DOCTOR OF PHILOSOPHY

The role of Cdc7 and cyclin-dependent kinases in DNA replication and S phase

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Abstract

The cell cycle is a highly orchestrated developmental process that eventually leads to the reproduction of a cell. In metazoans, it is driven by the successive activation of cyclin-dependent kinases (Cdk) and proper coordination of cell cycle transitions and processes ensure genomic stability. DNA replication takes place during S phase to faithfully duplicate a cell's genetic material. In eukaryotes, S phase onset involves the initiation of numerous licensed replication origins across the genome and requires the activities of two protein kinases, S phase-Cdk and Cdc7. In this thesis, I present work relating to the role of the S phase-promoting kinases in DNA replication and S phase regulation.

Using the cell-free system of *Xenopus* egg extracts, a small molecule inhibitor of Cdc7 was characterised. PHA-767491 was then used to demonstrate that Cdc7 executes its activity early in S phase before the Cdk-dependent step. Cdc7 is not rate limiting for the progression of the replication timing programme once its essential function has been executed, unlike S-Cdk whose activity is required throughout S phase. Protein Phosphatase 1 (PP1) was identified as a modulator of Cdc7 activity in egg extracts, which rapidly reverses Cdc7-dependent phosphorylation of chromatin-bound Mcm4 and likely functionally lowers Cdc7 activity during an etoposide-induced checkpoint response. This provides a novel mechanism for regulating Cdc7 by counteracting its activity on essential replication substrates in the event of replicative stress.

In the second part of the thesis, the design strategy for generating a *Cdc7*-conditional knockout mouse (cko) is outlined and results from the screen for a transgenic founder are presented. A *Cdc7*-cko mouse will be a valuable tool to further dissect Cdc7 function and regulation in mammalian cells. In the final section, S phase entry and progression in mouse embryonic fibroblasts lacking both Cdk1 and Cdk2 was examined. Contrary to expectations, Cdk1/Cdk2 double knockout cells can enter S phase in the absence of detectable S phase-Cdk activity. S phase progression, however, was inefficient. Cdc6 and cyclin E1 proteins were found to accumulate in high levels in these cells although the exact function(s) and mechanism(s) for these observations remain to be discovered. With this work, I hope to provide additional insight into the roles and regulation of S phase kinases in eukaryotic DNA replication.

Table of Contents

List of Figures

List of tables

List of abbreviations

4-OHT	4-Hydroxytamoxifen
APC	Allophycocyanin
APC/C	Anaphase Promoting Complex/Cyclosome
ASK	Activator of S phase kinase
ATM	Ataxia telangietasia mutated
ATP	Adenosine triphosphate
ATR	ATM and RAD3-related
ATRIP	ATR-interacting protein
BAC	Bacterial artificial chromosome
BCA	Bicinchoninic acid
bp	Base pairs
BRCA	Breast cancer
BRCT	BRCA1 C-terminus
BSA	Bovine serum albumin
CDC	Cell Division Cycle
Cdk	Cyclin-dependent kinase
CHO	Chinese hamster ovary
cko	Conditional knockout
CLB	Cyclin B type
CLL	Chronic lympholytic leukemia
CMG	Cdc45-Mcm-GINS complex
Cy3-dUTP	Cyanine 3-conjugated deoxy-Uridine triphosphate
dATP	Deoxy-ATP
Dbf4	Dumbbell forming 4
DDK	Dbf4/Drf1-dependent kinase
DIG	Digoxigenin
DKO	Double knockout
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DN	Dominant negative
DNA	Deoxyribonucleic acid
Drf1	Dbf4-related factor 1
DSB	Double strand break
DTT	Dithiothreitol
DUE-B	DNA unwinding element B
DYRK	Dual-specificity tyrosine phosphorylation-regulated kinase
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethylene glycol tetra-acetic acid
ELYS	Embryonic large molecule derived from yolk sac
ETO	Etoposide
FCS	Fetal calf serum
FLP	Fippase
FRT	Flippase Recognition Target
g	G-force or relative centrifugal force
GEMC	Geminin coiled-coil-containing
GFP	Green fluorescence protein
GINS	Go-Ichi-Nii-San (5-1-2-3 = Sld5, Psf1, Psf2, Psf3 complex)
HEPES	Hydroxyethyl piperazine ethanesulfonic acid
HotSHOT	Hot sodium hydroxide and Tris
HSV-TK	Herpes Simplex virus-thymidine kinase

HU	Hydroxyurea
IC_{50}	Half-maximal inhibitory concentration
IP	Immunoprecipitation
K1 KO	Cdk1 knockout
K2 KO	Cdk2 knockout
kb	Kilobases
K_i	Dissociation constant of an inhibitor
K_M	Michaelis constant
MAPK	Mitogen Activated Protein Kinase
Mcm	Mini chromosome maintenance
MEF	Mouse embryonic fibroblasts
MMS	Methyl methanesulfonate
MPF	Maturation Promoting Factor
MRN	Mre11-Rad50-Nbs1 complex
mRNA	Messenger RNA
NDT	Non dityrosine
NSD	N-terminal serine/threonine domain
ODP	Origin decision point
ORC	Origin Recognition Complex
PBS	Phosphate-buffered saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PI	Propidium iodide
PIP	PP1-interacting protein
PMSF	Phenylmethylsulfonyl fluoride
PP1	Protein Phosphatase 1
PP1c	Protein Phosphatase 1 catalytic subunit
PRK	Proliferation-related kinase
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative Real-time PCR
RAD	Radiation
Rb	Retinoblastoma
RECQL	Recombination Q-like
RNA	Ribonucleic acid
RPA	Replication Protein A
Rpm	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
shRNA	Short hairpin RNA
SPF	S phase promoting factor
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
tg	Transgene
TOPBP	Topoisomerase binding protein
Topo II	Topoisomerase II
UVC	Ultraviolet, electromagnetic subtype C
WGA	Wheat germ agglutinin
WT	Wild type

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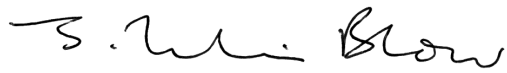
Finally, I thank my family for always being there for me.

Declaration

I declare that I am the author of this thesis and that, unless otherwise stated, all references cited have been consulted, that I have performed the work of which this thesis is a record and that this thesis has not been previously accepted for a higher degree; provided where the thesis is based upon joint research, the nature and extent of my individual contribution has been defined.

Wei Theng Poh

We declare that the conditions of the relevant Ordinance and Regulations for supervised postgraduate research have been fulfilled in the completion of this thesis.



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Chapter 1 – Introduction

1.1 The cell division cycle

The cell cycle is a highly orchestrated developmental process that eventually leads to the reproduction of a cell. In eukaryotic cells, it is classically divided into four phases in which genetic material is first duplicated during S (Synthesis) phase and then segregated during M (Mitosis) phase. S and M phase may each be preceded by a gap phase, the G1 and G2 phase respectively. Faithful replication of chromosomes followed by accurate segregation of sister chromatids are crucial to ensure that the two daughter cells each inherit a perfect copy of the genetic material that was originally carried in the mother cell, thus ensuring continued genomic stability (Blow and Dutta, 2005). For sustained cell proliferation, this chromosomal cycle is tightly coordinated with the growth cycle in which other cell constituents are also duplicated and divided into daughter cells (Nasmyth, 2001). Adaptions to the four-stage cell cycle also occur in nature, for instance, rapid cleavage cycles consisting only of alternating S and M phases take place during embryogenesis in order to produce large numbers of cells within a short span of time (Duronio, 2012).

1.1.1 A short history

The mechanisms underlying the cycle of cell growth and division remained largely unknown until the 1970s and 80s, when development of techniques in molecular biology quickly allowed advances to be made in the areas of cell biology, biochemistry and genetics. Details about basic control components and mechanisms revealed that processes are highly conserved across eukaryotic organisms (Nurse et al., 1998).

Cell cycle genetics was pioneered by Hartwell's seminal work in screening temperature-sensitive mutants of *Saccharomyces cerevisiae* (Hartwell et al., 1970). In

a series of papers published in the early 70s, Hartwell isolated and characterised the *cdc* (cell division cycle) collection of mutants, setting the stage for the discovery of important regulators of the eukaryotic cell cycle (Culotti and Hartwell, 1971; Hartwell, 1971a, b; Hartwell et al., 1970; Hartwell et al., 1973). Because a yeast cell's bud grows as it goes through the cell cycle, bud size provides a convenient measure of cell cycle position. Yeast mutants that were defective in passage through the chromosome or division cycle would arrest with characteristic shapes. By studying mutant yeast morphology after a shift to the restrictive temperature, it was then possible to determine a period of the cell cycle during which a particular temperature-sensitive event occurs at the permissive temperature, termed the execution point of this event, before the mutant finally arrested at the termination point with a distinctive morphology (Hartwell et al., 1970). Successful cell division only occurred when cell cycle events occur in the proper sequence. The orderly manner in which earlier events are successfully completed before later events can take place showed that there was great interdependence in between the events controlled by various *cdc* genes (Hartwell et al., 1974).

Inspired by Hartwell's work with the budding yeast, genetic studies in the fission yeast *Schizosaccharomyces pombe* by Nurse and colleagues soon led to other key insights to the cell cycle. Measurement of cell length could be conveniently used as a gauge of cell growth in fission yeast owing to its cylindrical shape and growth by lengthwise extension. Nurse and Thuriaux initially identified several fission yeast *cdc* mutant strains that divided at half the normal cell size at a restrictive temperature, which they named the "wee" mutants (Nurse, 1975; Thuriaux et al., 1978). One such mutant, the *wee2-1* strain was found to be allelic to the fission yeast *cdc2* gene and encodes a key regulator required for mitosis (Nurse and Thuriaux, 1980). Based on these features, Nurse suggested that *cdc2* must be a critical cell cycle regulator because while necessary for mitotic entry, if mutated, it could also accelerate this process. Evidence

that *cdc2* was central to cell cycle regulation was also supported by the discovery that it was required for G1/S as well as G2/M progression (Nurse and Bissett, 1981). Subsequent work identified the *cdc2* gene product to be a protein kinase (Simanis and Nurse, 1986). With the advent of cloning techniques by complementation, *S. pombe cdc2* was discovered to be the homologue of budding yeast *CDC28* (Beach et al., 1982). This finding suggested for the first time that the cellular mechanisms of cell cycle were conserved. The degree of conservation leapt from yeast to humans when the human homologue of *CDC28* and *cdc2* was isolated by heterologous complementation using a mammalian cDNA library (Lee and Nurse, 1987).

At the same time, important discoveries were also made using biochemical approaches. A protein-like substance termed the maturation promoting factor (MPF) had been purified from amphibian oocytes which could drive oocyte maturation (Masui and Markert, 1971). Since this involves progression through meiosis during maturation, this meant that MPF was able to induce the onset of M phase in a cell (Nurse, 1998). Development of an *in vitro* system derived from *Xenopus laevis* eggs (Lohka and Masui, 1983) provided the assay system required for biochemical purification of MPF, leading to the observation that MPF was made up of two proteins with molecular masses of 32 kDa and 45 kDa (Lohka et al., 1988). Meanwhile, a protein had been discovered in sea urchin embryos whose abundance oscillated in synchrony with cell division; this protein was named cyclin, the first of its family to be discovered (Evans et al., 1983). This report by Hunt and his colleagues was the first to describe periodic protein degradation over the course of the cell cycle. Cyclin genes were cloned and sequenced shortly after (Swenson et al., 1986). It was not long before the two components of MPF were identified as cyclin B and Cdc2, a cyclin-dependent kinase (Cdk) (Gautier et al., 1990; Gautier et al., 1988; Labbe et al., 1989).

Subsequent studies eventually led to an understanding that these cyclin-dependent kinases, whose activities fluctuate cyclically as a result of periodic cyclin transcription and degradation, are essential regulators of the cell cycle (Glotzer et al., 1991; Minshull et al., 1989a; Minshull et al., 1989b; Murray and Kirschner, 1989; Murray et al., 1989). From these early stages, starting from different approaches and using a variety of model organisms, the study of cell cycle regulation has since grown to encompass much greater complexity as more components are identified and greater molecular definition add layers of controls to existing mechanisms.

In the next sections of this Introduction, aspects of eukaryotic DNA replication will be discussed in some detail (Section 1.2), with a focus on the S phase-promoting kinase, Cdc7 (1.3) and ending with an overview of metazoan Cdk control of cell cycle transitions (1.4).

1.2 Chromosome replication

1.2.1 Eukaryotic DNA replication

Much of cell cycle research had focused on the mechanisms that drive the chromosome cycle rather than the growth cycle (Nasmyth, 2001). The physiological significance of a requirement for perfect fidelity in DNA replication is emphasised by the types of human genetic diseases that may result from under- or over-replication, including cancers, birth defects, developmental abnormalities and a group of diseases collectively known as the “chromosome instability syndrome” which result from defects in proteins for the assembly and monitoring of the replication fork (DePamphilis, 2006; Sclafani and Holzen, 2007). Regulatory mechanisms exist that enforce the strict alternation of DNA replication and chromosomal segregation and allow DNA replication to take place only once in each cell cycle.

DNA replication in eukaryotic cells occurs in a series of ordered events that begins with origin selection, followed by assembly of pre-replicative complexes around origins (pre-RC) (Section 1.2.2) and initiation of licensed origins (Section 1.2.3). Replication origins are chromosomal elements that bind initiator proteins, which promote local unwinding of the DNA double helix and recruitment of additional protein components, eventually leading to the assembly of the replisome at origins. It is now known that a common set of initiation proteins assembles at origins whose activities are regulated by specific kinases. Following replication initiation, two sister replication forks are established at each origin. Each strand of DNA is then replicated in a semi-conservative manner as replisomes progress bi-directionally away from the origin. Replication forks terminate when they encounter another fork head-on in the opposite direction (Bell and Dutta, 2002; Blow and Dutta, 2005; Kelly and Brown, 2000; Sclafani and Holzen, 2007).

1.2.2 Licensing DNA origins

Due to the large sizes of eukaryotic genomes, ranging from 10^7 to more than 10^9 base pairs (bp), replication is simultaneously initiated from numerous origins located on multiple chromosomes, typically spaced 30 – 100 kilobases (kb) apart (Bell and Dutta, 2002; Blow and Dutta, 2005; Kelly and Brown, 2000; Sclafani and Holzen, 2007). In order that this process is not only fast but also accurate, replication must be strictly regulated to ensure that no segment of the chromosome is duplicated more than once per cell cycle.

The early cell fusion experiments of Rao and Johnson first established that cells in different phases of the cell cycle differ in their capacity to undergo the initiation reaction (Rao and Johnson, 1970). When a G1 phase cell was fused to a G2 phase cell, the unreplicated G1 nucleus entered S phase directly while the DNA of G2 cell did not replicate again until the hybrid cell had undergone mitosis. Fusion of an S phase cell to

a G1 cell accelerated the latter's progress into S phase; this was not observed in the S/G2 hybrid. These results indicate that initiation of DNA replication required a positive, diffusible S phase-promoting signal that a cell in G1 but not G2 phase can respond to (Arias and Walter, 2007; Blow and Dutta, 2005). Working with the cell-free system of *Xenopus* egg extract, a "licensing" model was subsequently proposed, in which a licensing factor is required for replication initiation, binding to chromatin during late mitosis to G1 that is later removed as DNA is replicated (Blow and Laskey, 1988). This model effectively divides the process of DNA replication into two mutually exclusive stages, the first of which is permissive to licensing and the second to initiation.

The idea that DNA exists in two distinct states was validated when *in vivo* foot-printing experiments in yeast showed that origins formed a "pre-replicative" pattern in G1 cells, in contrast to origins that exhibited a "post-replicative" pattern from initiation until the end of mitosis (Diffley et al., 1994). Other experiments had in the meantime provided evidence that in fission yeast, Cdk activity is required to limit DNA replication to a single round in each cell cycle and that inhibition of Cdk activity in G2 causes origins of replication to revert to a pre-replicative state that is amenable to re-initiation (Broek et al., 1991; Hayles et al., 1994). These experiments neatly set DNA replication back into the broader context of the cell cycle, such that two-step initiation model is closely correlated to the oscillations of Cdk activity. The first step, licensing, occurs soon after M phase as Cdk activity decreases upon mitotic exit while replication initiation is triggered when S phase Cdk activity rises at the G1/S transition.

In molecular terms, origin selection occurs when the Origin Recognition Complex (ORC) binds to DNA. ORC serves as a landing pad for two other proteins, Cdc6 and Cdt1, to assemble onto origins which then coordinately load a head-to-head double hexamer of the mini-chromosome maintenance 2-7 (Mcm2-7) protein complex around origin DNA to form the pre-replicative complex (pre-RC) (Figure 1.1A) (Evrin et al.,

2009; Gambus et al., 2011; Gillespie et al., 2001; Remus et al., 2009). In *Xenopus* egg extracts, the Mcm2-7 complex remains bound to DNA even if ORC and Cdc6 are subsequently removed. Since DNA replication can proceed under these circumstances, this suggests that the essential functions of the ORC complex and Cdc6 in initiation are completed upon Mcm2-7 recruitment to DNA (Hua and Newport, 1998; Rowles et al., 1999). Indeed, following Cdt1 and Mcm2-7 association to chromatin, ATP hydrolysis followed by dissociation of Cdc6 triggers dissociation of Cdt1, stimulating the tight binding of Mcm2-7 to DNA (Randell et al., 2006). This stable association of Mcm2-7 complex on DNA is referred to as licensing. ATPase activity from the ORC is essential for iterative loading of Mcm2-7 to support pre-RC formation (Bowers et al., 2004).

Pre-RC assembly is restricted to late M and G1 phase, in the presence of low Cdk activity, whereas Mcm2-7 activation can only occur in the presence of high Cdk activity, which also serves to prevent *de novo* pre-RC assembly. Since the loading of the Mcm2-7 complex constitutes origin licensing, the displacement of Mcm2-7 from chromatin as replication forks proceed along DNA means that the “licence” is never associated with DNA that has already been replicated (Arias and Walter, 2007; Blow and Dutta, 2005). Upon entry in to S phase, multiple mechanisms ensure that no new pre-RCs can form and no re-replication takes place. In budding yeast, these depend largely on Cdk-dependent phosphorylation of pre-RC components (Arias and Walter, 2007; Chen and Bell, 2011; Nguyen et al., 2001), whereas in metazoans a major pathway to prevent relicensing is the inhibition of Cdt1 activity through degradation or by its inhibitor geminin (Li and Blow, 2004; Wohlschlegel et al., 2000). Although the mechanisms to ensure once-per-cycle chromosomal duplication differ between yeasts and metazoans, the outcome centers on the inhibition of Mcm2-7 rebinding to replicated DNA. This provides a molecular basis to the original licensing hypothesis.

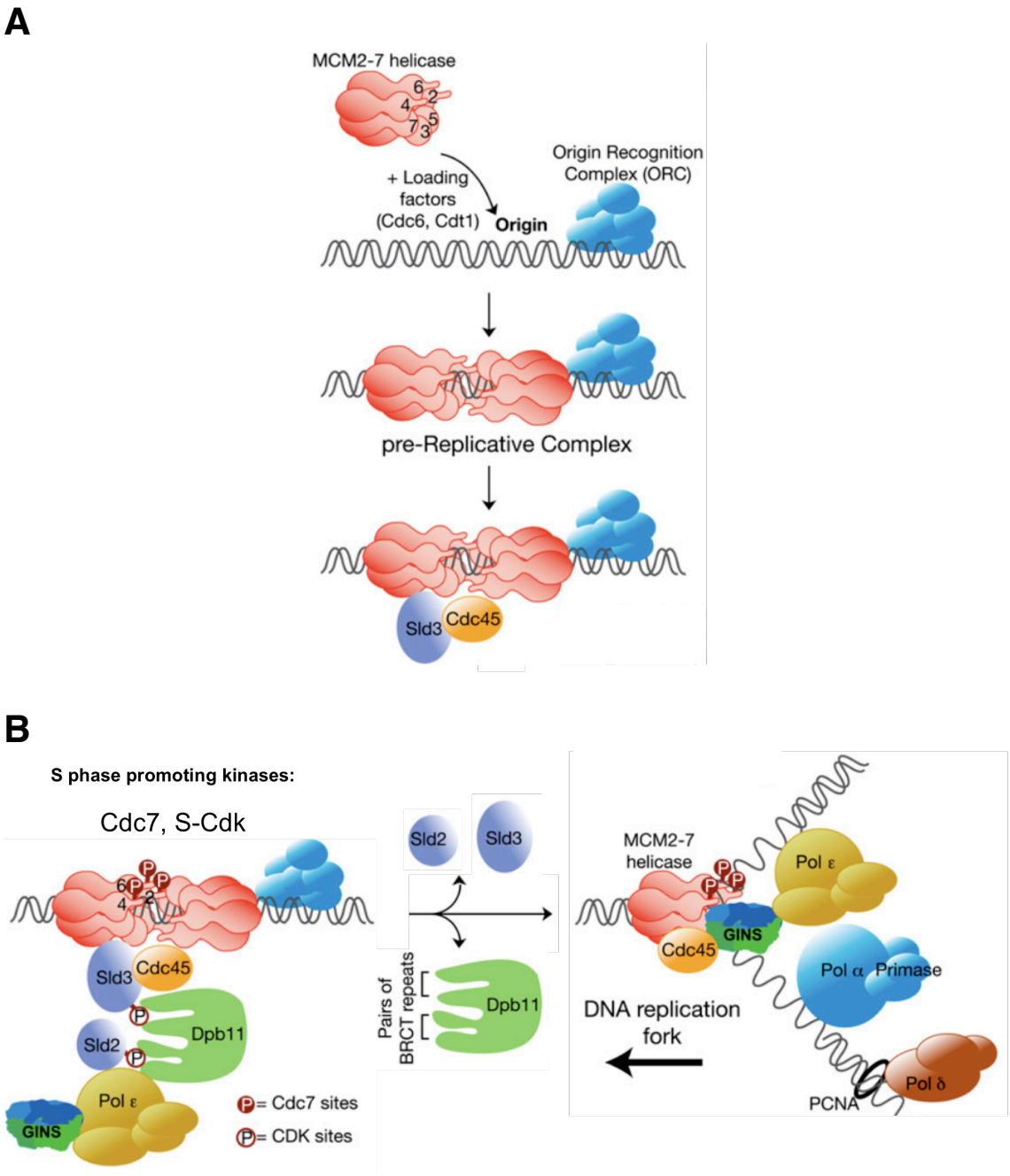

Figure 1.1. Licensing and initiation of replication origins in *S. cerevisiae*.

(A) In G1 phase of the cell cycle, origin selection begins by the binding of ORC to chromatin. Cdc6 and Cdt1 subsequently bind to ORC and then recruit Mcm2-7 to origins, forming the pre-RC. Stable loading of a double hexamer of Mcm2-7 is known as licensing. (B) At the G1/S transition, the activity of Cdc7 and S-Cdk are required to phosphorylate several components of the pre-RC and recruit other replication factors, including Cdc45 and GINS, to form the replisome. In budding yeast, Sld2 and Sld3 have been identified as the minimum set of Cdk targets essential for DNA replication. Initiation of origins results in the formation of a pair of replication forks moving bidirectionally away from the origins, replicating DNA in a semi-conservative manner. Adapted from (Labib, 2010).

1.2.3 Initiation of licensed origins

The initiation of licensed origins to trigger active DNA replication requires the activity of two S phase promoting kinases: Cdk and the Dbf4/Drf1-dependent, Cdc7 kinase (DDK) (Figure 1.1B) (Jares and Blow, 2000; Pacek and Walter, 2004; Walter, 2000). The actions of these protein kinases stimulate the recruitment and stable association of other protein components, including Cdc45 and the GINS complex, with the Mcm2-7 complex, which is important for *in vivo* helicase function (Labib, 2010).

While the Mcm2-7 complex consists of six ATPase subunits like other DNA replicative helicases and the sub-complex of Mcm4-6-7 possesses limited helicase activity in several eukaryotic species (Ishimi, 1997; Lee and Hurwitz, 2000; You et al., 1999), evidence suggests that Mcm2-7 only unwinds DNA as part of a larger functional unit *in vivo*. Cdc45, first identified in budding yeast, was shown to be required for the progression of the replication fork together with Mcm2-7 (Aparicio et al., 1997; Tercero et al., 2000). This was later found to be true also for its orthologue in *Xenopus* (Pacek and Walter, 2004; Shechter et al., 2004). Subsequent work determined that Cdc45 actually binds Mcm2-7 as part of an even larger assembly, together with the GINS complex, forming a Cdc45-Mcm-GINS (CMG) complex, at origins (Aparicio et al., 2006; Ilves et al., 2010; Moyer et al., 2006). The stable association of Cdc45 with an origin coincides with its temporal order of initiation and as such, Cdc45 recruitment is a marker of origin activation (Aparicio et al., 1999; Zou and Stillman, 2000). The GINS (Go-Ichi-Nii-San or 5-1-2-3, referring to the component subunits Sld5, Psf1, Psf2 and Psf3) complex is required to maintain the association of Mcm2-7 and Cdc45 within the CMG complex (Gambus et al., 2006). CMG complexes isolated from extracts of early *Drosophila* embryos were found to have DNA helicase activity and components of the CMG complex were among the components identified in a replicative helicase on replicating DNA in *Xenopus* extracts (Moyer et al., 2006; Pacek et al., 2006). These

lines of evidence suggest that the CMG complex is the active form of the eukaryotic helicase. With its ATPase activity, Mcm2-7 serves as the engine in this molecular machine to unwind origin DNA (Ilves et al., 2010).

1.2.3.1 S-Cdk activity is required for replication initiation

Early work in the cell cycle has shown that Cdk activity is required for DNA replication in eukaryotes. *Xenopus* extracts depleted of Cdks and yeast mutants of Cdks and cyclins cannot initiate DNA replication (Blow and Nurse, 1990; Hartwell, 1971a; Nurse and Bissett, 1981). It was first shown in budding yeast and the later in metazoans that Cdk activity is required throughout S phase for the activation of replication origins (Donaldson et al., 1998b; Thomson et al., 2010). However, the essential Cdk substrates for replication initiation have only been recently determined in *S. cerevisiae*, thereby allowing for a mechanistic description of Cdk requirement in this process (Figure 1.1B). The Sld2 and Sld3 proteins are proposed to be the “minimum set” of Cdk targets (Tanaka et al., 2007; Zegerman and Diffley, 2007). Upon Cdk phosphorylation Sld2 binds to the C-terminal pair of the four BRCT (BRCA1 C terminus) repeats of the Dbp11 protein (Masumoto et al., 2002; Tak et al., 2006), while Cdk phosphorylation of Sld3 promotes its association to the N-terminal pair of BRCT repeats of Dbp11 (Tanaka et al., 2007; Zegerman and Diffley, 2007). Previous work had shown that Sld3 and Cdc45 may associate with origins weakly during G1 and Sld3 is subsequently displaced from origins and is not required for the elongation process (Kamimura et al., 2001; Kanemaki and Labib, 2006). A model was thus proposed that Sld3 first associates with a component at origins, helping to recruit Cdc45, while Dbp11 then acts a bridge for phosphorylated Sld2 and Sld3, leading to the recruitment of GINS (Araki, 2010; Heller et al., 2011). This model is supported by the observation that yeast cells expressing a phosphomimetic mutant of Sld2 and a fusion of Sld3 to the C-terminal pair of BRCT repeats of Dbp11 can replicate DNA in the absence of Cdk

activity (Zegerman and Diffley, 2007). A mutated Cdc45 allele can also replace the Sld3-Dbp11 fusion protein in a similar assay (Tanaka et al., 2007). Based on sequence similarity, the metazoan homologue of Sld2 may be RecQL4 (Sangrithi et al., 2005) while three proteins, DUE-B (DNA unwinding element) (Casper et al., 2005), Treslin/ticrr (TopBP1-interacting, replication-stimulation protein) (Kumagai et al., 2010) and GEMC1 (Geminin coiled-coil-containing protein 1) (Balestrini et al., 2010) have been postulated to be the functional equivalent of yeast Sld3. Treslin/ticrr shares sequence similarity with Sld3 and its interaction with TopBp1 (metazoan Dbp11) is Cdk-dependent (Boos et al., 2011; Kumagai et al., 2011; Sanchez-Pulido et al., 2010).

1.3 The Cdc7 kinase

The other S phase-promoting kinase required for replication initiation is Cdc7, an essential serine/theonine kinase that is conserved from yeast to humans. *cdc7* was identified to encode a gene product involved in controlling the cell cycle in the original search for temperature sensitive *cdc* mutants in *S. cerevisiae* (Hartwell et al., 1970) and was determined to be required for the initiation of DNA synthesis (Hereford and Hartwell, 1974). Cdc7 was demonstrated to be a kinase capable of phosphorylating serine and/or threonine residues (Hollingsworth and Sclafani, 1990). Its fission yeast homologue, Hsk1 (homologue of Cdc-seven kinase 1), and homologues in *Xenopus*, mouse and human were isolated subsequently (Kim et al., 1998; Masai et al., 1995; Sato et al., 1997). Depleting Cdc7 from the cell-free system derived from *Xenopus* eggs resulted in the complete loss of DNA replication, indicating an essential role of *Xenopus* Cdc7 for replication activity (Jares and Blow, 2000; Walter, 2000). Cdc7 activity is also essential in mammalian cells (Jiang et al., 1999; Kumagai et al., 1999) and the deletion of *muCdc7* results in early embryonic lethality in mice (Kim et al., 2002).

In the following sections, the activators (Section 1.3.1) and substrates (1.3.2) of Cdc7 will be discussed, as well as its roles in the replication timing programme (1.3.3.1), intra-S checkpoint (1.3.4), other chromosome transactions (1.3.5) and its relevance to clinical applications (1.3.6).

1.3.1 Cdc7 regulators – Dbf4 and Drf1

The activity of the Cdc7 kinase is regulated by association with its activation subunits. In yeasts, only one regulator has been identified – Dbf4 (dumbbell forming 4) in *S. cerevisiae* and Dfp1 (dbf four in pombe 1) in *S. pombe*. In budding yeast, the Dbf4 protein accumulates during S phase and is targeted for ubiquitin-mediated degradation by the anaphase-promoting complex at the end of mitosis (Ferreira et al., 2000; Oshiro et al., 1999; Weinreich and Stillman, 1999). While Cdc7 is expressed at an approximately constant level throughout the cell cycle, kinase activity peaks in at the G1/S transition when it binds to and is activated by Dbf4/Dfp1 (Jackson et al., 1993; Kitada et al., 1992; Masai et al., 1995; Takeda et al., 1999). In human cells, the correlation between Cdc7/Dbf4 complex formation and oscillation of Cdc7 kinase activity has also been observed (Jiang et al., 1999; Kumagai et al., 1999). In human and *Xenopus*, a second regulator of Cdc7 has been identified. Drf1 (Dbf4-related factor 1) is a Dbf4-related protein which also activates Cdc7 (Montagnoli et al., 2002; Takahashi and Walter, 2005; Yanow et al., 2003; Yoshizawa-Sugata et al., 2005) and is important for promoting DNA replication (Silva et al., 2006; Takahashi and Walter, 2005). The active Cdc7/Dbf4 and Cdc7/Drf1 kinase complexes are collectively referred to as the Dbf4/Drf1-dependent kinase (DDK). The significance of two Cdc7 regulators in vertebrates is currently unclear. Experiments in *Xenopus* egg extracts indicate that both Dbf4 and Drf1 are only found in complexes with Cdc7 and that Cdc7/Drf1 is found in greater abundance over the Cdc7/Dbf4 complex (Silva et al., 2006; Takahashi and Walter, 2005). The expression of XDrf1 and XDbf4 is differentially regulated during

development and the primary Cdc7-binding partner that drives replication in early *Xenopus* embryos appears to be Drf1 (Silva et al., 2006; Takahashi and Walter, 2005). In human cells, both components accumulate and regulate the kinase activity of Cdc7 during the cell cycle (Yoshizawa-Sugata et al., 2005). Based on these observations, it may be possible that two regulators are required to fine-tune Cdc7 kinase activity temporally or in different biological processes, analogous to cyclin specification of Cdk activity in metazoans.

1.3.2 Cdc7 substrates for replication initiation

It has been shown that Cdc7 is able to phosphorylate several components of the replicative machinery, including multiple subunits of the Mcm2-7 complex (Jares and Blow, 2000; Jiang et al., 1999; Lei et al., 1997; Masai et al., 2000; Sato et al., 1997), Cdc45 (Kihara et al., 2000; Nougarede et al., 2000) and DNA polymerase α (Weinreich and Stillman, 1999). Of these proteins, Mcm2-7 appears to be the essential Cdc7 target during DNA replication. Genetic and biochemical data indicate that Cdc7 initiates DNA replication by binding to Mcm2-7 at origins of replication and phosphorylating several subunits of the hexamer. Evidence for the interaction between Cdc7 and the Mcm2-7 complex was first provided by the isolation of *bob1*, a mutant allele of *MCM5* in yeast, as a bypass suppressor for *cdc7* and *dbf4* (Hardy et al., 1997). It was proposed that Cdc7 phosphorylation results in a conformational change that activates the helicase, while *mcm5-bob5* mutations produce several protein conformations of which one is permissive for origin firing, thus bypassing a requirement for Cdc7 kinase activity though at reduced initiation efficiency (Hoang et al., 2007).

It was shown that yeast Mcm2 is an important downstream target of Cdc7/Dbf4 kinase (Brown and Kelly, 1998; Lei et al., 1997) and a *dbf4* mutant was identified as an allele-specific suppressor of the temperature sensitive *mcm2-1* allele (Lei et al., 1997).

Studies reported that Mcm2 is efficiently phosphorylated *in vivo* and *in vitro* by Cdc7 in fission yeast, mammalian cells as well as *Xenopus* egg extracts (Masai and Arai, 2002; Masai et al., 2006). The phosphorylation of Mcm2, classically detected as differential mobility in SDS-PAGE, appears to occur in a cell cycle-dependent manner. Though several S phase regulating kinases are known to phosphorylate Mcm2, mapping of specific phospho-sites on the human Mcm2 protein revealed that the observed cell cycle-dependent phosphorylations coincided almost exactly with Cdc7-dependent positions (Montagnoli et al., 2006). A similar study by Tsuji et al. showed that Mcm2 phosphorylation by Cdc7/Dbf4 is essential for the initiation of DNA replication in human cells (Tsuji et al., 2006). In *Xenopus* egg extracts, Cdc7 is specifically recruited to chromatin containing Mcm2-7, and this recruitment is mediated by its activating subunit (Jares and Blow, 2000; Jares et al., 2004; Walter, 2000). A docking region for Cdc7 has been reported in Mcm2 and that phosphorylation of Mcm2 by Cdc7 is essential for replication (Bruck and Kaplan, 2009). Mcm3 and Mcm7 have also been reported to be phosphorylated by Cdc7 *in vitro* (Weinreich and Stillman, 1999); Mcm5 is the only Mcm subunit that has not been shown to be a substrate of Cdc7 in *S. cerevisiae* and other species (Labib, 2010).

The N-terminus of eukaryotic Mcm2, Mcm4 and Mcm6 contain relatively unstructured extensions of up to a few hundred amino acids, which may be major substrates for Cdc7, as well as for Cdk (Labib, 2010). In *Xenopus*, Mcm4 hyper-phosphorylation was found to be Cdc7-dependent (Pereverzeva et al., 2000; Takahashi and Walter, 2005). Putative Cdc7 phosphorylation sites have been reported in the N-terminal tails of Mcm4 and Mcm6 in human cell lines and these sites may play important but overlapping roles in recruiting Cdc45 to chromatin. Full length Mcm4 protein is a better Cdc7 substrate than shorter fragments of the Mcm4 N-terminal tail, indicating that the kinase may require a docking site in this region (Masai et al., 2006). Such a Cdc7 docking region was described in the budding yeast N-terminal structured sequence adjacent to the

unstructured NSD (N-terminal Ser/Thr-rich domain) of eukaryotic Mcm4, known to contain multiple targets for Cdc7 phosphorylation (Sheu and Stillman, 2006). Hyper-phosphorylated Mcm4 was found enriched in the Cdc45-Mcm2-7 complex, suggesting that this is the activated form of the helicase. In a more recent study by the same group, it was proposed that the essential function of Cdc7 phosphorylation is to relieve an inhibitory activity residing in the N-terminal tail of Mcm4 (Sheu and Stillman, 2010). In cells lacking this inhibitory region, Cdc7 activity is no longer required for viability. However, these cells grew slowly, as did the *mcm5-bob1* mutants, suggesting that the formation or stability of the Cdc45-Mcm2-7 complex may be defective. The NSD may therefore have additional positive roles in promoting cell survival. Cells that lacked the entire NSD region were viable but defective in S phase in the absence of Cdc7 (Sheu and Stillman, 2010).

While the Mcm2-7 complex is likely the physiological target of Cdc7 for DNA replication, it is also phosphorylated by Cdk (Masai et al., 2000; Tenca et al., 2007). Consequently, mechanisms should exist that coordinate Cdk- and Cdc7-dependent events (Heller et al., 2011). Several studies indicate that sequential phosphorylation events target Mcm2 during the cell cycle in the budding yeast and that the proper pre-phosphorylation of the specific protein residues is required for Cdc7/Dbf4 to utilise Mcm2 as a substrate (Francis et al., 2009; Kihara et al., 2000; Lei et al., 1997). Using *Xenopus* egg extracts, it was determined that DNA replication can only occur when chromatin is sequentially exposed first to Cdc7 and then to the Cdk2 kinase. Cdc7 activity can be completed in the absence of significant Cdk activity whereas Cdk2 cannot execute its function before Cdc7 (Jares and Blow, 2000; Walter, 2000). Subsequent experiments using extracts from budding yeast gave results consistent with this (Heller et al., 2011). However, the preference of Cdc7 for serine and threonine residues in an acidic context may be fulfilled by having substrates located adjacent to a Cdk phosphorylation sites (Cho et al., 2006; Masai et al., 2000; Masai et al., 2006;

Montagnoli et al., 2006). Prior phosphorylation by Cdk may prime these substrates, thereby enhancing Cdc7 activity. It has been shown that Cdk modification can create Cdc7 target sites (Masai et al., 2006; Wan et al., 2008), indicating close coordination between the two S phase-promoting kinases in initiating origins of replication. In *Xenopus* egg extracts, pre-phosphorylation of Mcm2-7 by Cdks may occur during the preceding mitosis (Pereverzeva et al., 2000).

1.3.3 The replication timing programme

DNA replication initiates at numerous origins throughout the eukaryotic genome and subsets of origins become sequentially activated through S phase, following a temporal progression. In budding yeast, activation of pre-RCs proceeds in a genetically determined order on the chromosome and early- and late-firing origins have been clearly identified. Origins located near telomeres fire late, possibly as a result of differences in chromatin structure. Specific sequences have been well defined that can cause early replicating origins to fire late (Czajkowsky et al., 2008; Ferguson and Fangman, 1992; Friedman et al., 1996; Raghuraman et al., 2001; Stevenson and Gottschling, 1999). In contrast, the regulation of replication timing in metazoans is less well understood. Local chromatin environment, nuclear positioning of chromosome loci, patterns of histone modifications, transcriptional regulation and checkpoint regulation have been found to be related to the determination of replication timing (Chagin et al., 2010; Zink, 2006). Experiments in budding yeast indicate that replication origins become established as late-firing during G1 phase in the cell cycle (Raghuraman et al., 2001). This programming event appears to be similar to the “timing decision point” described in metazoans, which is a time in early G1 phase after which somatic cells can replicate according to a normal temporal programme if driven into S phase by *Xenopus* egg extracts *in vitro* (Dimitrova and Gilbert, 1999).

Analyses of the dynamics of DNA replication at a cellular level became possible when methods of labelling on-going DNA synthesis were developed to detect sites of DNA replication within the nucleus. Radioactive thymidine and antibodies against halogenated thymidine analogues were first used to analyse nuclear structures of replication (Aten et al., 1992; Gratzner, 1982; Milner, 1969; Nakamura et al., 1986; Nakayasu and Berezney, 1989; O'Keefe et al., 1992). Subsequently, fluorochrome-conjugated nucleotides and the expression of replication factors tagged to fluorescent markers provided ways to observe the progress of DNA replication through live cell microscopy (Cardoso et al., 1997; Leonhardt et al., 2000; Schermelleh et al., 2001). With these approaches, DNA replication was determined to occur at sub-nuclear sites termed replication foci, which accumulate replication factors and cell cycle proteins (Cardoso et al., 1997; Cardoso et al., 1993). Sequential patterns of DNA replication can be observed over the course of S phase, which can be categorised into five different phases of replication (named Type I – V) (O'Keefe et al., 1992). These characteristic spatial-temporal patterns are formed when replication foci localise to characteristic regions of the nucleus during specific times in the S phase. They remain stable over multiple cell divisions, indicating a close relationship between the spatial positioning of DNA in the nucleus and its replication timing in during S phase (Zink, 2006). Chromatin modification of the regions surrounding replication origins may play a part in the establishing the timing programme (Thomson et al., 2010).

Profiles of origin initiation have been characterised across the entire genome of the budding yeast (Raghuraman et al., 2001), fission yeast (Eshaghi et al., 2007; Hayashi et al., 2007; Heichinger et al., 2006; Mickle et al., 2007), *Drosophila* (MacAlpine et al., 2004; Schübeler et al., 2002) and mammalian cells (Cohen et al., 2006; Farkash-Amar et al., 2008; Hiratani et al., 2008; Jeon et al., 2005; White et al., 2004; Woodfine et al., 2005; Woodfine et al., 2004). These genomic approaches have identified relatively large domains of contiguous DNA that replicate at characteristic times over S phase

(Hiratani et al., 2008; Watanabe et al., 2002; Woodfine et al., 2004). Such timing domains are functionally compartmentalised replication zones that span several megabases in size. Their sizes suggest that they are comprised of one or several adjacent replicon clusters, which are groups of one to ten origins that initiate synchronously (Berezney et al., 2000; Gillespie and Blow, 2010; Hiratani et al., 2008; Jackson and Pombo, 1998; MacAlpine and Bell, 2005; Zink, 2006).

The exact relationship between (1) replication foci – subcellular structures observed through visualisation of cells undergoing replication, (2) replicon clusters – functional groups of initiating origins and (3) timing domains – zones defined by genomic analysis of replication timing, remains unclear, as does the understanding of their regulation during S phase (Gillespie and Blow, 2010). In higher eukaryotes, replication timing appears to be regulated at a global level, so that the loss of a single origin does not affect the overall timing programme (Hayashida et al., 2006). According to one model, origin firing is stochastic and can produce the defined patterns of replication observed on average in cells, if origins inherently have different relative firing probabilities and if the firing probabilities of all origins increase during S phase (Rhind, 2006; Rhind et al., 2010). Other studies show that checkpoint signalling pathways may modulate the extent to which the initiation of certain origins is delayed in response to DNA damage or replicative stress in a perturbed S phase (Santocanale et al., 1999; Shirahige et al., 1998), or by regulating origin activation differentially at the level of replication clusters depending on whether they are actively undergoing DNA synthesis (Blow et al., 2011; Ge and Blow, 2010).

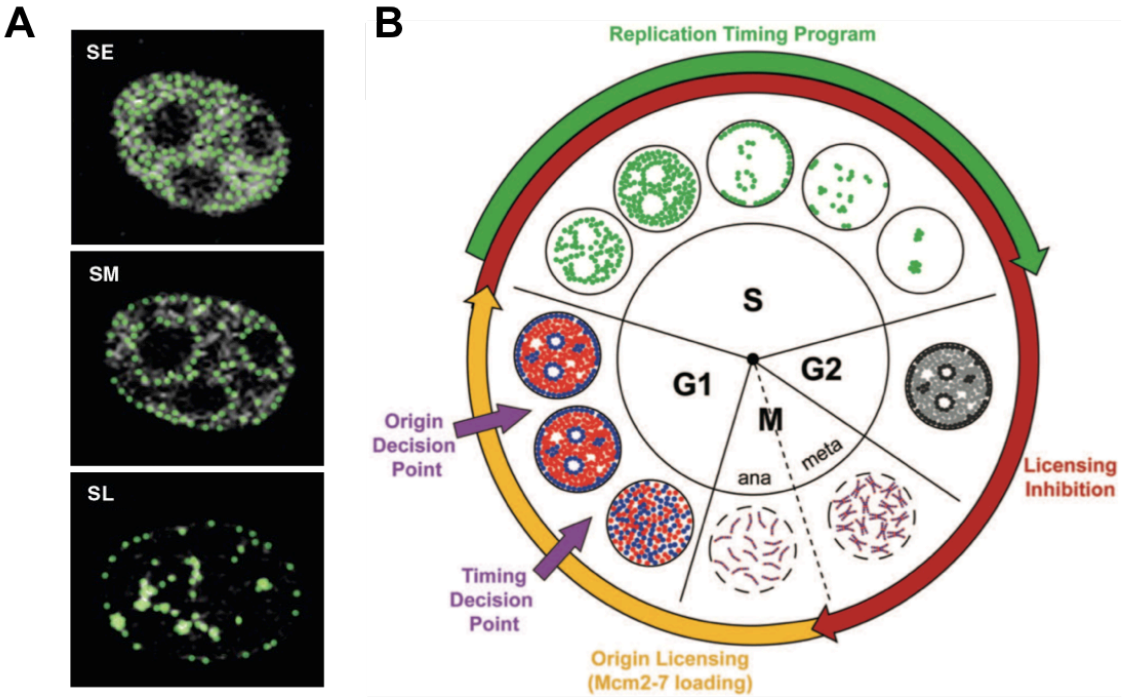

Figure 1.2. The replication timing programme.

(A) The progress of DNA replication can be visualised in human HeLa cells stably expressing GFP-tagged PCNA. Snapshots of a time-lapse confocal microscopy movie show that during early S phase, sites of active DNA replication are found in small foci distributed throughout the nucleus, mostly corresponding to euchromatic regions. The DNA replication machinery loads at perinuclear and perinucleolar heterochromatin regions in mid S phase and then at large constitutive heterochromatic chromosomal regions at the end of S phase. This temporal replication program is recapitulated at each cell cycle. SE: S early/ SM: S mid/ SL: S late. Adapted from (Chagin et al., 2010). (B) Schematic of the establishment and execution of the timing programme in the cell cycle. Cell cycle phases are denoted in the inner circle while stages of DNA replication are shown on the outer circle; yellow and red arrows represent periods of licensing competence and inhibition respectively and the green arrow indicates execution of the replication timing programme; representative nuclear transitions are illustrated. During mitosis, paired sister chromatids align on the metaphase plate before separation to opposite poles of dividing cells at anaphase onset. Chromosome decondensation and nuclear envelope reformation takes place. In early G1, euchromatin (red) and heterochromatin (blue) are initially randomly dispersed but become localised to specific domains in the nucleus. Replication nuclei (green) are then activated according to the timing programme over the course of S phase. Post-replication, nuclei pass through G2 into mitosis. Chromosome condensation and nuclear envelope breakdown marks entry into mitosis for another round of sister chromatid separation. Adapted from (Gillespie and Blow, 2010).

1.3.3.1 Cdk and Cdc7 in replication timing programme regulation

Since the firing of replication origins require phosphorylation events mediated by Cdk and Cdc7, it is likely that they are involved in regulating the replication timing programme.

Cdk activity is required throughout S phase for origin initiation in *S. cerevisiae*, in which S phase Cdk activity is provided by Cdc28 (Cdk1) binding to the B-type cyclins, Clb5 and Clb6. Earlier studies reported that in budding yeast cells lacking Clb5, S phase is lengthened and only early- but not late-firing origins are initiated (Donaldson et al., 1998b; Epstein and Cross, 1992; Schwob and Nasmyth, 1993). It was later determined that while Clb5 and Clb6 can both drive S phase, Clb6 is the minor S phase cyclin that is only expressed early in S phase. The defect in *clb5* mutants can be restored by the expression of a non-degradable Clb6 construct and S phase proceeded with a wild type temporal pattern (Gibson et al., 2004; Jackson et al., 2006; McCune et al., 2008). In *Xenopus* egg extracts, the execution of replication timing programme requires on-going activity of S-Cdk. Addition of roscovitine at different times throughout S phase to inhibit Cdk activity rapidly prevented origin firing within 2.5 min of treatment (Luciani et al., 2004). However, elongation of nascent DNA strands from origins fired before roscovitine addition was not affected.

Using the experimental system first developed by Gilbert et al., in which the replication of mammalian G1 nuclei is driven by incubation in *Xenopus* egg extracts (Dimitrova and Gilbert, 1999; Gilbert et al., 1995), it was demonstrated that Cdk activity is required throughout S phase for progression through the replication timing programme (Thomson et al., 2010). Activation of replication factories/clusters was found to be highly sensitive to Cdk activity, whereby reduction of Cdk activity was associated with a reduced number of active replication factories, while the number of forks within each

factory remained largely unchanged. Progression through different replication timing patterns was also not sensitive to modest changes to Cdk levels. This study provided evidence that Cdk drives progression through S phase in different ways, differentially affecting the timing program, factory activation, and replication initiation. A similar effect of Cdk activating replicon clusters was also reported when sperm nuclei were used as replication substrates in *Xenopus* egg extracts (Krasinska et al., 2008). In human cells, expression of a constitutively active mutant fusion construct of Cdk1-cyclin A resulted in the premature appearance of DNA replication at late origins in early S phase, again establishing a role for Cdk in regulating replication timing (Katsuno et al., 2009).

