled replication track.*

DNA combing can be used to determine kinetic rates of DNA replication. Replication fork velocity can be measured, using the length of the second nucleotide in replication tracks that are duel labelled. If tracks are only labelled with one nucleotide, it cannot be certain that replication forks were moving at the beginning of the labelling period. The length of this track, known pulse labelling period and uniform extension of combed DNA can be used to quantify replication fork velocity. Commonly in cells with activated oncogenes replication fork progression is slowed, for example cyclin E overexpression and Ras overexpression both induce replication stress and replication fork progression is slowed. (Jones et al., 2013; Maya-Mendoza et al., 2015)

Another alteration in the DNA replication programme that occurs when cells are under replication stress is replication fork stalling, this will cause activation of the DDR due to building up of RPA coated ssDNA (Ciccia & Elledge, 2010). Replication fork stalling rate can be measured using DNA combing, by measuring changes in replication track length from replication forks originating from the same origin. Briefly; lengths of replication tracks progressing bi-directionally from an origin can be measured, if replication forks stall frequently the sister replication tracks will be different lengths. This leads to an asymmetric extension from a single replication origin (Figure 4.3). The higher the frequency of replication fork stalling, the higher the correlation between sister replication track lengths. Fork asymmetry has been used to measure replication stress by increased fork stalling rates in Topoisomerase 1 and MCM3 deficient cells (Alvarez et al., 2015; Tuduri et al., 2009;).

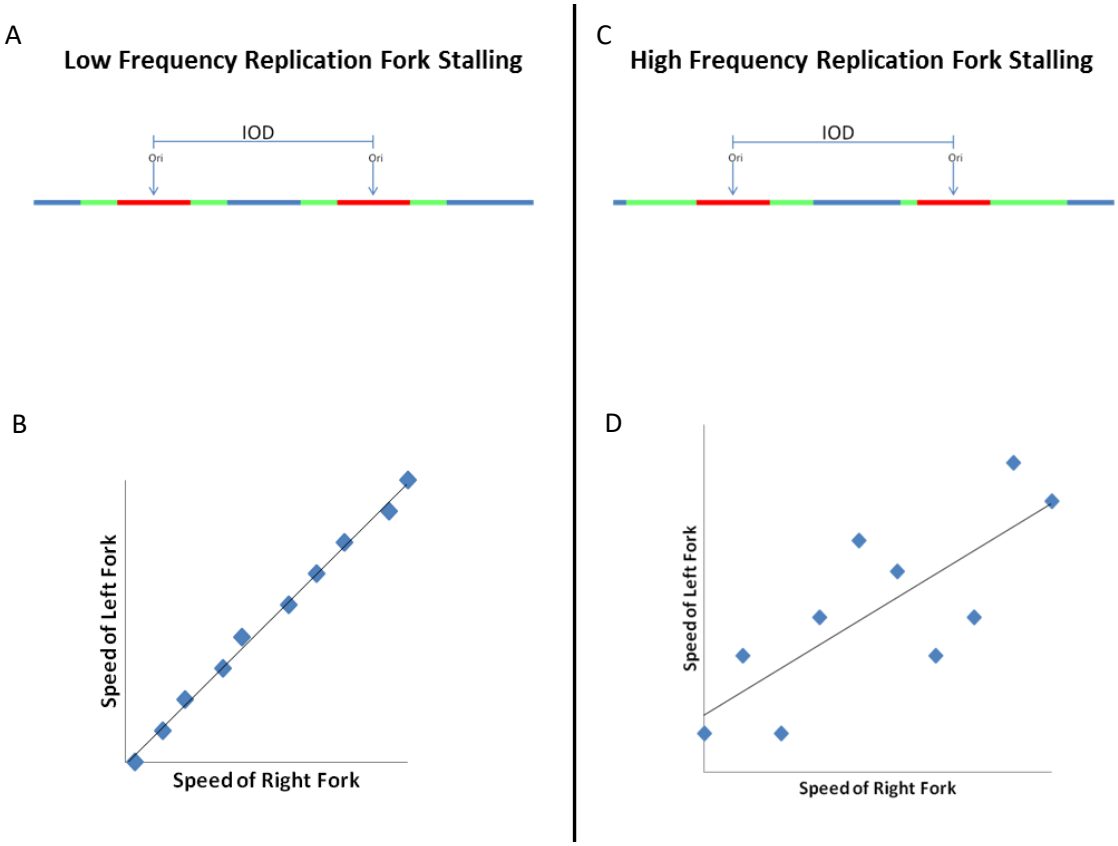


*Figure 4.3-**Using Replication Fork Asymmetry to Measure DNA Replication Fork Stalling Rate**: Measuring replication fork stalling rates by comparing asymmetry in sister replication tracks. The lower the correlation between sister fork progression the higher the frequency of replication fork stalling. A and B display schematics for symmetric and asymmetric origins. C and D display graphs showing a decrease in correlation of sister replication fork speed and increased fork stalling.*

DNA combing can be used to measure the distance between adjacent origins or inter origin-distance (IOD). Activation of oncogenes often caused changes in the replication programme that result in reduced IOD (Macheret & Halazonetis, 2015). This is possibly a result of activation of dormant origins in response to replication stress to ensure complete genome duplication (Ge et al., 2007). If IOD is too large, replication may not be completed or cells may not be able to compensate for stalled forks (Alver et al., 2014). IOD is simply measured by comparing distances between origins on the same fibre, Figure 4.4 shows a schematic for measuring IOD.

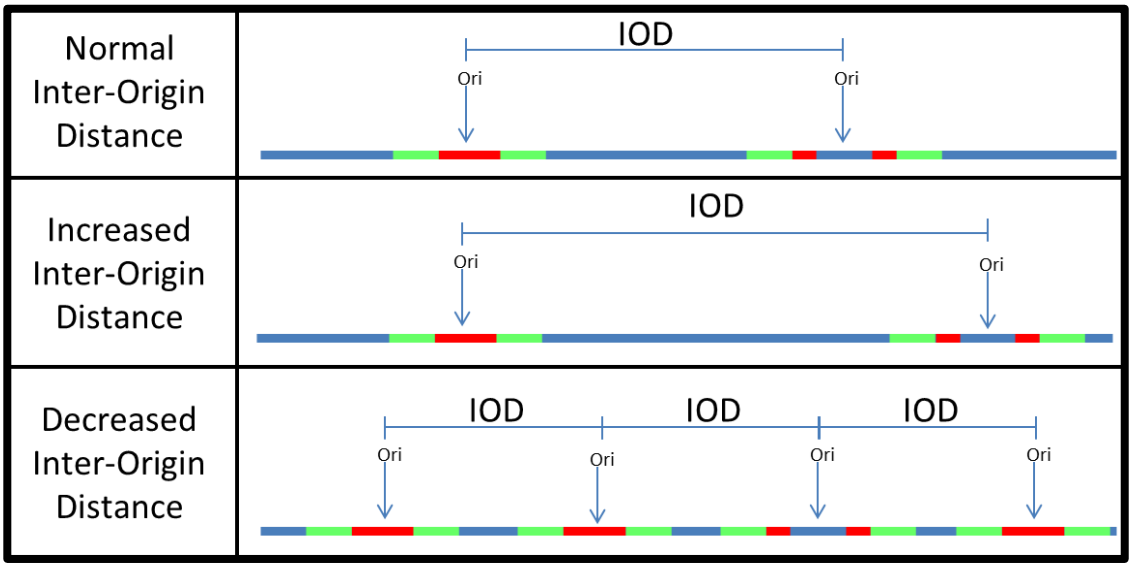


*Figure 4.4-**Quantifying changes in IOD using DNA combing:** A schematic displaying changes in IOD including increased IOD (middle), and decreased IOD (bottom)*

Re-replication occurs when control of DNA replication is aberrant and is indicative of replication stress, this can be measured using DNA combing. A similar single molecule analysis technique called DNA spreading has been used to measure re-replication. Cells are pulsed labelled with IdU and CldU with a half hour gap in between. Tracks could then be labelled and some regions of DNA were labelled with both nucleotides. This technique was used by Dorn et al. (2009) to show overexpression of preRC protein CDT1 and removal of its inhibition induced re-replication (Figure 4.5).

