Analysis of cyclin-CDK mediated regulation of the G1/S transition in post quiescent murine fibroblasts

PhD Biomedical and Life Sciences

By

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(M.Res., 2013; M.Tech., 2011)

To

Lancaster University

A Thesis submitted in fulfilment of requirement for the degree of Doctor of Philosophy

November 2017
Declaration

This dissertation is the result of my own work and includes nothing, which is the outcome of work done in collaboration except where specifically indicated in the text. It has not been previously submitted, in part or whole, to any university of institution for any degree, diploma, or other qualification.

Signed:______________________________________________________________

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Urvi Thacker
((M.Tech, M.Res.)
Dedication

This Thesis is dedicated to My Grandfather Shri Purushottam Velji Thacker
Abstract

Eukaryotic cells have evolved a complex set of regulatory proteins to maintain genome stability by regulating DNA replication once and once per cell cycle. Despite intensive investigation, a precise understanding of the proteins that are phosphorylated during the initiation phase of DNA replication remains to be identified. Here we investigate the roles of cyclin E and cyclin A in the G1 to S-phase transition and reconstitute initiation of DNA replication in cyclin depleted nuclei using an *in vitro* DNA replication system.

Using a murine 3T3 fibroblast model, chemical and genetic inhibition of CDK activity was used to evaluate the role of specific cyclin complexes in post-quiescent cells. CDK inhibition reduced levels of CDC6, MCM2 and PCNA in all contexts. Evaluation of the role of individual cyclin-CDK complexes was performed by siRNA mediated depletion. Cyclin E1/E2 depletion showed that Cyclin E regulates CDC6, MCM2 and PCNA at the transcriptional level; whereas Cyclin A regulates their accumulation though protection from the ubiquitin -proteasome system in late G1 phase. Furthermore, ubiquitination of CDC6 and MCM2 was confirmed using His-tagged ubiquitin pulldown assays suggesting that the CDK and the UPS regulate their levels late G1 phase.
Comparison of replication competence was performed using *in vitro* cell free DNA replication assays. Cyclin E depleted nuclei were unable to initiate DNA replication in S phase extracts; whereas cyclin A2 depleted nuclei retained full activity. Crucially, cyclin E depleted nuclei were unable to load CDK/DDK activated MCM2 proteins onto chromatin in G1 phase that correlated with replication competence *in vitro*. The data suggests that sustained cyclin activity is required to maintain pre-RC protein levels and facilitate formation of replication complexes in G1 phase. These results further suggest that perturbations in the CDK network results in destabilisation of replication proteins CDC6, MCM2, Ciz1 and PCNA that blocks replication complex assembly on chromatin, contributing to genomic stability.
Acknowledgements

I would like to express my deepest sense of gratitude to my supervisor Dr. Nikki Copeland for his patience, knowledge and excellent advice throughout my PhD. I am fortunate to work under his supervision. Constructive criticism helped me advance each day and work hard to achieve precision.

I would like to acknowledge all my friends and the members of lab specially, Daria, Tekle and James for making my working environment so pleasant. I would like to specially thank Dr. Jane Andrea for her help with confocal microscope.

Lastly, I would mention my deepest gratitude to my husband Kalyan Nathadwarawala, my parents and family members, for keeping faith in me, showing uncanny love and support to me to achieve my dream. Words are not enough to thank my grandfather for inspiring me and making me strong enough to achieve my goals. Thank you for your blessings! I remain indebted to all the people mentioned above, as no words can thank for their help.
Table of Contents

Abstract........................................................................................................................... iii

Acknowledgements ........................................................................................................ v

Table of Figures ............................................................................................................... xi

List of Tables .................................................................................................................. xiv

Abbreviations ................................................................................................................ xv

Chapter 1: General Introduction .................................................................................... 1

1.1 The cell cycle is divided into four phases................................................................. 2

1.2 Cyclin dependent kinases are the master regulators of the cell cycle. .............. 4

1.3 Regulation of Restriction point and the G1/S transition .................................... 6

1.4 CDK activity is regulated by multiple pathways to facilitate increasing activity through the cell cycle................................................................. 10

1.5 Temporal regulation of DNA replication complex assembly. ............................ 12

1.5.1 Specification of replication origins .................................................................. 13

1.5.2 Formation of the pre-replication complex (pre-RC) ....................................... 14

1.5.3 Formation of Pre-Initiation Complex (Pre-IC) .............................................. 18

1.5.4 Helicase activation and Replisome Formation .............................................. 22

1.6 Proteasomal degradation pathway plays important role in cell cycle regulation ................................................................................................. 25

1.7 Proteasome mediated regulation of DNA replication.......................................... 30

1.8 The quantitative model of CDK mediated cell cycle regulation ......................... 35

1.9 Specificity and degeneracy in cyclin E and cyclin A activities .......................... 38

1.9.1 Role of cyclin E-CDK2 in cell cycle progression .......................................... 38

1.9.2 Role of cyclin A-CDK2: .................................................................................. 41

1.10 Aims and Objectives ......................................................................................... 45

Chapter 2: Material and Methods .............................................................................. 47
2.1 Cell Culture ...........................................................................................................................................47
2.2 Quiescence mediated Cell Synchronization by contact inhibition and serum starvation in 3T3 cells.................................................................48
2.3 Synchronization of HeLa cells in S-Phase using double thymidine block ........................................................................................................49
2.4 SiRNA Transfection in 3T3 cells ...........................................................................................................49
2.5 Subcellular fractionation .......................................................................................................................51
2.6 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting ........................................52
2.7 EdU Labelling ...........................................................................................................................................57
2.8 Confocal imaging .....................................................................................................................................58
2.9 RNA Isolation .........................................................................................................................................58
2.10 Quantitative Real Time Polymerase Chain Reaction (q RT-PCR) ....................................................59
2.11 His(6) tagged ubiquitin plasmid generation ..........................................................................................60
2.12 His(6)-Ub mediated pulldown ............................................................................................................61
2.13 Chemical inhibition of CDK2 and DDK ..............................................................................................62
2.14 Cell free DNA replication assay .........................................................................................................64

Chapter 3 .....................................................................................................................................................68
3.1 Cell synchronization for analysis of temporal regulation of DNA replication........................................69
3.2 Chapter aims ..........................................................................................................................................72
3.3 Experimental approach synchronization of mouse fibroblasts to monitor cell cycle re-entry from G0......................................................................73
3.4 Synchronisation of mouse fibroblasts to monitor cell cycle re-entry from G0 .......................................75
3.5 Analysis of transcript levels and protein levels of Replication assembly proteins from synchronous cell population .................................................77
3.6 CDK2 inhibition destabilises replication assembly of proteins and blocks S phase entry in synchronised cell population........................................81
3.7 DDK inhibition destabilises replication complex assembly of proteins and blocks S phase entry from quiescence........................................84
3.8 Cyclin E –CDK2 regulates transcription of replication complex assembly proteins MCM2, CDC6, PCNA and MCM10.................................87
3.9 Cyclin A –CDK2 does not regulate transcription of replication complex assembly proteins MCM2, CDC6, PCNA and MCM10. ..................90
3.10 Inhibition of CDK2 or DDK reduces CDC6, MCM2, PCNA and cyclin expression by inhibition of E2F mediated transcription.........................93
3.11 Distinct roles for Cyclin E and A regulation of CDC6, MCM2 and PCNA abundance ...........................................................................96
3.12 Discussion .....................................................................................................99

Chapter 4 ...........................................................................................................102
4.1 Introduction: Ubiquitin proteasome system regulates G1 to S transition .................................................................................................103
4.2 APC/C^cdh1 mediated ubiquitination in G1 phase....................................106
4.3 Chapter aims .................................................................................................107
4.4 Proteasomal inhibition promotes stabilisation of MCM2, CDC6 and PCNA in G1 phase after CDK2 inhibition........................................108
4.5 Cyclin A depletion destabilises replication complex proteins (CDC6, PCNA, MCM2, MCM10), by proteasomal mediated degradation........113
4.6 Analysis of ubiquitination of MCM2, CDC6 and PCNA............................116
4.7 Cdh1- potential E3 ligase regulating degradation of MCM2, CDC6 and PCNA in absence of cyclin dependent kinases..............................118
4.8 Discussion .....................................................................................................124
4.8.1 Analysis of Cdh1 activity in regulation of CDC6, MCM2 and PCNA. ................................................................................................................................................. 127

Chapter 5 .................................................................................................................................................................................. 128

5.1 Ciz1 Discovery and structural characterization ................................................................. 129
5.2 Ciz1 function in initiation of DNA replication during G1/S transition 130
5.3 Chapter aims................................................................................................................................. 134
5.4 Ciz1 protein and transcript levels remain constant during G1/S transition: .................. 135
5.5 Ciz1 is destabilised in absence of CDK2 during G1-S transition ................. 138
5.6 Cyclin inhibition destabilizes Ciz1 during G1/S transition: .......................... 140
5.7 Ciz1 is proteasomally regulated in absence of kinase activity during G1-S transition .......................................................................................................................... 143
5.8 Ciz1 interacts with other cell cycle regulators including DHX9 ................. 146
5.9 Discussion ........................................................................................................................................................................... 150
5.9.1 Preliminary characterization of DHX9 in regulation of the G1/S transition ........................................................................................................................................................................ 153

Chapter 6 .................................................................................................................................................................................. 154

6.1 Introduction to cell-free replication assay: ................................................................. 155
6.2 Chapter aims........................................................................................................................................................................... 163
6.3 Analysis of replication complex assembly and initiation of DNA replication in vitro ........................................................................................................................................................................ 164
6.4 Cyclin E depleted nuclei are replication incompetent .............................................. 168
6.5 Cyclin A2 depleted nuclei are replication competent ............................................. 173
6.6 Active kinases in S phase extract are required for initiation of DNA replication in cell-free replication assays .................................................................................................................................................. 178
6.7. Cyclin E1/E2 depletion deregulates MCM2 Compartmentalisation in G1. .................................................................................................................. 181
6.8 Cyclin E is required for phosphorylation of MCM2 required for initiation of DNA replication ................................................................. 187
6.9 Cyclin A2 depleted nuclei can initiate DNA replication when supplemented with recombinant cyclin A-CDK2 ......................................................... 192
6.10 Discussion ................................................................................................. 195
6.10.1 CDK activity regulates MCM2 compartmentalisation in G1 phase. 199
6.10.2 Cyclin E1/E2 depletion prevents efficient phosphorylation of MCM2 in G1 phase ......................................................................................... 200

Chapter 7: General Discussion ........................................................................ 202
7.1 Chemical inhibition of CDK2 and DDK destabilizes pre-RC and replisome proteins. ................................................................. 204
7.2 Cyclin E-CDK2 regulates transcription of replication assembly of proteins ......................................................................................... 206
7.3 Role of UPS in maintaining protein stability in absence of kinase activity during G1/S transition ......................................................... 208
7.4 Ciz1 is regulated by UPS in absence of Cyclin-CDK2 during G1/S transition ......................................................................................... 212
7.5 DHX9 contributes to regulation of the G1/S transition ......................... 213
7.6 In vitro analysis of initiation of DNA replication ..................................... 214
7.7 Cyclin E1/E2 expression is required for phosphorylation of MCM2 to license DNA replication ................................................................. 217
7.7.1 Working Model ....................................................................................... 218
7.8 Future work ............................................................................................... 223
7.9 Concluding Remarks .................................................................................. 224

Chapter 8: References ..................................................................................... 226
### Table of Figures

<table>
<thead>
<tr>
<th>Figure 1</th>
<th>Figure 2</th>
<th>Figure 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1Cell cycle stage specific cyclin-CDK complexes regulate cell cycle transitions.</td>
<td>Transcriptional regulation of cyclin expression bypasses restriction point.</td>
<td>Experimental overview for analysis of cell cycle progression from G1 to S phase.</td>
</tr>
<tr>
<td>Transcriptional regulation of cyclin expression bypasses restriction point.</td>
<td>Replication Licensing.</td>
<td>Establishing cell cycle re-entry kinetics after synchronization in G0.</td>
</tr>
<tr>
<td>Ubiquitin mediated Proteasomal degradation of target proteins.</td>
<td>APC/C and SCF: major E3 ubiquitin ligases regulating cell cycle progression.</td>
<td>Transcription of E2F target genes after release from quiescence.</td>
</tr>
<tr>
<td>DNA replication licensing and initiation is controlled by cyclin – CDK and ubiquitin ligases.</td>
<td>Quantitative model of CDK activity defining cell cycle transitions.</td>
<td>Analysis of replication assembly of proteins in cells entering cell cycle from quiescence.</td>
</tr>
<tr>
<td>APC/C and SCF: major E3 ubiquitin ligases regulating cell cycle progression.</td>
<td></td>
<td>CDK2 inhibition with roscovitine destabilises replication complex assembly of proteins (MCM2, CDC6, PCNA and MCM10) and blocks S phase entry.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DDK inhibition prevents DNA replication and destabilises replication complex assembly of proteins.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cyclin E inhibition reduces replication complex assembly proteins CDC6, MCM2, MCMC10, PCNA abundance.</td>
</tr>
</tbody>
</table>
Figure 3-8 Cyclin A is required to promote accumulation of pre-RC proteins in post quiescent fibroblasts..............................................................91
Figure 3-9 Transcript analysis of replication assembly proteins after CDK2 and DDK inhibition.................................................................94
Figure 3-10 Transcript analysis of replication assembly proteins after siRNA mediated depletion of cyclin E1/E2 and cyclin A2.................................97
Figure 4-1 APC/C and SCF mediated G1/S transition.................................................105
Figure 4-2 Proteasome inhibition recovers destabilised proteins in absence of CDK2.................................................................................................109
Figure 4-3 Proteasome inhibition recovers destabilised proteins in absence of DDK. ..............................................................................................111
Figure 4-4 Destabilized replication proteins are degraded by proteasome mediated system in absence of cyclin A.................................................114
Figure 4-5 His6-Ub mediated pull down of MCM2, CDC6 and PCNA with MG132.................................................................................................117
Figure 4-6 Cdh1 a potential E3 ligase destabilising replication complex assembly in absence of cyclin A.........................................................120
Figure 4-7 CDK2 inhibition and Cdh1 depletion in synchronized cell population..............................................................................................121
Figure 4-8 Cdh1 transcript and protein levels in cells released from quiescence..............................................................................................123
Figure 5-1 The structural representation of Ciz1......................................................130
Figure 5-2 A Model for the role of Ciz1 in initiation of DNA replication. ....132
Figure 5-3 Ciz1 transcript levels remain constant during G1/S transition. ....136
Figure 5-4 Ciz1 is destabilised in absence of CDK2 activity during G1-S transition..............................................................................................138
Figure 5-5 Ciz1 transcript levels are upregulated by cyclin E.............................142
Figure 5-6 Ciz1 protein is proteasomally regulated in absence of CDK2........143
Figure 5-7 Ciz1 is proteasomally regulated in absence of Cyclin A.............144
Figure 5-8 Ciz1 interacts with cell cycle regulator DHX9..........................148
Figure 6-1 Mammalian cell-free DNA replication assay.............................166
Figure 6-2 Cyclin E depleted nuclei are replication incompetent....................171
Figure 6-3 Cyclin A depleted nuclei are replication competent........................176
Figure 6-4 CDK2 activities are required for initiation of DNA replication but not for loading of proteins MCM2, CDC6 and PCNA on to chromatin........179
Figure 6-5 Compartmentalization of MCM2 is regulated by cell cycle stages. ..................................................................................................................................................183
Figure 6-6 MCM2 compartmentalisation defective in cyclin E1/E2 depleted nuclei in cell free replication assays.................................................................185
Figure 6-7 Distinct cyclin E-CDK2 dependent phosphorylation of MCM2 correlates with replication competency.................................................................189
Figure 6-8 Cyclin A2 depleted nuclei can initiate DNA replication in addition of recombinant cyclin A-CDK2.................................................................193
Figure 6-9 Replication licensing......................................................................196
Figure 7-1 Postulated mechanism of action for effect of Cyclin A or CDK depletion in synchronised mouse fibroblasts.........................................................210
Figure 7-2 Working models of distinct mechanisms involved in initiation of DNA replication in G1 nuclei, Cyclin E1/E2 depleted nuclei and cyclin A2 depleted nuclei........................................................................................................220
List of Tables

Table 2-1 List of siRNAs used in siRNA knockdown ...........................................51
Table 2-2 List of antibodies used in western blot ....................................................56
Table 2-3 List of TaqMan primers used in qRT-PCR ..............................................60
Table 2-4 List of Drugs used in experiments as indicated ..........................................63
Table 2-5 Components of pre-mix used in cell free replication assay ....................64
**Abbreviations**

ARS: Autonomous replicating sequence
APC: Anaphase Promoting Complex
CDC6: Cell division cycle protein 6
Cdc20: Cell division cycle protein 20
Cdc45: Cell division cycle Protein 45
CDK: Cyclin dependent Kinase
CMG: Cdc45- MCM 2-7-GINS complex
CSK: Cytoskeletal buffer
Ciz1: Cip1- interacting zinc finger protein
DMEM: Dulbecco’s Modified Eagle Media
DMSO: Dimethylsulfoxide
EdU: Ethynyl deoxyuridine
Emi1: Early mitotic inhibitor 1
MCM: Minichromosome maintainance
ORC: Origin Recognition Complex
PBS: Phosphate Buffer Saline
PBST: Phosphate buffer saline Tween 20
PCNA: Proliferating Cell Nuclear Antigen
Pre RC: Pre–Replication Complex
Pre-IC: Pre-Initiation Complex
Pre-LC: Pre-Licensing Complex
Q-RT PCR: Quantitative Real Time Polymerase chain reaction
SCF: Skp-Cullin –F-Box complex
SDS: Sodium Dodecyl sulphate
siRNA: Small interfering RNA
SKP: S-phase Kinase associated protein
UPS: Ubiquitin proteasome system
WCE: Whole Cell Extract
Chapter 1
General Introduction
Chapter 1: General Introduction

Eukaryotic cells have evolved complex regulatory networks that control cellular growth and division. This process is called the cell cycle and incorporates an ordered system of biochemical switches regulated and governed by cyclin dependent kinases (CDKS). Through phosphorylation of target substrates CDKs drive each phase of the cell cycle to duplicate and segregate identical genetic components into daughter cells (Sclafani and Holzen, 2007). This process must be achieved with high fidelity and utilizes intricate intracellular signaling pathways and regulation of transcriptional programs to precisely regulate cellular division (Rhind and Russell, 2012). The cell cycle control system is of paramount significance in maintenance of cell numbers and tissue organization within the body. Understanding how these processes are regulated is important, as precise control of cellular division is required for stem cell maintenance (Furutachi et al., 2015), coordination of developmental processes (Jukam et al., 2017), and longevity (Kubben and Misteli, 2017) as deregulation of the cell cycle underpins cancer biology (Cecchini et al., 2012).
1.1 The cell cycle is divided into four phases

The process of cellular division is an ordered series of events that can be divided into two main phases (i) Interphase, where the cell increases in size and duplicates its chromosome. (ii) Mitotic phases, where the chromosomes are resolved and segregated equally into two genetically identical daughter cells. Interphase can be further subdivided into G1, S and G2 phases (Figure 1.1). In G1 phase, cells are responsive to mitogenic and anti-mitogenic signaling. The balance of mitogenic/anti-mitogenic signals dictates whether cells continue through the cell cycle or enter a reversible non-proliferative state called quiescence (Daignan-Fornier and Sagot, 2011). In an anti-mitogenic environment, cells enter a state of inactivity or rest termed quiescence, where cells are in a metabolically quiet state (Figure 1.1). In mammals, >95% of cells are in a quiescent state, demonstrating the importance for maintenance of a non-proliferative population for homeostasis (Cheung and Rando, 2013; Mendelson and Frenette, 2014).

In a mitogenic environment, cells exit G1 phase and replicate their DNA in the synthesis (S)-phase. After S-phase cells enter G2 phase or GAP2 phase where the cell proofreads its genetic material to ensure that DNA replication has been successfully completed and prepares for cell division in M phase. Finally, cells enter the mitotic phase (M Phase) that is further divided into five distinct phases namely prophase, prometaphase, metaphase,
anaphase and telophase which is intrinsically linked to cytokinesis (Coffman, 2004; Ivanchuk and Rutka, 2004; Jongsma et al., 2015; Kang et al., 2017; Schafer, 1998)

To ensure that cell cycle transitions occur at the right time, cells utilise the activity of cyclin dependent kinases, where specific cyclin-CDK complexes regulate the transition between each phase of the cell cycle. In addition, to ensure this process is completed accurately, cell cycle checkpoints assess each cell cycle phase is complete prior to cellular division. In eukaryotic cells, the cell-cycle control system controls cell-cycle progression at four major regulatory checkpoints (Bisteau et al., 2014; Lim and Kaldis, 2013). The first checkpoint is the restriction point in late G1 phase, where the cell commits to cell- cycle entry and chromosome duplication (Rhind and Russell, 2012). The second is the intra S-phase checkpoint mediated by ATR at sites of DNA damage leading to recruitment of factors to repair DNA prior to fork re-start (Iyer and Rhind, 2017; Rhind and Russell, 2012). G2/M checkpoint is the third checkpoint whereby cells arrest prior to early mitotic events that lead to chromosome alignment on the spindle in metaphase (Barnum and O’Connell, 2014). Finally, the metaphase to anaphase transition is mediated by the APC\textsuperscript{Cdh1} ubiquitin ligases that promote sister-chromatid separation, leading to the completion of mitosis and cytokinesis. The checkpoint mechanisms ensure that intra and extra-cellular signalling is adequate to promote cellular division
cycle, and that the genome is precisely duplicated prior to segregation into daughter cells (Lim and Kaldis, 2013).

1.2 Cyclin dependent kinases are the master regulators of the cell cycle.

The core members of the cell cycle control system are the cyclin and cyclin dependent kinases (CDKs). In mammalian cells, there are four principle cyclin proteins that play a central role in regulation of the cell cycle: cyclin D, cyclin E, cyclin A and cyclin B. Each cyclin has a specific CDK binding partner. Cyclin D which is an early G1 cyclin forms a complex with CDK4 or CDK6 (Nurse, 1997). Cyclin E interacts with CDK2, which governs the G1-S-phase transition (Stumpf et al., 2013). Cyclin A interacts with CDK2 and CDK1 and regulates S-phase which monitors DNA replication and ensures that the genome is copied once and only once, per cell cycle. Finally, cyclin B-CDK1 governs the transition from G2 into M phase. The unidirectional progression through the mitotic cell cycle is achieved through oscillations in cyclin levels with sequential accumulation and destruction of each cyclin subunit (Figure 1.1) (Enders, 2012; Obaya and Sedivy, 2002).
A: Activity of different cyclins and CDK complexes in the four phases of cell cycle. Early G1 phase is regulated by cyclin D-CDK4/6; whereas late G1 phase regulated by cyclin E-CDK2; S-Phase is regulated by Cyclin A-CDK2 before cyclin A-CDK1 regulates late S-phase and G2 phase; G2 phase to metaphase is regulated by Cyclin B-CDK1.

B: The Cell cycle is divided into four phases namely G1, S, G2 and M phase. Interphase consists of G1, S and G2 phases where cells prepare itself to divide into two daughter nuclei. G1 phase cell prepares itself to undergo DNA replication. In S-phase, the genome is duplicated. In G2 phase post DNA replication cells prepare to enter M Phase. In M-Phase (mitosis) cell divides into 2 daughter cells. (i) Somatic cells are able to enter reversible (quiescent) or irreversible (senescent and differentiated) G0 states from the G1 phase of the cell cycle before the restriction point (R-point). Once cells reach the R-point, they are committed to the next round of the cell cycle (Cheung and Rando, 2013) or (ii) Following mitosis, cells either maintain Rb hyper-phosphorylation and stay in the cell cycle or they lose Rb phosphorylation and enter a transient G0-like state (Cappell et al., 2016)

Figure 1-1 Cell cycle stage specific cyclin-CDK complexes regulate cell cycle transitions.
1.3 Regulation of Restriction point and the G1/S transition

For cell division to occur, extracellular signalling through mitogen signalling is required. For example EGF receptor is activated through EGF ligand binding, autophosphorylation and activation of the Ras GTPase pathway. Ras then promotes activation of the mitogen activated protein kinase (MAPK) cascade leading to activation of c-myc and cyclin D expression (Johnson and Skotheim, 2013; Larsson et al., 1985). Activation of Myc promotes cyclin D expression and enables formation of Cyclin D-CDK4 or CDK6 complexes (Cyclin D-CDK4/6) to form. Cyclin D-CDK4/6 is a principle regulator of the restriction point that ensures that cell divide only in suitable mitogenic signalling environments (Leontieva and Blagosklonny, 2013).

The G1 checkpoint or restriction phase is regulated by the Rb-E2F pathway. Rb binds and inactivates the E2F1-3 family transcription factors and E2F activation is dependent upon CDK mediated phosphorylation of the retinoblastoma protein (Figure 1.2) (Bertoli et al., 2013; Giacinti and Giordano, 2006; Hatakeyama and Weinberg, 1995; Weinberg, 1989). Cyclin D-CDK4/6 mono-phosphorylates one of the 14 sites of Rb in early G1-phase (Narasimha, 2014). Mono-phosphorylation of the retinoblastoma protein (Rb) enables weak transcription of E2F1-3 family regulated genes, required for DNA replication, including cyclin E (Choi et al., 2012; Geng and Sicinski, 2013; Narasimha et al., 2014). Cyclin E binds to and activates CDK2 required to hyper-phosphorylate
Rb and to fully activate E2F1-3 forming a positive feedback loop promoting S-phase gene expression. This results in an increase in E2F–dependent genes that are required for S-phase entry and the initiation of DNA replication (Deshpande et al., 2005; Hanashiro et al., 2008; Herrera et al., 1996; Narasimha et al., 2014; Williams et al., 1994; Yu and Sicinski, 2004).
Sustained mitogen stimulation enables Cyclin D-CDK4/6 mediated mono-phosphorylation of Rb. Monophosphorylation of Rb enables low level activation of E2F mediated transcription of cyclin E along with other proteins involved in G1-S transition and initiation of DNA replication. Cyclin E-CDK2 further hyper-phosphorylates Rb enabling smooth transition of cells post restriction point by origin licensing and feedback mechanism degrading CDK2 inhibitor protein p27. As the cells enter cell cycle from quiescence (G0) and progress into S-phase from early G1 to late G1 kinase activity regulating E2F and Rb phosphorylation allows smooth passage of cells through restriction point. (Adapted from Matson and Cook, 2017)
The restriction point is a bistable switch mechanism that requires increasing CDK activity to bypass the checkpoint imposed by the Rb-E2F pathway, and CDK inhibitor proteins p21 and p27 (Kollarovic et al., 2016; Zhang, 2013). To bypass the restriction point mitogenic stimulation must be sustained to enable an increase in CDK activity that inactivates Rb and leads to degradation of p27, leading to an increase in CDK activity. p27kip1 is a CDK2 regulator that directly inhibits CDK2 kinase activity. p27 is a substrate of cyclin E/CDK2 that is abundant in quiescent and G1 cells. p27 levels are reduced at the G1/S transition, as increasing cyclin E-CDK2 activity promotes phosphorylation of p27 leading to its degradation by polyubiquitylation by the Skp Cullin F-box (SCF) complex (Chen et al., 2012). The inactivation of Rb and the degradation of p27 enforce bypass of the restriction point and facilitates the quantitative increase in cyclin dependent kinase activity that regulates all other cell cycle phases, independently of mitogen stimulation (Kollarovic et al., 2016; Narasimha et al., 2014; Zhang, 2013).
1.4 CDK activity is regulated by multiple pathways to facilitate increasing activity through the cell cycle.

Multilevel regulation of DNA replication by Cyclin-CDK complex ensures that DNA replication takes place once and only once per cell cycle. Mechanisms include (i) Transcriptional regulation of G1 cyclin expression by the Rb-E2F pathway (ii) Regulation of CDK inhibitor proteins levels e.g. p21, p27 (Lim and Kaldis, 2013). (iii) Inhibitory phosphorylation of CDK1 and CDK2 by Wee1 which inactivates the complex (Hengstschläger et al., 1999). (iv) Activation of CDK by CDK activating Kinases (CAK) (v) Ubiquitin - Proteasomal mediated degradation of cyclin subunits via coordinated action of APC/C and SCF E3 ligases maintain cyclin CDK activity (Nakayama and Nakayama, 2006).

Cyclin dependent kinases serve as the key regulators of replication licensing by limiting it to G1 phase. During G1 phase CDK activity remains low, allowing replication factors to load at putative origins. During the transition from G1 to S-phase CDKs activate the factors necessary for origin firing there by initiating DNA replication in S-Phase. High CDK activity is inhibitory to the formation of new pre-RCs (Fisher, 2011; Pacek et al., 2006). CDK is therefore responsible for both removing the license and preventing relicensing in S-phase and beyond.
In addition to the CDKs, cells also utilize the activity of the Dbf4 dependent kinase (DDK), Cdc7 that requires Dbf4 binding for activation (Alver et al., 2017; van Deursen et al., 2012) analogous to the catalytic activation of CDKs through cyclin binding. Periodic stabilization of and destruction of the DBF4 protein during cell cycle regulates DDK activity, with increasing levels during S-phase transition (Blow and Laskey, 2016; Poh et al., 2014). Cdc7, which appears to be already bound on to the origin is activated by Dbf4, as DDK acts at individual origins throughout S-phase to activate early and late-firing origins (Miyazawa-Onami et al., 2017; Tanaka and Araki, 2013).
1.5 Temporal regulation of DNA replication complex assembly.

DNA replication is a sophisticatedly regulated process that imposes the once per cell cycle duplication of the genome. Eukaryotic and prokaryotic DNA replication machinery are loosely conserved, but there are significant differences in the organisation and structure of their genomes. Prokaryotic genomes are covalently closed, forming a circular chromosome that can be duplicated often with a single origin of replication (Nielsen and Lobner-Olesen, 2008). This approach would be inefficient and time consuming when considering the respective genome as *E. coli* has a ~4Mbp genome versus a 3 gigabase human genome. To overcome the logistical challenge of copying large, linear genomes, eukaryotes utilise multiple replication origins that are typically spaced ~ 50-100 kilobases apart (Cayrou et al., 2015; Cvetic and Walter, 2005; Gilbert, 2004). There are approximately 30-50,000 putative replication origins in the human genome (Chagin et al., 2016; Leonard and Mechali, 2013; Nasheuer et al., 2002) ensuring that the entire genome is replicated efficiently. To guarantee that the genome is accurately replicated, cells have established mechanisms that separate replication origin specification, licensing and activation. The separation of these steps is regulated by cyclin dependent kinase activity and the activity of inhibitory proteins such as geminin that prevents replication origin specification in all phases of the cell cycle except G1 phase. Geminin is also supported by the
ubiquitin proteasome system that regulates protein accumulation and restricts key regulator expression to specific stages of the cell cycle.