Several lines of evidence implicate a role for Cdc7 in the regulation of replication timing as well. In budding yeast, Cdc7 was shown to be required early as well as late in S phase for the initiation of early- and late-firing origins respectively (Bousset and Diffley, 1998; Donaldson et al., 1998a; Pasero et al., 1999). Patel et al. showed that Hsk1 is a rate-limiting factor for origin firing in fission yeast (Patel et al., 2008). Origin efficiency increased or decreased correspondingly with Hsk-Dfp1 levels and physically tethering the kinase near an origin increases its firing efficiency, indicating a possible effect of local concentration. These results are consistent with a study by Wu and Nurse who provided evidence that increased levels of Hsk1 or Dfp1 accelerated firing of both efficient and inefficient origins across the genome (Wu and Nurse, 2009). In a recent report, the association of the Sld3-Sld7-Cdc45 complex with origins, an event dependent on Cdc7 activity, was reported to be a key step in regulating origin firing in *S. cerevisiae*. Sld3, Sld7 and Cdc45 are normally present at low levels in budding yeast and increased levels of these proteins allow late firing origins to fire early in S phase. Increasing the levels of Cdc7 or Dbf4 also allowed late firing origins to fire early, suggesting that Cdc7 activity may be rate limiting for replication initiation (Tanaka et al., 2011a). These findings are consistent with another report showing that Dbf4 is in low abundance in the budding yeast and over-expression of Dbf4, the two essential Cdk

substrates Sld3 and Sld2 plus their binding partner Dpb11 is sufficient to allow late firing origins of replication to initiate early (Mantiero et al., 2011). These studies in yeast suggest that Cdc7 plays a role in promoting initiation at individual replication origins to drive the replication timing programme. A possible hypothesis for the role of Cdc7 in regulating replication timing may be that Cdc7 preferentially associates with Mcm2-7 at early-replicating origins in early S phase, and is displaced to activate later firing origins only when early firing origins have replicated. As yet, the role of Cdc7 in regulating metazoan replication timing remains to be elucidated.

1.3.4 Cdc7 in the intra-S phase checkpoint response

For the successful completion of DNA replication, cells must possess ways to constantly monitor the integrity of on-going replication forks and be able to initiate a DNA damage response as required during the course of S phase. The progression of replication forks may be hindered in a number of ways including the presence of protein-DNA complexes, depletion of the nucleotide pool, collisions between the replisome and transcription machinery and external DNA damaging agents. These elements induce the pausing or completely block the progression of replication forks, thereby increasing the possibility of DNA breakage events. The intra-S checkpoint ensures completion of replication before cells enter mitosis by slowing the rate of replication in response to DNA damage (Finn et al., 2011) and regulating the firing of uninitiated replication origins, including late-firing and dormant origins (Blow et al., 2011). The replication checkpoint is also involved in regulating subsequent steps related to fork restart processes (Branzei and Foiani, 2005, 2010).

Checkpoint signalling occurs through a network of sensors and transducers that detect and amplify the DNA damage or replicative-stress signal. Members from the family of phosphoinositide 3-kinase (PI3K)-related protein kinases, including ATM (ataxia-

telangiectasia mutated) and ATR (ATM and Rad3-related), act as major upstream regulators of the DNA damage response. ATR is activated in response to single-stranded DNA (ssDNA) coated with multiple subunits of RPA (replication protein A) – ssDNA-RPA. ATR binds to RPA through ATRIP (ATR-interacting protein) at the same time that the 9-1-1 complex (Rad9-Rad1-Hus1) is independently recruited to ssDNA-RPA. TopBP1 (topoisomerase-binding protein 1) then binds and activates ATR, causing phosphorylation of the downstream Chk1 kinase and other ATR effectors. ATR activation inhibits origin firing and replication factory activation, induces cell cycle arrest and stabilises and allows stalled forks to re-initiate. On the other hand, the ATM checkpoint kinase is recruited to sites of double-stranded DNA damage, binding to the MRN (Mre11-Rad50-Nbs1) complex and phosphorylating the histone variant H2AX. Activated ATM phosphorylates Chk2 and other downstream targets, leading to inhibition of origin firing, cell cycle arrest and repair of double strand breaks (Cimprich and Cortez, 2008; Masai et al., 2010).

Under replicative stress, replication forks are first paused or slowed down. In *S. cerevisiae*, proteins such as Mrc1 (Claspin in metazoans) and Tof1 (Swi1/Timeless), which are conserved replication fork factors, limit progression of the replisome under such circumstances (Katou et al., 2003). It was shown in budding yeast that *mec1* (homologue of ATR) or *rad53* (homologue of Chk2) mutations repress late origin firing in the presence of hydroxyurea (HU) (Santocanale et al., 1999; Shirahige et al., 1998). By analysing *S. cerevisiae* mutants, it was proposed that checkpoint proteins stabilise stalled forks and prevent disassembly of the replisome, thereby preventing replication fork collapse (Cobb et al., 2003; Katou et al., 2003; Lucca et al., 2004). However, this view is challenged by a recent study which revealed that the replisome remained stably associated with replication forks following replication stress, even in the absence of Mec1 and Rad53 (De Piccoli et al., 2012). The authors proposed the function of checkpoint kinases is to phosphorylate components of the stalled replisome,

maintaining it in a state competent for DNA synthesis. This explains why checkpoint-deficient cells fail to resume replication despite the presence of intact replicative machinery at replication forks (Berens and Toczyski, 2012; De Piccoli et al., 2012).

There is substantial evidence that Cdc7 kinase is a direct target of the S phase checkpoint. In budding yeast, checkpoint-dependent phosphorylation of Dbf4 by Rad53 restricts origin firing (Lopez-Mosqueda et al., 2010; Zegerman and Diffley, 2010). Dfp1 also undergoes Cds1 (homologue of Chk2)-dependent phosphorylation in fission yeast (Snaith et al., 2000). Chromatin association of Dbf4 is reduced in a Rad53-dependent manner after HU treatment (Pasero et al., 1999) and Rad53 phosphorylation of Dbf4 was found to attenuate Cdc7 kinase activity (Weinreich and Stillman, 1999). Genetic studies also suggest that Cdc7 is important in preserving fork integrity and recovery from fork stalling (Matsumoto et al., 2005; Takeda et al., 1999). In *Xenopus* egg extracts, initial studies suggested that the Topoisomerase II (Topo II) inhibitor etoposide causes checkpoint-mediated inhibition of Cdc7-Dbf4 complex formation and kinase activity (Costanzo et al., 2003). Similar results were obtained with human leukemia cells treated with etoposide (Dierov et al., 2004). However, other studies in both *Xenopus* egg extracts and mammalian cells provided evidence that the expression, complex formation, chromatin association and kinase activity of Cdc7 and associated regulators remain intact in cells during S phase checkpoint responses (Heffernan et al., 2007; Silva et al., 2006; Takahashi and Walter, 2005; Tenca et al., 2007; Tsuji et al., 2008), raising the possibility the Cdc7 may also play active roles in mediating checkpoint recovery.

In metazoans, some studies indicate that Drf1 may be more important in the S phase checkpoint than Dbf4 (Silva et al., 2006; Takahashi and Walter, 2005). Drf1 accumulates on chromatin in an ATR- and Claspin-dependent manner in *Xenopus* egg extracts during replication arrest (Yanow et al., 2003) and Cdc7 could phosphorylate

and interact with Claspin in extracts and human cells (Gold and Dunphy, 2010; Kim et al., 2008), suggesting a potential regulatory relationship during a perturbed S phase. Mammalian cells were defective in Chk1 and Claspin phosphorylation and became sensitive to replicative stress caused by HU when Cdc7 was knocked down with small interfering RNA (siRNA) (Kim et al., 2008). However, a recent report had described that Dbf4 is a direct downstream target of ATM and ATR upon checkpoint activation in human cell lines. ATM/ATR phosphorylation sites on Dbf4 were identified which are important in restricting origin firing without inactivating Cdc7 kinase activity, reconciling the apparent conflict broached by other studies. Indeed, Cdc7 kinase activity is required to prevent fork collapse and the formation of double stranded breaks upon replication stress (Lee et al., 2012). There is still much to be learnt about the role and mechanisms Cdc7 activity in the intra-S checkpoint response.

1.3.5 Cdc7 in other chromosome transactions

Cdc7 is an important regulatory molecule not only in DNA replication but also in chromosomal cohesion and meiosis. In *S. pombe*, Hsk-Dfp1 was found to function in the same pathway as Swi6 (fission yeast heterochromatin protein 1), required at centrosomes for cohesion between sister chromatids, to promote heterochromatin-mediated cohesion. This role for Hsk-Dfp1 was proposed to be distinct from its replication initiation function (Bailis et al., 2003). However, a later study suggests that Swi6 stimulates Sld3 recruitment to origins, which in *S. pombe* is dependent on Hsk1-Dfp1, and is required for early replication of two heterochromatic regions (Hayashi et al., 2009). In *Xenopus* egg extracts, Cdc7-Drf1 was found to be in a stable complex with Scc2-Scc4, a complex required for cohesin recruitment to chromosomes, and Scc2-Scc4 was unable to bind to chromatin in the absence of Cdc7 activity. Cdc7 activity is required to tether Scc2-Scc4 to pre-RCs to establish functional cohesion between replicated sister chromatids (Takahashi et al., 2008); it is currently unclear

whether this is mediated by Cdc7 phosphorylating Mcm2-7 or other proteins.

Cdc7 was found to play multiple roles in events that promote meiosis I segregation (Marston, 2009). While the *bob5* mutant allele allows a bypass of Cdc7 deletion in budding yeast, it could not rescue a prophase I arrest due to the failure in inducing transcription of NDT80, a global meiotic transcriptional regulator that is required for exit from prophase I and progression into meiosis (Lo et al., 2012; Lo et al., 2008; Sasanuma et al., 2008). Cdc7-Dbf4 is also required for the initiation of meiotic recombination (Matos et al., 2008; Sasanuma et al., 2008; Wan et al., 2008; Wan et al., 2006) and to localise the monopolin complex to kinetochores to specify mono-orientation (Lo et al., 2008; Matos et al., 2008). As in replication, Cdc7 appears to coordinate with Cdk phosphorylation events to execute its meiotic functions (Lo et al., 2012; Marston, 2009).

1.3.6 Cdc7 in clinical and translation applications

Given its essential function in regulating DNA replication, it is perhaps unsurprising that Cdc7 may be regulated differently in cancer cells, in which sustained proliferative signalling contributes towards tumour development. Cdc7 expression has been found to be up-regulated in several cancer cell lines and primary tumours while remaining relatively low or absent in healthy and/or non-proliferative cells (Bonte et al., 2008; Hess et al., 1998). Intriguingly, however, this does not correlate with the cells' proliferative status. Over-expression of Cdc7/Dbf4 either has no significant effect on cell cycle progression (Sato et al., 2003), arrests cells in G1 or appreciably retards S phase progression for cells already in S phase (Guo et al., 2005). Increased Cdc7/Dbf4 may instead confer a survival advantage for cancer cells through its functions in recovery or repair of stalled forks (Bonte et al., 2008; Hess et al., 1998).

The analysis of Cdc7 expression can provide prognostic information on malignant progression and is a powerful marker of clinical outcomes in cutaneous melanoma (Clarke et al., 2009) and epithelial ovarian carcinoma (Kulkarni et al., 2009). In several cancer types, Cdc7 over-expression correlated with advanced tumour stage and poor clinical prognosis. In breast cancer, Cdc7 deregulation leading to high expression levels has been associated with acquisition of a malignant, hyper-proliferative phenotype (Rodriguez-Acebes et al., 2010). Higher levels and an increased number of cells staining positive for Mcm2 phosphorylation, indicative of high Cdc7 kinase activity, were also found in many cancer cell types such as skin basalioma, laryngeal squamous cancer and colon adenomas and adenocarcinomas (Montagnoli et al., 2010). These clinical observations argue for a role of Cdc7 in the pathology of cancer aside from serving as a biomarker.

In addition to providing diagnostic information, the central role that the Cdc7 kinase plays in DNA replication identifies it as an attractive target in cancer therapy. Cdc7 depletion using siRNA provided the first indications that cancer cells lines behave differently from normal cell in response to replicative stress. Loss of Cdc7 in tumour cell lines resulted in an abortive S phase, leading to aberrant mitosis or p53-independent apoptosis. In contrast, a robust p53-dependent checkpoint response actively prevented normal fibroblasts from entering a lethal S phase in the absence of Cdc7 (Montagnoli et al., 2004). These results are consistent with a later study showing that fibroblasts depleted of Cdc7 arrest before entry into S phase in a checkpoint-dependent manner. The nature of this proposed pre-S phase arrest is currently unknown. Subsequent recovery of proliferative capacity suggested that this G1 arrest in normal cells is reversible (Tudzarova et al., 2010). The apoptotic cell death observed in cancer cells following Cdc7 depletion was suggested to be mediated by ATR-dependent activation of the p38 MAP kinase signalling pathway (Im and Lee, 2008). This was confirmed in a recent study which demonstrated that even p53-positive cancer cells were susceptible

to cell death as a result of Cdc7 depletion. The authors propose that Cdc7 depletion-dependent cell death can occur through multiple mechanisms and that p53 status affects the eventual mode of cell death in different cells (Ito et al., 2012).

These studies support the notion that differential expression of Cdc7 and other checkpoint defects may cause cancer cells to be selectively sensitive to a new class of drugs that targets the initiation of DNA replication (Blow and Gillespie, 2008). In this regard, several small chemical molecules have recently been reported to be potent and selective inhibitors of Cdc7 kinase function and to possess anti-tumour activity (Ermoli et al., 2009; Koltun et al., 2012; Menichincheri et al., 2010; Menichincheri et al., 2009; Shafer et al., 2008; Vanotti et al., 2008; Woods et al., 2012; Zhao et al., 2009). These drugs represent a novel approach to cancer therapy by targeting mechanisms regulating replication initiation, upstream of traditional anti-cancer therapeutics that inhibit elongation. Montagnoli et al. first reported that a Cdc7 inhibitor prevented replication initiation but does not impede fork progression or trigger the DNA damage checkpoint in cancer cells (Montagnoli et al., 2008). Several years on, potential candidates NMS-1116354 (Nerviano) (Montagnoli et al., 2010) and XL-413/BMS-863233 (Exelixis and Bristol Myers Squibb) (Koltun et al., 2012) are currently being tested in clinical trials. A better understanding of Cdc7 *in vivo* will undoubtedly be of great impact towards the development and utilisation of clinical applications targeting Cdc7 function in the cell cycle.

1.4 Control of cell cycle events by Cdks

A eukaryotic cell has to complete a series of physiological processes in a timely manner over the course of a cell cycle to successfully divide. Three distinct transitions mark the onset of S phase and the entry and exit of mitosis and the heterodimeric complexes of Cdks with their cyclin partners mediate progression through these

different events. An active Cdk complex functions as a serine/threonine kinase and is made up of a catalytic subunit from the Cdk family associated with a cyclin subunit, which confers periodicity and substrate specificity to the kinase (Bloom and Cross, 2007; Malumbres, 2011; Morgan, 1995). Up to six conserved Cdks exists in the budding yeast *S. cerevisiae*, but only one of them, Cdc28 (Cdk1), is necessary and sufficient to drive the cell cycle. Cdc28 associates with the G1 cyclins, Cln1, Cln2, and Cln3 for activities required in G1 phase and the B-type cyclins, Clb5 and Clb6, for S phase functions. In the absence of Clb5 or Clb6, other B-type cyclins, Clb1-4, can drive initiation (Donaldson et al., 1998b). Clb1-4 are required for mitotic events but must be down-regulated for mitotic exit and the completion of the cell cycle (Bloom and Cross, 2007). In mammals, 20 different Cdks exist (Malumbres et al., 2009), of which five are clearly associated with cell cycle control and have partners which are actual cyclins – proteins that are synthesised and degraded in cyclical manner every cell cycle. These are Cdk1, Cdk2, Cdk3, Cdk4 and Cdk6 (Malumbres, 2011; Malumbres and Barbacid, 2005).

Members of the Cdk and cyclin families combine to form different cyclin-Cdk complexes in mammalian cells. Of the five cell cycle-associated Cdks, Cdk1 is known as the mitotic Cdk for its essential roles in mitosis. Cdk1 associates with A- (G2) and B-type (late G2 and M) cyclins. Recent studies have also shown that Cdk1 can bind to interphase (D- and E-type) cyclins. This interaction is more pronounced in cells lacking interphase Cdks such as Cdk2 and Cdk4, which are the normal binding partners for these cyclins (Aleem et al., 2005; Malumbres et al., 2004; Santamaria et al., 2007). Cdk2, 3, 4 and 6 are known as the interphase Cdks. Only D-type cyclins are known to bind to Cdk4 and Cdk6. Cdk4- or Cdk 6-cyclin D complexes are the major sensors for mitogenic signals, mediating cell cycle entry. Cdk2 and Cdk3 are very similar to Cdk1 but do not possess its mitotic functions. Both Cdk2 and Cdk3 bind to E- and A-type cyclins and Cdk2-cyclin E and -cyclin A complexes play important roles in DNA

replication. Most laboratory mice do not have functional Cdk3 due to a spontaneous mutation that generate a premature stop codon in the *Cdk3* allele (Ye et al., 2001). There is no phenotype associated with the loss of Cdk3 and the functions of Cdk3 remains poorly studied (Malumbres, 2011; Malumbres and Barbacid, 2005).

Earlier analysis of the cell cycle Cdks led to a widely accepted 'classical' model of mammalian cell division in which specific Cdks are responsible for driving each of the cell cycle phases. Experiments in which kinase-dead mutants were expressed in human cells caused G1 arrest in CDK2-mutants and G2 arrest in CDK1-mutants (van den Heuvel and Harlow, 1993). Injection of antibodies and antisense oligonucleotides led to the interpretation that specific cyclin-Cdk combinations mediate particular cell cycle transitions (Pagano et al., 1993). Biochemical analysis revealed the preferred binding partners of various Cdks and cyclins at different stages of the cell cycle (Sherr, 1993). In this 'classical' model, cells may exit the cell cycle and become quiescent (G0) or enter G1 phase following cytokinesis. During G1 phase, D-type cyclin synthesis is induced by mitogenic signalling and active Cdk4-cyclin D and Cdk6-cyclin D complexes phosphorylate members of the retinoblastoma (Rb) protein family, which act as repressors of the E2F transcription factors when hypo-phosphorylated. Cdk phosphorylation of Rb proteins inactivate them, allowing transcription of E2F-regulated proteins required later in the cell cycle, including E-, A- and B-type cyclins (Dyson, 1998). Irreversible inactivation of Rb proteins is then completed by the action of Cdk2-cyclin E, through positive feedback (Harbour et al., 1999; Lundberg and Weinberg, 1998). At this point, cells are committed to the cell cycle, becoming independent of mitogenic signals; this corresponds to the 'restriction' or R point in the cell cycle. Cdk2-cyclin E is required to promote S phase entry and Cdk2-cyclin A and Cdk1-cyclin A are then responsible for progression through and completion of S phase. A-type cyclins associate with Cdk1 at the end of S phase but are degraded by ubiquitin-mediated proteolysis in pro-metaphase during mitosis. B-type cyclins are synthesised during S

and G2 phase and Cdk1-cyclin B complexes are essential to trigger and mediate progress through mitosis, being involved in different regulatory and structural processes including chromosomal condensation and breakdown of nuclear lamina. Degradation of B-type cyclins by the anaphase-promoting complex finally inactivates Cdk1-cyclin B complexes, allowing mitotic exit (Hochegger et al., 2008; Malumbres and Barbacid, 2005).

In the past decade or so, the advent of gene targeting techniques in mice has allowed a genetic approach in studying mammalian Cdks. Findings from these studies challenge the orderly scheme of the 'classical' model and suggest that there may be more plasticity and evolutionary conservation of the cell cycle than previously thought. From Table 1.1, the systematic knockdown of *Cdk* loci in the mouse germline has shown that Cdk2, Cdk4 and Cdk6 are not essential for the cell cycle in most cell types. The loss of each Cdk resulted in developmental defects only in specific cell lineages. The dispensability of Cdk2 in the mitotic cell cycle came as a surprise to the cell cycle field as it was believed to be essential for G1/S transition. The redundant activity that controls essential S phase functions appears to be supplied by Cdk1, which can associate with cyclin A and cyclin E, and in some cells by Cdk4 (Aleem et al., 2005; Hochegger et al., 2007; Jablonska et al., 2007; Krasinska et al., 2008; Santamaria et al., 2007). *Cdk4* null mice are viable but are diabetic and exhibit hypo-pituitarism due to defective proliferation of pancreatic β-cells and endocrine cells (Rane et al., 1999; Tsutsui et al., 1999) while Cdk6 ablation causes defects only in cells of the erythroid lineage (Malumbres et al., 2004). The lack of major cell cycle phenotypes in cells lacking individual interphase Cdks cannot be fully attributed to compensation by another interphase Cdk as loss of multiple combinations of interphase Cdks only enhanced the individual phenotype (Malumbres and Barbacid, 2009). Only the loss of Cdk1 had resulted in a lethal phenotype, despite the mutant cells carrying a full complement of the interphase Cdks (Diril et al., 2012). This clearly illustrates that

interphase Cdks cannot compensate for the absence of Cdk1. Cdk2 expressed from the Cdk1 locus also failed to rescue early embryonic lethality (Satyanarayana et al., 2008). The importance of Cdk1 is underscored by the observation that mouse embryos can develop normally to mid-gestation in the absence of all interphase Cdks, indicating Cdk1 alone is sufficient to drive a functional cell cycle in most cell lineages (Santamaria et al., 2007). Cdk1 can bind to all cyclins, resulting in the phosphorylation of pRb and the expression of genes regulated by the E2F transcription factors. Embryonic lethality eventually occurred as a result of defective haematopoiesis (Santamaria et al., 2007).

Cdk knockout	Viability	Phenotype	References
Cdk1	Early lethality before E3.5	-	(Diril et al., 2012)
Cdk2	Viable	Male and female sterility from meiotic defects	(Berthet et al., 2003; Ortega et al., 2003)
Cdk4	Viable	Lack of proliferation of postnatal pancreatic β-cells and pituitary lactotrophs	(Rane et al., 1999; Tsutsui et al., 1999)
Cdk6	Viable	Defective erythroid-lineage development	(Hu et al., 2009; Malumbres et al., 2004)
Cdk2 and Cdk4	Embryonic lethality at E15.5	Heart defects due to decreased numbers of cardiomyocytes	(Berthet et al., 2006; Malumbres et al., 2004)
Cdk2 and Cdk6	Viable	Similar phenotype to single mutants	(Malumbres et al., 2004)
Cdk4 and Cdk6	Late embryonic lethality at E14.5 – E18.5	Reduced proliferation of committed haematopoietic precursors	(Malumbres et al., 2004)
Cdk2, Cdk4 and Cdk6	Embryonic lethality at E12.5 – E15.5	Similar to Cdk2 and Cdk4 knockout with greater severity	(Santamaria et al., 2007)

Table 1.1. Gene targeting of Cdks in mice.

Altogether, these observations suggest that Cdks display significant plasticity, leading to the proposal of an ‘essential’ model of the cell cycle in which Cdk1 alone is sufficient to support proliferation of all cell types, similar to the yeast cell cycle (Malumbres and

Barbacid, 2009; Santamaria et al., 2007). Increased numbers of Cdks have evolved in metazoans to supply additional regulation and to control proliferation of a wide repertoire of cell types in accordance to requirements at the tissue or organism level. Although Cdk1 is sufficient to support a functional cell cycle during the embryonic development, a most active period of cell division, it is eventually unable to compensate in certain specialised cell types. Interphase Cdks may phosphorylate distinct substrates in this case. Emerging evidence suggests that tumour cells may also require specific interphase Cdks for proliferation. It is not clear if non-canonical Cdk-cyclin pairings occur under physiological contexts (Merrick and Fisher, 2012). Multiple Cdks probably only evolved at a late stage of cell cycle evolution, which was then already characterised by considerable complexity (Malumbres, 2005; Malumbres and Barbacid, 2009). As such, greater understanding of the complex Cdk network in mammalian cells, with its high levels of compensatory and shared functions between the Cdks or with other proteins, is required. This is especially essential in order to apply this knowledge to therapeutic ends, in developing Cdks as druggable targets.

1.5 Concluding remarks – this thesis

In this thesis, various aspects of S phase entry and progression will be addressed in two model systems. In the first section, I have analysed aspects of Cdc7 function in *Xenopus* egg extracts using PHA-767491 (Montagnoli et al., 2008; Natoni et al., 2011), a small molecule inhibitor of Cdc7. The point in the cell cycle in which Cdc7 executes its function was investigated as well the requirement of Cdc7 activity through S phase in relation to progression through the replication timing programme. The role of *Xenopus* Cdc7 in a perturbed S phase was also analysed. Following the biochemical characterisation of *Xenopus* Cdc7 activity, I present the design for generating a conditional *Cdc7* knockout mouse, which will be a useful tool to dissect mammalian regulation of Cdc7 function, and results from the screening process. Finally, preliminary

work in characterising Cdk1/Cdk2 double-knockout in mouse embryonic fibroblasts will be described in the last part of the this thesis, providing additional insights into the regulation of G1/S transition and S phase progression in mammalian cells.

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Chapter 2 – The role of Cdc7 in DNA replication and the intra-S checkpoint in *Xenopus* egg extracts

Introduction

Cdc7 is an essential kinase required for the initiation of DNA replication. It has been found to be up-regulated in tumor cell lines, including colon, lung, ovary, breast, leukemia, and prostate cancers (Bonte et al., 2008; Montagnoli et al., 2010). Genetic and other studies employing the use of siRNAs to block Cdc7 activity report disruption of S phase progression, leading to cell cycle arrest and subsequent p53-independent cell death through apoptosis (Ito et al., 2012; Kim et al., 2008; Montagnoli et al., 2004). Consequently, Cdc7 presents an attractive target in the development of novel cancer therapeutics. With the aim of developing a drug useful in a clinical setting, several small molecules belonging to different chemical classes have been reported to be potent inhibitors of Cdc7 activity as a result of large scale screening of chemical compound libraries (Ermoli et al., 2009; Koltun et al., 2012; Menichincheri et al., 2010; Menichincheri et al., 2009; Shafer et al., 2008; Vanotti et al., 2008; Woods et al., 2012; Zhao et al., 2009).

Pyrrolopyridinones and pyrido-thieno-pyrimidines are two classes of chemical compounds reported to be selective and potent inhibitors of Cdc7 activity. PHA-767491 and Compound 6d are ATP-mimetic, competitive small molecule inhibitors from these two classes respectively (Vanotti et al., 2008; Zhao et al., 2009). PHA-767491 was the first potent and selective Cdc7 inhibitor to be reported and characterised. It has a low half-maximal inhibitory concentration (IC_{50}) of 10 nM and exhibited 20-fold selectivity over other targets when tested in a panel of 38 human serine/threonine kinases. In cell-based assays using numerous tumour cell lines, PHA-767491 was found to inhibit Mcm2 phosphorylation at specific Cdc7-dependent sites, impairing origin firing without

affecting fork progression. Also, consistent with siRNA knockdown of Cdc7 (Montagnoli et al., 2004), PHA-767491 did not result in DNA damage or trigger a DNA replication stress response. It was found to have anti-tumour properties in rodent models, marking it as a promising prototype anti-proliferative agent (Montagnoli et al., 2008). Similar to PHA-767491, Compound 6d was determined to have a low dissociation constant (K_i) of 2 nM and an IC_{50} of 20 nM against Cdc7, indicating high substrate affinity and potency. It exhibited more than 500-fold selectivity over a panel of 11 kinases, including Cdk2-cyclin E. Compound 6d also inhibited Mcm2 phosphorylation in experiments using proliferating HT116 colon cancer cells and inhibited proliferation in a dose-dependent manner when tested on the HCT116, RKO, and SW480 human colon cancer cell lines (Zhao et al., 2009).

In this study, Compound 6d and PHA-767491 were synthesised in-house at the University of Dundee, based on published chemical structures (Vanotti et al., 2008; Zhao et al., 2009). I then tested these compounds in the *Xenopus* cell-free system in order to characterise and use them as molecular tools to probe Cdc7 function and regulation in the cell cycle. Cell-free extracts prepared from *Xenopus* eggs were used as an *in vitro* model to examine the molecular events occurring during DNA replication. This system takes advantage of cell cycle proteins and mRNA present in the *Xenopus* egg, which support 12 synchronous rounds of cell division in the absence of transcription and growth following fertilisation. Extracts can support all cell cycle activities occurring in the intact egg and are able to initiate and complete DNA replication of exogenous DNA templates in a manner consistent with that present *in vivo*. Biochemical manipulations can be readily performed and results obtained are applicable to other eukaryotic organisms as the basic mechanisms of the cell cycle appear to be highly conserved (Blow, 2001). As such, this is a suitable *in vitro* system for the purposes of this study.

Results

2.1 Compound 6d did not inhibit DNA replication in Xenopus *egg extracts*

Cell-free extracts were prepared from *Xenopus* eggs and activated by the addition of calcium to release them from meiotic metaphase II arrest, allowing them to proceed into interphase of the first embryonic cell cycle. When demembranated sperm nuclei are incubated in activated interphase egg extract, sperm chromatin first decondenses and is assembled into interphase nuclei. Licensing of replication origins occurs and S phase begins shortly after nuclear assembly (Blow, 2001). Figure 2.1 shows a typical replication profile. Little incorporation of [α-^{32}P]-dATP was detected in the initial 30 minutes of incubation during which time the sperm chromatin decondenses, becomes licensed for subsequent replication, and is assembled into a functional interphase nucleus. Replication starts at 30 minutes and DNA synthesis proceeds at a constant rate until 90 minutes, when almost all DNA template would be replicated. In these extracts, protein synthesis has been inhibited by the addition of cycloheximide, which prevents accumulation of cyclin B and progression from G2 into mitosis. Complete DNA replication is not perturbed by the inhibition of protein synthesis (Blow and Laskey, 1988). Cycloheximide-treated egg extracts therefore support a single complete round of DNA synthesis.

Compound 6d (Figure 2.2A) was titrated into *Xenopus* egg extract and its effect on DNA replication was assessed by quantifying the amount of DNA synthesised at 90 min. As shown in Figure 2.2B, no significant reduction in DNA replication was seen at any concentration of Compound 6d used, up to 200 μM, compared to the control sample. One possible explanation for this lack of effect is that Cdc7 may be active and able to phosphorylate essential substrates in solution prior to the addition of the inhibitor. To address this possibility, *Xenopus* egg extract was pre-incubated with

Compound 6d for varying intervals to inactivate Cdc7. Sperm nuclei were then added and DNA synthesis quantified. As shown in Figure 2.2C, DNA replication in samples pre-treated with Compound 6d also did not appear to be significantly different from control samples at both pre-incubation intervals tested. In delaying DNA template addition, there was a loss in replicative capacity as Cdt1 present in extracts become degraded, resulting in a decrease in total DNA synthesis with increased pre-incubation time (Li and Blow, 2005). As such, origin licensing and initiation were adversely affected and extracts were no longer efficient in supporting DNA replication *in vitro*. From these experiments, Compound 6d does not seem to be effective in inhibiting DNA replication in this system. The reason for this is not clear, but include the possibilities that the compound was incorrectly synthesised here or that Compound 6d is significantly less active against *Xenopus* Cdc7 than the mammalian Cdc7 it was developed against, perhaps due to sequence or structural differences in the ATP-binding pocket. No further experiments were performed with Compound 6d in this study.

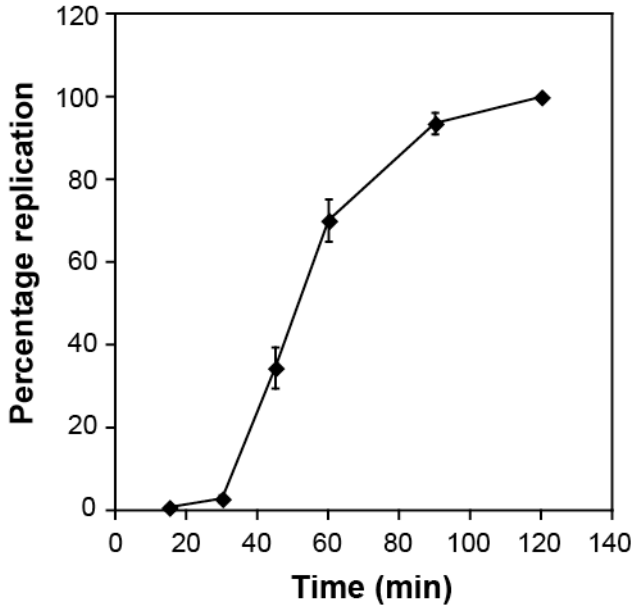

Figure 2.1. Replication time-course of demembranated sperm in *Xenopus* egg extract.

Demembranated *Xenopus* sperm was incubated in activated egg extract supplemented with [α-^{32}P]-dATP. Samples were taken at specified time-points and the amount of [α-^{32}P]-dATP incorporated into newly synthesised DNA was determined by scintillation counting. DNA synthesis was expressed as percentage of the amount synthesised by 120 min. The error bars represent S.E.M. from 14 experiments.

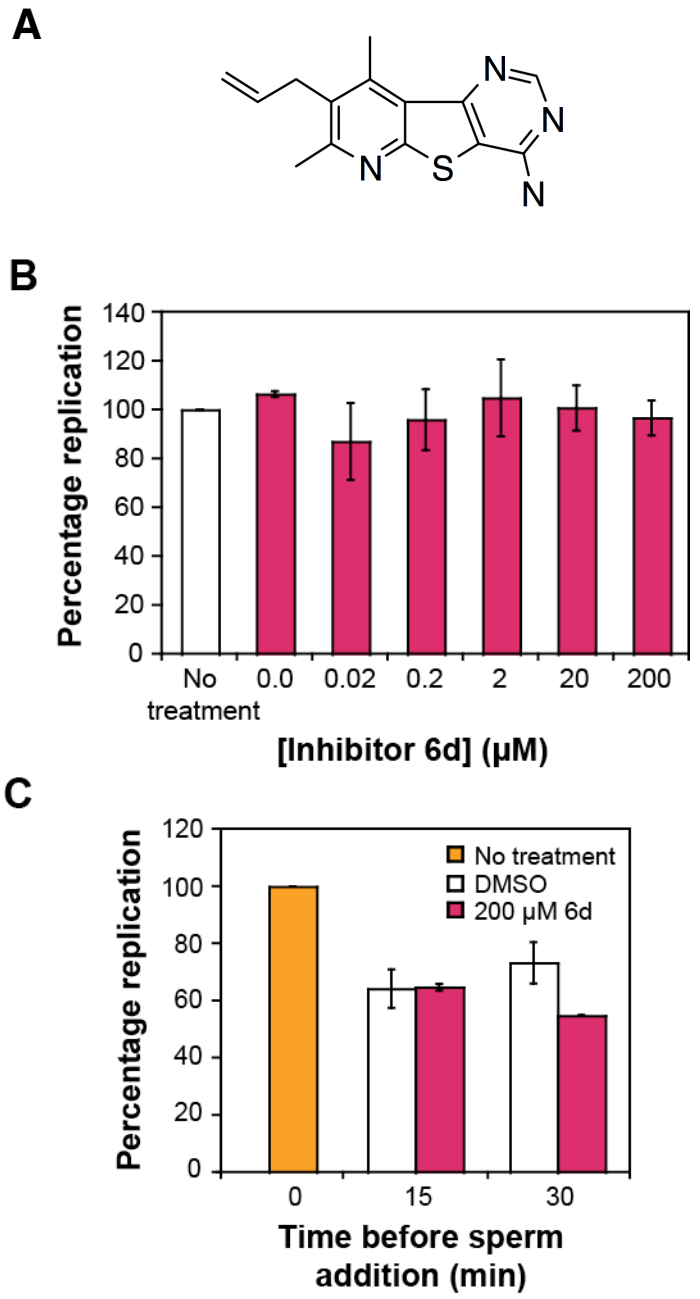

Figure 2.2. Compound 6d does not inhibit replication in *Xenopus* egg extract.

(A) Chemical structure of Compound 6d (Zhao et al., 2009). (B) Compound 6d was titrated into *Xenopus* egg extracts supplemented with [α-^{32}P]dATP, using demembranated sperm nuclei as DNA templates. The amount of DNA synthesised was determined by scintillation counting at 90 minutes after sperm addition. DNA synthesis was expressed as percentage of the amount synthesised by 90 min in untreated extract. (B) Compound 6d was added to *Xenopus* egg extract and incubated for the indicated times before sperm addition. DNA synthesis was determined and percentage replication calculated as in (A). The error bars represent S.D. from 2 experiments.

2.2 PHA-767491 inhibits DNA replication by blocking an essential Cdc7 activity

2.2.1 PHA-767491 inhibits DNA replication

As with Compound 6d, PHA-767491 was first titrated into *Xenopus* egg extracts and its effect on DNA synthesis measured. When extracts were treated with PHA-767491, DNA synthesis was inhibited when either demembranated *Xenopus* sperm or permeabilised CHO nuclei were used as DNA templates (Figures 2.3B and C). Since DNA replication cannot take place if initiation of DNA replication is inhibited, this observation is consistent with an inhibition of Cdc7 activity in *Xenopus* egg extracts. PHA-767491 appears to be slightly more potent when permeabilised CHO nuclei were used as templates as DNA synthesis was completely inhibited at 20 μM; a higher concentration above 50 μM was required for a similar inhibition with sperm nuclei.

In *Xenopus* egg extracts, the assembly of an intact nuclear envelope is first required before initiation of replication can occur, after the recruitment of pre-RC components to origins (Blow and Laskey, 1986; Newport, 1987; Sheehan et al., 1988). To test if PHA-767491 inhibition of DNA synthesis is due to non-specific effects on nuclear assembly, hence leading to a defect in DNA synthesis, sperm nuclei was incubated with interphase egg extract for 40 min and stained with Hoechst 33258 for visualisation. Nuclear envelope formation still occurred in the presence of PHA-767491, indicating that PHA-767491 does not affect nuclear assembly (Figure 2.4). Nuclei of comparable size and structure formed around sperm DNA in the presence of PHA-767491 and chromatin decondensation could be observed, similar to that of sperm incubated in control extract. PHA-767491 caused a reduction in the fine-scale dispersal of chromatin, which may be a consequence of inhibiting DNA replication (Blow, 1993).

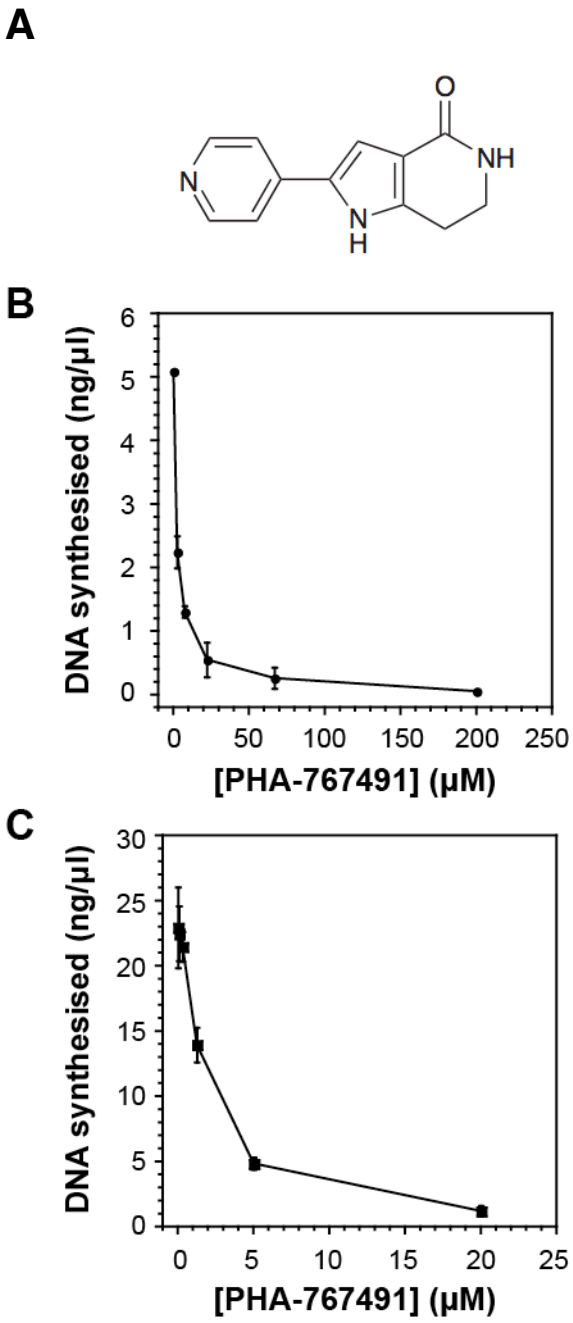

Figure 2.3. PHA-767491 inhibits replication in *Xenopus* egg extract.

(A) Chemical structure of PHA-767491 (Montagnoli et al., 2008). (B, C) PHA-767491 was titrated into *Xenopus* egg extracts supplemented with [α-^{32}P]dATP. Demembranated sperm nuclei (B) or permeabilised somatic mammalian nuclei (C) were used as DNA templates. The amount of DNA synthesised was determined by scintillation counting at 90 minutes after sperm/nuclei addition. The error bars represent S.D. from 3 experiments.

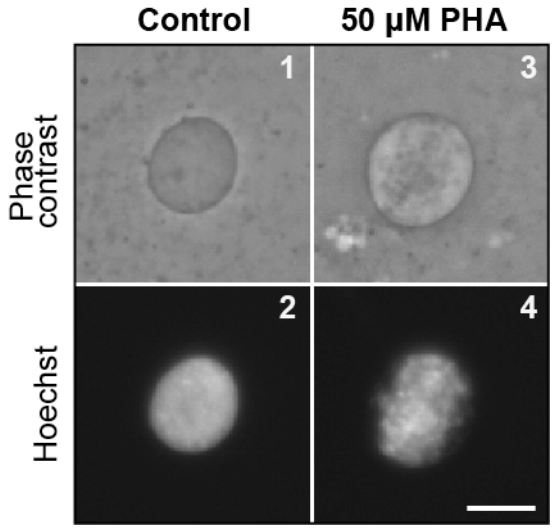

Figure 2.4. PHA-767491 does not inhibit nuclear envelope formation in egg extracts.

Sperm nuclei were incubated at 6 ng DNA/µl in egg extracts with (3 & 4) or without (1 & 2) 50 µM PHA-767491 for 40 min. Nuclei were stained with Hoechst 33258 and visualised by phase contrast (top) or fluorescence microscopy (bottom). The scale bar represents 10 µm.

2.2.2 PHA-767491 inhibits hyper-phosphorylation of chromatin-bound Mcm4

Mcm4 is a Cdc7 substrate and becomes hyper-phosphorylated as DNA replication occurs (Sheu and Stillman, 2006, 2010; Takahashi and Walter, 2005). This can readily be observed as a mobility shift of Mcm4 with SDS-PAGE (Figures 2.5A and B, "control" lanes). The effect of PHA-767491 was tested using Mcm4 hyper-phosphorylation as read-out for Cdc7 activity. As shown in these figures, hyper-phosphorylation of chromatin-bound Mcm4 was inhibited when extracts were treated with 50 µM PHA-767491. This suggests that PHA-767491 inhibits Cdc7 activity in *Xenopus* egg extracts. Mcm4 hyper-phosphorylation was not affected when Cdk activity was specifically blocked with 100 nM $p27^{kip1}$. This result shows that a Cdc7-dependent event can be completed in the absence of Cdk activity and is consistent with previous reports that the essential replication function of Cdc7 is executed before that of Cdks (Heller et al., 2011; Jares and Blow, 2000; Walter, 2000). Cdc45, a pre-initiation/replication fork protein which is recruited downstream of both Cdc7 and Cdk activities, was not detected on chromatin in both PHA- and $p27^{kip1}$-treated samples, indicating initiation of replication origins was inhibited.

When PHA-767491 was titrated into extract and chromatin was isolated in mid-S phase, phosphorylation of chromatin-bound Mcm4 was inhibited in a dose-dependent manner (Figures 2.5C and D). Cdc45 and PCNA, components of the replication fork, were also detected at lower levels with increasing PHA-767491 concentrations. Replication-dependent re-association of Cdc6 with chromatin was inhibited in a similar dose-dependent manner.

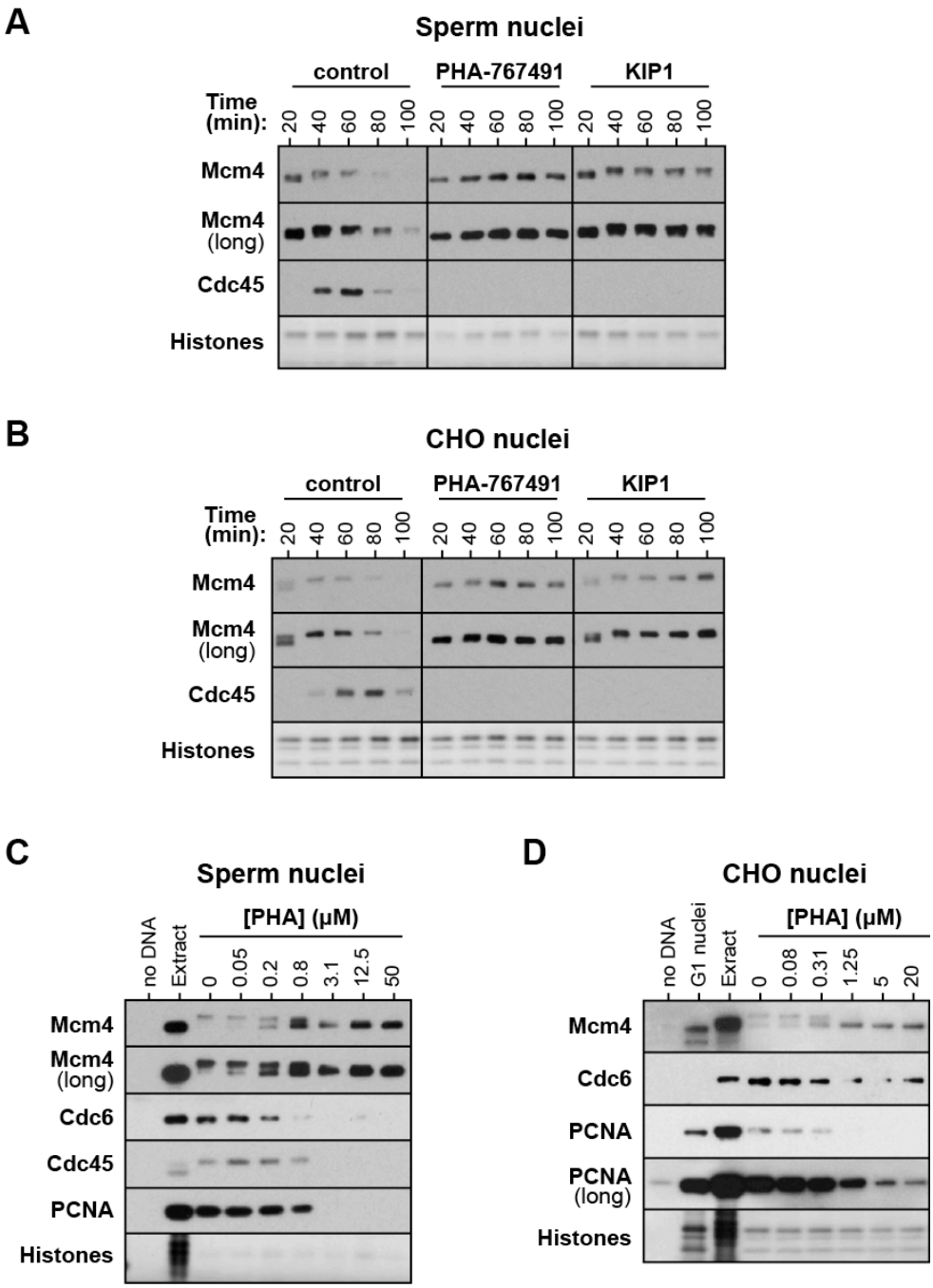

Figure 2.5. PHA-767491 inhibits hyper-phosphorylation of chromatin-bound Mcm4.

(A, B) Chromatin was isolated from replicating extracts, which were untreated (Control) or treated with PHA-767691 or $p27^{kip}$ (KIP1), at the indicated time-points after DNA addition and immuno-blotted for Mcm4 or Cdc45 to monitor S phase progression. 10 ng DNA/μl sperm nuclei and 50 μM PHA-767491 (A) or 60 ng DNA/μl mammalian nuclei and 20 μM PHA-767491 (B) were used. 100 nM $p27^{kip1}$ was used for both experiments. (D, E) PHA-767491 was titrated into *Xenopus* egg extract containing 10 ng DNA/μl sperm nuclei (C) or 60 ng DNA/μl mammalian nuclei (D). Chromatin was extracted in mid-S phase and immuno-blotted for Mcm4, Cdc6, Cdc45 and PCNA. Histones were coomassie-stained and used as loading controls.