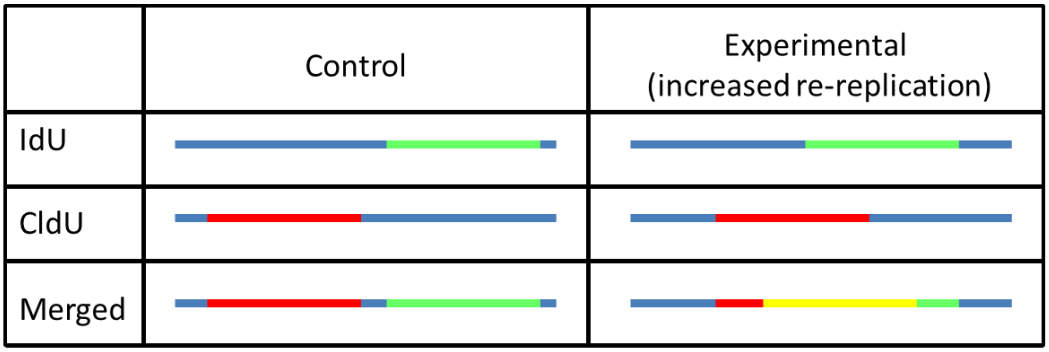


*Figure 4.5-**Measuring re-replication using DNA fibre analysis:** A schematic for measuring re-replication. Cells are pulse labelled with IdU and CldU with a half hour gap. Fibres that have no re-replication have no overlapping (yellow) tracks. Merged image shows a representative image where there is no re-replication (left) and there is re-replication (right).*

DNA combing provides a way to investigate replication dynamics at high resolution. It can be used to measure changes in the replication programme providing powerful insight into how cancer changes alter replication programs and induce asymmetry in replication forks. When coupled with cell-free replication experiments through synchronisation at the G1/S checkpoint it could be used to investigate the effects of Ciz1 levels and phosphorylation on DNA replication dynamics.

4.1.2 Aims

This chapter aims to establish the techniques required to perform DNA combing. This includes: preparation of silanized coverslips and purification of mouse fibroblast DNA. This chapter will also establish the pulse labelling and detection techniques required to generate duel labelled stretches of mammalian DNA, kinetic data will be generated regarding replication fork progression in mouse NIH3T3 cells including: replication fork rate, measuring replication fork stalling.

As a first step towards a detailed analysis of replication origin usage. Initially, the preparation of hydrophobic coverslips is discussed, quality control and preliminary work to develop this methodology. Stretching of λ phage DNA, dsDNA, ssDNA and analysis of pulse labelled DNA is determined. The long term aim is to integrate *in vitro* DNA replication techniques (Chapter 3) with DNA combing to determine effects of increased CDK activity on replication fork dynamics. Finally, we aim to integrate synchronised cell-free DNA replication assays (Chapter 3) with DNA combing. If successful, this would enable careful dissection of replication licensing. This approach could be used to study CDK activity on replication fork dynamics, the effect of Ciz1 on this process, and evaluation of Ap4A's activity on replication fork dynamics.

4.2 Preparation of Materials for DNA Combing

Before replication dynamics of mammalian DNA replication could be measured and investigated a variety of materials needed to be prepared and tested: These included hydrophobic coverslips for stretching DNA as well as antibodies and verification of the combing method.

4.2.1 Testing of Silanized Coverslips

First, hydrophobic silanized coverslips were prepared through extensive washing and overnight silanization. To ensure coverslips were correctly silanized hydrophobicity was tested. This was achieved simply through dropping water on one coverslip, water droplet ran straight off demonstrating that coverslips were hydrophobic. Now coverslips could be tested for their ability to adhere to DNA and allow stretching once the force from a receding air water meniscus was applied. This required optimisation of pH and cell number. Allemand et al. (1997) identified pH 5.5 as the optimum for stretching DNA fibres. However, here pH 5.5 allowed DNA binding to the hydrophobic coverslip but not stretching. pH was increased identifying pH 5.7 as the optimum for stretching DNA fibres. pH 5.7 was used in all subsequent experiments.

To test whether DNA could be successfully stretched onto the surface of the silanized coverslips lambda phage DNA was used as a positive control. Lambda DNA was bound to and combed onto the surface, slides were mounted with mounting media mixed with YOYO1 (Figure 4.6).

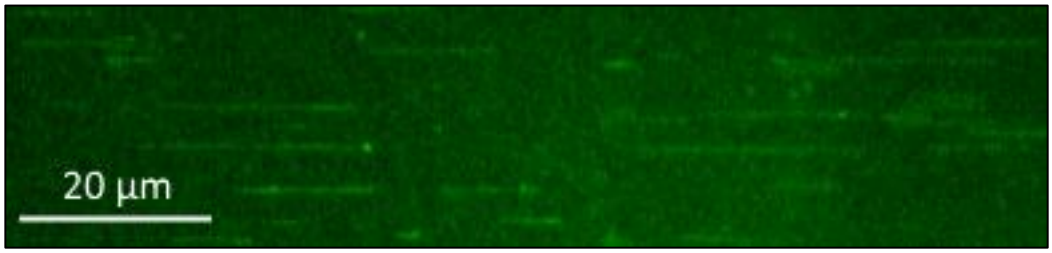


*Figure 4.6 – **Combed Lambda Phage DNA:** Lambda phage DNA stretched onto a silanized coverslip and dsDNA strands stained with YOYO1 (green). Confocal fluorescence microscope image, scale bar= 20 µm*

Lambda phage DNA was stretched as seen by parallel regions of DNA. Combed lambda phage DNA confirmed that the silanization process was successful and allowed for stretching of DNA fibres. The process could now be tested on longer genomic mammalian DNA. To ensure that DNA from mammalian cell lines could be effectively stretched coverslips were combed with NIH 3T3 genomic DNA. NIH3T3 cells were cultured as normal, and grown to approximately

70% confluence and cells harvested. Naked genomic DNA was purified, combed onto silanized coverslips and then probed with YOYO1 to stain dsDNA fibres. (Figure 4.7).

This was tested using 1 x 10^7 cells/ml stored in three agarose plugs. In one of the agarose plugs, the DNA had degraded during the purification procedure, highlighting the requirement to purify DNA separately multiple times from the same sample.

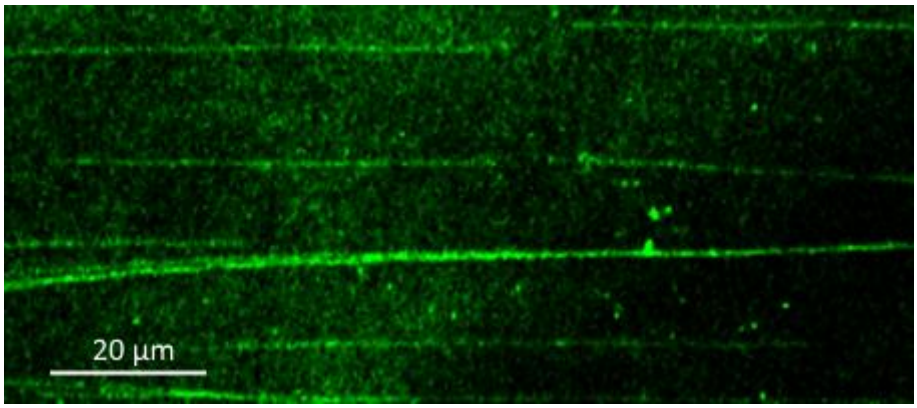


*Figure 4.7-**Combed NIH 3T3 DNA:** Confocal Fluorescence microcopy image of Combed mouse fibroblast genomic DNA, adhered to a silanized coverslip and stained with Yoyo1. Scale bar = 20 µm*

Genomic DNA was successfully purified from NIH3T3 cells; Figure 4.7 shows long fibres parallel and straight, fibres were stretched in the direction that coverslips were pulled from the DNA solution. This confirmed that the cell number being used was acceptable for DNA combing, as DNA was not too sparse, nor too close that it formed frequent bundles of DNA. Other studies have used 1 x 10^6 cells/ml of different cell types (Bartkova et al., 2006), however 1 x 10^7 cells/ml seemed to generate the sufficient density of DNA for the fibroblasts used here. Having established the correct well density and solution pH for adherence of DNA onto silanized coverslips, the denaturation step was performed and ssDNA was detected by immunofluorescence.

