Cdc7/ASK kinase as a novel target for anti-cancer drug development programmes

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I, Hye Kyung Hong confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

DEDICATION

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This thesis is dedicated to my parents and family.

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ABSTRACT

Targeting Cdc7, a kinase essential for DNA replication initiation, results in potent cancer cell killing. Cancer cells in which CDC7 is silenced by RNAi enter an abortive S phase followed by apoptosis due to loss of a functioning DNA replication origin activation checkpoint. This checkpoint prevents normal cells from entering S phase (reversible G1 arrest) if the DNA replication initiation machinery is perturbed. The pre-clinical anti-cancer effects of CDC7 silencing have highlighted this kinase as an important target for new drug development. Expanding on published reports, I performed further target validation using molecular tools generated in the work of this thesis, including an affinity-purified antibody to the Cdc7 regulator ASK and functional recombinant Cdc7/ASK kinase complex. Making use of fibroblast and HL60 tissue culture model systems, I show that Cdc7 and ASK are amongst a group of essential replication initiation factors that are tightly downregulated to suppress proliferative capacity during exit from cycle into quiescent and differentiated states. This finding is further supported by low expression levels in normal liver and oral squamous epithelium and the lack of Mcm2 phosphorylation at serine 53, a well known Cdc7 target. In liver carcinoma and oral squamous cell carcinoma, on the contrary, the majority of cancer cells are expressing Cdc7 and ASK and show Mcm2 phosphorylation at Ser-53. Thus it can be postulated that Cdc7 inhibitors should selectively kill cancer cells, while normal proliferating cells are reversibly arresting in G1 and quiescent and differentiated cell populations are not affected due to downregulation of the target protein. To screen for compounds that selectively inhibit Cdc7, I developed a sensitive *in vitro* kinase assay and contributed to the successful transfer of this assay to a high-throughput screening platform and the generation of a structural model of the Cdc7 kinase domain allowing *in silico* predictions of the most potent inhibitors. On completion of the work for this thesis, the HTS assay and structural model fromed the core of an ongoing drug discovery programme run by Cancer Research Technology. Two series of novel, selective small molecule inhibitors which exhibit low nM activity against Cdc7 and cellular efficacy (apoptosis) have been developed and are currently being tested in mouse xenograft models. The work presented in this thesis provides a strong rationale for targeting the DNA replication initiation pathway, and in particular Cdc7. Future intend to treat clinical trials will establish the potential of pharmacological Cdc7 inhibitors for selective cancer cell killing in patients.

TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

ABBREVIATIONS

1,25 Vti D3	1,25 dihydroxyvitamin D3
3D	Three-dimensional
6-MP	6-mercaptopurine
ACS	Autonomously replicating sequence consensus sequence
ADME	Absorption, distribution, metabolism and excretion
APC	Anaphase-promoting complex
ARS	Autonomously replicating sequence
ASK	Activator for S-phase kinase
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia related protein
BrdU	Bromodeoxyuridine
C	Confluent
CDK	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
CRT	Cancer research technology
CIN	Cervical intraepithelial neoplasia
CLL	Chronic lymphocytic leukaemia
DAB	3'3'-diaminobenzidine tetrahydrochloride
DAPI	4', 6'-diamidino-2-phenylindole
DC	Differentiated compartment
DMF	N,N,dimethylformamide
DHFR	Dihydrofolate reductase
DSB	Double-strand breaks

E	Elution fraction
EGFR	Epidermal growth factor receptor
EM	Electron microscopy
FITC	Fluorescein isothiocyanate
G0	Quiescence/quiescent
HER-2	Human epidermal growth factor receptor-2
HNSCC	Head and neck squamous cell carcinoma
IMZ	Imidazole
MCM	Minichromosome maintenance
MDR	Multi-drug resistance
MOI	Multiplicity of infection
MTX	Methotrexate
NSCLC	Non-small cell lung carcinoma
NLS	Nuclear localisation sequence
ORC	Origin recognition complex
ORF	Open reading frame
pASK	phosphorylated ASK
Pgp	P-glycoprotein
PI	Propidium iodide
pRb	Retinoblastoma protein
Pre-IC	Pre-initiation complex
Pre-RC	Pre-replicative complex
PP	λ- phosphatise
RLF	Replication licensing factor
RPA	Replication protein A

SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TdT	Terminal deoxynucleotidyl transferase
TPMT	Thiopurine methyltransferase
WCE	Whole cell extract
VCA	Vincristine mitotic accumulation

CHAPTER ONE

Introduction

The eukaryotic cell cycle consists of four phases - G1, S, G2 and M (Murray & Hunt 1993). Critical events in the cell cycle are the DNA synthesis phase (S), in which DNA replication takes place and mitosis (M), in which the replicated genome is subsequently segregated as sister chromatids into the two daughter cells. Preparation for S phase takes place in the preceding G1 phase and, similarly, the cell prepares for mitosis in the second gap phase, G2. Dependent on environmental and developmental signals, cells in G1 phase can reversibly leave the cell cycle and enter a quiescent state, known as G0, wherein cells are metabolically active but not embarking upon cell division (Pardee 1989). Alternatively, cells can withdraw irreversibly from the cell cycle through the processes of terminal differentiation and replicative senescence (Myster & Duronio 2000; Hayflick & Moorhead 1961; Campisi 1996).

DNA replication can be regarded as a final and critical step in growth control that lies downstream of growth signalling pathways and therefore is of potential importance in tumourigenesis. Indeed, cancers are characterised by uncontrolled cell proliferation which results from aberration in the complex growth signalling pathways that control cell cycle phase transitions, DNA synthesis and mitosis. A topical issue in drug discovery and development is to identify how cancer cells differ from normal cells, and how those differences can be exploited therapeutically. Several studies of the G1 events involved in the DNA replication initiation pathway suggest that inhibition of DNA replication initiation may provoke a differential response in normal and cancer cells (Shreeram et al. 2002; Feng et al. 2003; Montagnoli et al. 2004). This suggests

that various points in the DNA replication initiation pathway may be considered as potential targets for drug discovery, and in particular for cancer therapy.

In the work of this thesis, I studied the cell cycle dependent regulation of the Cdc7/ASK kinase complex in human cells with the aim of exploiting this essential kinase which is required for G1-S transition and S phase progression as a potential therapeutic target. In the following sections, the literature relevant to this work is reviewed, focussing in the first part on conventional chemotherapy and cyclin-dependent kinase inhibitors as an example for current anti-cancer drug discovery programmes. In the second part the DNA replication initiation pathway and its potential as a therapeutic target is reviewed.

1.1. Conventional cancer chemotherapy

Before 1950, cancer therapy largely depended on surgery which is the longest established treatment for most solid tumours. Radiotherapy became an effective treatment for the control of local and regional tumours after 1960 (reviewed in Chabner & Roberts Jr 2005). Despite the success of surgery and radiotherapy in controlling local tumours, they cannot cure metastatic cancer. Chemotherapy is a systemic treatment which can be used to kill cancer cells in every organ in the body. Therefore, it became an essential mode of treatment for metastatic cancer, and the focus for current anti-cancer drug development programmes. Conventional chemotherapeutic agents, which revolutionized the treatment of many types of cancer during the last 50 years, are shown in Figure 1.1 (Baguley 2002; Chabner & Roberts

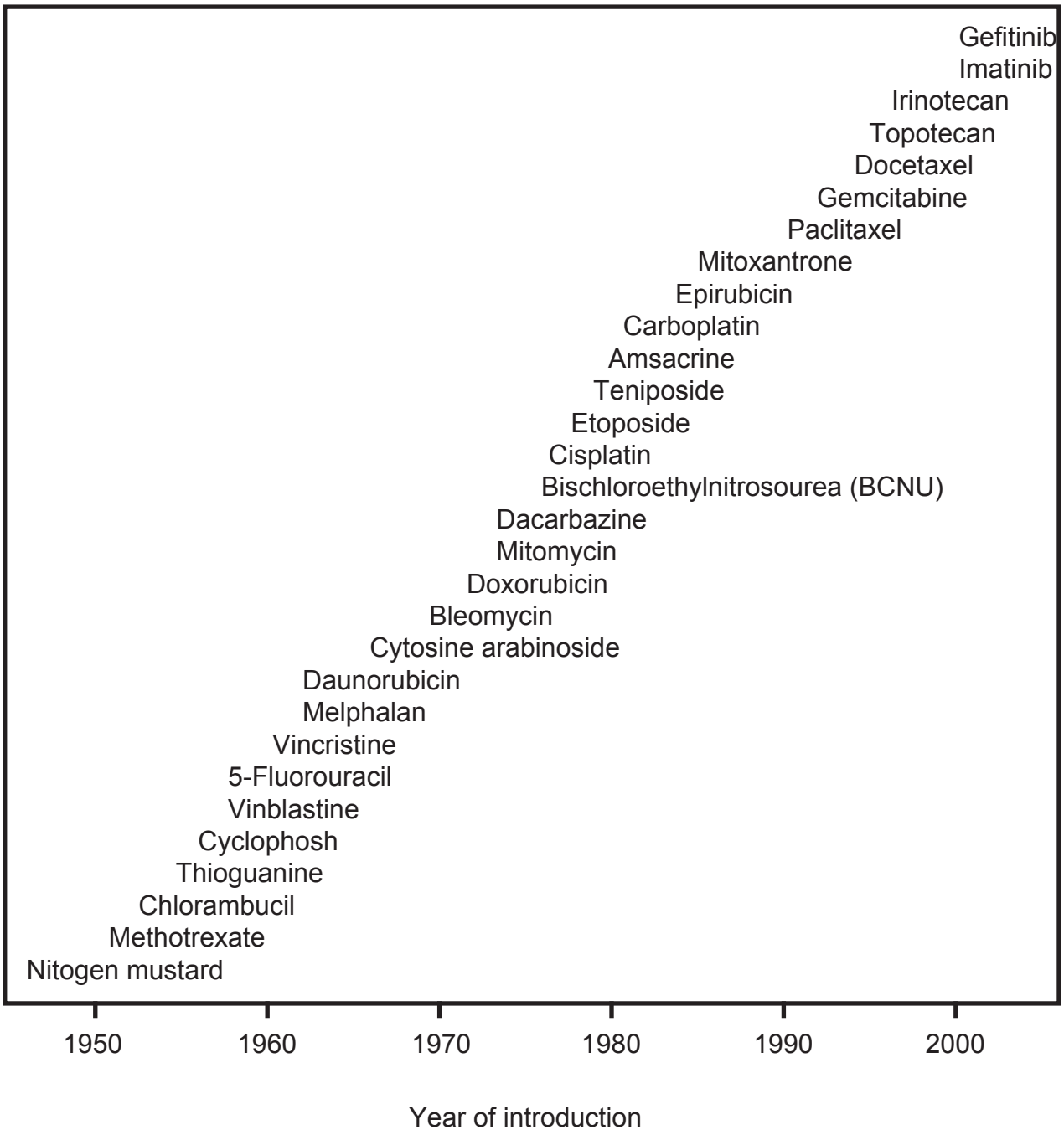


Figure 1.1: Chronology for the development of some of the anti-cancer drugs commonly in use today. The modern era of chemotherapy began in the 1940s with the first uses of nitrogen mustards and anti-folate drugs. Anti-cancer drug development since then has been dominated by the development of genotoxic (cytotoxic) drugs which act by targeting DNA synthesis or function. For example, nitrogen mustards and other alkylating agents covalently interact with DNA and induce DNA damage. Other agents which inhibit tumour growth by interfering with DNA synthesis or function include anti-metabolites, anti-tumour antibiotics, topoisomerase inhibitors and platinum compounds. Further strategies for inhibiting tumour growth were developed in the form of microtuble inhibitors and immunotherapies. Recent anti-cancer drug development has focused on targeted therapy that specifically inhibits and/or modifies tumour specific molecular biological targets including growth factors, signalling molecules, cell-cycle proteins, modulators of apoptosis and molecules that promote angiogenesis (Baguley 2002).

Jr 2005). In general, these agents can be classified by their mode of action. For example, the anti-cancer activity of nitrogen mustards and other derivatives, including melphalan and cyclophosphamide, is coupled to the ability of these compounds to covalently interact with DNA. Other agents which inhibit tumor growth by disrupting DNA synthesis incude anti-folate compounds, anti-tumor antibiotics, topoisomerase inhibitors and platinum compounds. In addition, further agents for inhitbiting tumor growth were developed in the form of microtubule inhibitors and immunotherapies.