Using a recently characterised antibody against *Xenopus* Cdc7 (Poh et al., manuscript in preparation), Dr Gaganmeet Singh Chadha performed a follow-up experiment to determine if PHA-767491 treatment affected Cdc7 association with DNA. As shown in Figure 2.6, PHA-767491 did not inhibit Cdc7 recruitment to chromatin. Cdc7 is thought to be recruited to chromatin by direct interaction with the Mcm2-7 double hexamer (Jares and Blow, 2000; Jares et al., 2004) and so it did not associate with chromatin when licensing was blocked by geminin. In contrast, PHA-767491 appears to slightly increase Cdc7 chromatin association. In addition, PHA-767491 did not promote the association of Cdc6 with chromatin. Cdc6 binds to chromatin in *Xenopus* extracts only when Mcm2-7 is not present on DNA. In control extracts, this means that high levels of Cdc6 are seen on DNA in G2 when all Mcm2-7 have been displaced as a consequence of DNA replication having completed (Oehlmann et al., 2004; Rowles et al., 1999; Sonneville et al., 2012). Cdc6 also strongly associates with chromatin when licensing is blocked by addition of geminin. Taken together, these results indicate that PHA-767491 does not affect licensing and Cdc7 recruitment to chromatin. The inhibition of DNA replication occurs by blocking an essential kinase activity of Cdc7 to phosphorylate Mcm4.

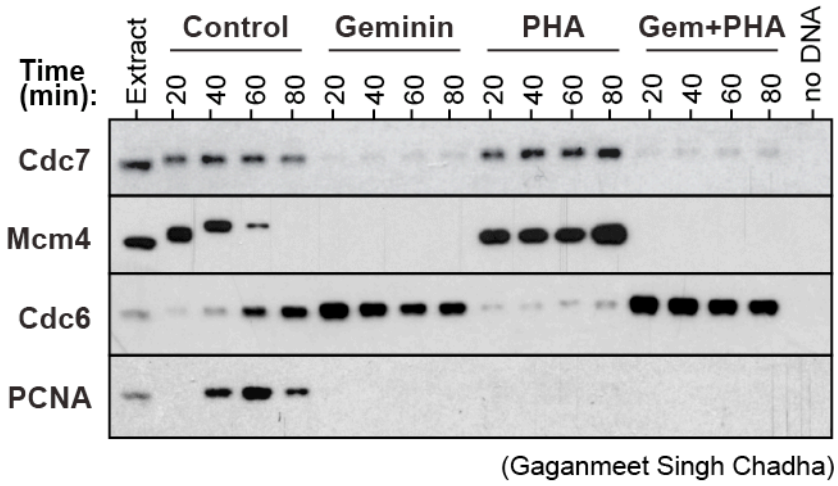

Figure 2.6. PHA-767491 does not inhibit Cdc7 binding to chromatin.

Sperm nuclei were incubated in extracts optionally treated with geminin or 50 μM PHA-767491. Chromatin was isolated at the indicated times and immuno-blotted for Cdc7, Mcm4, Cdc6 or PCNA. This experiment was performed by Gaganmeet Singh Chadha.

2.3 PHA-767491 causes rapid loss of Mcm4 hyper-phosphorylation, which is reversed by protein phosphatase 1

As PHA-767491 is a small molecule inhibitor, it is expected to act quickly on its target. This was tested using Mcm4 hyper-phosphorylation as a read-out. Sperm chromatin was incubated in *Xenopus* extract for 60 minutes in the presence of $p27^{kip1}$. This time interval allows Mcm4 at licensed origins to become maximally hyper-phosphorylated while $p27^{kip1}$ treatment causes hyper-phosphorylated Mcm4 to accumulate on chromatin since origin firing cannot occur. PHA-767491 was then added to the extract. Within 15 minutes of PHA-767491 addition, chromatin-bound Mcm4 had become largely dephosphorylated (Figure 2.7A, lanes 1-4).

This rapid turnover of Mcm4 hyper-phosphorylation suggests that a phosphatase may be acting in close proximity to Cdc7 such that when Cdc7 activity is inhibited by PHA-767491, equilibrium quickly shifts towards de-phosphorylation. In order to test this hypothesis and to identify the potential phosphatase, different phosphatase inhibitors were tested, each added at the same time as PHA-767491 treatment, while monitoring Mcm4 hyper-phosphorylation. When high concentrations (2 µM) of okadaic acid were added along with PHA-767491, Mcm4 dephosphorylation was largely abolished (Figure 2.7A, lanes 11, 12); however, lower concentrations (0.25 - 1 µM) failed to block a major proportion of Mcm4 dephosphorylation (Figure 2.7A, lanes 5-10). Okadaic acid is an inhibitor of protein phosphatases in the PP1 and PP2A classes with a higher potency towards PP2A such that sub-micromolar concentrations typically inhibit PP2A but not PP1 (Cohen, 1989). Therefore these results suggest that PP1 may be responsible for the rapid dephosphorylation of Mcm4 observed here.

To test this idea, extracts were pre-treated with Inhibitor-2 (I-2), a protein regulator that specifically inhibits PP1 activity (Huang and Glinsmann, 1976; Hurley et al., 2007).

Endogenous PP1 acts as a holo-enzyme. It is made up of a catalytic subunit which interacts with a regulatory subunit that modulates its substrate specificity and enzymatic activity (Cohen, 2002; Shi, 2009). Effective I-2-inhibition of PP1 activity is time-dependent, necessitating a 15-min pre-incubation of I-2 in *Xenopus* egg extract to allow for the formation of inactive PP1c•I-2 complexes. As seen in Figure 2.7B, I-2 pre-treatment before PHA-767491 addition largely prevented the dephosphorylation of Mcm4 in the presence of PHA-767491, indicating that PP1 is the main phosphatase acting on hyper-phosphorylated Mcm4 in *Xenopus* extracts. Consistent with this conclusion, PP1 has recently been shown to physically interact with Mcm2-7 hexamers bound to chromatin in *Xenopus* egg extracts (Gambus et al., 2011). This suggests that PP1 is recruited to chromatin-bound Mcm2-7 to modulate the dephosphorylation of Mcm4, and potentially of other Mcm2-7 subunits. This may occur as part of a larger protein complex.

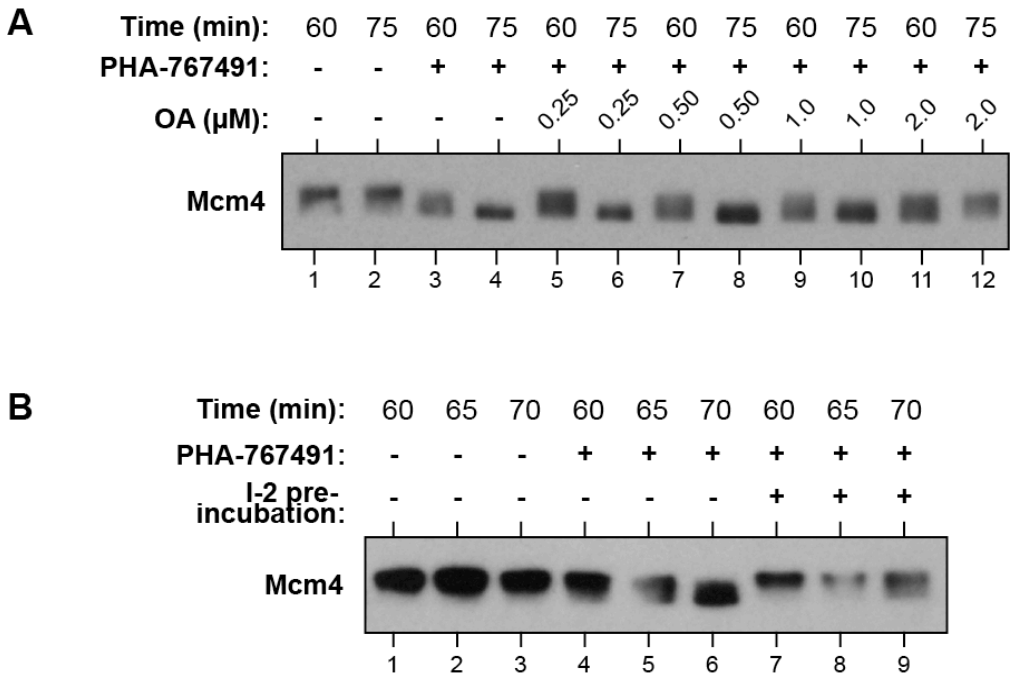

Figure 2.7. PP1 inhibition prevents Mcm4 dephosphorylation in the presence of PHA-767491.

(A) 10 ng DNA/μl sperm nuclei were incubated for 60 min in extracts treated with $p27^{kip1}$ to allow Mcm4 hyper-phosphorylation to occur. 50 μM PHA-767491 and okadaic acid (OA) at the indicated concentrations were added simultaneously and chromatin was isolated immediately (60 min) or 15 minutes later (75 min) and immuno-blotted for Mcm4. (B) Similar to Fig 2.7A, sperm nuclei was incubated for 60 min in extracts treated with $p27^{kip1}$. At 45 min after sperm addition, extracts were first pre-incubated for 15 min with 1.2 μM I-2, a protein inhibitor of PP1, before 50 μM PHA-767491 was added at 60 min. Chromatin was isolated immediately (60 min), after 5 min (65 min) and after 10 min (70 min) and immuno-blotted for Mcm4.

2.4 PHA-767491 is a selective inhibitor of Cdc7 kinase

2.4.1 Kinase profiling

To determine the selectivity of PHA-767491, it was tested against a panel of 95 protein kinases, performed through the kinase profiling service available at the University of Dundee Protein Phosphorylation Unit. In this assay, 1 μM PHA-767491 gave more than 90% inhibition of only 7 other protein kinases, namely DYRK1A, 2 and 3, PRK2, GSK3β, p38δ MAPK, and CK1 (Table 2.1), broadly in line with previous analyses of PHA-767491 specificity (Montagnoli et al., 2008; Natoni et al., 2011). None of these kinases have been implicated in the initiation of DNA replication and the use of specific inhibitors against these kinases did not significantly inhibit DNA replication in egg extracts (data not shown). The only kinase previously implicated in DNA replication that is affected by PHA-767491 is Cdk2-cyclin A, whose activity was inhibited by 72% under these assay conditions.

2.4.2 Pre-incubated sperm nuclei can replicate in PHA-767491-treated extracts

Since Cdk2 and Cdc7 are both required for the initiation of DNA replication, act at a similar time in this process and share common substrates, it is important to establish that PHA-767491 does not exert a significant cross-reactivity towards Cdk2 activity. In order to test the extent to which PHA-767491 inhibits DNA replication by inhibiting Cdk2 activity, a chromatin transfer experiment was designed based on the observation that Cdc7 acts before Cdks to promote DNA replication (Jares and Blow, 2000; Walter, 2000). The schematic is outlined in Figure 2.8A. Sperm nuclei were incubated in extract containing $p27^{kip1}$, which allows Mcm4 to be hyper-phosphorylated while preventing replication by inhibiting S phase Cdks. I-2 was added to this extract to minimise dephosphorylation of Mcm4. Chromatin was then isolated and transferred to fresh extract containing [α-^{32}P]-dATP and optionally containing 20 μM PHA-767491 and/or I-

2. The presence of PHA-767491 in the second extract prevents further Cdc7-dependent Mcm4 phosphorylation so that replication is dependent on chromatin-bound Mcm4 phosphorylated prior to the time of transfer. Replication in the second extract requires functional Cdk activity since $p27^{kip1}$ in the first extract had prevented Cdk-dependent events from occurring. Therefore, if S phase Cdk activity were inhibited by 20 μM PHA-767491, no replication will take place.

Before this experiment could be carried out, conditions for isolating intact nuclei for replication were tested. When chromatin isolation is carried out for immuno-blotting of chromatin-bound proteins, extract containing sperm nuclei is diluted with buffer and under-laid with the same buffer containing sucrose. This is spun at low speed to separate soluble proteins from chromatin. Following the removal of soluble proteins in the supernatant and a cushion wash, chromatin is then compacted with high-speed centrifugation. For the purpose of isolating nuclei for replication in a second extract, however, the hard spin is omitted to avoid damaging the nuclei. Nuclei also have to be further cushioned in addition to the sucrose solution during centrifugation. As shown in Figure 2.8B, under-laying the sucrose cushion with 30% glycerol allowed for isolation of nuclei that replicated most efficiently in the second extract. This was thus used in all further experiments to isolate nuclei for replication.

Figures 2.8C and D show that pre-incubated chromatin replicated efficiently to approximately 60% of control levels in extract treated with PHA-767491 whilst replication of naive sperm nuclei lacking hyper-phosphorylated Mcm4 was profoundly inhibited. This provides evidence that the Cdk-dependent step in DNA replication can be performed in the presence of PHA-767491 concentrations that inhibit replication. Consistent with Cdc7 being the key target of PHA-767491, addition of I-2 to the second extract enhanced the replication of pre-incubated chromatin. Taken together, these data strongly suggest that specific inhibition of Cdc7 kinase activity is the major reason

that PHA-767491 inhibits replication in the *Xenopus* system. This establishes PHA-767491 as a reasonably selective inhibitor of Cdc7 in the initiation of DNA replication and is suitable for use in analysing Cdc7 function in *Xenopus* egg extracts.

Kinase activity profiling - 1 μM PHA-767491

No.	Kinase	Activity (%)	SD
1	DYRK2	3.9	0.4
2	PRK2	4.1	0.9
3	DYRK3	4.4	0.3
4	DYRK1A	5.3	0.0
5	p38d MAPK	6.3	1.7
6	GSK3b	8.4	0.3
7	CK1	9.4	1.8
8	ERK8	12.2	0.2
9	ROCK 2	13.8	1.6
10	p38g MAPK	14.3	0.6
11	AMPK	24.5	1.3
12	CDK2-Cyclin A	27.8	0.8
13	MINK1	28.9	2.2
14	GCK	30.7	1.2
15	PRAK	35.3	2.1
16	PKD1	38.1	3.4
17	IRR	39.0	1.9
18	S6K1	39.5	4.1
19	HIPK2	42.6	4.2
20	PLK1	49.2	2.4
21	MNK2	49.8	4.8
22	CHK2	50.1	2.7
23	RIPK2	53.2	5.8
24	RSK1	54.9	0.9
25	BRSK2	56.9	3.1
26	MSK1	57.0	4.1
27	TTK	57.0	3.7
28	MKK1	58.1	0.7
29	MARK3	60.0	0.4
30	MAPKAP-K2	60.3	2.0
31	CAMKKb	62.2	1.3
32	ERK2	62.9	3.9
33	ERK1	67.1	6.3
34	LKB1	70.0	2.8
35	MARK2	70.1	12.6
36	BRSK1	71.6	0.2
37	SmMLCK	71.7	6.4
38	NUAK1	74.4	1.1
39	IKKb	80.8	3.9
40	MELK	80.9	1.6
41	SGK1	82.5	2.4
42	MARK4	83.2	10.1
43	PKCa	84.8	3.6
44	PIM1	85.0	0.5
45	CK2	87.6	11.2
46	PKCz	88.0	2.7
47	RSK2	88.2	2.0
48	IKKe	88.6	1.7

No.	Kinase	Activity (%)	SD
49	PIM3	88.9	6.8
50	MST4	89.7	6.8
51	PKA	89.8	2.4
52	PKBa	91.6	1.3
53	CHK1	93.0	5.8
54	MST2	93.1	0.5
55	PAK2	94.6	3.0
56	PAK5	95.9	10.2
57	IRAK4	96.1	2.0
58	JNK1	97.6	6.4
59	PDK1	98.3	3.6
60	MAPKAP-K3	99.2	6.2
61	JNK3	99.3	13.0
62	Lck	99.5	2.1
63	NEK6	100.2	1.6
64	TBK1	101.3	9.3
65	PAK6	101.4	5.1
66	IR-HIS	101.5	24.4
67	VEG-FR	101.9	3.0
68	MLK3	103.3	6.3
69	Src	104.7	1.4
70	Aurora A	105.0	0.5
71	PIM2	105.0	1.3
72	HIPK3	105.2	1.7
73	p38a MAPK	105.8	4.9
74	EPH A2	105.9	0.3
75	JNK2	106.4	3.1
76	MNK1	106.5	3.0
77	IGF-1R	106.6	7.8
78	PAK4	107.3	6.2
79	MLK1	108.5	0.6
80	EF2K	108.7	0.0
81	HER4	108.8	18.0
82	PKBb	111.3	0.6
83	NEK2a	111.5	0.5
84	p38b MAPK	111.9	3.2
85	PHK	112.6	11.1
86	BTK	114.0	21.6
87	CAMK1	114.7	10.1
88	SYK	114.7	1.3
89	SRPK1	114.7	0.1
90	Aurora B	115.4	7.0
91	YES1	116.6	5.8
92	EPH-B3	118.0	11.3
93	FGF-R1	120.8	24.2
94	CSK	121.9	0.6
95	HIPK1	159.4	13.1

Table 2.1. Kinase assay profile of PHA-767491.

1 μM PHA-767491 dissolved in DMSO was tested against a panel of 95 kinases using a radioactive (^{33}P-ATP) filter-binding assay, at an ATP concentration at or below the calculated K_M for ATP of each kinase. The kinase activity detected was expressed as a percentage relative to 100% control activity and the S.D. calculated for duplicate values. This assay was carried out by the MRC Protein Phosphorylation Unit, University of Dundee.

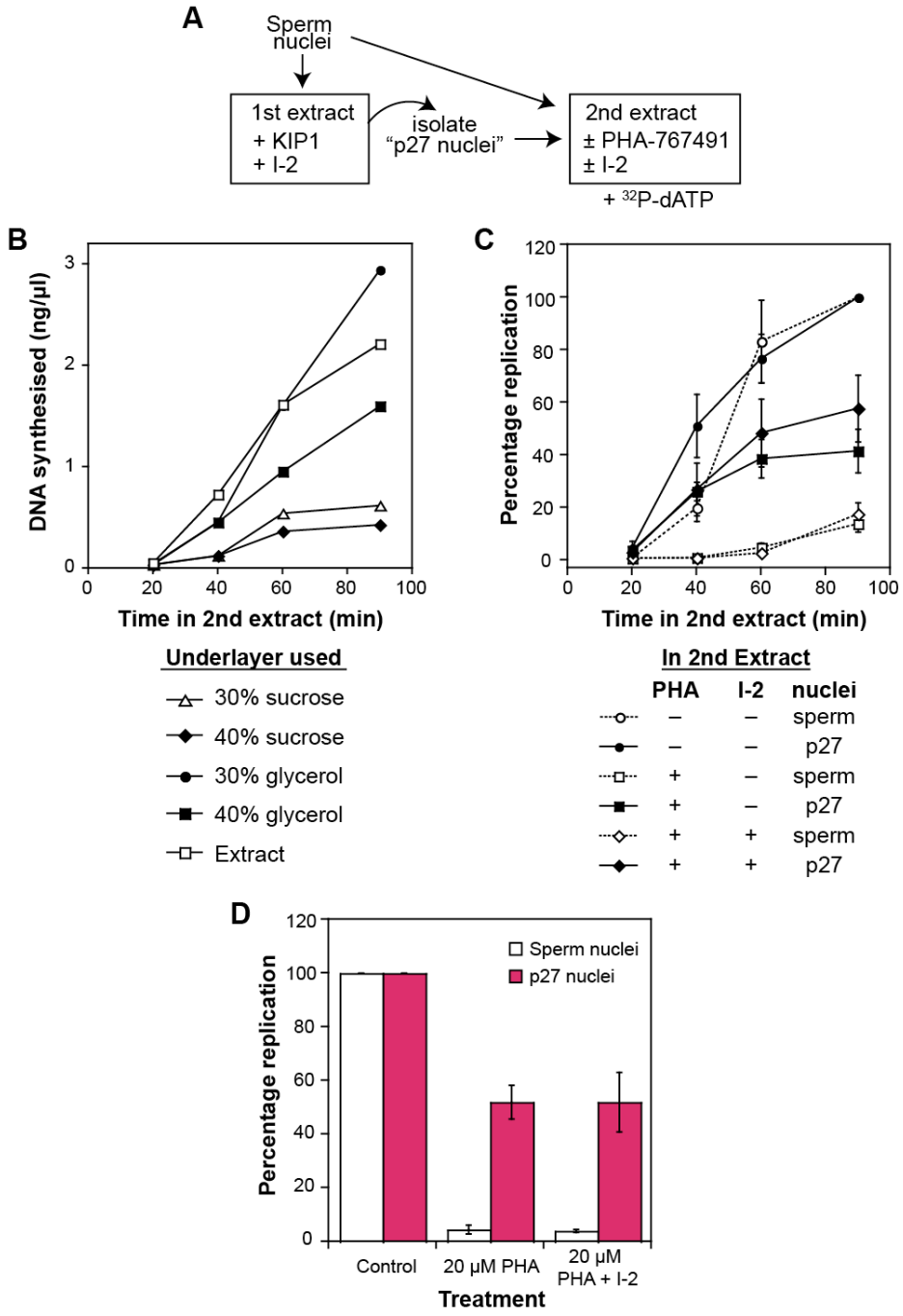

Figure 2.8. Pre-incubated chromatin templates can replicate in the presence of PHA-767491.

(A) Schematic of the chromatin transfer experiment: sperm nuclei were incubated in interphase extract with $p27^{kip1}$ and the PP1 inhibitor, I-2. During early S phase, nuclei were isolated ("p27 nuclei") and transferred to a second extract. The second extract was untreated, treated with PHA-767491 alone or treated with both PHA-767491 and I-2. DNA synthesis in the second extract was then measured and compared to DNA synthesis using naive sperm nuclei as templates. (B) Sperm nuclei were incubated in *Xenopus* egg extract. Nuclei isolation was carried out using different

buffers or egg extract to underlay the sucrose cushion. Extracted nuclei were transferred into a second extract containing [α-^{32}P]dATP. and samples were taken at the indicated time-points to assay for total DNA synthesis. (C) Sperm nuclei were incubated at 20 ng DNA/μl in interphase extract with p27^{kip1}. During early S phase (40 min), nuclei were isolated and transferred to untreated fresh extracts (filled circles) or fresh extracts treated with 20 μM PHA-767491 (filled squares) or 20 μM PHA-767491 and 1.2 μM I-2 (filled diamonds). Nuclei were added at a final concentration of 10 ng DNA/μl to the second extract and extracts were supplemented with [α-^{32}P]dATP. Total DNA synthesis was measured at the indicated times after transfer. Filled symbols represent replication of transferred “p27 nuclei” and the corresponding replication reactions in which naive sperm nuclei were used are represented by open symbols. Percentage replication was calculated by taking the amount of DNA synthesised at 90 minutes to be 100%. (D) At 40 minutes after DNA addition, nuclei that were pre-incubated had replicated DNA up to 50% of the untreated sample in the presence of PHA-767491 while naive sperm nuclei had barely synthesised any DNA. DNA synthesis expressed as percentage of the amount synthesised in control. The error bars represent S.D. from 3 experiments.

2.5 The execution point of Cdc7 activity in replication initiation

2.5.1 Nuclear assembly is required for Cdc7 hyper-phosphorylation of Mcm4

In *Xenopus* egg extracts and early embryos, origin licensing occurs only between anaphase onset and the completion of nuclear envelope assembly, while initiation of replication only occurs after nuclear assembly within a nucleoplasmic environment (Blow, 2001; Jares and Blow, 2000). To investigate the execution point of Cdc7 activity with respect to nuclear assembly and pre-RC formation, an experiment was performed to determine if Mcm4 hyper-phosphorylation occurs only on DNA-bound Mcm2-7 or whether it can also occur on soluble Mcm2-7 complexes. Figure 2.9A shows that unlike Mcm4 that was bound to DNA, soluble Mcm4 was not hyper-phosphorylated and its mobility on SDS-PAGE was not significantly changed when extracts were treated with PHA-767491, suggesting that Cdc7 only phosphorylated DNA-bound Mcm2-7.

Although Cdc7 can be recruited to licensed chromatin prior to nuclear assembly (Jares and Blow, 2000), this may occur more efficiently after nuclear assembly has occurred when Cdc7 is enriched within the nucleoplasm (Walter, 2000). The degree of Mcm4 hyper-phosphorylation in the absence of full nuclear assembly was therefore examined, using wheat germ agglutinin (WGA) to disrupt nuclear pore function (Finlay et al., 1987). As previously reported, concentrations of WGA that inhibited nuclear import and nuclear envelope formation were also found to inhibit DNA replication (Figures 2.9B, C) (Cox, 1992). Increasing concentrations of WGA progressively inhibited Mcm4 hyper-phosphorylation (Figure 2.9C). Taken together, these results indicate that Cdc7 does not phosphorylate its substrates in the cytoplasm and an intact nuclear envelope is required for maximal Mcm4 phosphorylation, possibly as a result of enhancing Cdc7 nuclear import and subsequent binding and phosphorylation of chromatin-bound Mcm2-7.

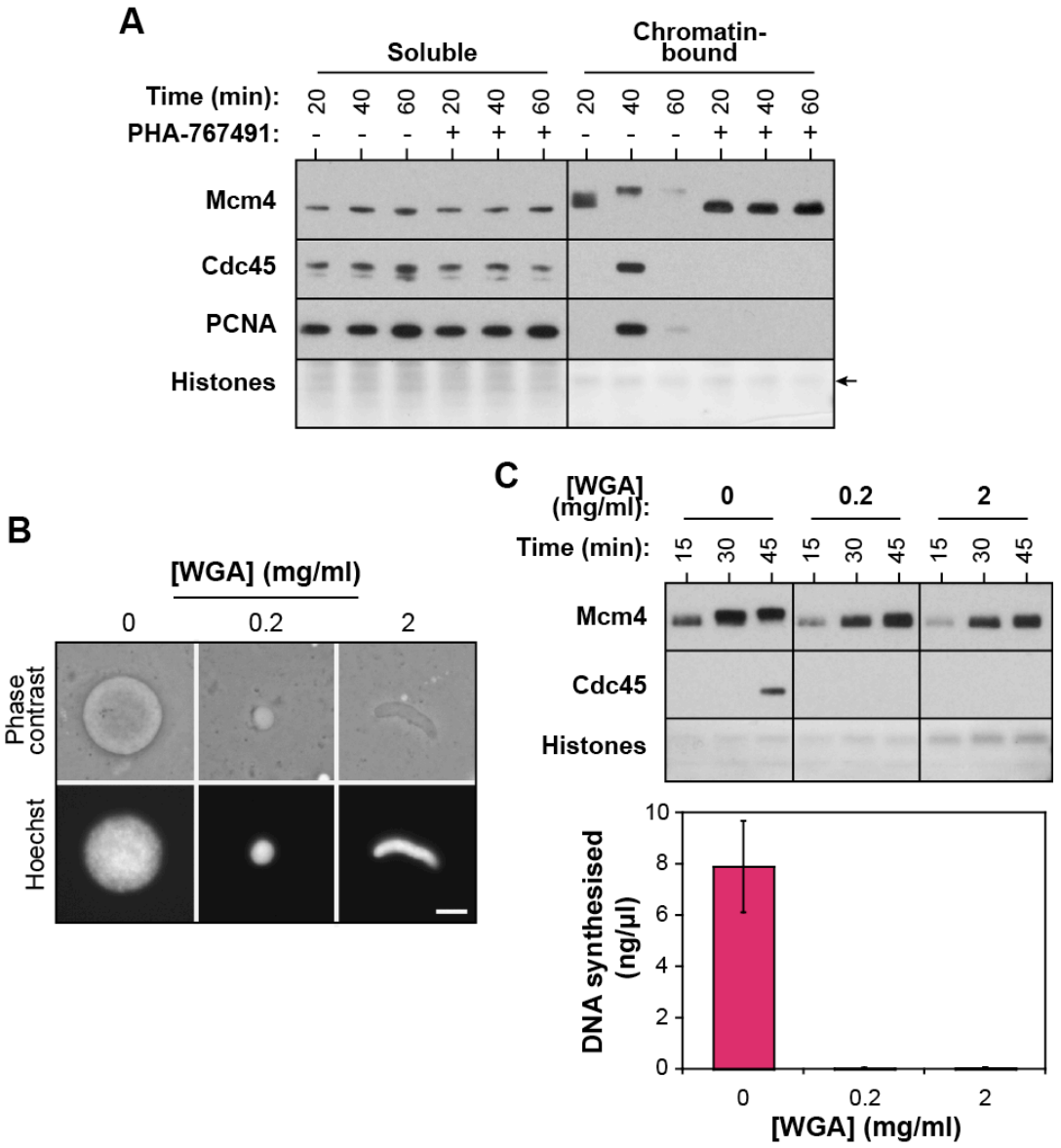

Figure 2.9. Mcm4 hyper-phosphorylation does not occur in the absence of nuclear envelope formation.

(A) Sperm nuclei were incubated at 10 ng DNA/µl in interphase extract in the absence or presence of 50 µM PHA-767491. At the indicated time-points, chromatin was isolated and immuno-blotted for Mcm4, Cdc45 and PCNA. 0.5% (v/v) of the supernatant was also loaded on the same SDS-PAGE gel to blot for soluble proteins. (B) Sperm nuclei were incubated in interphase extract supplemented with 0.2 or 2 mg/ml WGA. At 40 minutes, nuclei were stained with Hoechst 33258 and visualised by phase contrast for the nuclear envelope (top) or fluorescence microscopy for DNA (bottom). (C) Extracts were treated with WGA as in (B) and chromatin was isolated at the indicated time-points for immuno-blotting of Mcm4 and Cdc45. Extracts were optionally supplemented [α-^{32}P]dATP and total DNA synthesis was determined at 90 minutes by scintillation counting. Error bars represent S.D. from 3 experiments.

2.5.2 Cdc7 acts very early in S phase to initiate replication

Replication origins typically initiate at different times during S phase. When sperm nuclei replicate in *Xenopus* egg extracts, initiation occurs over the course of 20 to 30 minutes (Blow, 2001; Labit et al., 2008; Luciani et al., 2004). In order to determine whether Cdc7 acts on all origins early in S phase, or whether it acts on late-firing origins only later in S phase, a 'hybrid system' where mammalian somatic nuclei from cells synchronised in G1 are incubated in *Xenopus* extracts was used (Dimitrova and Gilbert, 1999; Thomson et al., 2010). These nuclei replicate in egg extract according to the same overall timing programme as mammalian replication *in vivo*, though compressed into an interval of approximately 120 minutes. This system provides a longer S phase compared to sperm nuclei, making it easier to distinguish between different stages of S phase. Here, this 'hybrid system' was used to establish the execution point of Cdc7, to address whether Cdc7 acts on all origins very early leaving them competent for replication, or whether it is required to act on late-firing origins later in S phase.

When nuclei from G1 CHO cells were added to *Xenopus* egg extract, chromatin-bound Mcm4 became maximally hyper-phosphorylated between 20 to 40 minutes from the start of the incubation, slightly slower than was seen with *Xenopus* sperm nuclei as substrate (Figure 2.10A). However, on both templates, maximal Mcm4 hyper-phosphorylation occurs in early S phase, suggesting that by this time Cdc7 has acted on both early and late origins. To confirm this interpretation, PHA-767491 was added at different times after CHO nuclei addition. I-2 was present in all experiments to minimise Mcm4 dephosphorylation. Figure 2.10C shows that when PHA-767491 was added at 30 minutes, before DNA replication had started, replication subsequently occurred to more than 60% of control levels. When PHA-767491 was added at 50 minutes, in very early S phase, subsequent DNA synthesis occurred to approximately 90% of control

levels. This supports the idea that the essential function of Cdc7 in replication is completed by early S phase and correlates well to the time of maximal Mcm4 hyper-phosphorylation.

These results are in contrast to experiments assessing the execution time of Cdks, which showed that even late in S phase, inhibition of Cdk activity promptly prevented further initiation (Luciani et al., 2004; Thomson et al., 2010). To verify this difference between Cdk and Cdc7 execution points, the effect of PHA-767491 was compared directly with a Cdk inhibitor, roscovitine, using the same approach outlined in Figure 2.10B. When either PHA-767491 or roscovitine was added to extract at the same time as CHO nuclei, DNA replication was completely inhibited (Fig 2.10D, open symbols). However, when the inhibitors were added 35 min later in early S-phase, replication proceeded to more than 60% of control levels in the presence of PHA-767491 but only to less than 20% in the presence of roscovitine (Figure 2.10D, filled symbols). This indicates that Cdc7 executes its function before Cdk and suggests that once Cdc7 has completed its essential function, it is no longer the rate-limiting factor in driving the replication timing programme.

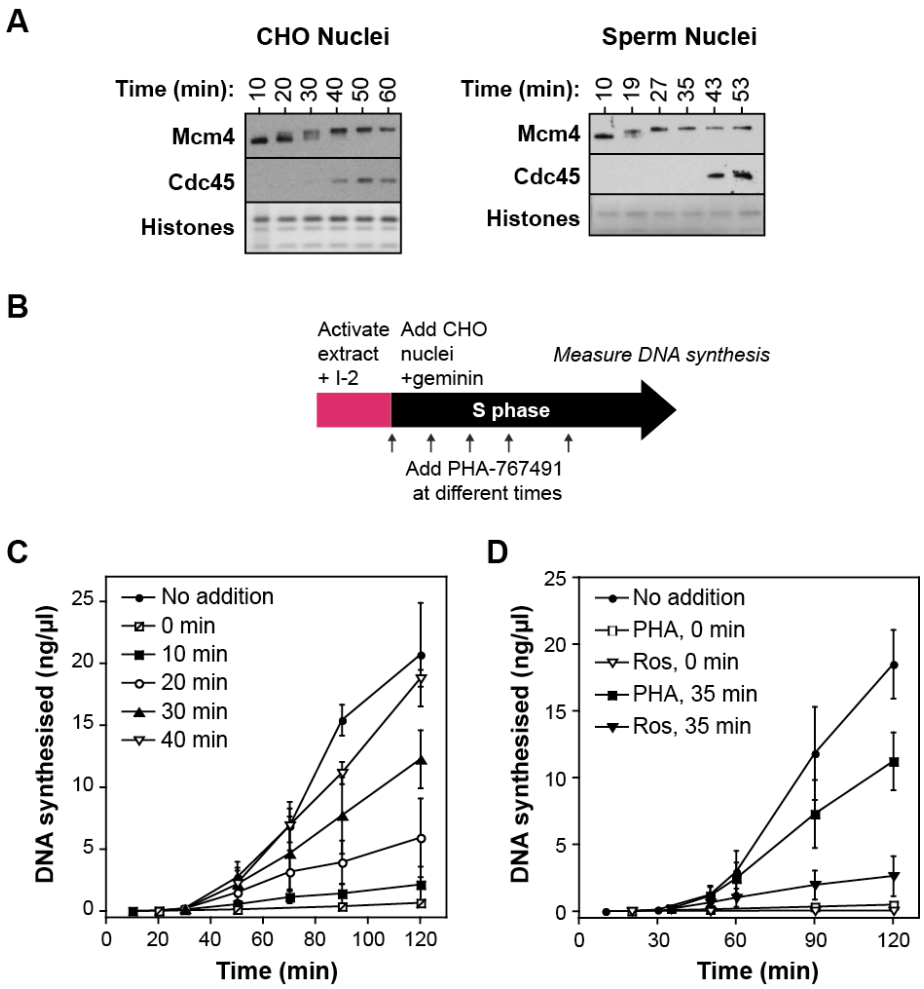

Figure 2.10. Cdc7 executes its essential function early in S phase, distinct from the Cdk execution point.

(A) Permeabilised G1 nuclei from somatic Chinese Hamster Ovary (CHO) cells were incubated at 60 ng DNA/µl in interphase *Xenopus* extract, supplemented with geminin to ensure that only origins licensed *in vivo* were used in replication. Sperm nuclei was incubated 10 ng DNA/µl in untreated interphase extract. Chromatin was isolated for immuno-blotting for Mcm4 and Cdc45 at the indicated times. (B) Schematic of time-of-addition experiments: *Xenopus* interphase extracts were first activated by the addition of $CaCl_2$ before geminin and permeabilised CHO nuclei were added. 20 µM PHA-767491 was added at different times throughout S phase and aliquots were sampled for total DNA synthesis after that. I-2 was added from the beginning of the reaction to prevent any de-phosphorylation of Mcm4. (C) PHA-767491 was added at 0, 10, 20, 30, and 50 minutes after the nuclei addition. At the indicated times, samples were removed and assayed for total DNA synthesis. (D) Nuclei were incubated as described in (A). At 0 or 35 minutes, extracts were supplemented with 20 µM PHA-767491 or 1 mM roscovitine and [α-^{32}P]dATP. Samples were assayed for total DNA synthesis at the indicated times. The error bars represent S.D. from 3 experiments.

2.6 The role of Cdc7 in the replication timing programme

In order to study the role of Cdc7 in the progression through the replication timing programme, the different patterns formed by active replication factories/clusters as nuclei progress through S phase *in vitro* were analysed (Dimitrova and Gilbert, 1999; O'Keefe et al., 1992; Thomson et al., 2010). G1 CHO nuclei were incubated in extract and PHA-767491 or roscovitine were optionally added at 35 minutes. Extracts were pulsed with Cy-3 dUTP at 60 or 90 minutes to allow the visualisation of actively replicating factories/ clusters. Nuclei in each condition and timepoint were then scored according to the stage in S phase they were in (Figure 2.11A).

When no inhibitor was present, most nuclei isolated at 60 minutes showed the Type III/IV replication pattern typical of mid – late S phase. This proportion subsequently increased to 60% at 90 minutes (Figure 2.11B). When roscovitine was added at 35 minutes, the replication profile was significantly slowed relative to control, showing that continued Cdk activity is required for normal progression through the replication timing programme (Thomson et al., 2010). In contrast, when PHA-767491 was added at 35 minutes, the overall replication pattern progression from 60 to 90 min was largely similar to controls. Continued Cdc7 activity therefore appears not to be required for progression through the replication timing programme once origins have been initiated, if Mcm dephosphorylation is prevented.

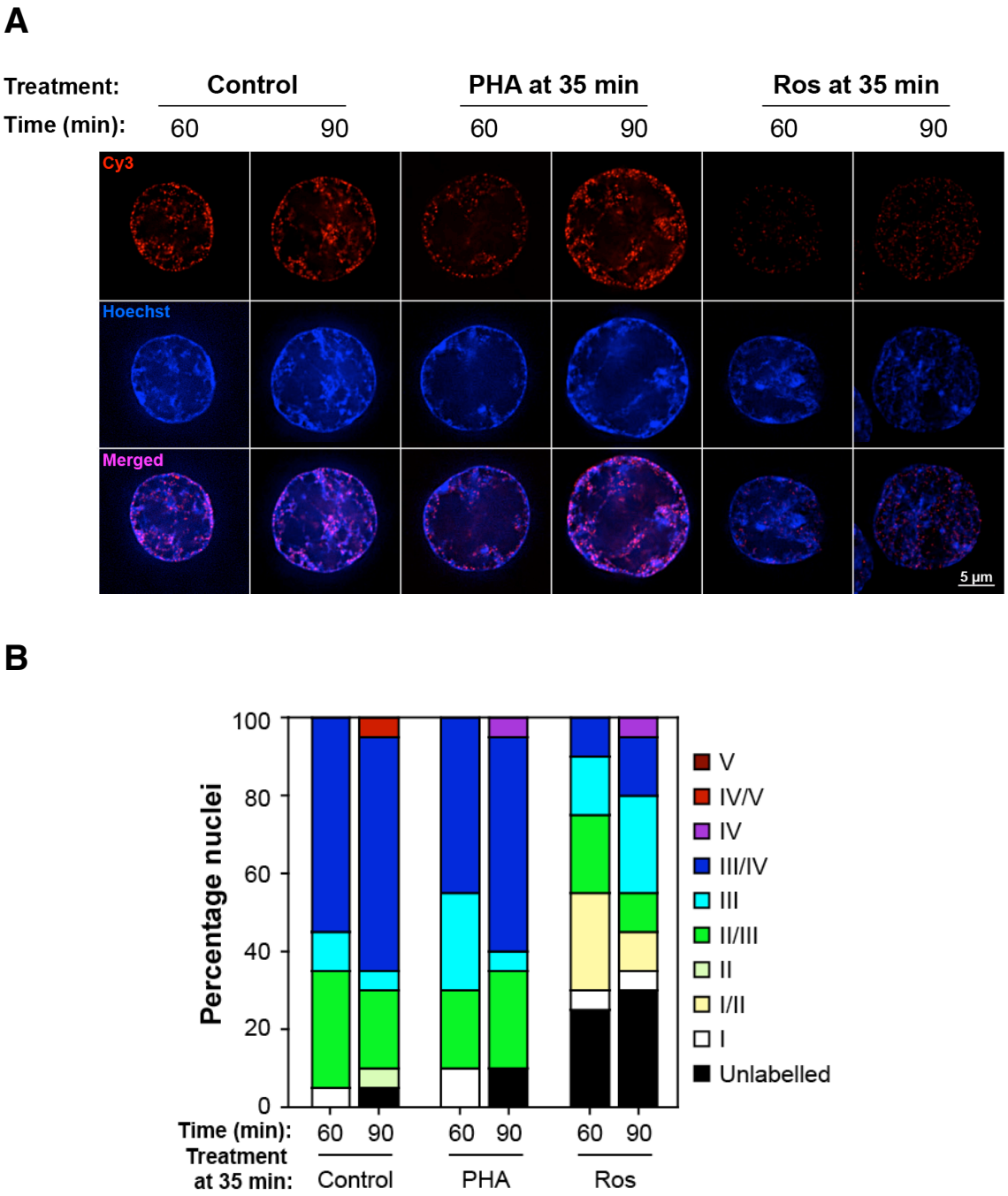

Figure 2.11. PHA-767491 does not affect progression through replication timing stages.

(A) Permeabilised mammalian nuclei were incubated in interphase extracts as in Figure 2.10D. During early S phase (35 min), PHA-767491 or roscovitine was added to the replicating extract. At 60 min or 90 min, extracts were supplemented with 25 µM Cy3-dUTP to label actively replicating origins. Nuclei were fixed and DNA stained with Hoechst 33258 for visualisation. (B) Nuclei were scored for the stage of replication based on the categories devised by Thomson et al (Thomson et al., 2010). The experiment was repeated 3 times and at least 20 nuclei were scored for each treatment at each timepoint. Similar trends were observed in all experiments; this bar chart represents data from one experiment.

2.7 Etoposide treatment and the role of Cdc7 in the checkpoint response

2.7.1 Multiple proteins modulate Cdc7 activity upon etoposide treatment

Previous studies have implicated Cdc7 in the intra-S checkpoint response to etoposide, an inhibitor of topoisomerase II (Topo II) that causes accumulation of covalently-linked protein-DNA complexes between Topo II and chromatin (Costanzo et al., 2003; Silva et al., 2006; Tsuji et al., 2008). However, the role of Cdc7 in this response remains controversial. To first establish if Cdc7 activity is regulated by checkpoint kinases in this system, *Xenopus* extract was treated with 300 μM etoposide prior to addition of *Xenopus* sperm chromatin. As shown in Figure 2.12A, the addition of etoposide caused a decrease in the levels of Mcm4 hyper-phosphorylation compared to controls. When caffeine was added to abolish ATM and ATR checkpoint kinase activities, Mcm4 hyper-phosphorylation and DNA synthesis were restored to control levels in etoposide-treated extracts. The checkpoint response on Cdc7 activity is not dependent on on-going replication, as Mcm4 hyper-phosphorylation was still inhibited when etoposide was added to extracts in the presence of $p27^{kip1}$ (Figure 2.12B).

Because caffeine is a relatively non-specific inhibitor of ATM/ATR family kinases, specific inhibitors KU55993 and NU7441, were used to bypass the effect of the ATM and DNA-PK kinases respectively. From Figure 2.12C, KU55993 and NU7441 were able to restore high levels of Mcm4 hyper-phosphorylation. The total amount of DNA synthesised was also slightly rescued. This suggests that both ATM and DNA-PK play a role in etoposide-induced inhibition of Mcm4 hyper-phosphorylation. When PP1 was inhibited by pre-treatment of extract with the I-2 inhibitor, similar results were obtained, although the rescue of DNA synthesis was negligible in this case (Figure 2.12D). This implies etoposide-induced inhibition of Mcm4 hyper-phosphorylation is sensitive to PP1

activity levels. The lack of replication rescue could suggest that other checkpoint targets are involved in this response, for instance Cdk2 which had been shown to be targeted by ATM in *Xenopus* egg extracts to inhibit DNA replication (Costanzo et al., 2000).

Upon etoposide treatment, there appears to be some variation in the level of replication inhibition, which may reflect some biological variation between extract preparations. Comparing Figures 2.12A – D, replication was inhibited to 30 – 50% of control levels. Looking at the level of Mcm4 hyper-phosphorylation in Figure 2.12A, a distinct inhibition of phosphorylation could be observed, where the maximal level of phosphorylation was not attained at any timepoints assayed in etoposide-treated extracts. In contrast, the highest level of Mcm4 phosphorylation was attained in the presence of etoposide in the experiments shown in Figures 2.12B – D, although the overall level of phosphorylation was lower in all instances, as evident in the Mcm4 smears, representing less phosphorylated protein complexes with higher mobility. This variation is also reflected in the Cdc45 blots: a marked decrease in chromatin-bound Cdc45 is perceptible in Figures 2.12A and D while differences in levels are subtler in Figures 2.12B and C. However in all experiments, there was a consistent delay in the Cdc45 recruitment to DNA in etoposide-treated extracts compared to controls, indicating delayed replication initiation and/or less efficient replication.

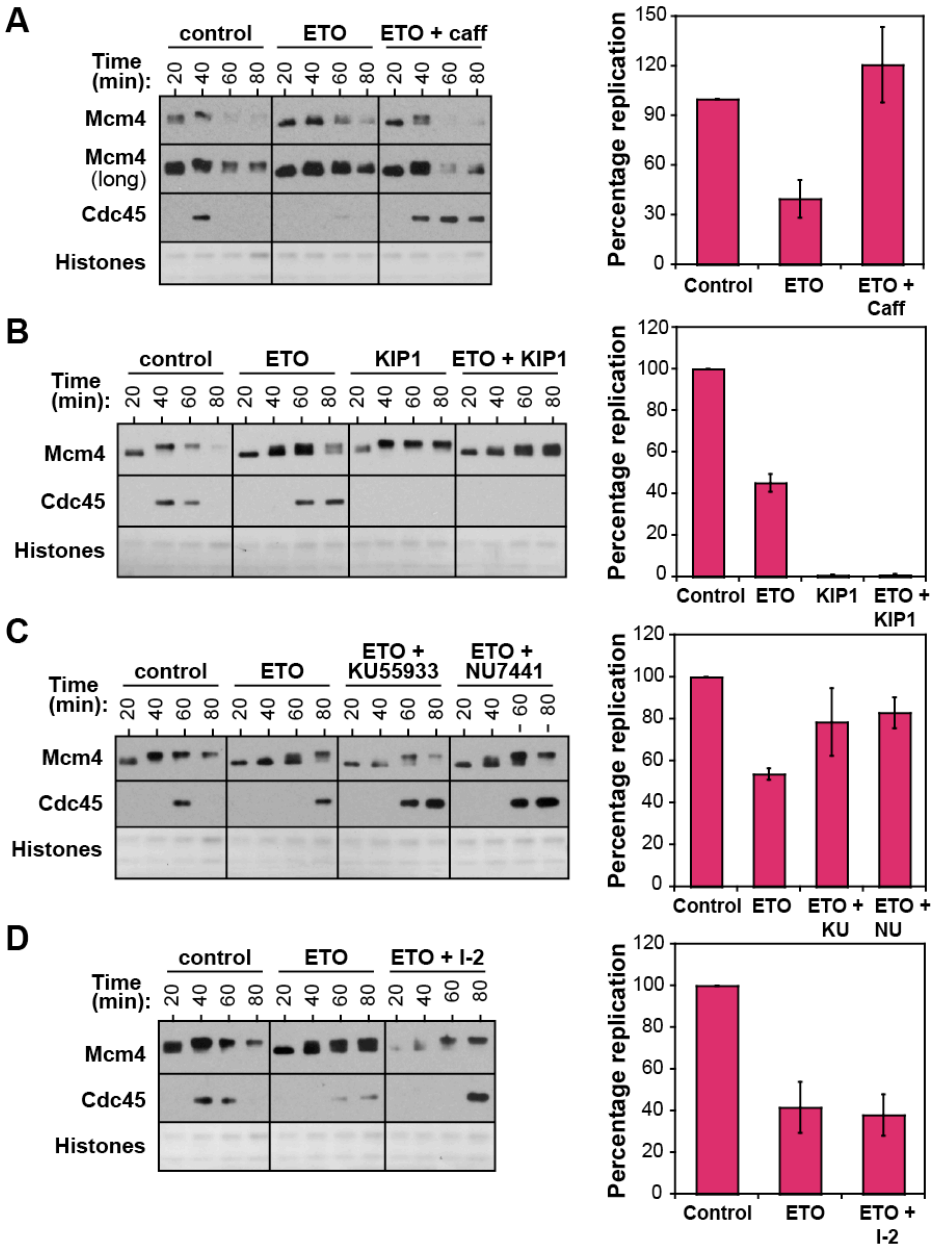

Figure 2.12. Etoposide treatment induces a checkpoint-dependent effect on DNA replication in *Xenopus* egg extracts.