4.2.2 Labelling Denatured DNA

To measure halogenated nucleotide inclusion during DNA replication by DNA combing, denaturation of DNA with NaOH is required to expose epitopes on IdU and CldU bases for immunofluorescent labelling. As a control to test whether denatured DNA remained stretched onto the prepared silanized cover slips an ssDNA antibody was tested. NIH3T3 cells were grown to approximately 70% confluence, yielding a 10^7 cell/ml concentration, harvested and genomic DNA purified. DNA was stretched onto the prepared silanized coverslips, dried onto

the coverslips, then denatured using sodium hydroxide to break the hydrogen bonds between strands. Denatured combed DNA was then immunofluorescently labelled using the ssDNA antibody as a primary antibody (Figure 4.8).

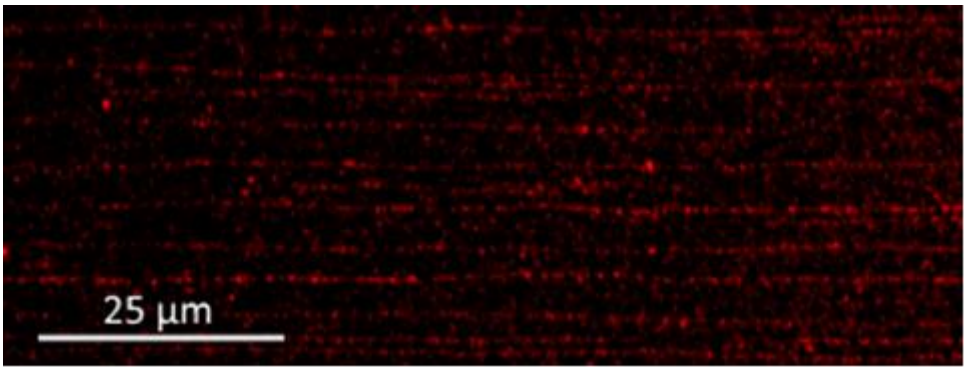


*Figure 4.8-**Detection of ssDNA on Silanized Coverslips**. DNA was purified from cycling NIH3T3 cells, combed and denatured. DNA was detected with a ssDNA antibody and fluorescently labelled with an alexafluor 568 conjugated secondary antibody. Scale bar = 25 µm*

DNA was successfully denatured and remained stretched and intact on silanized coverslips, shown by parallel straight lines of DNA (Figure 4.8). DNA was successfully stretched and visualised by immunofluorescence. This allowed testing that halogenated nucleotides could be included in growth medium, incorporated into DNA and then visualised on combed DNA through immunofluorescence.

4.2.3 Labelling Replicating DNA

When labelling DNA for analysis by DNA combing halogenated nucleotides were used. To test that halogenated nucleotides could be successfully incorporated into replicating DNA and labelled on stretched coverslips NIH3T3 cells were grown and pulse labelled in growth media containing IdU for 20 minutes, followed by thymidine for 1 hour to ensure labelled region was representative of 20 minutes of replication (Figure 4.9a). DNA was then purified by suspending cells in agarose plugs to avoid DNA damaging forces, DNA was combed onto silanized coverslips at 300 µm/sec and denatured with NaOH to expose the IdU epitopes. Fibres were probed with and anti-IdU antibody and visualised by immunofluorescence (Figure 4.9).

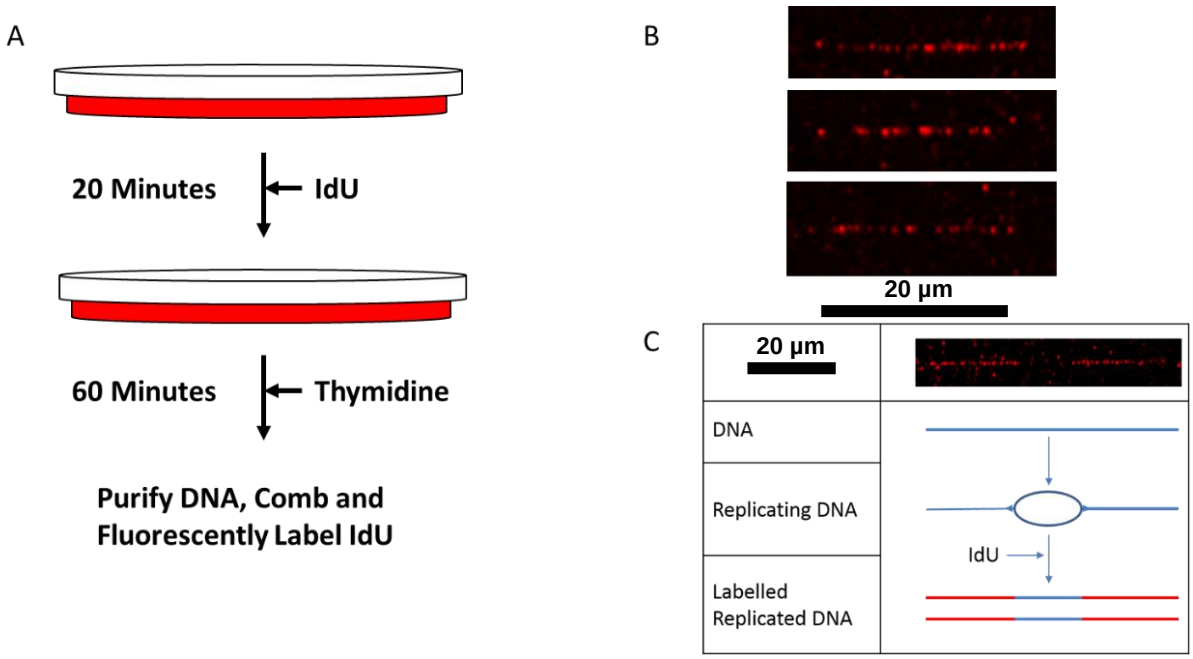


*Figure 4.9-**Labelling DNA Replication:** Combed genomic DNA purified from NIH3T3 cells labelled with IdU A) Schematic representation of the nascent DNA labelling through pulse labelling by addition of IdU for 20 minutes and addition of excess thymidine to stop labelling. Replication tracks were visualised B) Confocal microscope images of immunofluorescence labelled replicating DNA as well as replication origins C) Schematic showing a replication origin firing after the IdU pulse labelling. Scale bars=20 µm*

These results are displayed in Figure 4.9. Labelled replication tracks were uniformly stretched and aligned with the direction coverslips were removed from the well. DNA was also well preserved, of an equivalent length and easily visualised confirming that halogenated nucleotides could be incorporated into replicating DNA through inclusion in growth media. The combed DNA fibres allow the study of replication dynamics such as replication fork velocity and fork stalling experiments. Figure 4.9c displays a single origin that had fired during the 20 minute pulse labelling with IdU but after the labelling period had begun. This resulted in two adjacent replication tracks of an equal length with a gap in between where DNA was replicated before the modified nucleotide was included, this is displayed in the schematic in Figure 4.9c. This displayed that this technique could be used for studying the dynamics of origin firing, such as using fork asymmetry to measure replication fork stalling rates.

Single labelling of mammalian cells does not provide sufficient detail to garner much data. Without counterstaining of DNA it cannot be known whether adjacent replication tracks are on the same fibre. Additionally, it provides no information as to when the DNA in labelled replication tracks began replicating. Because of this it cannot be used to generate data regarding replication fork speed or information about origins. Because of this duel labelling is

used to give information as to when each track began replicating, if it is a fired origin, a termination event or a progressing replication fork.

4.3 Measuring Replication Parameters

4.3.1 Duel Labelling of Combed DNA and Estimation of Replication Fork Rate.

For accurate measurement of replication dynamics by DNA combing such as replication fork speed it is required that combed DNA is labelled with 2 modified nucleotides. To measure origin firing parameters such as IOD DNA needs to also be probed for ssDNA to ensure that adjacent origins fired from the same fibre. To ensure that DNA could be labelled with three different antibodies and used for analysis of replication dynamics NIH3T3 cells were grown and pulse labelled for 20 minutes with IdU, then thymidine to wash out IdU, then CldU (figure 4.10a). DNA was then purified, combed and then probed with three different primary antibodies. Figure 4.10 displays nascent replicating DNA that could be used for analysis of replication dynamics.

DNA combing can be used to measure replication fork velocity: DNA is uniformly stretched and pulse labelled with modified nucleotides for a known length of time. Lengths of replication tracks are therefore proportional to a replication fork velocity and can be therefore measured in kilobasepairs per minute (kbp/min). Replication tracks that are duel labelled but not replication termination events can be considered progressing replication forks. The length of tracks representing the second incorporated nucleotide in a duel labelled replication track can be used to calculate replication fork velocity. This single fibre data can be expanded to include a large number of replication tracks to estimate the average replication fork speed for a cell type. This ensemble data provides the distribution of fork rates and enables estimation of average for speeds (Figure 4.10).