1.5.1. Specification of replication origins

The specification of DNA replication origins begins by recognition of a putative replication site by binding of a complex of proteins called the origin recognition complex in eukaryotes. This is a conserved feature of DNA replication and other examples include the OriC sequence of *E. coli* and the Autonomously Replication Sequences (ARS) in *S. cerevisiae* (Leonard and Mechali, 2013). Bacterial and yeast replication origins show DNA sequence specificity, whereas metazoan origins do not display high sequence specific requirements (Evertts and Coller, 2012; Leonard and Mechali, 2013; Zhou et al., 2005). OriC and ARS sites can direct replication initiation if they are moved to ectopic sites in the genome, indicating that these initiation sites are genuine replication origins (DePamphilis, 1999; Aladjem, 1998; Altman et al., 2004; Robinson et al., 2005; Zhou et al., 2005; Cayrou et al., 2012). Metazoan origins are similar to fission yeast origins but lack a defined consensus sequence and putative origins of replication are encoded by sequences from one to a few kilo-bases in length (Aladjem et al., 1998; Evertts et al., 2012). There are studies reporting that metazoan origins are AT rich sequences (MacAlpine, 2004; Cayrou, 2012). DNA sequences are not the only factors governing recruitment of ORC, other factors including chromatin structure
and gene transcription have significant influence on the selection of DNA replication origins and timings when origins are activated to initiate DNA replication (Aggarwal et al., 2004; Kohzaki et al., 2005; Leonard et al., 2013).

In budding yeast, the ARS specifies the site that ORC associates with to promote replication complex assembly. In contrast mammalian replication origins do not have specific ARS motifs and display asymmetric AT-rich sequences. Unlike yeast systems purified human ORC complexes display limited sequence specificity and ATP hardly affects the interaction of ORC to DNA. The binding of metazoan ORC to DNA is regulated by chromatin conformation and epigenetic modifications (Vashee et al., 2003; Remus et al., 2004; Rampakakis et al., 2009; Dorn et al., 2011; MacAlpine et al., 2013; Leonard et al., 2013).

1.5.2 Formation of the pre-replication complex (pre-RC)

The ORC complex promotes loading of additional factors that mark putative replication origins and facilitate the higher order protein complex assembly required to copy DNA (Yeeles et al., 2015). Replication origin specification requires low CDK activity. Hence, origin licensing can begin as early as telophase, as nuclear envelopes begin to form around the segregated mitotic chromosomes, although it is not clear if licensing begins at this stage in all species or cell types (Dimitrova et al., 2002; Xouri et al., 2007; Symeonidou et al., 2013). Licensing continues throughout G1 and
ceases at the G1/S-phase transition. The minimal factors contributing to pre-
RC assembly are Origin Recognition complex (ORC), Cell division cycle 6
(CDC6), Minichromosome maintenance proteins 2-7 (MCM2-7) and CDT1
(Maine et al., 1984; Cocker et al., 1996; Gillespie et al., 2001; Remus et al.,
2009; Riera et al., 2017).

Eukaryotic ORC is a heterohexamer composed of six distinct
subunits, Orc1 through Orc6. ORC is the only licensing component that
directly binds origin DNA, and it is required for the nucleation of the pre-
RC (Bryant and Aves, 2011; Patel et al., 2006; Stillman, 2005). CDC6 is a
monomeric protein that is recruited to DNA by protein–protein interactions
with ORC (Bryant and Aves, 2011; Leonard and Mechali, 2013). CDC6 and
the Orc1–Orc5 subunits are members of the AAA+ family of ATPases which
are prevalent in many DNA metabolic processes (Li et al., 2008; Bell, 2014;
Deegan et al., 2016). ORC recruits CDC6 to chromatin leading to
recruitment of CDT1 bound to the MCM complex completing formation of
the pre-RC (Masai et al., 2010; Siddiqui et al., 2013). CDT1 interacts directly
with the MCM complex in solution and with both ORC and CDC6 (Cook et
al., 2004; Chen et al., 2007; Zhang et al., 2010; Fernandez-Cid et al., 2013). In
the absence of CDT1, MCM complexes are never recruited to DNA (Coster
et al., 2014; Mehanna and Diffley, 2012; Yuan et al., 2017). In that regard,
one likely role for CDT1 in replication licensing is as a molecular bridge or
‘courier’ to deliver soluble MCM complexes to DNA-bound ORC/CDC6. In support of that model, recent single molecule studies using purified yeast licensing proteins discovered that CDT1 is rapidly released upon successful loading of each MCM complex. By following fluorescently labelled proteins (Ticau et al., 2015) showed CDT1 and CDC6 release between the two rounds of MCM loading. This rapid shuttling between the bound and soluble states for both CDT1 and CDC6 suggests that each molecule could participate in many origin licensing events. Perhaps for this reason, the levels of both CDC6 and CDT1 are highly regulated during the cell cycle to prevent inappropriate origin licensing.

The correct loading of MCM double hexamers renders an origin competent for subsequent replication initiation, or ‘firing’, during S-phase (Deegan and Diffley, 2016). MCM2-7 does not display helicase activity in G1-phase as additional factors are required to facilitate helicase activation. Somewhat surprisingly, eukaryotic cells load many more MCM double hexamers than the number of DNA-bound ORCs (Edwards et al., 2002). At least 10-fold more origins can be licensed than are strictly required for complete replication under normal circumstances, though the degree of origin licensing likely varies among cells, tissues, and species (Woodward et al., 2006; Powell et al., 2015).
The MCM complex is a hexameric ring in solution before it is loaded (Ilves et al., 2010; Boskovic et al., 2016). MCM loading is therefore not a process of assembling the hetero-hexamers on DNA from their component subunits, but rather, loading pre-assembled hexamers onto DNA (Yuan et al., 2017). DNA passes through a side “gate” between the Mcm2 and Mcm5 subunits, and much speculation currently swirls around the mechanism and dynamics of MCM gate opening and closing (Costa et al., 2008; Bochman et al, 2010; Samel et al., 2014). Moreover, the MCM double hexamer central channels contain double-stranded DNA in G1 but the active MCM helicase at replication forks encircles single-stranded DNA and displaces the second strand (Boos et al., 2012; Graham et al., 2016).

In vitro, loaded MCM double hexamers can slide along DNA away from ORC, leaving space near ORC for another round of MCM loading (Ervin et al., 2009; Remus et al., 2009; Coster et al., 2014), and recent results suggest that MCMs may also slide in vivo (Di Gregorio et al., 2015; Powell et al., 2015). In a typical S-phase, some MCM complexes that had been loaded in G1 are activated as part of the regular replication program, whereas others initiate replication in response to nearby stalled or damaged replication forks to ensure replication completion. Origins that are only utilized under the latter conditions of replication stress are termed “dormant” origins, and they
safeguard the genome against under-replication (Ibarra et al., 2008; McIntosh et al., 2012).

1.5.3 **Formation of Pre-Initiation Complex (Pre-IC)**

The mechanism for pre-IC formation is conserved in eukaryotes. The pre-IC consists of Cdc45, MCM2-7 and GINS (CMG) complex that constitute the replicative helicase, which is associated with the replisome during S phase (Villa et al., 2016). The loading of the CMG complex is facilitated by MCM10, which associates directly with MCM2-7 in a low affinity complex. This complex then serves to facilitate associations with the CMG complex resulting in formation of a higher affinity complex (Douglas and Diffley, 2016). Association of MCM10 to the pre-RC complex facilitates recruitment of initiation factors leading to the formation of pre-initiation complex (pre-IC). Therefore, MCM10 coordinates the assembly and activation of the helicase via CDK2 and DDK mediated phosphorylation of the helicase. This critical mechanism ensures that MCM2-7 double hexamer complexes are assembled and activated in a coordinated manner (Douglas and Diffley, 2016; Perez-Arnaiz et al., 2016).

In *S. cerevisiae* Dbf4-dependent kinase activates the CMG complex (Heller et al., 2011) and facilitates higher order complex formation that enables CMG recruitment. CDK-mediated phosphorylation of Sld2 and Sld3 promotes Dpb11 binding, CMG loading and activation of MCM2-7 helicase
activity (Chuang et al., 2009; Takeda et al., 2005; Tanaka et al., 2007; Zegerman and Diffley, 2007). Sld3 (Treslin- homologue) coordinates CDC45 recruitment to MCM2-7 with DDK phosphorylation of MCM2 during S phase (Bruck and Kaplan, 2015). These mechanisms are conserved in Metazoa as the Sld3 homologue Treslin, binds to TopBP1 (Dpb11) after CDK-mediated phosphorylation in human and Xenopus systems (Kumagai et al., 2010; Kumagai et al., 2011; Boos et al., 2011).

Origin firing requires phosphorylation events from CDKs and a replication-specific kinase, DDK. These kinases promote the recruitment of additional essential helicase components, CDC45 and GINS, to activate DNA unwinding (Labib 2010; Ilves et al., 2010; Tanaka et al., 2013). The loading of the CMG complex is a critical step in the regulation of origin activation. Therefore, higher eukaryotes have evolved degenerate mechanisms to complete pre-IC formation mediated by DUE-B, GEMC1 and MDM2 binding protein (MTBP). DUE-B interacts with CDC45 in a DDK regulated process to promote recruitment of the CMG complex to chromatin (Chowdhury et al., 2010; Gao et al., 2014). Similarly, GEMC1 recruits CDC45 to chromatin in a cyclin E-CDK2 dependent step required for initiation of DNA replication (Balestrini et al., 2010). The efficiency of CMG recruitment is enhanced by MTBP, which is required for efficient CMG loading and initiation of DNA replication (Boos et al., 2011; Boos et al., 2013). Each
mechanism appears to be required for the efficient loading and activation of the replisome and contribute to mechanisms that ensure that DNA replication occurs once per cell cycle.
DNA replication is a multi-step process involving loading and unloading of various proteins in a timely manner in G1 phase. First step involves formation of pre-RC which is initiated by recruitment of ORC onto putative replication origins. CDC6 and CDT1 then interact with ORC in an ATP-dependent manner in order to recruit MCM2-7 complex (Helicase) onto the chromatin leading to the formation of pre-RC. MCM10, CDC45 along with GINS are recruited onto pre-RC to form Pre-initiation complex. Recruitment of these proteins is facilitated by cyclin E-CDK2. Completion of the pre-RC at the origin is referred to as replication licensing. Cyclin E-CDK2 phosphorylates CDC6 protecting from APC/C^Cdh1 mediated ubiquitination. Cyclin E-CDK2 along with Dbf4 dependent kinase (DDK) enables recruitment of replisome factors which leads to helicase activation by phosphorylating MCM 2-7 complexes. Cyclin A-CDK2 then facilitates loading of polymerase with PCNA and results in Replisome formation. Ciz1 binds to the pre-replication complex via CDC6 interactions during G1 phase and coordinates with cyclin A-CDK2 to promote initiation of DNA replication (adapted from Pauzaite, 2016).
1.5.4 Helicase activation and Replisome Formation

The CDK–mediated recruitment of DNA polymerases and accessory factors is the final step in initiation of DNA replication. The process of DNA replication requires three replicative polymerases—Pol α, Pol δ, and Pol ε— which associate with the processivity factor proliferating cell nuclear antigen (PCNA) (Stillman, 2005; Takeda et al., 2005). RNA primers on both leading strand and lagging strands are synthesised by DNA polymerase α (Pol α) that can then be utilized by processive DNA polymerases δ and ε. DNA polymerases α and ε perform leading-strand synthesis and Pol α and Pol δ perform lagging-strand synthesis with high nucleotide selectivity and efficient proofreading (Simon et al., 2014). Ctf4 is required for sister chromatid adhesion and for the stable assembly of the CMG complex and polymerases in the replisome (Samora et al., 2016; Takeda et al., 2005). The leading strand Pol ε is positioned ahead of CMG helicase, whereas Ctf4 and the lagging-strand polymerases Pol α/δ are behind the helicase (Takeda et al., 2005; Villa et al., 2016; Stillman et al., 2015; Sun et al., 2016). DNA polymerases have a semi closed hand structure, which allows them to load onto DNA and translocate (Leman and Noguchi, 2013; Meli et al., 2016). This structure permits DNA polymerases to hold the single-stranded template, incorporate dNTPs at the active site, and release the newly formed double
strand. However, the conformation of DNA polymerases does not allow for their stable interaction with the template DNA (Leman et al., 2013).

DNA polymerases require additional factors to support DNA replication in vivo and strengthen the interaction between template and polymerase, DNA sliding clamps, aid template binding and increase processivity of the polymerases. In eukaryotes, this sliding clamp is a homo-trimer known as proliferating cell nuclear antigen (PCNA), which forms a ring structure (Bell and Dutta, 2002; Saner et al., 2013). The PCNA ring has polarity with a surface that interacts with DNA polymerases and tethers them securely to DNA. PCNA-dependent stabilization of DNA polymerases has a significant effect on DNA replication because it enhances polymerase processivity up to 1,000-fold (Bravo et al., 1987; Prelich et al., 1987; Zhuang, 2010; Leman, 2013). Such stimulation of replication activity makes PCNA an essential cofactor in vivo. PCNA also has the distinction of being one of the most common interaction platforms in the replisome to accommodate multiple processes at the replication fork (Moldovan et al., 2007). Therefore, PCNA is often viewed as a regulatory cofactor for DNA polymerases (Bell, 2014; Duzdevich et al., 2015).

PCNA fully encircles DNA and must be loaded onto DNA at the replication fork. At the leading strand, this is a relatively infrequent process, because DNA replication is essentially continuous until termination (Leman
and Noguchi, 2013; Stillman, 2015). However, at the lagging strand, Pol δ is continually loaded at the start of each Okazaki fragment (Balakrishnan and Bambara, 2011). This constant initiation of Okazaki fragment synthesis requires repeated PCNA loading for efficient DNA replication. PCNA loading is accomplished by the replication factor C (RFC) complex, which is comprised of five AAA+ ATPases (Downey et al., 2010; Cai et al., 1998). RFC recognizes primer-template junctions and loads PCNA at these sites (Podust et al., 1995; Bloom et al., 2009). The PCNA homotrimer is opened by RFC using energy from ATP hydrolysis and is then loaded onto DNA in the proper orientation to facilitate its association with the polymerase (Zhang et al., 1999; Yao et al., 2006; Bloom et al., 2009; Thompson et al., 2009). Clamp loaders can also unload PCNA from DNA, an important mechanism during DNA replication termination (Yao et al., 2006; Thompson et al., 2009). Together these complexes mediate high fidelity duplication of the genome.
1.6 **Proteasomal degradation pathway plays important role in cell cycle regulation**

To enforce the unidirectional progress through the cell cycle, cyclin dependent kinase activity is coordinated with the ubiquitin–proteasome system (UPS) leading to smooth transition between cell cycle phases. The combined activity of the UPS and cyclin–CDK network guarantees timely duplication of the genetic material and its equal segregation into daughter cells, maintaining genome integrity and cell viability. Deregulation of protein ubiquitination or degradation processes can lead to aberrant cell proliferation and cancer (Nakayama and Nakayama, 2006).

Ubiquitin is a small (8kD) protein consisting of 76 amino acids and is highly conserved. Ubiquitin is covalently attached to target substrates by a cascade of enzymatic reactions leading to polyubiquitination and targeting to the 26S proteasome for degradation. Ubiquitin activating enzyme (E1) covalently autoubiquitylates itself to begin the process of transferring ubiquitin via E2 and E3 ligases to target substrates (Yu, 2011). Ubiquitin conjugating enzyme (E2) then transfers the activated ubiquitin molecule to the lysine residue on substrate with the help of ubiquitin ligase enzyme (E3). In some cases, E3 ligases work in coordination with set of adaptor proteins or substrates known as cullins (Choudhury et al., 2016). These ligases contain a RING (really interesting new gene)-finger domain, which is responsible for
interaction with E2 (Micel et al., 2013). Multiple ubiquitination cycles result in poly-ubiquitinated substrates by linking ubiquitin to Lysine-48 (K48, or K-11-another potential target lysine) (Grice and Nathan, 2016) resulting in poly-ubiquitin chain on the substrate which is recognized by proteasome. The multi-subunit 26S proteasome recognizes poly-ubiquitinated substrates and unfolds the polypeptide leading to its degradation into small peptides (Lub et al., 2016). This reaction takes place within the cylindrical core of proteasomal complex in ATP dependent manner (Bassermann et al., 2014; Teixeira and Reed, 2013).

Figure 1-4 Ubiquitin mediated Proteasomal degradation of target
Ubiquitin molecules are transferred to E1 enzyme, in an ATP dependent process. Ubiquitin is then transferred to E2 enzyme and subsequently transferred to target protein with the help of E3 enzyme. Where the target protein is poly-ubiquitinated and it is subjected to degradation via 26S proteasome complex. This is the general mechanism of Ubiquitin- proteasome pathway.
Cell cycle regulation via proteasomal mediated pathway involves one large subfamily of RING-finger E3 ligases comprising the cullin-RING ligases (CRLs). This includes two structurally similar enzymes: (a) the anaphase promoting complex/cyclosome (APC/C) and (b) Skp/cullin/F-box containing complexes (SCF) (Figure 1.6).

The APC/C complex is a prominent E3 ubiquitin ligase involved in cell cycle regulation. It consists of fourteen different subunits including APC/C11 RING-finger protein that interacts with an E2 enzyme to which APC/C2 cullin-like subunit serves as scaffold. Ube2S is one of the enzymes responsible for elongation and specificity of ubiquitin chains on APC/C substrates (Zhao et al., 2013). APC/C has two co-activators: Cdc20 (also known as Slp1 and Fzy) and Cdc20 homolog 1 (Cdh1) (also known as Fzr1), which define substrate
specificity and associate with the APC/C core at defined stages of the cell cycle. While SCF ligases can be active throughout the cell cycle, APC/C activity is restricted to the time between metaphase and the end of the next G_1 phase (Bassermann et al., 2014).

One of the mechanisms by which adaptor proteins work is by recognition of short destruction motifs (D-box and KEN-box) on target substrates through C-terminal domains composed of WD40 repeats. Cdc20 preferentially recognizes D-box, whereas Cdh1 recognizes both D-box and KEN-box motifs. APC/C is further regulated by APC/C inhibitors, such as Emi1 and phosphorylation of the APC/C core (Nakayama and Nakayama, 2006).

Among various targets of APC/C^{Cdh1} DNA replication protein CDC6 is one of the proteins that is degraded via this proteasome machinery (Mailand and Diffley, 2005). This further regulates the transition of G1/S phase by ensuring that replication licensing occurs and CDK activity is maintained at a low level. Cyclin D1 levels are controlled by APC/C^{Cdh1} by negatively regulating Ets2, a transcription factor involved in Ras-Raf–MAPK signalling (Bassermann et al., 2014). APC/C^{Cdh1} activity also poly-ubiquitinates positive cell cycle regulators including Skp2 (Bashir and Pagano, 2004). Specifically, Skp2 (S-phase kinase-associated protein, aka Fbl1) is targeted for proteolysis.
to avert premature formation of the SCF$^{\text{Skp2}}$ complex, which promotes cell cycle progression by mediating the degradation of CKIs (Bassermann et al., 2014). Importantly, the Cdc25A phosphatase, which promotes S-phase entry and mitosis by dephosphorylating and thus activating CDK2 and CDK1, is also kept at low levels during early G1 phase by APC/C$^{\text{Cdh1}}$ (Bassermann et al., 2014; de Boer et al., 2016).
1.7 Proteasome mediated regulation of DNA replication

Regulation of DNA replication relies precisely on following principles. First, the assembly of pre-RCs is restricted to a period of high APC/C and low CDK activity. APC/C promotes pre-RC assembly by eliminating cyclins and, in metazoans, geminin. Second, pre-RC assembly is generally inhibited by multiple mechanisms. Cells have evolved multiple mechanisms to ensure inhibition of pre-RC assembly including: UPS mediated proteolysis, nuclear export, inactivation by CDK mediated phosphorylation and inhibitor binding e.g geminin mediated inhibition of replication complex assembly. Therefore, cells use multiple pathways to prevent licensing at other phases of the cell cycle as this will result in re-replication of the genome. The combined activities of the CDK network and the UPS ensure that key regulators accumulate and are destroyed according to need. These mechanisms ensure that replication licensing is restricted and occurs only from telophase to restriction point to maintain genome stability.

Orc2–6 (together with CDC6) remain bound to chromatin during G1, S and G2 phases, the Orc1 subunit is released from chromatin during S-phase (Kreitz et al., 2001; Mendez et al., 2002; Li et al., 2002). Orc1 was degraded in a SCF\textsuperscript{Skp2}-dependent manner which presumably requires CDK phosphorylation of ORC (Mendez et al., 2002). Using a hamster cell line, Orc1 was not degraded, but instead was released from chromatin during S-phase when it
accumulated as either a mono-ubiquitinated or di-ubiquitinated form (Li et al., 2002). Finally, overexpression of cyclin A or K cyclin from Kaposi sarcoma-associated herpesvirus induces the relocalisation of Orc1 to the cytoplasm (Laman et al., 2001).

In mammals, the APC/C plays an important role in regulating pre-RC assembly. CDC6 is absent from early G1 and quiescent cells in many (but not all) cell lines (Mendez et al., 2000; Petersen et al., 2000; Alexandrow et al., 2004). Overexpression of Cdh1 is sufficient to induce CDC6 degradation in human cells (Petersen et al., 2000; Mailand and Diffley, 2005), suggesting that APC/C\textsuperscript{Cdh1} is important in regulating CDC6 proteolysis. CDKs also appear to play a role in inhibiting inappropriate pre-RC assembly. When CDKs are inhibited in G2 phase, either by treatment of cells with 6-DMAP or genetic ablation of CDK1, MCM2-7 rebinds and chromatin is re-licensed (Coverley et al., 1996; Coverley et al., 1998; Fujita et al., 1998; Diffley, 2004). In addition to being an APC/C substrate, CDC6 can also be targeted for proteolysis by cyclin A–CDK2 (Coverley et al., 2000). This proteolysis only affects soluble forms of CDC6, while forms that are bound to chromatin persist through S and G2 phases (Coverley et al., 2000; Fujita et al., 1999; Mendez et al., 2000; Alexandrow et al., 2004). CDKs can also regulate the nuclear localisation of ectopically expressed CDC6 (Delmolino et al., 2001), which is nuclear in G1 phase but redistributes to the cytoplasm in S and G2 phases. This
relocalisation requires CDK sites in the amino terminus of CDC6. Expression of cyclin A (but not cyclin E) regulates relocalisation of CDC6. Thus, the pool of CDC6 which is not bound to chromatin may be targeted for proteolysis and/or nuclear export by CDKs.

**Figure 1-6 DNA replication licensing and initiation is controlled by cyclin – CDK and ubiquitin ligases.**

Origins are licensed by the DNA loading of MCM complexes in late G1. During early G1, MCM loading is blocked through the APC$_{Cdh1}$-mediated destruction of CDC6. CDK mediated phosphorylation of CDC6 prevents its degradation by APC$_{Cdh1}$ mediated polyubiquitination. In late G1, MCM complexes are loaded at origins by the combined action of ORC, CDC6, and CDT1. Once cells enter S-phase, CDC6 is phosphorylated at Ser106 phosphorylation, which promotes CDC6 nuclear export. Three overlapping mechanisms suppress CDT1 activity during S-phase (Figure adapted from Rizzardi and cook, 2012).

CDT1 is regulated by two mechanisms, first by proteolytic degradation by SCF$_{Skp2}$ (Sugimoto et al., 2004; Nishitani et al., 2004) and second, by inhibiting a novel DNA binding activity of CDT1 (Sugimoto et al., 2004). CDK
inactivation in anaphase promotes the re-accumulation of CDT1 on chromatin (Sugimoto et al., 2004) and overexpression of CDT1 together with CDC6 induces re-replication (Vaziri et al., 2003). These examples demonstrate the importance of the UPS in regulation of the pre-RC proteins to ensure that replication licensing can only occur in G1 phase of the cell cycle (Figure 1.7).

Another E3 ubiquitin ligase required for proper cell cycle progression is CRL4Cdt2, which is known to have role in regulation of PCNA (Rizzardi et al., 2015). This complex is composed of Cul4, Roc1/2, an adaptor protein DDB1, and a substrate receptor or DCAF (DDB1- and Cul4-associated factor) that interacts directly with DDB1. There are at least 20 known DCAs encoded in the human genome (Havens and Walter, 2011). The DCAF Cdt2 (Cdc10-dependent transcript 2) is unique among DCAs in that its interaction with substrates is dependent on the substrate first interacting with DNA-loaded PCNA (Havens and Walter, 2011). Proteins that interact with PCNA contain a motif known as a PCNA-interacting protein box (PIP box) (Burgess et al., 2013; Masuda et al., 2015). Interestingly, PCNA must be loaded onto DNA by the RFC complex to promote interaction between the substrate and Cdt2, although the basis for substrates to distinguish soluble PCNA from DNA-loaded PCNA is not yet clear (Havens et al., 2009; Shiomi et al.,
2012). Through the requirement for substrate interaction with PCNA, CRL4<sup>Cdt2</sup>-mediated proteolysis is coupled to DNA replication during S-phase.

In contrast to APC/C, the SCF<sup>Skp2</sup> ubiquitin ligase complex becomes active later at the G1/S transition and remains active throughout S-phase. The SCF complex is composed of only 4 subunits: Cul1 (cullin scaffold), Roc1 (RING-domain protein), Skp1 (adaptor protein), and one of many F-box protein substrate adaptors. Skp1 associates with many different F-box proteins that confer substrate specificity. During G1, APC<sup>Cdh1</sup> inhibits SCF<sup>Skp2</sup> activity through ubiquitination and degradation of Skp2; this degradation is dependent on the N-terminal D-box of Skp2 (Bashir et al., 2004; Rodier et al., 2008). Later in G1, however, Skp2 is phosphorylated at Ser64/72 by Cyclin E-CDK2, and Skp2 continues to be phosphorylated, but by Cyclin A-CDK2 in S-phase. Phosphorylation at Ser64, and to a lesser extent at Ser72, prevents Skp2 ubiquitination by APC<sup>Cdh1</sup> (Wei et al., 2004; Bashir et al., 2004; Rodier et al., 2008). Once active, SCF<sup>Skp2</sup> promotes S-phase progression and contributes to the inhibition of origin licensing during S-phase.
1.8 The quantitative model of CDK mediated cell cycle regulation

Cyclin-dependent kinases are the key regulators of the cell cycle and their oscillating activity contributes to separation of replication licensing from replication origin firing, thereby restricting replication of their genome once per cell cycle (Reusswig et al., 2016). In cycling cells, pre-RC assembly begins during late mitosis (Diffley et al., 1994; Rowley et al., 1994) as the CDK activity drops due to anaphase promoting complex/cyclosome activation, cyclin degradation and phosphatase activation. In quiescent cells re-entering the cell cycle, pre-RC formation is facilitated by the activity of CDK and DDK during G1 phase (Bruck and Kaplan, 2015; Coverley et al., 2002; Geng et al., 2007; Mailand and Diffley, 2005). In both quiescent and cycling cells, separation of the replication licensing phase from the activation of replication origins is mediated by rising CDK activity (Figure 1.8). This quantitative model of CDK function defines thresholds that demarcate boundaries within, and between, stages of the cell cycle (Coudreuse and Nurse, 2010; Uhlmann et al., 2011).
Cyclin–CDK activity oscillates through the cell cycle defining key transition thresholds, threshold at S-Phase ($T_S$) and mitosis ($T_M$) that mark the G1/S transition and G2/M transition respectively. CDK activity also regulates temporal regulation of the replication licensing phase from the replication initiation phase where licensing is actively inhibited by high CDK activity. (Figure adapted from Uhlmann et al., 2011).

CDK activity temporally regulates replication origin specification and pre-RC assembly, restricting these events to early G1 phase when CDK activity is low. As CDK activity rises during mid- to late-G1 phase this process is inhibited. Inhibition of pre-RC formation is mediated by steric exclusion of pre-RC assembly by direct cyclin binding (Chen and Bell, 2011; Mimura et al., 2004; Wilmes et al., 2004) or CDK phosphorylation of the pre-RC components Orc2/6, CDC6 and CDT1 (Diril et al., 2012; Mimura et al., 2004; Takara and Bell, 2011). Phosphorylation of Orc2/6, CDC6 and CDT1 results in relocalization to the cytosol or ubiquitin proteasome system-mediated destruction that prevents inappropriate re-licensing at high CDK activity (Chen and Bell, 2011; Drury et al., 2000; Johansson et al., 2014; Walter...
et al., 2016). In addition, if CDK activity is too high, phosphorylation of DNA pol α prevents DNA replication at the G1/S transition (Voitenleitner et al., 1997; Voitenleitner et al., 1999). Therefore, CDK activity contributes to the regulation of origin assembly, activation of replication origins and prevention of re-replication at non-permissive concentrations in late stages of the cell cycle.
1.9 Specificity and degeneracy in cyclin E and cyclin A activities.

The quantitative model of CDK activity suggests that an increase in CDK activity should be sufficient to progress through the cell cycle (Nurse, 1996). In fission yeast this was demonstrated using a cdc13-cdc2 fusion protein that could recapitulate all events in the mitotic cell cycle in *S. pombe* (Coudreuse and Nurse, 2010). In mammalian contexts, the quantitative model is more complex as no single cyclin-CDK complex can regulate the entire cell cycle. However, there are degenerate activities mediated by CDK1 as the CDK2/4/6 ablated mouse fibroblasts were able to complete a mitotic cell (Santamaria et al., 2007).

1.9.1 Role of cyclin E-CDK2 in cell cycle progression

Cyclin E-knockout mouse models have been one of the key sources of experimental evidence in understanding the role of cyclin E-CDK2 in cell cycle progression (Parisi et al., 2003; Geng et al., 2003). These studies identified the essential function of cyclin E in development as supporting endo-replication in the generation of poly-ploid cells like megakaryocytes, which generate platelets and trophoblast giant cells, necessary for placental formation (Parisi et al., 2003; Geng et al., 2003); conversely using tetraploid complementation, cyclin E is dispensable for development of the embryo proper (Geng et al., 2007). Notably, the critical substrate for cyclin E-CDK2 during endo-replication is not known, in part because a full accounting of the
molecular determinants of endo-mitoses relative to diploid cell cycles is not yet established. It should be noted that cyclin E-knockout embryos also exhibited frequent, severe developmental defects affecting the atrioventricular cushions and aortic arches (Geng et al., 2003). Further, since knockout mice were unable to be studied into adulthood, a reasonable speculation is that other abnormalities would have been observed as a result of cyclin E ablation, especially in continuously proliferating tissues, such as hematopoietic and epithelial cells.