Alkylating agents

The beginning of the modern era of chemotherapy is linked to the discovery of nitrogen mustard as an effective treatment for cancer in 1942 (Gilman 1963). Nitrogen mustard is a prototype for alkylating agents, which are selectively interacting with DNA and induce DNA damage (Papac & Wood 1962; Roberts et al. 1968; Thomas et al. 1978; Erikson et al. 1980; Garcia et al. 1988). The effects of nitrogen mustard were first noted in autopsy findings from soldiers dying of exposure to sulphur mustard gas during the First World War (Krumbhaar 1919), and the first clinical trial began to treat a patient with non-Hodgkin's lymphoma (Gilman 1963). The response of the patient indicated that its therapeutic value is the induction of temporary regression in certain malignant diseases, and that the drug could be administered systemically to induce tumour regression. Thus, the work of Alfred Gilman with nitrogen mustard led to clinical use of the first chemotherapeutic alkylating agent and eventually to the production of a range of compounds with similar modes of action. Other improved alkylating agents, including cyclophosphamide and chlorambucil were chemically stabilized and became standard components of regimens used to treat patients with

lymphomas, leukaemias and some solid tumours (Gale and Foon 1985; Black and Livingston 1990). However, it has been noted that tumours quickly became resistant to these drugs (Behrens et al. 1987), and that these drugs are extremely toxic to rapidly proliferating normal tissues, invoking a number of side effects including nausea, vomiting, bone marrow depression and alopecia (Smith 1989).

Anti-folate compounds (Methotrexate)

A second approach to chemotherapy began shortly after the Second World War when the effects of folic acid on patients with leukaemia were investigated by Sydney Farber (Farber et al. 1948). Folic acid is needed to synthesize the DNA base for DNA replication and is important during periods of rapid cell division and growth (Mitchell et al.1941). Folic acid was identified as the factor that is deficient in patients with megaloblastic anaemia (Wills & Evans 1937), and it seemed to stimulate proliferation of acute lymphoblastic leukaemia (ALL) cells when administered to children with this cancer. This led to the synthesis of folate analogues, which block the function of folate-requiring enzymes and became the first drugs to successfully induce remission in children with ALL by suppressing proliferation of malignant cells (Farber et al. 1948; Bertino 1979). One of the first anti-folate compounds to be developed for clinical trial was methotrexate (4-amino-10-methyl-folic acid, MTX) which proved to have anti-tumour activity in breast cancer, osteogenic sarcoma and leukaemias (Jonsson & Kamen 1991; Stoller et al. 1977). The action of methotrexate depends on active transport into cells through the reduced-folate transporter 1 (RFT-1), its conversion to a long-lived intracellular polyglutamate, and its binding to dihydrofolate reductase (DHFR), which leads to inhibition of the synthesis of thymidylate and

purines and the induction of apoptosis (Jolivet et al. 1983; Allegra et al. 1990; Schweitzer et al. 1990). Mutation of RFT-1, amplification or mutation of DHFR, loss of polyglutamation, and defects in the apoptotic pathway have been shown to reduce efficacy leading to drug resistance (Bertino et al. 1996). Methotrexate was also the first drug that is used to monitor drug clearance (Stoller et al. 1977), and is still primarily used to treat patients with ALL, as well as those with certain lymphomas, osteosarcoma and choriocarcinoma (reviewed in Chabner & Roberts Jr 2005).

Platinum compounds

Cisplatin, a platinum based compound that was discovered by Barnett Rosenberg (Rosenberg et al. 1965), was approved for clinical use by the FDA in 1978 (reviewed in Desoize & Madoulet 2002). Cisplatin interacts with DNA producing inter- and intrastrand cross-links, which induce apoptosis through the inhibition of DNA replication and transcription (Pil & Lippard 1992). Cisplatin showed anti-cancer activity both in testicular cancer and in ovarian cancer in early phase I trials, however, further trials were stopped because of renal toxicity. This renal toxicity was reduced by combining cisplatin with high-volume fluid hydration (Lebwohl & Canetta 1998; Lokich & Anderson 1998; O'Dwyer et al. 2000). Cisplatin is now an important drug which is used to treat cancers including sarcoma, small cell lung cancer, germ cell tumours, lymphoma and ovarian cancer (O'Dwyer et al 2000; Desoize & Madoulet 2002), and is frequently given as part of a combination chemotherapy regimen with other drugs (Parmar et al. 2003; Stordal et al. 2007; reviewed in Muggia 2009). Subsequently, cisplatin derivatives, carboplatin and oxaliplatin were also developed and showed broad anti-tumour activity and comparatively less nephrotoxicity (reviewed in Rabik & Dolan 2007). In addition, fludarabine phosphate, a purine

analogue that inhibits DNA synthesis by interfering DNA polymerase, has been developed and has become a mainstay in the treatment of patients with chronic lymphocytic leukaemia (Rai et al. 2000).

Toposiomerase inhibitors

The discovery of taxanes (Wani et al. 1971) and camptothecins (Wall et al. 1966) generated interest in natural products as anti-cancer agents. Paclitaxel (Taxol) is the first of a novel class of microtubule stabilizing agents that lead to cell cycle arrest at G2/M followed by apoptosis (Schiff et al. 1979; Horwitz 1992; Jordan et al. 1996; Blagosklonny & Fojo 1999; Wang et al. 2000). In early preclinical studies, paclitaxel showed strong *in vivo* anti-tumour activity in the P1534 leukemia model and human tumour xenografts in nude mice (Fuchs & Johnson 1978; Suffness 1993). This moved Taxol into further preclinical studies and clinical trials, and it showed consistent efficacy in the treatment of patients with ovarian cancer, breast cancer, colon cancer, non-small cell lung cancer (NSCLC), head and neck cancer, and AIDS-related Kaposi's sarcoma (Horwitz 1992; Spencer & Faulds 1994; Rowinsky & Donehower 1995; Alshowaier & Nicholls 1997). Camptothecin, derived from a Chinese ornamental tree, inhibits topoisomerase I that allows DNA unwinding and strand passage (Hsiang et al. 1985). Camptothecin displayed *in vitro* cytotoxicity against human oral epidermoid carcinoma (9KB cell line) and *in vivo* anti-tumour activity (Wall et al. 1976). Despite showing their promise in preclinical studies, both classes of drug encountered significant problems in drug development. Paclitaxel proved difficult to synthesize and could only be obtained from the bark of the Pacific Yew tree. Furthermore, it was virtually insoluble and had to be formulated in a lipid emulsion that caused hypersensitivity reactions in some patients. To resolve these

problems, various approaches were employed including water-soluble prodrugs (Vyas et al.1995; Burt et al. 1995), non-prodrug water-soluble analogues (Uoto et al. 1998) and orally active analogues (Malingre et al. 2001). Some of them have been shown to improve solubility and finally underwent clinical evaluation (Singer et al. 2003; Sparreboom & Verweij 2003). Currently, paclitaxel and its semi-synthetic analog docetaxel are used to treat patients with various cancers and have become one of the most important anti-cancer agents presently used in cancer therapy (reviewed in Ganesh 2007). In the case of camptothecin, the drug had little anti-tumour activity in early clinical trials and was toxic to kidneys due to instability of its lactone ring at neutral pH, causing renal tubular damage. First generation analogues of camptothecin, topotecan (Hycamtin) and irinotecan (Camptosar), which are water-soluble and more stable derivatives of camptothecin, were discovered and passed Food and Drug Administration (FDA) approval for the treatment of colon cancer and are also used to treat lung and ovarian cancers (Saltz 1999; Noda et al. 2002; Bodurka et al. 2003).

Other effective drugs include anthracyclines and epipodophyllotoxins, both of which inhibit the action of topoisomerase II which is crucial for DNA replication, transcription and repair (Minocha & Long 1984; reviewed in Deweese & Osheroff 2009). However, despite rapid and continuous advances in anti-cancer drug development, these drugs are compromised by their lack of specificity with the resulting death and damage to normal tissue. Moreover, cancer cells can rapidly acquire resistance to anti-cancer drugs due to their inherent genetic instability (Gottesman et al. 2002; Luqmani 2005; Broxterman et al. 2009; Liu 2009). Furthermore, there are additional problems related to their acute and long-term toxicities. Although the toxic side effects of conventional anti-cancer drugs can

potentially be managed by lowering the dosage of the drug that is used for treatment, the ability of cancer cells to become resistant to different drugs remains a significant impediment to successful chemotherapy. It has become apparent that resistance develops against every effective drug, even the newest (Gottesman et al. 2002).

Drug resistance

Drug resistance can be either intrinsic to a subpopulation of heterogeneous cancer cells or acquired as a cellular response to drug exposure. In general, acquired drug resistance is the main cause of treatment failure in cancer patients following an initial good response (Kim and Tannock 2005). Multidurg resistanse (MDR) is a well recognised phenomenon of acquired resistance to chemotherapeutic drugs (reviewed in Liu 2009). There are two classes of transporter proteins at the cellular surface which are responsible for MDR genes in cancer cells. One is the ATP-binding cassette (ABC) transporter superfamily which fuctions as an ATP-dependent efflux pump for the drugs. The other is the solute carrier transporter superfamily which mediates the cellular uptake of chemotherapeutic drugs (Huang 2007). Resistance to chemotherapeutic drugs may occur either by increased levels of efflux transporters or by decreased activity of uptake transporters.

P-glycoprotein (P-gp; MDR1/ABCB1), the first identified ABC transporter, is encoded by human *MDR1/ABCB1* gene (Sarkadi 2006). P-gp is expressed in primarily in variety of cell types in the liver, pancreas, kindney, colon and jejunum (Thiebaut et al. 1987). Overexpression of P-gp results in increased drug efflux, thereby lowering the intracellular concentration of the drug and reducing sensitivity to treatment (Gottesman & Pastan 1996). Cancers derived from tissues which normally show low

expression of P-gp can acquire increased levels of the protein following treatment (Kohno et al. 1989), particularly when treatment involves anti-tumor antibiotics (Diddens et al. 1987), topoisomerase inhibitors (Rasheed & Rubin 2003) and microtubule inhibitors (Geney et al. 2002). Resistance to platinum compound is associated with increased expression of the copper efflux transporter, ATP7A (Samimi et al. 2004). Different types of drug resistance mechanism occur with anti-metabolites. Resistance to methotrexate which is an anti-folate compound can occur by a variety of mechanisms, including impaired transport of drug into the cell via the RFC, an increase in DHFR due to gene amplification or increased transcription, and diminished interacellular retention secondary to decreased polyglutamylation (Bertino et al. 1996). In addition to the mechanisms of resistance described above, reduced sensitivity to drug can be occur in variety of ways, including alterations in the activity or level of target enzyme (Pommier et al. 1986; Sullivan et al. 1989), decresed drug activation due to conjugation with increased glutathione (Behrens et al. 1987), enhanced DNA repair (Rixe et al. 1996), and failure to apoptosis pathway as a result of mutated cell cycle protein such as p53 (Selivanova 2001).

1.2. Targeted therapy

Over time understanding of cell biology and the development of new technologies has vastly increased and uncovered new signalling transduction pathways which regulate cellular activities such as cell proliferation and survival. This has led to a refinement of the concept for the ideal cancer target, allowing the establishment of rational drug discovery programmes. Notably, many key factors which are involved in the multiple steps of growth signalling pathways have been found to be frequently altered in cancer cells, and have therefore become the focus of target-based drug discovery

programmes (Sherr 2000; Sausville et al. 2000; Monga & Sausville 2002). The new targets include growth factors, signalling molecules, cell-cycle proteins, modulators of apoptosis and molecules that promote angiogenesis (Hanahan & Weinberg 2000; Yarden & Sliwkowski 2001; reviewed in Goldblatt & Lee 2010). Target-based drugs can be classified into small molecules that have specific molecular weights and structural formulas, and macromolecules that include monoclonal antibodies (reviewed in Saijo et al. 2003; Hait & Hambley 2009).