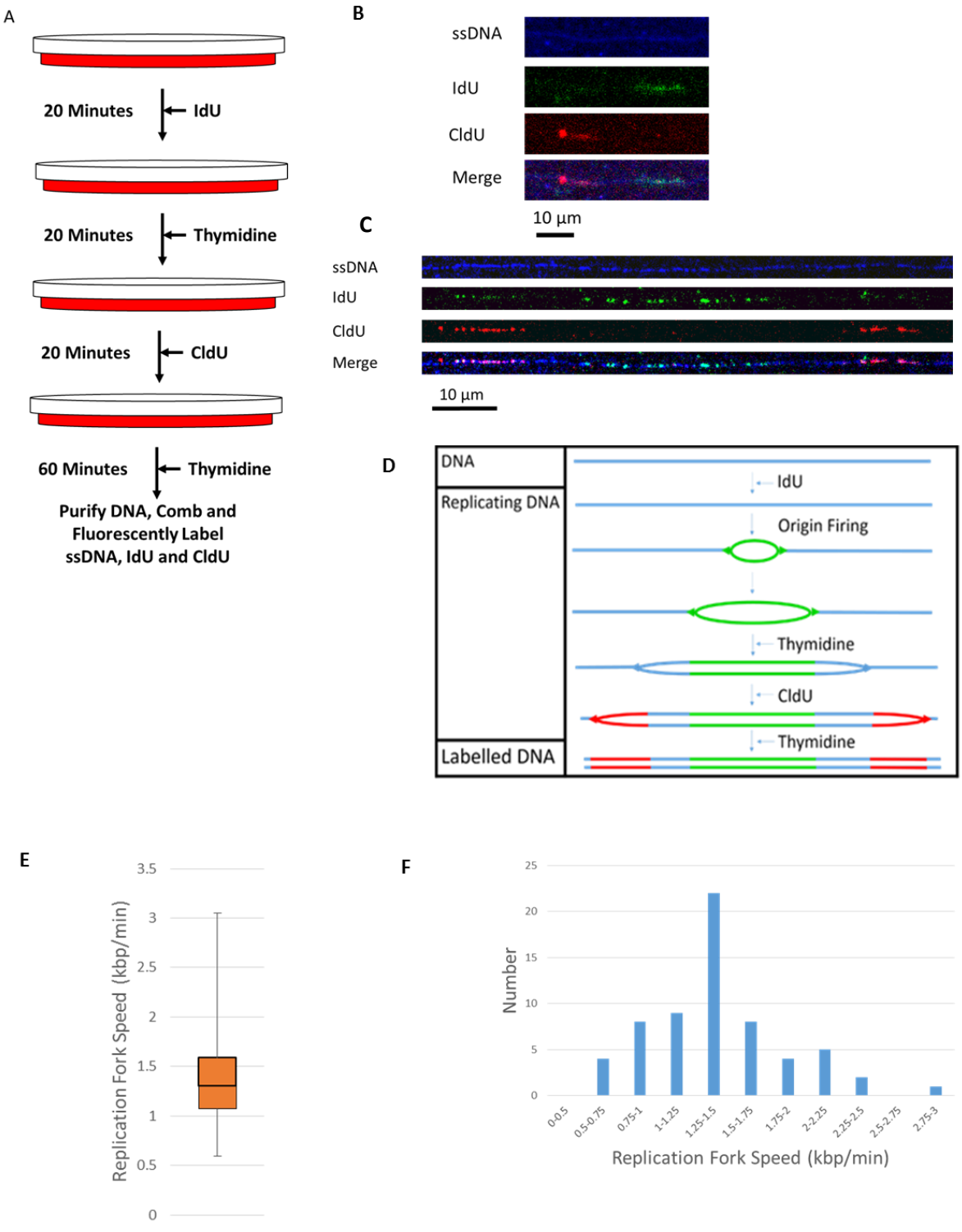


*Figure 4.10-**Dual labelling mouse fibroblasts with IdU and CldU.** A. Experimental overview of the labelling used in this experiment B) A duel labelled DNA fibre showing progression of a replication fork. C.) A Duel labelled replication origin that fired during the first pulse labelling period. D) A schematic for a replication origin that fired during the first pulse labelling as in C. E) A box and whisker plot showing the median replication fork rate, interquartile range and extreme values . F) Frequency distribution plot of measured replication fork rates.*

Figure 4.10b displays a labelled progressing replication fork. Replication was progressing along the DNA (blue) from right to left, this is seen by the sequential inclusion of a green track which represented the IdU labelling period followed by a gap which represented the thymidine labelling period followed by a red replication track which represents the CldU labelling period. The replication fork speed could be measured here simply by dividing the length travelled by the red replication track by the labelling period. Data from multiple progressing replication forks could be used to quantify the replication fork velocity of these cells (Figure 4.10e/f). Additionally, previous experiments have identified that DNA combing stretches DNA uniformly at 2kbp:1µm (Bensimon et al., 1994). This value can be used to calculate the replication fork velocity in kbp/min.

Figure 4.10c displays a labelled origin that fired during the first IdU pulse-labelling period. DNA (blue) began replicating whilst IdU was included in growth medium, replication progressed bidirectionally from the centre of the green replication track. The green replication track is flanked by two regions of unlabelled DNA (blue) representing the thymidine labelling period which are then flanked by two red sister replication tracks which represented the CldU labelling period. This showed that origin firing could be visualised by DNA combing. A schematic for this is shown in Figure 4.10a.

Here DNA combing was used to estimate replication fork speed for NIH3T3 cells. DNA was pulse labelled sequentially with IdU, thymidine, CldU (figure 4.9a). Only duel labelled replication forks on single fibres of DNA flanked by regions of blue DNA, on a constantly stretched fibre (Figure 4.10b) were used for estimating replication fork speed.

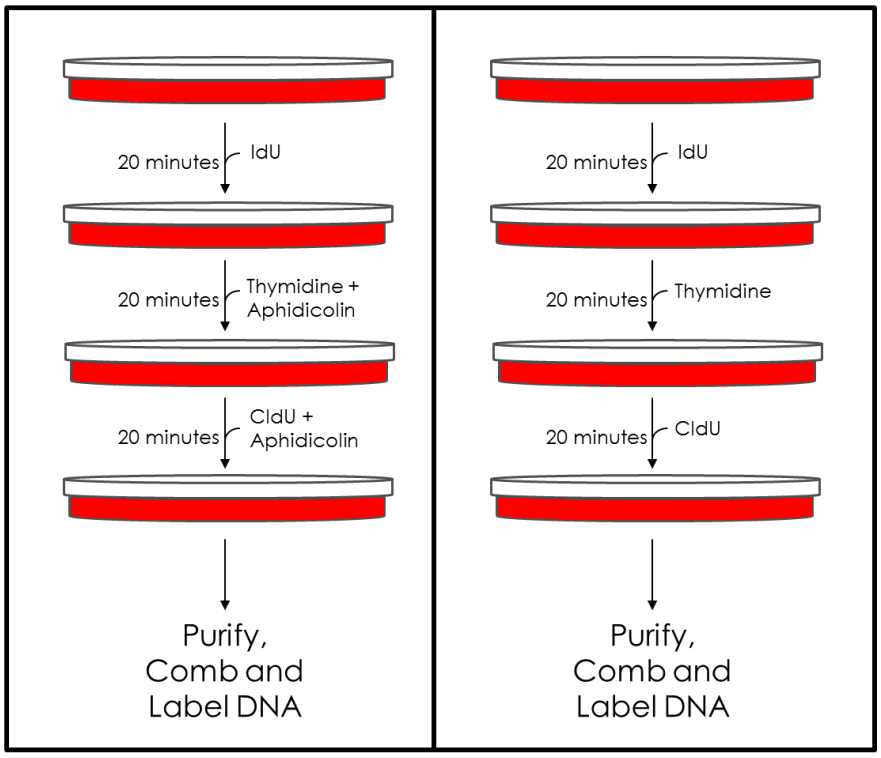
Median replication fork rate was 1.31 kbp/min, a rate consistent with other cell types (Table 4.1). Figure 4.10f shows the distribution of replication fork velocity centralised around the median value. Figure 4.10e displays a box and whisper plot of replication fork speed. This method could be used to compare replication fork speed between control and experimental samples for example cells could be made to overexpress Ciz1 or knockouts developed and replication fork velocity could be analysed to investigate Ciz1's role in DNA replication and if it induces replicative stress.