Cyclin E-knockout mouse embryonic fibroblasts (MEFs) proliferate normally under conditions of continuous cell cycling; however, these cells are unable to re-enter the cell cycle from the quiescent state due to impaired minichromosome maintenance (MCM) protein loading onto replication origins during G0-to-S-phase progression (Geng et al., 2003). In subsequent work, the Sicinski group sought to identify the critical function of cyclin E that supports cell cycle re-entry from G0. They found that an engineered cyclin E mutant defective for CDK activation was competent for loading MCM2 onto chromatin during the Go-to-S-phase transition (Geng et al., 2007). Thus, these results indicate that the essential cyclin E function in cell cycle progression past quiescence is kinase-independent. Reed and co-workers have contended that cyclin E-CDK2 kinase activity is required for
MCM2 loading by promoting the accumulation of CDC6 and Cdc7 mRNAs, possibly through cyclin E-CDK2-mediated E2F transcriptional activation (Chuang et al., 2009; Barr et al., 2016). They further reported that Dbf4-Cdc7 kinase phosphorylation of MCM2 at serine-5 is critical for its loading onto chromatin during G0-to-S transit.

Cyclin E-CDK2 directly promotes origin licensing by phosphorylating CDC6, thus protecting it from APC/C-dependent proteolysis (Mailand and Diffley, 2005). These findings are also at odds with the results obtained from studies of cyclin E-null MEFs, which show no defects in chromatin loading of CDC6 and CDT1 during G0-to-S progression (Geng et al., 2007). Moreover, CDC6 and mitogen-activated protein kinase (MAPK) in some cells appear to cooperate in recruiting active cyclin E-CDK2 complexes to chromatin to stimulate S-phase entry (Lunn et al., 2010). Differences in cell types, experimental manipulations of cyclin E expression and/or synchronization strategies may explain some of the disparate results among these studies. Ultimately though, the models proposed for a critical cyclin E function in regulating S-phase entry following quiescence that is kinase-dependent vs. one in which it is kinase-independent seem mutually exclusive. Further impetus to understand the basis of kinase-independent functions of cyclin E is provided by another provocative finding that emerged from the studies of
cyclin E-knockout MEFs; namely, that their resistance to Ras-induced transformation is reversed by re-expression of the kinase-dead cyclin E mutant (Geng et al., 2007). Determining the critical function of cyclin E in supporting transformation has obvious therapeutic implications, as envisioning a therapy to inhibit a kinase-independent function of cyclin E likely presents greater challenges than one that targets CDK2 activity.

1.9.2 Role of cyclin A-CDK2:

Cyclin A is important in the metazoan cell cycle for the onset of both DNA replication and mitosis. *Cyclin A* genes have been found in all multicellular organisms, including humans (Pines and Hunter, 1990). While only a single *cyclin A* gene is present in the genomes of C. *elegans* and *Drosophila*, mammalian cells express two A-type cyclins, A1 and A2 (Nieduszynski et al., 2002). Cyclin A1 is expressed almost exclusively in the testes and during meiosis in the male germline (Sweeney et al., 1996; Yang et al., 1997) and male knockout mice lacking cyclin A1 are sterile because of an arrest in meiotic prophase at the diplotene stage, just before the first meiotic division (Liu et al., 1998). The second mammalian A-type cyclin, cyclin A2 is ubiquitously expressed in all proliferating cells and is generally considered to be the mammalian S-phase cyclin (Pine and Hunter 1990; Yam et al., 2002; Hochegger et al., 2008). Elimination of cyclin A function by microinjecting antibodies against cyclin A or by expressing antisense cyclin A
RNA has been shown to inhibit DNA synthesis and mitosis (Girard et al., 1991; Pagano et al., 1992). Also, precocious expression of cyclin A in G1 cells accelerated their S-phase entry, indicating that cyclin A is rate limiting for the G1-to-S (G1/S) transition (Resnitzky et al., 1995). Deletion analyses of Xenopus laevis cyclin A (Kobayashi et al., 1992) have shown that it can be separated into three domains: the N-terminal domain, which is essential for periodic degradation of the cyclin A protein; the central cyclin box domain; and the C-terminal domain, whose function is largely unknown. X-ray crystallography of the human cyclin A-CDK2 complex has revealed that cyclin A interacts with CDK via the cyclin box domain and that the C-terminal domain is basically free of the CDK interaction (Jeffrey et al., 1995). In addition, a conserved substrate-docking site in the cyclin box domain has been identified (Schulman et al., 1998). This site is implicated in binding to various cyclin-CDK substrates through their cyclin-binding motifs (RXL motifs) but is not likely to contribute to substrate specificity, since the docking site and the RXL motifs are widely conserved among different cyclins and substrates (Miller et al., 2001; Roberts et al., 2012). In contrast, the C-terminal domain contains several clusters of charged amino acid residues on the protein surface and could therefore be involved in the interaction with a specific target protein.

During cell-cycle progression, cyclin A2 is induced at the beginning of the S-phase (Erlandsson et al., 2000; Girard et al., 1991). Once induced, cyclin
A2 binds and activates its catalytic partners, cyclin-dependent kinases CDK2 and CDK1. These cyclin A2-CDK complexes phosphorylate critical proteins that play role in DNA synthesis, thereby driving S-phase progression (Yam et al., 2002). Cyclin A2 is expressed throughout the S and G2 phases, and is rapidly degraded upon entry of cells into mitosis (Geley et al., 2001). CDK2 null mouse embryonic fibroblasts (MEFs) have decreased cyclin A kinase activity when compared with wild type MEFs after release from quiescence (Berthet et al., 2003), suggesting that cyclin A is not compensating for CDK2 at the G1/S. In addition, cyclin A associated kinase activity alone is not able to rescue the deficiency of cyclin E after release from quiescence. Cyclin E1/-/E2-/- MEFs do not enter the cell cycle despite that their cyclin A activity is similar as in wild-type MEFs (Geng et al., 2003). A more striking observation is the fact that cyclin A associated kinase activity is present in CDK2 null spleen extracts from young mice and in primary CDK2 null MEFs but is undetectable in CDK2 null spleen extracts from adult mice and in immortalized CDK2 null MEFs (Berthet et al., 2003). Similarly, when CDK2 is knocked down in human immortalized colon cancer cell lines there is no detectable cyclin A associated kinase activity (Tetsu and McCormick, 2003). This suggests that in embryos (or primary embryonic cells) the regulation of cell cycle is dependent on cyclin A, while in adult mice (or immortalized cell lines), new pathways evolve that replace the essential function of cyclin A or its associated kinases.
Indeed, cyclin A2 is essential for mouse development and its ablation results in embryonic lethality (Murphy et al., 1997).

While redundancy may provide the means for change, what is less clear is whether the different regulation seen in different cells has any deeper significance. It is likely that differences in cell cycles may require different types of regulation. For example, in early metazoan embryos, the first divisions occur without transcription, eliminating one level of potential regulation. Additionally, differences in the regulation of quiescence in different cell types may be important in understanding differences in pre-RC regulation. It may be more important for metazoans to ensure that pre-RCs do not assemble at origins in cells during quiescence, as the quiescent state may need to be maintained for many years. Whereas reassembly of pre-RCs upon re-entry into the cell cycle appears to be straightforward in budding yeast, because CDC6 and Mcm2–7 are re-synthesized prior to the synthesis of the G1 cyclins (Coster et al., 2014; Yeeles et al., 2017), the same does not appear to be true in mammalian cells, where licensing in mitosis differs from that in cells re-entering the cell cycle from quiescence in the requirement for cyclin E (Geng et al., 2007).
1.10 Aims and Objectives

The cell cycle is controlled by multiple cyclin dependent kinases (CDKs) that ensure that the genome is duplicated once and only once per cell cycle. Cyclin E and cyclin A show both functional degeneracy and functional specificity in certain contexts. Using a combination of cell based approaches and cell free DNA replication assays this project aims to:

1. Evaluate specific cyclin-CDK2 complexes in regulation of G1/S transition using quiescent mouse embryonic fibroblasts.
2. Investigate how cyclin E and cyclin A promote accumulation of pre-RC proteins in G1 phase
3. Evaluate whether the ubiquitin proteasome system regulates accumulation of replication proteins
Chapter 2
Materials and Methods
2.1 Cell Culture

NIH 3T3 cells were freshly revived from frozen stocks provided by Dr. Nikki Copeland and maintained in (D-MEM) Dulbecco’s modified eagle medium from GIBCO is supplemented with 10 % fetal bovine serum and 1 % Antibiotic (GIBCO DMEM (1x) Glutamax (TM) supplemented with 1g/L D-glucose and Pyruvate). To passage NIH 3T3 cells, media was removed, cells were washed with PBS (NaCl 0.138 M; KCl - 0.0027 M; pH 7.4), followed by incubation with 1X trypsin- EDTA (0.05% w/v trypsin, 0.2g EDTA GIBCO) for maximum of 5 minutes. Depending upon the cell density cells were then passaged 1:2 – 1:4 into 15 cm Nunclon delta dishes, maintaining cells at a density of 30-60 % confluence. Cells were passaged at least once every 48 hours. HeLa cells were cultured similar to NIH3T3 cells, with DMEM media supplemented with 10 % fetal bovine serum and 1% penicillin / streptomycin (GIBCO).
2.2 *Quiescence mediated Cell Synchronization by contact inhibition and serum starvation in 3T3 cells.*

3T3 cells were passaged and allowed to reach 100% confluency within 48 hours passaging. Once cells reached confluence fresh media was added onto the plate and cells cultured for a further 48 hours to 72 hours. Cells were then released by passaging one confluent plate into four 15 cm plates and one 9 cm plate with coverslips to reduce the cell density and allow the cells to re-enter cell cycle. Cells were grown on coverslips for analysis of S phase population using incorporation of EdU a thymidine analogue. In addition, for analysis of transcript levels, cells were grown on 9 cm plate were harvested for RNA isolation used for quantitative PCR.
2.3 Synchronization of HeLa cells in S-Phase using double thymidine block

To prepare a synchronised S-phase population of HeLa cells 20 X 15 cm dishes of cells were cultured at 30%-40% confluency and incubated with 2.5 mM thymidine (Sigma Aldrich). HeLa cells were incubated with media containing 2.5 mM thymidine for 24 hours, cells washed with 1x PBS and fresh DMEM media added without Thymidine for 8 hours. Subsequently, cells were then incubated with DMEM containing 2.5 mM thymidine for second thymidine incubation for 16-20 hours, cells washed with 1x PBS and fresh DMEM media added for 1 hour to release synchronised cells into S phase from thymidine block. These cells were then used for preparation of S–phase cytosolic extracts used in cell free replication assay (Section 2.14).

2.4 SiRNA transfection in 3T3 cells.

Mouse fibroblasts 3T3 cells were synchronised as described (section 2.2) and transfected with siRNA using Lonza nulceofector 2B with pre-designed validated silencer select siRNAs (Ambion/Life Technologies) by addition of 50 μM siRNA suspended in Kit R reagent (Lonza). Details for siRNAs are shown in Table 2.1 for cyclin A (Ccna2), Cyclin E (Ccne1 and Ccne2), Cdh1 and DHX9. Cells were prepared for transfection using the following method: cells were washed with 1x PBS and incubated with 2x Trypsin-EDTA for 5 minutes. To detach the cells were mixed with equal volume of media and cells were harvested by centrifuging at 500 x g for 5
minutes. Supernatant was carefully decanted, cells centrifuged at 500 x g for 1 minute to remove excess medium and cells were then re-suspended in transfection mix. Synchronised transfection was optimised to use one confluent plate in 2 parallel reactions; (control siRNA and target siRNA); whereas for asynchronous cells an 80 % confluent cycling plate was used per transfection. Transfection mix consists of 100µL of transfection reagent and 1 µM of (max volume 5µL) siRNA. Cells with transfection mix were transferred into cuvettes used for electroporation. The electroporation unit used for this transfection was Lonza 2b Nucleofector™ on pre-set program for NIH 3T3 cell line (U-30). Cells were then immediately transferred into fresh media and equally distributed between plates for Western blot analysis, RNA extraction/quantitation and EdU assays.
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<td>Silencer®-Ambion®</td>
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Table 2-1 List of siRNAs used in siRNA knockdown

2.5 Subcellular fractionation

Mouse 3T3 cells were washed with ice cold Cyto-Skeletal (CSK) buffer (CSK Buffer: 0.5M PIPES pH6.8, 300mM Sucrose, 100mM NaCl, 1M MgCl₂, 1mM DTT, 1X Complete Protease Inhibitor (Roche)). 1mM DTT and complete protease inhibitor cocktail tablet were added fresh just before harvesting cells. In addition to protease inhibitor, phosphatase inhibitor sodium orthovanadate (sigma Aldrich) of stock concentration 200mM was added to cell lysate to make up the final concentration of 1mM. Cells were then incubated in the CSK buffer for 5 minutes on ice and further incubation of 5 minutes at an angle to remove excess buffer. Cells were scrape harvested.
and volumes standardised to maintain equal protein loads. Cell lysates were split into 2 tubes for total protein (whole cell extracts) and detergent soluble and insoluble (chromatin) fractions by incubating with 0.1 % Triton x 100 for 5 minutes and harvested by centrifugation at 12000 rpm for 5 minutes. Supernatant was collected in fresh tube labelled as supernatant fraction containing cytosolic extract or pellet fraction containing chromatin fraction and mixed with 100µL 4X SDS Loading Buffer. All fractions were boiled at 95°C for 10 minutes and stored at -20 °C until further analysis is performed by resolving on SDS –PAGE.

2.6 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

Cell lysates with protein samples prepared in 1X Loading buffer were resolved on pre-cast 4-15% gradient gels, ran at 200V using Mini-PROTEAN 3 system (Bio-Rad) for 30-40 minutes until samples resolved appropriately. Samples were run on 4-15% gel with 1X Tris –Glycine-SDS buffer (Running buffer). Samples were loaded on to gel with pre-stained protein marker precision plus protein standard-all blue (Bio-Rad). Resolved gels were then transferred on to nitrocellulose membrane with semi-dry transfer system. Protran Whatman nitrocellulose membrane and filter paper were hydrated in Transfer Buffer (60 mM Tris Base, 1mM CAPS, 10% Ethanol, 0.2 % SDS) is sandwiched from bottom to top in four layers of filter paper, equilibrated
nitrocellulose membrane, SDS-PAGE gel and four layers of filter paper. To transfer the proteins onto nitrocellulose 0.8mA/m² current is applied for 2 hours. Nitrocellulose membrane was washed and incubated with blocking buffer for 30 minutes at room temperature or overnight at 4 °C. Post blocking, the blot was incubated with primary antibody with appropriate dilution ranging from 1:250-1:1000 (Table 2.2), for 2 hours at room temperature or overnight at 4 °C with constant agitation. The membrane was than washed thoroughly in 1X TBST (Tris buffered saline + 0.1% v/v Tween20) with 1% BSA for 3 x 5 minutes which was followed by incubation with secondary antibody conjugated with horseradish peroxidase with the dilution of 1:5000 for 1 hour at room temperature. The nitrocellulose membrane was washed 3 times with TBST before visualisation of WESTAR CYANAGEN EtaC Chemiluminescent Substrate for Western Blot was added to the membrane and imaged using a Chemidoc (BIO-RAD) for varying exposure times depending upon the antibody. The saturation tool within ImageLab was used to ensure levels of protein are within the dynamic range for this equipment. Material used in SDS page and Western blotting are as follows:

1) SDS Polyacrylamide gel (Huang et al.): Bio-RAD mini- PROTEAN TGX™ gradient gels (4-15%) with 15 wells comb with maximum volume of 15 µL.
2) Membrane: Whatman Protran Nitrocellulose membrane from Healthcare Life Sciences

3) 4X SDS- Loading buffer: 50mM Tris-HCl pH 6.8, 4% (v/v) SDS, 20% (v/v) glycerol, 1 % (v/v) β-mercaptoethanol, 0.06% (W/v) Bromophenol Blue dissolved in dH2O.

4) 10 x Tris-Glycine SDS buffer: 250mM Tris Base, 1.92 M Glycine, 1% SDS pH 8.3.

5) 1 x Transfer Buffer: 60mM Tris Base, 1mM CAPS, 10% Ethanol, 10% SDS.

6) Blocking Buffer: 1% (W/V) Bovine Serum albumin in 1x Tris Buffer Saline and 0.1% Tween 20 (W/V).

7) Wash Buffer: 1x Tris Buffer Saline and 0.1% (W/V) Tween 20.
   
   a. Detection Kit: WESTAR CYANAGEN Chemilluminescent Substrate for Western Blot 250mL kit.

8) List of Antibodies used in Western blot is described in (Table 2.2)
# Chapter 2: Materials and Methods

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Table 2-2 List of antibodies used in western blot.
2.7 EdU Labelling

To determine the number of S-phase cells, cells were labelled with ethynyl deoxyuridine (EdU) and labelled Click-iT imaging kit (Life Technologies). Cells were incubated with 10 µM (EdU) for minimum of 30 minutes for first time point post release of cells from serum starvation. Cells cultured on coverslips are fixed at different time points post release ranging from 15 to 24 hours. At time points cells grown on coverslips were harvested, washed twice in PBS before fixing with 4 % paraformaldehyde (Pfander and Matos) by incubating it for 20 minutes at room temperature and subsequently washed with PBS. These coverslips were fixed and stored at -20 °C until further processed. Coverslips were then washed 3 times with blocking buffer consisting of 3% BSA in PBS. Cells were permeabilised with 0.5% Triton X-100 in PBS for 20 minutes. Post permeabilisation cells were washed with blocking buffer and incubated in 20µL EdU labelling cocktail (1X Click-iT EdU reaction Buffer, Copper Sulphate (CuSO₄), 1XClick-iT EdU Additive Buffer and Alexa Flour azide 555) for 30 minutes in dark in a humidified chamber. Post incubation coverslips were washed thrice with blocking buffer and twice with 1XPBS. Coverslips were mounted with Vectashield with DAPI was used as nuclear counter stain. The slides were then used to count the positive nuclei using fluorescent microscope. Confocal Microscope was used to count
minimum of 100 nuclei per coverslip with DAPI and 568 filters on microscope to visualise blue nuclei and red spots within nuclei respectively.

2.8 Confocal imaging

Cells labelled with EdU were imaged and counted under Ziess LSM 510 Meta system upright confocal microscope in first two years of study and in final year Ziess LSM 880. EdU being incorporated in DNA marks proliferating cells when stained with Alexa fluor azide 555 emits red light when excited by 568 nm laser from the microscope. The red bright nuclei with DNA replication foci were termed as positive nuclei. Approximately 100 nuclei for each experiment were counted as they were counter stained with DAPI and could be distinguished as positive or negative nuclei respectively.

2.9 RNA Isolation

Transfected cells were harvested 24 hours post-transfection by washing them once with PBS, incubated in 1X trypsin to disassociate cells from the dish. Trypsin was quenched by addition of an equal amount of DMEM media supplemented with 10% serum and 1% antibiotic. This cell suspension was centrifuged at 500 x g for 5 minutes and supernatant was discarded. Pellet consisting of cells were frozen at -80 °C until RNA isolation was performed using PureLink RNA minikit (Ambion) as per manufacturer’s instructions.
The isolated RNA was measured using Nanodrop and used transcript abundance measured by RT-qPCR.

### 2.10 Quantitative Real Time Polymerase Chain Reaction (q RT-PCR)

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) was performed using Express one-step SuperScript qRT-PCR SuperMix (Life Technologies). This kit is composed of 2 modules, Express RT module containing Reverse Transcriptase and RNAseOUT™, and an EXPRESS qPCR SuperMix module with ROX. This approach was used to analyse the transcript levels of Cyclin A, MCM2, MCM10, PCNA, and CDC6. GAPDH or 18s rRNA was used as internal control for transcript levels. Each reaction consists of 1x superscript SuperMix, 50 ng-100 ng of Template RNA, 1x RT mix, Primers and DEPC water to make it up to the volume of 20µl. This reaction was made in triplicate for each of the genes and distributed in the 96 well qPCR plates. This reaction was mixed well before and ran it for 40 cycles on qPCR thermal cycler. All qRT-PCR data is performed with 3 technical replicates for each experiment. Three independent experiments were used, with relative quantitation of transcript abundance relative to GAPDH showing mean and standard deviation is shown (n=3) as observed in chapter 3. The list of TaqMan primers used are listed in Table 2.3.
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Table 2-3 List of TaqMan primers used in qRT-PCR.

2.11 His\textsubscript{(6)} tagged ubiquitin plasmid generation

His-(6)-ubiquitin plasmid was obtained from Dr. Elaine Taylor. This plasmid was amplified by transformation and purified by QIAGEN miniprep. Transformation of plasmid was carried out by adding 1 µl of the plasmid to competent cells for 30 minutes on ice followed by heat shock for 2 minutes at 42 °C, cells incubated for 5 minutes on ice and 250 µl pre-warmed SOC medium added to competent cells with incubation at 37 °C for 1 hour. Transformed bacteria were selected on LB agar plates with 100 µg/ml ampicillin. Colonies were picked next day and 5 ml LB broth with ampicillin
were inoculated for overnight mini-prep cultures. Qiagen mini-prep plasmid extraction was performed and isolated His\textsubscript{(6)}-tagged ubiquitin plasmid was transfected as described in following section.

\textbf{2.12 His\textsubscript{(6)}-Ub mediated pulldown}

3T3 cells were synchronized by contact inhibition and serum starvation. Synchronized cells were released into fresh DMEM medium and transfected with His-tagged ubiquitin plasmid. These cells were allowed to enter cell cycle and transition from G1 phase to S phase. These cells were then incubated with Proteasome inhibitor for 4 hours prior to harvest. 24 hours after transfection cell were washed in 10 ml of lysis buffer, and incubated for 5 minutes, following which plate was placed at angle of 45° and excess buffer was removed and cells were scrape harvested. Cells were collected in a 1.5 ml microfuge tube and volume made up to 200 µl with buffer, with 1 µl of PMSF (200 mM stock) and 10 µl of a 10 % triton X-100 solution. Incubated on ice for 5 minutes and spun at 10000 x g for 5 minutes. Supernatant was collected in a second tube labelled low salt and pellet was suspended in 200 µl of the high salt buffer and incubated on ice for 5 minutes followed by centrifuge at full speed for 5 minutes. Spin columns were prepared with 30 µl Ni\textsuperscript{2+}-NTA resin for each reaction. Beads were washed 3 times with 500µl of wash buffer prior to addition of the cell lysates. 200 µl of wash buffer was added to the cell extracts (total volume now ~400 µl) and 20 µl was removed for analysis on the
gel. This was the protein load (5% total). Bind for 1 hour on the mixer in cold room and wash the beads. Beads were washed in 500 µl of wash buffer and excess buffer was spun through into a collection tube at 1000 x g for 1 minute. Washing step was repeated 4 times. 60 µl of 4 x SDS-PAGE loading buffer was added to the beads in the tube, boiled for 10 minutes and allowed to cool before removing plugs and collecting the denatured proteins in a clean collection tube. This was the bound fraction for analysis by western blotting.

2.13 Chemical inhibition of CDK2 and DDK

3T3 cells were synchronized by contact inhibition and serum starvation. Cells were incubated with CDK2 inhibitor (Roscovitine) or DDK inhibitor (PHA 767491) after restriction point at around 20 hours post release. Roscovitine (30 µM) or PHA 767491 (10 µM) were incubated with cells for fours before cells were harvested in CSK buffer and fractionated into total fraction, cytoplasmic and chromatin associated fraction (Section 2.5). These fractions were then suspended in 4 x SDS loading buffer, boiled at 95°C and samples analysed on western blot.
Table 2-4 List of Drugs used in experiments as indicated.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Target</th>
<th>Supplier</th>
<th>Code</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roscovitine</td>
<td>CDK2 inhibitor</td>
<td>Sigma Aldrich</td>
<td>R7772</td>
<td>Working conc. 30µM; stock 20mM</td>
</tr>
<tr>
<td>PHA 767491 dihydrochloride</td>
<td>DDK (cdc7/cdk9) inhibitor</td>
<td>Sigma Aldrich</td>
<td>PZ0178</td>
<td>Working conc. 10µM; stock 10mM</td>
</tr>
<tr>
<td>MG132 (carbobenzyoxy-Leu-Leu-leucinal)</td>
<td>Proteasome inhibitor</td>
<td>Sigma Aldrich</td>
<td>C2211</td>
<td>Working conc. 10µM; stock 10mM</td>
</tr>
<tr>
<td>Nocodazole</td>
<td>Mitotic inhibitor</td>
<td>Sigma Aldrich</td>
<td>R17934</td>
<td>Working conc. 100ng/mL; stock 5mg/mL</td>
</tr>
<tr>
<td>Thymidine</td>
<td>DNA Replication block</td>
<td>Sigma Aldrich</td>
<td>T-9250</td>
<td>Working conc. 2mM</td>
</tr>
</tbody>
</table>
2.14 Cell free DNA replication assay

Cell free replication assay involves reconstitution of minimal components required to initiate DNA replication *in vitro*.

i. **Pre-Mix:** Pre-mix consists of Buffers and dNTPs required for replication assay. Pre-Mix is stored at -80 in 15 µl aliquots. dNTPS were purchased from sigma Aldrich with concentration of 100 mM and were stored at -20 until used. Composition of Pre-Mix is as followed:

<table>
<thead>
<tr>
<th>Stock</th>
<th>10x</th>
<th>Working concentration</th>
<th>Volume (total 1mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M HEPES pH7.8</td>
<td>400mM</td>
<td>40mM</td>
<td>400µL</td>
</tr>
<tr>
<td>1M MgCl₂</td>
<td>70mM</td>
<td>7mM</td>
<td>70µL</td>
</tr>
<tr>
<td>1M DTT</td>
<td>10mM</td>
<td>1mM</td>
<td>10µL</td>
</tr>
<tr>
<td>2M Phosphocreatine</td>
<td>400mM</td>
<td>40mM</td>
<td>200µL</td>
</tr>
<tr>
<td>0.5M ATP</td>
<td>30mM</td>
<td>3mM</td>
<td>60µL</td>
</tr>
<tr>
<td>0.1M GTP</td>
<td>1mM</td>
<td>0.1mM</td>
<td>10µL</td>
</tr>
<tr>
<td>0.1M CTP</td>
<td>1mM</td>
<td>0.1mM</td>
<td>10µL</td>
</tr>
<tr>
<td>0.1M UTP</td>
<td>1mM</td>
<td>0.1mM</td>
<td>10µL</td>
</tr>
<tr>
<td>0.1M dATP</td>
<td>1mM</td>
<td>0.1mM</td>
<td>10µL</td>
</tr>
<tr>
<td>0.1M dGTP</td>
<td>1mM</td>
<td>0.1mM</td>
<td>10µL</td>
</tr>
<tr>
<td>0.1M dCTP</td>
<td>1mM</td>
<td>0.1mM</td>
<td>10µL</td>
</tr>
<tr>
<td>Ultra pure Water</td>
<td></td>
<td></td>
<td>200µL</td>
</tr>
</tbody>
</table>

Table 2-5 Components of pre-mix used in cell free replication assay.
ii. **Creatine Phosphokinase (CPK)** (Calbiochem) with stock concentration of 5 KU. This was stored in low milligram quantities at -20 and re-suspended in CPK buffer (40mM HEPES pH 7.8 and 1mM DTT) fresh every time for the reaction.

iii. **Biotin -16-dUTP**: Commercially purchased from Roche diagnostics at concentration of 50 nmol and was stored at -20.

iv. **Nuclear and cytosolic extract**:

Late G1 phase nuclear extract was prepared by synchronizing 3T3 cells by contact inhibition and serum starvation and harvested by homogenization in hypotonic buffer (1M HEPES pH 7.8, 1 mM MgCl$_2$, 3 mM potassium acetate, 1 mM DTT) at around 17.5 hours post release. Nuclei were separated from cytosolic extract by centrifuging at 6000 rpm for 5 minutes at 4°C. Pellet fraction was re-suspended in equal quantities of hypotonic buffer and frozen into beads using liquid nitrogen for later use. G1 cytosolic extract was the supernatant fraction and was also stored into beads using liquid nitrogen ready to be used later. Cyclin E depleted and cyclin A depleted nuclei were prepared by transfection cyclin E1/E2 siRNA and cyclin A2 siRNA of synchronized 3T3 cells as described previously. Transfected cells were harvested after 24 hours of transfection and nuclear extracts were prepared in
hypotonic buffer and stored into beads by freezing in liquid nitrogen and stored for later use. S phase cytosolic extract was prepared by synchronizing HeLa cells in S phase by double thymidine block as mentioned previously. The cytosolic extract was stored in beads by freezing in liquid nitrogen and ready to be used later.

v. **Cell free Replication assay:**

Cell-free replication assay was performed as described by (Coverley et al., 2002; Coverley et al., 2005) and Copeland et al., 2010; Copeland et al., 2015). Each reaction was constituted with nuclear extract and cytosolic extract along with master mix consisting of (1:10) pre-mix, (1:50) biotinylated dUTP (Roche), (1:50) CPK (Millipore) and (1:50) MgCl₂. Each of the reaction was incubated for 30 minutes at 37 °C. After the reaction was complete 1/10th of the reaction mix was fixed with 4 % paraformaldehyde and transferred onto poly-D-lysine (Sigma-Aldrich) coated coverslips by spinning the reaction mix at 1500 rpm onto 30 % sucrose cushion. These coverslips were then washed with 1 x PBS three times, followed by washes with antibody buffer (detergent mix and 1% BSA). Coverslips were incubated with streptavidin-Alexa fluor 555 (1:10000) for 30 minutes in dark. After the incubation coverslips were washed three times with antibody buffer (specify components) and three times with PBS. Coverslips were then mounted with Vectashield with DAPI.
and analysed on Zeiss confocal microscope. The remaining reaction was incubated with 0.1 % Triton X-100 for 5 minutes on ice and fractionated into chromatin and cytoplasmic fractions by centrifugation at 14,000 rpm for 5 minutes. Pellet fraction suspended in 4x SDS loading buffer and samples were analysed by western blot. Further to this, nuclear matrix fractionation was performed by suspending pellet fractions after first spin with high salt buffer (0.5 M NaCl in PBS) for 5 minutes on ice and spinning at 14,000 rpm. The pellet fraction of this spin was then suspended in 4 x SDS loading buffer and labelled as nuclear matrix associate fraction and analysed on western blot.
Chapter 3
Analysis of the role of cyclin E and cyclin A-CDK2 in post-quiescent fibroblasts
3.1 Cell synchronization for analysis of temporal regulation of DNA replication

The cell cycle is a series of regulated molecular events that govern DNA replication and mitosis resulting in generation of daughter cells from single parent cell. Each cell can either continue to proliferate or exit cell cycle via reversible process known as quiescence thereby entering G0 phase of the cell cycle (Coller, 2011; Zetterberg and Larsson, 1985). Quiescent cells are non-proliferative that continuously repress transcription of genes required for cell cycle progression, but actively express anti-apoptotic, anti-senescence and anti-differentiation genes (Coller, 2011; Zetterberg et al., 1995).