Antibodies aside, the majority of recently developed target-based drugs are small molecules that specifically inhibit and/or modify cancer-specific biological changes. Innovations in technology and combinatorial chemistry are providing thousands of unique structures for *in vitro* screening, increasing the success rate in the identification of targeted small molecule inhibitors of specific targets. The first example of targeted therapy is the development of imatinib mesylate (also known as Glivec, Gleevec and ST1571) (Druker et al. 1996; Druker & Lydon 2000; Zang et al. 2003). Imatinib is a small molecule inhibitor of the Bcr-Abl fusion kinase in chronic myeloid leukaemia (CML). It also inhibits the KIT tyrosine kinase and platelet derived growth factor receptor-β (PDGFR-β) tyrosine kinase, extending its treatment potential to gastrointestinal stromal tumours and hypereosinophilic syndromes (Chabner & Roberts Jr 2005). However, in the acute leukaemia phase of CML, imatinib showed only brief remissions with treatment leading to a rapid outgrowth of drug-resistant cells displaying mutations in the catalytic kinase domain of Abl (Gorre et al. 2001; Shah & Sawyers 2003; Apperley 2007). Some patients with advanced gastrointestinal stromal tumors acquired resistance through the emergence of imatinib-insensitive c-KIT kinase variants (Sleijfer et al. 2007).

A second example of a targeted inhibitor is gefitinib (Iressa), a small molecular weight synthetic anilinoquinazaline, targeting the ATP binding pocket of the tyrosine kinase domain of the epidermal growth factor receptor (EGFR), which blocks signaling pathways involved in the proliferation and survival of tumour cells (Herbst et al. 2004). Gefitinib showed partial remission in 10-15% of patients with non-small-cell lung cancer (NSCLC), but does not outperform conventional chemotherapies in the number of tumour responses (Herbst et al. 2002; Ranson et al. 2002; Kris et al. 2003; Giaccone 2004). Sequence analysis of the EGFR in primary NSCLC tumors identified mutations within the tyrosine kinase domain that correlated with sensitivity to gefitinib (Lynch et al. 2004; Paez et al 2004). These mutations include in-frame deletions in amino acids 747-750 and point mutations at position 858 (L858R) (Irmer et al. 2007) or position 790 (T790M) (Kobayashi et al. 2005), which may accout for the differential response in patients.

Further targeted therapies have been developed, including Herceptin (trastuzamab). Herceptin is a recombinant monoclonal antibody targeted against human epidermal growth factor receptor-2 (HER-2), which is overexpressed in 20-30% of breast cancers (Slamon et al. 2001; Emens & Davidson 2004; Hudelist et al. 2004; Emens 2005; Neyt et al. 2005). Binding of Herceptin to the extracellular domain of HER-2 results in downregulation of HER-2 by inducing receptor internalisation, inhibition of cell cycle progression and stimulation of antibody-dependent cellular cytotoxicity (reviewed in Harries & Smith 2002). Results from several clinical trials showed that Herceptin is effective as a single agent in previously treated and untreated patients with metastatic breast cancer (Cobleigh et al. 1999; Vogel et al. 2002). Moreover,

combination of chemotherapy and Herceptin revealed a prolonged survival of patients with HER-2 positive metastatic breast cancer compared with chemotherapy alone (Slamon et al. 2001). However, the cardiac dysfunction was seen in some patients who received an anthracycline-cyclophosphamide regimen together with Herceptin, and prospective monitoring of cardiac function is an integral part of current Heceptin combination studies (reviewed in Harries & Smith 2002).

1.3. Development of Cyclin-dependent kinase inhibitors

Although many kinases have emerged as key regulators of most signalling pathways in cancer cells, the cyclin-dependent kinases (CDKs) have attracted special attention as drug targets because of their direct relevance to cell proliferation in cancer development (Malumbres & Barbacid 2001; Shapiro 2006). CDKs are serine/threonine kinases that are activated by association with a regulatory subunit, the cyclin. The activity of CDKs is negatively regulated by direct interaction with CDK inhibitors (CDKIs) which are divided into two major families: the INK4 (inhibitor of CDK4) family, including p16, p15, p18 and p19, which inhibit cyclin D associated kinases; and the Cip/Kip (kinase inhibitor protein) family, including p21, p27 and p57, which inhibit most CDKs (Sherr & Roberts 1999; Grana & Reddy 1995; Morgan 1997; Pines 1995). Accumulated evidence has shown that dysregulation of molecules controlling cell cycle progression plays an important role in tumour pathogenesis. For example, alterations in the pathway involving CDK4, CDK6, cyclin D, INK4, pRb and E2F have been observed in more than 80% of human cancers (Sherr & McComick 2002; Morgan 2007). Furthermore, genetic abnormalities of other CDKs (CDK1, 2 and 7), cyclins (A and E) and CDKIs (p21 and p27) have also been reported in many human cancers (Bardelli et al. 2003; Greenman et al. 2007).

There are several strategies to inhibit CDK activity for therapeutic intervention, and these strategies can be divided into efforts to directly target the catalytic CDK subunit or to manipulate CDK activity indirectly by targeting the regulatory pathways. The direct CDK inhibitors are ATP competitive compounds which are designed to interact specifically with the ATP-binding site of the catalytic domain of CDK (De Azevedo et al. 1997; Meijer & Kim 1997; Senderowiz et al. 2000; Senderowicz & Sauville 2000). Flavopiridol (Flavo), one of the most characterized CDK inhibitors, showed consistent anti-tumour activity in preclinical animal models and subsequently underwent several clinical trials as a single agent and in combination with other chemotherapeutic agents (Senderowicz & Sausville 2000; Innocenti et al. 2000; Stadler et al. 2000; Shapiro et al. 2001; Senderowicz 2003; Schwartz et al. 2005). Flavopiridol inhibits most CDKs; CDK1/cyclin B, CDK2/cyclin A and E, CDK4/cyclin D and CDK6/cyclin D and CDK7/cyclin H (Carlson et al. 1996; Losiewicz et al. 1994; Senderowicz 1999; Worland et al. 1993; Sedlacek 2001). Crystallographic studies have indicated that flavopiridol inhibits the activity of most CDKs by directly occupying the ATP-binding site (Hardcastle et al. 2002). Flavopiridol has been reported to repress the expression of cyclin D1 and D3, leading to loss of CDK4 activity in a breast cancer cell line (Carlson et al. 1999) and also the expression of VEGF (vascular endothelial growth factor) in human monocytes (Melillo et al. 1999). Flavopiridol also inhibits P-TEF (positive transcription elongation factor, also known as CDK9/cyclinT) which is critical for the function of RNA polymerase II, suggesting that transcriptional regulation of cyclin D and VEGF by flavopiridol may be a consequence of P-TEF related process (Chao & Price 2001). The inhibition of CDKs by flavopiridol leads to cell-cycle arrest and apoptosis in a

range of different tumour cell lines (Kaur et al. 1992; Drees et al. 1997; Byrd et al. 1998; Arguello et al. 1998; Parker et al. 1998; Senderowicz 1999; Sedlacek 2001). p53-independent apoptosis was observed in squamous head and neck cell lines with flavopiridol treatment (Patel et al. 1998). In addition, flavopiridol-induced apoptosis in leukaemia cell lines is associated with early activation of the MAPK kinase family (MEK, p38 and JNK), and this activation may lead to activation of caspases (Lahusen et al. 2000). *Kitada et al.* reported that the apoptotic effects of flavopiridol are associated with down-regulation of the anti-apoptosis proteins XIAP, Mcl-1 and BAG-1 in B cell chronic lymphocytic leukaemia (BCLL) cell lines, suggesting that mechanisms other than inhibiting CDK activity may be involved in flavopiridol-induced apoptosis (Kitada et al. 2000).

Despite promising results from preclinical studies, however, their efficacy in clinical trials has been modest due to their lack of selectivity affecting cancer cells, problems related with their pharmacokinetic properties, and appropriate schedule of administration (Schwartz et al. 2001; Shapiro et al. 2001; Aklilu et al. 2003). Flavopiridol showed anti-cancer activity in some patients with renal cell carcinoma, colon carcinoma, non-Hodgkin lymphoma, gastric carcinoma and mantel cell lymphoma in phase I clinical trials (Senderowicz et al. 1998; Tan et al. 2002; Thomas et al. 2002; Senderowicz 2003; Sausville et al. 2003). However, the activity of this drug administered alone in phase II trials in several epithelial tumours has been less than that encountered in preclinical studies (Senderowicz et al. 2000; Shapiro et al. 2001; Schwartz et al. 2001; Aklilu et al. 2003). Therefore, combination with standard chemotherapeutics such as paclitaxel and gemcitabine has been undertaken in preclinical and clinical trials and showed some synergistic effects in patients with

prostate, lung and oesophageal cancers (Schwartz et al. 2002; Cartee et al. 2002; Nahta et al. 2002).

Other CDK inhibitors have been developed, and of these, several have been evaluated for anti-cancer activity in preclinical studies. Purine-based CDK inhibitors showed selectivity for CDKs 1, 2 and 5, but demonstrated no inhibitory activity to CDKs 4 and 6. Some of these inhibitors have been reported to have potent anti-cancer activity, including olomoucine, purvalanols and CGP74514A (reviewed in Knights & Pestell 2009). These inhibitors are widely used in academic laboratories, however, as yet none of these compounds appear to have advanced to clinical trials for cancer therapy. Other CDK inhibitors (e.g. UCN-01, CYC202, BMS-387032) are also reported to show potent and selective inhibition of CDK, and anti-cancer effects in xenograft models (reviewed in Knights & Pestell 2009). Clinical trials of these inhibitors have been conducted with patients in phase I and II trials, but have yet to yield an objective therapeutic response (Sausville et al. 1998, 2001; Fuse et al. 1998). In contrast UCN-01 has been evaluated in combination with cisplatin, topotecan, fluorouracil, carboplatin and irinotecan (Lara et al. 2005; Kortmansky et al. 2005; Welch et al. 2007; Edelman et al. 2007; Jimeno et al. 2008) for the treatment of leukemia and advanced solid tumours, with early clinical trials showing promising results (Deep & Agarwal 2008).

A large number of novel chemotherapeutic strategies have been developed to reverse cancer progression, but there are still many limits in cancer treatment that have yet to be overcome. The drugs presented here represent some of the more successful and highly developed chemotherapeutic strategies at preclinical/clinical stages in drug

discovery and development and in clinical use. Many other chemotherapeutic strategies are currently undergoing investigation and evaluation, and new cancer targets are continually sought.

1.4. The anti-cancer drug discovery and development process

The drug discovery and development process is both complex and expensive, requiring many years and resources from an initial therapeutic concept to a new drug application. The process of drug discovery involves the identification and validation of targets, assay development, HTS (high-throughput screening), hit validation, lead generation/optimization and assessment of therapeutic efficacy. Once a compound has shown its value in this process, it will begin the process of drug development involving preclinical and clinical studies (phase I, phase II, phase III) prior to final registration as a drug (Figure 1.2) (Bleicher et al. 2003).

Three interrelated elements are essential to the success of the targeted drug discovery and development process. First, there must be good reason to believe that a given target, a specific gene or protein against which a drug will be developed, has causal relevance to cancer. The relevant data must evaluate the effects of modulating the activity of a given target in available experimental models. The process of gathering such functional information about the target is called ‘target validation’. Second, the process of finding a new drug against a chosen target usually requires a well optimized biochemical assay which can be adapted to HTS, wherein large libraries of chemicals are tested for their ability to modify the target (Walters & Namchuk 2003). In parallel with the HTS process, *in silico* screening (or virtual high throughput screening), where screening is done using computer-generated models and attempting

to "dock" virtual libraries to a target, is also often used to improve the efficiency of HTS by enhancing the overall quality and minimizing the number of hits-to-leads. Third, once a lead compound has been established with sufficient target potency and selectivity, this lead compound must be clinically assessed in preclinical studies involving *in vitro* and *in vivo* (animal) studies and in clinical trials. Clinical trials, commonly classified into four phases, are used to test the efficacy and safety of a candidate drug. In phase I trials, the drug is administered to a small number (20-80) of healthy volunteers to test for toxicity and side effects and for correct dosage levels. In phase II trials which focus on determining the optimal dosing of the drug, the test is repeated in a large number (100-300) of appropriate cancer patients, followed by a pivotal phase III trial against a comparator product (Roberts et al. 2003; Molzon 2003). If the drug successfully passes through phases I, II, and III, it will usually be approved by the national regulatory authority for use in the general population. Phase IV is ‘post-approval’ studies.