(A) 10 ng DNA/μl sperm nuclei were incubated in extracts treated with 300 μM etoposide or 300 μM etoposide and 5 mM caffeine. Chromatin was isolated for immuno-blotting at the indicated timepoints. (B) Extracts were set up as described in (A), treated with 300 μM etoposide or 300 μM etoposide and 100 nM $p27^{KIP1}$. Chromatin was isolated from immuno-blotting or extracts were supplemented with [α-^{32}P]dATP to assay for total DNA synthesis. (C) Extracts were set up as described in (A) and the specific inhibitors of ATM (KU55933) and DNA-PK (NU7441) were used at final concentration of 10 μM. (D) Extracts were set up as described in (A) and PP1 I-2 was pre-incubated for 15 min before sperm nuclei addition, at a final concentration of 1.2 μM. The error bars represent S.D. from 3 experiments in (A) and (D) and from 4 and 2 experiments respectively in (B) and (C).

2.7.2 Addressing the mechanism of the checkpoint response

2.7.2.1 ATM signalling

Etoposide can potentially induce extensive double strand breaks when replication forks encounter inhibited Topo II. To examine if ATM is the main checkpoint kinase responsible for this response, the restriction enzyme EcoRI was added to egg extracts to create double-stranded breaks in chromatin. ATM is primarily responsible for the signalling cascade in response to double-stranded lesions, involving phosphorylation of histone H2AX, whereas sensing of single-stranded breaks normally occurs through ATR. Figure 2.13A shows that etoposide treatment reduced Mcm4 hyper-phosphorylation but did not recruit γ-H2AX to chromatin (Figure 2.13A). This is in contrast to EcoRI treatment, which resulted in high levels of chromatin-bound γ-H2AX but had little effect on Mcm4 hyper-phosphorylation. DNA synthesis was completely inhibited, unlike etoposide treatment, which had allowed some degree of DNA replication. When KU55933 and caffeine were used to bypass checkpoint signalling, both inhibitors caused a similar decrease in the levels of γ-H2AX in response to EcoRI treatment. However, while caffeine was able to rescue DNA replication to some extent, KU55933 addition did not reverse inhibition of DNA replication (Figure 2.13B). These results show that DSB generation with EcoRI clearly triggers a different checkpoint response compared to etoposide. As such, the checkpoint response to etoposide that inhibits Mcm4 hyper-phosphorylation is unlikely to be solely mediated through the formation of double stranded breaks and ATM signalling.

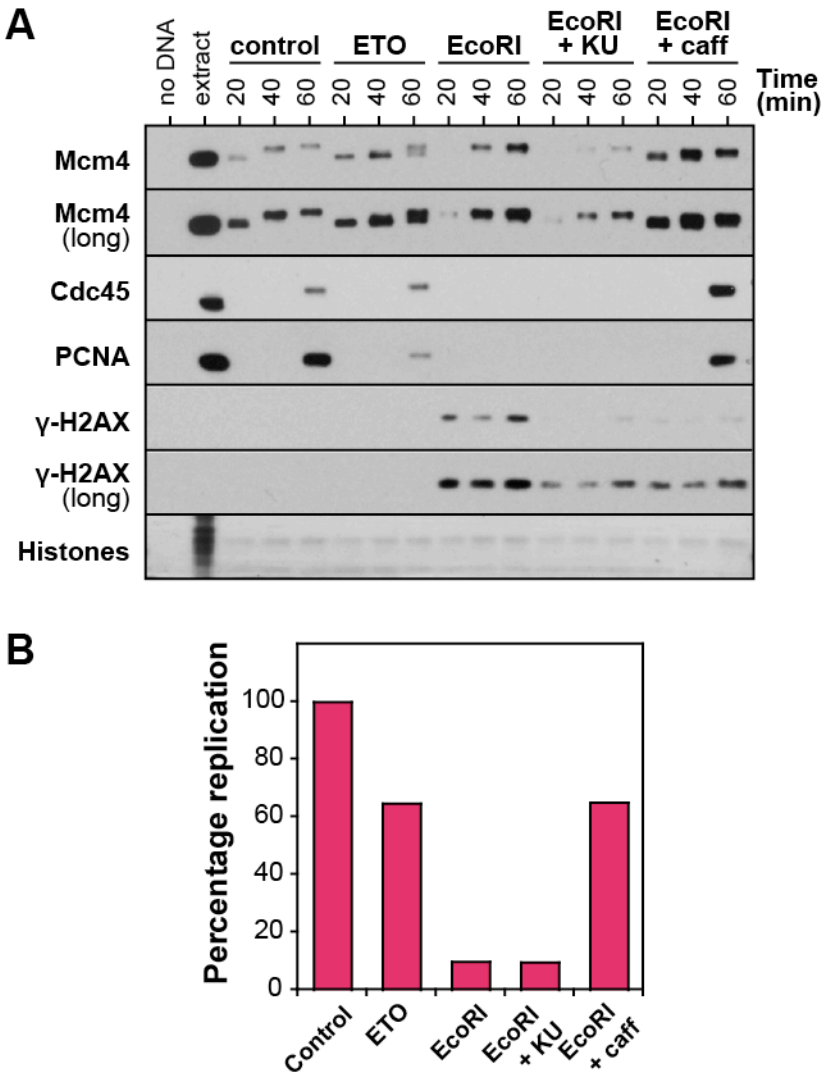

Figure 2.13. Etoposide does not cause a checkpoint response on Cdc7 activity solely through DSBs.

(A) 10 ng DNA/μl sperm nuclei were incubated in extracts treated with 300 μM etoposide, EcoRI alone or EcoRI supplemented with either 5 mM caffeine or 10 μM KU55933. Chromatin was isolated for immuno-blotting at the indicated time-points. (B) DNA synthesis was quantified at 90 min.

2.7.2.2 Addressing kinase down-regulation or phosphatase up-regulation

It was shown in an earlier section (Section 2.3) that PP1 is involved in modulating Mcm4 hyper-phosphorylation in concert with Cdc7 activity and that inhibition of PP1 by I-2 pre-treatment was able to reverse the loss of hyper-phosphorylation upon etoposide treatment (Figure 2.7). It is thus possible that this checkpoint response may be executed through down-regulating Cdc7 activity, up-regulating PP1 function or a combination of both. An experiment was designed to distinguish between these possibilities by assessing the activity of one enzyme (PP1) in the absence or presence of caffeine while the activity of the counteracting enzyme (Cdc7) is acutely blocked by the addition of a specific inhibitor (PHA-767491), under conditions of checkpoint activation. If PP1 activity were the main target of this checkpoint response, a difference in Mcm4 dephosphorylation would be observed between extracts with or without caffeine treatment. As shown in Figure 2.14, extracts were set up that were untreated, treated with etoposide or etoposide plus caffeine and sperm nuclei were incubated for 60 minutes with $p27^{kip1}$ addition to allow accumulation of maximally phosphorylated Mcm4 on chromatin. In mid-S phase, PHA-767491 was added (Time = 0 min) and chromatin rapidly isolated to assess the loss of Mcm4 phosphorylation under different conditions. If the checkpoint response is mediated through Cdc7 down-regulation, no difference was expected between extracts treated with etoposide whether caffeine was present added or not.

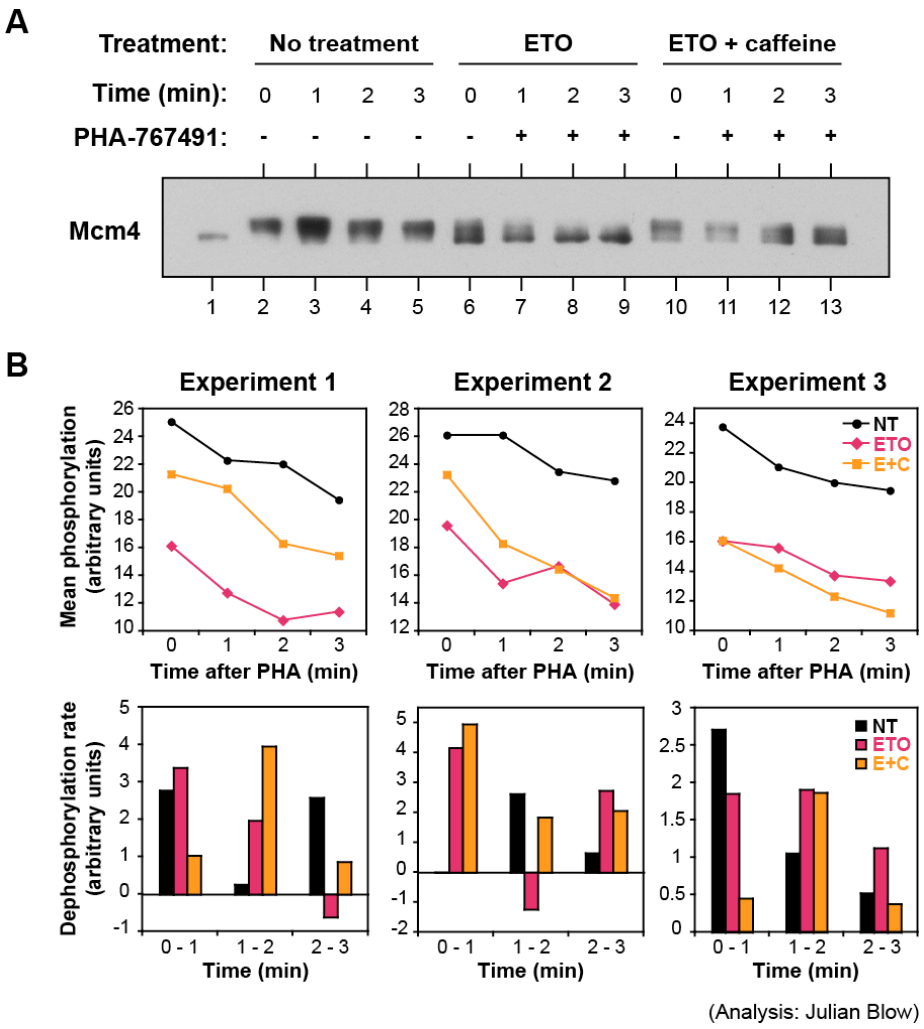

Figure 2.14. Rate of loss of phosphorylation in etoposide-treated extract

(A) 10 ng DNA/µl sperm nuclei were incubated in extracts treated with 300 µM etoposide or etoposide supplemented with 5 mM in the presence of $p27^{kip1}$. At 60 min, 50 µM PHA-767491 was added and an aliquot was immediately taken and diluted in cold isolation buffer. Aliquots were taken at the indicated time-points and chromatin was isolated and blotted for Mcm4. Whole extract was loaded in lane 1 as a control showing unphosphorylated Mcm4. (B) Mean phosphorylation (top row) was calculated by multiplying the densitometric quantification of Mcm4 band intensity, measured from bottom to top (low to high phosphorylation), with pixel position number, also measured from bottom to top, to take into account phosphorylation state. The black line represents untreated samples, pink: etoposide-treated and orange: etoposide with caffeine. Dephosphorylation rate (bottom row) was calculated by the change of mean phosphorylation over time, normalised to each initial mean phosphorylation value, to take into account the availability of substrates for dephosphorylation at each timepoint. Quantification was carried out for 3 experiments, as labelled above the graphs. The blot shown in (A) corresponds to Experiment 1. Julian Blow performed the densitometric quantification of blots and analyses shown in (B).

In control samples, Mcm4 was maximally phosphorylated at 0 min (Figure 2.14A). Over the course of the experiment, the major proportion of Mcm4 remained maximally hyper-phosphorylated. Some smearing of Mcm4 could be observed from 1 – 3 min, indicating that some dephosphorylation may be occurring during chromatin isolation. When extracts were treated with etoposide, Mcm4 did not reach maximal phosphorylation, indicating that a checkpoint response had been induced. Mcm4 then became dephosphorylated upon Cdc7 inhibition by PHA-767491, so that the major proportion of chromatin-bound Mcm4 was already dephosphorylated by 2 min. In the presence of caffeine, Mcm4 was more highly phosphorylated at 0 min, as expected, and subsequently also became dephosphorylated when PHA-767491 was added, noticeable in the increased intensity of the non-phosphorylated lower Mcm4 band.

The differences in the Mcm4 phosphorylation levels at 0 min of the experiment introduce considerable difficulty in determining the rate of dephosphorylation over the time-course. This is compounded by differently phosphorylated Mcm4 species presenting as a continuous smear in immuno-blotting. An attempt was made to measure the rate of dephosphoryation by quantifying the density of the Mcm4 bands in relation to the position of the band, which corresponds to the level of phosphorylation. This allows the derivation of a mean phosphorylation value for each band, as shown in Figure 2.14A. The rate of dephosphorylation is the decrease of this value from one time-point to the next. If this value were then normalised against the total phosphorylation of each sample, it would take into account substrate availability, that is, the amount of phosphorylated Mcm4, for PP1 to act on at the time of dephosphorylation. However, as shown in Figure 2.14B, no consistent trend can be observed over 3 independent experiments. A conclusive interpretation about the mechanism of checkpoint-mediated Mcm4 dephosphorylation could not be drawn from this quantification approach. An alternative approach using radio-active [γ-^{32}P]ATP may prove to be more amenable to the quantification of phosphorylation levels and rates.

2.7.2.3 Etoposide affects nuclear envelope formation and Cdc7 association to chromatin

Thus far, attempts to delineate the etoposide-induced checkpoint response have been met with non-conclusive results. However, with the availability of a Cdc7 antibody, Dr Gaganmeet Singh Chadha was able to carry out further experiments to elucidate the mechanism of this checkpoint response in egg extracts.

Consistent with the earlier report (Costanzo et al., 2003), the amount of Cdc7 associated with chromatin was reduced in the presence of etoposide (Figure 2.15A). Although caffeine addition rescued both Mcm4 hyper-phosphorylation and DNA replication (Figure 2.12), abolishing ATM and ATR checkpoint kinase activity did not restore normal levels of Cdc7 on chromatin. Densitometric quantification confirms that etoposide has two effects on Cdc7 activity: a caffeine-dependent inhibition of Mcm4 phosphorylation and a caffeine-insensitive inhibition of Cdc7 chromatin association. As such, inhibition of checkpoint kinases can restore Mcm4 hyper-phosphorylation even as levels of chromatin-associated Cdc7 remain low.

In *Xenopus* egg extract, Topo II is rapidly loaded onto sperm chromatin as it undergoes decondensation and has been implicated in the process of nuclear envelope assembly (Khoudoli et al., 2008; Lucas et al., 2001; Takasuga et al., 1995). It is possible that etoposide, a topoisomerase II inhibitor, interferes with normal nuclear assembly, hence leading to inefficient loading of Cdc7 onto chromatin. As shown in Figure 2.15C, nuclei assembled in the presence of 300 μM etoposide (“ETO at 0 min”) were significantly smaller than control nuclei. However, when etoposide was added 30 min after sperm addition, after the bulk of DNA decondensation had already occurred, nuclei subsequently attained normal sizes (“ETO at 30 min”). Consistent with this, later additions of etoposide trapped less topo II on DNA, allowed more Cdc7 to bind to

chromatin and allowed normal levels of Mcm4 hyper-phosphorylation (Figure 2.16D). As mentioned earlier, Cdc7 is enriched within the nucleoplasm when nuclear assembly occurs (Walter, 2000), allowing efficient Mcm4 hyper-phosphorylation prior to initiation of DNA replication. Taken together, it appears that the checkpoint-independent response on Cdc7 binding due to etoposide treatment is the consequence of disruption to nuclear envelope assembly. Activation of checkpoint-mediated responses may then involve PP1, possibly through recruitment to chromatin, to further functionally suppress Cdc7 activity against chromatin-bound Mcm2-7.

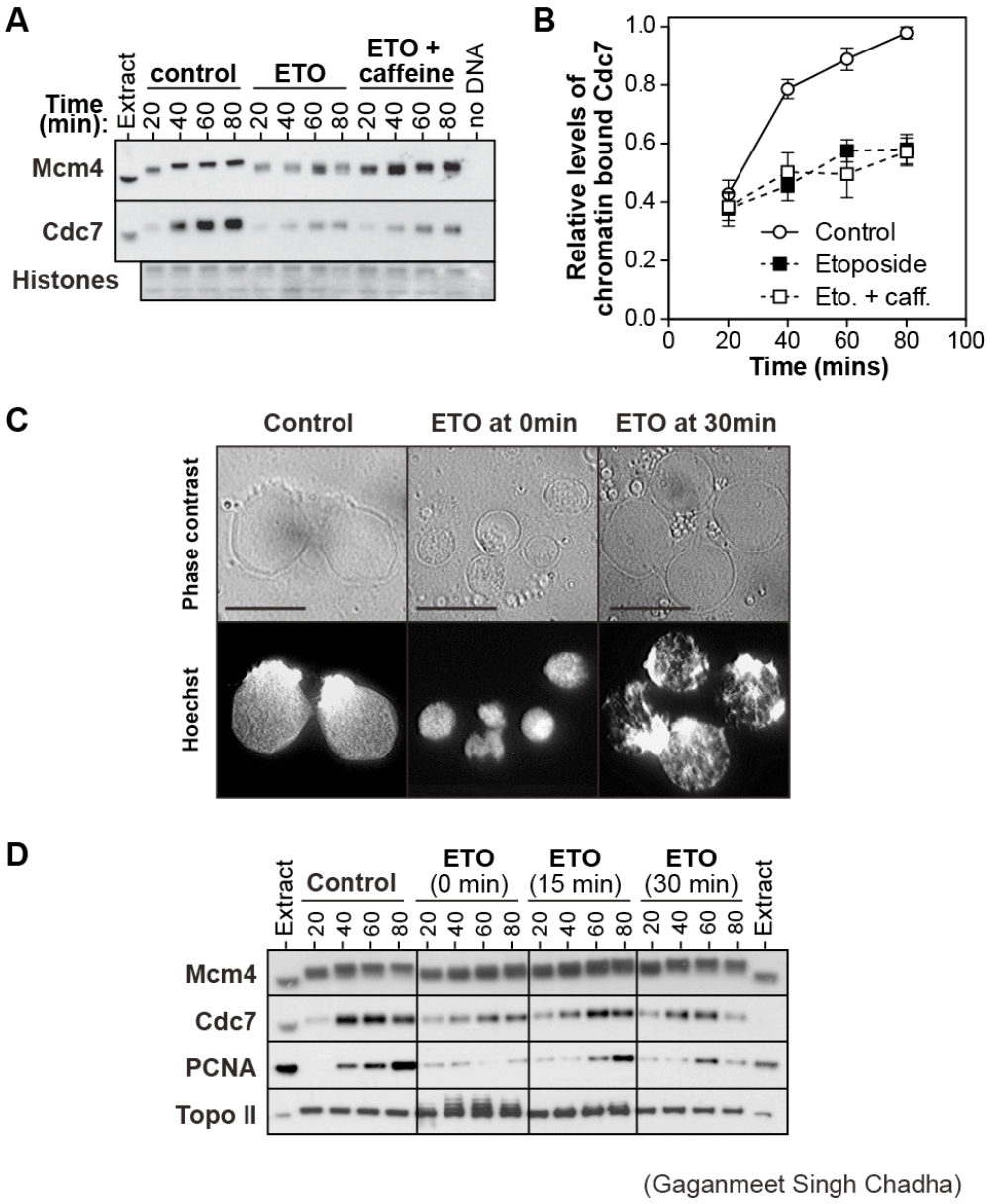

Figure 2.15. Early addition of etoposide inhibits nuclear envelope assembly and reduces Cdc7 activity on chromatin.

(A) 10 ng DNA/μl sperm nuclei were incubated in extracts treated with 300 μM etoposide or 300 μM etoposide and 5 mM caffeine. Chromatin was isolated for immuno-blotting at the indicated time-points. (B) Cdc7 levels were quantified and expressed as a proportion of the peak value. Mean and S.E.M. for 6 independent experiments are shown. (C) Extracts were supplemented with 300 μM etoposide at 0, 15 or 30 min following sperm nuclei addition. After 90 min, nuclei were stained with Hoechst 33258 and visualised by phase contrast (top) or fluorescence microscopy (bottom). Scale bars represent 25 μM. (D) Chromatin was isolated for immuno-blotting for Mcm4, Cdc7, PCNA and Topo II at the indicated time-points. Gaganmeet Singh Chadha performed the experiments presented in this figure.

Discussion

Previous studies of Cdc7 function in the *Xenopus* system were carried out by immuno-depleting Cdc7 and/or its regulatory subunits from egg extracts (Jares and Blow, 2000; Jares et al., 2004; Kundu et al., 2010; Pereverzeva et al., 2000; Silva et al., 2006; Takahashi et al., 2008; Takahashi and Walter, 2005; Yanow et al., 2003). This approach generally involves substantial incubation intervals with specific antibodies and result in the dilution of extracts used in the experiments. Non-specific protein absorption to beads may also occur that affects the amounts and types of protein removed from the extract (Silva et al., 2006). Using specific chemical inhibitors to block Cdc7 activity potentially introduces fewer disturbances into the biochemical environment for DNA replication, providing greater precision in defining Cdc7 activity and execution points in the cell cycle in this model system.

The effect and specificity of PHA-767491

PHA-767491 was reported to be a small molecule inhibitor that is specific for Cdc7 (Montagnoli et al., 2008). In order to analyse its selectivity, PHA-767491 was characterised across a panel of 95 protein kinases, out of which 14 had been included in the assay in an earlier study (Montagnoli et al., 2008). Results from the two screens were reasonably consistent, in which higher significant cross-reactivity was detected with GSK3-b and Cdk2. While GSK3-b has not been reported to be required in the initiation of DNA replication, Cdk2-cyclin A and Cdk2-cyclin E can both induce initiation of DNA replication in *Xenopus* egg extracts. In interphase egg extracts treated with protein synthesis inhibitors, Cdc2 (Cdk1) is inactive and initiation of replication depends on Cdk2 activity (Strausfeld et al., 1996). Since S-Cdk and Cdc7 are both required for the initiation of DNA replication, it was important to establish that PHA-767491 did not inhibit DNA replication due to significant cross-reactivity towards S-Cdk activity.

Functional assays were thus carried out to test the specificity of PHA-767491 in the context of DNA replication in *Xenopus* egg extracts.

Since nuclear assembly is required for DNA replication to occur, it was first established that PHA-767491 did not perturb nuclear envelope formation (Figure 2.4). Mcm4 hyper-phosphorylation has been shown to be dependent on Cdc7 activity (Takahashi and Walter, 2005) and the essential function of Cdc7 may be to relieve an inhibitory activity in the amino terminal domain of Mcm4 (Sheu and Stillman, 2010). Looking at the effect on chromatin-bound proteins, it was observed that PHA-767491 inhibited Mcm4 hyper-phosphorylation at concentrations where DNA replication was inhibited. This is consistent with PHA-767491 inhibition of an essential Cdc7 function, hence preventing chromosomal replication. PHA-767491 appears to inhibit the kinase activity of Cdc7 without perturbing its association to chromatin, consistent with its chemical characterisation as an ATP-analogue (Vanotti et al., 2008).

Chromatin containing hyper-phosphorylated Mcm4 but which has not undergone the essential Cdk-dependent step in initiation can replicate efficiently in extract treated with PHA-767491 whilst naive sperm nuclei could not (Figure 2.8). Since Cdc7 activity is required before Cdk for replication in egg extracts (Jares and Blow, 2000; Walter, 2000), this experiment provided evidence that the Cdk-dependent step in DNA replication can take place in the presence of PHA-767491. While a full rescue of DNA replication did not take place, Cdk-dependent replication occurred in the presence of PHA-767491 to more than 60% of control levels, suggesting that even under the unlikely assumption that all residual inhibition of replication was due to inhibition of Cdk activity, this only accounts for less than half of the inhibitory activity of PHA-767491 observed. The failure of a complete rescue could be attributed to several reasons. Firstly, phosphorylation of other Cdc7 substrates that are not present on chromatin at the time of transfer, such as Cdc45, may be required for efficient replication. Additional

or accessory Cdc7 phosphorylation events may also require the prior action of Cdk, which would not have occurred at the time of transfer. Previous studies have indicated that phosphorylation of Mcm2 on certain phospho-sites by S-Cdk may enhance Cdc7-dependent kinase activity (Masai et al., 2000; Montagnoli et al., 2006). Second, weakly associated replication factors that could enhance replication efficiency may not be transferred under these experimental conditions. Although the omission of detergent during nuclei isolation preserves the integrity of the nuclear membrane, these isolated nuclei are 'leaky' and soluble nucleoplasmic proteins will largely be lost when nuclei are removed from extract into buffer (Gillespie et al., 2012). Lastly, some degree of Mcm2-7 dephosphorylation is inevitable even in the presence of I-2 in the extract and the possibility of PHA-767491 exerting an inhibitory effect on other targets cannot be completely ruled out.

Comparing the effects of PHA-767491 and roscovitine (Figure 2.10D), a well-characterised Cdk inhibitor, it is clear that both kinases act on targets with different execution times. Addition of PHA-767491 to extracts in early S phase allowed the majority of DNA synthesis to occur, in contrast to Cdk inhibition which almost completely blocked replication when added at this time. Also, since protein synthesis is inhibited by cycloheximide in these extracts, cyclin A is absent. All S phase promoting Cdk activity is dependent on Cdk2-cyclin E1 complexes (Strausfeld et al., 1996). This provides more evidence that cross-reactivity towards Cdk2-cyclin A is unlikely to be responsible for PHA-767491 inhibition of DNA replication. Looking at the kinetics of DNA synthesis (Figure 2.8C, compare whole and dashed lines), pre-incubated nuclei incorporated [α-^{32}P]dATP at a higher rate than naive sperm. This is consistent with the interpretation that replication origins in pre-incubated nuclei were competent for initiation, with pre-RCs assembled on chromatin and the essential Cdc7-dependent activity already executed in the first extract. In contrast, a lag was initially observed in naive sperm before DNA synthesis proceeded more efficiently after 40 minutes. Taken

together, PHA-767491 largely inhibits DNA replication by acting specifically on Cdc7 activity in *Xenopus* egg extracts.

PP1 reverses Cdc7-dependent phosphorylation of Mcm4

Protein phosphatase 1 (PP1) belongs to the phosphoprotein phosphatase superfamily of protein serine/threonine phosphatases, which also includes PP2A, PP2B and PP4-7. The specific activity and subcellular localisation of PP1 depend on the interaction of a PP1 catalytic subunit (PP1c) with one or sometimes two regulatory subunits. In mammalian tissues, four PP1 catalytic subunits, PP1α, PP1γ_1, PP1γ_2 and PP1β/δ, are encoded by three genes (Ceulemans and Bollen, 2004; Ceulemans et al., 2002; Cohen, 2002; Heroes et al., 2012). More than 200 PP1-interacting proteins (PIPs) have been identified in vertebrates (Heroes et al., 2012), thus allowing for the formation of a vast array of PP1 holo-enzymes with distinct substrate sets and differential regulation. This greatly contributes to and accounts for the diverse functions mediated by serine/threonine phosphatases, despite their relatively small number in the genome (~40) compared to serine/threonine kinases (~400) (Ceulemans and Bollen, 2004; Cohen, 2002; Moorhead et al., 2007).

PIPs bind to PP1 through short docking motifs of four to eight residues in length. Through a unique combination of binding motifs, each PIP can interact with the surface grooves of a particular PP1 catalytic isoform in a highly specific manner (Bollen et al., 2010; Heroes et al., 2012). Several docking motifs have been functionally characterised, of which RVxF is regarded to be the canonical PP1-binding motif. It is present in many but not all PIPs. Other PIPs that do not possess an RVxF motif can nevertheless interact with the same region of a PP1 catalytic subunit. Other docking motifs include the SILK-type binding motif and the myosin phosphatase N-terminal element (MyPhoNE) (Cohen, 2002; Heroes et al., 2012).

I-2 was the first protein phosphatase regulator to be identified (Huang and Glinsmann, 1976). Its expression is conserved from yeast to human (Ceulemans et al., 2002) and the protein ranges from 25 – 32 kDa in size across different species (Li et al., 2007). Incubation of I-2 with PP1 results in a slow inactivation of PP1 catalytic activity (Bollen and Stalmans, 1992). The crystal structure of the PP1c•I-2 complex revealed that the interaction of I-2 with PP1cγ occurs at three major sites. While I-2 binding does not result in a conformational change in PP1cγ, amino acid residues 148-151 from I-2 are directly positioned across the active site of PP1c and I-2 Tyr^{149} occupies the phosphoamino acid binding site of PP1cγ such that one or more metal ions required for PP1 enzymatic function is displaced from the active site, thereby inhibiting PP1 activity (Hurley et al., 2007).

Upon addition of PHA-767491, hyper-phosphorylation of chromatin-bound Mcm4 was lost very rapidly (Figure 2.7). This effect could be reversed using I-2, identifying PP1 to be responsible for Mcm4 dephosphorylation. Previous data have shown that a PP1 catalytic subunit physically interacted with Mcm2-7 complexes that were immuno-precipitated from chromatin assembled in *Xenopus* egg extracts (Gambus et al., 2011). Together, this is consistent with a scenario of PP1 being dynamically recruited to chromatin during S phase, thereby being ideally localised to modulate the kinase activity of chromatin-bound Cdc7. This is the first report of a phosphatase acting with Cdc7 to modulate an essential activity required for DNA replication.

PP1 has been shown to be involved in cell cycle progression, regulating mitotic events such as chromosome condensation, spindle formation, chromosome separation and cytokinesis (Ceulemans and Bollen, 2004). PP1 also plays multiple roles in regulating the *Xenopus* early embryonic cell cycle including being required for chromatin decondensation before DNA replication (Walker et al., 1992). This report of a

phosphatase acting in concert with the Cdc7 kinase and its identification as PP1 has other implications for the regulation of Cdc7 activity in DNA replication, which would be discussed greater detail in later sections.

Cdc7 acts early in S phase and is not rate-limiting for DNA replication

In eukaryotic cells, DNA replication occurs through a series of ordered events leading to the loading of a double hexamer of the Mcm2-7 complex around origin DNA to form the pre-RC, thereby licensing the origin for replication in the subsequent S phase (Evrin et al., 2009; Gillespie et al., 2001; Remus et al., 2009; Remus and Diffley, 2009). In this study, I used PHA-767491 to define precisely when Cdc7 acts in the sequence of events leading to replication initiation in *Xenopus* egg extracts

In egg extracts, decondensed chromatin becomes surrounded by a functional nuclear envelope, which is stimulated by licensed origins promoting the assembly of the nuclear pore precursor ELYS (Gillespie et al., 2007). Comparison of soluble and chromatin-bound Mcm4 showed that soluble Mcm4 was not hyper-phosphorylated (Figure 2.9A), consistent with observations from earlier studies (Masai et al., 2006; Pereverzeva et al., 2000; Sheu and Stillman, 2006). The association of Mcm3 (and by inference, the Mcm2-7 complex) with chromatin has been shown to be independent of nuclear envelope assembly (Chong et al., 1995; Jares and Blow, 2000) and consistent with this observation, Mcm4 was detected on chromatin in the absence of nuclear assembly (Figure 2.9C). In contrast, the recruitment of Cdc45 occurred only when Mcm4 was maximally hyper-phosphorylated (Figures 2.5A and 2.9A) and did not take place when WGA was present (Figure 2.9C). *In vitro* studies in *Drosophila* provided evidence that Mcm2-7 must be associated with Cdc45 and the GINS complex in order to be active as a helicase (Ilves et al., 2010; Moyer et al., 2006). Since Cdc45 recruitment to chromatin is Cdc7-dependent and first requires nuclear envelope

formation (Jares and Blow, 2000), this is consistent with Cdc7 executing its essential function only after nuclear envelope formation in late M/early G1 phase. Although not strictly required, nuclear envelope assembly enhances Cdc7 association with chromatin (Silva et al., 2006; Walter, 2000). This is in line *in vitro* data from reconstituting replication initiation and elongation in yeast extracts, showing that Cdc7 function is required in late G1 to drive the formation of a complex between Sld3 and Cdc45. This complex associates with early origins prior to S-Cdk-dependent recruitment of Sld2, Dpb11 and GINS, which is then stabilised upon S-Cdk action (Heller et al., 2011). Once Cdc7-dependent phosphorylation of the Mcm2-7 complex has occurred, S phase-Cdk activity can promote initiation by phosphorylating its substrates (Heller et al., 2011; Jares and Blow, 2000; Walter, 2000).

Cdc7 phosphorylation of Mcm2-7 does not affect the stability of the double hexameric Mcm2-7 complex, believed to the active conformation of the helicase, before replication initiation (Gambus et al., 2011). This means that even if Cdc7 acts on all origins early, hyper-phosphorylated Mcm4 complexes may be maintained on chromatin throughout S phase, remaining primed for firing to occur according to the replication timing programme.

The precise duplication of eukaryotic genomic DNA takes place over a replication timing programme with origins organised in spatially distinct clusters that fire at different times throughout S phase. Although origin usage within clusters may vary significantly from cell to cell, the stage of S phase when specific clusters replicate is reproducibly consistent between different cells. S phase progression can be followed by visualising these actively replicating clusters (Chagin et al., 2010; Gillespie and Blow, 2010; Zink, 2006). It has been proposed that the replication of genomic DNA may be organised into three levels of regulation, namely, at the level of individual origin firing, clusters of origins firing and progression through different timing stages

(Thomson et al., 2010), although the molecular basis of the hierarchical differences and regulatory mechanisms is currently poorly understood.

A plausible model is that S phase Cdk acts as a global regulator to phosphorylate soluble replication factors while Cdc7 preferentially associates with Mcm2-7 at early-replicating origins early in S phase until these origins fire, thereby displacing Cdc7 to initiate later-firing origins. Cdc7 acts to fine tune the timing programme at a local level. It has been reported that while activation of new replication factories is highly sensitive to Cdk activity, initiation of origins within factories and progress through new timing stages is not (Thomson et al., 2010). This fits with the idea that Cdk regulates origin firing at a global level, perhaps through phosphorylating soluble substrates, Sld2 and Sld3 in yeast (or their metazoan equivalent), while another regulator operates at the level of individual origins. As Cdc7 associates with chromatin through its interactions with the Mcm2-7 complex, it is ideally localised to exert a local effect on individual origins.

However, the data in this study show that this is not how the replication timing programme is driven in *Xenopus* egg extracts. Here, Cdc7 phosphorylates and activates both early- and late-firing origins early in S phase. This is consistent with earlier work showing quantitative phosphorylation of Mcm2-7 in *Xenopus* egg extracts, which occurs even though only a fraction of these origins normally initiate in S phase (Edwards et al., 2002; Pereverzeva et al., 2000; Woodward et al., 2006). This conclusion appears to be inconsistent with studies in yeast which show that DDK activity is rate-limiting for origin initiation. Cdc7 was shown to be required throughout S phase for the initiation of early and late-firing origins in budding yeast (Donaldson et al., 1998a). In fission yeast, Patel and co-workers showed that changing levels of Hsk-Dfp1, the *S. pombe* homologue of Cdc7-Dbf4, corresponded with a change in origin efficiency. Physically tethering Hsk-Dfp1 near an origin also increased firing efficiency,

indicating that the effective local concentration of the kinase regulates origin firing (Patel et al., 2008). Recent reports examining temporal regulation of origin firing in budding yeast proposed that limited Cdc7 (DDK) activity regulates the timing of replication (Mantiero et al., 2011; Tanaka et al., 2011a), due to limited levels of Dbf4 (Mantiero et al., 2011). Over-expression of Dbf4 facilitated late origin firing in both studies.

Taking into account the observation Cdc7-dependent phosphorylation of Mcm4 is rapidly reversed by PP1, one interpretation that can reconcile these differences may be that although Cdc7 can execute its essential function early in S phase, sustained kinase activity is still required for normal S phase progression because of constant dephosphorylation. The requirement of Cdc7 in throughout S phase may be explained thus: in late G1/early S phase, Cdc7 executes its essential function to hyper-phosphorylate Mcm4, relieving an inhibitory effect of the Mcm4 NSD on DNA replication and activating the Mcm2-7 through other phosphorylation events. PP1 is closely and dynamically associated to chromatin and constantly dephosphorylates Mcm4. In an unperturbed S phase, the equilibrium is maintained towards net (hyper-) phosphorylation of Mcm4. Origins thereby remain competent for replication throughout S phase as long as Cdc7 activity is present. If Cdc7 activity is inhibited during S phase, the equilibrium shifts towards dephosphorylation by PP1 and overall Mcm4 hyper-phosphorylation is reduced. The NSD exerts its inhibitory function and origins that have not initiated can no longer be fired. This argument is consistent with the observation that inhibition of PP1 activity enhances rescue of replication in the chromatin transfer experiment (Figure 2.8).

A direct comparison between the effects of PHA-767491 and roscovitine indicates that Cdk inhibition continued to inhibit DNA synthesis whereas replication could take place relatively efficiently in the presence of PHA-767491 when both inhibitors were added to

the extract in early S phase (Figure 2.10). Previous studies have shown that S phase-Cdk activity (Cdk2-cyclin E and Cdk2-cyclin A) is needed throughout S phase for the initiation of replication when demembranated sperm nuclei were used as templates for replication but is not required for fork progression (Krasinska et al., 2008; Luciani et al., 2004; Strausfeld et al., 1994). The data in this study show that Cdc7 executes its function before Cdk; this supports the argument that once it has executed its function, Cdc7 activity is not the rate-limiting step in initiating origins throughout S phase. When the progress of nuclei undergoing replication was examined, PHA-767491 addition in early S phase did not prevent the appearance of later stage temporal patterns. This is also in agreement with the hypothesis that progression through the timing programme can be uncoupled from absolute level of DNA synthesis (Thomson et al., 2010) and shows that Cdc7 activity is not required for the advancing through the timing programme. If Mcm dephosphorylation is prevented, the early activity of Cdc7 is sufficient to allow approximately normal progression through the replication timing programme. In contrast, Cdk activity is required throughout S phase for new initiation events to occur (Luciani et al., 2004) and for progression through the replication timing programme (Thomson et al., 2010).

To sum up, while rate-limiting DDK activity contributes to the regulation of origin use during S phase in yeasts, the rapid phosphorylation of all replication origins by Cdc7 in *Xenopus* egg extracts may represent an adaption for the extremely short S phases that occur in an early embryonic environment. A residual timing programme may still be operative in *Xenopus* egg extracts and in this case, it appears to depend on rate-limiting Cdk activity or on limiting availability of Cdk substrates required for replication initiation (Krasinska et al., 2008; Labit et al., 2008; Luciani et al., 2004; Thomson et al., 2010).

Cdc7 in the intra-S checkpoint response

Previous studies have been carried out to investigate the checkpoint response triggered by the topoisomerase II inhibitor etoposide in *Xenopus* egg extracts. However, results from these studies were not consistent. Costanzo and co-workers reported that etoposide treatment inhibited replication, which could be reversed by the addition of caffeine. This checkpoint response was attributed to ATR signalling and down-regulated Cdc7 kinase activity by impeding Cdc7 association with Dbf4. However, chromatin-bound Cdc7 was still detected in etoposide-treated extracts. The authors proposed that etoposide generated single-stranded DNA structures, which RPA bind to and subsequently recruit ATR, thus triggering the checkpoint response (Costanzo et al., 2003). In a different study, etoposide was found to induce an ATR-dependent pathway through a similar mechanism as a result of slowed replication forks. However, etoposide treatment had no effect on the binding of the Drf1-Cdc7 or Dbf4-Cdc7 complexes to chromatin (Silva et al., 2006). Tsuji et al. also found that DNA replication was inhibited in the presence of etoposide and Chk1 phosphorylation was dramatically increased, indicating active ATR-Chk1 signalling. The authors showed that addition of Dbf4-Cdc7 to extracts treated with a low concentration of etoposide reduced Chk1 phosphorylation and restored DNA replication. They interpreted these results as Cdc7 playing an active role to attenuate the checkpoint response rather than being targeted for down-regulation (Tsuji et al., 2008). Studies in human cells also show that Cdc7 remains active in response to replicative stress and may be important in recovery functions (Heffernan et al., 2007; Silva et al., 2006; Takahashi and Walter, 2005; Tenca et al., 2007; Tsuji et al., 2008)

In this study, it was observed that Mcm4 hyper-phosphorylation and DNA replication was reduced when extract was treated with etoposide. This response is checkpoint-dependent as it is reversible upon caffeine treatment. When ATM or DNA-PK was

specifically inhibited using KU55933 or NU7441 respectively, some degree of rescue was evident at the level of Mcm4 phosphorylation, though not to the levels of the control sample. Also, since the reduction of hyper-phosphorylation did not depend on on-going replication, this checkpoint-dependent response is not majorly mediated by ATR (Figure 2.13). Taken together, ATM, ATR and DNA-PK signalling pathways may all be involved to some extent and it is likely that etoposide did not simply elicit a straightforward response through single- or double-stranded DNA breaks. This interpretation differs from previous studies where ATR/ATR-Chk1 signalling was found to be primarily responsible for etoposide inhibition of DNA replication (Costanzo et al., 2003; Silva et al., 2006; Tsuji et al., 2008). It also differs from a previous study where Cdc7 was found not to play a role in the checkpoint response to double stranded breaks (Petersen et al., 2006).

Looking at the levels of chromatin-bound Cdc7, two etoposide-dependent effects on Cdc7 were evident in the present study. There was a caffeine-insensitive inhibition of Cdc7 association to chromatin and a caffeine-dependent inhibition of Mcm4 phosphorylation. This is consistent with the report from Costanzo et al. in that the etoposide checkpoint down-regulates Cdc7 kinase activity (Costanzo et al., 2003) but is not supported by studies by Silva et al. and Tsuji et al. who found that chromatin association, complex formation and kinase activities of both Dbf4-Cdc7 and Drf1-Cdc7 were unaltered in etoposide-treated extract (Silva et al., 2006; Tsuji et al., 2008). Silva et al. also reported that Mcm4 phosphorylation was not affected (Silva et al., 2006). In the mammalian systems, neither Dbf4-Cdc7 nor Drf1-Cdc7 complexes were released from chromatin in human cells treated with etoposide. In fact, Mcm4 phosphorylation was induced upon drug treatment and was abolished by siRNA depletion of Cdc7 (Tenca et al., 2007).

The differences in the response to etoposide observed in this study compared to previous studies could be due to variation in experimental conditions. While low speed supernatants which support nuclear envelope formation were used in all studies, the concentration of etoposide used in the other studies (30 μM) did not affect replication or reduce Mcm4 phosphorylation in this study (data not shown). In contrast, replication was reportedly inhibited up to 90% (Silva et al., 2006). It is possible that due to variations in extract preparation method, the low speed supernatant used in this study was more robust towards replicative insults. As such, there may be a different checkpoint response towards this comparatively lower-grade damage. In addition, while an inhibition to nuclear assembly was observed in this study, this was not directly addressed in the other reports. As previously mentioned, etoposide appears to be causing a complex response, possibly signalling through several pathways. The data presented here may describe a distinct mechanism of etoposide action from those investigated in other studies.

Disparate observations may be the result of different signalling pathways in response to the intra-S disruption during the cell cycle. ATM and ATR are members of the phosphatidylinositol kinase-related family of protein kinases that function at or near the top of the signalling pathways of various checkpoint regulatory pathways in response to DNA damage (Zhou and Elledge, 2000). While ATM is mainly involved in the response to double-stranded breaks, ATR plays a key role in detecting lesions that generate single-stranded breaks, such as those arising from stalled replication forks (Harrison and Haber, 2006). In this signalling cascade, two other protein kinases, Chk1 and Chk2, act downstream of ATM and ATR respectively (Zhou and Elledge, 2000). While this describes the general response to DNA damage, ATM and ATR may also respond to common DNA lesions and exert their effects on downstream targets directly. In *Xenopus* egg extracts, it was reported that EcoRI treatment resulted in a major reduction of DNA replication which was completely reversible by the addition of

caffeine, in line with the data presented in Figure 2.13 (Yoo et al., 2004). Removal of either ATM or ATR by immuno-depletion allowed a restoration of DNA replication up to 20 to 30% of control levels while depletion of both checkpoint kinases restored replication to approximately 70% of normal levels. Similar depletions of Chk1, Chk2 or both Chk1 and Chk2, however, had no effect on EcoRI inhibition of DNA replication. The authors concluded that ATM and ATR act cooperatively to inhibit DNA replication in response to double-stranded breaks and that the they exert their effects directly or through downstream effectors apart from the Chk1 and 2 kinases (Yoo et al., 2004). In another study, DNA replication in an extract depleted of Chk1 was still found to be sensitive to aphidicolin treatment and could be rescued by caffeine addition. This indicated that checkpoint signalling was still activated and that Chk1 was not an essential component in this response (Luciani et al., 2004). Given that chromosomal replication is a dynamic process involving the coordination of many proteins complexes over large stretches of DNA, impediments to replication fork progression may result in different physical intermediates, which trigger mixed checkpoint responses.

On the other hand, different DNA damaging agents may also converge on a common mechanism to trigger checkpoint response through the formation of a common DNA structure. Lupardus and colleagues had showed that UV, MMS and aphidicolin treatment slowed down replication forks in *Xenopus* egg extracts, stalling DNA replication in a caffeine-insensitive manner. They proposed that slowed replication forks caused the formation of ssDNA structures which were insufficient to trigger a replication checkpoint response but necessary as templates for the association of additional proteins, like RPA and DNA polymerase α, to generate a checkpoint-activating structure. On-going replication is required in this process (Lupardus et al., 2002). This is consistent with the model in which the uncoupling of DNA unwinding by the Mcm helicase and DNA replication by replicative helicases in the presence of DNA

damage generates large regions of unwound ssDNA to allow accumulation of RPA and ATR on chromatin (Byun et al., 2005).

Given the complex nature of the intra-S checkpoint signalling cascade, it is therefore important to consider the mode of action of etoposide in order to understand the Cdc7-dependent response elicited. Etoposide is an anticancer agent that is used clinically in the treatment of human malignancies. In mammalian cells, it acts by poisoning topoisomerase II, stabilising covalent cleavage complexes that the enzyme transiently forms with double-stranded nicks to resolve DNA supercoils. This gradually increases the level of enzyme-mediated double-stranded breaks in the genome leading to cell death (Burden and Osheroff, 1998). Costanzo et al. had argued that while topoisomerase II is covalently bound to the 5' terminus of cleaved DNA, the 3' end remains free and is not equivalent to the conformation of a double-stranded break. Instead, they proposed that the intermediate is first processed into a single-stranded structure which loads RPA to activate an ATR-dependent checkpoint (Costanzo et al., 2003). In the present study, a substantial amount of replication was still observed in the presence of etoposide (approximately 40% of control, see Figure 2.12), unlike previous reports (Costanzo et al., 2003; Silva et al., 2006; Tsuji et al., 2008). Here, in the presence of on-going replication, several checkpoint mechanisms may be active simultaneously, generating and responding to single-stranded intermediates and other double-stranded structures created, whether as a consequence of prior single-stranded processing or not. This means that ATM and ATR could both be involved in a complex signalling response. The involvement of Chk1 or 2 kinases has not been addressed yet by this present study, so the question of whether ATM/ATR signal through the Chk kinases or act directly on distinct downstream targets remains to be clarified.

While the checkpoint signalling process remain ambiguous, it is clear that Cdc7 function is restrained in response to etoposide treatment. In principle this could occur

either by inhibition of Cdc7 kinase activity or by promoting Mcm dephosphorylation. The observation that caffeine affects Cdc7 binding and Mcm4 phosphorylation differently shows that the amount of Cdc7 on chromatin is not limiting for DNA replication and implies that another factor may be regulating Mcm4 hyper-phosphorylation in response to the checkpoint. Given that Mcm4 hyper-phosphorylation is reversed by PP1 (Figure 2.7) and I-2 addition can restore Mcm4 hyper-phosphorylation in etoposide-treated extracts (Figure 2.12), an attractive explanation is that PP1 activity is up-regulated by the checkpoint response, thereby functionally lowering Cdc7 activity. Consistent with this hypothesis, unpublished experiments by Dr Gaganmeet Singh Chadha show that etoposide promotes the chromatin association of PP1α and PP1γ in a checkpoint-dependent manner (Poh et al., manuscript in preparation).

Regulators of PP1 have been reported to be involved in modulating PP1 activity in response to DNA damage (Kuntziger et al., 2011). In *Xenopus* egg extracts, Peng *et al.* reported that one such regulator, Repo-Man (Recruits PP1 onto Mitotic chromatin at Anaphase), recruits PP1γ to chromatin. Repo-Man associates with ATM and PP1 through distinct domains, targeting PP1γ to antagonise ATM activation and negatively regulate DNA damage-induced signal transduction. Inhibition of PP1 results in a sensitized response to DNA damage caused by double-stranded breaks and Repo-Man dissociated from active ATM at sites of DNA damage, both on chromatin in extract or on DNA fibres prepared from mammalian cells. The authors propose that Repo-Man provides specificity and localisation to PP1, acting as an inhibitory mechanism to prevent checkpoint response activation by a sub-threshold level of DNA damage (Peng et al., 2010). While these results are different from the findings in this study, most likely because this specific PP1 regulator is mediating a distinct PP1 response, it demonstrates that PP1 can be mobilised to associate with DNA in response to checkpoint signalling and to modulate signal transduction. With PP1 being able to form

complexes with 50 or possibly more regulatory subunits in a mutually exclusive manner (Cohen, 2002), it is difficult to speculate on the identity of the regulator acting in the etoposide-induced response without more data.