The decision to commit to cell cycle or to remain in quiescence is dependent on a functional restriction point. The Restriction point was first described by (Pardee, 1974), evaluating the timing of DNA synthesis in hamster fibroblasts after a cell cycle block and release. Restriction point or R-point is defined as the point in the cell cycle when the cell commits irreversibly to S-Phase entry as extracellular stimulus is no longer required for completion of the cell cycle and hence also known as ‘point of no return’ (Pardee, 1974; Zetterberg et al., 1995). Prior to this point the cell requires sustained mitogen stimulus to proliferate and exit quiescence.

Progression from past the G1 restriction point requires sustained mitogen signalling and a collection of interconnected and reinforcing
mechanisms that stimulate CDK2 activation leading to Cyclin E/CDK2 activation resulting in activation of transcription of replication assembly proteins. CDK2 is believed to be cytoplasmic in some quiescent cells, which prevents its phosphorylation by nuclear kinases (Geng et al., 2007).

To study the cell cycle, pharmacological agents that arrest cells at different phases are widely used. Mimosine, (Dijkwel, 1992), the plant derived drug which prevents initiation of DNA replication results in arrest in G1, is used as one of the synchronisation method. However, it affects the intrinsic levels of p53 and p21 and hence alters the analysis. Another example of drug used for synchronisation of cells in G1 phase is lovastatin (Javanmoghadam-Kamrani, 2008), a mevalonate synthesis inhibitor; however, there are reports that it induces apoptosis. Hydroxyurea (Gerenday, 1997) or double thymidine (Galgano, 2006) treatment synchronise cells in S phase by targeting ribonucleotide reductase inducing replication stress. Nocodazole and colcemid arrest cells in M phase by acting as microtubule-disrupting agents (Olmsted et al., 1989). These drugs provide synchronous cell populations but each approach induces potential artefacts related to their mechanism of action that include metabolic perturbations and toxicity. Pharmacological agents used in manipulating the cells to synchronise have been shown to result in dissociation of nuclear and cytoplasmic cell-cycle processes, disruption in
metabolic state of the cell and cell death (Levenson and Hamlin, 1993; Merrill, 1998). The artefactual nature of the drug treatments limits their use for analysis of physiologically relevant process.

Several drug free methods have been established for synchronising cells from asynchronous cell populations. Centrifugal elutriation can isolate cells at any phase of cell cycle, and mitotic shake off can be used to isolate mitotic cells; however, yield of the cells is very low and hence cannot be used for variety of experiments. When choosing a synchronisation method it is necessary to consider cell type, the cell cycle phase, doubling time and the cell cycle duration (Langan et al., 2017; Memili et al., 2004; Jackman et al., 2001). Here we use the physiologically relevant contact inhibition and serum starvation approach to generate a synchronised post-quiescent population of cells to study cell cycle progression (Coverley et al., 2002; Copeland et al., 2010).
3.2 Chapter aims

The cell cycle is controlled by multiple cyclin dependent kinases (CDKs) that ensure that the genome is duplicated once and only once per cell cycle. The proteins that are phosphorylated by cyclin E-CDK2 and cyclin A-CDK2 remain to be fully identified. The aims of this chapter are to investigate the role of cyclin E-CDK2 and cyclin A-CDK2 in G1/S transition. Here, synchronised 3T3 cells are stimulated to re-enter the cell cycle. During cell cycle re-entry CDK2 activity is inhibited and the effects on pre-RCs, pre-IC and replisome will be monitored. In addition, effects on cell cycle regulation by DBF4 dependent kinase will also be monitored. For comparison protein levels and transcript levels by qRT-PCR will be monitored. These approaches will give a better understanding of the role of cyclin-CDK2 and DDK activity in late G1 phase and their role in replication complex assembly in the G1/S transition.
3.3 Experimental approach synchronization of mouse fibroblasts to monitor cell cycle re-entry from G0

This study utilises synchronisation of the cells at the G0 stage of the cell cycle by contact inhibition and serum starvation (Coverley et al., 2002; Copeland et al., 2010; Copeland et al., 2015; Langan, 2011). Quiescent cells are released from starvation and passage into diluted subsequent concentration of cells. Synchronisation of cells was confirmed by incorporation of EdU, a nucleotide analogue that allows monitoring of the number of cells in S-phase. In addition, as a control to demonstrate synchronisation; expression levels of proteins were studied by western blot and transcript levels were analysed by quantitative real time polymerase chain reaction (qRT-PCR) (Figure 3.1).
Mouse fibroblasts (3T3) are synchronised by contact inhibition and serum starvation. Cell-cell contacts are disrupted by trypsinization and cells are diluted to facilitate cell-cycle re-entry. Comparison of siRNA transfected/chemical inhibitor treated sample and control cells enable comparison of replication complex assembly by western blot and S phase entry (EdU) at defined time-points in G1 and S phase.

Figure 3-1 Experimental overview for analysis of cell cycle progression from G1 to S phase.

Mouse fibroblasts (3T3) are synchronised by contact inhibition and serum starvation. Cell-cell contacts are disrupted by trypsinization and cells are diluted to facilitate cell-cycle re-entry. Comparison of siRNA transfected/chemical inhibitor treated sample and control cells enable comparison of replication complex assembly by western blot and S phase entry (EdU) at defined time-points in G1 and S phase.
3.4 Synchronisation of mouse fibroblasts to monitor cell cycle re-entry from G0

To investigate cell cycle progression in a mammalian system, the physiologically relevant quiescence release method is used. This drug free protocol enables study of the cell cycle between G0 and S-phase to monitor the early steps in DNA replication using cell-based assays and to produce material for cell-free DNA replication assays (Copeland et al., 2010; Coverley et al., 2002). Regulation of the cell cycle by reversible cell cycle exit into a quiescent state is a key regulatory event in stem cells and somatic tissues. This approach yields tight synchronized populations of cells to enable temporal analysis of stages within G1 that facilitate that mediate DNA replication complex assembly to proceed. Isolation of the cell population at defined time points (Figure 3.2.A), enables analysis of the transcript (Figure 3.3) and protein levels (Figure 3.4) throughout G1 phase and early S phase.
A: Experimental overview identifies time points for analysis and quantification of EdU time points. B: Line graph showing percentage of cells in S-phase at indicated time points where N=3+/−S.D. C: Representative images of the EdU labelled nuclei from 10 hour to 24 hour with increase in EdU positively labelled nuclei from 18 hour post release.
3.5 Analysis of transcript levels and protein levels of Replication assembly proteins from synchronous cell population:

Transcriptional regulation of regulators of DNA replication is mediated by E2F family of transcription factors. As a control to ensure synchrony of cell population a selection of E2F regulated genes were analysed at transcript and protein levels. Expression of E2F target genes were followed from G0 to S-Phase at 0, 4, 8, 12, 16, 20 and 24 Hrs. Samples were processed and qRT-PCR performed (Section 2.10). Transcript level of pre-RC proteins CDC6, MCM2, Pre-IC protein MCM10, cell cycle regulators cyclin E1/E2 and cyclin A2 as well as the replisome protein PCNA were monitored for 24 hours post release from quiescence. There is steady increase in transcript levels with a peak at 20 hour time point and going down again at 24 hour post release from quiescence.
Chapter 3: Results

Relative quantification of E2F target genes standardised to GAPDH. Cells were harvested at indicated time points and transcript levels quantified by qRT-PCR for E2F target genes. Cumulative of each of the E2F gene transcripts (Cyclin A2, CDC6, Cyclin E1, Cyclin E2, PCNA, MCM2, MCM10) show same trend. Data shows mean, where N=3.

Similarly, to detect changes in protein levels in cells re-entering the cell cycle from G0, cells were harvested at different time points ranging 0-24hrs. Cells were harvested and samples were prepared and analysed by western blotting (Figure 3.4). Expression of proteins appears to have increased with increasing time post release, consistent with the synchronised population of cells preparing to enter S–Phase and commit to DNA replication.
Whole cell extracts were prepared at indicated time points and analysed by western blot. Replication assembly proteins including MCM2, PCNA, CDC6 and cell cycle regulator cyclin A start to accumulate from around 16 hour time point post release from quiescence.

Protein analysis during G1/S transition (Figure 3.4) reveals cyclin E starts to accumulate as soon as 8 hours after release from G0 phase, prior to the restriction point. This is consistent with temporal analysis of cyclin E expression (Figure 3.3), and with the existing literature suggesting that cyclin E – CDK2 complex was responsible for early G1/ S transition events, such as transition through the restriction point (Foster et al., 2010), and for pre-replication complex activation at the end of G1 phase (Coverley et al., 2002). Additionally, the regulatory protein, for CDC7 kinase activation, Dbf4 was shown to accumulate prior the restriction point, around 12 hours after release.

Figure 3-4 Analysis of replication assembly of proteins in cells entering cell cycle from quiescence

Whole cell extracts were prepared at indicated time points and analysed by western blot. Replication assembly proteins including MCM2, PCNA, CDC6 and cell cycle regulator cyclin A start to accumulate from around 16 hour time point post release from quiescence.
from quiescence. Dbf4 along with CDC7 as Dbf4 dependent kinase (DDK) was also proposed to be involved in G1/S transition and DNA replication initiation (Sheu and Stillman, 2006; Tanaka and Araki, 2010), thus the data were consistent with a tightly synchronised population of cells. Interestingly, cyclin E–CDK2 and DDK are expressed at 12 hours where they function to promote activation of DNA replication complex assembly in G1 phase (Bruck et al., 2015; Deegan and Diffley, 2016; Nougarede et al., 2000). Importantly, cyclin A accumulates post-restriction point, 16 hours after release from G0 phase, which is distinct from its transcript levels (Figure 3.3). This is due to APC/C^{Cdh1} mediated degradation of cyclin A in G1 phase (Choudhury et al., 2016). Cyclin A–CDK2 complex is responsible for DNA replication firing in the early S phase that occurs around 18 hours after release from quiescence (Coverley et al., 2002).

The data presented here shows that the synchronisation of cells by serum starvation and contact inhibition enables synchronous transcript accumulation, protein levels and S-phase entry demonstrating that this approach enables reproducible cell cycle re-entry after release from quiescence. This method of cell synchronisation and release therefore is an effective method to study pre-replicative proteins and their regulation by cyclin-CDK2 in G1-S transition and initiation of DNA Replication.
3.6 CDK2 inhibition destabilises replication assembly of proteins and blocks S phase entry in synchronised cell population.

Protein phosphorylation by kinases plays a very important role in regulation of various cell processes including, proliferation, cell cycle, differentiation and apoptosis. Cyclin dependent kinases (CDK) play an important role in control of cell cycle and proliferation. Initially, the CDK1/2 specific inhibitor Roscovitine (Meijer, 1997) was used to assess how CDK2 activity promotes cell cycle progression in an asynchronous population of cells. Using an asynchronous population of cells at 70-80% confluency, cells were treated with Roscovitine for 4 hours, scrape harvested and whole cell extract analysed by Western blot (Figure 3.5). Analysis of the protein samples revealed a reduction in replication complex assembly proteins (CDC6, MCM2, PCNA) in absence of CDK2 activity.
Chapter 3: Results

Figure 3-5 CDK2 inhibition with roscovitine destabilises replication complex assembly of proteins (MCM2, CDC6, PCNA and MCM10) and blocks S phase entry.

A: Western blot analysis of the MCM2, PCNA and CDC6 in asynchronised cell system with roscovitine treatment of 4 hours. B: As for A using a synchronized population of cells released from quiescence +/- roscovitine for 4 hours and cells harvested at 24 hours post release and analysed by western blot analysis for MCM2, PCNA and CDC6. C: Histogram showing S phase cells at 24 hour post release and CDK2 inhibition by roscovitine where N=3+/- S.D.
As roscovitine is both a CDK1 and CDK2 inhibitor, this effect could be caused by either kinase, in any phase of the cell cycle. As MCM2, PCNA and CDC6 accumulate in mid G1 phase, we next examined whether CDK2 inhibition affects protein accumulation in a synchronized late G1 population of 3T3 cells (Figure 3.5.B). Synchronization in G1 phase also examined the activity of CDK2 more precisely as CDK1 is inhibited at this phase of the cell cycle. Consistent with the results using asynchronous population of cells, inhibition of CDK2 activity reduced CDC6, MCM2 and PCNA levels following addition of roscovitine, which correlates with a reduction in S-phase cells (Figure 3.5.C).
Chapter 3: Results

3.7 DDK inhibition destabilises replication complex assembly of proteins and blocks S phase entry from quiescence

DDK is a two subunit Serine-threonine kinase composed of CDC7 kinase and DBF4 regulatory subunits. DDK is a key regulator of changes in MCM2-7 helicase via phosphorylation leading to helicase activation and template unwinding (Hardy et al., 1997; Hoang et al., 2007). In cells re-entering cell cycle from quiescence, DBF4 protein levels peak during G1/S transition and is associated with CDC7 kinase for substrate recognition (Labib, 2010). Chemical inhibition of CDK2 by roscovitine (Section 3.5), demonstrates reduction in regulatory proteins of DNA replication in both asynchronous and synchronized cell population (Figure 3.5) Interestingly, CDK2 inhibition also blocks S phase progression of quiescently release cells there by inhibiting DNA replication.

To test whether DDK activity is required for accumulation of CDC6, PCNA and MCM2 in late G1 phase a chemical inhibitor of DDK PHA-767491 (PHA)(Poh, 2014) was used in an asynchronous population of cells. Asynchronous cells were treated with DDK inhibitor PHA-767491 and harvested 4 hours after treatment. Western blot analysis of these protein samples revealed a reduction in replication complex assembly proteins (DBF4, cyclin A, CDC6, MCM2 and PCNA) (Figure 3.6.A). Additionally, DBF4 one of the components of pre-initiation complex is destabilised in absence of DDK.
There was also an effect of DDK inhibition on cyclin A protein levels but not on cyclin E and PCNA levels in asynchronous cell system.

**Figure 3-6** DDK inhibition prevents DNA replication and destabilises replication complex assembly of proteins.

**A:** Western blot analysis of the MCM2, PCNA and CDC6 in asynchronous cell system with roscovitine treatment of 4 hours. **B:** Western blot analysis of the MCM2, PCNA and CDC6 in synchronised cell system with roscovitine treatment of 4 hours. **C:** Histogram showing percentage of S phase cells at 20 and 24 hours timepoints post release and DDK inhibition by PHA 767491.
Quiescent cells were released into fresh medium and incubated with PHA 767491 at 16 and 20 hours post-release and harvested after 4 hours of treatment. Addition of PHA 767491 reduced the number of cells entering S-phase at 20-24 hour time points (Figure 3.6.C), that correlated with a reduction in replication complex assembly proteins MCM2, Dbf4 and Cyclin A at 20 hours. However, little effect was noted at the 24 hour time point (Figure 3.6.B).

Formation of pre-initiation complex (pre-IC) requires activation of MCM2-7 helicase by DDK and Cyclin E-CDK2 mediated phosphorylation (Labib, 2010). The results presented here showing inhibition of CDK2 (Figure 3.5) and DDK (Figure 3.6) reveal destabilisation of replication complex assembly of proteins (MCM2, MCM10, CDC6 and PCNA).
3.8 Cyclin E–CDK2 regulates transcription of replication complex assembly proteins MCM2, CDC6, PCNA and MCM10.

Cell synchronisation may enable dissection of the role of Cyclin dependent kinases in replication complex assembly and initiation of DNA replication. Chemical inhibition of CDK2 by roscovitine and DDK inhibition by PHA 767491 suggest that phosphorylation mediated by CDK2 and DDK is required for replication protein accumulation and progression to S phase in post-quiescent cells. However, since CDK2 inhibition and DDK inhibition were carried out by chemical inhibitors, a genetic approach was also used to exclude off target effects. In order to determine if the inhibition of specific CDK2 complexes promote accumulation of replication complex assembly proteins, cyclin isoforms E1 and E2 were simultaneously depleted. Targeted depletion of cyclin E (cyclin E1/E2) was performed by siRNA transfection, and the effect on the G1 to S phase transition monitored at the protein and RNA level, and by EdU incorporation to determine the number of S phase cells.

Cyclin E knockdown was confirmed by transcript analysis performed for cyclin E1 and cyclin E2 by qRT-PCR with reduction of 90% transcript of cyclin E1 and 80% reduction of Cyclin E2 (Figure 3.7.A). Cyclin E knockdown significantly reduces the number of cells entering S-Phase (Figure 3.7.B). Approximately 40% of cells were found to be in S phase from the proliferation
assay performed on control cells, whereas cells transfected with Cyclin E siRNA fail to enter S-phase as less than 5% cells entered S-Phase (Figure 3.7.B).

Figure 3.7.C demonstrates that reduction of cyclin E-CDK2 activity leads to a reduction of DNA replication complex proteins CDC6, PCNA, MCM10, cyclin A and MCM2 by western blot analysis post cyclin E1 and E2 siRNA transfection. Cyclin E levels appear to have reduced post cyclin E1/E2 siRNA transfection at both timepoints (20 hours and 24 hours) post release.
Chapter 3: Results

A: Transcript levels for control and siRNA treated cells were determined by quantitative reverse transcriptase PCR confirming effective depletion of Cyclin E1 and Cyclin E2.

B: Control and Cyclin E1 and Cyclin E2 siRNA treated cells were labelled with EdU to determine number of cells in S-phase at indicated time points (15hrs-24hrs).

C: Western blot showing an effect of siRNA mediated depletion of Cyclin E at two different time points 20hrs and 24hr, compared to control cell lysates prepared from untreated cells. Replication complex assembly of proteins MCM2, MCM10, CDC6 and PCNA appear to be reduced at 24 hour time point.

Figure 3-7. Cyclin E inhibition reduces replication complex assembly proteins CDC6, MCM2, MCMC10, PCNA abundance.

A: Transcript levels for control and siRNA treated cells were determined by quantitative reverse transcriptase PCR confirming effective depletion of Cyclin E1 and Cyclin E2. B: Control and Cyclin E1 and Cyclin E2 siRNA treated cells were labelled with EdU to determine number of cells in S-phase at indicated time points (15hrs-24hrs). C: Western blot showing an effect of siRNA mediated depletion of Cyclin E at two different time points 20hrs and 24hr, compared to control cell lysates prepared from untreated cells. Replication complex assembly of proteins MCM2, MCM10, CDC6 and PCNA appear to be reduced at 24 hour time point.
Chapter 3: Results

3.9 Cyclin A –CDK2 does not regulate transcription of replication complex assembly proteins MCM2, CDC6, PCNA and MCM10.

Both CDK2 inhibition and cyclin E depletion have resulted in a reduction in replication assembly proteins (MCM2, CDC6, MCM10 and PCNA) and exit from G1 phase. Next the contribution of cyclin A-CDK2 activity in regulation of accumulation of replication proteins was assessed by siRNA mediated depletion of Cyclin A. Quiescent cells were treated with cyclin A2 siRNA and stimulated to re-enter the cell cycle. Cyclin A2 transcript levels were reduced by >95% (Figure 3.8.A) and cyclin A protein levels were undetected. The percentage of S-phase cells was ~45 % in control cells to less than 10 % for cyclin A depleted cells (Figure 3.8.B). Furthermore, there was a clear reduction in CDC6, MCM2 and PCNA at 20 and 24 hours post-release and siRNA transfection. The results are consistent with CDK2 inhibition by roscovitine (Figure 3.5) and siRNA mediated depletion of cyclin E1 and E2 (Figure 3.7). In addition, there was a reduction in DBF4 at 20 hour that was less apparent at 24 hours. Importantly, cyclin A2 depletion did not affect accumulation of cyclin E suggesting that cyclin A2 activity is also required for accumulation of CDC6, PCNA and MCM2 in fibroblasts.
Figure 3-8 Cyclin A is required to promote accumulation of pre-RC proteins in post quiescent fibroblasts.

A: Transcript levels for control and siRNA treated cells were determined by quantitative reverse transcriptase PCR confirming effective depletion of Cyclin A.

B: Western blot showing an effect of siRNA mediated depletion of Cyclin A2 at two different time points 20 hours and 24 hours, compared against control cell lysates.

C: Control and Cyclin A siRNA treated cells were labelled with EdU to determine number of cells in S-phase at indicated time points (15hrs-24hrs).

D: Immunoblot showing replication protein abundance in control and cyclin A2 depleted cells.
The apparent decrease in CDC6, PCNA and possibly DBF4 was unexpected after depletion of cyclin A. The reduction in protein levels for each protein could prevent licensing of replication origins and prevention of DNA replication, as seen in the reduced number of cells that can accumulate in S-phase after depletion of cyclin A (Figure 3.8.C). CDC6 is a key component of the pre-RC and is essential for replication complex assembly formation (Mailand and Diffley, 2005) as reduction in CDC6 could reduce assembly of Pre-RC and restrict replication licensing. Dbf4 is required for the regulation and activation of the replicative helicase MCM2-7. In addition, depletion of the DNA polymerase sliding clamp PCNA would drastically reduce processivity of DNA polymerases (Kath et al., 2014; Takeda and Dutta, 2005). Reduction in protein levels for CDC6, Dbf4 and PCNA may provide a mechanistic basis for the failure of cells to enter S-Phase after cyclin A2 and cyclin E1/E2 depletion. This apparent reduction in protein level could conceivably be due to transcriptional down regulation or potentially a destabilisation of the protein level post-translationally, perhaps by proteasomal degradation.
3.10 Inhibition of CDK2 or DDK reduces CDC6, MCM2, PCNA and cyclin expression by inhibition of E2F mediated transcription.

As a first step to identifying the mechanism by which CDC6, MCM2 and PCNA levels are regulated, the transcript levels were compared for control cells and cells after CDK or DDK inhibition.

To assess whether CDK2 inhibition and DDK inhibition affects transcription qRT-PCR was performed on cells 20 and 24 hours post release after roscovitine and PHA treatments. Roscovitine reduced transcription of cyclin E1, E2, MCM10 and CDC6 by 80%; MCM2, cyclin A2 and PCNA transcript levels were less affected. Similarly, DDK inhibition reduced cyclin E1, E2, CDC6 and MCM10 by approximately by 75% and cyclin A2, MCM2 and PCNA were less affected. These results suggest kinase inhibition results in reduced protein levels by reducing transcription of target genes (Figure 3.9).
Chapter 3: Results

Figure 3-7 Transcript analysis of replication assembly proteins after CDK2 and DDK inhibition.

Transcript analysis of replication proteins MCM2, CDC6, PCNA and MCM10 along cell cycle regulatory proteins cyclin E1/E2 and cyclin A2 post CDK2 inhibition by Roscovitine at 20 hour time point (A) and 24 hour time point (B). Transcript analysis of replication proteins MCM2, CDC6, PCNA and MCM10 along cell cycle regulatory proteins cyclin E1/E2 and cyclin A2 post DDK inhibition by PHA 767491 at 20 hour time point (C) and 24 hour time point (D). Data shows Mean, Where N=3.

The chemical inhibitors used to block CDK2 and DDK are known to have off target effects, for example roscovitine is potential CDK1 inhibitor as well as CDK2. Even though the CDK1 is inhibited in early G1 phase and hence roscovitine effects are likely to be primarily due to CDK2 inhibition.
DDK inhibitor PHA 767491 on the other hand is a CDK2 inhibitor ($K_i = 230$ nM) in addition to its DDK inhibitor activity. Consequently the inhibitory effect on transcript levels using PHA 767491 is potentially via inhibition of CDK2 mediated regulation of the Rb-E2F pathway. Therefore, we do not have direct evidence of a role for DDK activity in regulation of transcription of the E2F pathway due to potential crosstalk with CDK2 activity at concentrations used here.

Targeted depletion of individual cyclins (E1/E2 and A2), have a more detrimental effect on cell cycle progression of post-quiescent cells (Figure 3.7.B and Figure 3.8.B) through destabilisation of replication assembly proteins MCM2, CDC6, MCM10 and PCNA. Transcript analysis of these cyclins post siRNA mediated knockdown shows 90% reduction in cyclin E1/E2 or A2 transcript levels (Figure 3.7.A and Figure 3.8.A). This suggests that targeted depletion of cyclin E1/E2 and cyclin A2 and transcript analysis of potentially affected proteins would enable us to understand the possible mechanism behind regulation of these proteins in absence of Cyclin-CDK activity during G1/S transition in mammalian system.
3.11 Distinct roles for Cyclin E and A regulation of CDC6, MCM2 and PCNA abundance

To test whether reduction of replication complex proteins is mediated at the level of transcription in absence of cyclin E1 and cyclin E2, quiescent cells were transfected with cyclin E1/E2 siRNAs. 24 hours post-transfection cells were harvested and transcript levels for E2F regulated genes MCM2, MCM10, CDC6, PCNA were determined by qRT-PCR (Figure 3.10). Consistent with its role in regulation of the Rb-E2F pathway, cyclin E1/E2 depletion led to the reduction in E2F regulated genes PCNA, MCM2, MCM10 and CDC6, that correlates with protein expression levels (Figure 3.10.A).

The data presented demonstrate that CDK2 inhibition by roscovitine (Figure 3.9.A, B) and cyclin E1/E2 depletion reduced expression of E2F pathway genes. The canonical role of cyclin E-CDk2 is well established for cyclin E1/E2-CDK2 but cyclin A-CDK2 is not required for Rb inhibition, rather cyclin A-CDK2 further supresses the activity of Rb through S-phase but is not required for bypass of restriction point (Caldon et al., 2010).
Chapter 3: Results

Figure 3-10 Transcript analysis of replication assembly proteins after siRNA mediated depletion of cyclin E1/E2 and cyclin A2.

Transcript analyses of replication proteins MCM2, CDC6, PCNA and MCM10 along regulatory proteins cyclin E1/E2 post Cyclin E knockdown (A). Transcript analyses of replication proteins MCM2, CDC6, PCNA and MCM10 post Cyclin A knockdown (B).
To determine the role of cyclin A-CDK2 in regulation of E2F target genes, cyclin A knockdown was performed by transfecting synchronous quiescent cells with Cyclin A siRNA. Cells were harvested 24 hours post transfection with Cyclin A siRNA and transcript levels determined by quantitative reverse transcriptase PCR (Figure 3.10.B). The transcript levels of genes PCNA, MCM2, MCM10 and CDC6 are unaffected by cyclin A2 depletion. Importantly, the detected protein levels for CDC6, MCM2, MCM10 and PCNA were found to be reduced by Cyclin A knockdown. This suggests that cyclin A is required for accumulation of CDC6, PCNA, MCM2 via a distinct mechanism from cyclin E1/E2. There is a need to better understand how cyclin A2 contributes to the accumulation of important regulators of the replication licensing system. These data suggest that their abundance is regulated at the level of post-translational modification, potentially via the UPS.
3.12 Discussion

Cell synchronisation studies mediated by contact inhibition and serum starvation enabled the study of DNA replication complex assembly in late G1 to S-Phase (Copeland et al., 2010; Coverley et al., 2002). Initial studies on cell synchronisation were performed to determine the correct time course for subsequent experiments (Figure 3.2). Monitoring the proportion of cells in S-phase and protein levels enabled correlation of cell cycle stage with replication complex assembly. Synchronised cells were released from quiescence and synchrony confirmed by monitoring transcript levels, protein expression and S–Phase entry. This approach serves as the basis to analyse and dissect the function of cyclinE-CDK2, cyclin A-CDK2 and DDK in late G1.

The apparent reduction in CDC6 protein levels after inhibition of CDK2 activity is consistent with previous works that have identified that CDK2 regulates CDC6 levels at the transcriptional level and post-translationally by the ubiquitin proteasome system (UPS) (Mailand and Diffley, 2005). CDK2 activity has been shown to be required for both CDC6 and Cdc7 expression in cell re-entering the cell cycle from quiescence (Chuang et al., 2009). To evaluate the contribution of transcriptional regulation on CDC6 protein levels quantitative real time PCR (qRT-PCR) was performed monitoring MCM2, MCM10, CDC6 and Cyclin A after CDK2, DDK and individual Cyclin depletion. This approach demonstrated that transcripts
levels remain unchanged after siRNA mediated depletion of cyclin A2, but inhibition of CDK2 kinase activity or cyclin E1/E2 depletion revealed a reduction in transcription of these proteins mediated by the Rb-E2F pathway.

Inhibition of DDK also blocks S phase entry in quiescent release cells (Figure 3.9). However, the DDK inhibitor used in these experiments is PHA 767491, which is also a CDK2 inhibitor (Albanese et al., 2010). The data presented here cannot determine that PHA is not inhibiting transcription through inhibition of CDK2 activity. Consequently, the datasets are similar for both roscovitine and PHA767491 with respect to transcriptional regulation, cell cycle progression and protein abundance (Figure 3.9). In future studies use of an alternative DDK inhibitor such as XL 413 (Sasi et al., 2017; Sasi et al., 2014) or depletion of Dbf4 or Cdc7 would potentially resolve whether DDK activity contributes to transcriptional regulation of the Rb-E2F pathway.

Chemical inhibition and genetic inhibition of CDK2 activity lead to a reduction in protein levels of CDC6, MCM2 and PCNA. More detailed analysis identified that CDK2 inhibition by roscovitine and cyclin E1/E2 depletion led to a reduction in transcript levels, potentially identifying the mechanism that reduced protein levels. However, cyclin A2 depletion reduces S-phase entry and reduces CDC6, PCNA and MCM2 levels by a yet to be identified mechanism (Figure 3.10.B). A striking observation in Figure 3.8
showed that cyclin A2 depletion does not affect cyclin E levels. As cyclin E is unaffected by cyclin A2 depletion this suggest that cyclin E-CDK2 could mediate hyperphosphorylation of Rb, thereby promoting expression of E2F regulated genes. Surprisingly, MCM2, CDC6 and PCNA levels are much reduced relative to controls, despite apparently normal transcript levels (Figure 3.10). This suggests that cyclin E and cyclin A may perform specific functions in G1 phase as cells re-enter the cell cycle from quiescence. Cyclin A-CDK2 activity appears to contribute to accumulation of DNA replication, potentially by protection from degradation mediated by Ubiquitin proteasome system. This forms the hypothesis for the next chapter, where the role of the UPS in degradation of the replication assembly proteins is assessed in absence of Cyclin-CDK2 activity.
Chapter 4
The Ubiquitin-Proteasome system and cyclin A-CDK2 regulates CDC6 and MCM2 levels in late G1 phase
4.1 Introduction: Ubiquitin proteasome system regulates G1 to S transition

Global analysis of transcript and protein abundance reveal that approximately 50% of proteins correlate with their transcript abundance, suggesting that the proteome is more dynamic than the transcriptome during cell cycle (Weintz et al., 2010; Ning, 2012). This highlights that intrinsic protein stability and the role of UPS mediated degradation contribute to regulation of protein levels within the cell.