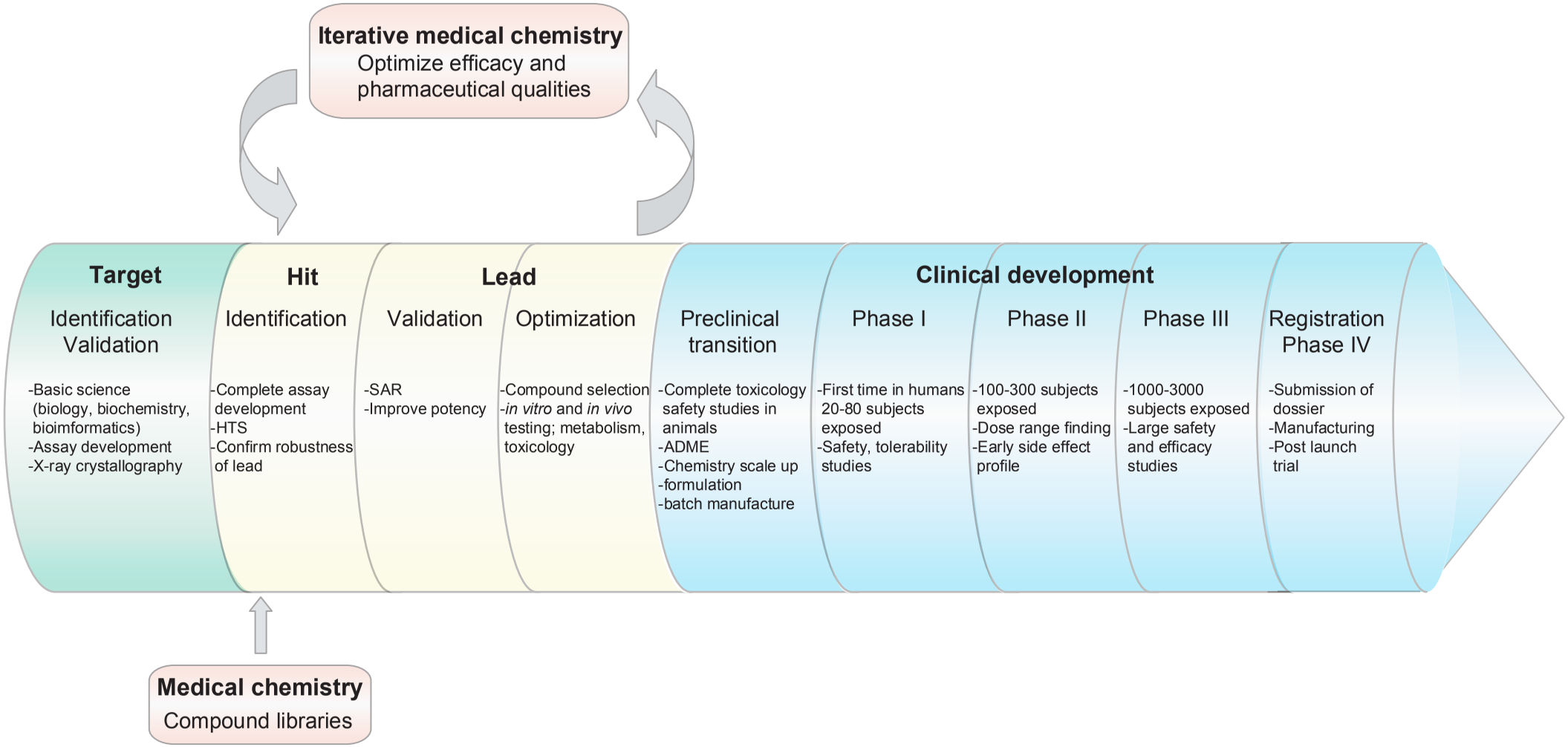


Figure 1.2: Schematic diagram of the drug discovery and development process. The diagram shows how various technologies and key tasks are employed from initial research through to the clinical trials stage. Target identification and validation is undertaken with the use of basic science. Techniques in chemistry such as combinatorial chemistry and chemiinformatics are used to generate a compound library which is tested against the targets using HTS. Promising drug candidates arising from screening in conjunction with computational biology and structure based design are being subjected to pharmaceutical properties (ADME) and toxicology studies during the preclinical stage. Having passed successful preclinical studies, a drug candidate is tested further in clinical trials involving both healthy and cancer patients. (HTS - high through-put screening; SAR - structure activity relationships; ADME - adsorption, distribution, metabolism and excretion) (Bleicher et al. 2003)

1.5. The DNA replication initiation machinery as a potential anti-cancer target

Despite advances in our understanding of cell biology and new technologies, drug discovery is still a long process with a low rate of novel therapeutic agents. The study of the molecular basis of cancer, as well as the availability of the complete sequence information of the human genome, has led to the expectation that the discovery and development of new cancer drugs might produce more predictable and efficient cancer therapies (Benson et al. 2006). Accordingly, a new strategy for cancer therapy has emerged in the form of targeted therapy which is the development of drugs that target the action or activity of a specific signalling pathway in cancer cells (Benson et al 2006). Although various target-based drugs have shown promising efficacy in early drug development programmes, their clinical use has been limited by the associated toxic side effects and the development of drug resistance (Deep & Agarwal 2008; Hait & Hambley 2009).

The initiation of DNA replication is a crucial decision point in cell proliferation that lies at the convergence point of complex and potentially redundant oncogenic signalling and transduction pathways (Williams & Stoeber 1999). Unlike these numerous pathways, there appears to be only a single mechanism of DNA replication initiation, which is tightly controlled through the assembly and activation of the pre-replication complex (pre-RC), and the basic components of this mechanism are conserved in all eukaryotic species studied (reviewed in Sclafani & Holzen 2007; also see 1.5.1.2. DNA replication initiation pathway). Importantly, the position of the

DNA replication initiation pathway at the final step of growth signal transduction pathways may provide a reductionist solution to the major challenge of developing effective therapeutic strategies, given the complexity of addressing multiple mutations that occur at all levels of signal transduction pathways for each tumour type and subtype. Moreover, inhibiting the initiation of DNA synthesis should minimize genomic damage and therefore circumvent the problems of infertility and further neoplastic transformation associated with the use of DNA damaging agents and drugs targeting elongation DNA synthesis. A further criterion which must be considered is the specificity of therapeutic agents targeting the DNA replication initiation pathway, especially since DNA replication is essential to all dividing cells and agents targeting elongation DNA synthesis are known to invoke severe toxic side effects in normal cells. Prior to addressing this issue, I will provide an overview of the DNA replication initiation pathway and our current understanding of a specific kinase - Cdc7 - in this pathway.

1.5.1. The DNA replication initiation pathway in eukaryotic cells

The DNA replication initiation pathway is a complex process involving multi-protein enzymatic reactions and the basic components of this pathway are conserved in all eukaryotes (reviewed in Sclafani & Holzen 2007). Consequently, findings based on genetics in yeast, biochemistry in *Xenopus* eggs and in tissue culture model systems using mammalian cells can be brought together to generate a molecular understanding of the DNA replication initiation pathway (reviewed in ven den Heuvel 2005).

1.5.1.1. Origins of replication

Origins of replication are the places at which DNA replication starts (Jacob 1993). Generally, replication starts at an origin and proceeds bidirectionally to form a single replicon (reviewed in Sclafani & Holzen 2007). Many attempts to identify putative replication origins have relied on assaying the ability of DNA fragments to facilitate the so-called autonomous replication of plasmids in *Saccharomyces cerevisiae* (Stinchcomb et al. 1980). Using 2D-gel analysis, those Autonomously Replicating Sequence (ARS) were shown to be *bona fide* origins in yeast, both in plasmids and in chromosomal DNA (Brewer & Fangman 1987; Huberman et al. 1988). The consensus ARS is approximately 100 bp long and consists of a 17 bp A domain region with an ACS (ARS consensus sequence) and flanked by a less conserved B domain (Newlon 1996). In *Xenopus* egg extracts, replication occurs at regular intervals of approximately 10 kb (Lucas et al. 2000; Blow et al. 2001). Studies of replication origins in mammalian cells have revealed that replication can occur at regions referred to as 'zones' that vary in size from 6 to 55 kb (Dijkwel & Hamlin 1995), and are dependent on chromatin and nuclear structure, sharing little sequence similarity except for the presence of AT-rich elements and DNA unwinding elements (DePamphilis 1999).

1.5.1.2. DNA replication initiation pathway

The mechanism of DNA replication initiation occurs in two steps; the first stage is started by the formation of the pre-replicative complex (pre-RC) which comprises six subunits of the origin recognition complex (ORC), the loading factors Cdc6 and Cdt1, and the putative DNA helicase Mcm2-7 at replication origins. This assembly process occurs during late mitosis and early G1 and results in the origin becoming 'licensed'

for DNA replication. In the second stage, the licensed origins are activated by S phase-specific cyclin-dependent kinases (CDKs) and the Dbf4-dependent kinase (DDK, Cdc7/ASK) (Figure 1.3; reviewed in Sclafani & Holzen 2007).

Pre-RC assembly

In our current understanding of eukaryotic DNA replication, the origin is marked by ORC which provides a "landing-pad" for other replication proteins (Diffely & Cocker 1992; reviewed in Duncker et al. 2009). ORC is a six protein complex comprised of the Orc1-6 subunits that are structural and functional conserved in all eukaryotes (Quintana & Dutta 1999). ORC was first discovered by identification of proteins that bound to the ARS core sequence of the A-domain in *Schizosaccharomyes cerevisiae* origin (Bell & Stillman 1992). Human (Vashee et al. 2003), Xenopus (Gillespie & Blow 2001) and *S. pombe* (Chuang & Kelly 1999; Kong & DePamphilis 2001) ORCs prefer an A-T rich sequence, but no consensus sequence has been identified. Except for Orc6, most ORC subunits are in the superfamily of AAA^+ ATPases (ATPases Associated with various cellular Activities) with conserved Walker A, B, C and D motifs (Koonin 1993; Bell & Dutta 2002). The ATP binding activity of human Orc1, Orc4 and Orc5 subunits is required for replication in the *Xenopus* cell free system (Giordano-Coltart et al. 2005). Orc1 ATPase is activated by Cdc6 binding, which is also an AAA^+ ATPase, inducing a conformational change in the ORC-Cdc6-DNA complex which increases specificity (Speck et al. 2005; Speck & Stillman 2007). This suggests that ORC-Cdc6 might act as a clamp loader for the MCM complex, allowing the MCM complex to encircle double stranded DNA (Neuwald et al. 1999).