4.3.2 Stalling Replication Forks with Aphidicolin

Having established that DNA combing could be used to measure DNA replication fork progression, to ensure that alterations in fork progression could be detected we used aphidicolin to block DNA replication fork progression. Aphidicolin was used to stall replication forks by inhibition of DNA polymerases (Baranovskiy et al., 2014). To test the ability of aphidicolin to stall DNA replication forks and to determine whether this is measurable through DNA combing, NIH3T3 cells were pulse labelled with IdU for 20 minutes, media was replaced with thymidine containing media with aphidicolin for 20 minutes, then replacing media with CldU and aphidicolin for 20 minutes. This could be compared to a control where aphidicolin was not included (Figure 4.11a) if replication fork stalling increased the proportion of replication tracks labelled with both nucleotides should decrease. Figure 4.11 displays the results of these experiments.

This provided a positive control to establish that replication fork stalling could be detected. Additionally, in the future it could be modified to measure DNA replication fork restart after stalling, or the ability to measure stalling of elongating forks rather that initiation when combining DNA combing and the *in vitro* replication system described in Chapter 3.

A



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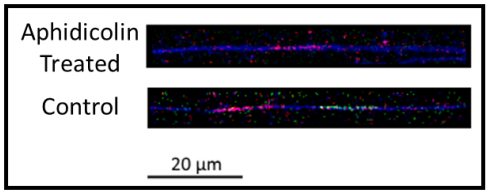

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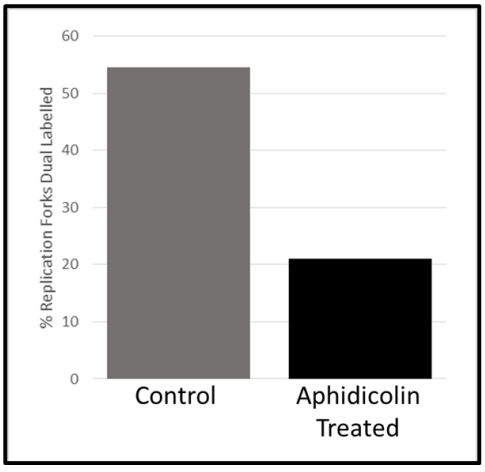

*Figure 4.11-**Observing Replication Fork Stalling** Replication fork stalling by treatment with aphidicolin. A displays a schematic for the treatment and labelling of cells. B displays replication tracks representative of the samples. C displays the proportion of duel labelled replication tracks. (N=2)*

Figure 4.11a displays a schematic for the treatment of cells before DNA was isolated, combed and labelled, experimental samples were treated with aphidicolin to stall replication forks, control samples were labelled without inclusion of aphidicolin. Figure 4.11b is a representative image of labelled DNA from each of the samples, replication tracks in control cells were frequently duel labelled with both nucleotides, whereas cells treated with aphidicolin tended to only be labelled with IdU (red) but not the second modified nucleotide CldU (green). Figure 4.11c quantifies the proportion of replication tracks that were duel labelled across the aphidicolin treated samples and the control. This showed a reduction in the amount of duel labelled tracks. A reduction in duel labelled tracks indicated that at many progressing replication forks after treatment by aphidicolin replication fork progression had been halted preventing the inclusion of the CldU. This confirmed that aphidicolin mediated replication fork stalling could be measured by DNA combing, displaying the usefulness of the technique in measuring DNA replication stress.

4.4 Combing DNA from *in vitro* Replication Experiments

As discussed earlier, CDK deregulation induces DNA replication stress (Section 1.7). The results described in Chapter 3 demonstrated that in *in vitro* replication models Ciz1-N471 can both broaden and amplify the DNA replication initiation activity of recombinant cyclin A/CDK2 (Figure 3.7; 3.9). This activity perturbation could constitute CDK activity deregulation if it acts the same *in vivo* allowing cells to enter S phase at canonically non-permissive kinase levels it could induce DNA replicative stress. Combining cell-free DNA replication assays and DNA combing by repeating cyclin A/CDK2 titration experiments and investigating the effects of replication at non-permissive kinase levels could help identify whether Ciz1 can induce replication stress, which could help to explain its apparent oncogenic function.

Combining cell-free experiments using replication competent nuclei from quiescent release synchronised cells could be a powerful tool for investigating replication stress in mammalian replication. Replication fork deceleration and replication fork stalling are the definition of replication stress (Zeman & Cimprich., 2014). To be able to measure this directly is key in identifying factors that induce stress. To test if DNA from nuclei harvested post quiescence release cell-free replication assays were prepared as in chapter 3. DNA was purified from 20µl of isolated nuclei, DNA was combed onto silanized coverslips and stained with YOYO1 (Figure 4.12).

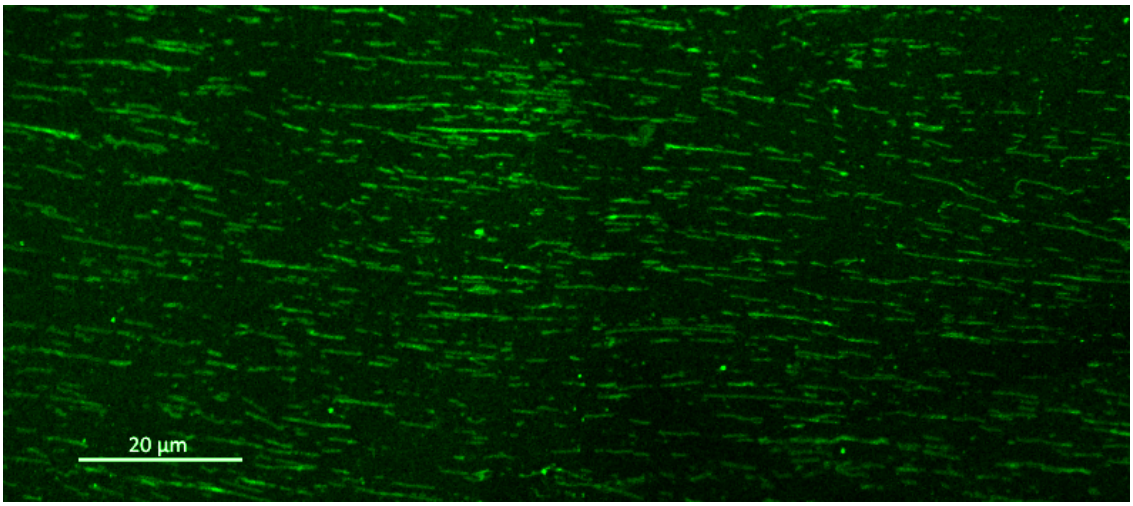

*Figure 4.12 –**Combing DNA from in vitro Replication Experiments:** Combed genomic DNA purified from nuclei isolated from NIH3T3 cells and stained with YOYO1 (green).*

DNA combed from *in vitro* replication assays and stained with YOYO1 consistently resulted in short fragmented stretches of DNA, typically shorter than 20 µm, this is displayed in figure 4.8. DNA of this quality could not be used for analysis of DNA replication. To successfully use *in vitro* replication experiments in DNA combing the technique will need to be modified to ensure that DNA purified is of a high enough quality to get enough data to measure replication dynamics. It is not clear why DNA from post quiescent nuclei would produce fragmented DNA, this could be due to DNA damage introduced during the nuclei isolation, or storage.

4.5 Chapter Discussion

DNA combing is a powerful tool for the study of DNA replication origin usage, fork rates and for investigating replicative stress. Here, the basic steps in establishing the technique were completed and a rudimental analysis of DNA fork progression rate determined. In addition, measurements of replication fork stalling, and initial attempts to combine post-quiescence *in vitro* replication method and DNA combing.

We have established DNA combing as a tool for the investigation of DNA replication dynamics we can begin to obtain quantitative data to study DNA replication stress in the future. Here the replication fork speed of NIH3T3 cells was estimated using DNA combing to be 1.31 kbp/min. This seems a plausible replication fork speed with other studies providing a fork rate between 1-2.1 kbp/min, other replication fork rates seem to vary around this amount.