The proteasome contributes to regulation of the cell cycle and facilitates temporal organization of the replication complex assembly as cells exit mitosis (Chang et al., 2015; Sivakumar and Gorbsky, 2015). Replication licensing is tightly regulated, as new licensing during late S phase and G2 phases can lead to re-replication that ultimately promotes genome instability. The consequences of re-replication include gene amplification and aneuploidy that drive oncogenesis (Shen et al., 2012). Therefore, it is imperative that cells prevent re-replication. Origin licensing is restricted to G1 phase, primarily through low CDK activity that enables pre-RC formation and ubiquitin-mediated proteolysis of licensing components and cell cycle regulators in later phases of the cell cycle (Chen et al., 2011; Diril et al., 2012; Walter et al., 2016; Johansson et al., 2014; Bassermann et al., 2014).

The transition from G1 to S-phase results from decreasing APC/C<sup>Cdh1</sup> activity, activating the SCF complex leading to decreased CKIs.
levels, rising cyclin expression, and therefore increasing CDK activity, phosphorylation and inactivation of Rb (retinoblastoma) protein family members, and activation of E2F transcription factor family members (Bassermann et al., 2014; Yang et al., 2012). The inactivation of APC/C\textsuperscript{Cdh1} at the G1/S transition is critical for the accumulation of cyclin A for S-Phase progression. Cyclin A levels accumulate in part due to E2F regulated expression of Emi1, an APC/C pseudosubstrate that inhibits its activity in late G1 phase (Prosser et al., 2012; Yang et al., 2012). Emi1 is degraded following phosphorylation by PLK1 and ubiquitination by SCF and this allows activation of APC/C that is critical for progression through M Phase and G1 Phase (Hatano and Sluder, 2012; Machida and Dutta, 2007) (Figure 4.1).
The UPS controls the cell cycle. The cell division cycle is regulated primarily by the activity of cyclin-dependent kinases (CDKs) and protein degradation by the ubiquitin–proteasome system (UPS). Each CDK complex contains one of many activating subunits, termed cyclins, the levels of which oscillate during the cell cycle. CKIs (CDK inhibitors), such as p27 and p21, inhibit CDK activity and promote cell cycle arrest and/or delay. SCF complexes and the APC/C (anaphase-promoting complex/cyclosome) provide the specific, rapid and timely proteolysis of cell cycle regulators, which ultimately controls CDK1 and CDK2 to finely modulate their activities during cell cycle progression.

**Figure 4-1 APC/C and SCF mediated G1/S transition.**

The UPS controls the cell cycle. The cell division cycle is regulated primarily by the activity of cyclin-dependent kinases (CDKs) and protein degradation by the ubiquitin–proteasome system (UPS). Each CDK complex contains one of many activating subunits, termed cyclins, the levels of which oscillate during the cell cycle. CKIs (CDK inhibitors), such as p27 and p21, inhibit CDK activity and promote cell cycle arrest and/or delay. SCF complexes and the APC/C (anaphase-promoting complex/cyclosome) provide the specific, rapid and timely proteolysis of cell cycle regulators, which ultimately controls CDK1 and CDK2 to finely modulate their activities during cell cycle progression.
4.2 APC/C\textsuperscript{cdh1} mediated ubiquitination in G1 phase

Cdh1 levels remain low from G2 and early mitosis due to phosphorylation (Oakes et al., 2014; Choi et al., 2014). Cdh1 is dephosphorylated at mitotic exit and is activated resulting in ubiquitination of Cdc20 (Huang et al., 2001), Aurora kinases (Littlepage et al., 2002; Stewart et al., 2005) and polo-like kinase 1 (PLK1) (Lindon et al., 2004) ensuring low kinase activity for mitotic exit (Singh et al., 2014; Kao et al., 2014; Zhang et al., 2016). During G1 phase, APC/C\textsuperscript{cdh1} polyubiquitinates many proteins including mitotic cyclins CDC25A (Donzelli et al., 2002), Skp2 and Cks1 (Bashir et al., 2004) leading to proteasome mediated degradation. In addition, APC/C\textsuperscript{cdh1} regulates destruction of replication regulators including Geminin (McGarry et al., 1998) and CDC6 (Mailand and Diffley, 2005); as well as its own E2, Ube2C and UbcH10 (Rape et al., 2004; Williamson et al., 2009). This leads to its inactivation of APC/C\textsuperscript{cdh1} and resulting stabilization of cyclin A (Oakes et al., 2014). This suggests that APC/C\textsuperscript{cdh1} plays an important role in regulation of protein stability during G1/S phase.
4.3 Chapter aims

This chapter aims to investigate whether CDK activity contributes to regulation of protein abundance through inhibition of the Ubiquitin Proteasome System (UPS). Chapter 3 identified that inhibition of CDK2 and DDK with Roscovitine and PHA 74756 along with genetic inhibition of cyclin E and cyclin A individually have shown to block cell cycle progression and S phase entry in cells released from quiescence. Inhibition of CDK2 activity in G1 phase leads to reduction in pre-RC proteins MCM2, CDC6; Pre-IC protein MCM10 and replisome protein PCNA in absence of cyclin –CDK2 and DDK activity. As observed in chapter 3, Cyclin E-CDK2 appeared to regulate transcription of MCM2, MCM10, CDC6 and PCNA; however, it is not clear how cyclin A –CDK2 depletion led to a reduction in MCM2, CDC6, MCM10 and PCNA abundance.

The aim of the chapter is to investigate the potential role of ubiquitin –proteasome system in degradation of proteins in the absence of cyclin-CDK2 and DDK activity. Secondary aim of the chapter is to investigate whether the E3 ligase Cdh1, that is active throughout G1 phase, contributes to the destabilization of MCM2, CDC6 and PCNA in G1 phase after inhibition of cyclin-CDK activity.
4.4 Proteasomal inhibition promotes stabilisation of MCM2, CDC6 and PCNA in G1 phase after CDK2 inhibition.

The observation that inhibition of cyclin-CDK2 and DDK activity reduces the abundance of DNA replication proteins (MCM2, CDC6 and PCNA) suggests that CDK and potentially DDK mediated phosphorylation in G1 phase regulate protein. The reduction in protein levels suggest that there are two possible mechanisms that mediate the reduction these proteins in absence of kinase activity.

Transcriptional regulation was tested in Chapter 3 and it was observed that cyclin E-CDK2 regulated the transcription of replication proteins (MCM2, CDC6, MCM10 and PCNA) via E2F mediated pathway. The focus of this chapter is to test the second hypothesis involvement of ubiquitin proteasome system (UPS) and its potential role in regulation of CDC6, MCM2 and PCNA abundance.

To test whether the UPS contributes to protein stability, a proteasomal inhibitor (MG132) was used. Initially, cycling asynchronous or synchronous 3T3 cells were co-incubated with the CDK2 inhibitor roscovitine with or without MG132 in independent experiments (Figure 4.2).
Chapter 4: Results

A: Asynchronous 3T3 cells were incubated with 30µM roscovitine and 10µM of MG132 for four hours and harvested in lysis buffer and analysed by western blot for recovery in destabilized MCM2, CDC6 and PCNA with proteasomal inhibition.

B: Synchronous 3T3 cells were incubated with 30µM roscovitine +/- 10µM of MG132 for four hours from at 20 hours and harvested in lysis buffer at 24 hours post release and analysed by western blot for recovery in destabilized MCM2, CDC6 and PCNA with proteasomal inhibition.

Inhibition of CDK2 activity in asynchronous cells, levels show a reduction in MCM2, CDC6 levels and PCNA to a lesser degree (Figure 4.2.A) consistent with observations from chapter 3. Importantly, in lane 3 (Cell lysate prepared from co-incubation with Roscovitine and MG132) of this western blot we see recovery in destabilized MCM2, CDC6 and PCNA with proteasome inhibition by MG132. Lane 4 shows proteasome inhibition and accumulation of MCM2, CDC6 and PCNA in absence of CDK2 inhibitor indicating positive activity of proteasome inhibitor MG132 (Figure 4.2A).

**Figure 4-2** Proteasome inhibition recovers destabilised proteins in absence of CDK2 activity.

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Inhibition of CDK2 activity in asynchronous cells, levels show a reduction in MCM2, CDC6 levels and PCNA to a lesser degree (Figure 4.2.A) consistent with observations from chapter 3. Importantly, in lane 3 (Cell lysate prepared from co-incubation with Roscovitine and MG132) of this western blot we see recovery in destabilized MCM2, CDC6 and PCNA with proteasome inhibition by MG132. Lane 4 shows proteasome inhibition and accumulation of MCM2, CDC6 and PCNA in absence of CDK2 inhibitor indicating positive activity of proteasome inhibitor MG132 (Figure 4.2A).
Similarly, to determine whether the proteasome potentially degrades this approach was repeated in a synchronised late G1 population using CDK and proteasomal inhibitors (Figure 4.2.B). The western blot shows a reduction of pre-RC proteins MCM2, CDC6 and replisome protein PCNA with incubation of roscovitine and a partial recovery with co-incubation with MG132. These results are consistent with a proteasomal mediated degradation of MCM2, CDC6 and PCNA after CDK2 inhibition.
Figure 4-3 Proteasome inhibition recovers destabilised proteins in the absence of DDK activity.

A: Synchronous 3T3 cells were incubated with 10µM PHA 74756 and 10µM of MG132 for four hours post restriction point around 20 hours and harvested in lysis buffer at 24 hours post release and analysed by western blot for recovery in destabilized MCM2, CDC6, CDC45 and PCNA with proteasomal inhibition.

For comparison with roscovitine mediated CDK2 inhibition, PHA 767491 (PHA) a DDK/CDK2 inhibitor was added to a synchronized late G1 population of cells. PHA inhibits DDK when used at 10µM and destabilizes pre-RC proteins MCM2, CDC6; pre-IC proteins CDC45, MCM10 and
replisome protein PCNA (Figure 4.3). Interestingly, DDK inhibition also affects cyclin A accumulation but not cyclin E. Similarly, inhibition of DDK activity reduces MCM2, MCM10, CDC6 and PCNA (Figure 4.3) that is reversed by inhibition of the proteasome by MG132. This suggests that CDK2 and potentially DDK mediated phosphorylation may promote stabilisation by direct phosphorylation or indirectly by potential regulation of E3 ligase and by inhibition of ubiquitination. Interestingly, CDC45 appears to be destabilized with DDK inhibition. Individual inhibition of CDK2 and DDK affects accumulation of pre-RC proteins MCM2, CDC6; Pre-IC proteins CDC45, MCM10 and replisome protein PCNA required for initiation of DNA replication and G1/S transition. Importantly, in both asynchronous and synchronous G1 population cells CDK/DDK inhibition appears to promote UPS mediated degradation that can be reversed by inhibition of the proteasome.
4.5 Cyclin A depletion destabilises replication complex proteins (CDC6, PCNA, MCM2, MCM10), by proteasomal mediated degradation

The use of small molecule inhibitors of CDK2 and DDK suggests that both CDK and DDK kinase activity promotes and facilitates accumulation of replication proteins in mid-late G1 phase and that this can be reversed by proteasomal inhibition (Figure 4.2 and Figure 4.3). In principle, roscovitine may destabilize the regulatory proteins by inhibition of cyclin E–CDK2 and cyclin A-CDK2. To ensure that effects observed were due to reduction in CDK2 mediated phosphorylation and not due to off target inhibition, siRNA mediated targeted depletion of cyclin A was performed in synchronized cells.

Cyclin A knockdown destabilized replication proteins CDC6, PCNA, MCM as observed in Chapter 3 (Figure 3.8, 3.10) potentially suggesting proteasomal regulation of proteins. To test this hypothesis, the proteasomal inhibitor MG132 was used and the effects on protein stability monitored. Cells were synchronized in G0 and transfected with cyclin A siRNA or control siRNA. Each reaction was performed in parallel; a control reaction and MG132 treated sample that were monitored as cells re-entered the cell cycle. There is a clear reduction in cyclin A2 transcript levels as determined by q-RT-PCR (Figure 4.4.A). Consistent with these observations there was a significant decrease in the number of cells that entered S-phase as determined by EdU labelling staining positively replicating cells (Figure 4.4.B).
A: Histogram generated from q-PCR analysis indicating cyclin A transcript depletion. B: Histogram showing block in S phase analysed by EdU labelling. C: Western blot analysis showing recovery in destabilized proteins with addition of MG132.

Figure 4-4 Destabilized replication proteins are degraded by proteasome mediated system in absence of cyclin A.

Analysis of protein levels was performed by western blot. Depletion of cyclin A show inhibition of cyclin A-CDK2. The results presented here shows destabilization of DNA replication proteins CDC6, PCNA and MCM2 (Figure 4.4). Again, consistent with earlier results using CDK2 and DDK inhibition
this destabilisation in reversed by addition of proteasomal inhibitor MG132. Interestingly, Cyclin E, p27, DBF4 remain unaffected in absence of cyclin A and that protein levels of these proteins remain consistent even with addition of MG132. These data suggest that cyclin A-CDK2 activity prevents degradation by ubiquitin proteasome system in late G1 phase in post-quiescent cells.
4.6 Analysis of ubiquitination of MCM2, CDC6 and PCNA.

The data presented suggest that the proteasome may degrade CDC6, MCM2 and PCNA after inhibition of CDK2 activity in late G1 phase. To determine whether CDC6, MCM2 and PCNA are poly-ubiquitinated a plasmid expressing a hexa-histidine-tagged ubiquitin (His<sub>6</sub>-Ub) was transfected with 3T3 cells to enable enrichment and isolation of ubiquitinated substrates. Detection of multiple bands would be consistent with poly-ubiquitination and suggest that UPS regulates their abundance.

Cell lysates were prepared from GFP transfected cells to ensure efficient transfection and, and from His<sub>6</sub>-Ub transfected cells with and without MG132. The addition of MG132 will enrich for poly-ubiquitinated proteins through inhibition of their degradation. Ubiquitinated proteins are then partially purified using Ni<sup>2+</sup>-NTA resin, washed extensively and bound proteins analysed by immunoblotting (Figure 4.5).

For both CDC6 and MCM2, GFP transfected and His-Ub transfected cells recovered little protein, but addition of MG132 lead to an increase in recovered proteins, consistent with an UPS mediated degradation. Furthermore, addition of MG132 uncovered the number of bands that were detected between 110-150 kDa for MCM2 (Figure 4.5.A) and 60-70 kDa for CDC6 (Figure 4.5.B), consistent with poly-ubiquitination.
Chapter 4: Results

3T3 cells were transfected with GFP or His-Ub and cell lysates produced 24 hours after transfection. His-6- Ub labelled proteins were recovered by Immobilised metal affinity chromatography (IMAC) +/- MG132. Ubiquitinated proteins were detected by western blot A: for MCM2, B: CDC6 and C: PCNA.

As Ub-PCNA was not detected it remains to be determined how PCNA levels are regulated. Multiple experiments show that inhibition of CDK2 activity reduces PCNA levels, as it could not be directly detected here using a His tagged ubiquitin, perhaps PCNA stability is regulated by an alternative mechanism. Nevertheless, this approach could detect poly-ubiquitinated MCM2 and CDC6, consistent with UPS mediated degradation.

Figure 4-5 His6-Ub mediated pull down of MCM2, CDC6 and PCNA with MG132.

3T3 cells were transfected with GFP or His-Ub and cell lysates produced 24 hours after transfection. His-6- Ub labelled proteins were recovered by Immobilised metal affinity chromatography (IMAC) +/- MG132. Ubiquitinated proteins were detected by western blot A: for MCM2, B: CDC6 and C: PCNA.

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117
4.7 Cdh1- potential E3 ligase regulating degradation of MCM2, CDC6 and PCNA in absence of cyclin dependent kinases.

To further support a UPS mediated degradation of MCM2 and CDC6, preliminary experiments were performed targeting a G1 ubiquitin E3 ligase. E3 ligases transfer ubiquitin moieties to the target proteins and marking them for degradation via 26S proteasome. There are more than 600 E3 ligases (Li et al., 2008) that regulate cellular homeostasis and contribute to mechanisms that maintain DNA replication once and once per cell cycle.

Cyclin dependent kinases mediated phosphorylation of CDC6 could be responsible for stabilization. CDC6 is a substrate of cyclin E-CDK2 and cyclin A-CDK2 mediated phosphorylation at three serine residues (Herbig et al., 2000); two of these lie in the close proximity of APC/C destruction boxes. Serine 54 is next to RXXL-type destruction box and serine 74 is near a KEN type destruction box (Petersen et al., 2000). APC/C<sup>Cdh1</sup> recognizes the KEN box motif for destruction and that CDC6 is regulated by APC/C<sup>Cdh1</sup> mediated degradation in absence of kinase activity (Mailand and Diffley 2006).

Figure 4.6 shows co transfection of Cdh1 siRNA with cyclin A siRNA in synchronized cell system in comparison with proteasomal inhibitor MG132 with cyclin A siRNA transfection. Relative quantitation of cyclin A and Cdh1 was tested post transfection relative to GAPDH (Figure 4.6.A). Cyclin A transcript levels were depleted in both cyclin A transfected alone and co
transfected with cyclin A and Cdh1 siRNA (Figure 4.6.A). Similarly, Cdh1 transcript levels were depleted as well in Cdh1 siRNA transfection alone and with co-transfection of cyclin A and Cdh1. The cell cycle kinetics in post-quiescent cells was determined after cyclin A, Cdh1 and co-transfection cells (Figure 4.6.B). Depletion of cyclin A alone reduced S-phase entry to the greatest extent, less than 20 % of cells in S-phase at 24 hours after release. However, Cdh1 and Cdh1 co transfection with cyclin A had an intermediate effect with less than 30% of cells in S phase compared to 45% in control reactions. Analysis of protein levels after cyclin A2 or Cdh1 siRNA transfections show efficient depletion of cyclin A but Cdh1 was not detected.
Figure 4-6 Cdh1 a potential E3 ligase destabilising replication complex assembly in absence of cyclin A.

A: Transcript analysis of cyclin A, Cdh1 in individual transfection and co-transfection of cyclin A and Cdh1. B: Histogram showing number of S phase cells in absence of cyclin A, Cdh1 and in combination of cyclin A and Cdh1. C: Western blot analysis for combination of cyclin A and Cdh1 co-transfection.

To assess whether Cdh1 depletion prevents loss of CDC6, MCM2 or PCNA quiescent cells were transfected with Cdh1 siRNA and roscovitine was used to inhibit CDK activity (Figure 4.7).
Figure 4.7 CDK2 inhibition and Cdh1 depletion in synchronized cell population.

A: Transcript analysis of Cdh1 after Cdh1 depletion. B: EdU analysis of cells re-entering cell cycle and in S phase with Cdh1 depletion in comparison with control cells. C: Western blot analysis of CDC6, MCM2, PCNA for samples prepared in reactions with CDK2 inhibitor and Cdh1 depletion. The inconclusive western blot with Cdh1 depletion as protein levels could not be equalised due to low cell recovery post-transfection after Cdh1 depletion.

Figure 4.7.A shows effective Cdh1 depletion with a 95% reduction in transcript levels post Cdh1 siRNA transfection in synchronized cells. To assess whether Cdh1 depletion affects cell cycle progression EdU labelling of cells was performed. As observed in Figure 4.7.B shows nearly 35% of cells enter S phase in absence of Cdh1 compared to 45% of cells in S phase in
control transfections. To determine whether CDK2 inhibition destabilises CDC6, MCM2 or PCNA after Cdh1 depletion roscovitine was used. Western blot analysis showed MCM2 was effectively reduced by addition of roscovitine, whereas CDC6 and PCNA were not affected. Addition of MG132 and roscovitine increased CDC6 and MCM2 levels (Figure 4.2) appear to be affected with CDK2 inhibition and recovery in the destabilized proteins with MG132. However, with Cdh1 depletion was inconclusive because there was a reduction in cell number after Cdh1 depletion and it was not possible to balance the protein loads in multiple experiments.

To assess whether the expression of Cdh1 correlates with cell cycle stage where cyclin A-CDK2 is active, the mRNA and protein levels of Cdh1 were determined in a synchronised population of 3T3 cells (Figure 4.8.A). RT-qPCR of cyclin A2 and Cdh1 were in synchronised 3T3 cells re-entering cell cycle every 4 hours post-release. Cyclin A expression was determined in Chapter 3. Its transcript levels increase throughout G1 phase and peak after restriction point around 18-20 hours post release. This contrasts with Cdh1, which is transcribed in early G1 phase and is reduced prior to restriction point. With transcript levels of Cdh1 appear to be at the peak when cells are in quiescence or G0 and the levels appear to fall with the cells re-entering cell cycle and S-phase.
Chapter 4: Results

A: Transcript levels of Cyclin A and Cdh1 in cells re-entering cell cycle from quiescence.

B: western blot analysis of Cdh1 and cyclin A over the time course of release from quiescence indicating increase in protein levels of cyclin A with a peak at 24 hours post release and Cdh1 levels decrease as the cells re-entering cell cycle from quiescence.

Western blot analysis of Cdh1 and cyclin A show opposing results, Cyclin A levels increase with cells re-entering cell cycle and peak at 24 hour time point post release, whereas Cdh1 levels decrease as cells re-entering cell cycle. The temporal expression of Cdh1 does not correlate with expression of cyclin A2 suggesting that Cdh1 is not abundant during late G1 phase where we detect a UPS mediated degradation in late G1, suggesting that Cdh1 is unlikely to be the E3 ligase involved in regulation of protein abundance in late G1 phase.

Figure 4-8 Cdh1 transcript and protein levels in cells released from quiescence.
4.8 Discussion

In this chapter, the role of Ubiquitin proteasome system in absence of active kinases during G1/S transition and potential mechanism by which cyclin A-CDK2 regulate the replication assembly proteins was assessed. APC/C and SCF being the key E3 ligases regulating cell cycle progression (Rikardi and Cook, 2012), and that absence of kinase activity upregulates the APC/C activity to degrade substrates to promote transition from G1 to S phase.

As observed in Chapter 3 and Figure 4.2 inhibiting CDK2 with roscovitine reduced DNA replication proteins CDC6, MCM2 and PCNA similar to the effect of cyclin A knockdown (Figure 4.5). This suggests that Cyclin A-CDK2 complex is responsible for appropriate accumulation of CDC6 and MCM2 during late G1 phase. Roscovitine predominantly inhibits CDK1, CDK2 and in G1 phase CDK1 is inactive due to inhibitory phosphorylation (Jang et al., 2016). Inhibition of CDK2 in late G1 phase reduced CDC6, MCM2 and PCNA levels but addition of MG132 reverses protein degradation, suggesting involvement of the UPS in their degradation. These experiments lead to intriguing area of research to understand mechanism of ubiquitin ligases in maintaining and regulating the process of replication licensing.

The G1/S transition is regulated by two main ubiquitin proteasome complexes, APC/C\(^{Cdh1}\) and SCF regulating the protein degradation during cell
cycle (Araki et al., 2005). APC/C and SCF work in coordinated fashion in regulating the protein concentration depending upon the requirement of cell cycle (Bashir et al., 2004). Understanding mechanism by which cyclin A functions in coordination with ubiquitin system was studied in detail in this chapter. The cyclin A2 specific role in destabilisation of CDC6 and MCM2 was restricted to late G1 to S-phase transition in post-quiescent cells. As protein stability can be increased by addition of MG132 suggests a role of ubiquitin proteasome mediated regulation in stabilizing replication assembly proteins MCM2, CDC6, PCNA during G1/S transition and DNA replication.

In addition to CDK2 inhibition, there was also a reduction of CDC6, MCM2 and PCNA when incubated with the DDK inhibitor PHA 767491. As this inhibitor also inhibits CDK2 and due to the similarity between the results with roscovitine and PHA, we cannot exclude that PHA is inhibiting the CDK2 network leading to the reduction in CDC6, MCM2 and PCNA. Nevertheless, for both roscovitine and PHA mediated inhibition respectively, the reduction in protein levels could be reversed by addition of MG132. Consistent results indicate a role for the UPS in regulation of CDC6, MCM2 and PCNA abundance in absence of kinase activity during G1/S transition. To further support the UPS mediated degradation His-tagged ubiquitin was used to identify ubiquitinated substrates. After CDK2 and proteasomal inhibition, there was an enrichment of poly-ubiquitinated CDC6 and MCM2
Chapter 4: Results

(Figure 4.5) but PCNA was not detected using this approach. The failure to detect PCNA suggests that PCNA may be destabilised by a different mechanism that is yet to be identified.

Taken together this data demonstrates that Cyclin A2 depletion prevents G1 exit in post-quiescent cells (Figure 3.7, Figure 4.4). This observation is distinct from those of the cyclin A2 null mouse fibroblasts that showed no requirement for cyclin A2 for cell cycle progression (Geng et al., 2007). These differences may be related to technical differences in our approaches or perhaps relate to the differences in cycling and post-quiescent populations. Here we are using post-quiescent cells to investigate cyclin A2 cell cycle regulation. The results clearly show that depletion of cyclin A2 prevents initiation of DNA replication in post-quiescent cells. These data presented here suggest that cyclin A-CDK2 prevents UPS mediated degradation of CDC6 and MCM2 that appears to be required for efficient initiation of DNA replication in post-quiescent cells.
4.8.1 Analysis of Cdh1 activity in regulation of CDC6, MCM2 and PCNA.

Preliminary experiments aimed at investigating the role of Cdh1 in degradation of MCM2 and CDC6 were unsuccessful due to a consistent loss of cells following Cdh1 transfection. Other approaches would be required to address this question. However, temporal analysis of Cdh1 and cyclin A expression found an inverse correlation, consistent with Cdh1 mediated destruction in G1 phase and cyclin A-CDK2 mediated destruction of Cdh1 at the G1/S transition. The temporal analysis of Cdh1 expression suggests that another E3 ligase is responsible for poly-ubiquitination of CDC6 and MCM2. Further analysis is required to identify and characterise the E3 ligase that regulates accumulation of pre-RC proteins in G1 phase.
Chapter 5
Analysis of Ciz1 and DHX9 in synchronized post-quiescent mouse fibroblasts
5.1 Ciz1 Discovery and structural characterization

Ciz1 (Cip1-interacting zinc finger protein) is a protein unique to vertebrates and is conserved in mammals. It was first identified as Cip1-p21 interacting protein in *S. cerevisiae* yeast two hybrid assay (Mitsui et al., 1999). Ciz1 not only interacts with p21 but also to cyclin E-CDK2, cyclin A, and CDC6 resulting in contribution to regulation of cellular proliferation and transcriptional regulation (Coverley et al., 2005; Copeland et al., 2010; Copeland et al., 2015).

The domain structure of Ciz1 is consistent with the role in spatial coordination of DNA replication complex assembly (Ainscough 2007). The C-terminus contains nuclear matrix binding domains (Copeland et al., 2010) and N terminus contains the binding sites for pre-RC protein CDC6 and cyclin A-CDK2 and the nuclear matrix (Copeland et al., 2015). The C-terminal domain contains three C2H2-type zinc finger, an acidic patch, with a nuclear matrix association domain similar to matrin 3 (MH3 domain) (Mitsui et al., 1999; Warder and Keherly, 2003) Figure 5.1.A.
Figure 5-1 Structural representation of Ciz1

A: The figure shows the regions corresponding to the glutamine-rich domain (QD1 and QD2), the acidic domain (AD), the zinc finger domains (ZF), and MH3 domain (MH3) of the Ciz1. Black dots indicate the phosphorylation sites of Ciz1 (Mitsui et al., 1999). B: the phosphorylation sites in the N-terminal are presented in black (6 out of total 14 are presented), cyclin-binding domains are shown in light blue ovals, and the dark blue oval indicates the interaction site of cyclin E and cyclin A, required for the regulation of DNA replication function of Ciz1 (Copeland et al., 2015). The minimal functional fragment of Ciz1, named N471, is depicted as yellow and red parts combined.

5.2 Ciz1 function in initiation of DNA replication during G1/S transition

It was determined that Ciz1 is involved in cell cycle regulation and potentially carcinogenesis by interacting with p21-Cip1 and regulating its availability at G1/S checkpoint. Consecutive experimentation determined that mRNA of Ciz1 was abundant in many human cell lines and in a variety of tissues. Ciz1 mRNA was most abundant in kidney and testis tissues in mice (Mitsui et al., 1999). Later, it was independently isolated from human medulloblastoma and described as a DNA replication factor and potential
driving force in tumorigenesis (Warder and Keherly, 2003). The Ciz1 is known to associate with the consensus DNA sequence ARYSR(0–2)YYAC where, (A: adenine; R: purine; Y: pyrimidine; S: either purine or pyrimidine; C: cytocine) (Liu et al., 2016; Warder and Keherly, 2003).

Ciz1’s association with nuclear matrix contributes to temporal and spatial regulation of DNA replication (Berezney et al., 1975; Ainscough et al., 2007). The nuclear matrix is the insoluble structure that associates with cell cycle regulators and enzymes required for DNA replication (Berezney et al., 1975). Replisomes are directly associated with the nuclear matrix and retain activity after nuclear fractionation (Radichev et al., 2005; Munkley et al., 2011). Ciz1 is localized to the nuclear matrix by matrin-like zinc finger domains in the C-terminus of Ciz1 (Mitsui et al., 1999; Ainscough et al., 2007), where it colocalizes with PCNA in S-phase cells, contributing to the G1/S transition and increased cellular proliferation (Coverley et al., 2005). The localization of Ciz1 proximal to active replication factories in G1 phase and colocalization with PCNA during S phase (Ainscough et al., 2007) suggests that Ciz1 may contribute to localization and recruitment of cell cycle regulators for efficient initiation of DNA replication at the nuclear matrix.
Ciz1 binds to the pre-replication complex via CDC6 interactions during G1 phase. Cyclin A (A) displaces cyclin E formerly bound to Ciz1, bringing activated kinase CDK2 (K2) proximal to its targets. Increasing local concentrations of cyclin A – CDK2 promotes initiation of DNA replication and inactivation of Ciz1 by hyper-phosphorylation. This provides a window of opportunity whereby Ciz1 contributes to activation of the replisome and hyper-phosphorylation prohibits re-replication (Copeland et al., 2015).