In this study, the inhibition of Cdc7 chromatin loading was dependent on etoposide being present in extract at the time of chromatin decondensation, and correlated with the ability of etoposide to trap Topo II on DNA and inhibit full chromatin decondensation (Figure 2.15). Taken together with observation that nuclear assembly is disrupted by etoposide treatment, a model of this checkpoint response involving Cdc7 and PP1 is proposed, as outlined in Figure 2.16. In this scheme, Mcm phosphorylation is the central event required for replication initiation to proceed. The net actions of a kinase (Cdc7) and phosphatase (PP1) regulate the equilibrium of Mcm phosphorylation status. Nuclear assembly and origin licensing occur before phosphorylation takes place. The introduction of etoposide results in the disruption of nuclear envelope assembly, which reduces Cdc7 recruitment and activity, triggering a checkpoint kinase-mediated recruitment of PP1 to chromatin via a specific regulator. Together, these events reduce total Mcm phosphorylation and inhibit replication initiation. While checkpoint bypass with caffeine cannot restore nuclear architecture to allow Cdc7 recruitment to chromatin, it is able to reverse the effect on PP1 recruitment. As such, etoposide-induced inhibition of Mcm4 phosphorylation can be rescued without restoring normal levels of chromatin bound Cdc7. However, due to checkpoint effects on other targets, such as Cdk, the addition of I-2 alone cannot rescue the level of DNA synthesis.

This model presents a novel mechanism through which Cdc7 activity is affected by etoposide. Unlike previous reports in the *Xenopus* system that position Cdc7 downstream of and inactivated by checkpoint activity (Costanzo et al., 2003) or others which maintain that Cdc7 remains active to mediate a recovery response (Silva et al., 2006; Tenca et al., 2007; Tsuji et al., 2008), this schematic places Cdc7 outside of the

direct effect of checkpoint kinases when Mcm4 activity is taken as a readout. Instead, PP1 is the proposed target of checkpoint activity while Cdc7 function is restricted by lack of nuclear formation. Checkpoint kinases counteract Cdc7 function at least in part by promoting PP1 chromatin association, thereby reducing Cdc7-mediated Mcm phosphorylation, which in turn affects recruitment of Cdc45 to chromatin and DNA replication. Prevention of hyper-phosphorylated Mcm4 from PP1 dephosphorylation with caffeine to bypass checkpoint kinase activity can therefore rescue the distinct delay in the recruitment of Cdc45 to DNA in etoposide-treated extract, allowing initiation of replication origins.

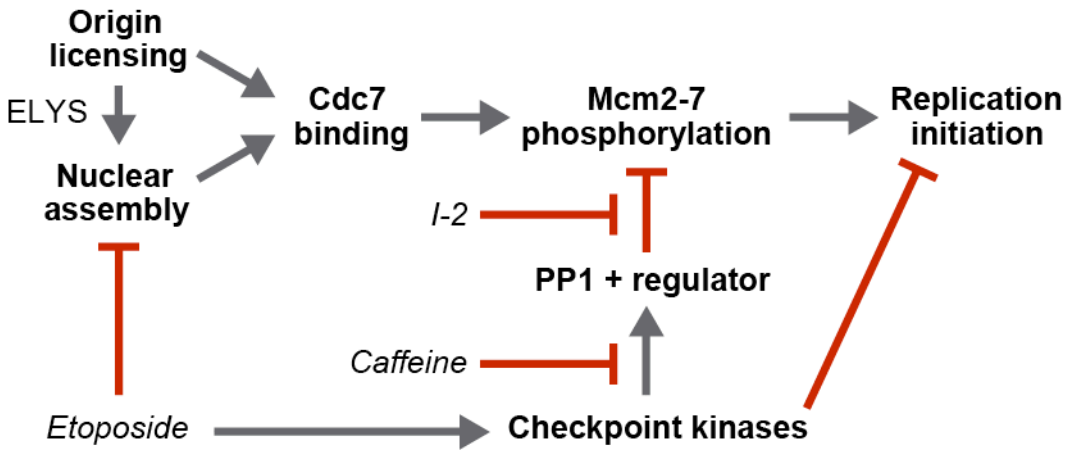

Figure 2.16. Proposed mechanism for the checkpoint-dependent response triggered by etoposide treatment in *Xenopus* egg extracts.

In late M phase of the cell cycle, nuclear envelope reassembles following chromosome segregation. Origin licensing then takes place from late M to early G1 phase, when Mcm2-7 complexes are recruited to chromatin to form pre-RCs. Nuclear assembly enhances recruitment of Cdc7 to chromatin-bound Mcm2-7, thereby promoting Cdc7 phosphorylation of Mcm2-7 at the onset of S-phase. Cdc7 and S-Cdk phosphorylation of the Mcm2-7 complex is required for the activation of the helicase. PP1 modulates the hyper-phosphorylation of Mcm4. When etoposide is added to *Xenopus* egg extracts, nuclear envelope assembly is disrupted. This reduces the amount of Cdc7 that can be recruited to chromatin and also triggers a checkpoint response through checkpoint kinase to recruit PP1γ to chromatin. The result is an overall decrease in Mcm2-7 phosphorylation, which prevents initiation of replication from taking place. Caffeine can bypass the checkpoint kinase action on PP1, thus restoring Mcm4 hyper-phosphorylation, allowing rescue of DNA replication.

Concluding remarks

Through the course of this chapter, I have presented data from the characterisation of the small molecule inhibitor, PHA-767491, on Cdc7 activity in *Xenopus* egg extracts and showed that it inhibits DNA replication largely by blocking an essential Cdc7 activity. Making use of the rapid action of a chemical inhibitor, I have used PHA-767491 in experiments to determine the time of execution of Cdc7 for its S phase functions and identified that Cdc7-dependent Mcm4 hyper-phosphorylation is quickly reversed by the action of PP1 phosphatase activity.

I had examined whether Cdc7 is involved in the progression of the replication timing programme and my results indicate that in the absence of Mcm4 dephosphorylation, Cdc7 is not rate-limiting for the progress through the replication timing programme once it has executed its essential function for initiation early in S phase. To address the role of Cdc7 in the intra-S checkpoint, I used etoposide in *Xenopus* egg extracts and explored its effects and mechanism of action. Finally, based on my work and results from Dr Gaganmeet Singh Chadha (Poh et al., manuscript in preparation), I propose a novel intra-S checkpoint mechanism in which checkpoint regulated PP1 activity functionally restrains Cdc7 activity upon etoposide treatment in *Xenopus* egg extracts.

While the present study has provided additional insight into Cdc7 function and regulation in *Xenopus*, more work remains to be done to address several aspects of the replication timing programme and of the intra-S checkpoint response. These include determining how Cdc7 or PP1 may be involved in the regulation of early- and late-origin firing, how this is affected by the intra-S checkpoint and also to look in greater detail how PP1 activity is regulated by checkpoint kinases. The biochemical evidences presented here may provide the starting points to begin answering these questions.

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Chapter 3 – Generating a *Cdc7* condition knockout mouse

Introduction

Many studies on the function of Cdc7 have been done using yeast or *Xenopus* cell free extracts as model systems. These systems are versatile and amenable to genetic or biochemical modifications, greatly facilitating experimental design. Also, since Cdc7 is conserved from yeast to human, findings from such model systems are likely to be relevant to mammalian studies. To complement data from *in vitro* models, genetic studies in a mammalian system can provide additional information about developmental roles and other *in vivo* functions of the protein at the level of tissues and the whole organism.

The development of technologies enabling the introduction of any mutation into the mouse genome through gene targeting in embryonic stem (ES) cells offers a powerful approach to investigate gene function in the whole organism. However, in the case of essential genes, the complete absence of the gene product results in early embryonic lethality, preventing studies in later development or adult stages. From the perspective of clinical studies, the complete inactivation of a gene may not reflect the full spectrum of its function in a physiological context, as disease phenotypes may arise from genetic changes arising only in a subset of cells or during a certain temporally-specific stage (Lobe and Nagy, 1998).

To address these issues, a conditional knockout (cko) strategy may thus be employed. The basic idea is to insert a pair of *lox*P sites flanking opposite ends of the gene of interest. The gene can then be inactivated by the expression of the Cre (Causes recombination) recombinase, which catalyses recombination of the *lox*P sites in animals carrying this cko allele. Another level of control can be achieved by placing the

Cre gene under the control of tissue-specific and/or inducible promoters (Hayashi and McMahon, 2002) or to selectively introduce the *Cre* gene via viral delivery systems (Liu et al., 2003; Lobe and Nagy, 1998). Conditional knockouts can also be generated using the Flp/FRT system in which the *S. cerevisiae* derived Flp (flippase) recombinase mediates excision between FRT recognition sites. However since the bacteriophage P1 derived Cre is optimally functional at 37ºC, it is more widely used in conditional mutagenesis than the more thermolabile Flp enzyme (Buchholz et al., 1996).

Genetic studies of Cdc7 in the mouse model have previously been published. Kim et al. reported that deletion of murine *Cdc7* resulted in embryonic lethality between E3.5 and E6.5. This observation was not unexpected since *Cdc7* is an essential gene. E3.5 *Cdc7*$^{-/-}$ blastocysts that were isolated and cultured *in vitro* failed to form an inner cell mass. Using a plasmid construct encoding a functional *Cdc7* gene flanked by *lox*P sites, the group then generated and characterised conditional *Cdc7*-deficient ES cells lines carrying this Cre-removable *Cdc7* transgene (*Cdc7*$^{-/-}$tg). Upon the loss of *Cdc7*, *Cdc7*$^{-/-}$ cells rapidly ceased DNA replication and arrested with G2/M DNA content. Rad51 foci were observed in these cells, indicating a G2/M checkpoint response and the cells death eventually occurred through p53-dependent apoptosis (Kim et al., 2002). In a follow up study, mice generated with the *Cdc7*$^{-/-}$tg genotype were found to be sterile due to a defect in spermatogenesis in males and abnormal oogenesis in females. These mice also developed at a slower rate than wild type mice and *Cdc7*$^{-/-}$tg MEFs displayed decreased proliferative capacity with a smaller population of cells in S phase. These phenotypes were attributed to hypomorphic expression of the Cdc7 protein, which was five times lower than in wild type mice. By introducing a second copy of the transgene (*Cdc7*$^{-/-}$tg/tg), Cdc7 expression was increased to two-thirds of wild type levels and these defects were rescued. These mammalian genetic studies revealed that Cdc7 is essential for early embryogenesis, highlighting the importance of a critical level of Cdc7 expression in mammalian development and an essential role for

Cdc7 in germ cell development (Kim et al., 2003). A related study in which *ASK*, the mammalian homologue of the Cdc7 activator Dbf4, was deleted in a mouse ES cell line showed that murine ASK is required for Cdc7 kinase activation *in vivo* and confirmed the essential role of Cdc7 for cell proliferation and DNA synthesis in ES cells (Yamashita et al., 2005).

In the present study, a *Cdc7* cko allele was constructed to generate transgenic mice in which Cdc7 loss can be induced conditionally. These mice represent a comprehensive model in which the contribution of targeted Cdc7 loss can be examined in different tissues, cell types and developmental stages. They will be useful models in tumorigenesis and other translational studies. The strategy for conditional *Cdc7* knockout is outlined in the following sections and results from the screenings done to identify the transgenic founder will be presented. The design and construction of this targeting vector was done in collaboration with Dr Zheng Xinde. I was involved only in the very preliminary stages of the construction process. Xinde designed and initiated the Southern blot strategy subsequently used in the screening process. I carried out and completed the final stages of the screening.

Method

Creating a conditional knockout targeting vector requires the insertion of *lox*P/FRT sites and positive and negative selection markers into the DNA sequence of the gene of interest cloned into bacterial artificial chromosomes (BAC). A major drawback of using conventional cloning techniques to create gene-targeting constructs is the dependence on suitably located, unique restriction sites within or in the proximity of the gene of interest. The development of the recombineering method of chromosome engineering (Copeland et al., 2001) has greatly facilitated this process, offering the flexibility to

precisely modify BAC DNA and shortening the time required (Liu et al., 2003). The recombineering technique is used in creating this *Cdc7* targeting vector.

Recombineering is a tightly regulated homologous recombination-based system based in *Escherichia coli* (Copeland et al., 2001). Efficient homologous recombination can be achieved by three proteins (Exo, Bet and Gam) encoded by the Red genes of the bacteriophage λ, allowing linear double-stranded DNA (dsDNA) molecules to be integrated into DNA cloned in BACs. The *exo* gene encodes a 5' – 3' exonuclease which digests the ends of the dsDNA fragment to produce 3' single stranded overhangs. A pairing protein encoded by *bet* then binds to these overhangs and promotes annealing to a complementary sequence on the cloned DNA. Since linear dsDNA is unstable in *E. coli* due to the activity of endogenous RecBCD, a third phage-encoded protein Gam is required to inhibit the bacterial exonuclease (Poteete, 2001; Stahl, 1998). The λ-phage recombination genes can be expressed from a plasmid (Murphy et al., 2000) or from a defective prophage integrated into the bacteria genome (Yu et al., 2000).

Design of the Cdc7 conditional knockout (cko) targeting vector

The murine *Cdc7* gene is located on the mouse chromosome 5E5 and consists of 12 exons, encoding 564 amino acids (Figure 3.1A) (Kim et al., 1998). The first step in designing a targeting construct is to identify the sequence of interest. The targeting strategy is based on the genomic sequence of murine *Cdc7* in the NCBI database (NC_000071, Region 107 393 341 – 107 413 450). In order to decide which exons to target for knockout, several considerations were taken into account. The loss of the floxed exons should (1) result in loss of a protein function, (2) generate a frame-shift mutation and (3) result in the expression of a truncated protein so that the protein product is functionally inactive. Attention was paid to minimise the distance between

*lox*P sites, as the efficiency of recombination decreases with increasing distance, that flanking introns should be large enough to accommodate *lox*P sites and facilitate genetic manipulation and promoter- and enhancer-rich introns are avoided (Bouvier and Cheng, 2009).

In this study, exons 3 and 4, encoding kinase domains I and II of murine Cdc7 were chosen. These exons are relatively small, separated only by a 217-bp intron, and are flanked by two very large introns of 3743 and 3452 bp. This would allow positioning of the two *lox*P sites close to each other and also provide a large distance to work with for the insertion of targeting construct elements. Alternative splicing of exons 2 and 5 due to the loss of intervening exons 3 and 4 would not yield an in-frame variant protein, hence fulfilling the criterion of a frame-shift mutation. In the event that exon 2 splices to exons 6 through 12, only the exon 2-11 and 2-12 combination will result in a product in the same reading frame as the full-length protein. However, since these resulting peptides will be very small and missing important conserved domains, they are unlikely to be functional.

In this targeting strategy, a gene trap in the form of the engrailed-2 splice acceptor (eng2SA) was introduced in the 5' homology arm of the targeting construct. As illustrated in Figure 3.1B, the linear targeting vector consists of a 5' homology arm containing the FRT-*lox*P-eng2SA-Neo-FRT-*lox*P cassette and a lonely *lox*P site after exon 4, followed by the 3' homology arm. With the inclusion of a splice acceptor, exon 2 will be aberrantly spliced into eng2SA, resulting in a truncated message and hence protein product. Mutant mice carrying one copy of the correctly targeted allele would be constitutively hypomorphic for *Cdc7* expression while mice homozygous for the targeted allele are expected to be phenotypically equivalent to a *Cdc7* null mouse, which would be lethal. The homology arms were selected so that they were from regions of the genome that are free from repetitive sequences and they should not

contain restriction sites for at least one unique restriction enzyme that cuts the vector backbone so that this enzyme can later be used to linearise the targeting vector for electroporation into ES cells.

The targeting construct is introduced into the wild type *Cdc7* locus by homologous recombination in a gene-targeting event, giving rise to the mutant allele (Figure 3.1C). The FRT-*lox*P-eng2SA-Neo-FRT-*lox*P cassette can subsequently be excised from the allele by FLP-mediated recombination between the two FRT sites. This generates a conditional knock-out allele with exons 3 and 4 flanked by *loxP* sites (Figure 3.1D) – this is the floxed allele. Expression of *Cdc7* from this allele is expected to be comparable to that from a wild type locus. Mice carrying the floxed allele may be crossed with mouse strains carrying tissue-specific and/or inducible Cre recombinase. This then allows selective deletion of exons 3 and 4 by Cre-mediated recombination as required, resulting in the *Cdc7*-null knock-out allele (Figure 3.1E).

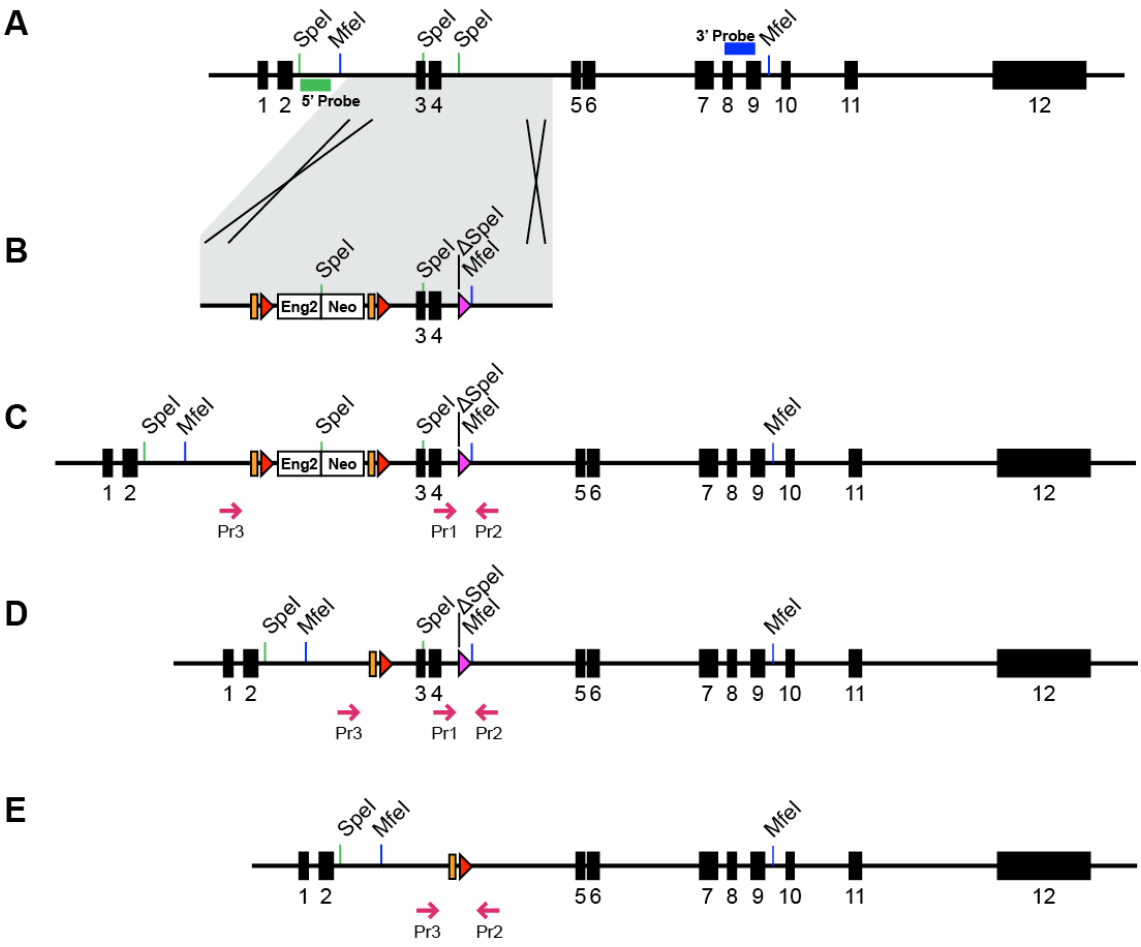

Figure 3.1. Schematic of the targeting strategy for creating the *Cdc7* cko allele.

(A) The *Cdc7* gene is located on the mouse chromosome 5E5 and consists of 12 exons. Exons 3 and 4 were selected for gene targeting. The positions of relevant restriction enzyme sites and the 5' and 3' probes for Southern blotting are indicated. (B) The linearised targeting construct would be integrated into the genomic locus by homologous recombination. (C) The targeted allele results when the linear targeting construct is successfully integrated. Pr1, 2 and 3 indicate positions of PCR primers designed to differentiate wild type (A), floxed (C, D) and null (E) alleles for genotyping. (D) The engrailed-2 splice acceptor (Eng2) and the Neo^r cassette can be excised by recombination between the FRT sites to leave a floxed, conditional knockout allele. (E) Mice carrying the floxed allele may be crossed with mouse strains expressing tissue-specific and/or inducible Cre recombinase. Selective deletion of exons 3 and 4 by Cre-mediated recombination results in a *Cdc7*-null allele.

Construction of the targeting vector

Figure 3.2 illustrates the schematic for constructing the targeting vector. The construction of the linear targeting vector begins with the retrieval of a suitable length of genomic sequence from a BAC in which modifications can be introduced. This is accomplished by gap repair recombination with a linearised retrieval vector, bearing homology arms to 5' and 3' regions flanking the desired genomic region, and a suitable BAC containing the region of interest (RP23-24K16) (Figure 3.2). The retrieval vector is constructed by modifying a pBlight-TK vector backbone (PKB782) (Warming et al., 2006) to contain a 413-bp 5' retrieval arm and a 397-bp 3' retrieval arm. The 5' arm was PCR amplified from the BAC template with the primers PKO1187 and PKO1188 (see Material and methods for primer sequences), gel purified, digested with BamHI and XhoI and ligated into PKB782. The 3' arm was PCR amplified from the BAC template with the primers PKO1185 and PKO1186, gel purified, digested with HindIII and SpeI and also ligated into PKB782. To retrieve the genomic 6527-bp fragment, recombineering-competent BAC clones harbouring pSim18-Hygro (Lee and Liu, 2009) were shifted from 32ºC to 42ºC for 15 min to induce expression of the genes required for recombination, namely *exo*, *bet* and *gam*. The retrieval vector, linerised with XhoI and HindIII, was introduced by electroporation. Ampicillin-resistant clones were selected and presence of the retrieved *Cdc7* locus can be confirmed by restriction digest analysis.

The retrieval vector backbone includes a herpes simplex virus-thymidine kinase (HSV-TK) cassette, situated outside the homology arms. It encodes the enzyme thymidine kinase which converts ganciclovir into a toxic product and functions as a negative selection marker against random integration events during gene targeting. Only ES colonies that have correctly undergone homologous recombination, hence losing the HSV-TK gene, will survive in the presence of ganciclovir.

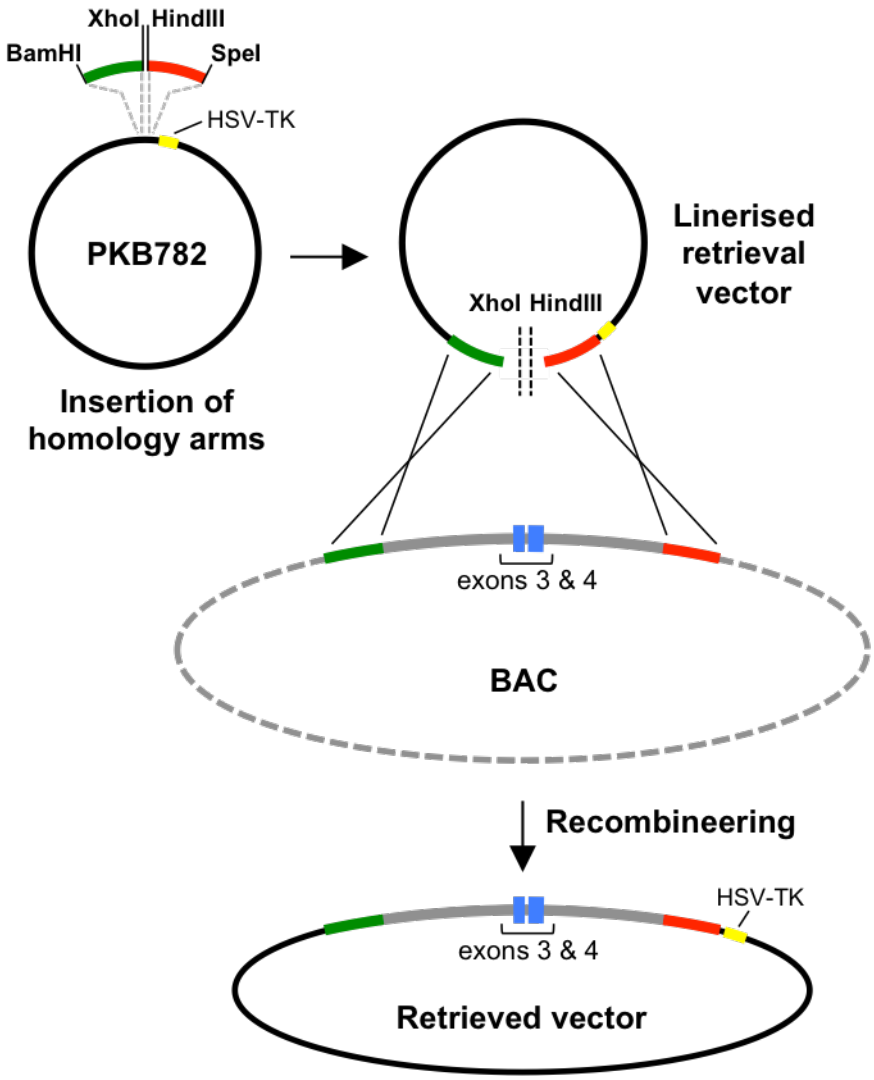

Figure 3.2. Schematic for constructing the retrieval vector.

Homology arms (green, red) were amplified by PCR using the primer pairs PKO1187 and PKO1188 (5'), and PKO1185 and PKO1186 (3') with the corresponding restriction enzyme sites. These were cloned into the pBlight plasmid PKB782, after which the plasmid was linerised and transformed into recombineering-competent BAC-containing bacteria. The BAC sequence between the homology arms was "retrieved" onto the retrieval vector by gap repair. ES cells in which the targeting vector is correctly integrated into the *Cdc7* locus through homologous recombination will lose the HSV-TK gene carried on the plasmid backbone, enabling their positive selection with ganciclovir.

The next step is the introduction of the lonely *lox*P site into the retrieved locus. First, a plasmid bearing the *lox*P-Neo-*lox*P cassette (PKB781, pL452) (Liu et al., 2003) was modified to contain homologous regions flanking the targeted *lox*P insertion site within the intronic sequence between exons 4 and 5 (Figure 3.3A and C, pink segments). The 397-bp 5' arm was amplified from the BAC template with the primers PKO1197 and PKO1198, gel purified, digested with ApaI and EcoRI and ligated into PKB781. The 263-bp 3' arm was likewise generated and added using the primers PKO1202 and PKO1238 and enzymes BamHI and NotI. An MfeI restriction site was included in PKO1202 after the BamHI site for future Southern Blot analysis. Recombineering-competent bacterial cells containing the retrieved sequence were heat-shocked at 42ºC for 15 min as before. The modified *lox*P-Neo-*lox*P cassette was released from its vector backbone with ApaI/NotI and introduced by electroporation. Antibiotic-resistant clones were again selected and insertion verified by restriction digest. The floxed Neo cassette could then be removed to leave a single *lox*P site by introducing this construct into Cre-induced competent cells (StrataClone SoloPack competent cells, #200185-41) by heat-shock at 42ºC for 15s. Ampicillin resistant clones were selected and insertion verified by restriction digest.

The final stage of the construction is then to introduce the FRT-*lox*P-eng2SA-Neo-FRT-*lox*P cassette. Homology arms were cloned into PKB996 (pLTM330), generously provided by Dr Lino Tessarollo from the National Cancer Institute-Frederick (Figure 3.3B and C, blue segments). The 253-bp 5' homology arm was PCR amplified with PKO1193 and PKO1194, digested with SalI and EcoRV and ligated into PKB996. The 3' homology arm was amplified with PKO1201 and PKO1217, digested with SacII and ligated into PKB996. A ScaI restriction site was added to the 3' end of this insert via PKO1217 for verifying that the insertion of this homology arm into the single SacII cut site occurred in the right direction. A SpeI site was also added inside the SacII site for

the purpose of future Southern Blot analysis. Recombineering-competent bacterial cells containing the retrieved sequence modified with the lonely *lox*P site were heat-shocked at 42ºC for 15 min as before. The modified FRT-*lox*P-eng2SA-Neo-FRT-*lox*P cassette was excised from its vector backbone with SalI and ScaI and introduced by electroporation. Kanamycin-resistant clones were selected and correct insertion verified by restriction digest. This completes the construction of the targeting vector (PKB1086).

The ability to delete the *lox*P- and FRT-flanked sequences in this cassette was verified. The vector was sequenced on both strands at the *lox*P and FRT boundaries to ensure integrity of the targeting construct. Finally, the vector was linerised with PvuI and sent to Dr Lino Tessarollo at the National Cancer Institute-Frederick for electroporation into ES cells.

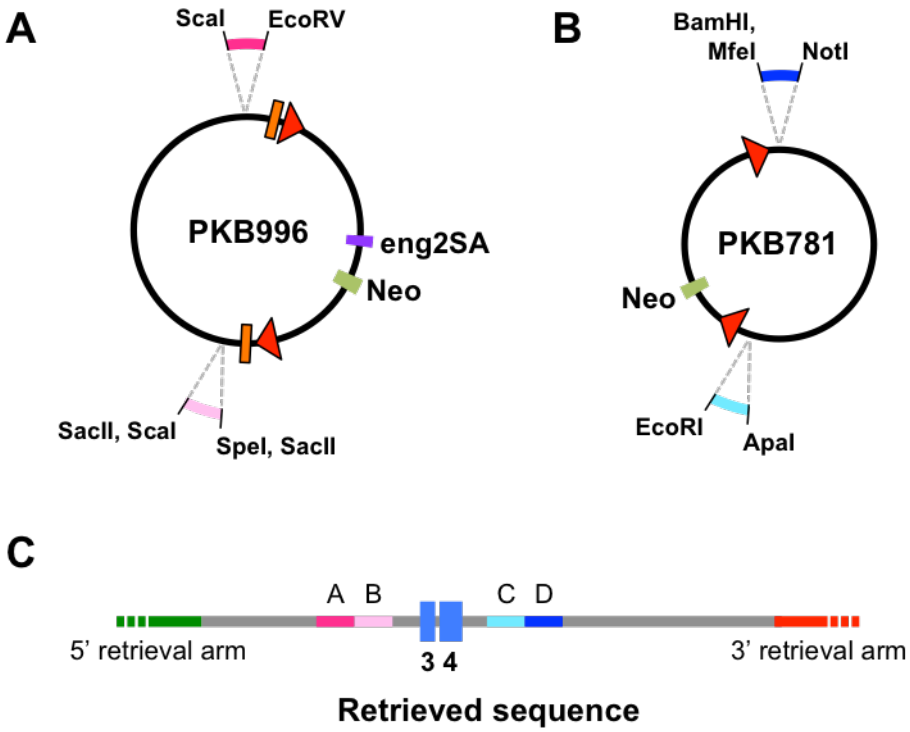

Figure 3.3. Schematic for constructing the targeting vector.

(A) Homology arms (dark and light pink) corresponding to sequences within the retrieved BAC sequence (Figure C, regions A, B) were generated by PCR amplification using the primer pairs PKO1193 and PKO1194 (5'), and PKO1201 and PKO1217 (3') with the indicated restriction enzyme sites. These were digested and ligated into PKB996. The plasmid was linearised with SalI and ScaI to yield the FRT-*lox*P-eng2SA-Neo-FRT-*lox*P cassette (Orange box: FRT; Red arrowhead: *lox*P). (B) Homology arms (dark and light blue) corresponding to sequences within the retrieved BAC sequence (Figure C, regions C, D) were likewise generated by PCR amplification using the primer pairs PKO1197 and PKO1198 (5'), and PKO1202 and PKO1238 (3') with the indicated restriction enzyme sites. These were digested and ligated into PKB781. The *lox*P-Neo-*lox*P cassette was released from the plasmid backbone with ApaI and NotI digestion. (C) *lox*P-Neo-*lox*P was first inserted into between regions C and D. Following Cre recombination to leave the lonely *lox*P site, FRT-*lox*P-eng2SA-Neo-FRT-*lox*P was then inserted between regions A and B.

Results

3.1 Screening for Cdc7*-targeted mice*

A Southern blot strategy was designed to distinguish the correctly targeted allele from wild type in screens of electroporated ES cell and, subsequently, tail lysates from crosses between chimeric mice. The positions of the 5' and 3' probes are illustrated in Figure 3.1A. The 5' probe recognises a fragment of 3.6 kb in wild type and 5.6 kb in targeted samples upon SpeI restriction digest. The 3' probe identifies a fragment of 8.6 kb in wild type and 5.7 kb in the targeted samples upon MfeI digest.

Following the successful generation of mice carrying the targeted allele, an alternative PCR-based method also has to be available to rapidly establish the genotypes of mice from this colony. To this end, a set of 3 primers was designed (Figure 3.1, C – E). Primers 1 and 2 flank the lonely *lox*P site whereas Primer 3 is located much further upstream, before the FRT-*lox*P-eng2SA-Neo-FRT-*lox*P cassette. This arrangement allows a single PCR reaction to distinguish among wild type, targeted/floxed and null genotypes. Primers 1 and 2 will produce PCR products in wild type and targeted/floxed genotypes which differ by approximately 146 bp – the size of the *lox*P site plus residual nucleotides from the Cre/*lox* recombination. Primer 3 will not result in any PCR product, as the distance to Primer 2 is too large for efficient amplification by standard PCR techniques. In the null genotype however, a Cre/*lox* recombination would excise the intervening region up to the lonely *lox*P site, including the sequence recognised by Primer 1, bringing Primer 3 close enough to Primer 2 for efficient PCR amplification. Primer 3 is positioned such that the size of this PCR product is unique from the PCR product of Primers 1 and 2.

3.1.1 1st screening of mouse tail lysates

Figure 3.4 shows results from the first screening of tail lysates that I carried out to check for germline transmission of the correctly targeted allele from chimeric mice to their first generation offspring. Chimeric mice had been derived from positive clones from an earlier Southern blot screen of ES cell lysate following electroporation (results unavailable). From the 33 tail lysate samples received, a preliminary screen was first carried by PCR on HotSHOT-extracted genomic DNA. This PCR reaction amplifies a region found in the neomycin resistance cassette, which in principle would identify samples in which the gene locus had been correctly targeted. 14 out of 33 samples were found to be positive for the Neo cassette and were followed up with Southern blotting to confirm correct targeting at both the 5' and 3' ends.

Genomic DNA was first extracted using phenol-chloroform-isoamyl-alcohol and Southern blotting performed using non-radioactive using DIG-labelled probes following restriction digests using SpeI or MfeI. As shown in Figure 3.4B, all 14 samples were positive for the 5.6 kb band when probed at the 5' end. The 3.6 kb wild type was also present in all samples as any mouse carrying the targeted allele would be heterozygous at this stage. However, looking at the results from the 3' probe, although all samples were positive for the 8.6 kb wild type band, as expected, none of them tested positively for the 5.7 kb targeted band. Instead, a puzzling observation was that of a much larger band between 10 and 20 kb in size, present in all samples. This indicated that no sample was correctly targeted at both 5' and 3' ends. A likely explanation for the larger 3' band was that homologous recombination at the 3' end had occurred at an intervening region upstream of the lonely *lox*P site. Since the lonely *lox*P site spans only an extremely short interval of only 146 bp, there is a higher possibility of it being "lost" in this manner without a selectable marker to maintain it in transformed cells. To test this hypothesis, two sets of PCR were carried out. The top panel of Figure

3.4C shows the result from a genotyping PCR, which gives different PCR products for wild type and targeted alleles (WT: 329 bp/ Targeted: 444 bp). Only the wild type PCR product was observed in all tail lysate samples. The lower panel shows the result of PCR amplification using primers that overlap the lonely *lox*P site. Wild type sequences would not be amplified by this reaction. No PCR product was detected in all tail lysate samples. This confirms the hypothesis that the *lox*P site was not present in these genomic DNA samples. Accordingly, with only correct targeting at the 5' end without a corresponding 3' insertion of the lonely *lox*P sequence containing an engineered MfeI site, a restriction digest with MfeI would be expected to yield a fragment of approximately 13 kb. This is consistent with the Southern blot results observed.

From this screen, it was decided that a new round of ES cell electroporation would be carried out.

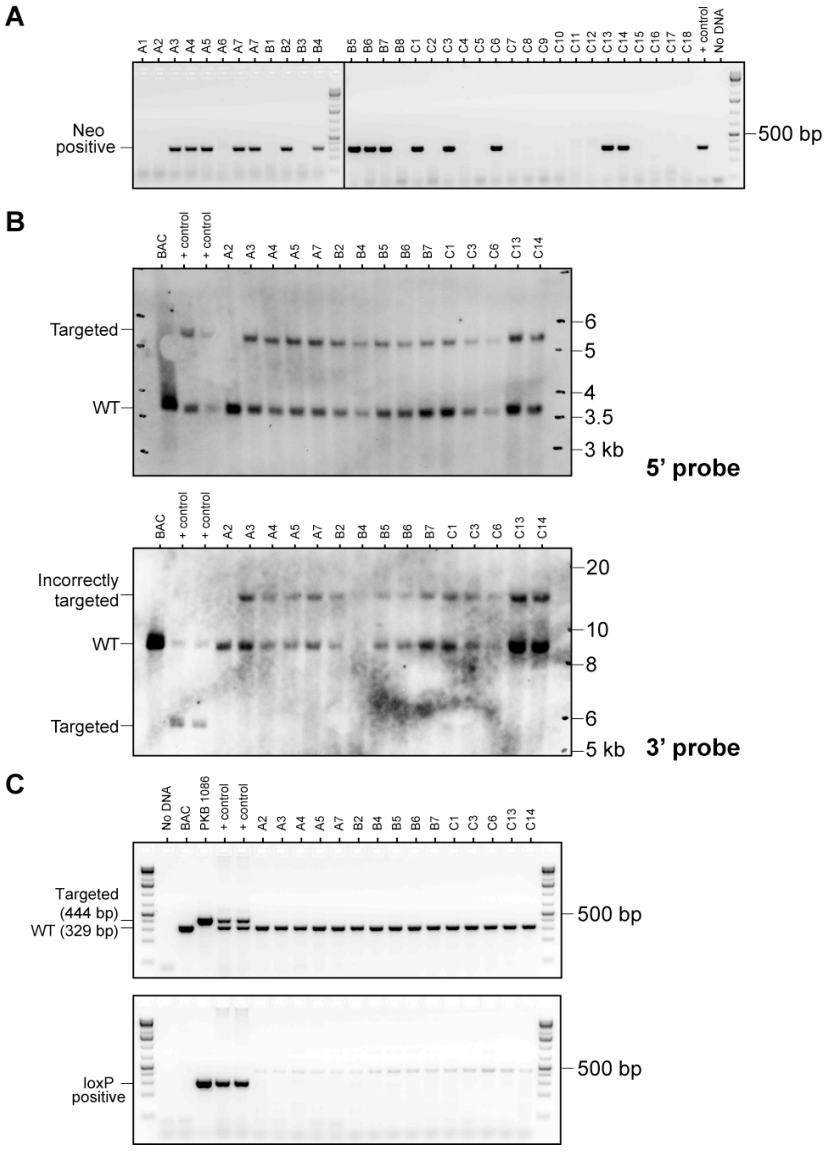

Figure 3.4. The targeting construct was only correctly integrated at the 5' end, losing the lonely *lox*P site in the 3' homology arm.

(A) Genomic DNA was extracted from tail lysate using the HotSHOT method and PCR amplification carried out to check for the presence of the Neo^r cassette using the primers PKO838 and PKO839. The expected PCR product is 458 bp. (B) For Southern blotting, genomic DNA was extracted with phenol-chloroform-isoamyl-alcohol and digested with SpeI for detection with the 5' probe or MfeI for detection with the 3' probe. The 5' probe was PCR amplified with PKO1502 and PKO1503 and the 3' probe with PKO1191 and PKO1192 and were DIG-labelled by random-primed labelling. Sample A2 was included as a negative control and correctly targeted ES cells samples from a previous screen were used as positive controls (+ control). Expected fragment sizes are indicated. (C) PCR amplification to distinguish between wild type and targeted alleles was carried out with primers PKO2282 and PKO2285 (top). The primer pair PKO2284 and PKO2286 was used to verify the presence of the lonely *lox*P site (bottom). The expected PCR product is 261 bp. The faint band visible at ~500 bp is non-specific.

3.1.2 Screening of ES lysates

Figure 3.5 shows the results the screening of a new set of ES cell lysates. 66 samples were received this time. Southern blotting with the 3' probe following MfeI digestion was carried out for all samples. Four samples, samples 2639, 2640, 2654 and 2669 were positive for the targeted band. 17 samples were found to be positive for the 13 kb band which results when homologous recombination had occurred before the lonely *lox*P site (Figure 3.5A).

Samples 2639, 2640 and 2650 – 2671 were also tested for positive targeting at the 5' end following SpeI digestion (Figure 3.5B). Nine samples were found to be correctly targeted, including the samples 2639, 2640, 2654 and 2669. This indicates that ES clones 2639, 2640, 2654 and 2669 had correctly integrated the targeting construct into the *Cdc7* locus at both 5' and 3' ends. Consequently, these were to be used in microinjections into blastocysts for the derivation of chimeric mice.

Only Southern blotting can be reliably used in the screening of ES cell lysates, as these lysates may have been prepared from mixed colonies following electroporation. As such, even a few cells carrying correctly targeted construct would yield a positive PCR result. If a mixed colony were subsequently used to derive chimeric mice, the efficiency of generating a desired transgenic animal would be lowered. There is also the possibility of successful electroporation but no recombination in the ES cell. In this scenario, PCR would yield a false positive result.

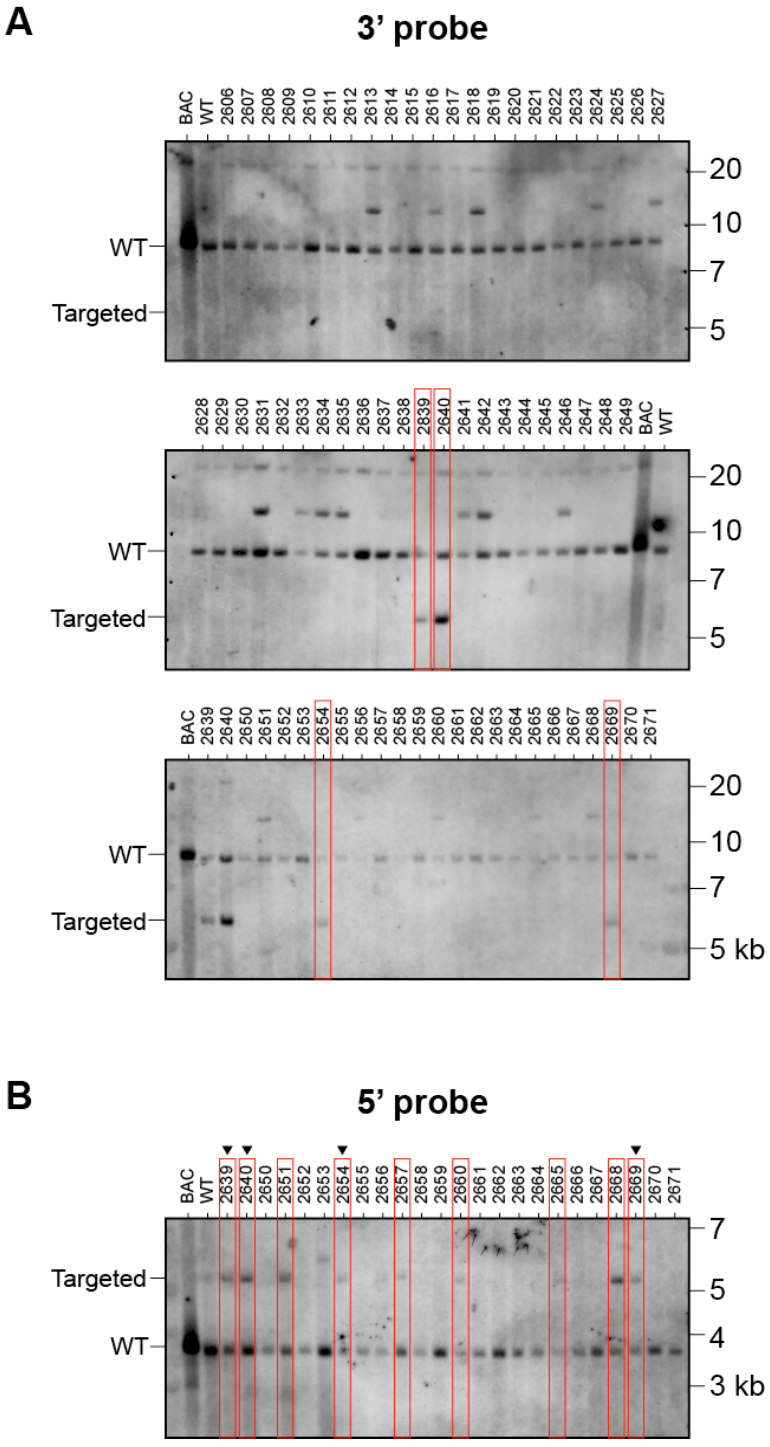

Figure 3.5. ES clones 2639, 2640, 2654 and 2669 are correctly targeted.

(A) Genomic DNA was extracted with phenol-chloroform-isoamyl-alcohol and digested with MfeI for detection with the 3' probe. Samples highlighted with red boxes are correctly targeted. (B) Correctly targeted samples together with a subset of samples were digested with SpeI for detection with the 5' probe. Samples highlighted with red boxes are correctly targeted at the 5' end. Samples with additionally marked with an arrowhead are correctly targeted at both ends.

3.1.3 2nd screening of mouse-tail lysates

Following ES cell injections into blastocysts, the chimeric mice born will be mated and their offspring have to be tested to identify animals carrying a germline transmission of the targeted allele. Nine samples were received for this second round of screening of mouse-tail lysates. As shown in Figure 3.6A, D5 tested positive for the targeted band at both the 5' and 3' end by Southern blotting. PCR analysis verified the presence of a floxed allele, the lonely *lox*P site and the Neo cassette, indicating that the targeting construct had indeed been correctly inserted. These positive results indicated a germline transmission of the cko allele in this mouse. From this screen, mouse D5 will be mated and the resulting embryos re-derived in order to establish a transgenic mouse colony under specific-pathogen-free conditions, in line with local animal facility requirements.

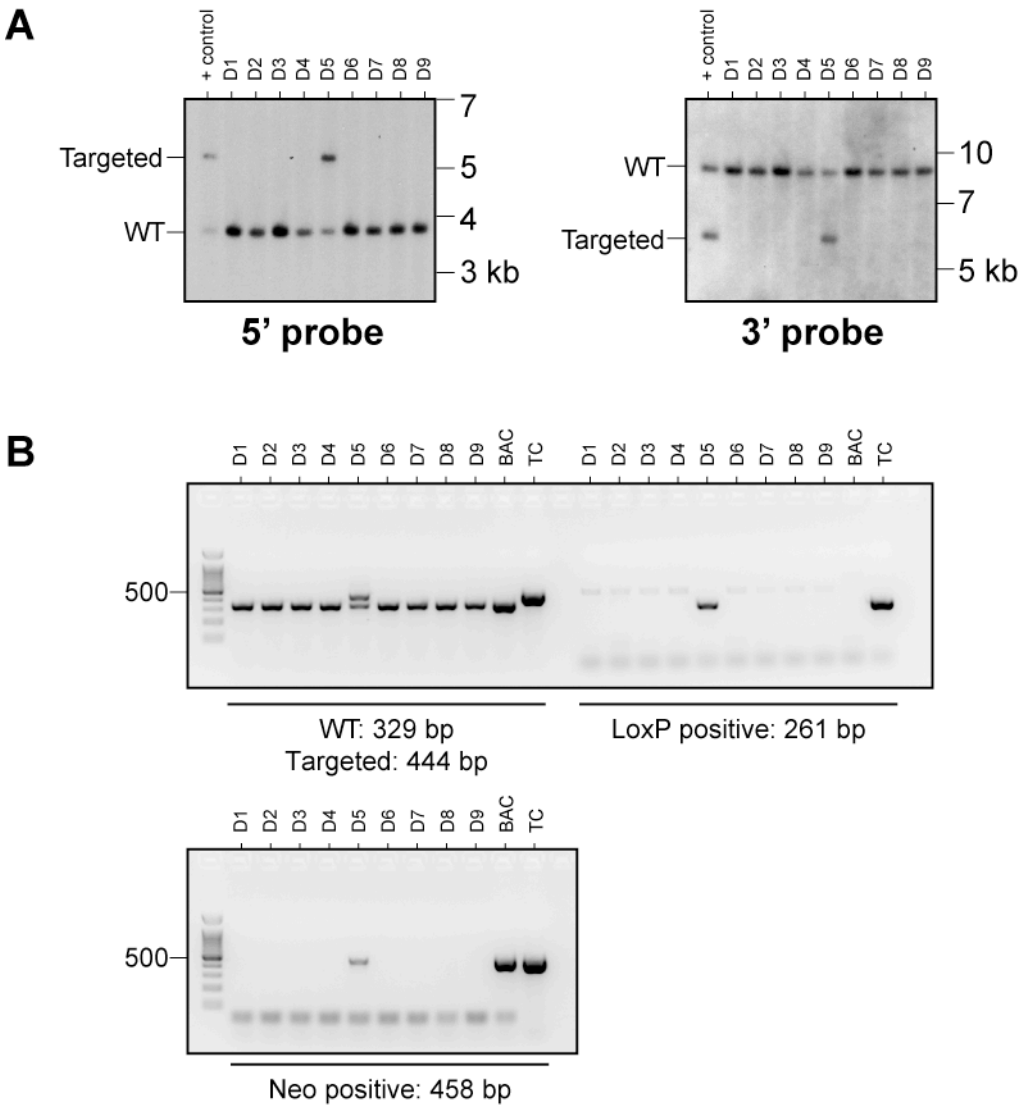

Figure 3.6. Mouse D5 is heterozygous for the *Cdc7* cko allele.