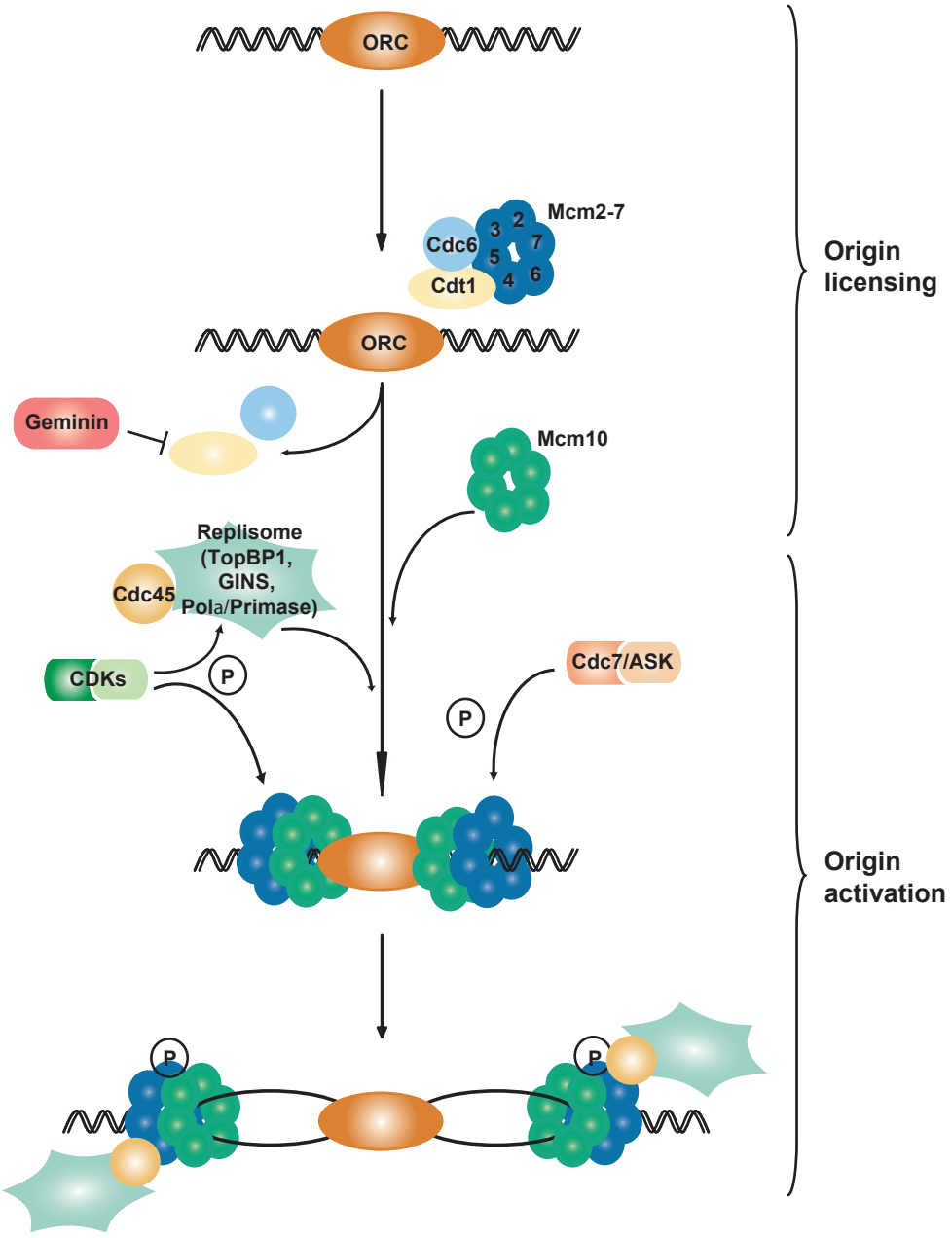


Figure 1.3: The eukaryotic DNA replication initiation pathway. After mitosis, pre-replicative complex (pre-RC) assembly begins with the loading of the origin recognition complex (ORC) onto chromatin at replication origins. ORC recruits Cdc6 and Cdt1, which in turn load the Mcm2-7 complex to origins of DNA replication to form pre-RCs. Once Mcm2-7 complexes are loaded at origins, Cdc6 and Cdt1 are removed from the origin and the origin licensing repressor geminin inhibits pre-RC re-formation by interaction with Cdt1. Cdc7/ASK and CDKs become active and phosphorylate Mcm2-7 proteins triggering a conformational change in the Mcm2-7 complex that is required for unwinding of DNA. In late G1, Mcm10, Cdc45 and the replisome that contains DNA polymerase-a/primase are recuited.

ORC is bound to origins throughout the cell cycle in *S. cerevisiae* (Liang & Stillman 1997), however in mammalian cells, Orc2-Orc6 remain chromatin-bound at all stages of cell cycle, whereas Orc1 is released from chromatin during S phase and restored during the subsequent G1 phase (DePamphilis 2003).

ORC recruits two more proteins, Cdc6 and Cdt1, to the origin. These proteins in conjunction with ORC are required for chromatin loading of the MCM complex, which is the putative DNA replicative helicase (Labib & Diffley 2001; Pacek & Walter 2004). In the presence of ATP, addition of Cdc6 to the ORC complex produces a ring-like structure around DNA that resembles the MCM complex in size and shape (Adachi et al. 1997; Fletcher et al. 2003; Gomez-Llorente et al. 2005; Kubota et al. 2003; Pape et al. 2003). Cdc6 has conserved Walker A and B motifs which are essential for ATP binding and hydrolysis. Mutations in these motifs abrogate Cdc6 functions regarding pre-RC assembly and/or recruitment of the MCM complex (Perkins & Diffley 1998). Cdt1 is also required to load the MCM complex during G1 phase and is negatively regulated by geminin which prevents re-replication to occur at already licensed origins (Maiorano et al. 2000; Nishitani et al. 2000; Wohlschlegel el al. 2000; Tada et al. 2001; Cook et al. 2004; Lee et al. 2004; Ferenbach et al. 2005). As Cdc6 ATPase is needed for Cdt1 binding to the origin *in vitro*, it has been proposed that a Cdt1-MCM complex is loaded onto the ORC-Cdc6-DNA complex during initiation (Randell et al. 2006). Cdc6 and Cdt1 then dissociate and ATP hydrolysis by ORC completes the MCM loading reaction (Randell et al. 2006; Speck et al. 2005; Speck & Stillman 2007).

MCM is a six subunit protein complex comprised of the Mcm2-7 subunits which are conserved in all eukaryotes (Kearsey & Labib 1998; Tye 1999; Tye & Sawyer 2000) and the six subunits generate a heterohexamer (Ishimi 1997; You et al. 1999; Fletcher et al. 2003; Pape et al. 2003). The identification of DNA helicase activity in an assembly of MCM proteins (Ishimi 1997), and the discovery that MCMs move along the DNA as the replication fork progresses (Aparicio et al. 1997; Labib et al. 2000) supported the notion that MCM acts as the replicative helicase at eukaryotic DNA replication forks. Human MCM subunits generate a subassembly including Mcm4-6-7, which was shown to have DNA-dependent ATP hydrolysis as well as weak helicase activity *in vitro*, although no such activities have been detected in the intact Mcm2-7 complex (Ishimi 1997; You et al. 1999; Kelman et al. 1999; Chong et al. 2000; Lee & Hurwitz 2001). Mcm2-7 complexes from *Xenopus* and yeast have ATPase but not DNA-binding or helicase activity (Adachi et al. 1997). Several reports have shown that excess Mcm2-7 complexes are loaded at origins with approximately 40 MCM hexamers per origin in *Xenopus* (Edwards et al. 2002) and 10 in *S. cerevisiae* (Donovan et al. 1997), and are distributed at significant distances away from where ORC is bound (Ritzi et al. 1998; Edwards et al. 2002; Harvey & Newport 2003). Various suggestions have been made for the function of the excess Mcm2-7 complexes, including roles in DNA pumping (Laskey & Madine 2003), checkpoint activation (Cortez et al. 2004; Tsao et al. 2004), transcriptional regulation (Yankulov et al. 1999; DaFonseca et al. 2001; Fitch et al. 2003), and chromatin remodelling (Burke et al. 2001; Dziak et al. 2003). Once the components of the pre-RC have been assembled, the next step is origin activation which involves Mcm2-7 helicase activation and recruitment of additional factors including Mcm10, Cdc45, Sld3, Sld2,

Dpb11 and the GINS complex (Takeda & Dutta 2005; reviewed in Sclafani & Holzen 2007).

In addition to the core pre-RC components, ORC, Cdc6, Cdt1 and Mcm2-7, a further MCM protein, Mcm10/cdc23 (*S. cerevisiae*/*S. pombe*) is also essential for DNA replication (Merchant et al. 1997; Aves et al. 1998; Homesley et al. 2000). Mcm10 is needed for the loading of the Cdc45 protein after pre-RC assembly and for stabilizing the replisome as shown in human cells (Izumi et al. 2000), *Xenopus* egg extract (Wohlschegel et al. 2002) and *S. cerevisiae* (Ricke & Bielinsky 2004; Sawyer et al. 2004). Mcm10 interacts with the Mcm2-7 complex (Merchant et al. 1997; Aves et al. 1998; Homesley et al. 2000), as well as Orc2 and Cdc7/Dbf4, and has been suggested to stimulate Mcm2-7 phosphorylation by Cdc7/Dbf4 (Lee et al. 2003). Mutations in *S. cerevisiae* Mcm10 show defects in completion of S phase after origins have fired, suggesting an addition role in elongation (Merchant et al. 1997; Homesley et al. 2000; Kawasaki et al. 2000; Gregan et al. 2003).

Further initiation factors involved in activation of the replication origin have been identified through genetic screening in *S. cerevisiae*, including Dpb11 (DNA polymerase B possible subunit) (Araki et al. 1995), its counterpart in *S. pombe* Cut5 (Saka & Yanagida 1993; Araki et al. 1995; McFarlane et al. 1997), Sld2 and Sld3 (Kamimura et al. 2001; Masumoto et al. 2002; Noguchi et al. 2002; Nakajima & Masukata 2002) and the GINS complex (composed of Sld5, Psf1, Psf2 and Psf3) (Kanemaki et al. 2003; Kubota et al. 2003; Takayama et al. 2003). Putative homologues of Dpb11 have been identified in higher eukaryotes including Mus101 in *Drosophila* (Yamamoto et al. 2000), TopBP1 in mammals (Makiniemi et al. 2001)

and Cut5/Mus101 in *Xenopus* (Van Hatten et al. 2002; Hashimoto & Takisawa 2003), based on their conservation of repeating BRCT protein motifs and their involvement in both DNA replication and checkpoint activation. Although all these factors are essential for initiation of DNA replication, precise mechanisms of assembly of the individual factors or their functions in DNA replication, and functional homologues of these factors in higher eukaryotes remain to be elucidated.

Origin activation

S phase-specific cyclin-dependent kinases (CDKs, CDK2/cyclin A and E) and Cdc7 kinase/Dbf4 (Dbf4-dependent kinase; DDK) are required for origin activation (Woo & Poon 2003). CDKs and Cdc7 kinase regulate different steps in activation of the replication origin. CDKs may function globally to initiate S phase, whilst Cdc7 has a more specific role in both G1-S transition and S phase progression and has been shown to phosphorylate various members of the Mcm2-7 complex *in vivo* and *in vitro* (Pasero et al. 1999; Murray 2004; Forsburg 2004; Ishimi et al., 2001; Tsuji et al., 2006; Cortez et al. 2004; Yoo et al. 2004; Montagnoli et al. 2006; Charych et al. 2008; Swords et al. 2010). Phosphorylation of the Mcm2-7 complex by Cdc7 results in a conformational change in the structure of the Mcm2-7 complex, which enables it to promote DNA unwinding and leads to the loading of Cdc45 in mammalian cells (Masai et al. 2006), in *Xenopus* egg extracts (Jares & Blow 2000; Walter 2000), *S. cerevisiae* (Sclafani et al. 2002; Zou & Stillman 2000; Sheu & Stillman 2006; Devault et al. 2008), and in *S. pombe* (Yabuuchi et al. 2006). CDK is also required for tight association of Cdc45 with chromatin in *S. cerevisiae* (Zou & Stillman 2000) and *Xenopus* egg extracts (Mimura et al. 2000). CDK's role in promoting origin activation has been recently determined. In the current model, the role of CDKs is to

phosphorylate Sld2 (Masuda et al. 2003) and Sld3 (Tanaka et al. 2007; Zegerman & Diffley 2007), enabling their binding to Dpb11, which then binds to the origin and recruits the replisome with Cdc45. Loading of the GINS complex, Cut5 and Cdc45 requires both CDKs and Cdc7 kinase, suggesting that both CDKs and Cdc7 are involved in two parallel pathways that are required for replication initiation (Yabuuchi et al. 2006). Similar to findings reported in yeast, in human cells the interactions between Cdc45, Mcm2-7, and GINS complex also requires both CDKs and Cdc7 kinase activity for the initiation of DNA replication (Im et al. 2009). The final step in replication initiation is the loading of the replicative polymerases, DNA polymerase α, δ and ε (Waga & Stillman 1998; Kawasaki & Sugino 2001; Hubscher et al. 2002), which occurs after origin unwinding has been stimulated by Cdc45 and RPA binding (Mimura et al. 2000; Walter & Newport 2000).

1.6. Dbf4-dependent kinase Cdc7

As discussed in Section 1.5., the regulation of pre-RC assembly and its activation at replication origins is essential for eukaryotic chromosomal DNA replication, which is central to cell growth, development, and the generation of tissues and organs. Cdc7 is an essential kinase required for origin activation to promote DNA replication. In this section, the main focus will be on the current understanding of Cdc7 regulation in eukaryotic cells, and the roles of this kinase in eukaryotic DNA metabolism.

1.6.1. Cell cycle control of Cdc7 kinase activity

The Cdc7 kinase/Dbf4-dependent kinase (DDK) was originally identified in *S. cerevisiae* as a mutant which, under non-permissive conditions, arrests with a defect

in S phase initiation (Hartwell 1973; Sclafani & Jackson 1994). Kinases related to Cdc7 have been identified in other species including *S. pombe* (Masai et al. 1995), mouse (Kim et al. 1998), *Xenopus* and *H. sapiens* (Jiang et al. 1997; Sato et al. 1997; Hess et al. 1998), suggesting a conservation of regulatory mechanisms of DNA replication by this family of kinases. Cdc7 is activated by binding a regulatory subunit called ASK (Activator for S phase Kinase) in *H. sapiens*, also called Dbf4 in *S. cerevisiae* and *Xenopus,* and Dfp1/Him1 in *S. pombe* (Diffley et al. 1995; Hardy & Pautz 1996; Stillman 1996; Masai & Arai 2002; Sclafani et al. 2000). A second ASK/Dbf4-related molecule, named Drf1, has also been identified in *H. sapiens,* and its possible homologue in *Xenopus* (Montagonoli et al. 2002; Yanow et al. 2003).