Ciz1 accumulates throughout the G1 phase, and its abundance correlates with accumulation of cyclin activity in G1 phase. siRNA mediated depletion of Ciz1 inhibits cell progression and blocks entry into S phase (Coverley et al., 2005). The requirement of Ciz1 in DNA replication was explained by its specific and sequential association with cyclin E and cyclin A on different sites, bringing them near their targets, such as CDC6, CDT1, and MCM further suggests active role in cell proliferation (Copeland et al., 2010; Copeland et al., 2015). Ciz1 is proposed to promote initiation of DNA replication by recruiting cyclin E and cyclin A-CDK2 to putative replication sites (Copeland et al., 2010).
The Ciz1 protein is phosphorylated by Cyclin – CDK complexes at various sites (Copeland et al., 2010; Copeland et al., 2015) (Figure 5.1. B). The phosphorylation of different sites contributes to the activation of Ciz1 or inhibition of its DNA replicative function (Copeland et al., 2015). The phosphorylation on T293 site by cyclin A – CDK2 was shown to inhibit Ciz1 function, thus contributing to mechanisms that prevent DNA re-replication (Copeland et al., 2015). There are 6 CDK phosphorylation sites in N471 minimal functional fragment of Ciz1, and their phosphorylation potentially provides different effects on Ciz1 (Ainscough et al., 2007, Coverley et al., 2005; Copeland 2015).

Importantly, Ciz1 can only associate with Cyclin A-CDK2 in a hypophosphorylated state to promote initiation of DNA replication, as phosphorylation at >3 sites prevent cyclin binding *in vitro* and *in vivo*. This contrasts with CDC6 binding that are independent of the phosphorylation status of Ciz1 (Copeland et al. 2015). Hyper-phosphorylation of Ciz1 prevents successive recruitment of cyclin A to replication origins that have already fired, preventing further initiation events (Copeland et al., 2015) (Figure 5.2). As Ciz1 can prevent replication initiation in a hyper-phosphorylated form, this suggests that phosphorylation of Ciz1 contributes to genome stability by regulation of initiation of DNA replication and to mechanisms that prevent re-replication (Copeland et al., 2015; Figure 5.2).
5.3 Chapter aims

Ciz1 is known to promote cell cycle progression. Here, the accumulation of Ciz1 in G1 phase will be investigated and the role of cell cycle kinases CDK and DDK in its accumulation will be investigated. In addition, the role of the UPS in regulation of Ciz1 abundance will be investigated.

A second objective is to characterise the role of DHX9 in regulation of the G1/S transition. Preliminary evidence demonstrated that Ciz1 and DHX9 associate in S-Phase extracts suggesting a common function. Therefore, cell cycle kinetics will be analysed in DHX9 depleted cells and replication complex assembly monitored.
5.4 Ciz1 protein and transcript levels remain constant during G1/S transition:

Ciz1 interacts with cyclin E-CDK2 and cyclin A-CDK2 and promote DNA replication (Copeland et al., 2010; Copeland et al., 2015). To determine the cell cycle dynamics of Ciz1 expression, 3T3 mouse fibroblasts were synchronized by contact inhibition and serum starvation. After release from quiescence the protein and transcript levels of Ciz1 were monitored during cell cycle re-entry from G1 to S-phase. Rabbit polyclonal antibody (N471) raised against the N-terminal domain of Ciz1 was used to monitor Ciz1 protein levels in cells released from quiescence (Figure 5.3.A.). Two phosphorylation sites within Ciz1 were also monitored to determine temporal regulation by CDK2. Cyclin A–CDK2 mediated phosphorylation on T293 site inhibits Ciz1 function and increases in S phase (Copeland et al., 2010; Copeland et al., 2015). However, phosphorylation on S331 site was selected as it was readily modified by cyclin A -CDK2 in vitro, but it did not inhibit DNA replication function (Copeland et al., 2010). Therefore, phosphorylation of Ciz1 at S331 may regulate alternative functions, such as Ciz1 activation or promotes its accumulation.
Figure 5.3 Ciz1 transcript levels remain constant during G1/S transition:

A: Graph showing an increase in S phase cells from 0-24 hour time points measured by EdU incorporation in positively replicating cells. B: Comparison of Ciz1 transcript levels with E2F regulated cyclin A and cyclin E, Ciz1 expression is consistent through G1 phase whereas cyclin E and cyclin A show a 10-fold increase in expression (N=3). C: Western blot analysis of Ciz1 along with phospho-specific sites S331, T293 in comparison with cyclin E and cyclin A after release from quiescence through 0-24 hour time course. D: Relative Quantification of the band intensities of the proteins presented in western blot (C).
Chapter 5: Results

Cell cycle synchronization was confirmed by EdU incorporation (Figure 5.3.A), with an increase in S phase cells with approximately 50% of cells in S phase at 24 hours post-release showing efficient synchrony. Cyclin E-CDK2 levels remain constant in cells released from quiescence (Geng et al., 2003; Geng et al., 2007; Chapter 3; section 3.4). However, cyclin A protein levels start accumulating from 16 hour onwards post release from quiescence as observed previously (Figure 3.4). Interestingly transcript levels of Ciz1 do not correspond to the protein levels as Ciz1 accumulates up to 12 hours post-release from quiescence despite little change in transcript abundance (Figure 5.3.B). Ciz1 transcript levels do not fluctuate in comparison with E2F regulated genes cyclin E1/E2 and cyclin A2, show that a large increase in transcript levels as cells progress into S-phase. These data suggest that Ciz1 accumulation is not mediated by transcriptional regulation but may be related to post-translations mechanisms, as Ciz1 protein levels and phosphorylation status Ciz1 corresponds to that of cyclin E and cyclin A levels (Copeland et al., 2010; Copeland et al., 2015).
5.5 Ciz1 is destabilised in absence of CDK2 during G1-S transition

To investigate whether Ciz1 accumulation is dependent upon CDK activity, cells were synchronized by contact inhibition and serum starvation. After release from quiescence Ciz1 protein levels and its phosphorylation status followed after CDK2 inhibition using roscovitine (Figure 5.4).

Figure 5-4 Ciz1 is destabilised in absence of CDK2 activity during G1-S transition.

A: Histogram showing block in S phase cells with inhibition of CDK2, with statistically significant (P<0.05) less than 10 % cells in S phase measured with EdU incorporation at 20 hour and 24 hour time point post release from quiescence. B: Western blot of whole cell extracts were prepared 4 hours after treatment with roscovitine at 20 and 24 hours post release from quiescence respectively. Ciz1 phospho-threonine 293 (pT293) and phospho-serine 331 (pS331) were monitored in synchronous G1 cells. CDK2 inhibition reduces both T293 and S331 phosphorylation.
Roscovitine addition reduces S phase cells progression at both time points (16hr-20hr and 20hr-24hr), as measured by EdU incorporation. Interestingly, (Figure 5.4.B) demonstrates that inhibition of CDK2 activity by roscovitine Ciz1 leads to a reduction in Ciz1 protein levels at both 20 and 24 hours but this effect is more evident in the 24 hour time-point. Importantly, the levels of CDK mediated phosphorylation of Ciz1 particularly at the T293 site is markedly reduced at both 20 and 24 hour time points. These data suggest that Ciz1 protein levels increase as a consequence of CDK mediated phosphorylation at multiple sites.
Chapter 5: Results

5.6 Cyclin inhibition destabilizes Ciz1 during G1/S transition:

Chemical inhibition of CDK2 by roscovitine inhibits S phase progression of cells released from quiescence. As observed in (Figure 5.4) CDK2 inhibition destabilizes Ciz1 protein levels as CDK2 inhibition blocks Ciz1 phosphorylation at S331 and T293 site. CDK2 is cognate partner to both cyclin E and Cyclin A and hence to ensure specific effect of CDK2 inhibition, targeted depletion of cyclin E and cyclin A was performed by siRNA mediated transfection.

Cyclin E-CDK2 and cyclin A-CDK2 activity, as discussed in chapter 3 and Chapter 4, promote accumulation of CDC6 and MCM2 by distinct mechanisms. Cyclin E-CDK2 promotes their transcription via the Rb-E2F pathway and cyclin A2 appears to regulate UPS mediate proteasomal degradation. (Figure 3.8-Figure 3.11, Figure 4.4). To further investigate the role of cyclin E and cyclin A in the regulation of Ciz1 accumulation, each cyclin was depleted by siRNA mediated transfection and the effect on Ciz1 protein levels analysed by western blot.

Figure 5.5.A shows the western blot analysis of depletion of cyclin E1/E2 in synchronized 3T3 cells. Ciz1 protein levels appear to be destabilized at both time points 20 hour and 24 hour post release and transfection, suggesting Ciz1 is destabilized in absence of cyclin E activity similar to replication assembly proteins MCM2, MCM10, CDC6, PCNA (Figure 3.10).
RT-qPCR was performed to determine the transcript analysis of cyclin E1/E2 and Ciz1 as observed in (Figure 5.5.B). Cyclin E1/ E2 transcript levels are effectively depleted by siRNA mediated depletion. Interestingly, Ciz1 transcript levels were moderately affected with depletion of cyclin E1 and E2 in comparison to cyclin A depletion. However, statistical analysis of the Ciz1 transcripts is not significant as P>0.05 calculated by performing T-test. This suggests that Ciz1 transcript levels are not regulated by cyclin E-CDK2 activity.
Chapter 5: Results

A: Western blots showing cyclin E1/E2 inhibition destabilizing Ciz1 protein levels. 

B: Transcript analysis performed by qPCR analysis of cyclin E1, E2 and Ciz1 showing Ciz1 transcript levels in absence of cyclin E1 and E2 at 24 hour time point post transfection and release from quiescence.

C: Western blots showing cyclin A2 inhibition destabilizing Ciz1 protein levels.

D: Transcript analysis performed by qPCR analysis of cyclin A2 and Ciz1 showing Ciz1 transcript levels not affected in absence of cyclin A2 at 24 hour time point post transfection and release from quiescence at 24 hour time point (N=3).

Interestingly, cyclin A2 depletion also destabilizes Ciz1 protein levels but does not affect the transcript levels, suggesting that cyclin E and cyclin A activity is required for Ciz1 protein accumulation during G1/S transition in synchronized post-quiescent mouse fibroblasts.

Figure 5-5 Ciz1 protein is destabilised in absence of cyclin E and cyclin A.
5.7 Ciz1 is proteasomally regulated in absence of kinase activity during G1-S transition

Cyclin E-CDK2 and cyclin A-CDK2 activity independently phosphorylate Ciz1 protein during G1/S transition indicating a significant role in initiation of DNA replication (Copeland et al., 2015). The data presented here suggest that CDK mediated phosphorylation of Ciz1 promotes its accumulation. To evaluate the role of the proteasome in regulation of Ciz1 abundance during G1 / S transition, MG132 was used to inhibit the proteasome concurrently with CDK inhibition (Figure 5.6).

**Figure 5-6 Ciz1 protein is proteasomally regulated in absence of CDK2.**

A: Western blot analysis of asynchronised cells co-incubated with roscovitine ±MG132. B: Histogram showing block in S phase cells with inhibition of CDK2, with statistically significant less than 10% cells in S phase measured with EdU incorporation at 24 hour time point post release. C: Western blot analysis of synchronised cells co-incubated with roscovitine ±MG132 20 hours after release from quiescence and harvested at 24 hours post release, showing recovery in Ciz1 protein levels with addition of MG132.
Figure 5-7 Ciz1 is proteasomally regulated in absence of Cyclin A.

A: Histogram showing cyclin A transcript levels depleted with cyclin A2 siRNA transfection. B: Line graph showing block in S phase cells with depletion of Cyclin A, with statistically significant less than 5 % cells in S phase measured with EdU incorporation at 24 hour time point post release. C: Cyclin A siRNA mediated depletion was performed by transfection and harvested at 24 hours post release from quiescence Western blot analysis of cyclin A2 depleted synchronised cells ±MG132. D: Quantification of intensity of Ciz1 abundance from western blot (C).
To avoid potential artefacts from the use of small molecule inhibitors, siRNA mediated depletion of cyclin A was also used to reduce CDK activity in late G1 phase of the cell cycle (Figure 5.7). Inhibition of CDK2 activity by addition of roscovitine (Figure 5.6A) and cyclin A depletion (Figure 5.7) promoted a reduction in Ciz1 protein levels consistent with earlier experiments (Figure 5.4 and Figure 5.5). siRNA mediated cyclin A depletion reduced transcript levels by more than 95% (Figure 5.7.A) and reduced the number of cells from approximately 45% to approximately 10% in cyclin A2 depleted cells. In parallel reactions monitoring cyclin A levels, siRNA mediated depletion reduced cyclin A levels to undetectable levels (Figure 5.7.C).

The reduction in cyclin A reduced Ciz1 levels relative to control samples, that was reversible by inhibition of proteasome, suggesting that ubiquitin mediated degradation regulates Ciz1 protein levels. MG132 increased Ciz1 levels for both roscovitine treated and siRNA depleted cells suggesting that CDK mediated phosphorylation of Ciz1 promotes its accumulation by prevention of UPS mediated degradation of Ciz1 in G1 phase.
5.8 Ciz1 interacts with other cell cycle regulators including DHX9

Proteomic analysis identifying Ciz1 interacting partner has been performed using S -Phase cellular extracts (Data not shown). One of the significant hits that appeared from this screen identified a DNA helicase (DHX9) that has been shown to associate with putative replication origins and contribute to regulation of DNA replication (Lee et al., 2014).

DHX9 is a DEXH box helicase exhibiting both RNA and DNA helicase activity. It also shows triple helical DNA unwinding activity (Zhang, 1994; Jain, 2010). It was originally isolated from bovine tissue. DHX9 structure consists of two RNA-binding domains at the N-terminus, a core helicase region consisting of seven conserved helicase motifs and a DNA-binding domain and nuclear localisation signal at the C terminus (Jacob et al., 1991; Zhang, 1997). DHX9 is a multifunctional protein with implications in several biological processes (Lee, 2014). Its interacting partners include EGF receptor, BRCA1, cAMP response element binding protein and RNA polymerase II (Nakajima et al., 1997; Huo et al., 2010).

Ciz1 and DHX9 have been implicated in contributing to accurate DNA replication (Copeland et al., 2010; Copeland et al., 2015; Coverley et al., 2005; Lee et al., 2014). DHX9 is associated with replication origins in cells and contributes to S phase progression (Lee et al., 2014). Ciz1 promotes initiation of DNA replication through interactions with cyclin A during the G1/S
transition and enhances cyclin A-CDK2 recruitment to chromatin during initiation of DNA replication (Copeland et al., 2010).

To assess the role of DHX9 in cell cycle regulation mouse fibroblasts were synchronized in G0 using contact inhibition and serum starvation and the requirement for DHX9 in entry into S-phase was determined (Figure 5.8). Quiescent 3T3 cells were transfected with control siRNA and anti-DHX9 siRNAs and the proportion of cells in S-Phase determined. Both DHX9 transcript (Figure 5.8.A) and protein levels (Figure 5.8.C) were efficiently depleted and this resulted in a moderate decrease in cells entering S-phase compared to control (Figure 5.8.B).
Figure 5-8 Ciz1 interacts with cell cycle regulator DHX9.
A: Transcript analysis of DHX9 post siRNA mediated depletion of DHX9 in synchronized mouse embryonic fibroblasts. B: Reduction in number of cells entering S phase post DHX9 depletion measured by EdU analysis. C: Western blot analysis showing DHX9 depletion, Ciz1 and cyclin A protein reduction in absence of DHX9.
Depletion of DHX9 leads to a reduction in cyclin A levels and a moderate reduction in Ciz1 levels. Both Ciz1 and cyclin A accumulate as cells transit from G1 to S-Phase (Copeland et al., 2015) (Figure 5.3), therefore the results are consistent with a delay in cell cycle progression as a result of DHX9 depletion. As prolonged depletion of DHX9 was previously shown to promote senescence via p53 activation (Lee et al., 2014), the levels of p53 were monitored (Figure 5.8.C). After acute depletion of 24 hours p53 levels were unaffected and p21 levels were reduced suggesting that p53 is not activated by acute depletion of DHX9 (Lee et al., 2014). Taken together these data suggest that Ciz1 and DHX9 share a common function in regulation of the G1/S transition and further studies are required to fully understand how they contribute to efficient cell cycle progression in murine fibroblasts.
**5.9 Discussion**

In mouse fibroblasts, Ciz1 interacts with temporal cell cycle regulators, including CDC6, cyclin E, cyclin A, and CDK2 proximal to the origin of replication (Coverley et al., 2005; Ainscough et al., 2007; Copeland et al., 2010; Copeland et al., 2015). The data presented here are consistent with earlier works that shows that Ciz1 accumulation is required for successful initiation of DNA replication and cell entry to S phase in normal cells (Coverley et al., 2005; Copeland et al., 2010). Ciz1 protein accumulated through G1 phase that mirrors E2F regulated genes (Figure 5.3). However, Ciz1 transcription levels are relative constant during the G1 to S-phase transition in mouse fibroblasts. The correlation between Ciz1 phosphorylation and its abundance suggest that Ciz1 accumulation may be CDK regulated. These data suggest that Ciz1 is regulated by CDK2 mediated phosphorylation to promote its accumulation as well as regulating its function in regulation of the initiation phase of DNA replication.

Targeted inhibition of CDK2, cyclin E and cyclin A individually lead to reduction in Ciz1 protein accumulation (Section 5.4-5.6). CDK2 inhibition mediated by roscovitine affects Ciz1 stability around 24 hours post-release from quiescence, whereas DDK inhibitor affected Ciz1 stability at around 20 hour time point post release. Interestingly, CDK2 inhibitor inhibited Ciz1 phosphorylation at T293 and S331 at 24 hour time point, suggesting
requirement of kinase activity for regulation of Ciz1 during G1/S transition. As observed in section 5.5, however, cyclin E appears to affect Ciz1 transcript levels more than cyclin A depletion. However, statistical analysis performed by T-test reveals P value > 0.05 and thus transcript reduction of Ciz1 is not statistically significant. It is however evident from section 5.5 that targeted inhibition of cyclin E and cyclin A independently destabilize Ciz1 protein levels and that phosphorylation mediated by cyclin E-CDK2 and cyclin A-CDK2 is required for Ciz1 stability during G1/S transition.

Additionally, Ciz1 levels could be recovered with addition of MG132 concomitantly with roscovitine, suggesting that Ciz1 protein is regulated by proteolytic degradation in absence of active kinases during G1/S phase. These observations suggest that Ciz1 levels are positively regulated phosphorylation, and negatively regulated proteolytic degradation. Further characterization of Ciz1 regulation by CDK and UPS mediated regulation. In addition, greater insight into the regulatory network that governs Ciz1 levels could be gained from identification of potential E3 ligase(s) that regulates Ciz1 levels.

The data presented here demonstrate for the first time that Ciz1 is regulated by phosphorylation leading to its accumulation in G1 phase most likely through protection by UPS mediated degradation in a low CDK kinase environment. The data are consistent with a model whereby Ciz1 accumulates
in response to increasing CDK activity in G1 phase. Phosphorylation of Ciz1 promotes Ciz1 accumulation that facilitates in recruitment of cyclin E-CDK2 and cyclin A-CDK2 to putative replication sites (Copeland et al., 2015). This model suggests CDK activity regulates Ciz1 accumulation, activation for DNA replication initiation, and the inactivation of Ciz1 by hyper-phosphorylation during S phase to prevent DNA re-replication (Ainscough et al., 2007; Copeland et al., 2010; Copeland et al., 2015; Coverley et al., 2005). Further investigation of the role of S331 and T293 in Ciz1 accumulation is required to identify whether there is a site specific temporal order to site phosphorylation in G1 phase.
5.9.1 Preliminary characterization of DHX9 in regulation of the G1/S transition

Unpublished data identified that Ciz1 interacts with DHX9 in late G1 phase and early S-phase. To better understand whether DHX9 contributes to regulation of cell cycle re-entry from quiescence, cell cycle kinetics was monitored in DHX9 depleted cells. Targeted depletion of DHX9 by siRNA mediated transfection in synchronized cells re-entering the cell cycle from quiescence produced a statistically significant decrease in the number of S-phase cells suggesting a defect in cell cycle progression. These data suggest that DHX9 and Ciz1 may be active within the same pathway as siRNA mediated depletion of Ciz1 also markedly reduces cell cycle progression (Coverley et al., 2005; Copeland et al., 2010). However, as we only monitored cell cycle progression in the cell cycle following transfection, our data is distinct from other studies monitoring the effects of prolonged DHX9 depletion (22 hours) that led to a stabilization of p53 and senescence (Lee et al., 2014). The data therefore suggest that DHX9 contributes to efficient cell cycle progression directly as acute depletion reduces cell cycle progression in post-quiescent mouse fibroblasts (Figure 5.8). These data further expand the cell cycle regulators that associate with Ciz1 during S-phase. Full characterisation of the Ciz1-DHX9 complex will aid our understanding of how Ciz1 contributes to regulation of the G1/S transition.
Chapter 6

*In vitro* analysis reveals distinct roles for Cyclin E and cyclin A regulation of initiation of DNA replication
6.1 Introduction to cell-free replication assay:

A long-standing goal is the full description of the molecular pathways that precisely regulate the duplication of the genome, as a failure to do this can promote accumulation of mutations, and genomic instability. The fundamental regulatory proteins that facilitate this process have recently been identified through a combination of genetic and biochemical approaches, identifying the minimal set of regulatory proteins and enzymes that are sufficient to accurately copy DNA in a semi-conservative manner \textit{in vitro} (Yeeles et al., 2015; Yeeles et al., 2017). In this system, template DNA with an ARS is incubated with ORC, CDC6, CDT1-MCM2-7 initially followed by DDK for another 20 minutes followed by firing factors (Sld3/7, Sld2, Dpb11, S-CDK (cyclin A-CDK2), GINS, CDC45, MCM10) and replication proteins (Topo II, Pol alpha, RPA, Ctf4) in replication buffers and this entire reaction is quenched and analysed on agarose gel. With this assay, the specific requirement of different polymerases in sequential leading and lagging strand synthesis and optimise the reaction rates with addition of PCNA with RFC in leading and lagging strand synthesis (Yeeles et al., 2017).

The biochemical reconstruction of this process defined the minimal protein chemistries that mark putative sites of replication, activation of replication origins and copy the genomic template in the absence of other components of the nuclear structure (Yeeles et al., 2015). This seminal work
suggests that this process could be reconstituted using mammalian proteins and enzymes in future. The increased complexities of mammalian genomes and the increased number of regulatory factors that contribute to the regulation of DNA replication in vitro, make reconstitution more challenging in a mammalian context. These considerations have necessitated an amalgamation of a semi-purified nuclei and cytosolic extracts that enable reconstitution of DNA replication in vitro and will be discussed in this section.

One of the widely used assays to understand the complex mammalian replication system is the cell-free in vitro replication assay (IVRA). IVRA can provide a snapshot of the regulatory mechanisms controlling replication in higher eukaryotes (Krude, 2000). Direct biochemical analysis of replication initiation in eukaryotic somatic cells has been impeded by the lack of an efficient mammalian cell-free DNA replication system to complement cellular and genetic approaches. Cell-free DNA replication assays were established approximately 30 years ago however, these early experimental in vitro replication assays had their limitations and new and improvised techniques have been developed henceforth.

Initially, metazoan cell-free systems derived from activated eggs of the frog Xenopus laevis can replicate vertebrate cell nuclei under early embryonic cell cycle control (Lohka and Masui, 1983; Blow and Laskey, 1986; Newport,
1987; Murray, 1989). These early embryonic cycles differ significantly from somatic vertebrate cells that are regulated by internal transcriptional networks. In the *Xenopus* system, both proteins and transcripts are present within the extracts that provide pools and *de novo* production of regulatory proteins that facilitate very rapid alternating S and M phases without intervening G1 and G2 phases. Therefore, key regulatory steps of the somatic cycle, the G1/S and the G2/M transitions, are formally absent in early embryonic cycle. The lack of extensive G1 and G2 phases enable an extremely rapid cell cycle of approximately 25 minutes, which is more than an order of magnitude shorter than in the somatic cell cycle that is typically 24 hours in length (Li et al., 2012). Although these cell-free systems are valuable for identifying enzymatic and structural aspects of DNA replication, they are limited when studying the somatic cell cycle control of initiation.

In higher eukaryotes, DNA replication initiates from developmentally controlled chromosomal sites that lack conserved sequence elements. Moreover, specific DNA sequences may be altogether dispensable for metazoan origin function, as origin usage is promiscuous during the early embryonic cleavage stages of *Drosophila, Xenopus* and during plasmid replication in mammalian cells (Spradling and Orr-Weaver, 1987; Hyrien et al., 1995; Schaarschmidt et al., 2004). Heterologous DNA templates replicate
efficiently in *Xenopus* (Harland and Laskey, 1980; Mechali and Kearsey, 1984), and artificial tethering of initiation factors to DNA can be sufficient to specify an origin in *Drosophila* and mammalian cells (Takeda et al., 2005; Crevel and Cotterill, 2012). The dependency of origin function on a conserved DNA sequence element, the ARS, in budding yeast has therefore often been contrasted to the heterogeneous origins of higher eukaryotes. The mechanistic basis for this difference is not clear, yet it may be unexpected given the general conservation of basic eukaryotic replication initiation factors.

The first mammalian *in vitro* cell-free DNA replication assay was derived from Simian Virus 40 (SV40) and its somatic host cell (Krude et al., 1997). The activation of bidirectional DNA replication requires the trans-acting initiator proteins T antigen and depends on essential viral control elements, namely cis-acting DNA viral sequences (reviewed by Challberg and Kelly, 1989; Stillman et al., 1989). Utilisation of viral control elements serve to overcome the strict once per cell cycle replication of the host chromosomal DNA replication in the infected cell nucleus. However, this system is limited in the functional analysis of the regulatory events of an unaltered cell cycle.

To gain more insight into the regulatory events that ensure once per cell cycle duplication of the genome several different approaches were developed to investigate events at the G1/S transition. Mammalian cell-free
DNA replication methodologies utilise alternative mechanisms to synchronise at precise stages of the cell cycle. There are three reconstituted DNA replication strategies that have been utilised for investigation the regulation of DNA replication.

(i) **G1 nuclei generated from post mitotic cells**: Template nuclei preparation utilizes the addition of Nocodazole to arrest cells and synchronize in mitosis. Utilization of this approach identified that early G1 nuclei initiated poorly compared to late G1 nuclei and G2 phase nuclei were completely inactive (Krude 1997). The ability to determine replication competence highlights the importance of cell-free DNA replication assays to investigate the temporal regulation of replication licensing (Krude 1997). The use of template nuclei from post-mitotic cells can be restrictive because of complex chemical synchronisation protocols and the need for tight synchrony to achieve good ratios of signal to noise (Krude et al., 1997, Krude 2006)

(ii) **G1 nuclei generated from mimosine arrested cells**: Mimosine is a rare plant amino acid derivative isolated from Leucaena seeds, can be used to synchronise cells at G1/S transition by preventing the formation of replication forks (Krude, 1997; Lalande, 1990). Efficient template nuclei for the initiation of chromosomal DNA replication *in vitro* are prepared from
human cells treated for 24 hr with 0.5 mM mimosine, or with an appropriately high concentration of other iron chelators (Krude, 1999; Szuts et al., 2004). Nuclei from mimosine-arrested late G1 phase cells are licensed to replicate and contain assembled pre-RCs. Nuclei isolated from the mimosine-arrested cells do not contain active DNA replication forks, as measured in nuclear run-on replication assays (Krude, 1999; Krude, 2000). In vitro DNA replication assays utilise the rapid initiation of DNA replication upon restoration of free iron levels in vivo and can be exploited for the study of chromosomal DNA replication in vitro. Furthermore, in vivo pre-labelling studies have shown that they do not contain stalled or abandoned DNA replication forks that were active during the mimosine treatment (Keller et al., 2002). These template nuclei have enabled the identification of Y-RNA in the initiation process of mimosine arrested nuclei (Krude et al., 2006; Langley et al., 2016).

(iii) G1 nuclei generated from post quiescent cells: Utilization of murine fibroblasts synchronised through contact inhibition circumvents potential artefacts mediated by chemical synchronisation approaches. In addition, contact inhibition enables isolation at any time point in G1 or S phase. This enables temporal regulation of cell cycle progression to be studied in detail (Chapter 3, Chapter 4). Synchronisation can be achieved by using confluent 3T3 cells arrested by contact inhibition in quiescence (G0), and
subsequent stimulation to re-enter the proliferative cell cycle by sub-
cultivation at lower cell densities (Stoeber et al., 1999). Template nuclei for
chromosomal DNA replication in vitro were therefore prepared from
quiescent and stimulated cells at various times after release. Initially,
mouse 3T3 cells were used (Stoeber et al., 1999), but similar results were
also obtained with human WI38 cells. (Stoeber et al., 2001) Quiescent
mammalian cells are characterized by loss of CDC6 protein and
dissociation of MCM proteins from chromatin (Stoeber et al., 1999; Stoeber
et al., 2001; Williams et al., 1999). Nuclei isolated from quiescent cells do
not support DNA replication in human cell extracts in vitro (Stoeber et al.,
1999; Stoeber et al., 2001).

The competence of isolated nuclei from stimulated quiescent cells to
initiate DNA replication in S phase cytosol (extract) arises gradually between
16 hours and 18 hours after stimulation, in late G1 phase, about 3 hour before
entry into S phase in vivo (Kingsbury et al., 2005). This gain of initiation
competence coincides with the restriction point in vivo, when cell cycle
progression becomes serum-independent (Coverley et al., 2002). The timing of
this ‘window of opportunity’ varies with experimental conditions, especially
serum concentration and batch, and requires being determined for each
preparation of template nuclei. Initiation competence correlates to validation
to avoid batch to batch variation with the maximal induction of CDC6 protein expression following stimulation, and it follows an association of the pre-RC proteins CDC6 and MCM2, 3, 5 and 7 with chromatin (Kingsbury et al., 2005, Coverley et al., 2000).