(A) Genomic DNA was extracted from mouse-tail lysates with phenol-chloroform-isoamyl-alcohol and digested with SpeI for detection with the 5' probe or MfeI for detection with the 3' probe. D5 is positively targeted when tested with both probes. ES cell lysate from sample 2640 (Figure 3.5) was used as a positive control. (B) PCR verification was done to confirm the presence of the targeted allele (PKO2282, PKO2285), the lonely loxP site (PKO2284, PKO2286) and the Neo^r cassette (PKO838, PKO839). The expected band sizes are indicated below each gel picture. The targeting vector (TC) was used as a positive control in these PCRs.

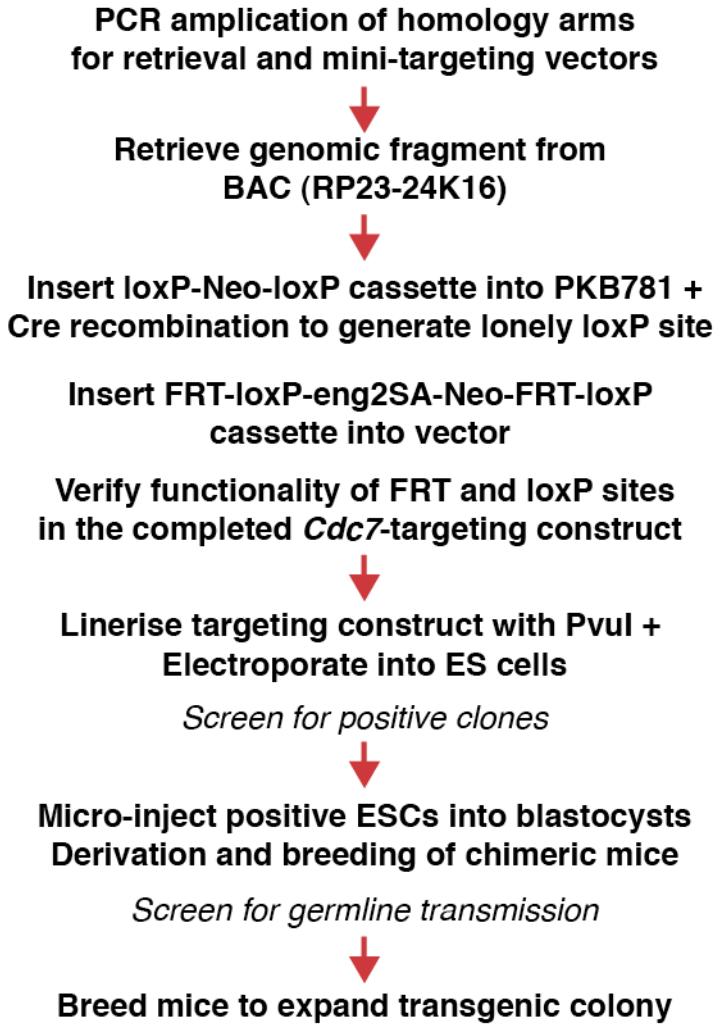

Figure 3.7. Workflow for creating the *Cdc7* conditional knockout mouse using the recombineering method.

Discussion

In this chapter, I have briefly outlined the design strategy for the generation of a conditional *Cdc7* knockout mouse. Results from the screening performed to identify a transgenic founder were presented. One mouse was found to carry a germline transmission of the conditional knockout targeting construct.

The recombineering technique for gene targeting

The workflow for creating a *Cdc7* cko mouse strain is summarised in Figure 3.7, making use the recombineering technique in various stages to construct the targeting vector. The use of recombineering simplifies the process compared to using traditional cloning methods which rely on restriction enzymes and DNA ligases for vector construction. Greater flexibility is afforded, as any region of the BAC is now amenable to modification (Copeland et al., 2001). The placement of genetic elements such as *lox*P/FRT sites and selection markers in the targeting vector can be precisely determined without being limited by having to make use of existing restriction sites in the regions of interest.

In the retrieval step, a selected portion of the BAC vector is subcloned into a high copy plasmid for target vector construction. This relies on the gap repair mechanism to recombine free ends of the retrieval vector with homologous sequences carried on the BAC. The retrieval vector could have been generated by PCR amplification, using chimeric primers that have at their 5' ends the BAC homology sequences (45 – 50 bp) and at their 3' ends sequences to prime and amplify the high copy plasmid (Copeland et al., 2001). However in this study, the method of cloning and inserting longer homology arms of 300 – 400 bp into the retrieval plasmid was chosen to increase subcloning efficiency (Liu et al., 2003). By subcloning only a selected portion of the

BAC sequence, the potential problem of removing existing *lox*P sites in the BAC backbone before introducing the *lox*P-Neo-*lox*P and FRT-*lox*P-eng2SA-Neo-FRT-*lox*P into the regions flanking exons 3 and 4 is circumvented (Liu et al., 2003).

The expression of the λ-phage genes *exo*, *bet* and *gam* for recombination can be placed under the control of a plasmid (Murphy et al., 2000) or from a defective prophage integrated into the E. coli chromosome (Yu et al., 2000). In the generation of the *Cdc7* cko targeting vector, bacteria strains were made recombineering competent by transformation with the pSim18 plasmid (Lee and Liu, 2009). When λ-Red genes are expressed from a defective prophage system, gene expression is driven by the strong λ P_L promoter, which in turn is under the control of the temperature-sensitive λ *cI857* repressor. At 32ºC, there are negligible levels of Exo, Bet and Gam proteins. Expression of *exo*, *bet* and *gam* can be rapidly induced to high levels simply by shifting the culture to 42ºC for 10 to 15 min (Liu et al., 2003). The pSim18 plasmid carried the Red genes under this heat-inducible control, combining the flexibility of tightly controlled gene expression with the ease of delivering recombineering functions into any BAC-containing bacteria strain by simple plasmid transformation (Lee and Liu, 2009).

Considerations in inserting the lonely lox*P site*

An important feature of this conditional knockout is the presence of *lox*P sites flanking exons 3 and 4. Owing to the small size of the lonely *lox*P site in comparison to the length of homology on either side, care must be taken to ensure that the whole targeting vector had been correctly integrated at both the 5' and 3' ends. To this end, an MfeI site was engineered into the *lox*P-Neo-*lox*P cassette so that an MfeI digest followed by Southern blotting would give a 5.6 kb product in a correctly targeted genomic sample. As described in Sections 3.1.1 and 3.1.2, this also allowed the

identification of constructs that had only incorporated the 5' end of the targeting vector correctly. Another precaution was to make use of PCR primers specific for the lonely *lox*P sequence to confirm its presence. As seen in Figure 3.5A, 17 out of 66 ES cell lysate samples did not integrate the lonely *lox*P, compared to 4 out of 66 samples which did so correctly, pointing to a low efficiency of introducing the small lonely *lox*P site into the gene locus. On hindsight, the efficiency of this recombination step could have been improved by having a longer 3' homology arm, starting from the lonely *lox*P site, of approximately 5 kb up to 10 kb in length (Bouvier and Cheng, 2009). By having the lonely *lox*P far from the ends of the linear targeting vector, it would be "safely" located in the middle portion of the targeting vector, which is exchanged intact into the genomic locus, following homologous recombination occurring much further downstream in the 3' direction.

Future perspectives for the Cdc7 conditional knockout mouse

The cellular effects of Cdc7 depletion have been characterised through siRNA knockdown models in various cancer cell lines. Experiments revealed that inhibition of origin firing through targeting Cdc7 triggers a tumour-cell specific, p53-independent apoptotic response following an abortive S phase, which is not observed in normal cells. In contrast, wild type p53 and intact checkpoint responses arrest normal cells reversibly in G1 following Cdc7 depletion, maintaining untransformed cells in a viable non-proliferative state (Montagnoli et al., 2004; Montagnoli et al., 2008; Tudzarova et al., 2010). The response of normal somatic cells to Cdc7 depletion appears to be similar to the response to licensing inhibition and it has been proposed that a licensing checkpoint may exist in G1 that senses an insufficient number of licensed origins prior to entry into S phase (Blow and Gillespie, 2008; Shreeram et al., 2002). This is different from an intra-S checkpoint response that arrests cells in S phase following replication fork stalling or DNA damage (Branzei and Foiani, 2009). However, Cdc7 depletion also

caused cell death in p53-positive cells, suggesting that p53 alone is insufficient to prevent cell death due to Cdc7 depletion in these cells. The authors investigated the mechanisms of cell death upon Cdc7 depletion and their results revealed that distinct cell-cycle responses were induced by Cdc7 depletion in p53-positive and -negative cells, with p53-negative cells arresting in G2 with an accumulation of mitotic regulators, followed by post-mitotic cell death after prolonged arrest, while p53-positive cells mostly die after entry into an aberrant S phase (Ito et al., 2012). The molecular mechanisms leading to cell death and the checkpoint response following Cdc7 depletion remain to be characterised.

Cells from this *Cdc7* conditional-knockout mouse may be useful in delineating the molecular differences in the putative licensing checkpoint operating in G1 and the intra-S checkpoint as cells expressing normal, low and no Cdc7 protein respectively may can be generated and characterised for experiments. Activation of the intra-S checkpoint requires that some origins be fired before insufficient initiation present as replicative stress, leading to fork stalling which may eventually result in cell death. This process may be recapitulated using cells with a low level of Cdc7 expression whose response may be expected to be different from cells in which *Cdc7* knockout has been induced prior to S phase, leading to signalling through the putative licensing checkpoint. By crossing the *Cdc7* conditional knockout strain with a p53-null mouse line, the role of p53 status in response to *Cdc7* knockout can also be studied. Alternatively, mouse embryonic fibroblasts with a $Cdc7^{flox/flox}$ genotype may be infected with shRNA to generate a stable p53-knockout cell line, which may be used in experiments to delineate p53-dependent and -independent checkpoint signalling pathways upon Cdc7 abrogation. These experiments mirror those reported in an earlier study carried out with siRNA knockdown of Cdc7 in a human fibroblast cell line (Tudzarova et al., 2010). This will be a useful means to validate the findings presented

in that study using a different and potentially more thorough and specific approach to abrogate Cdc7 expression in cells.

In the same way, cells expressing normal, low and no Cdc7 protein will also be useful to examine the role of Cdc7 in replication timing regulation. DDK in the fission yeast has been suggested to be a diffusible, catalytic, rate-limiting activator of origin firing (Patel et al., 2008). Through manipulating Hsk1-Dfp1 levels, it was demonstrated that increasing or decreasing origin firing rates caused increased genomic instability. The cells derived from this cko animal present an ideal model in which this hypothesis can be tested in mammalian cells.

Cdc7 and its regulator Dbf4 have been found to be upregulated in multiple cancer cell lines and primary tumours, including ovarian cancer (Kulkarni et al., 2009), malignant melanoma (Clarke et al., 2009; Nambiar et al., 2007) and breast cancer (Choschzick et al.). It is not clear how Cdc7 expression levels contribute to tumourigenesis and cell survival, however. Higher levels of Cdc7 expression do not always correlate with the proliferative status of cells; indeed, overexpression of Cdc7 and Dbf4 have been shown to cause cell cycle arrest in S phase (Guo et al., 2005). At the level of a whole organism, the importance of Cdc7 protein abundance or activity to tumourigenesis and cell survival may be studied if mice with the *Cdc7*$^{flox/flox}$ genotype were used to generate models of different cancers. The effect of Cdc7 depletion may then be characterised in different tissues via the expression of the Cre recombinase driven by tissue specific promoters or at different stages of disease development. Results from these studies may complement work from ongoing clinical trials (Koltun et al., 2012; Montagnoli et al., 2010) and other work using Cdc7 inhibitors as a potential anticancer treatments (Ermoli et al., 2009; Koltun et al., 2012; Menichincheri et al., 2010; Menichincheri et al., 2009; Shafer et al., 2008; Vanotti et al., 2008; Woods et al., 2012; Zhao et al., 2009).

Observations from such animal studies could have implications for how best to use Cdc7 inhibitors as anticancer agents in a physiological context.

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Chapter 4 – Characterisation of *Cdk1/Cdk2* double knockout mouse embryonic fibroblasts

Introduction

Cyclin-dependent kinases drive important transitions in the cell cycle. Highly conserved in metazoans and abundantly expressed in all dividing cells, Cdk2 was believed to be an essential regulator of mammalian S phase based on its activation timing, cyclin-binding preferences and dominant negative (DN) phenotypes. In an early study, *in vitro* injection of Cdk2-specific antibodies into cells caused an arrest in G1 phase of the cell cycle (Tsai et al., 1993). Similar cell cycle arrests were also observed with antibodies against cyclin A and cyclin E, the cyclin partners of Cdk2 (Ohtsubo et al., 1995; Pagano et al., 1992). By overexpressing DN alleles in which the Cdk is catalytically inactive while still retaining its cyclin-binding functions, it was determined that Cdk1 and Cdk2 regulate different processes in the cell cycle (Hu et al., 2001; Itzhaki et al., 1997; van den Heuvel and Harlow, 1993).

Cdk2 was thought to drive the G1/S transition in association with E-type cyclins, phosphorylating pRb to complete its inactivation after the initial action of Cdk4 and Cdk6 (Lundberg and Weinberg, 1998), and then allow progression through S phase with A-type cyclins as binding partner. However, this notion was challenged when it was reported that knockdown of Cdk2 with antisense DNA or siRNA oligonucleotides did not cause a G1 arrest or block proliferation in human cancer cell lines (Tetsu and McCormick, 2003). This study was followed by the generation of viable mice in which *Cdk2* has been disrupted by gene targeting, demonstrating that *Cdk2* is genetically dispensable for cell proliferation and the mitotic cell cycle *in vivo* (Berthet et al., 2003; Ortega et al., 2003). These results raised questions about the presumed specialised functions of Cdks in cell cycle events and transitions and it was of major interest to

delineate the Cdk activities needed for S phase entry and progression.

Primary mouse embryonic fibroblasts (MEFs) isolated from *Cdk2*$^{-/-}$ embryos proliferate in culture with similar kinetics to those derived from heterozygous or wild type embryos at low culture passages. These cells enter S phase with similar kinetics to wild type cells after release from serum starvation (Berthet et al., 2003; Ortega et al., 2003). Cells from later passages proliferate more slowly and exhibit a slight delay in S phase entry (Berthet et al., 2003; Ortega et al., 2003). In primary *Cdk2*$^{-/-}$ MEFs, immuno-precipitates obtained with antibodies specific to cyclin A contained kinase activity (Berthet et al., 2003; Ortega et al., 2003) and pRb was phosphorylated on Thr821, a residue thought to be a specific Cdk2-cyclin E substrate, indicating that another kinase can phosphorylate Cdk2 substrates.

In the absence of Cdk2, Cdk1-cyclin A was initially suggested to phosphorylate Cdk2-cyclin A targets (Berthet et al., 2003; Ortega et al., 2003). It was subsequently demonstrated that Cdk1 is able to bind cyclin E1 and these Cdk1-cyclin E complexes are active and responsible for promotion of S phase entry in the absence of Cdk2 (Aleem et al., 2005). In the study, proliferation of wild type MEFs was reduced following knockdown of Cdk1 with a short hairpin RNA (shRNA) construct. This effect was more pronounced in *Cdk2*$^{-/-}$ MEFs. The percentage of *Cdk2*$^{-/-}$ cells entering S phase following release from serum starvation was also reduced by a greater extent after Cdk1 knockdown, compared to wild type MEFs. These findings indicate that Cdk1 plays a role in S phase entry, although Cdk1-cyclin E activity is normally very low in wild type cells, presumably due to regulation by p27 (Aleem et al., 2005). Consistent with these results, mouse embryos lacking all interphase Cdks can develop to mid-gestation (Santamaria et al., 2007), with cell cycle progression being mediated by Cdk1, which was able to bind all cyclins, phosphorylate pRb and allow the expression of genes regulated by E2F transcription factors. Cdk1 thus appeared to have a previously

unstudied role in mediating G1/S entry and progression that becomes more important in the absence of Cdk2.

In order to study the importance of Cdk1 and Cdk2 activity in mammalian cells, in particular their roles in promoting S phase entry and progression, the loss of both Cdk1 and Cdk2 was analysed in cells derived from a conditional *Cdk1/Cdk2* double knockout mouse model. This is possible by taking advantage of the *Cdk1* conditional knockout mouse recently generated (Diril et al., 2012). Cdk1 loss can be induced in cells carrying the targeted construct by expression of the Cre recombinase. Induction of recombination in the *Cdk1* allele in a *Cdk2* null background (Berthet et al., 2003) then allows for *in vitro* analysis of cell cycle events in the absence of both functional Cdk1 and Cdk2 activities. In the following sections, results including cell cycle kinetics and biochemical characterisation are presented.

Results

4.1 Cdk1 loss is induced upon 4-OHT treatment to generate double knockout cells

Primary mouse embryonic fibroblasts (MEFs) were isolated from *Cdk1*$^{flox/flox}$/*Cdk2*$^{+/+}$ or *Cdk1*$^{flox/flox}$/*Cdk2*$^{-/-}$ embryos carrying the allele for inducible Cre/ESR1 expression. Supplementation of cell culture media with 4-hydroxytamoxifen (4-OHT) causes nuclear translocation of the Cre recombinase, hence excising exon 3 of *Cdk1* which is flanked by *lox*P sites (Figure 4.1) (Diril et al., 2012). Induction was carried out for 72 h, concurrent with serum starvation of the MEFs to synchronise them in G1 phase of the cell cycle (Figure 4.2A).

After 72 h serum starvation and induction, cells were harvested and genotyping PCR revealed that the floxed *Cdk1* allele was no longer detected at a significant level. The PCR product for the *Cdk1* null (*Cdk1* knockout; K1 KO) allele was observed instead (Figure 4.2B). qRT-PCR carried out using primers specific for regions spanning exons 3 and 4 also confirmed a loss of *Cdk1* transcript after 4-OHT treatment. As seen in Figure 4.2C, *Cdk1* transcript could be detected upon release from induction and serum starvation in wild type and *Cdk2* knockout (K2 KO) cells, which increased 2-fold by 24 h after release into full serum. In contrast, no *Cdk1* transcript was detected at both time-points in the *Cdk1* KO and *Cdk1*/*Cdk2* double knockout (DKO) cells that were exposed to 4-OHT during serum starvation. Together, this demonstrates that wild type *Cdk1* allele and expression is lost following 4-OHT treatment.

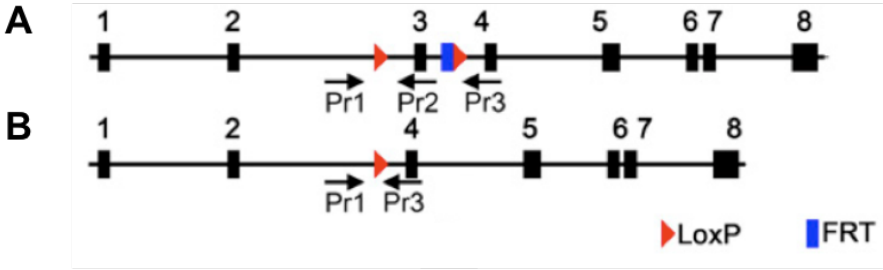

Figure 4.1. Schematic of the conditional *Cdk1* knockout construct.

(A) In the floxed *Cdk1* construct, exon 3 is flanked by a pair of *lox*P sites (red triangles). An FRT site (blue rectangle) is left behind after Flp-mediated recombination to remove a Neo^r selection cassette. (B) After Cre recombination, exon 3 will be excised, resulting in a frame-shift. Pr1, 2 and 3 indicate positions of PCR primers for genotyping verification. Modified from (Diril et al., 2012).

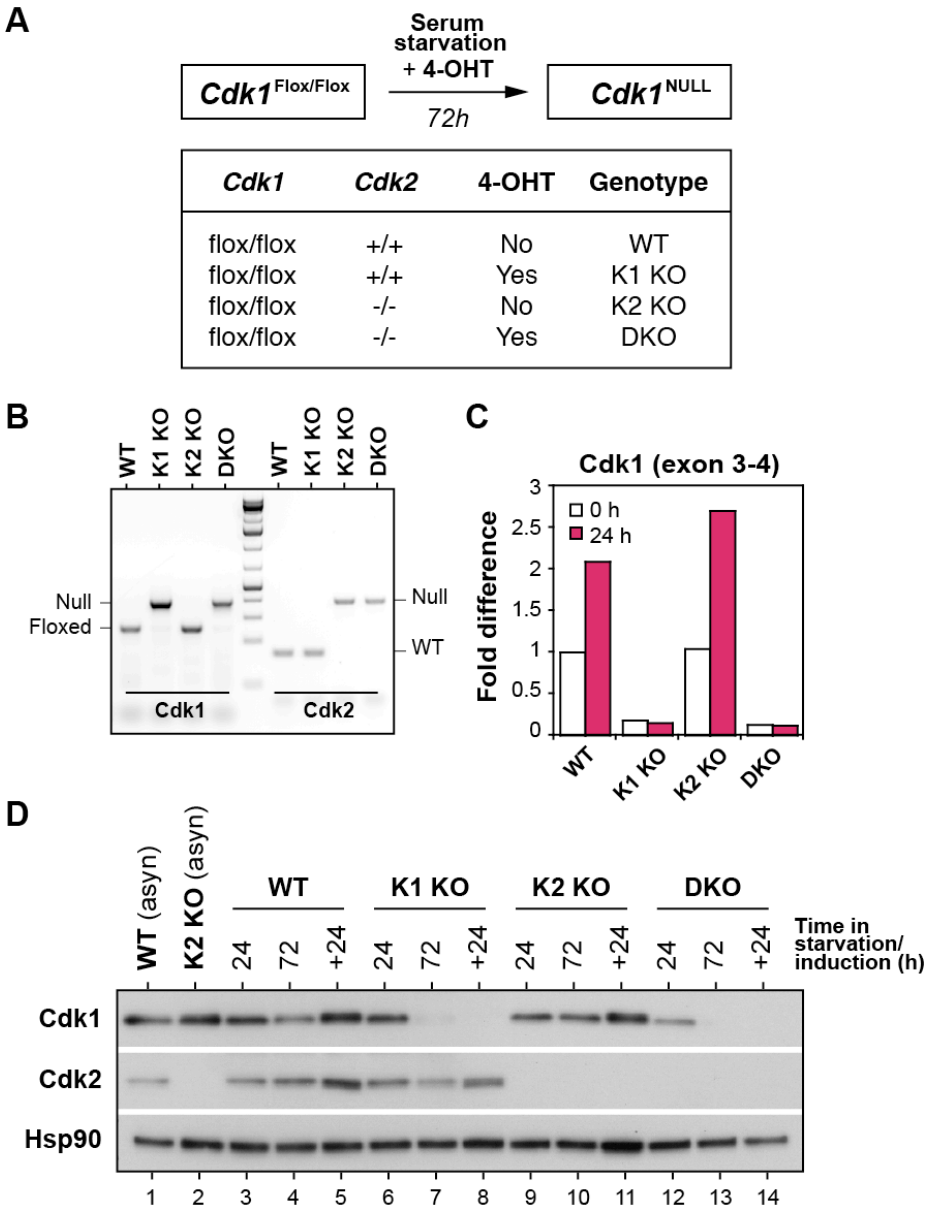

Figure 4.2. Treatment with 4-OHT induced loss of Cdk1 in MEFs.

(A) Schematic of experimental setup. MEFs homozygous for the floxed *Cdk1* locus became *Cdk1*-null upon 4-OHT induction. This occurred concurrently with 72 h serum starvation to synchronise cells in G1. (B) Cells were harvested upon release from serum starvation and DNA extracted for genotyping. (C) qRT-PCR was performed using primers specific for the floxed *Cdk1* exon. (D) Cells were collected before serum starvation and induction (lanes 1,2), during serum starvation (24, 72 h) and after release into full serum (+24 h). Whole cell lysates were immuno-blotted for Cdk1 and Cdk2. Hsp90 served as loading control.

To monitor the loss of Cdk1 protein following 4-OHT induction, cells were harvested over the course of serum starvation and 4-OHT induction (Figure 4.2D, “24” and “72”) and after release into full serum (“+24”). Whole cell lysates were blotted with antibodies specific for Cdk1 and Cdk2. During serum starvation in the absence of 4-OHT, the amount of Cdk1 protein decreased slightly from 24 to 72 h after starvation in wild type cells and increased when cells were released into medium containing full serum. This profile was also observed in *Cdk2* KO cells, although there was a smaller decrease in Cdk1 level from 24 to 72 h during serum starvation. This decrease likely represents a general down-regulation of protein expression as cells become quiescent, which is reversed upon serum stimulation. In MEFs treated with 4-OHT during the course of serum starvation (K1 KO and DKO), the amount of Cdk1 detected was drastically decreased from 24 to 72 h. Background levels of Cdk1 were detected at 72 h in *Cdk1* KO cells; however by 24 h after release into full serum Cdk1 was no longer detected in both *Cdk1* KO and DKO cells by immuno-blotting. These results corroborate with *Cdk1* transcript levels, demonstrating that 4-OHT effectively induces the loss of the *Cdk1*$^{flox/flox}$ allele, leading to loss of Cdk1 protein in MEFs.

Cdk2 levels remained constant during serum starvation in wild type cells and were increased in the presence of serum. Cdk2 levels decreased slightly during serum starvation in *Cdk1* KO cells and increased upon release from starvation. However, the level of Cdk2 detected at 24 h after release was lower in *Cdk1* KO cells than wild type. Cdk2 was not detected at all time-points in *Cdk2* KO and DKO cells, as expected.

4.2 Cdk1/Cdk2 DKO cells can enter S phase but cannot progress through DNA synthesis efficiently

Cdk1 was proposed to compensate for the loss of Cdk2 interphase functions in *Cdk2* knockout mice (Aleem et al., 2005). To determine if the loss of both Cdk1 and Cdk2 would affect S phase entry and cell cycle progression in MEFs, BrdU was added to culture media for 30 min before cells were harvested at different time-points following release from serum starvation to monitor DNA synthesis (Figure 4.3A). As shown in Figure 4.3, at 18 h after release from serum starvation, 56.6% of wild type cells had incorporated BrdU and appear to be in early S phase, displaying a total DNA content between 2N and 4N. By 24 h, the S phase population had decreased to 43% as cells progress into late S phase. Cells in G2 phase have completed one round of DNA synthesis and have 4N DNA content. From 18 to 24 h, the G2 cell population increased from 5.2% to 22.6% while the G1 cell population did not change greatly. At 48 h, the percentage of cells incorporating BrdU had decreased to 29.6%, G2 cells had decreased to 9.3% and the population of cells with 2N DNA content but did not incorporate BrdU had increased to 50.7%. This indicates that wild type MEFs have completed one round of DNA replication, undergone mitosis and cycled back to G1 by 48 h after release into full serum.

When Cdk1 was absent in MEFs, cells were still able to enter S phase. At 18 h, 47.5% of cells had incorporated BrdU. This proportion decreased to 28.4 and 5.6% at 24 and 48 h respectively, indicating that cells were largely not actively synthesising DNA by the end of the time-course. The G2 cell population steadily increased from 18.9 to 34.4 and finally to 61.7% at 48 h. At all time-points, the G1 population remained <20% of all cells. This indicates that *Cdk1* KO cells were able to enter S phase and complete one round of DNA replication but then become arrested in G2 cells due to a mitotic defect. This is consistent with previously published results (Diril et al., 2012). *Cdk1* KO cells

cannot initiate early events of mitotic entry such as cytoskeletal reorganization and rounding up of the cell body and therefore cannot divide following genome duplication. Also in agreement with the earlier report, a significant level of endo-reduplication was observed in *Cdk1* KO cells compared to wild type cells, resulting in a population of cells with 8N DNA content, which can be observed as a distinct peak in total DNA content. Endo-reduplication in *Cdk1* KO cells is attributed to unrestrained Cdk2-cyclin A2 activity (Diril et al., 2012).

Cdk2 KO cells behaved largely in a similar manner to wild type cells, entering and progressing through S phase from 18 h to 24 h and cycling back to G1 by 48 h. *Cdk2* KO cells were slightly delayed in S phase entry as only 37.9% was BrdU-positive at 18 h compared to 56.6% of wild type cells. This is consistent with previous reports (Berthet et al., 2003; Ortega et al., 2003). However, no difference was observed at the subsequent time-point, indicating that there was largely no significant difference in cell cycle progression in wild type and *Cdk2* KO MEFs.

Surprisingly, a population of DKO cells cells (14%) had incorporated BrdU at 18 h. This percentage had increased to 27.9% at 24 h with a corresponding decrease in the G1 cell population, suggesting that more cells had entered S phase by this time. Looking at the histogram of total DNA content, S phase entry was significantly delayed in DKO cells compared to wild type cells. The distribution of cells in G1, S and G2 phases subsequently remained relatively constant from 24 to 48 h, implying a lack of progress through the cell cycle. At 48 h, the intensity of BrdU incorporated had decreased by one order of magnitude compared to the intensity at 24 h, indicating that cells at this time were no longer incorporating nucleotides efficiently. Taken together, these results strongly suggest that while a small population of cells lacking Cdk1 and Cdk2 can enter S phase, the timing of entry is delayed, progress through S phase is inefficient and cells could not complete one round of DNA synthesis following synchronisation.

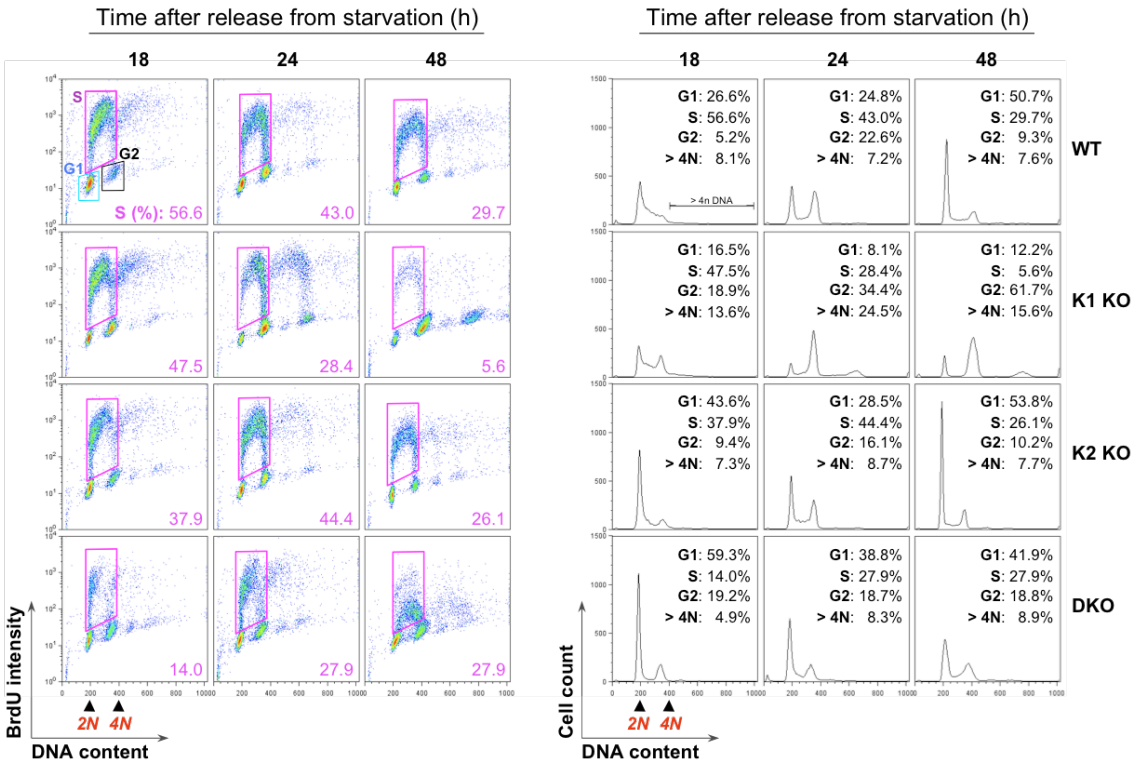

Figure 4.3. Cell cycle profile of MEFs following release from serum starvation.

Following serum starvation with or without 4-OHT induction, MEFs were labelled with BrdU for 30 min before harvest. Cells were also stained with PI for total DNA content and analysed by flow cytometry. This experiment has been performed 6 times and this is the cell cycle profile of one representative experiment.

From Figure 4.3, DKO cells that were able to enter S phase appeared to synthesise DNA inefficiently, as observed from the markedly reduced level of BrdU incorporated at 48 h. This observation may also be attributed to the unlikely scenario that some DKO cells had completed a first S phase normally and had entered a second aberrant S phase. To distinguish between the two scenarios, a BrdU pulse experiment was carried out. In this experiment, instead of labelling cells at the time of collection, BrdU was added to culture medium at 18 h after serum starvation when cells are entering S phase. BrdU was then washed off and the cell cultures maintained for varying durations until they were harvested at indicated time-points (Figure 4.4B). This scheme allows all cells entering S phase at 18 h to be labelled with BrdU and follows the progress of these cells through the time-course. Cell division following mitosis would reduce the intensity of the incorporated BrdU label by approximately half. In contrast, labelling cells with BrdU just before harvest (Figure 4.4A), as in the previous experiment, only captures the distribution of cells in different phases at the time of collection but does not provide information about the progress a particular cell population with time.

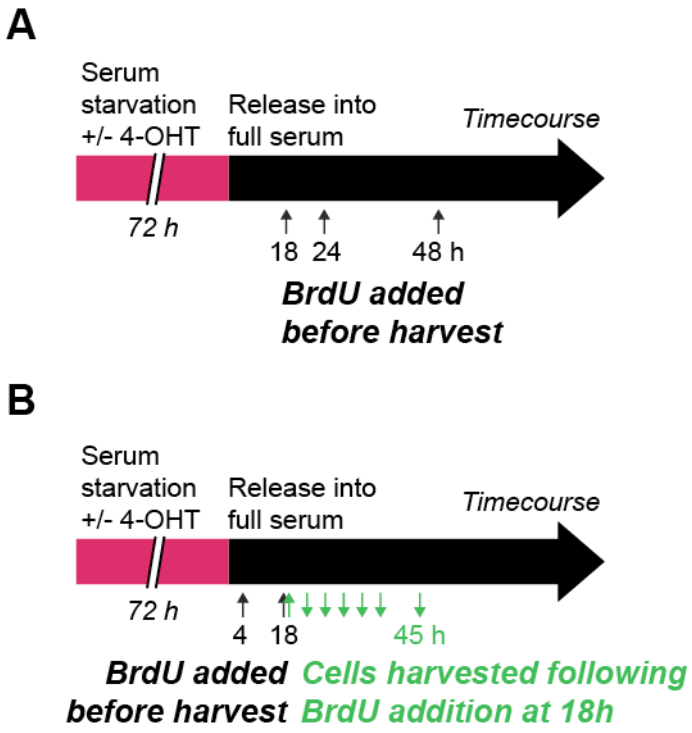

Figure 4.4. Schematics of BrdU treatment of MEFs to analyse cell cycle profile and cell cycle progression.

(A) To analyse the profile of different cell populations at individual time-points, MEFs were labelled with BrdU for 30 min before harvest, following release from serum starvation with or without 4-OHT induction. (B) In order to monitor the progress of one cell population through the time-course, cells were labelled for 30 min at 18 h following release from serum starvation with or without 4-OHT induction. BrdU was washed off and pulse-labelled cells allowed to progress through the cell cycle. Cells were collected at intervals following the BrdU addition.

The results from this pulse-labelling approach are presented in Figure 4.5. At 4 h after release from serum starvation, cells were well-synchronised in G1, with 57 – 69.9% cells displaying 2N DNA content, without BrdU label. At 18 h, cells in all genotypes entered S phase, with 59.4, 52.1, 35.2 and 10.6% from wild type, *Cdk1* KO, *Cdk2* KO and DKO respectively, having incorporated BrdU. This is consistent with results from Figure 4.3.

In an unperturbed S phase, wild type cells that were labelled at 18 h began to synthesise DNA, with the total DNA content of the labelled population increasing from 21 to 24 h. At 27 h, some cells have completed DNA synthesis and undergone cell division, resulting in the reappearance of cells with 2N DNA content and a lower intensity of BrdU labelling. This G1 population continued to increase till 29 h and then remained constant till 45 h, the final time-point in this experiment. The cell cycle progression of *Cdk2* KO cells is largely similar to wild type cells, with BrdU labelled cells having completed DNA synthesis and cycled back to G1 largely from 27 h after release from serum starvation. Again, this is consistent with previous reports that loss of Cdk2 does not affect the mitotic cell cycle (Berthet et al., 2003; Ortega et al., 2003).

A similar progression from S phase entry up to 27 h was observed in *Cdk1* KO cells, after which the proportions of cells with 4N and 8N DNA content remained constant. The proportion of cells with 2N DNA content remained low while the level of BrdU incorporated in cells that have completed S phase remained high until the end of the time-course. Taken together, this indicates that *Cdk1* KO cells did not undergo cell division to cycle back to G1.

Unlike the cells of the other genotypes which largely entered S phase at 18 h and then (for wild type and *Cdk2* KO) began to divide and cycle back to G1 by 27 h, DKO cells that had incorporated BrdU at 18 h only appeared to have substantially accumulated

4N DNA content by 29 h. There also appears to be a G2 arrest. These results suggest that the absence of both Cdk1 and Cdk2 prevented efficient DNA progression, leading to a defective S phase while the loss of Cdk1 then contributed to a subsequent mitotic defect and G2 arrest.

Overall, these results are also in line with proliferation assays, where it was observed that loss of Cdk2 did not affect cell proliferation whereas loss of Cdk1 alone or together with Cdk2 led to senescence and non-proliferation in MEFs (Figure 4.6). *Cdk2* KO cells proliferate like wild type cells.

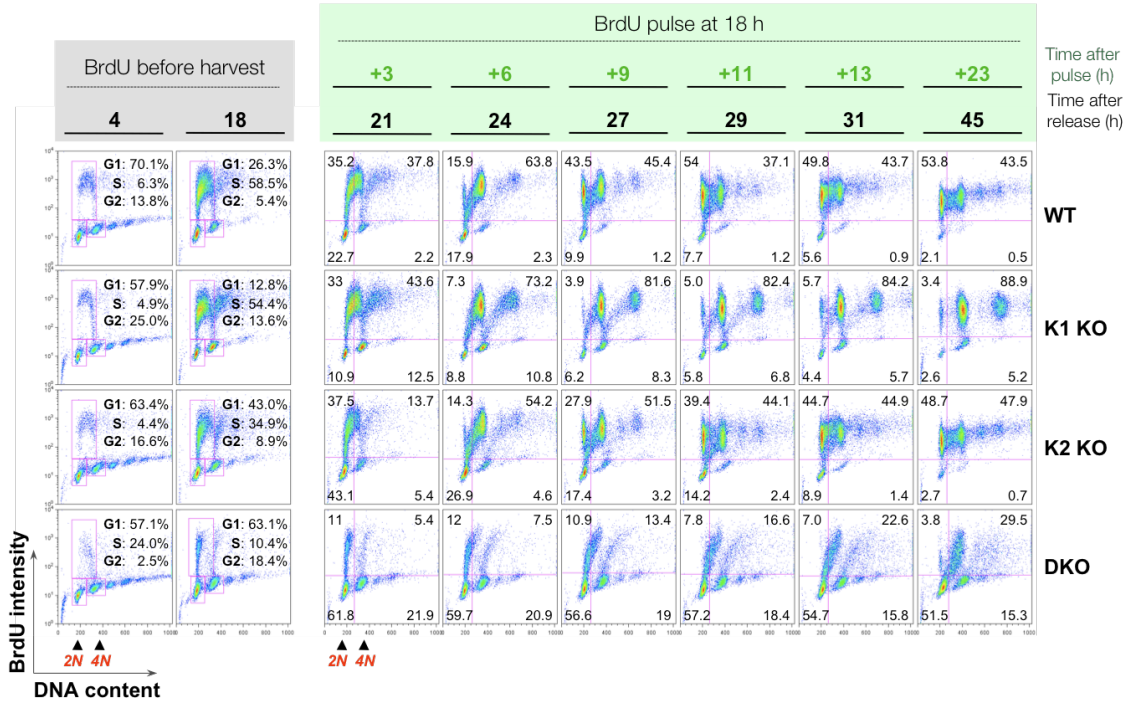

Figure 4.5. DKO cells enter but progress very inefficiently through S phase.

Following serum starvation and 4-OHT induction, cells were released into full serum. At 4 h and 18 h, cells were labelled with BrdU for 30 min before they were harvested (grey box). As outlined in Figure 4.4B, cells were pulse-labelled with BrdU for 30 min at 18 h. BrdU was washed off and cells were collected at the indicated time-points after labelling to monitor the progress of one population of cells (green box). Following collection, BrdU-labelled cells were analysed with flow cytometry. The labels in the top row (green text) indicate the time after the BrdU pulse while the labels in bottom row (black text) indicate the time after release into full serum. This experiment was performed twice with similar results; this is a representative plot.

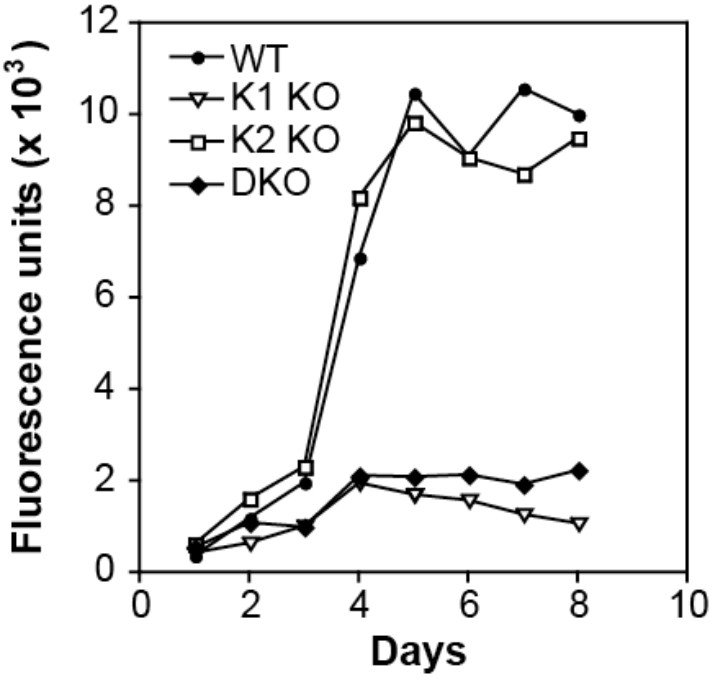

Figure 4.6. DKO cells do not proliferate.

Following serum starvation with or without 4-OHT, cells were plated at low density. Proliferation of MEFs was analysed using the alamarBlue assay every 24 h over the course of 8 days. This experiment has been performed three times and a representative plot is shown.

4.3 Cyclin E and Cdc6 protein levels are elevated in DKO cells

In order to explore the molecular basis for the cell cycle profiles observed, cells were collected at 24 h after release from serum starvation and whole cell lysates blotted for cell cycle regulators and components of the pre-RC. At this time-point, cells would be in mid-S phase. As shown in Figure 4.7A, Cdk1 was not detected in *Cdk1* KO and DKO cells, indicating efficient 4-OHT induced loss, and Cdk2 was not detected in *Cdk2* KO and DKO cells as expected. Cdk1 protein level appeared to be slightly lower in *Cdk2* KO cells. Cdk4 protein levels were relatively constant across cell types but may be slightly lower in *Cdk1* KO cells, as was the case for Cdk6. p27, a member of the Cip/Kip family of Cdk inhibitors, appeared to be lower in *Cdk1* KO and higher in cells lacking Cdk2 (*Cdk2* KO and DKO). This is in agreement with the report that Cdk2-cyclin E phosphorylation can down-regulate p27 (Sheaff et al., 1997). Asynchronous wild type and *Cdk2* KO cells appeared to express higher levels of Cdk1; there was no significant difference in the levels of other proteins.

Cyclin A2 was observed to be present in significantly higher levels in serum-released cells compared to asynchronous cells. This likely reflects the enrichment of S phase cells following synchronisation, compared to an asynchronous MEF population. Comparing across different genotypes, cyclin A2 was present at a lower level in cells lacking Cdk2: it was lower in *Cdk2* KO compared to wild type asynchronous cells (lanes 1 and 2) and in *Cdk2* KO and DKO compared to wild type and *Cdk1* KO synchronised cells (lanes 5 – 6 and 3 – 4). DKO cells had a significantly lower level of cyclin A2 compared to all genotypes. Cyclin B1 appeared to be present at a higher level in *Cdk2* KO and significantly lower in DKO, similar to cyclin A2. Cyclin D1 levels did not vary significantly across genotypes or between asynchronous and synchronous cells. In contrast, cyclin E1 was present in markedly increased levels in DKO cells

compared to the other genotypes. However, no significant difference was observed across the other cell types (Figures 4.7A and B).

The protein levels of components of the pre-replicative complex were examined to determine if an absence or deficiency of any components might be impeding S phase onset or progression in DKO cells. As shown in Figure 4.7A, there was no difference in the levels of Mcm2, Mcm4 and Cdc45 present across all cell types. Mcm2 may be marginally higher in DKO cells. In contrast to other pre-RC proteins, Cdc6 was present at an appreciably elevated level in DKO cells. There appeared to be slightly increased levels of Cdc6 in synchronised cells than asynchronous cells. Again, this may be due to higher proportion of cells undergoing S phase cells following synchronisation compared to a typical asynchronous MEF population.

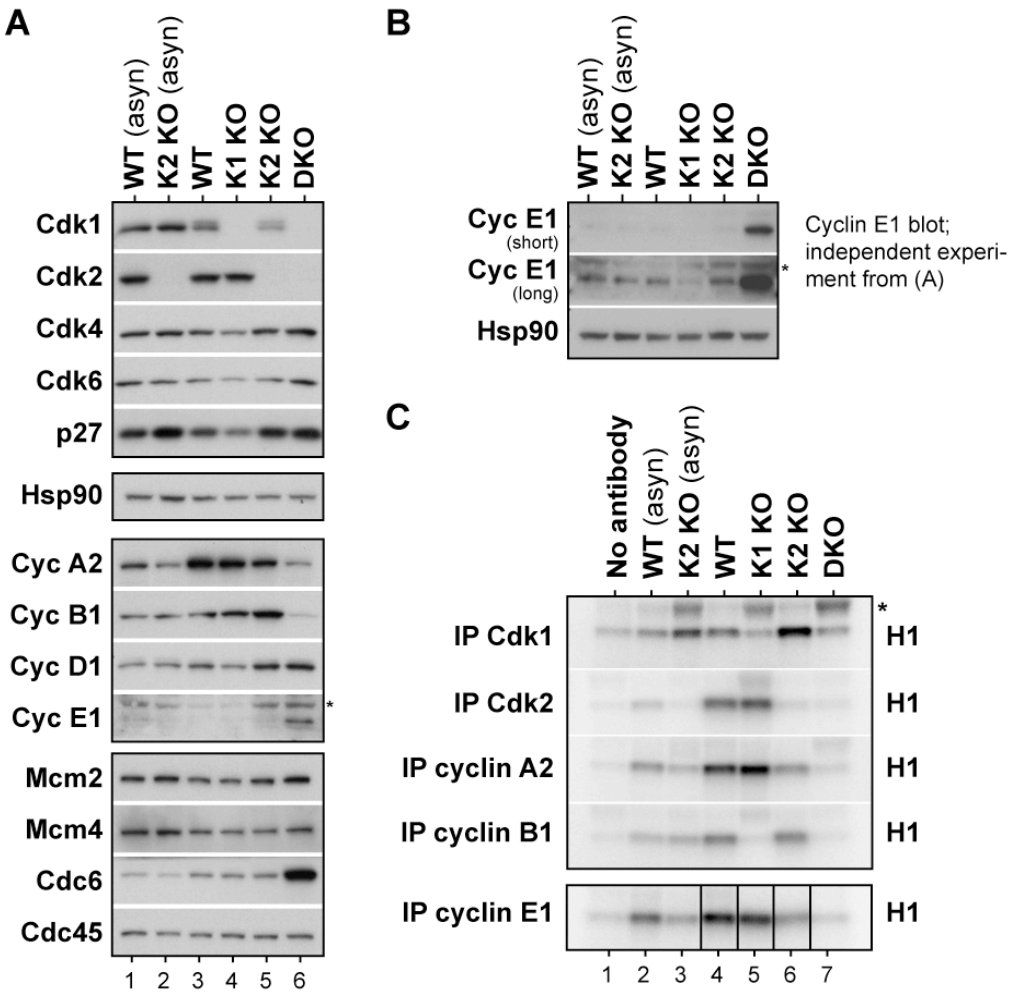

Figure 4.7. Cdc6 and cyclin E1 protein levels are upregulated in DKO.