Cdc7 kinase activity was reported to be cell-cycle-regulated from yeast to mammals (Jackson et al. 1993; Kumagai et al. 1999; Takeda et al. 1999; Brown & Kelly 1998; Oshiro et al. 1999; Weinreich & Stillman 1999; Jiang et al. 1999; Ferreira et al. 2000; Yamada et al. 2002). Although Cdc7 protein levels are approximately constant throughout the cell cycle, its activity is dependent upon and regulated by interaction with ASK which is regulated in cell-cycle dependent manner in mammalian cells (Kumagai 1999; Jiang et al. 1999; Masai & Arai 2002; Sato et al. 2003). In *S. cerevisiae*, Dbf4 is degraded in late mitosis and early G1 phase by the APC (anaphase-promoting complex) (Weinreich & Stillman 1999; Oshiro et al. 1999; Ferreira et al. 2000).

Biochemical and genetic studies indicate that the Mcm2-7 complex is a target of Cdc7 kinase (Ishimi et al., 2001; Tsuji et al., 2006; Montagnoli et al. 2006; Sheu & Stillman, 2006; Masai et al., 2006; Charych et al. 2008). Cdc7 kinase directly phosphorylates

Mcm2 (Lei et al. 1997; Kumagai et al. 1999; Jiang et al. 1999; Sclafani 2000; Masai & Arai 2002; Tsuji et al. 2006; Cho et al. 2006; Charych et al. 2008), Mcm4 (Masai et al. 2006; Devault et al. 2008; Sheu & Stillman 2010) and Mcm6 (Masai et al. 2006; Sheu & Stillman 2006), activating the Mcm2-7 helicase and stimulating association of Cdc45 with the Mcm2-7 complex.

S phase-specific cyclin-dependent kinases (CDKs, CDK2/cyclin A and E) and Cdc7 are regulated independently of each other during the cell cycle but by similar mechanisms, i.e. both kinases require their regulatory subunits, cyclin and ASK/Dbf4, for kinase activity (Masai & Arai 2002; Morgan 2007; Sclafani 2000). Although the requirement of CDKs and Cdc7 kinase/DDK for DNA replication initiation is conserved in eukaryotes, the order of execution points is controversial and may differ among species. CDKs function has been shown to be required before Cdc7 kinase in *S. cerevisiae* (Nougarede et al. 2000), whereas Cdc7 kinase/DDK must execute its function before CDKs for replication in *Xenopus* egg extracts and *S. pombe* (Walter 2000; Yabuuchi et al. 2006).

1.6.2. Role of Cdc7 kinase complex

Characterisation of *cdc7* mutants indicated that Cdc7 kinase is involved in several aspects of DNA metabolism (Sclafani & Jackson 1994). Cdc7 kinase is required for the initiation of DNA replication, and involved in checkpoint pathways (reviewed in Sawa & Masai 2008; Swords et al. 2010). A recent study in *Xenopus* egg extracts showed that Cdc7 kinase is also required for chromosomal segregation in mitosis through loading of Scc2-Scc4 and cohesin onto chromatin (Takahashi et al. 2008). In the meiotic cell cycle, Cdc7 kinase promotes meiosis I segregation by the initiation of

DNA recombination and the recruitment of monopolin to kinetochores for monoorientation through phosphorylating meiosis-specific proteins (Wan et al. 2006, 2008; Sasanuma et al. 2008; Matos et al. 2008; Marston 2008; Katis et al. 2010).

The mechanisms that ensure precise DNA replication during each cell cycle are not clearly understood, but are in part linked to cell cycle checkpoint pathways (Zhou & Elledge 2000). At least two types of aberrant events are monitored during S phase, failure of ongoing replication forks and DNA damage which immediately lead to activation of S phase checkpoints (Jares et al. 2000; reviewed in Segurado & Tercero 2009) (Figure 1.4). Several protein kinases involved in checkpoint pathways include ATM, ATR (*Xenopus* and mammalian), Mec1 (*S. cerevisiae*)/Rad3 (*S. pombe*) and Rad53 (*S. cerevisiae*)/Cds1, Chk1 (*S. pombe*) (reviewed in Niida & Nakanishi 2006; Segurado & Tercero 2009). As a consequence of checkpoint activation in S phase, further replication origin initiation is suppressed (intra S checkpoint) and progression into mitosis is blocked (G2/M checkpoint). Checkpoint pathways are composed of three phases; the sensor phase that is involved in detecting the presence of damaged chromosomes, the mediator phase that is involved in activating checkpoint kinases, and the effector phase which is involved in executing the checkpoint responses (Niida & Nakanishi 2006). Beyond the role of Cdc7 kinase in the initiation of DNA replication, there is evidence that Cdc7 kinase plays important roles in the checkpoint pathways (reviewed in Sclafani 2000; Duncker & Brown 2003; Sawa & Masai 2008; Swords et al. 2010).

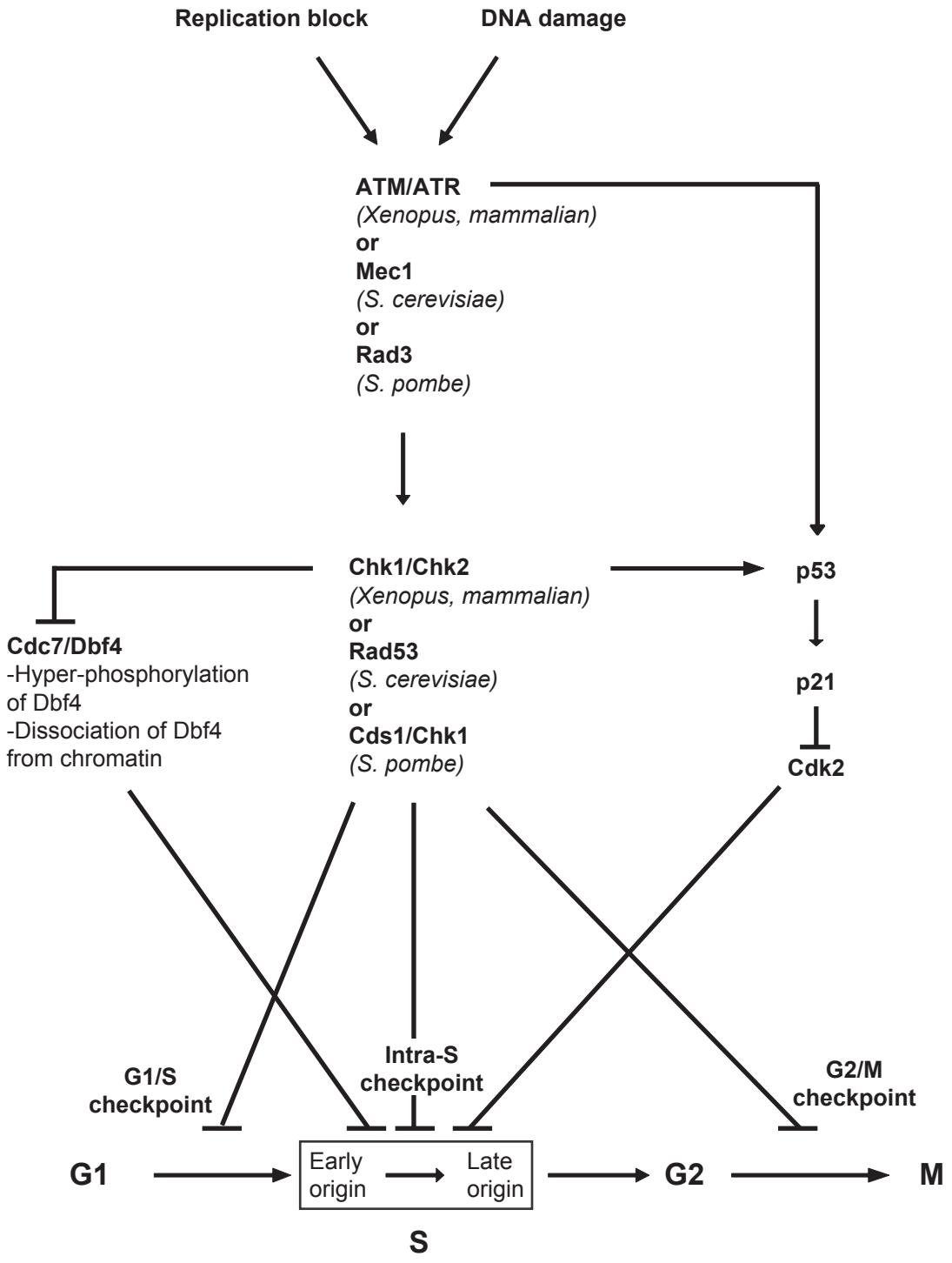


Figure 1.4: Schematic overview of checkpoint pathways and role of Cdc7/Dbf4 kinase in the intra-S checkpoint. Incomplete replication and DNA damage lead to activation of the checkpoint kinases; ATM/ATR or Mec1 or Rad3. Depending on where the cell happens to be in the cell cycle, this can cause cell cycle arrest via either the 'G1/S', the ' Intra-S' or the 'G2/M' checkpoint. As a consequence of Rad53/Cds1 activation, Cdc7/Dbf4 becomes hyper-phosphorylated and is displaced from chromatin which blocks further origins from initiating (reviewed in Jares et al. 2000). Activation of p53 induces p21 which blocks re-replication by inhibiting Cdk2, which is required for new origin firing.

Cdc7 kinase interacts with and phosphorylates Claspin, a checkpoint mediator protein, and is required for the activation of the ATR-Chk1 checkpoint pathway in response to stalled replication forks in mammalian cells (Kim et al. 2008). Similarly, Hsk1 kinase (the fission yeast Cdc7 homologue) phosphorylates Mrc1, the yeast equivalent of Claspin, in response to replication fork arrest, and this phosphorylated Mrc1 is required for the activation of Cds1 which is a yeast checkpoint effector kinase (Takeda et al. 1999). It has also been reported in budding yeast that Cdc7 kinase is required for continuous activation of the Rad53 checkpoint kinase in response to hydroxyurea (Ogi et al. 2008), suggesting that Cdc7 kinase plays a important role in activating checkpoint pathways through regulation of checkpoint mediator proteins. On the other hand, the role of Cdc7 in the effector phase of checkpoint pathways was suggested from different studies. In yeast models, Dbf4 (*S. cerevisiae*)/Dfp1 (*S. pombe*) is phosphorylated in a Rad53 (*S. cerevisiae*)/Cds1 (*S. pombe*) dependent manner when cells are treated with hydroxyurea (Brown & Kelly 1998; Weinreich & Stillman 1999; Kihara et al. 2000), and this phosphorylation reduces Cdc7 kinase activity by dissociation of Dbf4 from chromatin (Weinreich & Stillman 1999).

Cdc7 kinase is also inactivated in *Xenopus* egg extracts through the dissociation of the Cdc7/Dbf4 complex in response to etoposide, which activates the ATR-dependent checkpoint pathway (Costanzo et al. 2003), and the subsequent failure of chromatin binding of Cdc45 (Jares & Blow 2000). Furthermore, Cdc7 kinase is shown to be downregulated by etoposide in Bcr-Abl negative, ATR-proficient human leukemia cells (Dierov et al. 2004), suggesting that Cdc7 kinase is an essential target which is inactivated by checkpoint pathways to inhibit the initiation of DNA replication. However, on the contrary, several studies demonstrated that Cdc7 kinase activity is

not affected by replication fork arrest in *Xenopus* and human cells, suggesting the possibility that Cdc7 kinase in higher eukaryotes is not an essential target of the S phase checkpoint (Takahashi & Walter 2005; Liu et al. 2006; Silva et al. 2006; Heffernan et al. 2007; Tenca et al. 2007; Tsuji et al. 2008). A recent study showed that addition of purified Cdc7 kinase complex to *Xenopus* egg extracts or overexpression of ASK in cancer cells downregulates ATR-Chk1 checkpoint pathway in response to DNA damaging agents, suggesting that Cdc7 kinase is involved in regulating the S phase checkpoint by abrogating checkpoint pathway and activating reinitiation of DNA replication during the checkpoint recovery, although the target(s) of Cdc7 kinase in this mechanism were not revealed (Tsuji et al. 2008). Notably, downregulation of Cdc7 by small interfering RNA causes defective S phase progression leading to p53-independent apoptotic cell death without a significant checkpoint response in a variety of cancer cell lines (Montagnoli et al. 2004; Tenca et al. 2007), suggesting that loss of Cdc7 kinase in cancer cells leads to S phase arrest which results in DNA damage and cell death in the case of defective checkpoint activation (reviewed in Swords et al. 2010).