The mammalian cell-free systems have turned out to be highly versatile and adaptable experimental tools for biochemical investigations of the initiation of chromosomal DNA replication. Isolation of late G1 phase nuclei supports initiation of DNA replication (Coverley et al., 1998; Coverley et al., 2002; Copeland et al., 2010). This system established so far has led to the functional identification of novel DNA replication initiation factors, which can be characterised biochemically in vitro and in intact cells in vivo (Krude 2006). In addition, recombinant cyclin-CDK complexes can be utilized to facilitate replication licensing (Coverley et al., 2002) and initiation of DNA replication (Coverley et al., 2002; Copeland et al., 2010). This well characterized system enables temporal regulation of replication licensing and replisome assembly (Copeland et al., 2015). This system also enabled identification of Ciz1 as a factor that promotes cell cycle progression in complex with cyclin A-CDK2 (Coverley et al., 2005; Copeland et al., 2010) and characterisation of the adenosine dinucleotide Ap4A acting to inhibit the initiation phase of DNA replication (Marriot et al., 2015).
6.2 Chapter aims

In Chapter 3, evidence was presented that demonstrated that inhibition of cyclin dependent kinase activity promotes loss of pre-RC proteins CDC6, MCM2, MCM10 and replisome protein PCNA that indicated with a loss of Replication initiation. To identify the role of cyclin E-CDK2 and cyclin A-CDK2 in the initiation phase of DNA replication, nuclei will be prepared from cyclin E1/E2 and cyclin A2 depleted cells. The cyclin depleted nuclei will be assessed in their ability to initiate DNA replication. By dissecting requirement of individual cyclins using cell-free replication assays, we aim to identify whether cyclin E and cyclin A have distinct roles in the regulation DNA replication complex assembly and for initiation of DNA replication. The main aims of this chapter are:

1. Produce synchronized G1 extracts, S phase extracts and replication licensed nuclei.
2. Prepare cyclin depleted nuclei and assess whether S phase extract can induce initiation.
3. Evaluate whether there are cyclin specific events that enable initiation in cyclin depleted nuclei.
6.3 Analysis of replication complex assembly and initiation of DNA replication in vitro

The cell free system used here combines synchronized cell extracts prepared by the biologically relevant quiescence release method. This approach enables purification of nuclei from a late G1 population of cells that can initiate DNA replication. To initiate DNA replication nuclei are supplemented with recombinant cyclin A-CDK2 kinase (Coverley et al., 2002; Copeland et al., 2010) or S-phase cytosolic extracts that contain active cyclin CDK complexes (Krude, 1997; Coverley et al., 2002). This system has identified key components involved in DNA replication, specifically the distinct roles of cyclin E and cyclin A regulation of the replication licensing and initiation phases respectively (Coverley et al., 2002) that were later confirmed in mouse genetic ablation models (Geng et al., 2003; Geng et al., 2007).

To initiate DNA replication G1 nuclei are combined with S-phase extract, an energy regenerating system and nucleotides (dNTPS) (Section 2.14; Figure 6.1.A). This assay requires incorporation of control reactions to identify the proportion of nuclei that are already in S-phase. To do so G1 nuclei are incubated in G1 cytosolic extracts that are incapable of promoting initiation of DNA replication. Positive reactions in this context identify nuclei that have
engaged in run on synthesis i.e. nuclei that have initiated DNA replication and have engaged and processive replisome complexes are already formed.

Replication competent nuclei are activated by addition of S phase extract to licensed G1 nuclei, identifying nuclei that can initiate DNA replication in vitro (Figure 6.1.B). Analysis of the chromatin fraction before and after addition of S-phase cytosolic extract demonstrates that in vitro DNA replication assay facilitate the loading of replication factors (Figure 6.1.C). Consequently, quantitative analysis of protein abundance on chromatin after incubation G1 extract and S-phase extracts identifies loading factors that correlate with increased initiation. Incubation of replication licensed nuclei in S phase extract increased chromatin loading of replication assembly proteins MCM2, CDC6, PCNA along with cyclin A and cyclin E (Figure 6.1.C). The increased loading of replication factors in S phase extract, relative to parallel reactions in G1 extract suggests that licensed nuclei are deficient in MCM2, CDC6, PCNA, cyclin A and cyclin E rendering G1 nuclei incompetent of DNA replication initiation.
A: Experimental layout for preparation of G1 nuclei and G1 cytoplasmic extract and S phase extract. B: Histogram showing representative percentages for in vitro initiation of DNA replication with S phase extract relative to G1 phase extract. C: Western blot analysis of replication complex assembly proteins MCM2, CDC6, PCNA, Cyclin A and Cyclin E on chromatin in G1 control and S phase extract. D: Immunofluorescence images of replicating G1 nuclei with G1 extract and S phase extract stained against biotin-dUTP from the reaction.

Figure 6-1 Mammalian cell-free DNA replication assay.
Western blotting found that levels of MCM2 and CDC6 were unaffected by addition of S-phase extracts. However, phospho-specific antibodies against CDK and DDK sites S40, S41 respectively (denoted P-MCM2) were phosphorylated at a higher level in S-phase extracts that promote replication relative to G1 extracts cell free replication assays (Figure 6.1C). In addition, these data suggest that cyclin E, cyclin A and PCNA levels increase on chromatin during incubation in S-phase cytosolic extracts and correlate with initiation of DNA replication (Figure 6.1.C). Having produced replication competent G1 nuclei, these nuclei were then to be used as positive controls for comparison with cyclin depleted nuclei to further characterise alterations in chromatin bound fractions.
6.4 **Cyclin E depleted nuclei are replication incompetent**

Cyclin E –CDK2 is required for transcriptional regulation of MCM2, CDC6, PCNA and MCM10 via regulation of the E2F-Rb pathway (section 3.10). Cyclin E-CDK2 activity is required for loading of MCM2-7 complex facilitated by CDC6 (Coverley 2002) and for cell cycle re-entry from quiescence via defects in MCM2-7 recruitment (Geng et al., 2003; Geng et al., 2007). Initiation of DNA replication and progression to S phase in post-quiescent cells are blocked in cyclin E null cells due to an arrest in G1 phase due to a failure of MCM2-7 chromatin loading (Geng et al., 2007). Previously, it was established that nuclei at restriction point could initiate DNA replication after sequential addition of cyclin E-CDK2 and then cyclin A-CDK2 (Coverley et al., 2002). However, whether S phase extract is sufficient to initiate DNA replication in absence of cyclin E1/E2 remains to be studied *in vitro*.

Cyclin E depleted nuclei were prepared by transfecting G0 synchronized cells with mixed cyclin E1 and cyclin E2 siRNAs in cells released into fresh medium for 24 hours prior to harvesting nuclei. The cell free DNA replication assays are prepared by reconstituting cyclin E depleted nuclei with G1 phase extract and in second reaction with S phase extract (Figure 6.2.A). As the reactions are prepared in the same components as with G1 nuclei, positive replicating nuclei are counted by labelling with modified
nucleotide biotin conjugated dUTP. Cyclin E1/E2 depleted nuclei fail to initiate DNA replication with G1 phase extract (Figure 6.2.B). Interestingly, these nuclei fail to initiate DNA replication even with S phase extract which has both cyclin E-CDK2 and cyclin A-CDK2 kinases within the cytosolic extracts (Copeland et al., 2010). This supports observations that demonstrated that sequential cyclin E-CDK2 and cyclin A-CDK2 activity is required to promote initiation of DNA replication in vitro (Coverley et al., 2002).

Confocal immunofluorescence microscopy (Figure 6.2.C) of cyclin E depleted nuclei show minor distortion in nuclear shape, but nuclei retain ability to incorporate biotinylated dUTP with ~5-10% of cyclin E depleted nuclei in S-phase after cyclin E depletion in a G1 extract (Figure 6.2.C). The number of nuclei in S-phase after addition of S-phase extract is ~10% (Figure 6.2) compared with ~40% of G1 nuclei that are replication competent in control reactions (Figure 6.1). The failure to initiate DNA replication was previously attributed to a failure to load MCM complexes in the absence of cyclin E in in vitro replication assays (Coverley et al., 2002) or failure of MCM2-7 loading onto chromatin in a cyclin E null cell line (Geng et al., 2007). Western blot analysis of cell free replication assays showed that pre-replication proteins MCM2, CDC6, replisome protein PCNA and cyclin A and cyclin E appears to be efficiently loaded onto chromatin from S phase extract (Figure 6.2.D). This effect may be due to cyclin E-CDK2 activity in the S-phase.
extract used. This approach reveals that loading of DNA replication factors from S-phase extract was insufficient to promote initiation at similar levels seen in control reactions in cyclin E1/E2 depleted nuclei.

Western blot analysis revealed an inconsistent loading pattern for cyclin E in cyclin E depleted nuclei. The previous cyclin E antibody that was used (Abcam ab1979) was discontinued and other antibodies that were used contradict the qRT-PCR data for protein levels. Despite extensive antibody testing we were unable to get consistent protein and transcript levels. As such both protein and transcript levels are shown for each experiment. Furthermore, we cannot exclude that the differences are related to different time points for harvesting nuclei (17 hours for control vs 24 hours for cyclin E depleted) as cyclin E protein levels accumulate in G1 phase and decrease thereafter (Figure 3.7, 3.10) Despite this we found that S-phase extracts were able to promote chromatin loading of MCM2 and that this was insufficient to adequately license DNA for initiation and DNA replication.
Figure 6-2 Cyclin E depleted nuclei are replication incompetent.

A: Experimental overview for preparation of cyclin E depleted nuclei (αE) and G1 cytoplasmic extract and S phase extract. B: Histogram showing qRT-PCR of cyclin E1/E2 transcript analysis post cyclin E1/E2 siRNA transfection. C: Histogram depicting initiation of DNA replication with S phase extract as contrast to G1 phase extract in cyclin E depleted nuclei. D: Western blot depicting chromatin loading of replication complex assembly proteins MCM2, CDC6, PCNA, Cyclin A and Cyclin E from S phase extract. E: Immunofluorescence images of replicating cyclin E depleted (αE) nuclei with G1 extract and S phase extract stained against biotin-dUTP from the reaction.
These data demonstrate that cyclin E is required for efficient transition from G1 into S-phase. The loading of MCM2 and apparent phosphorylated CDC6 form in S-phase extract is insufficient to promote initiation. The data presented here are consistent with the requirement of cyclin E1/E2 for replication licensing at the level of CDC6 and MCM2-7 loading onto chromatin prior to exposure of cyclin A-CDK2 to initiate DNA replication (Coverley et al., 2002, Geng et al., 2003, Geng et al., 2007).
6.5 Cyclin A2 depleted nuclei are replication competent

Cyclin E-CDK2 levels accumulate prior to G1/ S transition and are degraded after entry to S phase promoting cyclin A accumulation. Cyclin A depletion blocks S phase entry of cells re-entering cell cycle from quiescence (Figure 3.8 and Figure 4.5). Ablation of cyclin A in fibroblasts does not affect the cell cycle proliferation (Kalaszczynska et al., 2009); however, we have found that siRNA mediated depletion of cyclin E1/E2 and cyclin A2 blocks fibroblast proliferation (Figure 3.7, Figure 3.8, Figure 4.4). The major technical difference between evidence from this study and mouse ablation studies is the utilisation of quiescence as a synchrony step was not assessed in other studies (Kalaszczynska et al., 2009; Spencer et al., 2013; Barr et al., 2016).

In post-quiescent mouse fibroblasts cyclin A depletion blocks S phase entry of cells (Chapter 3 section 3.8). The data showed a block in S phase cells that correlated with destabilization of pre-RC protein MCM2, CDC6, Pre-IC protein MCM10 and replisome protein PCNA as seen for cyclin E1/E2 depleted cells (Figure 3.7). Interestingly cyclin E levels remains unaffected after depletion of cyclin A2 (Figure 3.8) and consequently, transcript levels of E2F genes are unaffected. This suggests that cyclin A2 depleted nuclei may be replication competent. To identify whether cyclin A2 depleted nuclei retains replication competence, in vitro replication assays with cyclin A2 depleted nuclei were performed.
Chapter 6: Results

To analyse the replication competency of cyclin A depleted nuclei, cells were synchronized and siRNA was used to deplete cyclin A2 in quiescent cells. Cyclin A2 depleted nuclei were assessed to initiate DNA replication in cell-free replication assays. Comparison with cyclin A2 depleted nuclei and replication competent late G1 nuclei when incubated with G1 extracts as the negative control reaction, Figure 6.3 show that cyclin A2 depleted nuclei are restricted in their ability to initiate DNA replication. In fact, cyclin A2 depleted nuclei showed less than 5% of nuclei in S-phase when reconstituted with G1 extract. However, cyclin A2 depleted nuclei can initiate DNA replication when supplemented with S phase extract to a comparable level to control replication licensed nuclei with ~40% nuclei in S phase (Figure 6.3.C). Representative immunofluorescence assay images of cyclin A depleted nuclei reconstituted with G1 extract and S phase extracts show punctate incorporation of biotinylated dUTP consistent with replisome activity (Figure 6.3.E).

Parallel western blot analysis of chromatin fraction was performed to compare chromatin fractions of control and cyclin A2 depleted nuclei (Figure 6.3D). In cyclin A2 depleted nuclei MCM2, CDC6, PCNA and cyclin A protein levels were decreased relative to control reactions. The chromatin associated protein levels of the replication assembly proteins MCM2, CDC6 and PCNA increased by addition of an S-phase extract to greater than levels seen in
control reactions. Comparison of Cyclin E and Cyclin A depleted nuclei reveal that chromatin fractions are capable of efficient chromatin loading of CDC6, MCM2, PCNA and indeed cyclin E and cyclin A. As both cyclin E1/E2 depleted nuclei and cyclin A2 depleted nuclei have comparable loading of pre-RC proteins, consistent with efficient replication licensing and recruitment of the replisome component PCNA, it was unexpected that there were clear differences in the replication competence for cyclin E1/E2 and cyclin A2 depleted nuclei (Figure 6.2).
Figure 6-3 Cyclin A depleted nuclei are replication competent.
A: Experimental layout for preparation of cyclin A depleted nuclei (αA) and G1 cytoplasmic extract and S phase extract. B: Histogram depicting relative quantitation of cyclin A2 transcript levels for control and cyclin A2 depleted cells. C: Histogram showing percentage of nuclei in S-phase for control nuclei incubated in G1 extract and cyclin A2 depleted nuclei in G1 and S-phase extracts showing replication competence. D: Western blot depicting loading of replication complex assembly proteins MCM2, CDC6, PCNA, Cyclin A and Cyclin E on to chromatin from S phase extract. E: Immunofluorescence images of replicating cyclin A depleted (αA) nuclei with G1 extract and S phase extract stained against biotin-dUTP from the reaction.
These observations are consistent with earlier data showing a reduction in replication licensing as a consequence of CDK inhibition in G1 phase and reveal that there are specific activities of cyclin E and cyclin A in the replication licensing and initiation phase of DNA replication. The specific activities of cyclin E and cyclin A are consistent with earlier work using quiescence release to prepare replication competent late G1 phase nuclei (Coverley et al., 2002) and reveal differences with mitotic cell cycle in mouse cyclin A2 null fibroblast studies (Kalaszczynska et al., 2009).
6.6 Active kinases in S phase extract are required for initiation of DNA replication in cell-free replication assays.

To assess whether CDK activity was required for chromatin loading of replication factor cell free replication assays were performed using cyclin A2 depleted nuclei in presence and absence of CDK/2 inhibitor- roscovitine. The percentage of replicating cyclin A2 depleted nuclei with G1 phase extract, S phase extract and S phase extract with roscovitine in comparison with G1 nuclei with G1 extract (Figure 6.4.A). In cyclin A2 depleted nuclei, G1 extract identifies 5% of cells are in S phase, whereas addition of S-phase extract promotes initiation in 45% of nuclei, that could be inhibited by addition of roscovitine where ~7% were in S phase (Figure 6.4.A). Analysis of the chromatin bound fraction in parallel reaction a reduction in loading of MCM2, CDC6 and PCNA on to chromatin in cyclin A2 depleted nuclei with G1 extract (Figure 6.4.B). Addition of S phase extract to cyclin A2 depleted nuclei promotes chromatin loading of replication factors independent of CDK activity, demonstrating that CDK2 activity is not required for chromatin binding in this context. In addition, this suggests that loading of replication factors onto chromatin is CDK2 independent in this context but demonstrates that initiation of DNA replication required CDK2 activity.
Figure 6-4 CDK2 activities are required for initiation of DNA replication but not for loading of proteins MCM2, CDC6 and PCNA on to chromatin. 
A: histogram showing percentage of nuclei in S-phase in cyclin A2 depleted nuclei incubated with G1, S-phase and S-phase extracts + roscovitine. 
B: Western blot showing loading of MCM2, CDC6 and PCNA onto chromatin in cyclin A depleted nuclei with S phase extract and inactivated S phase extract. 
C: histogram showing percentage of nuclei in S-phase in cyclin E1/E2 depleted nuclei incubated with G1, S-phase and S-phase extracts + roscovitine. 
D: western blot showing loading of MCM2, CDC6 and PCNA onto chromatin in cyclin E depleted nuclei with S phase extract and inactivated S phase extract.
To test whether CDK2 was required for loading of MCM2, CDC6 and PCNA on to chromatin a Cyclin E1/E2 depleted nuclei reconstituted with G1 phase extract, S phase extract and S phase extract with roscovitine in comparison with G1 nuclei with G1 extract (Figure 6.4). Cyclin E depleted nuclei were incapable of initiating DNA replication (Figure 6.4.C).

Similarly, western blot analysis of chromatin fraction in cyclin E1/E2 depleted nuclei (Figure 6.4.D) showed loading of MCM2, cyclin A. However, CDC6 levels were equivalent in all cyclin E1/E2 depleted nuclei and PCNA was reduced in S phase extract with roscovitine relative to S phase alone. These data suggest that both cyclin E1/E2 depleted nuclei and cyclin A2 depleted nuclei efficiently load DNA replication factors from licensed S-phase cytosolic cellular extracts, even in absence of CDK2 activity but only cyclin A2 depleted nuclei retain replication competence suggesting that they contain ‘licensed’ replication origins.
6.7. Cyclin E1/E2 depletion deregulates MCM2 Compartmentalisation in G1.

The nuclear matrix contributes to nuclear compartmentalization of the factors responsible for temporal and spatial regulation of transcription and DNA replication (Berezney et al., 1997). The nuclear matrix associates with cell cycle regulators and enzymes required for DNA synthesis (Radichev et al., 2005; Munkley et al., 2011).

Localization of MCM2 to nuclear matrix occurs in late G1 phase and correlates with potential to initiate DNA replication. This compartmentalisation is transient and after initiation of DNA replication MCM2 is released from the nuclear matrix (Hesketh et al., 2015). Previous work (Coverley et al., 2002; Geng et al., 2003; Geng et al., 2007) and data presented here (Figure 6.2, 6.3) suggest that replication licensing in deficient in cyclin E1/E2 depleted nuclei and this leads to hypothesis that this may affect the localization of MCM2-7 complex.

To test this hypothesis using in vitro DNA replication assays, it is necessary to determine whether MCM2 nuclear matrix compartmentalisation is present in this context. Initially, in vitro assays were performed to determine whether the localization of MCM2-7 complex on the nuclear matrix is correctly regulated in this context. After reactions were complete, comparison between chromatin loading fraction and sub nuclear localisation was investigated by high salt fractionation in the presence and absence of
roscovitine (Figure 6.5). Comparison of chromatin loading of MCM2, CDC6, cyclin A in G1 nuclei with G1 extract, S phase extract with and without roscovitine are consistent with (Figure 6.4.A). Analysis of the nuclear matrix fraction in parallel reactions comparing G1/G1, G1/S and G1/S with roscovitine reveals an increase in MCM2 in nuclear matrix fraction in the presence of roscovitine. Importantly, MCM2 is enriched on the nuclear matrix prior to initiation and dissociated after initiation of DNA replication. Significantly, MCM2 was enriched in S-phase extracts after CDK2 inhibition that prevents initiation of DNA replication (Figure 6.5.B). As roscovitine effectively reduces initiation of DNA replication \textit{in vitro}, this demonstrates that MCM2 is retained on the nuclear matrix when initiation of DNA replication is inhibited (Figure 6.5) as seen elsewhere (Hesketh et al., 2015).
Figure 6.5 Compartmentalization of MCM2 is regulated by cell cycle stages.

A: Histogram showing inactivated S phase extract incubated with replication competent nuclei. B: western blot showing loading of MCM2, CDC6 and cyclin A onto chromatin in G1 nuclei with S phase extract and inactivated S phase extract. C: Western blot of salt fractionated chromatin loading of MCM2 in inactivated S-phase extract.

The data are consistent with a salt resistant MCM2 fraction in late G1 cells that can be released by initiation of DNA replication. Next to investigate the compartmentalisation of MCM2 in cyclin E1/E2 and cyclin A2 depleted nuclei an analogous approach was used. Cell free DNA replication assays using cyclin depleted nuclei were used for comparison. In cyclin E1/E2 nuclei there was a low level of cyclin E in G1 extracts on chromatin and the nuclear matrix fraction. Incubation of cyclin E1/E2 depleted nuclei in S-phase nuclei showed enrichment of MCM2 on chromatin fractions (Figure 6.6.A) and was absent from the nuclear matrix fraction. In parallel reactions with the addition of roscovitine, MCM2 loaded onto chromatin and the nuclear matrix
efficiently. These observations suggest that cyclin E1/E2 depleted nuclei are
defective in the loading of MCM2 onto the nuclear matrix fraction.

Comparison with cyclin A2 depleted nuclei revealed similar effects to
late G1 nuclei (Figure 6.5). Incubation in a G1 extract revealed a low level of
MCM2 on both chromatin (Figure 6.6.C) and nuclear matrix fractions (Figure
6.6.D). Incubation of cyclin A2 depleted nuclei in S-phase extracts increased
the levels of MCM2 on chromatin but not the nuclear matrix fractions.
Importantly, inhibition of CDK2 activity enriched MCM2 on the nuclear
matrix fraction (Figure 6.6.D) that correlated with a reduction of cells in S-
phase (Figure 6.4.C).
Figure 6-6 MCM2 compartmentalisation defective in cyclin E1/E2 depleted nuclei in cell free replication assays.

A: Comparison of western blot showing loading of MCM2, CDC6 and PCNA onto chromatin in cyclin E1/E2 depleted nuclei with S phase extract and inactivated S phase extract and salt fractionated loading. B: Nuclear matrix fraction shown on western blot. C: Comparison of western blot showing loading of MCM2, CDC6 and PCNA onto chromatin in cyclin A2 depleted nuclei with S phase extract and inactivated S phase extract. D: Nuclear matrix fraction shown on western blot.
Therefore, MCM2 appears to be associated with nuclear matrix when cells re-enter cell cycle from quiescence and are preparing for G1/S transition and initiation of DNA replication (Hesketh et al., 2015). Analysis of the localization of MCM2 reveals subtle differences between cyclin E1/E2 and cyclin A2 depleted nuclei and suggests that MCM2 is regulated by CDK activity and correlates with replication competence of depleted nuclei. This hypothesis suggests that CDK mediated phosphorylation of MCM2 may dictate its localisation and replication competence.
6.8 Cyclin E is required for phosphorylation of MCM2 required for initiation of DNA replication

To further investigate whether there may be differences in cyclin E1/E2 and cyclin A2 activities at the level of MCM2 localization and phosphorylation, MCM2 phospho-specific antibodies were used. The replicative helicase MCM2-7 complex is regulated by DDK and CDK mediated phosphorylation. Data presented here is consistent with the role of cyclin E during pre-RC activation. G1 nuclei are replication competent when reconstituted with S phase extract. An important feature of cyclin E1/E2 depleted nuclei is the block in cyclin A2 accumulation (Figure 6.2, Figure 6.5 and Figure 6.6.A). This in contrast with cyclin A2 depletion where cyclin E is present at high levels both cyclin E1/E2 depleted nuclei. In cyclin A2 depleted nuclei, there is a consistent reduction in MCM2, CDC6 and PCNA compared to control reactions. This accumulation of cyclin E in this context may determine the replication competence of the nuclei as cyclin E is required for activation of pre-RC (Coverley et al., 2002; Geng et al., 2003, 2007; Copeland et al., 2010). The key distinction between cyclin E1/E2 depleted nuclei and cyclin A2 depleted nuclei is that only cyclin A2 depleted nuclei can initiate DNA replication, suggesting a specific role for cyclin E1/E2 in replication licensing, perhaps at the level of MCM2 regulation. To determine whether the phosphorylation status of MCM2 is distinct for cyclin E1/E2 and cyclin A2
depleted nuclei, MCM2 phosphorylation was monitored in G1 and S phase extracts in cell-free DNA replication assays.

Cell-free DNA replication assays were performed using cyclin E1/E2 depleted nuclei and compared with cyclin A2 depleted nuclei. Western blot analysis of the chromatin loading of cyclin E, cyclin A, MCM2, CDC6, PCNA in G1 nuclei were compared to cyclin E1/E2 depleted nuclei and cyclin A2 depleted nuclei. Cyclin E levels are distinctly different in all three nuclei. G1 nuclei are harvested at 17.5 hour and cyclin E1/E2 and cyclin A2 depleted nuclei are harvested post 24 hours. Cyclin E protein levels in cyclin E depleted nuclei are considerably lower than control and cyclin A2 depleted nuclei (Figure 6.7.A).
Figure 6-7 Distinct cyclin E-CDK2 dependent phosphorylation of MCM2 correlates with replication competency.

A: Western blot analysis of comparison between G1 nuclei, cyclin E depleted nuclei and cyclin A depleted nuclei. B: Histogram showing cyclin E depleted nuclei are incompetent of initiation of replication when reconstituted with S phase, but cyclin A2 depleted nuclei can initiate replication when reconstituted with S phase extract. C: Western blot showing difference in chromatin loading of p-MCM2, MCM2, CDC6, PCNA in cyclin E depleted nuclei and cyclin A2 depleted nuclei with G1 extract and S phase extract.
Cyclin E depleted nuclei and cyclin A depleted nuclei were reconstituted with G1 phase extract and protein levels determined by western blotting (Figure 6.7.A). Consistent with earlier observations there is a reduction in chromatin associated MCM2, CDC6, PCNA in cyclin depleted nuclei compared to control late G1 nuclei (Figure 6.7A). Cyclin depleted nuclei were incubated in G1 extracts or S-phase extracts and the number of nuclei that are replicating their DNA was determined by incorporation of biotin –dUTP. This showed that only cyclin A2 depleted nuclei retain replication competence (Figure 6.7.B). Consistent with earlier observations cyclin E1/E2 depleted nuclei were not replication competent and failed to initiate in S-phase extract. In parallel reactions, the chromatin association of MCM2, CDC6, and PCNA were monitored. In addition, the phospho-specific antibody raised against the CDK2 and DDK site ser 40/41 was used for comparison. Incubation of cyclin depleted nuclei in S-phase extracts showed an increased chromatin association for MCM2, CDC6, and PCNA on chromatin. However, comparison between the levels of p-MCM2 (p-S40 and p-S41) that monitors DDK and CDK2 phosphorylation demonstrated that p-MCM2 was only present on chromatin in Cyclin A2 depleted nuclei. Addition of S-phase extract promoted loading of p-MCM2 onto chromatin at similar levels to those seen in cyclin A2 deleted nuclei.
These observations suggest that cyclin E1/E2 is required to enable phosphorylation of MCM2 in G1 phase to license DNA replication. Cell free DNA replication assays using cyclin E1/E2 depleted nuclei reveal that loading of p-MCM2 from S-phase extracts is insufficient to enable initiation of DNA replication. This suggests that depletion of cyclin E1/E2 prevents efficient phosphorylation of MCM2, potentially reducing MCM2-7 localization and activation in the pre-IC assembly. These data suggest that temporal separation of p-MCM2 loading from the initiation phase is a key regulatory event in the licensing and initiation process of DNA replication.
6.9 Cyclin A2 depleted nuclei can initiate DNA replication when supplemented with recombinant cyclin A-CDK2.

The specific activities of cyclin E and cyclin A studied here (Figure 6.2, 6.3 and 6.7) are consistent with earlier work using quiescence release to prepare replication competent late G1 phase nuclei (Coverley et al., 2002) and reveal differences with mitotic cell cycle in mouse cyclin A2 null fibroblast studies (Kalaszczyńska et al., 2009). Addition of recombinant cyclin A-CDK2 to cell free replication assay has previously been analysed by (Coverley et al., 2002 and Copeland et al., 2010) and these experiment indicate that late phase G1 nuclei can initiate DNA replication when supplemented with 0.1ng/µl recombinant cyclinA-CDK2 with G1 cytosolic extract in cell free replication assay.

To determine the efficiency of cyclin A2 depleted nuclei to initiate DNA replication in detail, recombinant cyclin A-CDK2 was added to G1 extracts at concentration range as indicated in the (Figure 6.8). Cyclin A2 depleted nuclei can initiate DNA replication when supplemented with S phase extract compared to control reactions using G1 nuclei (Figure 6.8.A). For comparison, recombinant cyclin A-CDK2 was added with G1 cytosolic extract at concentration range indicated in (Figure 6.8.B) and (Figure 6.8.C). Consistent with previous studies G1 nuclei can efficiently initiate DNA replication peak at 0.1nM of recombinant cyclin A-CDK2 and concentration
higher than 0.1ng/µl appear to be inhibitory as percentage of biotin-dUTP positive nuclei decreases with increasing concentration of recombinant cyclin A-CDK2.

Figure 6-8 Cyclin A2 depleted nuclei can initiate DNA replication in addition of recombinant cyclin A-CDK2.

A: Histogram showing percentage of nuclei in S-phase for control nuclei incubated in G1 extract and cyclin A2 depleted nuclei in G1 and S-phase extracts showing replication competence. B: Line graph showing initiation of DNA replication in G1 nuclei with addition of recombinant cyclin A-CDK2 in the following range (0.01ng/µl, 0.02ng/µl, 0.05ng/µl, 0.1ng/µl, 0.2ng/µl, 0.5ng/µl, 1.0ng/µl) with peak at 0.1ng/µl of recombinant cyclin A-CDK2 with G1 extract. C: Line graph showing initiation of DNA replication in cyclin A2 depleted nuclei with addition of recombinant cyclin A-CDK2 in the following range (0.01ng/µl, 0.02ng/µl, 0.05ng/µl, 0.1ng/µl, 0.2ng/µl, 0.5ng/µl, 1.0ng/µl) with peak at 0.1ng/µl of recombinant cyclin A-CDK2 with G1 extract.
Interestingly, cyclin A2 depleted nuclei when supplemented with recombinant cyclin A-CDK2 at range of concentration as indicated in (Figure 6.8.C), can initiate DNA replication and peak at 0.1ng/µl of recombinant cyclin A-CDK2 with G1 extract. This further suggests that cyclin A2 depleted nuclei can initiate DNA replication with addition of recombinant cyclin A-CDK2. In addition to peak at 0.1ng/µl concentration of recombinant cyclin A-CDK2, increasing concentration decreases the replication competency, indicating higher concentration of recombinant cyclin A-CDK2 works as inhibitory concentration to initiate DNA replication similar to observations in G1 nuclei (Figure 6.8.B) However, this is a preliminary observation and needs further analysis to understand the mechanism in detail.
Chapter 6: Results

6.10 Discussion

The data presented in this chapter demonstrates the requirement of cyclin E–CDK2 for efficient initiation of DNA replication. Cell-free DNA replication assays using replication competent late G1 phase extract reconstituted with S phase extract is long established (Krude et al., 1996). However, the work presented here shows for the first time that genetically depleted nuclei can be used in cell-free DNA replication assays; greatly increasing capacity in future experiments. Cyclin E1/E2 depleted nuclei and cyclin A2 depleted nuclei were prepared by siRNA transfection in synchronized fibroblasts and reconstituted with S phase extract to study replication initiation in absence of cyclin activity. The data presented in (Figure 6.1) suggests efficiency of the experimental system with the use of S phase extract to reconstitute initiation of DNA replication in G1 nuclei. In vitro replication assays using cyclin E1/E2 depleted nuclei revealed that in both G1 extract and S phase extract that cyclin E1/E2 depleted nuclei are not licensed correctly and cannot initiate DNA replication. However, S phase extract was sufficient to load replication factors onto chromatin (Figure 6.3, 6.7). This further suggests a strict requirement for cyclin E in initiation of DNA replication, consistent with previous observations by (Coverley et al., 2002; Geng et al 2007; Copeland et al., 2010). Cyclin E–CDK2 activity is required for formation and activation of the pre-initiation complex and is
required for recruitment of CMG complex onto chromatin. Formation of the pre-IC then leads to helicase activation leading to recruitment of polymerases and formation of replisome and DNA replication.