Hamster V79 fibroblast cells display a fork speed between around 1.6 kbp/min and 2.1 kbp/min (Wilhelm et al., 2016) and HeLa cell replication fork speed varies from less than 1 kbp/min through to 2 kbp/min (Técher et al., 2013). This highlights that this replication fork speed is plausible, but also highlights the high variability in replication fork speed even within the same cell line, meaning comparing specific fork speed results should only be done within experiments, to ensure meaningful results.

The analysis here has demonstrated that determination of fork progression fits with observed rates of other cell lines and species (Table 4.1). The results generated to date suggest that the IOD in an unperturbed system has guided few measurable inter-origin distances. DNA fibres are typically too short, and only a few IOD are detected. The technique should be modified to yield longer DNA fibres.

Species	Mean Replication Fork Speed (kb/min)	Reference
Human Primary Normal Keratinocytes	1.46	(Conti et al., 2007)
Hamster V-79 Cells	2.09	(Willhelm et al., 2016)
Saccharomyces cerevisiae	2.9 (median 2.3	(Raghuraman et al., 2001)
Schizosaccharomyces pombe	2.8	(Heichinger et al., 2006)
HeLa cells	1-2	(Técher et al., 2013)

Table 4.1-Replication Fork Rates of Different Cell Types

The DNA is likely to be sheared after it has been released into buffered solution due to low forces applied during movement of the solution. Modification of the procedure can be performed in several ways to reduce denaturation of DNA and DNA shearing. These included reducing movement between release of DNA from agarose plugs by melting plugs directly into the Teflon blocks, altering NaCl concentrations of DNA buffer solution and using a combing apparatus with lower friction. Each method saw a large increase in the length of DNA visualised by combing (Kaykov et al., 2016). Modifying the technique could allow for combing of longer DNA strands to determine more parameters of replication dynamics. Similar approaches can be trialled in the system used in this study to increase fibre length in the future. These modifications should also be used when attempting to increase DNA fibre length from *in vitro* DNA replication experiments.

Once the technique for combing of DNA from post quiescence *in vitro* replication experiments has been developed, it could be used to investigate replication stress in this system. An advantage of cell-free replication experiments is that it allows for incorporation of modified

nucleotides that have large tags such as biotin-16-dUTP and digoxigenin-11-dUTP. This allows for detection without denaturation of DNA, by counterstaining dsDNA with Yoyo1, reducing the number of antibody incubations, saving time. It would also be of interest to investigate replication dynamics in isolated nuclei from mitotically cycling cell compared to post-quiescent release nuclei.

There are a number of way nuclei could be duel labelled, Marheineke et al. (2005) duel labelled DNA from late G1 nuclei arrested by mimosine using biotin-16-dUTP and digoxygenin-dUTP and detected using fluorescently tagged avidins for biotin-16-dUTP and using fluorescent antibodies to detect digoxygenin dUTP. Another modified nucleotide often used to measure DNA replication is EdU; EdU was used in chapter 3 to monitor number of cells entering S phase, EdU can be fluorescently labelled using click it chemistry. EdU has been used in DNA combing experiments as a substitute for BrdU to avoid the DNA denaturation stage (Bianco et al., 2012). Due to no DNA denaturation being required for any of these detection methods DNA can be counterstained with YOYO1 or another fluorescent dsDNA binding fluorophore which would save significant amounts of time.

Chapter 5: General Discussion

5.1 Ciz1 Can Perturb the Replication Initiation Capacity of Recombinant Cyclin A/CDK2

Results shown earlier display that in *in vitro* replication assays an N terminal fragment of Ciz1 can alter the kinetics of DNA replication initiation by recombinant cyclin A/CDK2: both increasing the proportion of replication competent nuclei that initiate and expanding the range of concentrations that cyclin A/CDK2 can activate DNA replication. This is of particular interest for two key reasons: Ciz1-N471 can expand the replication initiation capacity of cyclin A/CDK2, and Ciz1 can modify the replication activating concentrations of cyclin A/CDK2.

As discussed earlier perturbations in the normal regulation of CDKs during DNA initiation often induces replication stress, for example by forcing replication with insufficient nucleotide pools (Bester et al., 2011). Addition of recombinant Ciz1-N471 alters the normal function of cyclin/CDK activity during DNA replication initiation at the G1/S transition in *in vitro* kinase experiments. If this is true in intact cells as well as *in vitro* in nuclei this may provide a framework for Ciz1 inducing replicative stress in cells which may help explain its role in a number of cancers, including prostate, colon and lung cancer (Higgins et al., 2012; Liu et al., 2014; Wang et al., 2014). The combination of *in vitro* DNA replication assays and DNA combing could, with refinement, provide a toolkit to investigate whether replication initiation at abnormal CDK2 concentrations promotes DNA replication stress. This could help to explain whether Ciz1 has a direct role in inducing replication stress and whether or not this underpins its apparent oncogenic activity.

Another reason is that DNA replication initiation in non-cancerous cells does not occur at the higher kinase levels found in the latter stages of the cell cycle, orchestrated through a variety of mechanisms discussed earlier, for example activation of ubiquitination of replication licensing proteins through cyclin/CDK activity (Laman et al., 2001; Méndez et al., 2002). Cancer cells have mutations that increase the basal kinase levels of cells for example cyclin D and E overexpression. Ciz1 could serve as an adapter protein allowing replication to occur at non-permissible kinase levels in cancer cells, this could explain its increased expression in certain tumour types, and cancer cells that display an overexpression of ciz1 could be displaying gene addiction to Ciz1 to allow continued replication.

If Ciz1 is inducing replication stress or facilitating replication through gene addiction in abnormal kinase conditions (Figure 3.12), then it could be a useful target for the development of future therapeutics and diagnostic tools against certain cancer types.

5.2 Ap4A Blocks Replisome Assembly

Results gathered here have demonstrated that Ap4A reduces the fraction of replication competent nuclei that can be initiated to replicate their DNA by an S phase extract. Additionally, replication licensing proteins and replication protein binding were shown to be reduced when Ap4A was included in a HeLa S phase extract. Ap4A appears to prevent DNA replication initiation by blocking the replication licensing process, thereby preventing the formation of preRCs, pre ICs and binding of PCNA. Ap4A may function through binding of the DNA primase enzyme DNA pol α (Baxi et al, 1994). If Ap4A inhibits DNA pol α it may act in a similar manner to aphidicolin which inhibits polymerase activity resulting in stalled replication forks (Krokan et al., 1981). If this is the case prevention of replication complex assembly could be mediated through replication fork collapse after sustained lack of replication initiation. Replication assays chould be set up utilizing aphidicolin to compare whether aphidicolin prevents replisome assembly in the same manner as Ap4A.

The effect of aphidicolin on already elongating replication forks would be interesting to investigate. Data obtained thus far does not provide insight into whether Ap4A prevents just initiation, or elongation. This could be studied by combining *in vitro* replication assays with DNA combing. Nuclei could be initiated with an S phase extract with Biotin-16-dUTP followed by centrifugation and extracts replaced with an S phase extract containing Ap4A and EdU. Data could be obtained from parallel reactions of both replisome assembly, through western blotting, and replication fork progression data, through DNA combing.

Ap4A has previously been shown to bind ATP binding domains as it has a structure similar to ATP (Maness et al., 1983). Ap4A could be competing with ATP for a number of proteins involved in the loading of the proteins here to DNA. PCNA is loaded onto DNA by replication factor C (RFC) a AAA ATPase (Thompson et al., 2012). PCNA binding is consistently blocked by Ap4A in the experiments shown here. This could be due to Ap4A blocking ATP binding to RFC.

MCM2 DNA binding is shown to be reduced in *in vitro* replication experiments in a manner that varies highly between nuclei batches. MCM2 is part of the heterohexameric MCM2-7 ring that binds DNA. MCM2-7 like PCNA binds DNA as a ring and requires ATP activity to assemble and encapsulate the DNA fibres and mediate interactions with DNA polymerases (Kang et al., 2014). If Ap4A is mediating its effect as an ATP competitive inhibitor it is possible that MCM2-7 DNA binding could be blocked by Ap4A competing for ATP binding. Similarly Ap4a may inhibit kinases in a similar manner which could prevent loading of the CMG, prevent

polymerase recruitment and PCNA loading. Preliminary work appeared to show that Ap4A could inhibit TAK1, but further analysis has cast doubt on this observation.