Takahashi et al. have reported the important role of Cdc7 kinase for chromosomal segregation in mitosis through the chromatin association of cohesion (Takahashi et al. 2008). A protein complex called cohesion is required to establish and maintain sister-chromatid cohesion which is essential for accurate segregation of chromosomes after DNA replication (Bailis et al. 2003; Nasmyth 2005). Cohesion is established by ring-shaped structures that consists of a four subunit complex; two structural maintenance of chromosome (SMC) proteins, Smc1 and Smc3, Rad21/Scc1, an α-kleisin subunit and SA/Scc3 (Losada 2007). Cohesion is loaded onto chromosomes to link replicated

sister chromatids, and a complex composed of Scc2 and Scc4 is required for this cohesion loading (Ciok et al. 2000; Gillespie & Hirano 2004; Seitan et al. 2006). A recent study in *Xenopus* egg extracts showed that Cdc7 kinase interacts with the Scc2/Scc4 complex, and it's kinase activity is required for chromatin association of Scc2/Scc4 complex, which promotes the chromosomal recruitment of cohesion (Takahashi et al. 2008). It has also been suggested that binding of Scc2/Scc4 complex to Cdc7 kinase allows the use of Mcm2-7 as a platform enabling cohesion assembly onto chromatin prior to the initiation of DNA replication (Takahashi et al. 2008; reviewed in Swords et al. 2010).

In addition to the important role of Cdc7 kinase in mitotic cell cycle, Cdc7 kinase has been identified as a key regulator in meiosis (reviewed in Marston 2009; Swords et al. 2010). Meiosis is a specialized cell cycle that generates haploid gametes from diploid cells through having one round of DNA replication followed by two consecutive chromosome segregations, meiosis I and II (reviewed in Morgan 2007; Marston 2009). The segregation of homologous chromosomes during Meiosis I requires three key modifications; meiotic recombination and formation of chiasmata between homologous chromosomes, generation of sister kinetochore monoorientation, and protection of centromeric cohesion (Petronczki et al. 2003; reviewed in Marston 2009). Several studies in budding yeast showed that inactivation of Cdc7 kinase in meiosis results in a delay in DNA replication and a prophase arrest with no meiotic recombination (Schild & Byers 1978; Valentin et al. 2005; Wan et al. 2006). Analysis using a temperature-sensitive (*ts*) mutant of *hsk1*also suggested that Hsk1 is essential for meiotic double strand break (DSB) formation during meiosis progression in fission yeast (Ogino et al. 2006). It was suggested that the initiation of meiotic recombination

is regulated by Cdc7 kinase activation together with Cdc28/Clb5 (S phase Cyclin-dependent kinase) via phosphorylation of Mer2 protein, one of the Spo11 endonuclease accessory factors that promotes the formation of DSB (Murakami & Keeney 2008; Wan et al. 2008; Sasanuma et al. 2008). Furthermore, the requirement of Cdc7 kinase for sister kinetochore monoorientaion during meiosis I analyzed in *bob1 cdc7Δ* mutants showed that homolog segregation fails during meiosis I because cohesion at centromeres holds bioriented sister chromatids together (Lo et al. 2008; Matos et al. 2008). Cdc7 kinase regulates monopolin complex localization to kinetochores together with Cdc5/Spo13 kinase through phosphorylation of the monopolin subunit Lrs4, which is essential for monopolar attachment (Matos et al. 2008). A recent study showed that Cdc7 kinase and casein kinase 1δ/ε (CK1δ/ε) are required for cohesion's α-kleisin subunit (Rec8) cleavage and meiosis I nuclear division (Katis et al. 2010). The role of Cdc7 kinase in chromosomal segregation in mitosis and homolog segregation in meiosis has mainly been studied in *Xenopus* and yeast, and thus, it remains to be determined whether Cdc7 kinase plays a similar role in human cells.

1.7. Inhibition of the DNA replication initiation pathway

Cell cycle specific drugs targeting DNA synthesis have already been used in modern chemotherapy. However, these drugs often cause genomic damage which activates the ATR/ATM dependent S-phase checkpoint pathway and mediates cellular responses involving the activation of DNA repair, cell cycle control and survival (Zhou & Elledge 2000). Unlike the cellular responses to inhibition of DNA elongation, the inhibition of the DNA replication initiation should minimize collateral damage to

DNA. Furthermore, uncontrolled cell growth in cancer cells and dysregulation of the replication initiation pathway in tumourigenesis (Williams et al. 1998; Hess et al. 1998; Freeman et al. 1999; Stoeber et al. 1999; Going et al. 2002; Stoeber et al. 2001; Stoeber et al. 2002; Karakaidos et al. 2004; Korkolopoulou et al. 2005; Bonte et al. 2008; Clarke et al. 2009) suggests that agents targeting the DNA replication initiation pathway may have high efficacy as anti-cancer agents (reviewed in Blow & Gillespie 2008).

As discussed in section 1.5.1, the initiation of the DNA replication process involves two steps, pre-RC assembly and origin activation (reviewed in Sclafani & Holzen 2007). In order to inhibit the replication initiation pathway for therapeutic intervention, several studies suggest that there are therapeutic opportunities to be gained by targeting either pre-RC assembly or origin activation (Shreeram et al. 2002; Feng et al. 2003; Montagnoli et al. 2004; Machida et al. 2005). The potential exploitation of the DNA replication initiation pathway by inhibiting pre-RC assembly as an anti-cancer target was initially shown in a study by *Shreeram et al.,* which suggested that inhibition of the licensing system may provoke a differential response in normal and transformed cells and cause cancer cell-specific killing (Shreeram et al. 2002) (Figure 1.5). Inhibition of pre-RC assembly by overexpressing a non-degradable form of geminin in U2OS (p53+/Rb+) and Saos2 (p53-/Rb-) cells resulted in progression into S or G2 phase after which cells undergo apoptosis. In contrast, ectopic expression of geminin in IMR-90 primary fibroblasts resulted in G1 arrest and did not induce cell death, suggesting that primary cells could respond to the inhibition of replication initiation by blocking S phase progression (Shreeram et al. 2002; Shreeam & Blow 2003; reviewed in Blow & Gillespie 2008).

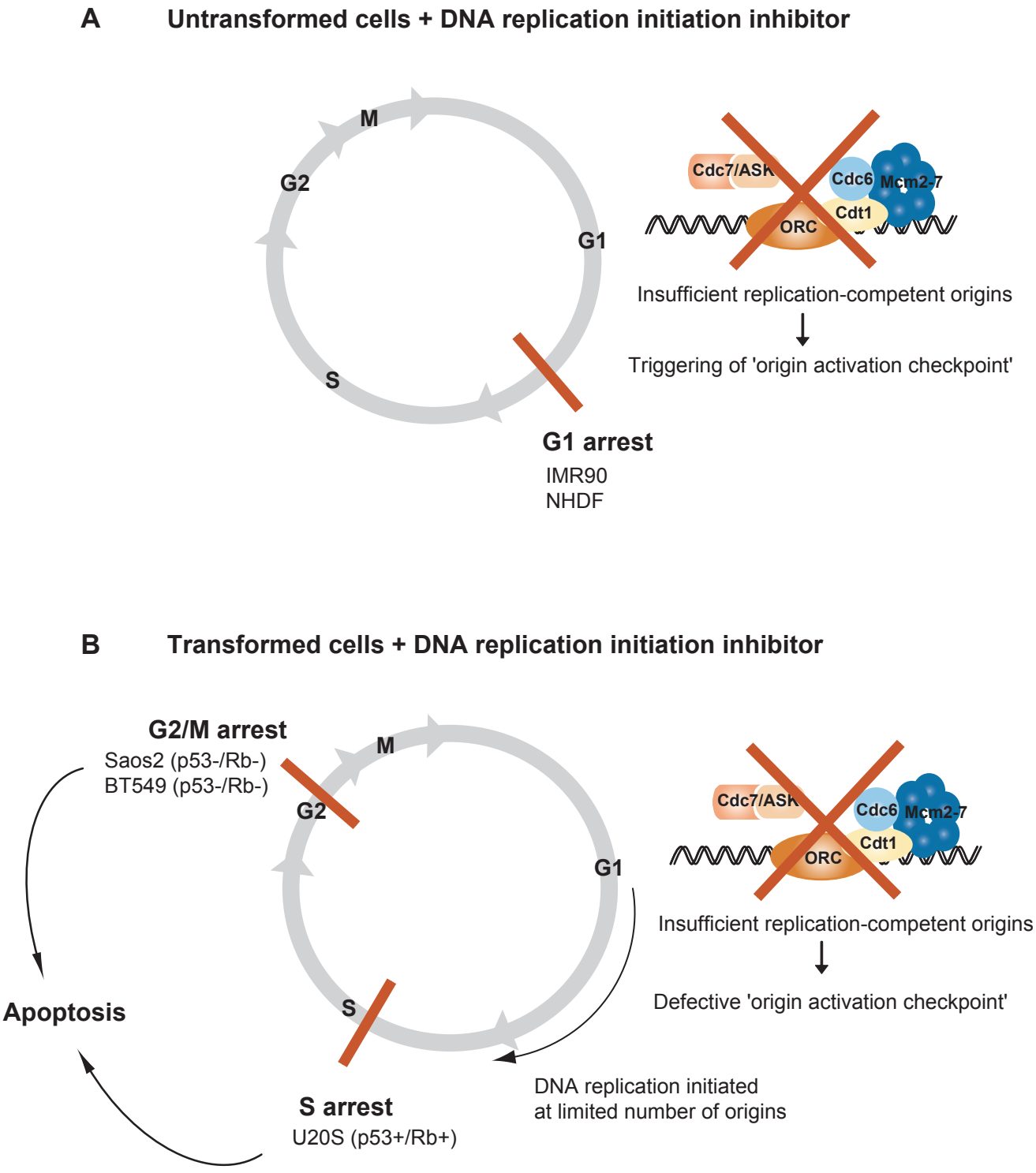


Figure 1.5: Differential response of cells to DNA replication initiation inhibitor. DNA replication begins at replication origins in early G1 with the formation of pre-RCs, which involves sequential assembly of ORC, Cdc6, Cdt1 and Mcm2-7 complexes, as a prerequisite for origin firing and DNA replication initiation. (A) When replication initiation is blocked in untransformed cells, the initiation of DNA replication is impeded by activation of a putative 'origin activation checkpoint' and cells arrest in G1 phase. (B) Transformed cells do not arrest in G1 due to a defective 'origin activation checkpoint'. Instead DNA replication is initiated from a few licensed origins, ultimately resulting in DNA damage and apoptosis (Shreeram et al. 2002 and Montagnoli et al. 2004).

Secondly, a similar difference between cancer and normal cells was also seen by inhibiting origin activation. *Montagnoli et al*. showed that inhibition of Cdc7 kinase by small interfering RNA in cancer cell lines caused an abortive S phase, leading to cell death by either p53-independent apoptosis or aberrant mitosis without eliciting a significant checkpoint response. However, in primary fibroblasts, a putative p53-dependent pathway actively prevents progression through a lethal S phase in the absence of Cdc7 kinase (Montagnoli et al. 2004).

The mechanisms of cancer cell specific death by inhibiting the replication initiation pathway are not clearly understood. In primary fibroblasts, inhibition of the replication initiation pathway which targets either pre-RC assembly or origin activation would cause replication fork arrest, but cells have a putative 'origin activation checkpoint' in G1 phase that monitors insufficient replication-competence and prevents lethal progression into S phase. By contrast, cancer cells with a deficient origin activation checkpoint response induce apoptosis after an abortive S phase (reviewed in Blow & Gillespie 2008; Swords et al. 2010). Because cancer cell-specific killing appears to be a unique feature of the inhibition of replication initiation, small molecule inhibitors of the replication initiation pathway would be expected to induce cancer specific cell death. Since protein kinases are quite selective in their actions, they have become exploitable targets for small molecule inhibitors that directly interfere with catalytic activity through ATP-competitive ligands in the ATP-binding site of the protein kinase (reviewed in Smyth & Collins 2009). As a member of the family of serine/threonine kinases, Cdc7 kinase contains conserved catalytic domains forming the ATP-binding site, which is a prime target for the design and development of selective inhibitors.