(A) Cells were collected before serum starvation and induction (lanes 1,2) or 24 h after release into full serum. Whole cell lysates were immuno-blotted for the indicated proteins. Hsp90 served as the loading control. (B) A blot with a longer exposure of cyclin E1 is presented from a different experiment, carried out as described in (A). (C) MEFs were treated and harvested as in (A). Immuno-precipitates for the indicated Cdks or cyclins were prepared from total cell lysate and subjected to a radioactive kinase assay using histone H1 as substrate. The panel presented for cyclin E1 kinase activity is taken from a single phospho-scan of samples that were run on the same gel. Lanes have been omitted where there are separating lines for side-by-side comparison of the lanes shown. The asterisk (*) indicates a non-specific band.

4.4 Cyclin E1-associated kinase activity is not detected in DKO

Kinase assays were carried out to compare the kinase activities associated with Cdk1, Cdk2 and cyclins from MEFs of different genotypes. Lysates prepared from asynchronous cells and synchronised MEFs collected 24 h after serum starvation were used in this experiment. Overall, IPs from asynchronous wild type and Cdk2 KO cell lysates contained less kinase activity than synchronised MEFs of the same genotype. This is likely to be due to the enrichment of S phase cells by synchronisation.

Histone H1 was phosphorylated by all IPs from wild type cell lysates while no kinase activity was detected with all IPs from DKO cell lysates (Figure 4.7B). As anticipated, there was no Cdk1 and cyclin B1 kinase activity from IPs from *Cdk1* KO and no *Cdk2* activity from IPs prepared with *Cdk2* KO cells. Significant background was detected in the Cdk1 kinase assay, even in the “No antibody” control. Despite this, a difference in Cdk1 kinase activity level could still be observed in cells that contained or lacked Cdk1 protein. Cdk1-associated kinase activity appears to be elevated in Cdk2 KO cells, although no corresponding increase was detected in the cyclin B1-associated kinase activity. This is consistent with the previous report that Cdk1 kinase activity compensates for the loss of Cdk2 (Aleem et al., 2005), so that Cdk1 kinase activity increases in the absence of Cdk2 activity by binding to cyclin A2 molecules, thereby maintaining constant total kinase activity.

A p13-suc1 IP could be used to verify this since both Cdk1 and Cdk2 bind to p13-suc1, representing the total Cdk activity associated with Cdk1 and Cdk2. According to this idea, little or no difference is expected between p13-suc1 IPs from both wild type and *Cdk2* KO. In wild type cells, cyclin A2 binds to Cdk2 predominantly in S phase (Diril et al., 2012), so p13-suc1 would mainly pull down Cdk1-cyclin B1 and Cdk2-cyclin A2 complexes. However in the absence of Cdk2, previously free Cdk1 molecules that were

not binding cyclin B1 can form active complexes with unoccupied cyclin A2, resulting in more Cdk1 protein being engaged as active kinase complexes. Cdk1-cyclin B1 activity would be expected to remain unchanged.

Cyclin A2 kinase activity appears slightly higher in *Cdk1* KO compared to wild type cells. This is consistent with the idea that Cdk1-cyclin A2 complexes having lower specific kinase activity than Cdk2-cyclin A2 complexes (Diril et al., 2012). In a normal cell cycle, cyclin A2 initially forms complexes with Cdk2. Rising levels of Cdk1 protein from late S phase act to sequester cyclin A2 molecules from Cdk2, quenching cyclin A2-associated kinase. However in *Cdk1* KO cells, the absence of Cdk1 allows cyclin A2 to remained bound within high activity Cdk2-cyclin A2 complexes. This then results in the detection of higher levels of H1 phosphorylation compared to wild type cells (Diril et al., 2012).

In *Cdk2* KO cells, kinase activity was still detected with cyclin A2 and cyclin E1, the major cyclin partners of Cdk2, likely due to complex formation with Cdk1, although the kinase activity detected was lower than wild type controls. Cdk1 is known to bind to both cyclins to form active complexes, with Cdk1-cyclin E complexes being shown to compensate for Cdk2 functions in the mitotic cell cycle in the absence of Cdk2 protein (Aleem et al., 2005). No cyclin E1 kinase activity was detected in DKO cells, indicating that the high levels of cyclin E1 proteins present in DKO cells are not part of active kinase complexes. Since Cdk1, Cdk2, cyclin A and cyclin B proteins are not detected or detected at very low levels in DKO cells at this time-point (Figure 4.7A), no kinase activity was detected in these IPs with DKO lysates.

4.5 Cdc6 *transcript levels remain high in DKO cells throughout S phase*

Since high levels of Cdc6 were present in total cellular protein in DKO MEFs, it was of interest to determine if increased expression was the reason for this observation. Total RNA was extracted from cells at 0, 18, 24 and 28 h after release from serum starvation. Following reverse transcription, qRT-PCR was carried out for *Cdc6* expression levels (Figure 4.8A).

Looking at *Cdc6* expression levels in wild type MEFs, transcript levels increased upon release from serum starvation, to three times of 0 h levels at the start of at 18 h, decreased to two-fold at 24 h and then increased to approximately 2.5-fold at 28 h. This is consistent with published data that expression of mammalian *Cdc6* peaks in G1 and is down-regulated during S phase (Hateboer et al., 1998). This trend was also observed in *Cdk1* KO cells, though with slightly lower transcript levels at 24 and 28 h compared to wild type cells. In *Cdk2* KO cells, transcript levels across the three timepoints were similar to 18 h wild type levels. This may be attributed to the slight cell cycle delay in *Cdk2* KO MEFs (Figures 4.3 and 4.5). As such expression levels may not yet have been down-regulated from higher G1 levels. In DKO cells, there was a greater increase in transcript levels that was maintained over the course of S phase at 24 and 28 h. It appears that the loss of Cdk1 or Cdk2 alone does not affect the pattern of *Cdc6* expression greatly but the absence of both somehow resulted in an up-regulation of *Cdc6* mRNA through the S phase time-points surveyed. The reasons for this increase remains to be further investigated.

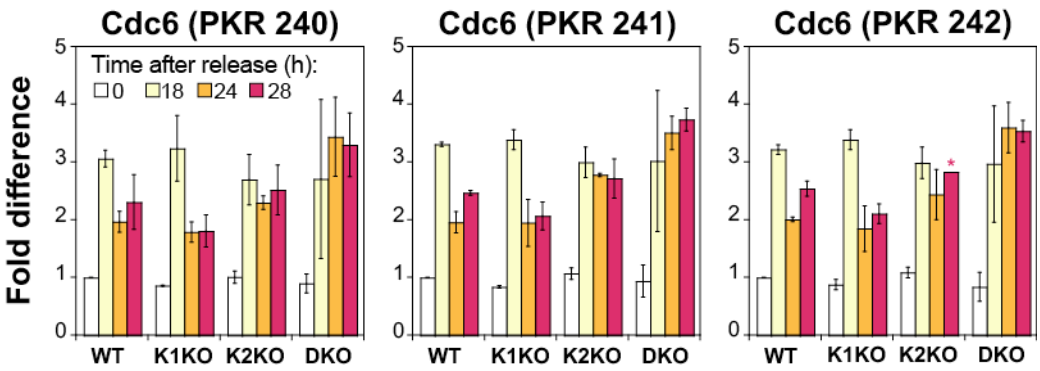

Figure 4.8. DKO *Cdc6* expression remains high through S phase.

Following serum starvation with or without 4-OHT induction, cells were harvested at the indicated time-points. Total RNA was extracted from cells and qRT-PCR performed with three primer pairs specific for *Cdc6* (PKR 240, 241, 242) following reverse transcription reaction. Values were normalised to housekeeping genes (β-actin, hsp90 and cyclophilin A) and the wild type 0 h sample to derive fold difference. Error bars represent S.D. from two experiments. The asterisk (*) indicates where a sample was lost and represents the value from only one experiment.

4.6 Increased levels of chromatin-bound Cdc6 in DKO cells

Cdc6 is a component of the pre-replicative complex that is loaded onto DNA during origin licensing from late M to G1 phase of the cell cycle. Together with Cdt1, it acts as a loading factor to recruit the Mcm2-7 helicase onto chromatin. Since the levels of Cdc6 were found to be much higher in whole cell lysate in DKO cells, the amount of Cdc6 bound to chromatin was also examined. Pre-RC components that have been assembled onto chromatin templates become resistant to extraction by detergents and can thus be fractionated from soluble cytosolic/nucleosolic proteins. Histone H3 or lamin A/C partition only with the insoluble/chromatin fraction and are used as loading controls.

Cells were serum-starved, treated with 4-OHT and harvested at 24 and 72 h during serum starvation (Figure 4.9A, “24” and “72”) or at 24 h after release into full serum (Figure 4.9A, “+24”). Cdc6 was not detected during serum starvation but was subsequently present in levels comparable to asynchronous cells at 24 h after release into full serum. Mcm2 and Mcm4 were present at all time-points, but at lower levels during serum starvation.

Both Mcm2 and 4 and Cdc6 were largely not detected on chromatin during serum starvation in MEFs from all genotypes. Upon release into full serum, both Mcm subunits and Cdc6 were detected on chromatin, as were seen in samples from asynchronous cells. These results indicate that origins are not licensed when cells are arrested in G0/G1 by serum withdrawal, consistent with previous results analysing licensing in quiescent cells. Pre-RC assembly subsequently takes place after release into full serum, an indication of the cells’ proliferative status (Stoeber et al., 2001).

The levels of chromatin-bound Cdc6 at 24 h after release appeared to be slightly decreased in *Cdk1* KO compared to wild type and were markedly higher in DKO cells. Chromatin-bound Cdc6 in *Cdk2* KO was similar to wild type amounts. Chromatin-bound Mcm4, on the other hand appeared to be present at lower levels in *Cdk1* KO cells compared to wild type and was detected in *Cdk2* KO and DKO cells at similar levels, higher than wild type. Since *Cdk1* KO cells can enter and complete a normal S phase following release from serum starvation (Figure 4.3 and 4.5), these results suggest that the decreased level of Mcm4 detected on chromatin in *Cdk1* KO MEFs was sufficient to support DNA synthesis and S phase progression. The distinctly higher level of Cdc6 bound to chromatin in DKO cells did not lead to the recruitment of a correspondingly larger quantity of Mcm4 and Mcm2 protein. This indicates that the large excess of Cdc6 protein may play a role independent of its pre-RC assembly function under these conditions. For instance, reports of Cdc6 involvement in delaying entry into mitosis upon S phase defect have been previously described (Liu et al., 2009a; Lu et al., 2009).

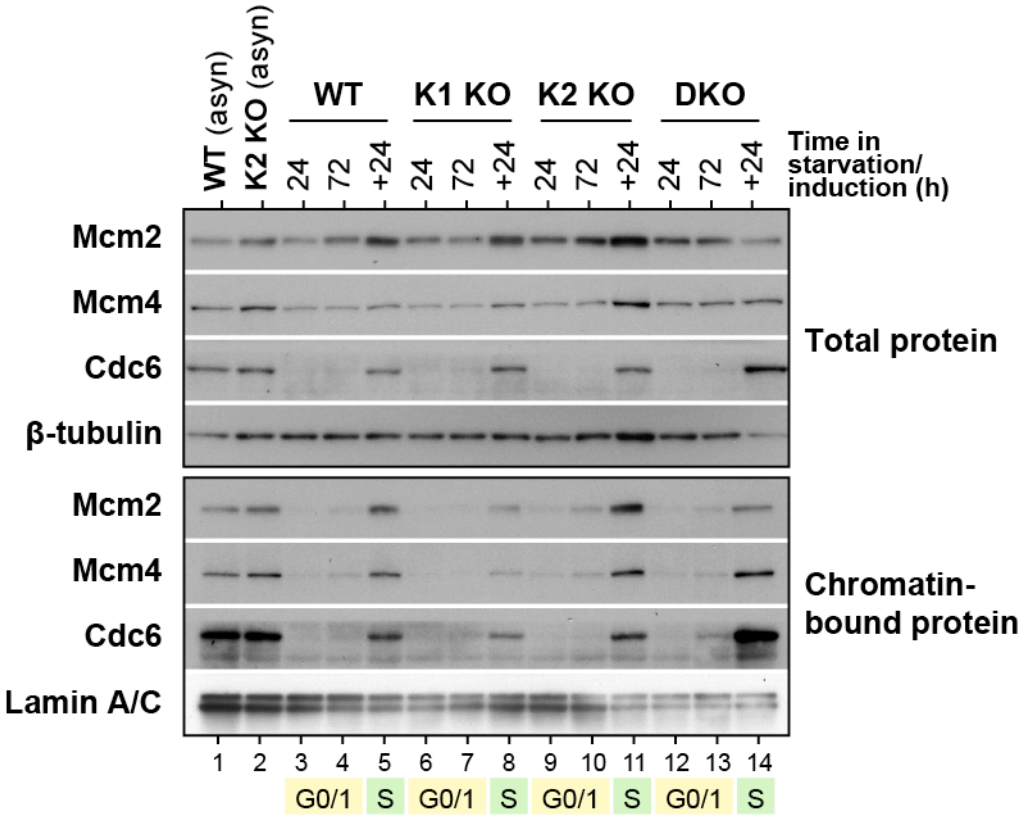

Figure 4.9. Chromatin-bound Cdc6 is increased in DKO cells.

Cells were collected before serum starvation (lanes 1,2), during serum starvation (24, 72 h) and after release into full serum (+24 h). Whole cell lysate was prepared from a fraction of the cells harvested and immuno-blotted for total cellular levels of Mcm2, Mcm4 and Cdc6, with β-tubulin as loading control (top panel). The remaining portion of cells collected was CSK-extracted and the insoluble chromatin-bound fraction was also blotted for the same proteins. Lamin A/C only partitions to the insoluble fraction and was used as loading control (lower panel).

4.7 Total and chromatin-bound Cdc6 protein levels remain high in DKO cells throughout S phase

In *Xenopus* egg extracts, Cdc6 levels on chromatin changes over the course of S phase, being displaced from chromatin following licensing and then re-loaded again later in S phase (Oehlmann et al., 2004). Overexpression has also been previously shown to cause changes in Cdc6 subcellular localisation in mammalian cells (Coverley et al., 2000; Delmolino et al., 2001; Pelizon et al., 2000; Petersen et al., 1999; Petersen et al., 2000; Saha et al., 1998). Thus Cdc6 partitioning within the cellular environment is related to its protein function. A detailed time-course was therefore carried out to characterise the changes in Cdc6 protein levels in MEFs over G1 and S phase, to determine if the high levels of Cdc6 in DKO cells are differently regulated and thereby hint towards a possible function.

Cdc6 was not detected at 12 h after release from serum starvation in cells of all genotypes (Figure 4.10A). In wild type and *Cdk1* KO cells, Cdc6 was synthesised prior to G1/S transition and could be detected from 16 h, with levels remaining unchanged up to 24 h in wild type and 21 h in *Cdk1* KO cells. Cdc6 levels were slightly lower at 24 h in *Cdk1* KO. Cdc6 was detected at much lower levels in both *Cdk2* KO and DKO cells at 16 h and remained low in *Cdk2* KO cells. This suggests that there may be slight delay in the onset of Cdc6 synthesis in the absence of Cdk2 activity or this may be an effect of a delay in cell cycle progression. However by 24 h, significantly more Cdc6 was detected in total protein in DKO cells. In a separate time-course looking at later time points, Cdc6 levels remained relatively constant in wild type and *Cdk2* KO cells. In *Cdk1* KO cells, levels decreased at 24 and 27 h during S phase and appeared to increase slightly at later times until 43 h. In DKO cells, a dramatic increase in protein levels was observed at 24 and 27 h, decreasing slightly at 30 h and again at 43 h.

Overall Cdc6 levels remained significantly higher compared to cells of other genotypes at all time-points.

The drastic increase in protein levels from 18 h after release from starvation is not in proportion to the increase in Cdc6 transcript levels in DKO MEFs. This suggests that while Cdc6 expression may be up-regulated in DKO cells, differences in degradation may also contribute to the accumulation of Cdc6 observed here.

Cdc6 has been previously shown to be an APC/C substrate (Mailand and Diffley, 2005; Petersen et al., 2000). It was proposed that Cdc6 is stabilised in G1 by Cdk2-cyclin E phosphorylation so that Cdc6 can accumulate earlier than licensing inhibitors like geminin and cyclin A, which are also APC/C substrates. This allows for an interval during which Cdc6 is available for pre-RC assembly before APC/C inactivation later in G1, which allows cyclin accumulation and also prevents licensing from taking place in S phase (Mailand and Diffley, 2005). In line with this, cyclin A only began accumulating from 18 h in wild type, *Cdk1* KO and *Cdk2* KO cells, several hours later than Cdc6 was detected (Figure 4.10A and C). Cyclin A was not detected in DKO cells. From Figures 4.9 and 4.10, it is clear that licensing can take place in cells from all genotypes. In *Cdk2* KO cells, Cdk1 is probably able to compensate for the absence of Cdk2 to stabilise Cdc6 by forming complexes with cyclin E, which still contained kinase activity (Figure 4.7B). In DKO cells, since there is no detectable S phase Cdk activity, the cellular environment is conducive for licensing in this respect.

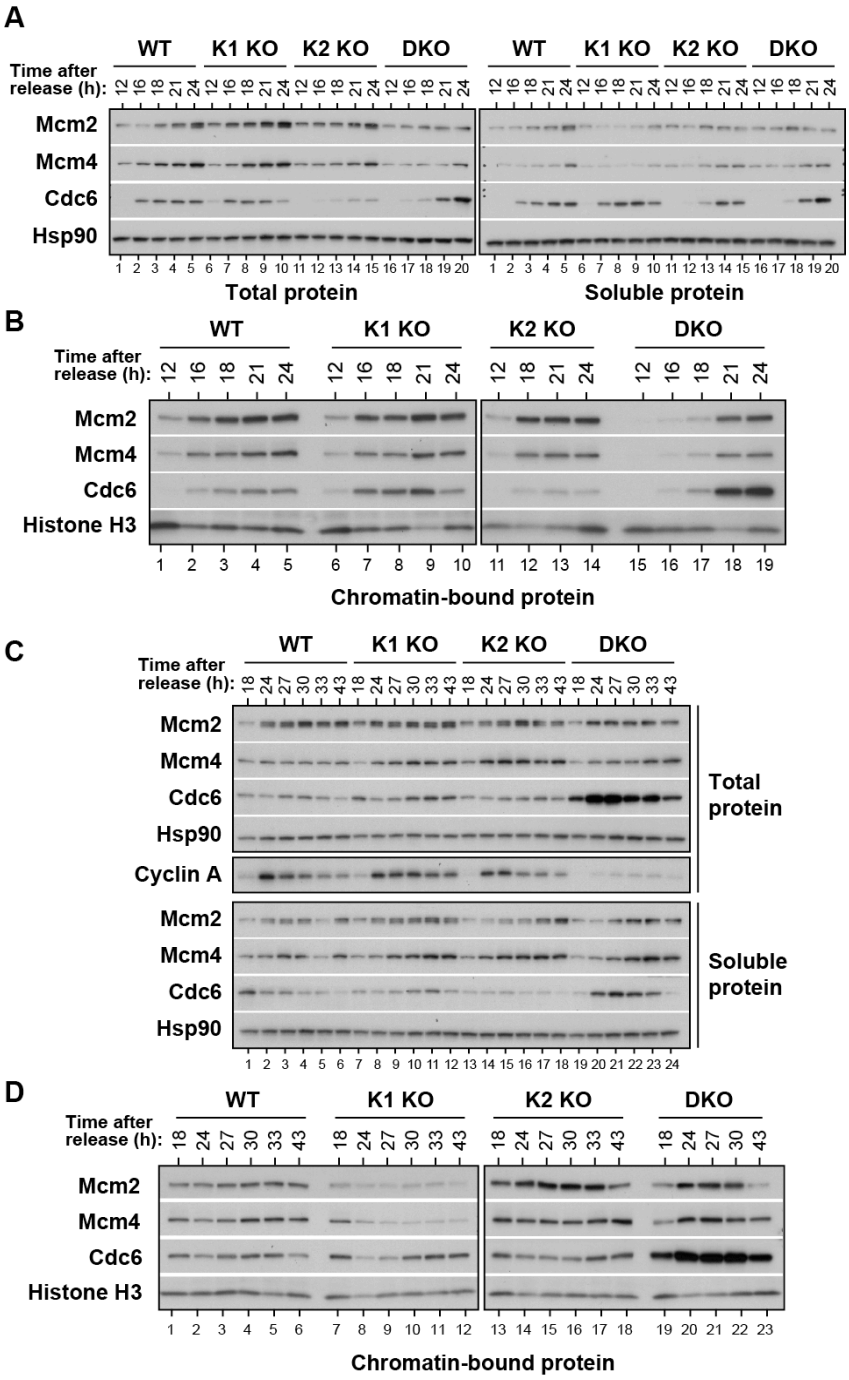

Figure 4.10. Chromatin-bound Cdc6 levels remained high throughout S phase.

MEFs were serum starved with or without 4-OHT for 72 h. Following release into full serum, cells were harvested at the indicated time-points prior to or following S phase entry. Whole cell lysate (Total protein), the soluble fraction from CSK extraction (Soluble protein) and the chromatin-bound fraction were immuno-blotted for the Mcm2, Mcm4 and Cdc6. Hsp90 and histone H3 were used as loading controls. Total cyclin A2 was also blotted for in the second time-course. Panels represent: (A) G1/S, total and soluble protein; (B) G1/S, chromatin-bound protein; (C) S/G2 and after, total and soluble protein; and (D) S/G2 and after, chromatin-bound protein.

Cdc6 and Mcm proteins were detected on chromatin from 12 – 16 h in wild type cells (Figure 4.10), consistent with other studies in which pre-RC assembly was detected from late G1 in mammalian cell lines that have been synchronised by serum starvation (Mailand and Diffley, 2005; Mukherjee et al., 2009). In DKO cells, the amount of Cdc6 on chromatin correlated closely with the total amount of protein detected in whole cell lysate over the entire time-course. The high levels of Cdc6 total protein in DKO corresponded with high levels chromatin-bound Cdc6 while the difference in soluble Cdc6 between cell types is less significant. This suggests that the excess Cdc6 is mostly bound to DNA in these MEFs. Cdc6 was loaded onto chromatin from 16 h after release into full serum and was detected at higher levels from 21 – 24 h. Cdc6 remained bound on chromatin at high levels throughout the time-course until 43 h after release from serum starvation. Increased levels of Cdc6 in DKO did not recruit a corresponding level of Mcm2 and Mcm4 to chromatin. Levels of chromatin-bound Mcm4 in *Cdk1* KO cells appear lower after 24 h although total protein levels remain comparable to other cells. The reason for this is not clear but this reduced level of Mcm was still sufficient for *Cdk1* KO cells to complete one round of DNA synthesis. This is consistent with the idea that more origins are licensed than required for complete DNA replication in an unperturbed S phase in normal cells (Ge et al., 2007; Santocanale et al., 1999; Woodward et al., 2006).

4.8 Cdc6 degradation is impaired in DKO cells

To characterise the contribution of degradation pathways to the high levels of Cdc6 protein observed in DKO cells, cycloheximide (CHX) was added to cells to inhibit protein biosynthesis at the onset of S phase. Cells were treated with CHX over a time-course followed by immuno-blotting for Cdc6 to determine its half-life. From Figure 4.11A, Cdc6 was rapidly degraded upon inhibition of protein synthesis, indicating active protein turnover in MEFs. In wild type and *Cdk2* KO cells, Cdc6 was no longer detected at 1 h after treatment while in *Cdk1* KO, Cdc6 was not detected after 30 min. In contrast, Cdc6 was still detected at 1 h in DKO cells, indicating that Cdc6 is degraded less rapidly, hence having a longer half-life. Examining Cdc6 protein levels over CHX-treatment of a shorter interval yielded similar results, with DKO cells having detectable levels of Cdc6 for the entire duration of the timecourse (Figure 4.11B). In contrast, Cdc6 was only stable up to 60, 20 and 45 min in wild type, *Cdk1* KO and *Cdk2* KO cells respectively.

When protein degradation was inhibited by the addition of the proteasome inhibitor MG132, comparing the amount protein detected at the end of the time-course to the protein detected in cells treated with both CHX and MG132 would reflect the amount of protein synthesised within this interval (4.11A, lanes 8 and 7; B, lanes 9 and 8). In the absence of protein degradation, comparable levels of Cdc6 appears to have been synthesised in all cell types by 75 min; this may be slightly higher in wild type cells (4.11B, lanes 9 and 8). By 3 h, slightly more Cdc6 has been synthesised in *Cdk2* KO cells than wild type and DKO cells while less protein has been synthesised in *Cdk1* KO cells (4.11A, lanes 8 and 7).

Having established this, a comparison of samples from DMSO-treated cells, in which synthesis and degradation processes were unperturbed, with those from cells treated

with both CHX and MG132, in which both synthesis and degradation were inhibited, would give an indication of the equilibrium between synthesis and degradation (4.11A, lanes 6 and 7; B, lanes 10 and 8). After 75 min, there were higher levels of Cdc6 in DKO cells treated with DMSO than cells treated with CHX and MG132 (Figure 4.11A). After 3 h, there were higher levels of Cdc6 in both *Cdk2* KO and DKO cells treated with DMSO than cells treated with CHX and MG132 (Figure 4.11B). These observations indicate that in *Cdk2* KO and DKO cells, degradation had proceeded less efficiently than synthesis, leading to an eventual accumulation of protein by 3 h. In DKO cells, the large accumulation can be clearly observed by mid S phase, 23 h after release into full serum (Figure 4.11B, lane 11). Taken together, these results suggest that Cdk2 plays a role in Cdc6 degradation while Cdk1 appears not to affect this process. From these experiments, it can be concluded that impaired degradation and increased expression both contributed to the high levels of Cdc6 protein in DKO cells. It is important to take into consideration that the differences in protein turnover observed in this experiment may reflect variations in cell cycle phases rather than an effect of Cdk loss *per se*, since cells of different genotypes progress through the cell cycle differently even when they have been synchronised by serum starvation.

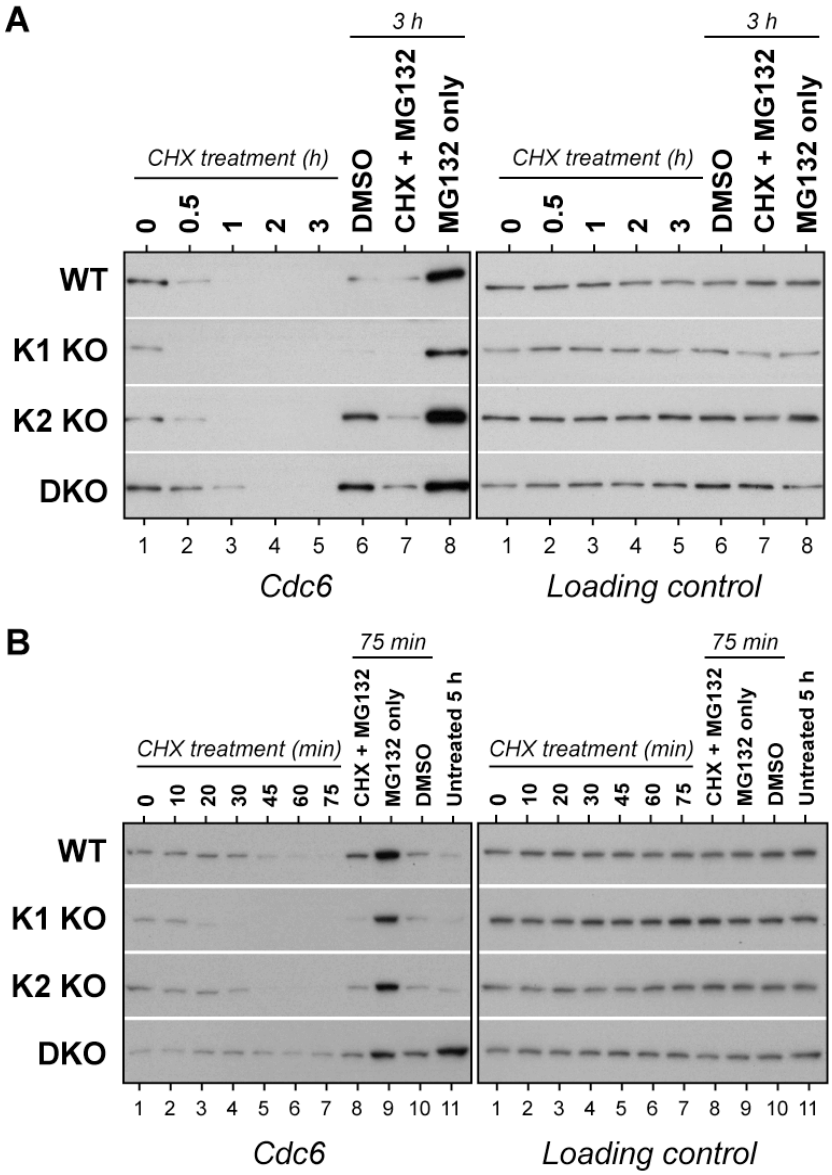

Figure 4.11. Cdc6 degradation is impaired in DKO cells.

(A, B) MEFs were serum starved with or without 4-OHT for 72 h. 18 h after release into full serum, cells were treated with cycloheximide (CHX) for the indicated intervals. Cells were also treated with DMSO, MG132 or both CHX and MG132 and harvested three hours (A) or 75 min (B) after treatment. (B) In addition, cells were harvested at 23 h after release into full serum (Untreated 5 h). All samples were lysed directly with sample buffer during collection and subsequently immuno-blotted for Cdc6. Hsp90 served as loading control.

4.9 Cyclin E1 *transcript levels are increased in DKO cells*

Addressing the other observation that total cyclin E1 levels are also higher in DKO cells, RNA extraction and qRT-PCR analysis were also carried out to determine if increased expression might account for the increase. Three primer sets and RNA samples from two experiments were used. PKR 023 and PKR 277 were each used on one sample set while PKR 215 was used for both sample sets. Expression profiles of *cyclin E1* generated from PKR 023 and PKR 277 were largely similar. The slight difference in the profiles obtained from using PKR 023 and PKR 277 is likely due to biological variation between experiments as this variation was also observed between the profiles generated using two sample sets and one primer pair, PKR 215.

From Figure 4.12, *cyclin E1* transcript levels were highest at 18 h in wild type cells, at approximately 2 times the level at 0 h. This coincided with entry into S phase. Transcript levels were reduced to close to 0 h levels at 24 h and 28 h. A similar profile was observed in *Cdk1* KO cells. In *Cdk2* KO cells, a greater increase from 0 to 18 h was observed, with transcript levels of approximately 2.5 times of 0 h levels. Transcript levels then decreased slightly at 24 and 28 h. In DKO cells, the transcript level at 0 h appears to be slightly higher than wild type. This was greatly increased to at 18 or 24 h and remained high at 3 to 4 times of wild type 0 h levels through 28 h.

It appears that a slight increase in *cyclin E1* transcript level correlated with the absence of Cdk2 while a greater increase correlated to the absence of both Cdk1 and Cdk2. This suggests that Cdk2 activity in late G1 may be involved in regulating *cyclin E1* expression. Increased transcript levels correlated to increased protein levels detected in DKO cells. A more detailed analysis of cyclin E1 protein level and also an examination of its degradation kinetics, similar to the experiment presented in Figure

4.11, would provide more data about how cyclin E1 is accumulating in DKO cells and suggest a possible function for this observation.

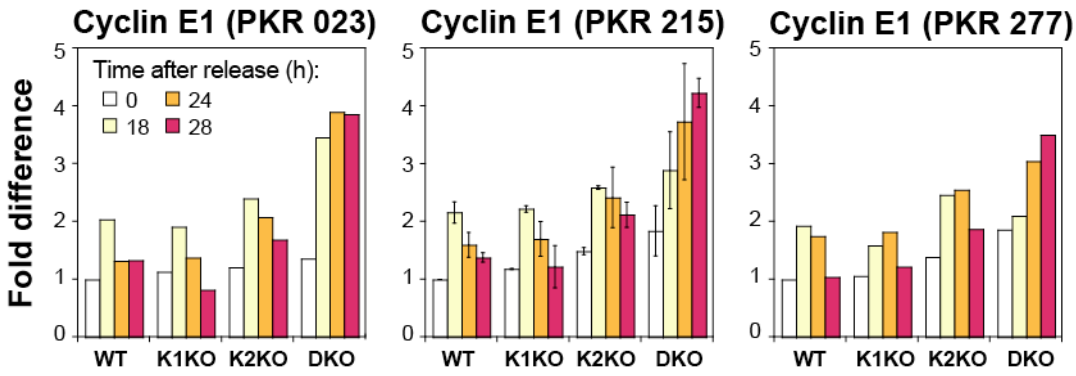

Figure 4.12. DKO *cyclin E1* expression is upregulated.

Following serum starvation with or without 4-OHT induction, cells were harvested at the indicated time-points. After total RNA extraction and reverse transcription, qRT-PCR was performed using primer pairs specific for *cyclin E1.* Three primer pairs (PKR 023, 215 and 277) were used with samples from two experiments: PKR 023 and PKR 277 were each used with one sample set while PKR 215 was used with both sample sets. Values were normalised to three housekeeping genes (β-actin, hsp90 and cyclophilin A) and the wild type 0 h sample to derive fold difference. Error bars represent S.D.

Discussion

In this chapter, the loss of both Cdk1 and Cdk2 were studied in mouse embryonic fibroblasts. Since the loss of Cdk1 results in embryonic lethality at an early blastocyst stage which cannot be further characterised, an inducible Cdk1 knockout model was used to obtain MEFs lacking both Cdk1 and Cdk2 (Diril et al., 2012). Following release from serum starvation to synchronise cells in G1, a proportion of DKO cells was able to enter S phase although the cells subsequently failed to progress through and complete one round of DNA replication. Consistent with this observation, DKO MEFs do not proliferate. At the protein level, it was observed that Cdc6 and cyclin E1 levels were higher in DKO cells compared to wild type or *Cdk1*- or *Cdk2*-single knockout MEFs. Cdc6, a protein involved in pre-RC assembly, was also detected at higher levels on chromatin although Mcm2 and Mcm4 levels did not increase correspondingly. Impaired Cdc6 degradation related to the absence of Cdk2 appears to contribute to this observation. *Cyclin E1* was also found to be up-regulated in DKO cells. No associated kinase activity was detected with cyclin E1 in DKO cells.

Conditional loss of Cdk1 in DKO MEFs

The loss of Cdk1 in the cells used this study is conditional upon 4-OHT treatment. While Cdk1 expression, protein and associated kinase activity were not detected in DKO cells, the possibility of some cells having residual kinase activity cannot be absolutely ruled out. It may be argued that this accounts for a small percentage of cells that are able to enter S phase since the overall level of kinase activity required for the events of S phase onset and progression is low compared to that required for mitotic entry. However, such cells will be expected to cycle with normal kinetics unlike the DKO cell cycle profile observed here. Nevertheless, an attempt was made to address this issue by making use of RO-3306, a selective chemical inhibitor of Cdk1 in human cells (Vassilev et al., 2006). MEFs were synchronised by serum starvation and induced

with 4-OHT after which cells were treated with RO-3306 and the vehicle control, DMSO. If 4-OHT had resulted in abrogation of Cdk1 activity, there should be no difference observed in the cell cycle profile in DKO cells treated with RO-3306 or DMSO. However, preliminary results indicate that RO-3306 is highly toxic and not sufficiently potent in MEFs (results not shown). As such further optimisation would be required to make use of this drug in this assay for a more conclusive outcome.

G1/S transition in the absence of Cdk1 and Cdk2 activity

Following 4-OHT treatment, recombination occurred efficiently in the targeted *Cdk1* locus and consequently, Cdk1 was not detected at the mRNA or protein level (Figure 4.2). At the G1/S transition, Cdk2-associated kinase activity has important functions in activating the transcriptional programme that commits cells to divide. This is mediated by Cdk hyper-phosphorylation of pRb (Lundberg and Weinberg, 1998). Cdk activity is also required for initiation of replication origins for DNA replication at the onset of S phase. Both Cdk2-cyclin A and Cdk2-cyclin E complexes have been implicated in the processes of pre-RC assembly and activation (Coverley et al., 2002; Mailand and Diffley, 2005). In the absence of Cdk2, Cdk1 can take over the function of Cdk2 in the mitotic cell cycle and is sufficient to carry out most cell cycle activities (Aleem et al., 2005; Santamaria et al., 2007). As such, it was expected that cells would arrest in G1 in the absence of both Cdk2 and Cdk1. However, surprisingly, a fraction of DKO cells was able to enter S phase (Figures 4.3 and 4.5).

In light of the apparent versatility of Cdk and cyclin functions, Cdk4 and Cdk6 most likely compensate for some of these functions in DKO cells in order to effect G1/S transition. One function that is likely to be adequately fulfilled by Cdk4 and Cdk6 is the phosphorylation and inactivation of pRb repression of E2F transcription factors. The accepted paradigm is that phosphorylation of pRb by D-type cyclin-Cdk4 or -Cdk6

complexes first causes partial inactivation to relieve transcriptional repression of E2F, allowing expression of a limited suite of genes needed to progress through G1, for instance the E-type cyclins. Cdk2-cyclin E activity then further phosphorylate pRb, completing its inactivation (Harbour et al., 1999; Lundberg and Weinberg, 1998). E2F-regulated genes required for S phase include cyclins A2, B1, E and Mcm subunits (Ishida et al., 2001; Polager and Ginsberg, 2003; Ren et al., 2002). However, contrary to this orderly pathway of activation, mice lacking Cdk2 and mice lacking both E-type cyclins are viable and cycling MEFs from these mice can enter S phase normally (Berthet et al., 2003; Geng et al., 2003; Ortega et al., 2003). pRb was phosphorylated in *Cdk2*$^{-/-}$ (Ortega et al., 2003) and *cyclin E1*$^{-/-}$/*E2*$^{-/-}$ MEFs (Geng et al., 2003) and known E2F targets such as Cdc6 and cyclin A2 were induced normally (Geng et al., 2003). Indeed, Cdk4 was shown to phosphorylate Cdk2-specific pRb sites in colon cancer cells depleted of Cdk2 (Tetsu and McCormick, 2003) and pRb was efficiently phosphorylated on Thr821, a residue thought to be specifically phosphorylated by Cdk2-cyclin E (Zarkowska and Mittnacht, 1997) in *Cdk2*$^{-/-}$ cells (Ortega et al., 2003). Taken together, these results argue that there is no stringent requirement pRb to be specifically phosphorylated by Cdk2-ctclin E in order for cells to enter S phase.

While Cdk1 can phosphorylate pRb in the absence of all interphase Cdks (Santamaria et al., 2007), the activity of just Cdk4 and Cdk6 may be sufficient to mediate this phase transition, even in the absence of Cdk2 kinase activity and the compensating Cdk1 activity in DKO cells. Cdk4 may be the more important Cdk in fulfilling this this function since the loss of both Cdk2 and Cdk4 leads to embryonic lethality in mice and pRb was found to be hypo-phosphorylated (Berthet et al., 2006), whereas mice lacking one of these Cdks (Berthet et al., 2003; Malumbres et al., 2004; Ortega et al., 2003; Rane et al., 1999; Tsutsui et al., 1999) or both Cdk2 and Cdk6 (Malumbres et al., 2004) are viable. This hypothesis is also consistent with the report that ablation of Cdk2 in *cyclin*

D-null cells prevents proliferation and cell cycle re-entry, whilst either perturbation alone has little effect (Kozar et al., 2004).

In the present study, *Cdc6* and *cyclin E* expression were robustly detected (Figures 4.8 and 4.12) in the absence of Cdk1 and Cdk2 activity in DKO cells. Since *Cdc6* and *cyclin E* are E2F-regulated genes (Hateboer et al., 1998; Ohtani et al., 1995; Ohtani et al., 1998; Yan et al., 1998), this suggests that Rb mediated transcriptional repression may have been sufficiently relieved for a subset of DKO cells to enter S phase. Malumbres et al. reported that MEFs could cycle with normal kinetics even though pRb was hypo-phosphorylated due to the absence of Cdk4 and Cdk6 (Malumbres et al., 2004). Compensation by non-cognate Cdks may thus be less efficient but still sufficient to promote G1/S entry. Alternative but not mutually exclusive to this interpretation, the gradual phosphorylation of pRb over G1 may represent a temporal or some other form of differential control of transcriptional de-repression, affecting functional subsets of E2F-controlled genes differently. In addition to gene products required for S phase, E2F-regulated genes also control events even later in the cell cycle, in mitosis (Henley and Dick, 2012). For instance, the mitotic checkpoint protein Mad2 is an E2F target and is aberrantly expressed in cells defective in the pRb pathway, being overexpressed in tumour cell types with high E2F activity (Hernando et al., 2004).

Mukherjee et al. had demonstrated that ongoing *de novo* mRNA synthesis in late G1 is dispensable for G1/S transition in murine and CHO cells, suggesting that transcription only in early G1, prior to maximal Rb hyper-phosphorylation, is sufficient for S phase entry (Mukherjee et al., 2009). As such, a possible explanation for normal cell cycle progression in the absence of full pRb phosphorylation could be that genes required for progression through an unperturbed S and subsequent phases of the cell cycle may be sufficiently expressed by early G1, whereas full Rb inactivation would allow expression of a full complement of proteins which become necessary under conditions of replicative or other stresses to the cell cycle. Further work to examine the kinase

activity of D-type cyclins, Cdk4 and Cdk6 using pRb as substrate will be required to verify these suggestions. Monitoring the level and kinetics of pRb phosphorylation in DKO cells will also be informative. The timing and expression of S phase-related genes and other cell cycle regulators under the control of E2F transcription factors should also be closely examined to gain a better understanding of the relationship between pRb inactivation by phosphorylation leading up to and after the G1/S transition.

Cdc6 regulation and function in DKO cells

In this study, pre-RC assembly appears to be unperturbed in DKO cells as Cdc6, Mcm2 and Mcm4 were detected on chromatin following fractionation of total cell lysates (Figures 4.9 and 4.10). However, DKO cells that entered S phase did not progress through S phase efficiently (Figures 4.3 and 4.5). There was a conspicuous increase of Cdc6 protein levels in total cell lysate and also on chromatin. These observations raised questions about the mechanisms and functional significance of this Cdc6 accumulation.

The regulation of Cdc6 abundance and sub-cellular localisation have been proposed to be important in the regulation of cell cycle and licensing activities in metazoans. In mammalian cells, chromatin-bound Cdc6 persists through S phase and G2 once licensing has occurred in G1. Several studies had established that non-chromatin bound Cdc6 is exported from the nucleus in a Cdk-dependent manner, serving as a mechanism to prevent inappropriate pre-RC formation during S phase (Coverley et al., 2000; Delmolino et al., 2001; Pelizon et al., 2000; Petersen et al., 1999; Petersen et al., 2000; Saha et al., 1998). However, in contrast to these data derived from over-expression studies, other studies demonstrated that a subpopulation of Cdc6 remains in the nucleus throughout this time despite not being associated with DNA (Alexandrow and Hamlin, 2004; Fujita et al., 1999; Mendez and Stillman, 2000; Okuno et al., 2001).

These apparently conflicting findings were resolved by the observation that endogenous Cdc6 is regulated in a different manner from exogenous proteins (Alexandrow and Hamlin, 2004). Native Cdc6 protein remains nuclear throughout the cell cycle while over-expressed proteins are subjected to nuclear-cytosolic translocation.

Consistent with the behaviour described for endogenous Cdc6, chromatin-bound Cdc6 was present at all time intervals leading up to and following entry into S phase in cells of all genotypes (Figure 4.10). The proportion of Cdc6 in soluble- and chromatin-bound pools appears to remain constant through the cell cycle and total protein levels correlated tightly with chromatin-bound protein. In wild type, *Cdk1* KO and *Cdk2* KO cells, levels of chromatin-bound Cdc6 may be slightly lower at 24 up to 30 h time-points. This is reminiscent of the regulation Cdc6 in *Xenopus* egg extracts in which Cdc6 is displaced from DNA following origin licensing but regains high affinity for origins once replication forks have initiated. Cdc6 reloading during S phase in this *in vitro* system is required to activate the Chk1 kinase in response to inhibition replication elongation (Oehlmann et al., 2004).

Phosphorylation by Cdk2-cyclin E was shown to be necessary to stabilise Cdc6 in G1, preventing its association with APC/C and protecting it from proteolysis (Mailand and Diffley, 2005). However, Cdc6 levels were unaffected in MEFs lacking type-E cyclins and Cdc6 was phosphorylated on Ser54 in these cells (Geng et al., 2007; Geng et al., 2003), indicating that other Cdk-cyclin complexes can adequately compensate for this function. Cdk2-cyclin A is likely to confer this function and in the case of *Cdk2*-null cell types, Cdk1 may take over. Consistent with this, Cdc6 levels are not noticeably different in *Cdk2* KO from wild type MEFs (Figure 4.7). According to this line of argument, it would be anticipated that Cdc6 could not be stabilised in DKO cells. Contrary to expectations, Cdc6 was detected in large excess in DKO MEFs and largely

localised to chromatin. Examining the profile of *Cdc6* expression and protein turnover in DKO cells (Figures 4.8 and 4.11), the large accumulation of Cdc6 protein appears to be a combination of the effects of increased expression and decreased degradation, although the mechanism for Cdc6 stabilisation remains as yet unclear. In opposition to published work (Mailand and Diffley, 2005), it appears that Cdk activity is required to degrade Cdc6 in *Cdk2* KO and DKO MEFs. It could be possible that this is an indirect effect of Cdk on another Cdc6 regulator.

In a normal cell cycle, timely proteolysis of cyclins governs the periodic oscillation of Cdk activity. In particular, cyclins A and B are degraded through the action of the APC/C ubiquitin ligase during mitosis, resulting in the low Cdk activity throughout G1 permissive for origin licensing. The activity of the APC/C requires interaction with either one of its activators, Cdc20 or Cdh1, and Cdh1 is primarily responsible for APC/C activity after mitotic exit up through G1 (Di Fiore and Pines, 2008; van Leuken et al., 2008). In turn, Cdh1 function is antagonised by Emi1 (Di Fiore and Pines, 2007; Hsu et al., 2002; Machida and Dutta, 2007). In a recent study, the continuous activity of APC/C^{Cdh1} as a result of Emi1 depletion resulted in the degradation of cyclin A and B in cells (Di Fiore and Pines, 2007). Cyclin E, which is not an APC/C substrate, was also found to accumulate to higher than normal levels in these cells. These findings are similar to those observed in DKO cells, in which cyclins A and B were found to be absent from total cell lysate (Figure 4.7). This suggests that Emi1 may be deregulated in DKO cells, a plausible explanation given that *Emi1* gene expression is under the control of E2F transcription factors (Hsu et al., 2002). The absence of Cdk1 and Cdk2 may have caused aberrant E2F regulation in these MEFs through effects on pRb. Monitoring the expression of Emi1 and probing for the protein in cells following release from serum depletion would provide more data required to substantiate this idea. In the study by Di Fiore and Pines, Cdc6 level was not significantly affected in the absence of Emi1 (Di Fiore and Pines, 2007), possibly due to (increased) protection from Cdk2-

cyclin E mediated phosphorylation. However, this does not explain the accumulation of Cdc6 in DKO cells since there is no detectable Cdk1- and 2-associated kinase activity here. This suggests that an active mechanism involved in ensuring proper cell cycle progression is responsible for preventing Cdc6 proteolysis and its accumulation in these MEFs.

In line with this notion, a previous study had reported that chromatin and total cellular levels of Cdc6 were markedly upregulated in cells when S phase arrest was induced by HU treatment (Liu et al., 2009a). While the exact mechanisms probably differ in mammalian cells, a function for Cdc6 and ATR interaction under conditions of perturbed S phase exists in yeasts. In both budding and fission yeast, cells lacking Cdc6 undergo mitosis despite failing to replicate their DNA (Kelly et al., 1993; Piatti et al., 1995), indicating that Cdc6 plays a role in coordinating successful S phase completion to entry into mitosis. Also in fission yeast, Cdc6 stabilised on chromatin acted as a receptor for the Rad3-Rad26 complex, homologues of mammalian ATR/ATRIP, which is required to maintain the block on mitosis for the interval of the arrest (Hermand and Nurse, 2007).

Liu et al. found that increased Cdc6 levels in mammalian cells coincided with activation kinetics of ATR. Cdc6 was phosphorylated by ATR, which then stabilised Cdc6 by attenuating proteasomal degradation (Liu et al., 2009a). In DKO cells, Cdc6 in was also more stabilised against proteolysis (Figure 4.11). Liu and colleagues further demonstrated that increased levels of chromatin Cdc6 did not reflect its role in pre-RC assembly, again consistent with observations in DKO cells (Figures 4.9 and 4.10), but instead served a purpose in delaying mitotic entry following S phase arrest due to HU-induced replicative stress (Liu et al., 2009a). This S/M checkpoint response is triggered when perturbed replication causes an accumulation of stalled replication forks in cells

(Borlado and Mendez, 2008). It may be possible that the accumulation of Cdc6 in DKO MEFs is a checkpoint response which may be regulated by ATR.