Ap4a is produced by cells in response to the DNA damaging agent mitomycin C (Marriott et al., 2015). Ap4A could form part of a response in cells to DNA damage that allows cells to prevent cell cycle progression allowing damaged DNA to be repaired to prevent generation of mutations and abnormal chromosome structures. This could be due to changes in gene expression, like those seen when NUDT2 expression is impaired (Marriott et al., 2016), which leads to down regulation of certain cancer promoting genes. Or this could be due to the direct activity of Ap4A. Figure 5.1 shows a schematic for a potential replication inhibitory pathway for Ap4a. The unknown mechanisms by which Ap4a prevents DNA replication in *in vitro* should be investigated further and it should be identified whether this is a novel pathway or whether Ap4a is involved in an existing pathway.

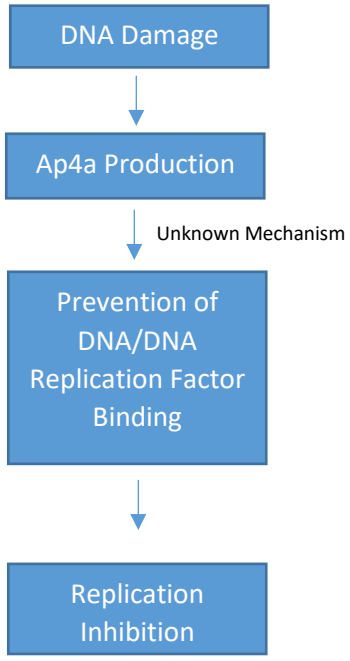

*Figure 5.1-**Model for Ap4A Replication Initiation Inhibition:** Proposed timeline for Ap4A production to prevent DNA replication in response to DNA damaging agents. Highlighting the unknown mechanism for ATP blockage of replication.*

5.3 DNA Combing

DNA Combing has been established here as a technique for measuring replication dynamics in mammalian cells to gain greater insight into the DNA replication programme in mammalian cells. Replication parameters were shown to be measurable and quantifiable using DNA combing including DNA fibre length, replication fork speed and replication fork stalling. Going forward these techniques will be useful in determining whether the oncogenic capacity of Ciz1 is through an abnormal Ciz1 activity inducing replication stress or abnormal DNA replication and to visualise DNA replication forks in Ap4A inhibited cells to gain greater insight into this process.

The major problem with DNA combing seen here was short DNA fragment lengths found in cell free experiments. Solutions to this problem were discussed earlier (Section 4.5). Another issue discovered with combing was inconsistency in the silanisation of coverslips sometimes leading to non-stretched DNA (occurring in around 1 out of 4 coverslips). This was resolved by stretching samples of DNA onto multiple coverslips to ensure that data could be obtained, this is fairly resource costly as the time and reagents that are required for generation of small numbers of silanized coverslips is high, making it essential to optimise the procedure as best as possible.

Inspection of coverslips during the cleaning and silanisation stages of the coverslips can be done to ensure sufficient drying of coverslips, no decolouration or debris build up and viscous acid is removed sufficiently. If a powder like build up appears on coverslips they must be discarded. The hydrophobicity of coverslips can be tested by dropping water onto coverslips, if coverslips are coated water will run straight off. Coverslips used for experiments could be tested by dropping water onto one side of the coverslip and combing DNA onto the other. Another solution to this issue that is discussed later (Section 5.3) is DNA spreading, a single fibre analysis technique that does not require the production of silanized coverslips.

5.4 DNA Combing replicability and accuracy

Whilst DNA combing can be used to measure the IOD of origins that fired during the first of two sequential halogenated nucleotide pulse labelling this likely undervalues the real IOD. This is due to origins that fire before, after the first pulse labelling or multiple neighbouring origins being masked when firing within the same labelling stage. (Técher et al., 2013) This means that the overall IOD cannot be concluded from DNA combing but an IOD representative of the labelling timeframe. However, changes to the overall IOD would cause

changes to the representative IOD so significant changes in IOD observed by DNA combing as an experimental response still provide useful information as to whether or not replication stress is being induced.

Variability in replication dynamics between different batches of cells could lead to false results. The same cell lines have observed different replication fork speeds on multiple occasions between studies (Técher et al, 2013). This could be due to different culture techniques in different labs. Due to these differences seen by others, it is essential to treat cells used in experiments in exactly the same manner for as long as possible before testing experimentally. For example, if an experiment was being designed in which a drugs effect on replication fork speed on an asynchronous culture of cells was being tested cells must be treated identically up to the point of the drug treatment. This would include reviving cells from the same stock, using the same media and serum during cell growth, and pooling trypsinised cells during any passages before splitting across multiple plates. These would reduce any artificial differences in replication dynamics created by differences in preparation leading to a more accurate experimental result.

To add further validity to DNA combing results, further methods of DNA stretching could be used. A prominently used alternative to DNA combing to stretch single DNA fibres is DNA spreading. DNA spreading was first used to map to hamster dihydrofolate reductase gene locus (Parra & Windle, 1993). DNA spreading is a technique in which cells are dropped onto slides, dried and lysed directly onto the slide surface. Slides are then tilted at a 15° angle along the X axis allowing the DNA to run down the slide and stretch, where it can be fixed and labelled (Jackson & Pombo, 1998).

DNA spreading has been since adapted to measure replication fork speed, by pulse labelling growing cells with IdU and CldU and detected in the same manner as used for DNA combing single fibre experiments. DNA spreading has been used to show that Fanconi anaemia proteins help protect forks halted by treatment with hydroxyurea through IdU incorporation, expanding their DNA repair role across further forms of DNA damage than interstrand crosslink repair. (Schlacher et al., 2012)

Obtaining results of replication dynamic parameters through both DNA spreading and DNA combing will identify any differences between the two DNA stretching methods and could provide further validity to any results gained using one of the methods. DNA spreading uses a smaller cell number than DNA combing; using 2 µl drops of a cell suspension in the 10^5 cells/ml range, depending on cell type (Nieminuszczy et al., 2016). This would equate to DNA

harvested from 200-2000 cells on each slide. DNA harvested from 330 µl of 1 x 10^7 cells/ml in the DNA combing method described here represents DNA from approximately 3.3 x 10^6 cells. It would be interesting to see if harvesting DNA from a smaller sample of cells introduces biases or differences when compared to the larger number used in DNA combing.

5.5 Future Techniques for Measuring Replication Parameters

DNA sequencing technology has advanced rapidly since the human genome was first sequenced by the year 2000 (Lander et al., 2001). Whilst the sequencing of the first genome took decades of international collaboration next generation sequencing technology has drastically cut the cost and time required for sequencing of long strands of DNA meaning that a human genome can now be fully sequenced for as little as $1,000 (Goodwin et al., 2016). Advances in DNA sequencing have long term benefits on personalised medicine and disease causing SNP analysis it could also be very useful for the study of DNA replication.

One of the most interesting developments in DNA sequencing is nanopore sequencing. The basis of nanopore sequencing is that DNA will cross a membrane due to an electrochemical gradient because of DNA's negative charge. As DNA crosses pore it produces a change in electrical signal that is different for each DNA. Nanopore sequencing has been miniaturised by Oxford Nanopore Technologies, DNA is fed through a protein pore by a processive helicase enzyme with a known DNA feed rate allowing for high throughput real time DNA sequencing (Eisenstein, 2012; Feng et al., 2015). This technique can be used to recognise modified nucleotides. For example, polymer tagged nucleotides have been developed that have a distinct signal from normal nucleotides (Fuller et al., 2016). Using modified nucleotides pulse labelled into growing cells it could be theoretically possible to measure DNA replication at the sequence level to provide high fidelity information regarding replication parameters at both the length and sequence level.

Deep sequencing techniques have been used to generate maps of origin usage frequency in yeast (Müller et al., 2013). Briefly, DNA copy number was sequenced and compared across S phase and G1 phase yeast cells. The higher the copy number showed increased origin usage. This technique was used to compare origin usage across diploid and haploid yeast. This technique would be more difficult to measure origin usage in mammalian populations due to less perfect cell synchronisation and increased genome size.

5.6 Methods for Obtaining More Consistent Cell Synchrony for *in vitro* Replication Assays

Budding and fission yeast cultures can be released into S phase by arresting in G1 to a much greater extent to mammalian cells. This means combing experiments can be achieved using only one modified nucleotide as it can be known when they initiated (Lebofsky & Bensimon, 2003). Sadly, this is not true for mammalian cells that require duel-labelling experiments to accurate determine replication kinetics.