**Figure 6-9 Replication licensing.**

DNA replication is multi-step process involving loading and unloading of various proteins in timely manner in G1 phase. First step involves formation of Pre-RC which is initiated by recruitment of ORC onto putative replication origins. CDC6 and Cdt1 then interact with ORC in ATP dependent manner in order to recruit MCM2-7 complex (Helicase) onto the chromatin leading to the formation of Pre-RC. MCM10, Cdc45 along with GINS are recruited on to Pre-RC to form Pre-initiation complex. Recruitment of these proteins is facilitated by cyclin E-CDK2. Completion of the Pre-RC at the origin is referred to as replication licensing. Cyclin E-CDK2 along with Dbf4 dependent kinase (DDK) recruit’s replisome factors leading to helicase activation. Cyclin A-CDK2 then facilitates loading of polymerase with PCNA and results in Replisome formation.
Cyclin A accumulates around restriction point in cells released from quiescence (Figure 3.2). Cyclin A-CDK2 is required for initiation of DNA replication as cyclin A depletion blocks in S phase entry in cells re-entering the cell cycle from quiescence (Figure 3.8, Figure 4.4). However, to understand the role of cyclin A-CDK2 in initiation of DNA replication, in vitro cell-free replication assay with cyclin A2 depleted nuclei was performed. Cyclin A2 depleted nuclei are replication competent and S phase extract is sufficient to load replication complex assembly proteins MCM2, CDC6 and PCNA onto chromatin and initiate DNA replication. In addition to S phase extract, cyclin A depleted nuclei were reconstituted with G1 extract supplemented with recombinant cyclin A-CDK2 (Figure 6.8) and 0.1 ng/µl concentration of cyclin A-CDK2 gave the highest percentage of replicating nuclei, with increase in concentration of recombinant cyclin A-CDK2 increase in replicating nuclei and peak at 0.1nM/µl. Interestingly, concentration above 0.1 ng/µl appeared to be inhibitory concentration as number of replicating nuclei decreased. This is similar to previous observations (Coverley et al., 2002; Copeland et al., 2010). This suggests that cyclin A-CDK2 above a certain concentration inhibits initiation of DNA replication. However, more analysis needs to be done in using recombinant cyclin A-CDK2 with cyclin A depleted nuclei to understand the role of cyclin A–CDK2 alone in initiation of DNA replication.
Initiation of DNA replication involves kinase independent loading of pre-RC proteins MCM2, CDC6, CDT1 once ORC is bound to replication origins leading to formation of pre-RC. Cyclin E-CDK2 mediated phosphorylation furthers helps recruitment of CMG complex and MCM10 leading to formation of pre-IC. Late G1 nuclei are harvested in a pre-licensed state and hence are replication competent and with addition S phase extract can initiate DNA replication (Figure 6.2, Figure 6.3). As observed in Figure 6.4 and 6.5 it is evident that loading of replication proteins MCM2, CDC6 from active S phase extract is not sufficient for initiation of DNA replication. Interestingly, inhibition of CDK2 activity in S-phase extracts prevents initiation of DNA replication but does not affect the chromatin loading of replication proteins (pre-RC) MCM2, CDC6 and replisome protein PCNA. This may suggest that CDK2 inhibition facilitates phosphorylated proteins from S phase extract to load on to chromatin.
6.10.1 CDK activity regulates MCM2 compartmentalisation in G1 phase.

The Nuclear matrix is an insoluble structure of chromatin that associates with cell cycle regulators to assist in DNA replication. Here, nuclear matrix association was determined by performing high salt washes on the chromatin fraction and revealed salt resistant MCM2 loading and accumulation of MCM2 in CDK2 inhibited reaction under high salt conditions. These observations were consistent with other studies performed on MCM2 association with nuclear matrix in late G1 phase (Hesketh et al., 2015).

Initially, the localisation of MCM2 to the nuclear matrix was investigated using cell-free DNA replication assays. In late G1 licensed nuclei, MCM2 efficiently associates with the nuclear matrix. Addition of an S-phase extract led to a dissociation of MCM2 from the nuclear matrix that was CDK2 dependent as roscovitine prevented its release (Figure 6.5). This analysis was extended using cyclin E1/E2 and cyclin A2 depleted nuclei. Cyclin E1/E2 depleted nuclei are replication incompetent that appears to due to defects in the licensing of MCM2-7 via CDK2 and DDK mediated phosphorylation in G1 phase (Figure 6.7). Comparison with Cyclin A2 depleted nuclei that are replication competent and express cyclin E at levels similar to control cells showed nuclear matrix localisation that was reduced by CDK2 activity as cells enter S-phase (Figure 6.6).
6.10.2 Cyclin E1/E2 depletion prevents efficient phosphorylation of MCM2 in G1 phase.

The data presented here demonstrate that Cyclin E1/E2 and cyclin A2 expression is required for G1 exit in fibroblasts re-entry in the cell cycle from quiescence. In this context Cyclin E and cyclin A have specific functions in stabilizing the regulatory factors required for efficient DNA replication. In particular, Cyclin E is required for correct regulation of MCM2-7 via phosphorylation at Ser40/41 of MCM2 (Figure 6.7) and phosphorylation at these sites correlate with replication competence \textit{in vitro}. In addition, Cyclin E contributed to transcriptional regulation of pre-RC (MCM2, CDC6), pre-IC (MCM10) and replisome protein (PCNA) and to the correct licensing of the MCM2-7 complex in G1. Our data suggest that only after cyclin E-CDK2 activity leads to p-MCM2 loading making nuclei replication competent to initiate DNA replication \textit{in vitro}.
Chapter 7
General Discussion
Chapter 7: General Discussion

Regulation of replication complex assembly of proteins is mediated by cyclinE-CDK2 and cyclin A-CDK2 (Reviewed Pauzaite et al., 2016). Here chemical and genetic inhibition of CDK2 activity was found to reduce accumulation of several DNA replication factors including: pre-RC components MCM2 and CDC6; Pre-IC protein MCM10 and replisome protein PCNA. siRNA mediated depletion of cyclin E in post-quiescent cells reduced transcription of E2F factor regulated genes CDC6, MCM2, MCM10 and PCNA. These observations are entirely consistent with canonical role of cyclin E-CDK2 in regulation of the Rb-E2F pathway. However, data presented here suggests an additional novel role for cyclin A-CDK2 in late G1 phase that protects components of the pre-RC from Ubiquitin proteasome system (UPS) mediated degradation. It remains to be addressed whether cyclin A-CDK2 phosphorylates CDC6, MCM2 directly or acts via inhibition of the E3 ligase that mediates polyubiquitination. Nevertheless, this additional layer of regulation could enable cells to respond to genetic stress post-restriction point in G1 phase. Further work is required to investigate whether induction of the p53 pathway via p21 expression could result in degradation of DNA replication factors and prevent S-phase entry.
Cell-free replication assays using late G1 phase nuclei supplemented with S phase extract revealed differences in MCM2 localization and licensing regulated by distinct cyclin-CDK2 complexes. Cyclin E1/E2 depleted nuclei are replication incompetent, whereas cyclin A2 depleted nuclei are replication competent when supplemented with S phase extract. Our data support a model where specific cyclin-CDK2 complexes regulate the temporal separation of replication licensing and initiation of DNA replication. These observations are consistent with earlier work using post-quiescent cells and *in vitro* DNA replication assays (Coverley et al., 2002). The data support the following model: cyclin E-CDK2 activates E2F regulated genes and activates the MCM2-7 helicase in coordination with DDK to license putative origins of DNA replication. These events render nuclei replication competent, but in post-quiescent mouse fibroblasts only the specific activity of cyclin A2-CDK2 can promote initiation of DNA replication.
7.1 Chemical inhibition of CDK2 and DDK destabilizes pre-RC and replisome proteins.

Synchronised post-quiescent mouse fibroblasts enable study of replication complex assembly in late G1 to S-phase (Copeland et al., 2010; Coverley et al., 2002). Initial studies on cell synchronisation were performed to determine the correct time course for subsequent experiments (Figure 3.2). Monitoring the proportion of cells in S-phase and protein levels enabled correlation of cell cycle stage with replication complex assembly. The expression level of protein remains consistent with transcript levels with time further confirms synchronisation of quiescent cells. This further serves as a basis to analyse and dissect the function of cyclinE-CDK2, cyclin A-CDK2 and DDK activities in late G1 phase.

Cyclin-CDK activity is critical for regulation of the initiation of DNA replication and suppressing re-replication of genome by inhibiting replication during S phase and later phases of the cell cycle. (Hua et al., 1997; Coverley et al., 2002; Mimura et al., 2004). As observed in Chapter 3, inhibition of CDK2 in both asynchronous and late G1 synchronized cells resulted in reduced protein levels for MCM2, CDC6 and PCNA. CDK2 mediated phosphorylation of CDC6 protects it from APCcydh1 mediated degradation (Mailand and Diffley, 2005), setting a precedent for this mechanism. Here this observation is extended to show that CDK2 mediated phosphorylation stabilizes MCM2,
PCNA and CDC6 during G1/S phase transition and promoting initiation of DNA replication. Similarly, inhibition of DBF4 dependent kinase (DDK) with PHA 767491 (PHA) synchronous late G1 cells revealed destabilization MCM2, DBF4, CDC6, CDC45, Cyclin A and a reduction in S-phase entry.

However, chemical inhibition of CDK2 and DDK can result in off target effects. Roscovitine is also a potent CDK1 inhibitor but as CDK1 is inhibited at that time point of cell cycle, roscovitine would inhibit CDK2 at the concentration used. The IC50 of roscovitine for cyclin E-CDK2 and cyclin A-CDK2 is 0.70µM (Cicenas et al., 2015; De Azevedo et al., 1997). Unfortunately the DDK inhibitor used here, PHA 767491, is also a CDK2 inhibitor (Albanese et al., 2010). PHA has IC50 for CDK2 at 270 nM. The data presented here cannot exclude that the effects of PHA may be due to off-target CDK2 inhibition. In future, this could be resolved through use of XL 413, a potent DDK inhibitor that does not affect CDK2 activity for comparison and depletion of Dbf4/Cdc7 to investigate this effect in detail.
7.2 Cyclin E-CDK2 regulates transcription of replication assembly of proteins

Destabilization of the replication assembly proteins suggests that phosphorylation mediated by kinases in G1/S transition is involved in maintaining stability and initiation of DNA replication. This analysis determined that CDK activity promotes accumulation of CDC6, MCM2 and PCNA by 2 mechanisms: (i) Rb-E2F mediated transcriptional regulation or (ii) post-translational modification by ubiquitin-proteasome mediated degradation.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) of replication assembly proteins MCM2, CDC6, MCM10, PCNA transcripts from drug mediated kinase (CDK2, DDK) inhibited cells revealed a reduction in transcription of each gene (Figure 3.9). Similarly, siRNA mediated depletion of cyclin E1/E2 reduced CDC6, MCM2, MCM10 and PCNA transcript levels, likely via the canonical cyclin E-CDK2 regulated RB-E2F pathway.

Next, we investigated the requirement of cyclin A in quiescently synchronized mouse embryonic fibroblasts. Contrary to the findings in cyclin E1/E2 depleted nuclei, siRNA mediated depletion of cyclin A2 in post-quiescent cells did not affect E2F1-3 regulated transcripts (Figure 3.10). Further investigation using proteasomal inhibitors reversed the loss of CDC6,
MCM2 and PCNA in cyclin A2 depleted (Figure 4.4) and CDK2 inhibited cells (Figure 4.2), consistent with a role for the proteasome in regulation of protein abundance. Therefore, this analysis has identified distinct mechanisms for cyclin E-CDK2 and cyclin A-CDK2 in regulation of the G1/S transition. In mouse fibroblasts re-entering the cell cycle from quiescence cyclin E-CDK2 is required for transcription of replication assembly proteins in order to maintain their stability during G1 phase and cyclin A promotes accumulation and stabilization of replication assembly of protein in late G1 phase.
7.3 Role of UPS in maintaining protein stability in absence of kinase activity during G1/S transition

The cell cycle is regulated by ubiquitin proteasome system (UPS) mediated degradation that contributes to irreversible cell cycle transitions. Here we investigated the role of ubiquitin proteasome system during the G1/S transition in absence of kinase activity in initiation of DNA replication as a potential mechanism of regulation of destabilized replication assembly proteins.

Inhibition of CDK2 and DDK using small molecule inhibitors and depletion of cyclin A2 by siRNA resulted in destabilization of replication complex assembly proteins and blocks S phase entry in post-quiescent cells. Two independent experiments support a role for the UPS in regulation of the pre-RC components CDC6 and MCM2. Importantly the reduction of CDC6 and MCM2 levels after CDK2 inhibition could be reversed by addition of proteasomal inhibitor MG132. In addition, using a His-tagged ubiquitin construct, pulldown of ubiquitinated proteins included poly-ubiquitinated MCM2 and CDC6 (Figure 4.5). These data suggest that if CDK2 activity is perturbed during G1 phase, cells could prevent S-phase entry prior to restriction point bypass by regulation by cyclin E-Rb-E2F network. In addition, these results suggest that cells have an additional regulatory control that responds to perturbations in CDK activity leading to degradation of
CDC6 and MCM2 post-restriction point in late G1. This could prevent inappropriate S-phase entry and contribute to mechanisms that maintain fidelity of the genome. This data presented here used manipulations in CDK activity to reveal the UPS system actively degrades replication proteins in the absence of CDK activity. This response could potentially be recapitulated by activation of p53 after genotoxic stress in post-restriction point cells. Induction of the DNA damage response and p53 mediated transcription of the CDK1 p21, may reduce CDK activity. Further work is required to establish whether this mechanism induces a G1 checkpoint in post-quiescent cells.

CDK2 activity in G1 and S phase regulates the loading and stabilization of the proteins involved in DNA replication (Choudhury et al., 2016; Mailand and Diffley, 2005). Here the depletion of cyclin A and inhibition of CDK2 was found to result in proteasomal mediated degradation CDC6 and MCM2 by direct ubiquitination (Figure 3.6) and stabilization by addition of a proteasomal inhibitor, MG132 (Figure 4.2). These results suggest that CDK prevents ubiquitin proteasome system mediated degradation by 2 potential mechanisms: (i) Kinases phosphorylate the replication complex assembly proteins there by stabilising them or protecting them from ubiquitin proteasome system (UPS) (Figure 7.1.A) (ii) Kinases phosphorylate E3 ligases there by marking them for self-destruction or by subsequent E3 ligases in the
cell system which in turn inhibits the activity on these proteins (Spruck and Strohmaier, 2002) (Figure 7.1.B).

![Diagram](image)

**Figure 7-1 Postulated mechanism of action for effect of Cyclin A or CDK depletion in synchronised mouse fibroblasts.**

Two models postulating the mechanism by which G1/S kinases regulate replication complex assembly proteins (CDC6, Ciz1, MCM2 and PCNA). In model A, CDK phosphorylates each protein, and the phosphoprotein is protected from ubiquitylation by the E3 ligase. This is consistent with CDK mediate protection of CDC6 from APC/C^cdh1 (Mailand and Diffley, 2005). In model B, CDK mediates phosphorylation of the E3 ligase directly, thereby inactivating it and protecting target substrates. This is consistent with phosphorylation of the E3 ligase Cdh1 that is regulated by cyclin A-CDK and Plk1 in S-phase (Spruck and Strohmaier, 2002).

Phosphorylation can regulate ubiquitination enzymes to influence E3-substrate interactions (Vittal et al., 2015). Phosphorylation can inhibit the E3 from interacting with E2, dynamically affecting the dynamics and specificity of the E3 (Craney et al., 2016; Brown et al., 2016). Phosphorylation can directly
influence the ubiquitination of the substrates (Zhu et al., 2005) or phosphorylation can enable recognition of the substrate later marked for degradation by SCF (Strack et al., 2000). Detailed analysis is the interplay between CDK and the UPS is required to distinguish between these possibilities in future work.
Chapter 7: General Discussion

7.4 Ciz1 is regulated by UPS in absence of Cyclin-CDK2 during G1/S transition

The data presented here demonstrate for the first time that Ciz1 is regulated by phosphorylation leading to its accumulation in G1 phase (Chapter 5). Ciz1 accumulates through G1 phase and correlates with expression of cyclin E and cyclin A (Figure 5.3). The protein levels for Ciz1 mirror those of E2F pathway genes but surprisingly, the transcription of Ciz1 is stable through G1 phase in comparison to E2F regulated genes (Figure 5.3.B). The data presented here show that Ciz1 is regulated by CDK2 activity and possibly by the UPS. CDK2 inhibition by small molecule inhibition or siRNA mediated depletion reduces Ciz1 levels that can be reversed by inhibition of the proteasome (Figure 5.4). The data are consistent with a model whereby Ciz1 accumulates in response to increasing CDK activity in G1 phase. Ciz1 is known to be a cyclin E-CDK2 and cyclin A-CDK substrate (Copeland et al., 2015). These data suggest that Ciz1 is regulated by CDK2 mediated phosphorylation to promote its accumulation as well as regulating its function in regulation of the initiation phase of DNA replication. Further investigation of the role of S331 and T293 in Ciz1 accumulation is required to whether there is a temporal order to Ciz1 site specific phosphorylation and to test how this regulates Ciz1 activity.
7.5. DHX9 contributes to regulation of the G1/S transition.

A proteomic screen identifying Ciz1 interaction partners, recovered DHX9 a DNA/RNA helicase that performs numerous functions including binding to replication origins (Lee et al., 2014). To better understand whether DHX9 contributes to regulation of cell cycle re-entry from quiescence siRNA mediated depletion of DHX9 was performed here. Reduction of DHX9 in synchronized post-quiescent cells produced a statistically significant decrease in the number of S-phase cells suggesting a defect in cell cycle progression. These data suggest that DHX9 and Ciz1 may be active within the same pathway as siRNA mediated depletion of Ciz1 also markedly reduces cell cycle progression (Coverley et al., 2005; Copeland et al., 2010). However, as we only monitored cell cycle progression in the cell cycle following transfection, our data is distinct from other studies monitoring the effects of prolonged DHX9 depletion that led to a stabilization of p53 and senescence (Lee et al., 2014). The data therefore suggest that DHX9 contributes to efficient cell cycle progression (Figure 5.8) and prolonged depletion of DHX9 induces a replication stress response leading to p53 activation and senescence (Lee et al., 2014).
7.6 In vitro analysis of initiation of DNA replication

Cell-free replication assays have previously identified that cyclin E-CDK2 was required for MCM2 and CDC6 loading on to chromatin to license replication origin activation by cyclin A-CDK2 (Coverley et al., 2002). Here, chemical and genetic inhibition of cyclin E-CDK2 and cyclin A-CDK2 resulted in reduction of MCM2, CDC6 and PCNA protein levels in G1 phase, thereby preventing S-phase entry. To further investigate whether cyclin depleted nuclei were ‘licensed’ to replicate i.e. retain replication competency a siRNA approach was used to deplete specific cyclins. After developing this approach cyclin E1/E2 depleted nuclei and cyclin A2 depleted nuclei were assessed to identify differences in replication complex assembly and replication competence.

Cell-free replication assay using late G1 phase nuclei reconstituted with S phase extract can initiate DNA replication and load replication assembly proteins on to chromatin (Figure 6.1). It is first time that genetically depleted nuclei are used in cell-free DNA replication assays. Cyclin E1/E2 depleted nuclei and cyclin A depleted nuclei were prepared by siRNA transfection in synchronized fibroblasts and reconstituted with S phase extract to study replication initiation in absence of cyclin activity. Cyclin E1/E2 depleted nuclei were unable to replicate when incubated in S-phase cellular extracts, whereas cyclin A2 depleted nuclei can initiate DNA replication when
supplemented with S phase extract containing active kinases and phosphorylated proteins. Evaluation of the chromatin fraction in cyclin E1/E2 and cyclin A2 depleted nuclei showed that both nuclei could ‘load’ CDC6, MCM2, PCNA, cyclin E and cyclin A onto chromatin in cell-free replication assays from S-phase extracts. These observations suggest that loading of these factors is not sufficient for DNA replication on Cyclin E1/E2 template nuclei. Interestingly, S phase extract is sufficient to load replication assembly proteins on to chromatin for both cyclin E1/E2 depleted nuclei and cyclin A2 depleted nuclei (Figure 6.2, 6.3).

As MCM loading is a key licensing event, the localisation of MCM2 was determined in Cyclin E1/E2 depleted nuclei. MCM2 loads onto chromatin in late G1 phase and early S–phase and is enriched on the nuclear matrix prior to initiation in late G1 phase (Hesketh et al., 2015). Cell free DNA replication assay was further used to investigate nuclear matrix association of replication assembly proteins. The Nuclear matrix is a sub-nuclear insoluble that associates with cell cycle regulators to assist in DNA replication. Here we found cyclin E1/E2 depleted nuclei retain MCM2 on the nuclear matrix in S-phase extract, whereas cyclin A2 depleted nuclei initiate and release MCM2, suggesting that cyclin E depleted nuclei are defective in MCM2-7 localisation to the nuclear matrix (Figure 6.6).
Further analysis of MCM2 regulation was performed by analysing the phosphorylation of MCM2 in cyclin A2 depleted nuclei. MCM2 levels are low on chromatin fractions and nuclear matrix fractions when incubated in G1 extract. MCM2 loads efficiently onto chromatin in S-phase extracts but as cells initiate DNA replication the nuclear matrix fraction remains low. Importantly, CDK2 inhibition prevents initiation of DNA replication in this context and shows an enrichment of MCM2 onto the nuclear matrix under these conditions. These data suggest that MCM2 localises to the correct sub-nuclear compartments during late G1 and early S-phase in cyclin A2 depleted nuclei (Figure 6.6), consistent with their replication competence.
7.7 Cyclin E1/E2 expression is required for phosphorylation of MCM2 to license DNA replication

DNA replication complex assembly is separated into distinct phases (Figure 7.2). The phosphorylation of MCM2-7 complex by the combined activities of DDK and CDK is an essential step in replication licensing through loading of the pre-IC (Tanaka and Araki, 2013). The phosphorylation status of MCM2 at a regulatory CDK/DDK (p-Ser40/p-Ser41) sites in the N-terminal region of MCM2 was evaluated. Depletion of cyclin E1/E2 prevents efficient phosphorylation of MCM2 on chromatin, whereas cyclin A2 depleted nuclei contained chromatin associated p-MCM2 (Figure 6.7). Therefore, the phosphorylation status of MCM2 correlates with the replication competence of cyclin depleted nuclei (Figure 6.7). The data suggests that cyclin E-CDK2 and DDK mediated phosphorylation of MCM2 is required to promote the formation of pre-IC (Tanaka and Araki, 2013), and this is a critical step in promoting replication competence in S-phase extracts.

In cyclin E1/E2 depleted nuclei that data suggest that the absence of cyclin E-CDK2 activity prevents formation of the pre-IC rendering cyclin E depleted nuclei replication incompetent. Conversely, in cyclin A2 depleted cyclin E-CDK2 levels are similar levels to unperturbed late G1 nuclei, leading to the cyclin E-CDK2 mediated phosphorylation of MCM2 at S40 and S41 (Figure 6.7). Cyclin A2 depleted template nuclei are therefore replication
competent and initiate DNA replication when supplemented with S phase extract (Figure 6.7.B).

However, from the Figure 6.6 we can interpret here that cyclin E-CDK2 mediated phosphorylation of MCM2 at S40 and S41 is required for initiation of DNA replication. This analysis is limited by the availability of phosphor-specific antibodies for the MCM2-7 complex and could be complimented by global phosphor-proteomic analysis of cyclin E-CDK2 and cyclin A-CDK2 substrates to identify the full repertoire of cyclin specific substrates. The identification of initiation specific CDK substrates remains elusive and would be far more informative on the precise role of each of the cyclin E and cyclin A initiation of DNA replication.

7.7.1 Working Model

With cell-free replication assay in cyclin E depleted nuclei and cyclin A2 depleted nuclei, we were able to identify that cyclin E depleted nuclei are replication incompetent. This leads to generation of working model as described in Figure 7.2. As cyclin E1/E2 depleted nuclei cannot carry out cyclin E mediated phosphorylation of MCM2, which is required for formation of pre-initiation complex, and that cyclin A depleted nuclei are replication competent as they have cyclin E mediated phosphorylation of MCM2
allowing the formation of pre-IC and active kinases from S phase extract are able to carry out DNA replication in cyclin A depleted nuclei.

Figure 7.2 shows distinction between the nuclei (late G1 nuclei, cyclin E1/E2 depleted nuclei and Cyclin A2 depleted nuclei) postulated from the observations in Figure 6.6 showing phosphorylation of MCM2 at S40 and S41 site mediated by cyclin E-CDK2 and DDK. Figure 7.2.A demonstrates possible mechanism by which G1 nuclei can initiate DNA replication when supplemented with S phase extract containing all active kinases and phosphorylated proteins involved in DNA replication. As observed in the Figure 7.2.A, G1 nuclei are replication competent as they have active cyclin E-CDK2 and cyclin A-CDK2 leading to formation of pre-IC and replisome leading to initiation of DNA replication when supplemented with S phase extract. Interestingly, in cyclin E1/E2 depleted nuclei we postulate from the observations of Figure 6.6 that, as it lacks cyclin E-CDK2 activity it does not have active pre-IC and replisome making it replication incompetent even when supplemented with S phase extract. Conversely, in cyclin A2 depleted nuclei, there is cyclin E-CDK2 activity leading to formation of active pre-IC complex with the phosphorylation status of MCM2 at S40 and S41 (Figure 6.6.), making Cyclin A depleted nuclei replication competent and can initiate DNA replication when supplemented with S phase extract.
Figure 7-2 Working models of distinct mechanisms involved in initiation of DNA replication in G1 nuclei, Cyclin E1/E2 depleted nuclei and cyclin A2 depleted nuclei.

**A:** Composition of G1 nuclei leading to formation of active pre-initiation Complex and replisome and when supplemented with S phase extract can initiate DNA replication.  
**B:** Composition of Cyclin E1/E2 depleted nuclei with lack of Cyclin E-CDK2 leading to inactivation of pre-IC due to loss of phosphorylation at S40 and S41 of MCM2 and thereby making Cyclin E1/E2 depleted nuclei replication incompetent even when supplemented with S phase extract.  
**C:** Composition of Cyclin A2 depleted nuclei with formation of active pre-Initiation complex with phosphorylation of MCM2 at S40+S41 making the nuclei replication competent when supplemented with S phase extract.
This analysis suggests that cyclin E-CDK2 and DDK mediated phosphorylation of MCM2 at Ser 40 and Ser 41 is indicative of the replication statuses of the template nuclei. In addition, cyclin E1/E2 is required to facilitate relocalization of MCM2 away from the nuclear matrix. These data strongly implicate MCM2-7 as being the major regulatory target of cyclin E-CDK2 consistent with other in vitro studies (Coverley et al., 2002) and in vivo analyses (Geng et al., 2003; Geng et al., 2007).

In further support of the formation of pre-IC in cyclin A2 depleted nuclei, it was found that addition of recombinant cyclin A-CDK2 could initiate DNA replication. Cyclin A2 depleted nuclei were reconstituted with G1 extract supplemented with recombinant cyclin A-CDK2 (Figure 6.8) and 0.1 ng/µl concentration of cyclin A-CDK2 gave the highest percentage of replicating nuclei, with increase in concentration of recombinant cyclin A-CDK2 increase in replicating nuclei and peak at 0.1 ng/µl. Interestingly, concentration above 0.1 ng/µl appeared to be inhibitory concentration as number of replicating nuclei decreased. This is similar to previous observations seen by Coverley et al., 2002 and Copeland et al., 2010. This suggests that cyclin A-CDK2 above a certain concentration works as inhibitory phosphorylation for initiation of DNA replication. It remains to be addressed whether recombinant cyclin E-CDK2 to cyclin E1/E2 depleted
nuclei would confer replication competency. Further analysis would help identify cyclin E-CDK2 substrates there by helping understand the process of DNA replication in detail.
7.8 Future work

Mechanism of action of DDK in initiation of DNA replication is not very well understood as the experiments presented here were performed using chemical inhibitor with off-target effects on CDK2. Targeted depletion of Cdc7-Dbf4 by siRNA mediated depletion would provide more insights into understanding mechanism by which DDK mediates regulation of initiation of DNA replication.

Further to understanding the role of UPS in absence of active kinases in initiation of DNA replication during G1/S transition. Identification of E3 ligases regulating ubiquitination of MCM2, CDC6 and PCNA would help understand the mechanism in detail. To identify potential E3 ligase a global siRNA screen or in vitro ubiquitination assays could be performed.

Another experimental procedure with Cdc7-Dbf4 inhibited nuclei in cell free replication assay would further help understand the process of initiation of DNA replication in detail. Additionally, recombinant cyclin E-CDK2 to cyclin E1/E2 depleted nuclei would confer replication competency. A global analysis of cyclin E and cyclin A substrates would be required to identify the full repertoire of substrates. This would be a far informative on the precise role of cyclin E and cyclin A in initiation of DNA replication.


7.9 Concluding Remarks

Genome duplication is the key objective for any dividing cell. All nuclear DNA must be duplicated accurately to ensure that the genetic information stays preserved for the next generation (Toueille and Hubscher 2004; Masai et al., 2010). In human cells, DNA replication has to accurately copy around $6 \times 10^9$ bases over approximately 10 hour during S phase (Goren and Cedar 2003); therefore, it must be spatiotemporally regulated at different levels. It is evident from the data presented in this thesis that sustained cyclin-CDK activity is required for initiation of DNA replication during G1/S transition in Mouse fibroblasts released from quiescence (G0). Cyclin E-CDK2 and cyclin A-CDK2 both are equally important in cells re-entering cell cycle and for initiation of DNA replication. Using genetically inhibited nuclei in cell free replication a novel experiment we demonstrated the temporal regulation of cyclin E and cyclin A in initiation of DNA replication.
Chapter 8
References
Chapter 8: References


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