In DKO cells, while G1/S transition may be accomplished by compensation by other Cdks so that licensing can occur, replication initiation and progression may be inadequately supported. Consequently, inefficient DNA replication in DKO cells could gradually constitute replicative stress to these cells, leading to up-regulation of Cdc6 in a checkpoint response. Inefficient replication may lead to slow-moving forks that stall and collapse soon after initiation, gradually accumulating single-stranded DNA or other intermediates that signal defective S phase progression. Increased Cdc6 as a result of checkpoint activation may also explain increased levels of cyclin E in DKO cells. It has been previously reported that cyclin E binds to replication origins and mediates Mcm loading in a kinase-independent manner (Geng et al., 2007). In cells treated with mitomycin C, a DNA-damaging agent, cyclin E and Cdc6 were retained on chromatin, slowing down S phase progression and activating the replication checkpoint (Lu et al., 2009). If the accumulation of Cdc6 in DKO cells were due to a checkpoint response, perhaps a portion of the BrdU incorporation observed in DKO cells was due to DNA repair synthesis rather than replicative DNA synthesis. Nevertheless, this will only account for a small amount of incorporation and is unlikely to be at levels similar to BrdU incorporation via replicative DNA synthesis.

If ATR were involved in this response, caffeine treatment would be expected to prevent Cdc6 accumulation above control levels. This would be a possible future experiment to do in continuing to define the mechanism(s) and function(s) underlying Cdc6 accumulation in MEFs lacking Cdk1 and Cdk2. To check if Cdc6 accumulation is a general effect of inefficient replication, wild type MEFs may be subjected to other modes of replicative stress such as HU or aphidicolin treatment. Also, to determine if Cdc6 stabilisation were an effect of a loss of S phase-Cdk activity, MEFs may be

treated with other Cdk inhibitors such as roscovitine and purvalanol-a. In addition, further work is required to substantiate the proposal that APC/C functions are perturbed in DKO cells and that up-regulation of Cdc6 and cyclin E signify involvement in a checkpoint response.

The role of Cdks in pre-RC initiation

At this preliminary stage of investigation, many questions remain with regards to cell cycle progression in DKO cells. Cdk activity is essential for origin initiation during G1/S transition. Following pre-RC assembly at origin DNA, the inactive Mcm2-7 complex needs to be activated by the action of S phase-promoting kinases, Cdk and Cdc7, to recruit Cdc45 and GINS to generate the CMG complex. This is believed to be the active replicative helicase (Labib, 2010). In *S. cerevisiae*, Cdk phosphorylation of Sld2 and Sld3 generates phosphosites on each protein that can interact with Dbp11 (Tanaka et al., 2007; Zegerman and Diffley, 2007). Sld3, together with Sld7, likely mediates Cdc45 association with the pre-RC while Sld2 chaperones the GINS complex to proteins at the origin (Muramatsu et al., 2010; Tanaka et al., 2011b). Less is known about this mechanism in vertebrates. Treslin/ticrr has been identified as the orthologue of Sld3, while the best candidate for Sld2 is RecQL4 (Kumagai et al., 2010; Matsuno et al., 2006; Sanchez-Pulido et al., 2010; Sangrithi et al., 2005). Functional analyses revealed Cdk-dependent regulation of Treslin/ticrr interaction with TopBP1, the human homologue of Dbp11, which mirrors Sld3-Dbp11 in yeast (Boos et al., 2011; Kumagai et al., 2011). Cdk phosphorylation of two conserved residues were identified to be important since non-phosphorylatable mutants at these sites could not rescue the inhibition of DNA replication in human cells when endogenous Treslin/ticrr was knocked down by siRNA (Boos et al., 2011; Kumagai et al., 2011). While RecQL4 is required for DNA replication, a requirement for Cdk phosphorylation in this function has not been identified. RecQL4 also appears to be dispensable for GINS recruitment,

suggesting that it is required for helicase initiation at a different step (Matsuno et al., 2006; Sangrithi et al., 2005).

Apart from BrdU incorporation as an indication of active DNA synthesis, there is little data at this point to verify that replication origins have initiated normally and that replication machinery are loaded and properly functioning. To address this, chromatin association of Cdc45, components of GINS and proteins forming the RPC can be probed for. Attempts to detect Cdc45 and cyclin E1 on chromatin by immuno-blotting have been unsuccessful so far, likely due to technical issues. Conditions used for protein fractionation and different antibodies would have to be tested to determine optimum conditions for detecting these proteins. DNA fibre analyses, examination of nascent strand synthesis and imaging of active sites of DNA replication could also be used to check that replication origins have initiated; the former two methods could also provide information about replication fork progression.

Given that pre-RC components and processes are conserved from yeast to human, it is unlikely that the DNA replication observed in DKO cells in the present study had occurred independently of Cdk activity. One explanation for the ability of these MEFs to enter S phase could be that the Cdk targets required for replication initiation, might be pre-phosphorylated prior to the loss of Cdk1 activity and remain stable through quiescence and up to the G1/S transition. Since Cdk appears to be responsible for the phosphorylation of soluble targets, there may be no requirement for its activity in pre-RC formation or other chromatin related functions. However, the pool of pre-phosphorylated targets would likely become limiting in the absence of continued kinase activity, leading to inefficient replication which cannot be sustained over the course of S phase. In *Xenopus* egg extracts, Cdk activity is required throughout S phase for new initiation events to occur (Luciani et al., 2004) and for progression through the replication timing programme (Thomson et al., 2010). Without Cdk activity, late origins

may not fire, for instance, leaving stretches of the genome unreplicated. Origin initiation from fewer origins also mean that replication forks have to travel greater distances, increasing the chance of fork stalling and resulting DNA strand breaks (Ge and Blow, 2009). As this stage, this hypothesis remains speculative and additional experiments and a better understanding of Cdk function in mammalian replication initiation at the molecular level will help resolve these outstanding issues.

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Chapter 5 – Summary and concluding remarks

The events of a cell cycle consist of a series of different and dynamic chromosome transactions that are tightly coupled to other cellular processes, with duplication of a cell's genetic contents occurring in S phase. Proper coordination of cell cycle transitions and regulation by checkpoint pathways ensure that genomic stability is maintained for cell survival. Cdks function as the engine to drive progression of cell cycle events by the successive activation of different Cdk-cyclin complexes. In particular, S phase onset, which involves initiation of DNA replication, is normally mediated by Cdk2 in metazoans together with Cdc7 activity.

In this thesis, I have presented work relating to the role of S phase-promoting kinases in DNA replication and S phase regulation. Using the cell free system of *Xenopus* egg extract, I first characterised the activity of PHA-767491, a small molecule inhibitor of Cdc7, in a biochemical context. I provide evidence that Cdc7 executes its activity before Cdk early in S phase and is not rate-limiting in the progression of replication timing programme once Cdc7 has executed its essential function. The phosphatase PP1 was identified as a novel modulator of Cdc7 activity, rapidly reversing Cdc7-dependent phosphorylation of chromatin-bound Mcm4. Finally, the role of Cdc7 in an etoposide-induced checkpoint response was investigated, uncovering a role for PP1 in checkpoint function.

Moving on from a cell-free system into mouse genetics, I then outlined the design strategy and screening process for the generation of a conditional *Cdc7*-knockout mouse. A single mouse bearing germline transmission of the conditional knockout targeting construct was identified. This conditional knockout mouse will be useful in

delineating the effects of Cdc7 depletion in specific developmental and tissue contexts. Cells derived from these mice can also provide the basis to investigate molecular mechanisms of Cdc7 function, such as in the checkpoint response. In the last part of the thesis, I made use of another conditional knockout mouse to examine the effect of *Cdk1* and *Cdk2* double knockout in MEFs. Contrary to expectations, DKO cells enter S phase in the absence of S phase Cdk activity. I find that Cdc6 and cyclin E1 proteins accumulate at high levels in these cells, although the exact function(s) and mechanism(s) for these observations remain to be discovered.

In this work, I have provided greater molecular detail about the regulation and function of Cdc7 in replication initiation, timing programme progression and checkpoint signalling in *Xenopus* and described a novel mechanism for PP1 in functionally lowering Cdc7 activity during an intra-S checkpoint response. As Cdc7 is believed to play key roles in these processes, it is hoped that these findings can contribute to a better understanding and provide fresh avenues for further investigation. On the other hand, the study of *Cdk1* and *Cdk2* knockout in mouse embryonic fibroblasts are still in the initial stages and primarily serves to identify the questions that need to be addressed.

5.1 The kinase and the phosphatase: partners at the replication checkpoint

It is clear from this and other studies in different model systems that Cdc7 has a role to play the intra-S checkpoint. However questions remain about the mechanisms of the signalling process. In this study, while total DNA synthesis is reduced upon etoposide-induced checkpoint response, it is not known how origin firing is affected here. In budding yeast, inhibition of late origin firing is a major effect of the intra-S checkpoint response (Santocanale and Diffley, 1998). Slowing down ongoing replication forks can

also reduce the total rate of DNA synthesis (Kumar and Huberman, 2009; Seiler et al., 2007). Yet another study proposed that the replication checkpoint retards S phase progression in a global manner rather than affecting a subset of origins, so that all origins are fired in the same order as in an unperturbed condition, only over a longer time interval (Alvino et al., 2007). Some experiments that may begin to address this issue include: (1) monitoring replication over time in the presence of etoposide to track overall rates of DNA synthesis through S phase, (2) nascent strand analysis or DNA fibre experiments to monitor new origin initiation and ongoing fork progression and (3) assessing the labelling patterns of active replication clusters in nuclei to determine if all characteristic S phase stages could be observed in the presence and absence of etoposide.

From the present study and other unpublished data (Gaganmeet Chadha, Poh et al., manuscript in preparation), checkpoint-mediated enhancement of PP1 activity was found to functionally lower Cdc7 activity. In this regard, either scenario of local or global effects on origins may be borne out through checkpoint-mediated action on PP1. PP1 localisation on chromatin presents the possibility that it can act locally at different origins to regulate origin initiation, hence allowing differential control over early- or late-firing origins. On the other hand, its activity may also be globally upregulated during a checkpoint response, perhaps through degradation or nuclear export of an inhibitory subunit (or the reverse for an activating subunit), hence changing the overall rate of origin firing but not affecting the pattern of initiation. The interplay of a kinase and a counteracting phosphatase, especially a holo-enzyme administered by a repertoire of regulatory subunits, affords abundant possibilities in coordinating origin firing during S phase. In the same vein, uncovering the identity of the PP1 regulatory subunit responsible for the checkpoint-sensitive up-regulation is another issue of interest. It is also not clear now if ATR/ATM signal through the Chk kinases in this response. These

upstream components of this checkpoint response, too, merit consideration to further the understanding of how Cdc7 activity is regulated.

5.2 The Cdk connection: same dance, different choreography

The molecular basis of how Cdk activity mediates the initiation of replication has been extensively studied in *S. cerevisiae*, having essential Cdk targets and their mechanisms of action minutely defined. However, how the Cdk cycle is coordinated with replication initiation remains elusive in metazoans. Much research of metazoan Cdk requirements at the G1/S transition has been focused on the restriction point and how cells activate the transcriptional programme required for cell cycle commitment. One hurdle in addressing the role of Cdk in initiation has been the difficulty in identifying its physiological substrates for origin firing. Multiple Cdks have evolved to play different roles in controlling the cell cycle, which presumably also evolved greater complexity to coordinate the tasks of replicating larger genomes and to integrate intercellular and other regulatory inputs at the level of tissues and the organism (Diffley, 2011; Malumbres, 2005).

Fundamental processes of replication may have remained conserved through evolution but specific roles may be performed by different protein components with inter-species variation (Labib, 2010), such that differences exist even between the budding and fission yeasts. For instance, Cdk phosphorylation of Sld3 is essential in promoting its association with Dbp11 in the budding yeast (Tanaka et al., 2007; Zegerman and Diffley, 2007) whereas both Hsk1 (fission yeast Cdc7) and Cdk activity are required for fission yeast Sld3 function in mediating CMG complex formation (Nakajima and Masukata, 2002). Another example is that metazoans have evolved important Cdk-independent mechanisms to prevent re-licensing unlike a major reliance on regulation through Cdk phosphorylation in *S. cerevisiae* (Arias and Walter, 2007). Besides Cdk

activity regulation of the initiation of replication, several studies have also uncovered a different relationship in that down-regulation of pre-RC components negatively regulates Cdk2-cyclin E activity in cells as a means to prevent S phase entry in the absence of sufficient numbers of licensed origins (Liu et al., 2009b; Machida et al., 2005; Nevis et al., 2009). More work will be required to delineate the relationship between Cdks and replication initiation at the molecular level. In this respect, past and recent advances in detailing the players using the yeast model system will continue to serve as a framework to guide studies in metazoan systems.

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Chapter 6 – Materials and methods

6.1 Methods for working with *Xenopus* cell-free extracts

6.1.1 Preparation and use of Xenopus *egg extract*

Metaphase-arrested *Xenopus laevis* egg extract was prepared as previously described with modifications (Chong et al., 1997; Gillespie et al., 2012). Unfertilised eggs were rinsed with MMR (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EDTA, 5 mM HEPES-KOH at pH 7.8; final pH adjusted to 7.8) to remove non-egg debris and dejellied for 5-10 minutes with 2% cysteine solution (w/v). Visibly activated eggs and apoptotic eggs were removed throughout the washing and dejellying procedure. Fully dejellied eggs, which form a tightly packed mass, were washed twice with XBE2 (100 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM HEPES-KOH pH 7.8, 5 mM EGTA, 17.1g/l sucrose) and once with XBE2 supplemented with protease inhibitors. Eggs were transferred to 14-ml Falcon tubes containing 1 ml of the same buffer but supplemented with 100 µg/ml cytochalasin D (Sigma, C8273) and packed by centrifuging at 3000 rpm in a swinging bucket rotor (Beckman Coulter, JS-13.1) for 1 minute. Excess buffer and lysed eggs, which would be white and float to the surface, were removed with a Pasteur pipette at this point.

The packed eggs were spin-crushed at 10 000 rpm for 10 minutes at 4ºC with the same rotor. The cytoplasmic fraction was extracted collected via side-puncture using a 20-gauge needle and 1 ml syringe and supplemented with protease inhibitors (1 µg/ml each of leupeptin, pepstatin and aprotinin), cytochalasin D to a final concentration of 10 µg/ml and 1 x ER mix [1 M phosphocreatine, 600 µg/ml creatine phosphokinase, 10 mM Tris-Cl pH 7.6) and 15% (v/v) LFB1/50 (50 mM KCl, 40 mM HEPES-KOH pH 8.0, 20 mM K_2HPO_4/KH_2PO_4 pH 8.0, 2 mM $MgCl_2$, 1 mM EGTA, 10% sucrose (w/v), 2 mM

DTT, and 1 µg/ml each of leupeptin, pepstatin and aprotinin]. This was clarified by spinning at 30 000 rpm in a swing bucket rotor for 17 minutes at 4ºC (Beckman Coulter, SW 55 Ti). The clear golden cytoplasmic extract was collected and 2% glycerol (v/v) added before being snap-frozen in 20-µl beads in liquid nitrogen.

All extracts were supplemented with 250 µg/ml cycloheximide, 25 mM phosphocreatine and 15 µg/ml creatine phosphokinase and incubated with 0.3 mM $CaCl_2$ for 10 minutes to allow release from metaphase arrest before use. For DNA synthesis reactions, sperm nuclei (Gillespie et al., 2012) was incubated to a final concentration of 6–10 ng DNA/µl or CHO nuclei to a final concentration of 10 000 nuclei/µl extract. When CHO nuclei were used, extracts were further supplemented with 150 µg/ml geminin and incubated for 10 min after $CaCl_2$ addition to prevent licensing from occurring.

6.1.2 Analysis of DNA replication in Xenopus egg extracts

DNA synthesis was assayed by measuring the incorporation of [α-^{32}P]-dATP (PerkinElmer, NEG512H250UC) into acid-insoluble material followed by scintillation counting as previously described (Blow and Laskey, 1986; Chong et al., 1997). For each reaction, sperm nuclei were incubated to a final concentration of 6 ng DNA/µl in 5 µl interphase extract supplemented with 50 µCi/ml [α-^{32}P]-dATP. At pre-determined times, the reaction was terminated by the addition of 160 µl Stop-C (20mM Tris-Cl pH 7.5, 5 mM EDTA, 0.5% SDS) containing 0.2 mg/ml Proteinase K and incubated at 37ºC for 30 minutes. This digested mixture was then precipitated with 4 ml of cold 10% trichloroacetic acid (w/v) (TCA)-2% $Na_4PO_2O_7.10H_2O$ (w/v) at 4ºC for 30 minutes. 40 µl of the TCA-reaction mixture was spotted onto a filter disk for measurement of total ^{32}P and the remainder of the sample was filtered under vacuum through a 25-mm-diameter glass microfibre filter (GF/C, Whatman). The GF/C filter was washed down once with 5% trichloroacetic acid (w/v) (TCA)-0.5% $Na_4PO_2O_7.10H_2O$ (w/v) and once with 70%

ethanol. The filter was dried under an infra-red lamp. ^{32}P on the filters was measured by liquid scintillation. The amount of DNA synthesised can then be calculated by multiplying the percentage of total counts in acid precipitable material by a factor of 0.654, assuming an endogenous dATP concentration of 50 µM in the extract. All incubations were carried out at 23ºC.

6.1.3 Chromatin and nuclear templates

For immunoblotting reactions, 12.5 µl of extract was diluted with 400 µl ice-cold NIB {50 mM KCl, 50 mM mM HEPES-KOH pH 7.6, 5 mM $MgCl_2$, 0.5 mM spermidine, 0.15 mM spermine, 2 mM DTT; supplemented with protease inhibitors, phosphatase inhibitors [25 mM β-glycerophosphate pH 7.6, 0.1 mM sodium orthovanadate and 0.1 µM microcystine-LR (Enzo Life Sciences, ALX-350-012)] and 0.1% Triton X-100} and under-laid with the 100 µl of the same buffer containing 20% sucrose (w/v). This was centrifuged in a swing bucket rotor at 2100g for 5 minutes at 4ºC (Eppendorf, A-8-11). NIB was removed and the top of the cushion was washed once with NIB before the rest of the cushion was removed, leaving 15 µl above the pellet. Chromatin was then compacted by spinning at 13000g for 2 minutes in fixed angle rotor (Eppendorf, FA-45-30-11). The remaining buffer was removed and the chromatin pellet was resuspended in SDS gel loading buffer for SDS-PAGE.

In nuclear transfer experiments, where chromatin was isolated and incubated in a second extract for a further round of DNA synthesis, 45-µl aliquots of the first extract containing 20 ng/µl sperm nuclei was diluted in 800 µl ice-cold NIB supplemented with protease and phosphatase inhibitors but not containing Triton X-100. This was under-laid with 100 µl of the same buffer plus 15% sucrose (w/v) and further under-laid with 5 µl 30% glycerol (w/v). After spinning at 2100g for 5 minutes at 4ºC in a swing bucket rotor, the top of the sucrose cushion was washed once and the cushion removed.

Chromatin was gently resuspended in the glycerol cushion and then added to the second extract to a final concentration of 10 ng/µl.

6.1.4 Immunoblotting and antibodies

Immunoblotting was carried out as per standard techniques. Chromatin pellets were re-suspended in SDS loading buffer and separated on 4-12% Bis-Tris gradient gels (Invitrogen). Proteins were transferred onto PVDF membranes (GE Healthcare, RPN303F) using a wet transfer system, blocked in PBS with 0.2% Tween-20 and 5% non-fat milk (Marvel). Membranes were incubated with appropriate primary and secondary antibodies before being developed using enhanced chemiluminescence detection (SuperSignal® West Pico Chemiluminescent; Thermo Scientific, 34087). To visualise histones as loading controls, the dye front was not allowed to run out of gels and the lower portion of gels were cut before transfer and stained with Coomassie Brilliant Blue.

The sheep anti-Mcm4 and sheep anti-Cdc45 affinity-purified antibodies and sheep anti-Cdc6 serum used in this study have been previously described (Gambus et al., 2011; Luciani et al., 2004; Oehlmann et al., 2004; Prokhorova and Blow, 2000). The PCNA (PC10) and PP1α and PP1γ antibodies are from Santa Cruz. The Cdc7 antibody was raised in sheep against a bacterially-expressed immunogen consisting of the C-terminal 99 amino acids of *Xenopus* Cdc7 by Peter Gillespie (University of Dundee). The antiserum was affinity purified prior to use (Poh et al., manuscript in preparation).

6.1.5 Recombinant proteins and other reagents

Geminin was synthesised and purified as previously described (Ferenbach et al., 2005). Briefly, *Escherichia coli* host strain BL21 carrying the His-tagged geminin^{DEL} (McGarry and Kirschner, 1998) expression construct was cultured in Luria-Bertani

medium containing the appropriate antibiotics at 37ºC. 1 mM isopropyl-β-thiogalactopyranoside was added when OD_{600} reached 0.4-0.5 and the culture was grown for a further 2 hours. Cells collected by centrifugation at 5500 rpm for 10 minutes at 4ºC and lysed using Bugbuster (Novagen, 70921) according to the manufacturer's instructions. The bacterial lysate was then incubated with Ni-NTA beads (Qiagen, 36113), pre-washed and equilibrated with PBS containing 300 mM KCl and 20 mM imidazole, for 1 hour at room temperature. The mixture was transferred to a column and beads were washed 3 times with the same buffer. Protein elution was carried out with the same buffer but containing 250 mM or 500 mM imidazole. Protein fractions collected were quantified using the Quant-iT Protein Assay Kit (Molecular probes, Q33212) according to the manufacturer's instructions. Recombinant geminin was stored in 100 µl aliquots at -80ºC. To prepare a working stock of His-geminin^{DEL}, the protein was diluted to 150 µg/ml with LFB1/50 supplemented with 2.5 mM Mg-ATP.

Full length p27^{kip1} was synthesised by Gaganmeet Singh Chadha (University of Dundee) from a pGEX-p27-KIP1 plasmid (a gift of J. Walter, Harvard Medical School) and purified from RosettaTM(DE3)pLysS cells (Novagen) using Glutathione-Sepharose. The PP1 I-2 protein inhibitor was generously provided by the DSTT, University of Dundee. I-2 was bacterially expressed, purified and initially solubilised in 50 mM Tris-HCl pH 7.5, 0.1 mM EGTA, 150 mM NaCl, 0.1% β-mercaptoethanol, 270 mM sucrose, 0.03% Brij-35, 1 mM Benzamidine and 0.2 mM PMSF. I-2 action is time-dependent and it was typically pre-incubated with extracts for 15 min prior to sperm/nuclei addition (see individual figure legends for specific conditions). Compound 6d (Zhao et al., 2009) and PHA-767491 (Montagnoli et al., 2008) were synthesised at the University of Dundee. Roscovitine was bought from Calbiochem (557360), caffeine from ICN Biochem (150114), NU7441 from Axon Medchem (1463) and KU55933 from Tocris (3544).

6.1.6 Protein Kinase Assay

The MRC Protein Phosphorylation Unit, University of Dundee tested PHA-767491 against a panel of 95 kinases. The assay was carried out using a radioactive (^{33}P-ATP) filter-binding assay. 0.5 µl of PHA-767491 dissolved in DMSO was assayed in a total reaction volume of 25.5 µl at a final concentration of 1 µM. The 95 kinases used were divided into two groups and tested at 5 or 20 µM ATP so that the ATP concentration used was at or below the calculated K_M for ATP for each kinase. The full panel of protein kinases tested in this assay can be accessed at http://www.kinase-screen.mrc.ac.uk/kinase-protein-kinase-panel.

6.1.7 Preparation of CHO nuclei

CHOC-400 cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, 31885-023) supplemented with nonessential amino acids, 10% fetal calf serum (Gibco, 10270-106), and 10 U/ml streptomycin/penicillin (Gibco, 15140) at 37°C in 5% CO_2. G1 cells were prepared as previously described (Gilbert et al., 1995; Thomson et al., 2010). Briefly, cells were treated with 50 ng/ml nocodazole (Sigma-Aldrich, M1404) for 4 h to block cells in metaphase before being released into G1 for 4 h in fresh medium to obtain post-origin decision (ODP) point cells.

CHO nuclei used for DNA synthesis were prepared as previously described (Thomson et al., 2010). Post-ODP cells were harvested by trypsin treatment and resuspended in 1 ml ice-cold transport buffer [20 mM HEPES, 110 mM K acetate, 5 mM Na acetate, 2 mM Mg acetate, and 1 mM EGTA pH 7.6]. Cells were permeabilised with 50 µg/ml digitonin (Sigma, D141) for 5 minutes on ice and the reaction was stopped by adding 3% BSA (w/v) in transport buffer. Nuclei were counted and the required number recovered by centrifugation at 1500 rpm for 5 minutes at 4ºC.

6.1.8 Replication pattern labeling and analysis

As previously described (Thomson et al., 2010), somatic nuclei replication patterns were labelled by supplementing extracts with 25 µM Cy3-dUTP (GE Healthcare, PA53022) for 2.5 minutes. The reaction was stopped by resuspending extracts (20 µl) in 400 µl ice-cold Buffer A [60 mM KCl, 15 mM Tris-HCl, pH 7.4, 15 mM NaCl, 1 mM β-mercaptoethanol, 0.5 mM spermidine, and 0.15 mM spermine] and under-laid with 1 ml Buffer A containing 10% sucrose (w/v). Nuclei were recovered by spinning at 1500 rpm for 5 minutes at 4ºC in a swing bucket rotor. Nuclei were resuspended in 200 µl paraformaldehyde (4%) and incubated for 10 minutes at 23ºC. After fixation, samples were carefully loaded into wells in a 24-well plate containing 12-mm poly-L-Lysine coverslips covered with 2 ml TBS (10 mM Tris-HCl, pH 7.5, and 0.15 M NaCl) plus 10% sucrose (w/v). Nuclei were spun down onto the coverslips on a swing plate rotor at 1500 rpm for 5 minutes at 4ºC. Coverslips were retrieved and washed twice with TBS containing 0.1% TX-100 (v/v) and twice with TBS. Nuclei were stained with Hoechst 33258 for 5 minutes at 23ºC. After 2 final washes with TBS, coverslips were mounted with Vectashield mounting medium and sealed for visualisation.

Images were acquired using a cooled camera (CoolSNAP HQ; Photometrics) on a restoration microscope (DeltaVision Spectris; Applied Precision) built around a stand (Eclipse TE200; Nikon) with a 100× 1.4 NA Plan Apo lens (Nikon). Images were taken every 0.25 µm and 22 sections optical sections were recorded for every nucleus. 3-dimensional datasets were deconvolved using the constrained iterative algorithm software (SoftWoRx; Applied Precision) and images were imported into the Open Microscopy Environment (OME) (Goldberg et al., 2005; Swedlow, 2003) for analysis. Timing patterns for this hybrid system of CHO nuclei in *Xenopus* extracts were analysed following the classification scheme detailed in a previous publication

(Thomson et al., 2010). Data was generated from 20 nuclei in each condition and time-point for each experiment. 3 independent experiments were carried out.

6.2 Methods for generating a *Cdc7* conditional knockout mouse

6.2.1 Primers used for the construction of the targeting vector

All PCRs were performed for 35 cycles under standard PCR conditions.

Vector component	Oligo no.	RE site added	Primer sequence
5' retrieval miniarm F	PKO1187	BamHI	5'- GCT GGA TCC GAC TTC TGG TGC ACA TT -3'
5' retrieval miniarm R	PKO1188	XhoI	5'- CGT CTC GAG CTG CTA AGA GCA CTG AC -3'
3' retrieval miniarm F	PKO1185	HindIII	5'- CGT AAG CTT GTC AGA GAT GGT CAA TG -3'
3' retrieval miniarm R	PKO1186	SpeI	5'- GCT ACT AGT CTC ATG TTC CAG ATA CG -3'
Engrailed 5' miniarm F	PKO1193	SalI	5'- CGT GTC GAC TCA CCC AGC TTT AGT CC -3'
Engrailed 5' miniarm R	PKO1194	EcoRV	5'- CGT GAT ATC GCC AGG GCT ATA CAG AG -3'
Engrailed 3' miniarm F	PKO1201	SacII, SpeI	5'- CGT CCG CGG ACT AGT GCT CAC TTT GTA GAC CA -3'
Engrailed 3' miniarm R	PKO1217	SacII, ScaI	5'- CGT CCG CGG AGT ACT GAA CTA ATG AGA TAC AGT CAG GG -3'
Lonely loxP 5' miniarm F	PKO1197	ApaI	5'- CTA GGG CCC CTG ATA GTC TTG TCT CC -3'
Lonely loxP 5' miniarm R	PKO1198	EcoRI	5'- CGT GAA TTC GTT AAG AGC ATC AAG GC -3'
Lonely loxP 3' miniarm F	PKO1202	BamHI, MfeI	5'- CGT GGA TCC CAA TTG TAG TGC ATT TCC ATA GC -3'
Lonely loxP 3' miniarm R	PKO1238	NotI	5'- CGT GCG GCC GCA TGG TGG TAT CTT CTG TAC AC -3'

Table 6.1. Primers for constructing the *Cdc7* targeting vector

6.2.2 Genomic DNA isolation

Genomic DNA was extracted from embryonic stem cell lysates or Proteinase K digested tail clips. Lysates were briefly centrifuged and 500 µl of phenol: chloroform: isoamyl-alcohol (Invitrogen, 15593-049) was added over the samples. This was gently vortexed for 15 min and then centrifuged at maximum speed in a microfuge for 5 min at room temperature. The upper aqueous phase was carefully removed into a fresh tube. 500 µl chloroform was added over the aqueous solution. Samples were then vortexed and centrifuged as described above. The upper aqueous phase was collected into a fresh tube. 800 µl isopropyl-alcohol was added to these samples. Tubes were gently inverted repeatedly until white DNA precipitates become visible. Samples were centrifuged at maximum speed for 2 min and the supernatant carefully aspirated, leaving 10 µl over the DNA pellet. The pellet was washed once with 100% ethanol (DNA grade) and once with 70% ethanol. The pellet was then air-dried for 30 min at 45°C. 50 to 100 µl nuclease-free water was added and the genomic DNA was resolubilised by shaking at 37°C at 1000 rpm in a thermoshaker. Total DNA was quantified with a spectrophotometer (Thermo Scientific, NanoDrop 8000).

6.2.3 Non-radioactive Southern Hybridisation

Probe preparation

Analysis of genomic DNA by southern blotting was carried out using non-radioactive digoxigenin-dUTP (DIG) labelled probes. DIG-labelled DNA probes were generated by random primed labeling using the DIG DNA Labelling Mix (Roche, 11277065910) with the following PCR conditions and primer pairs:

Component	Volume for 1 reaction (µl)
Nuclease-free water	14.5
10X Green Buffer for DreamTaq (Fermentas, B71)	2
10X DIG DNA Labelling Mix	2
DreamTaq DNA polymerase (Fermentas, EP0702)	1
Forward Primer	0.2
Reverse Primer	0.2
BAC DNA template	1
Total volume	20

Probe	Primers
5’	PKO1502: 5’- ATG TGA CTC GCT GTG TTA G -3’ PKO1503: 5’- CCT GTG ACA CTA AAC AGT GG -3’
3’	PKO1191: 5’- GTG TGG GTG CTG AGT TTT GC -3’ PKO1192: 5’- GCT TGA AGC AGC AAT ACC TTC TG -3’

Table 6.2. Primers for generation of DIG-labelled probes.

PCRs were run for 35 cycles with 61°C as the melting temperature.

A dot-blot was used to verify the successful generation of DIG-labelled probes.

Southern blotting

2 µg genomic DNA was digested with restriction endonucleases for 6 hours at 37°C.

Restriction digests were set up as follows:

For analysis with the 5' probe:

Component	Volume for 1 reaction (µl)
Nuclease-free water	11.8
10X NEBuffer 4	2
100X BSA (10 mg/ml)	0.2
Genomic DNA	2 µg
SpeI (NEB, R0133)	1
Total volume	20

For analysis with the 3' probe:

Component	Volume for 1 reaction (µl)
Nuclease-free water	12
10X NEBuffer 4	2
Genomic DNA	2 µg
MfeI (NEB, R0589)	1
Total volume	20

Digested DNA was resolved on 0.8% agarose gel overnight. The gel was briefly stained with ethidium bromide to visualise DNA and destained by washing with water. The gel was then treated with 0.25 M HCl for 15 min to depurinate DNA, rinsed with water for 15 min and treated with transfer buffer (0.5 M NaOH, 1.5 M NaCl) for 30 min. Gravity-aided transfer of DNA onto positively charged nylon membrane (GE Healthcare, RPN203B) was carried out for 6 h, following which the membrane was washed with 2X SSC (150 mM NaCl, 15 mM sodium citrate) for 5 min and allowed to air dry. DNA was cross-linked to the membrane with UV. The membrane was pre-incubated with hybridisation buffer (Ambion, AM8670) for 1 hour at 42°C. It was then incubated with fresh hybridisation buffer containing 5 ng/ml of labelled probe, which had first been denatured at 95°C for 5 min and chilled on ice, with gentle rocking at 42°C overnight. Following two washes with 2X SSC containing 0.1% SDS, 5 min each at room temperature, and two washes with 0.5X SSC containing 0.1% SDS, 15 min

each at 68°C, the membrane was further washed once with Buffer 1 (100 mM maleic acid, 150 mM NaCl, pH 7.5) for 5 min at room temperature and blocked with Buffer 2 [1% Blocking Reagent (Roche, 11096176001) in Buffer 1] for 45 min. Alkaline phosphatase conjugated antibody (Anti-DIG-AP, Fab Fragments, Roche, 11093274910) was diluted 1: 10000 in buffer 2 and incubated with the membrane for 30 min. The membrane was washed twice with Buffer 1 containing 0.3% Tween-20 for 15 min and once with Buffer 3 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) before hybridised probes were detected by chemiluminescence using CPD-*Star* Ready-to-use substrate for alkaline phosphatase (Roche, 12041677001).

6.2.4 Other PCR reactions

Primers were designed to amplify specific regions as required during the screening process for positive gene targeting. The primers used are listed in the Table 6.3. PCRs were run for 35 cycles with melting temperatures of 61ºC, 62ºC and 59ºC for the Neo cassette, Lonely *lox*P and $Cdc7^{FLOX/NULL}$ amplifications respectively.

PCR	**Primers**
Neo cassette	PKO0838: 5'- GTA TCC ATC ATG GCT GAT GC -3' PKO0839: 5'- CAG AAG AAC TCG TCA AGA AGG C -3'
Lonely *lox*P	PKO2284: 5'- GCT ATA CGA AGT TAT TAG GTC CC -3' PKO2286: 5'- GGT GGT ATC TTC TGT ACA CAG -3'
$Cdc7^{FLOX/NULL}$	PKO2282: 5'- CTC AGT GTG CCA TTT ACA GA -3' PKO2285: 5'- GTG AGG ATG CCA ATA AAG CT -3'

Table 6.3. Other primers used in the process of generating the *Cdc7* cko mouse.

6.3 Methods for mouse work and mammalian cell culture

6.3.1 Mouse strains and genotyping

Conditional *Cdk1* knockout mice with ER-CreEsr1 or Rosa26-CreERT2 (*Cdk1*$^{flox/flox}$) (Diril et al., 2012) were bred with with *Cdk2*$^{+/-}$ mice (Berthet et al., 2003) to generate the *Cdk1*$^{flox/flox}$ *Cdk2*$^{+/-}$ mouse strain. *Cdk1*$^{flox/flox}$ *Cdk2*$^{+/-}$ mice were then crossed to generate *Cdk1*$^{flox/flox}$ *Cdk2*$^{-/-}$ 4-hydroxytamoxifen (4-OHT) conditional double mutant animals. Mice were routinely genotyped with genomic tail DNA isolated using the HotSHOT method (Truett et al., 2000). Briefly, animal tissue was lysed with alkaline lysis buffer (25 mM NaOH, 0.2 mM disodium EDTA, pH 12) at 95°C for 25 min. The alkaline pH was neutralised by addition of an equal volume of neutralisation buffer (40 mM Tris-HCl). 1 µl of resultant genomic DNA was used in each 20-µl PCR reaction with primer sets as listed in Table 6.4. All PCRs were performed for 35 cycles with annealing temperatures of 68°C for Cdk1$^{FLOX/NULL}$ and 62°C for Cdk2^{NEO} and Cre respectively.

Strain	**Genotyping primers**
Cdk1$^{FLOX/NULL}$	PKO0844: 5'- TTT GTC AAG AAA TGC AGC AGG ACA C -3' PKO1006b: 5'- GCG GTG GTT TAA ACC CAT AAT CCC GGC AC -3' PKO1158: 5'- GCT CAC ATG TTC CTG AAT GGT GGA -3'
Cdk2^{NEO}	PKO1640: 5'- GTC TTC TTG TTC CCC AGG TG -3' PKO1641: 5'- CGC CTT CTA TCG CCT TCT TG -3' PKO1642: 5'- ATG GGT GGG CAG CAT AGA AC -3'
Cre	PKO1628: 5'- AGC ACT GGA AAT GTT ACC AAG GAA C -3' PKO1629: 5'- GGC TGG CTA AAC TCT GGC CCT ACA -3' PKO2122: 5'- CCA CCA GCC AGC TAT CAA CTC G -3' PKO2123: 5'- TAC ATT GGT CCA GCC ACC AGC -3'

Table 6.4. Primers used for genotyping.

All mice used in this study were of mixed C57BL/6 x 129S1/SvImJ background. Mice were group-housed under standard conditions with food and water available *ad libitum* and were maintained on a 12-hour light/dark cycle. Mice were fed a standard chow diet

containing 6% crude fat and were treated humanely in compliance with the Institutional Animal Care and Use Committee (IACUC) guidelines.

6.3.2 Isolation and culture of mouse embryonic fibroblasts (MEFs)

Primary MEFs were prepared from embryos as previously described (Berthet et al., 2003). A female pregnant mouse was sacrificed at 13.5 days post coitum. The uterus was removed and washed in PBS after which the yolk sacs were separated and embryos isolated carefully. The head and organs were removed and a small amount of tissue was collected from the tail for genotyping. The remaining embryonic tissue was finely chopped with a razor blade and trypsinised for 20 min at 37°C. Finally, tissue and cell clumps were dissociated by pipetting. Isolated cells were plated in a 10-cm tissue culture dish (passage 0) and grown in DMEM (Prime; Invitrogen, 12701-017) supplemented with 10% FCS (Hyclone, SH30070.03) and 1% penicillin/streptomycin (PAA, P11-010). Primary MEFs were cultured in a humidified incubator at 37°C with 5% CO_2 and 3% O_2.

6.3.3 Synchronisation and induction of MEFs

Synchronisation of MEFs at G1 phase of the cell cycle was carried out by serum starvation. MEFs were plated at high densities in 15-cm dishes and allowed to become confluent. Complete medium was replaced with starvation medium (DMEM containing 0.3% serum and 0.2% penicillin/streptomycin). After 72 h, cells were trypsinised and re-plated in complete medium to induce synchronous entry into cell cycle.

To induce Cre-mediated recombination of the *Cdk1*-floxed locus to achieve *Cdk1* knockout, 50 ng/ml 4-OHT (Sigma, H7904) was added during the entire starvation period. Cells were genotyped at the end of the induction period using the *Cdk1*$^{FLOX/NULL}$ PCR protocol.

6.3.4 Proliferation assay

Proliferation of MEFs was analysed using the alamarBlue assay (AbD Serotec, BUF012B). Cells were seeded in a 96-well plate at 1.5 x 10^3 cells/well, in triplicate per genotype per day. Complete medium with 10% alamarBlue was added to each well and fluorescent reading was carried out at 590 nm after 4 h.

6.3.5 Bromo-deoxyuridine (BrdU) labelling and analysis

To monitor S-phase progression, cells were labelled with BrdU and analysed by flow cytometry. 100 µM BrdU (BD Pharmingen, 550891) was added to the MEF culture 30 min before collection at different time-points. At each time-point, cells were trypsinised and collected by centrifugation. The cell pellet was washed once in PBS and thoroughly dissociated into single cells before being fixed with ice-cold 70% ethanol by gentle vortexing. Fixed cells were incubated in blocking solution (PBS-1% BSA) for 5 min at room temperature. DNA was denatured by treating cells with 2N HCl/0.5% Triton X-100 for 20 min at room temperature and neutralised with 0.1 M sodium tetraborate, pH 8.5. Cells were then washed with PBS-1% BSA-0.5% Triton X-100 and incubated with APC-conjugated anti-BrdU antibody (BD Pharmingen, 623551) for at least 30 min. Cells were washed twice with PBS-1% BSA and stained for 15 min at room temperature in PBS containing 2.5 µg/ml propidium iodide (PI) (Merck, 537059) and 20 µg/ml RNase A (Sigma-Aldrich, R6513). Cells were filtered through a 40-µm cell strainer (BD Falcon, 352340) to dissociate any cell aggregates before analysis on the FACSCalibur flow cytometer (BD Biosciences). Cell cycle profile analysis was performed using the FlowJo 8 software.

6.3.6 Protein extraction, chromatin fractionation and immuno-blotting

To prepare total cell protein lysate from MEFs, cells were first collected by trypsinisation and washed with PBS. The cell pellet was resuspended in EB buffer (80

mM beta-glycero-phosphate, 15 mM $MgCl_2$, 20 mM EGTA, pH 7.3) containing 0.5% NP-40, 150 mM NaCl and protease inhibitors [(10 µg/ml each of leupeptin, chymostatin, and pepstatin (Chemicon, EI8, EI6 and EI10)] and sonicated with using a sonicator bath (Diagenode Bioruptor; 15 s on/1 min off, total 10 min). The cell lysate was centrifuged at 14000g for 10 min at 4°C. Protein concentration was measured using the BCA protein assay (Thermo Scientific, 23225). Protein Lysates were snap-frozen in liquid nitrogen and stored at -80°C until required.

Chromatin fractionation was carried out as previously described (Klotz-Noack et al., 2012) with modifications. Briefly, cells were trypsinised and washed with PBS. The cell pellet was resuspended in CSK buffer (10 mM Hepes-KOH pH 7.4, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 0.25% Triton-X100), freshly supplemented with 1 mM PMSF, 0.1 mM Na_3VO_4 and 10 µg/ml each of leupeptin, chymostatin and pepstatin. Samples were incubated on ice for 5 min and centrifuged at 5000g for 5 min at 4°C. The supernatant containing soluble cytoplasmic and nucleoplasmic proteins were snap-frozen in liquid nitrogen and stored at -80°C. The pellet containing chromatin-bound proteins was resuspended and washed twice with CSK buffer and frozen in liquid nitrogen. To extract chromatin-bound proteins, the pellet was resuspended in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.5% Na-deoxychelate) supplemented with 1 mM PMSF, 0.1 mM Na_3VO_4, 0.1 mM NaF, 10 µg/ml leupeptin, chymostatin, pepstatin) and processed as for total protein lysate.

Immuno-blotting was carried out as follows: 5 to 10 ug of protein sample in SDS-sample buffer was separated on 8% or 10% polyacrylamide gels, transferred onto PVDF membranes (Millipore, IPVH0010) using a semi-dry transfer system and blocked in Tris-buffered saline with 0.2% Tween 20 and 5% non-fat milk (Biorad, 1706404). Membranes were probed with appropriate primary antibodies, followed by anti-mouse,

anti-rabbit or anti-goat secondary antibodies conjugated to horseradish peroxidase (Pierce, 0031432) and developed using enhanced chemiluminescence (Millipore, WBKLS0500).

Affinity purified antibodies against Cdk2 have been previously described (Berthet et al., 2003). Other antibodies are bought commercially: mouse anti-Cdc6 (Santa Cruz, sc-9964), rabbit anti-Mcm2 (Cell Signalling, 3619), goat anti-Mcm4 (Abcam, 3728), rabbit anti-Cdc45 (Cell Signalling, 3673), rabbit anti-Cdc2 (Cdk1) (Santa Cruz, sc-954), mouse anti-Cdk4 (Cell Signaling, 2906), mouse anti-Cdk6 (Cell Signaling, 3136), rabbit anti-cyclin A2 (Santa Cruz, sc-596), mouse anti-cyclin B1 (Cell Signaling, 4135), mouse anti-cyclin D1 (Cell Signaling, 2926), rabbit anti-cyclin E1 (eBioscience, 14-6714), mouse anti-p21 (Santa Cruz, sc-6246), mouse anti-p27 (BD Transduction, 610242), mouse anti-Hsp90 (BD Transduction, 610419), rabbit β-tubulin (Cell Signalling, 2128), rabbit anti-Lamin A/C (Cell Signalling, 4777) and rabbit anti-histone H3 (Cell Signalling, 9715).

6.3.7 Immunoprecipitation and kinase assays

Immunoprecipitation (IP) and kinase assays were carried out as previously described (Berthet et al., 2003) with modifications. For immunoprecipitation, 50 to 500 ug of whole cell lysate was incubated with the respective antibody/peptide overnight at 4°C in buffer EB containing 0.5% NP-40, 150 mM NaCl, protease inhibitors and 1 mg/ml ovalbumin. IP for cyclin A2 was performed using antibody-conjugated sepharose beads (Santa Cruz, sc-751AC). Otherwise, IPs were carried out using antibodies against Cdk1 (Clontech, s1190), Cdk2 (Berthet et al., 2003) or cyclin E1 (eBioscience, 14-6714) or serum against cyclin B1 (Berthet et al., 2003). Unconjugated antibodies were isolated with bound proteins by incubating with protein A beads (Roche, 11719408001) for 1 h at 4°C. Beads were washed twice with EB containing 0.5% NP-

40, 150 mM NaCl, protease inhibitors and 1 mg/ml ovalbumin and once with EB containing protease inhibitors and 1 mg/ml ovalbumin. Immunoprecipitated proteins were then eluted from beads with 1 x SDS-sample buffer and assessed for kinase activity using histone H1 (Roche, 11004875001) as substrate.

For kinase assays, immunoprecipitated proteins on beads were incubated in EB buffer supplemented with 10 mM DTT, 15 µM ATP, 2.5 µCi [γ-32P]-ATP (PerkinElmer, NEG502A) and 1.6 µg of histone H1 for 30 min at room temperature. Reactions was stopped by adding SDS-sample buffer, heated at 80°C for 5 min and separated by SDS-PAGE. Proteins were fixed and stained with Bismarck Brown/Coomassie blue and quantification of incorporated radioactivity was done using a phosphoimager (Fujifilm, FLA-7000).

6.3.8 RNA analysis

Total RNA was extracted from cells using the Qiagen RNeasy mini kit according to the manufacturer's protocol. First-strand cDNA was synthesised from 0.2 µg total RNA for each quantitative real-time PCR (qRT-PCR) reaction using the SuperScript III reverse transcriptase (Invitrogen, 18080-051) following manufacturer's instructions. qRT-PCR was carried out with the Maxima SYBR Green qPCR Master Mix (Fermentas, K0252) and primers as listed in Table 6.5. The Rotor-Gene thermal cycler (Corbett Research) was used to monitor reactions with the following programme: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec. All data were normalized to the expression levels of three housekeeping genes: β-actin, Hsp90 and cyclophilin A.

Gene	Primers
Cdk1 (PKR196)	F: 5'- AGC GAG GAA GAA GGA GTG CC -3' (PKO2004) R: 5'- CAT GAG CAC ATC CTG CAG GC -3' (PKO2005)
Cyclin E1 (PKR023)	Commercial primer set (SABiosciences, PPM02891B)
Cyclin E1 (PKR215)	F: 5'- GAG CTT CTA GAC CTG TGC GT -3' (PKO2316) R: 5'- CGC ACC ACT GAT AAC CTG AG -3' (PKO2317)
Cyclin E1 (PKR277)	F: 5'- ACA GCT TCG GGT CTG AGT TC -3' (PKO2875) R: 5'- CGG AGC CAC CTT CTT CTT TC -3' (PKO2876)
Cdc6 (PKR240)	F: 5'- TGG GCG ATG ACA ACC TGT GC -3' (PKO2689) R: 5'- GGG GAA GCC TTA AAT GTC GG -3' (PKO2690)
Cdc6 (PKR241)	F: 5'- GAT CGT GTT GGT GTT GGA CG -3' (PKO2691) R: 5'- GCT TCA AGT CUC GGC AGA AT -3' (PKO2692)
Cdc6 (PKR242)	F: 5'- GCT GGA TGT TTG CAG GCG AG -3' (PKO2693) R: 5'- CAC CCT GTT CCC ATC AAC TT -3' (PKO2694)
β-actin (PKR143)	F: 5'- ACG GCT CCG GCA TGT GCA AA -3' (PKO1630) R: 5'- TTC CCA CCA TCA CAC CCT GG -3' (PKO1631)
Hsp90 (PKR144)	F: 5'- ACT GCT CTG CTC TCC TCT GG -3' (PKO1632) R: 5'- CAT CGA TGC CCA GGC CTA GT -3' (PKO1633)
CypA (PKR195)	F: 5'- CCT TGG GCC GCG TCT CCT T -3' (PKO2002) R: 5'- CAC CCT GGC ACA TGA ATC CTG -3' (PKO2003)

Table 6.5. Primers used for qRT-PCR.

6.3.9 Other chemicals used

Cycloheximide (Sigma, C19988-1G) and MG132 (Calbiochem, 474790) were dissolved in DMSO as required.

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