Mammalian cell culture synchronization is not as simple. The methods used here provide approximately 45% release into S phase. However, there are inconsistencies in S phase entry between batches of nuclei. This can be caused by different times in reaching confluency, different incubation periods or simple cell to cell variability. These two factors highlight a need for more methods for synchronising mammalian cell populations. Using a synchronised cell population may help to enrich for replicating DNA in DNA combing; increasing the number of cells in S phase and therefore the number of labelled fibres. This may introduce certain biases though as the kinetics of DNA replication may be different at the start or end.

Another method that can be used to isolate cell cycle specific cell populations is through centrifugal elutriation. Centrifugal elutriation separates particles based on their size and can be used to differentiate between cell cycle stages. Different fractions can be isolated and analysed by flow cytometry to determine their cell cycle stage (Banfali, 2011).

Isolation of a population of late G1 nuclei through centrifugal elutriation followed by dounce homogenisation may provide a better signal to noise ratio in S phase extract initiation and more consistent protein loading results, as their flow cytometry profiles would always be verified. Additionally, consistency between nuclei isolated through centrifugal elutriation, and contact inhibition/serum depletion would help to confirm results. As well as potential identifying differences between mitotic cell cycle and cells re-entering the cell cycle from quiescence. Interestingly comparing nuclei harvested in the two different ways may highlight differences in initiation kinetics between cycling cells (centrifugal elutriation of an asynchronous population) and cells that are re-entering the cell cycle from G0. G0 cells have differences to cycling cells, origins are no longer licence with reductions in CDC6 and MCM proteins (Blow & Hodgson, 2002). It would be interesting to see any difference in initiation kinetics between nuclei harvested in these ways.

5.7 Future Work

5.7.1 Cell-free Combing Experiments Resulted in Short Fragmented DNA

The work done here to attempt to combine the post quiescence release *in vitro* replication system with DNA combing needs further development. Whilst DNA was combed onto a silanised coverslip surface it was short and fragmented, furthermore no labelled replication tracks could be detected. Further work should be done to modify the system to ensure longer strands of DNA, and labelling and detection methods should be modified. Greater fibre length would allow for analysis of replication fork speed and IOD.

The short fragmented DNA is likely due ssDNA nicks being introduced during preparation of the genomic DNA, nicks relatively close to each other in opposite strands of DNA will cause DNA to be pulled apart during the combing process, creating stretches of fragmented DNA. Introducing a higher frequency of nicks will lead to shorter more fragmented DNA (Kaykov et al., 2016). Clearly, there was significant DNA damage caused at some point when preparing nuclei. Damage caused during the DNA purification process was discussed earlier but briefly, it can be minimised by reducing movement and increasing combing speeds. DNA damage could have been induced during preparation, for example during the high forces during centrifugation. Alternatively, DNA from isolated nuclei could have been damaged during storage. They are stored in liquid nitrogen, so by the time they are used experimentally they will have passed through a freeze thaw cycle which could damage DNA.

To test if nuclei isolated from post-quiescent NIH3T3 cells have an increased number of ssDNA breaks a number of methods could be used: one such method is a comet assay, comet assays are used to measure ssDNA damage. Briefly, cells are lysed in agarose followed by being subjected to an electrical current. Damaged DNA migrates further so DNA damage can be measured by the length of DNA tails (Olive & Banáth, 2006). This assay could be modified to measure if nuclei have large amounts of ssDNA damage.

5.7.2 Further Work With Ciz1

Once *in vitro* replication assays and DNA combing have been successfully combined replication kinetics at abnormal cyclin A/CDK2 concentrations should be investigated. If Ciz1 is allowing replication to continue in a dysregulated CDK activity high replication stress environment (Figure 3.12) then this should be measurable using changes in fork rate, IOD and increased replication fork stalling. These factors are all measurable by DNA combing. This would provide an important insight into how Ciz1 promotes tumour growth.

In order to further investigate Ciz1's role in DNA replication models should be generated in which ciz1 activity is abnormal. One way to achieve this would be through genetic manipulation. Ciz1 could be mutated to be made inactive, mutated to change its activity e.g. phosphorylation sites, knocked out or be expressed through a different promoter to induce Ciz1 overexpression. Data acquired from abnormal Ciz1 activity may help to explain Ciz1 activity in DNA replication initiation and elongation

Genetic manipulation with CRISPR/Cas9 provides a powerful tool for modifying genomes of mammalian cells. This revolutionary method enables the modification of the genome of cells at a high efficiency (Cong et al., 2013). Crispr/Cas9 can be used to insert random mutations in an attempt to prevent gene function through DNA repair by NHEJ or precise point mutations by transfecting cells with homologous DNA sequence with the desired modified sequence (Doudna & Charpentier, 2014).

Using crispr cas9 NIH3T3 cells expressing CIZ1 could be modified. CRISPR/Cas9 could be used to generated Ciz1 knockout NIH3T3 cells. Ciz1 knockout cells generated though CRISPR/Cas9 gene editing will likely be viable as previously generated Ciz1 knockout mice grew normally (Nishibe et al., 2013). This indicated Ciz1 knockouts should be non-lethal and suggest that CRISPR-Cas9 deleted cell lines would provide a good model for investigating Ciz1's role in DNA replication in cell-based experiments.

Once mouse embryonic fibroblast Ciz1 knockouts are generated, the effect of loss of Ciz1 activity could be measured in a variety of ways: cell proliferation can be measured, changes in cell cycle profile could be measured by flow cytometry to determine the portion of cells that are in S phase. The ability of cells to synchronously release into S phase from G0 could also be measured using the synchronisation techniques discussed earlier, if there are any effects it could be measured whether or not they could be rescued by transfection with an expression plasmid containing the Ciz1 gene.

Replication competent nuclei could also be harvested from these cells providing a platform for measuring replication dynamics. Replication dynamics of normally cycling could also be measure using DNA combing and compared to Ciz1 knockout cells. Parameters that could be measured include replication fork velocity and if the DNA purification protocol can be refined to stretch longer strands of DNA, inter origin distance and replication fork asymmetry.

Knockout Ciz1 cells have displayed an increased tumorigenesis in response to DNA damaging agents (Nishibe et al., 2013). It is worth investigating if this phenotype would be true of

knockout cells generated by CRISPR/Cas9, this will be tested by treatment with DNA damaging agents such as hydroxyurea or aphidicolin. Changes in cell proliferation and cell invasiveness could be measures and replication dynamics after treatment could be quantified using DNA combing. This may help to explain how Ciz1 appears to have both a tumour suppressor and oncogenic function.

Ciz1 knockout cells should also be used to measure changes in cellular replication. Ciz1 appears to function to promote cells entering S phase through co-ordination of cyclin E and cyclin A/CDK2 during the G_1/S transition as proposed (Copeland et al., 2010). Knocking out this function may have an observable effect on cell cycle profiles using flow cytometric techniques. The DNA content of a population of cells can be measured using propidium iodide as a marker for DNA content. This then displays the proportion of cells in each phase of the cell cycle. Any change seen in the cell cycle profile in ciz1 knock out for example and increase in the $G_{0/}G_1$ population could be indicative of Ciz1s role. If a change is seen, it should be determined whether this can be rescued by transfection with a Ciz1 expression plasmid.

To add to results that could be gained from generating Ciz1 knockouts using CRISPR/Cas9 experiments should be done utilizing RNA interference to knock down Ciz1 and cyclin levels to investigate the interplay between these factors during DNA replication initiation as well as during ongoing DNA replication. Combining Crispr/Cas9 work with other genetic silencing techniques such as RNA interference will could provide a diverse set of evidence into the role of Ciz1s co-operation with cyclin/CDKs during the G1/S transition and whether alteration of this confers a replication stress phenotype.

5.8 Concluding Remarks

DNA Combing and *in vitro* replication assays are powerful tools for investigating DNA replication during both the initiation and elongation stages. Further refinements to both techniques including new methods and adapting existing methods for acquiring replication nuclei could improve both the quantifiable data and the reproducibility of this techniques. Further adaptation of the post-quiescent release method for nuclei isolation to couple with DNA combing.

Further work should be done to investigate whether Ap4a inhibits DNA replication *in vivo*. The mechanism by which Ap4a inhibits DNA replication in *in vitro* replication experiments should also be investigated, it should be determined whether or not this is through a novel mechanism or if it activates an existent mechanism that halts cell cycle progression.

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