Furthermore, the available structural information for CDK2, including crystal structure data with incorporated small molecule inhibitors (Fabbro et al. 2002), may provide a good starting point for the development of small molecule inhibitors of Cdc7 kinase. However, as shown recently, Cdc7 kinase is involved in several aspects of DNA metabolism, including DNA replication initiation, chromosomal segregation in mitosis and meiosis, and DNA damage response, suggesting that there is a possibility that Cdc7 inhibitors may affect somatic and germ line cell cycle progression and could cause toxic side effect to normal cells. Moreover, an important question which remains to be addressed is whether the G1 arrest observed in primary cells following inhibition of replication initiation is reversible. If primary cells were able to restart cell proliferation following removal of a replication initiation inhibitor, cytotoxicity would be significantly reduced. Our group has recently shown that G1 arrest following Cdc7 knockdown in primary cells is rapidly reversible, even after holding cells in a G1 arrested state for days, with recovery and cell cycle progression occurring within hours (Rodriguez-Acebes et al, in press). These data suggest that normal cells are likely to arrest temporarily in G1 phase in response to Cdc7 small molecule inhibitor and that the arrest is reversible when the inhibitor is removed, which may reduce toxic side effects to normal cells. Taken together, these findings support the hypothesis that inhibitors of the replication initiation pathway could potentially provide effective anti-cancer agents for the treatment of a wide range of cancers. The importance of Cdc7 kinase for the viability of cancer cells and the reversibility of cell proliferation following removal of Cdc7 inhibition in normal cells suggest that Cdc7 kinase is a novel target for anti-cancer drug development programmes.

1.8. Summary and aims of thesis

DNA replication is central to cell division, and understanding its fundamental mechanisms not only advances our understanding of cell proliferation, but also of more complicated processes such as development, differentiation and self-renewal of tissues (reviewed in Sclafani & Holzen 2007). The DNA replication initiation pathway has recently emerged as a potential target for a novel therapeutic anti-cancer regime. Among the factors involved in DNA replication initiation, Cdc7 has emerged as an exciting anti-cancer target because it is an essential kinase required for origin activation. Modern enzymatic assay screening platforms provide a powerful approach for the development of novel inhibitors. In the first part of this thesis, described in Chapter Three, I have focused on the generation of molecular tools, including affinity-purified antibodies and functional Cdc7/ASK recombinant proteins that are essential for an investigation of the role of this kinase in the cell cycle dependent regulation of DNA replication in human cells. In Chapter Four, I describe a detailed expression analysis of the replication initiation machinery and characterize the regulation of Cdc7/ASK kinase activity in *in vitro* tissue culture model systems of cycling cells and in the out-of-cycle states of quiescence and differentiation. These studies were extrapolated into normal and cancerous human tissues to validate Cdc7/ASK kinase as a potential therapeutic target and to predict how normal human tissues may respond to an inhibitor targeting DNA replication initiation. In Chapter Five, I describe the development of a robust and sensitive assay for Cdc7 kinase activity and the generation of a homology model of the Cdc7 active site to identify specific small molecule inhibitors. Finally in Chapter Six, I discuss how this assay has been transferred into a 384-well format for automated HTS. Using HTS, Cdc7/ASK kinase has been screened against compound libraries for hit identification, which has been

taken further to hit selection/validation and a hit-to-lead development programme. The work presented in this thesis provides a strong rationale for targeting the DNA replication initiation pathway as a new therapeutic approach to cancer treatment.

CHAPTER TWO

Materials and methods

2.1 General

2.1.1. Chemicals and reagents

Unless otherwise indicated all chemicals were obtained from Sigma (St. Louis, MO, USA). All solutions were made using deionised water, except where stated, and sterilised either by autoclaving at 121°C or passage through a sterile filter (0.22 μm). All enzymes and buffers for DNA manipulation were obtained from New England Biolabs Inc. (Beverly, MA, USA) or the Promega Corporation (Madison, WI, USA).

2.1.2. Buffers and solutions

4 X LAEMLLI SAMPLE BUFFER

Tris-Cl pH 6.8	33 mM
SDS	3.3% (w/v)
Glycerol	30% (v/v)
β-mercaptoethanol	17% (v/v)
Bromophenol blue	0.02% (w/v)

5 X TRIS-GLYCINE ELECTROPHORESIS BUFFER

Tris-base	125 mM
Glycine	1.25 M
SDS	0.5% (w/v)

COOMASSIE BLUE R-250 STAINING SOLUTION

Methanol	50% (v/v)
Glacial acetic acid	10% (v/v)
Coomassie Blue R-250	0.25% (w/v)

COOMASSIE GEL DESTAIN SOLUTION

Methanol	10% (v/v)
Glacial acetic acid	10% (v/v)

TRANSFER BUFFER

Tris-base	0.3 M
CAPS	10 mM
SDS	0.02% (w/v)
Methanol	10% (v/v)

GEL LOADING BUFFER

Glycerol	10% (v/v)
Bromophenol blue	0.025% (w/v)

TE BUFFER

Tris-HCl pH 8.0	10 mM
EDTA pH 8.0	1 mM

DNA EXTRACTION BUFFER

Na2Co3	100 mM
NaCl	170 mM
EDTA pH 10.9	10 mM

RIPA BUFFER

Tris-Cl pH 7.4	50 mM
NaCl	150 mM
NP40	1% (v/v)
Sodium deoxycholate	1% (v/v)
SDS	0.1% (w/v)
EDTA	1 mM

IMMUNOPRECIPITATION BUFFER

Tris-Cl pH 8.0	50 mM
NaCl	150 mM

EDTA	5 mM
NP40	0.5% (v/v)

LYSIS BUFFER FOR BACULOVIRAL-EXPRESSED RECOMBINANT PROTEIN

Tris-Cl pH 9.0	25 mM
KCl	250 mM
NP40	0.5% (v/v)
Glycerol	10% (v/v)
$MgCl_2$	10 mM

WASH BUFFER FOR BACULOVIRAL-EXPRESSED RECOMBINANT PROTEIN

Tris-Cl pH 9.0	25 mM
KCl	250 mM
NP40	0.5% (v/v)
Glycerol	10% (v/v)
$MgCl_2$	10 mM
Imidazole	5 mM

ELUTION BUFFER FOR BACULOVIRAL-EXPRESSED RECOMBINANT PROTEIN

Tris-Cl pH 9.0	25 mM
KCl	250 mM
NP40	0.5% (v/v)
Glycerol	10% (v/v)
$MgCl_2$	10 mM
Imidazole	250 mM

LYSIS BUFFER FOR BACTERIAL-EXPRESSED RECOMBINANT PROTEIN

Tris-Cl pH 7.5	25 mM
NaCl	250 mM
Glycerol	10% (v/v)

DENATURE BUFFER FOR BACTERIAL-EXPRESSED ASK FRAGMENT

Tris-Cl pH 7.5	25 mM

KCl	500 mM
Triton X-100	2% (v/v)
Urea	6M

RENATURE BUFFER FOR BACTERIAL-EXPRESSED ASK FRAGMENT

Tris-Cl pH 7.5	25 mM
NaCl	250 mM
Glycerol	10% (v/v)
Imidazole	20 mM

WASH BUFFER FOR BACTERIAL-EXPRESSED RECOMBINANT PROTEIN

Tris-Cl pH 7.5	25 mM
NaCl	250 mM
Glycerol	10% (v/v)
Imidazole	5 mM

ELUTION BUFFER FOR BACTERIAL-EXPRESSED RECOMBINANT PROTEIN

Tris-Cl pH 7.5	25 mM
NaCl	250 mM
Glycerol	10% (v/v)
Imidazole	250 mM

IN VITRO KINASE ASSAY BUFFER

Tris-Cl pH 7.5	50 mM
$MgCl_2$	10 mM
Dithiothreitol	1 mM
ATP	0.1 mM

ANTIBODY-BASED KINASE REACTION BUFFER FOR 96-WELL FORMAT

Tris-Cl pH 7.5	50 mM
$MgCl_2$	10 mM
Dithiothreitol	1 mM
ATP	0.1 mM

KINASE REACTION BUFFER FOR HTS

Tris-Cl pH 8.5	50 mM
$MgCl_2$	10 mM
Dithiothreitol	1 mM

WASH BUFFER FOR HTS

Tris-Cl pH 8	25 mM
NaCl	150 mM
Tween 20	0.05% (v/v)

DEVELOPMENT BUFFER FOR HTS

Diethanolamine pH 9.8	1M
$MgCl_2$	0.5 mM
PNPP (p-Nitrophenyl Phosphate)	4.5 mM

2.1.3. Culture media

All growth media were sterilised by autoclaving at 121ºC for 20 min, and media recipes are for 1 litre.

LB BROTH [AGAR]

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
[Bacteriological (No1) agar]	15 g
pH 7.0	

2 X TY BROTH [AGAR]

Tryptone	16 g
Yeast extract	10 g
NaCl	5 g

[Bacteriological (No1) agar]	15 g
pH 7.0	

NZY

N-Z-amine AS	10 g
Yeast extract	5 g
NaCl	5 g

SOC MEDIUM

Tryptone	2% (w/v)
Yeast extract	0.5% (w/v)
NaCl	10 mM
KCl	2.5 mM
$MgCl_2.6H_20$	10 mM
$MgSO_4$	10 mM
Glucose	20 mM
pH 7.4	

2.2. Molecular biology techniques

2.2.1. Bacterial strains

Genetic manipulations and expression of recombinant proteins were carried out in the following strains of *Escherichia coli* (Table 2.1.):

Table 2.1. Bacterial strains

Bacterial Strain	Antibiotic resistance	Partial genotype	Application	Supplier
Top10	-	F-*recA1 endA1*	DNA cloning	Invitrogen
XL1-Blue	Tetracycline	F'*proAB lac1*^{q} ZΔM15 Tn10 (Tet^{R})	Site-directed mutagenesis	Stratagene
Rosetta (DE3) pLysS	Chloramphenicol	F-*ompTdcm lacY1* (DE3) pLysS pRARE (Cam^{R})	Expression of His-*hs*Mcm2 (1-154)	Stratagene
Rosetta2 (DE3) pLysS	Chloramphenicol	F^{-}-*ompT hsdS*$_{B}$(r_{B}^{-} m_{B}^{-}) *gal dcm* (DE3) pLysSRARE2 (Cam^{R})	Expression of His-ASK (271-674)	Novagen
DH10Bac	Ampicillin	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K λ– *rps*L *nup*G/ pMON14272/pMON7124	Generation of Bacmid	Invitrogen

2.2.2. Transformation of E. coli

50 µl aliquots of chemically competent cells were thawed on ice. Purified plasmid DNA (1 – 100 ng) was added, mixed gently and incubated on ice for 5 min. Cells were heat-shocked in a pre-heated water bath at 42°C for 1 min and chilled on ice for 2 min. Cells were resuspended in 250 µl of SOC medium, except for XL1-Blue cells where NZY+ broth was used, allowed to recover at 37°C for 1 h with gentle shaking and spread over LB agar plates containing the appropriate selection marker(s). Plates were incubated overnight at 37°C.

2.2.3. Plasmid isolation

3-5 ml of LB medium was inoculated with a single bacterial colony and cultures grown overnight at 37°C with shaking at 200 r.p.m. Plasmid DNA was isolated using the QIAprep spin miniprep kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol and stored in TE buffer at -20°C.

2.2.4. Restriction endonuclease digestion

Digestion of DNA was carried out according to the manufacturer's protocol for the particular restriction endonuclease enzyme. Typically, 50 to 500 ng of DNA was digested in a reaction volume of 20 µl containing 10 U of restriction endonuclease for 1 h at 37°C.

2.2.5. Dephosphorylation of linearised vector DNA

Linearised vector DNA was incubated with calf intestinal phosphatase (0.5 U/µg vector DNA) for 30 min at 37°C for dephosphorylation of blunt, 3'- or 5'-overhanging ends.

2.2.6. Ligation of DNA

Ligation of insert DNA into plasmid was performed using T4 DNA ligase (Promega, Madison, WI, USA). Reactions containing a 3:1 molar excess of insert to vector, 10 U of T4 DNA ligase and 2 x rapid ligation buffer (Promega) in a final volume of 20 µl were incubated for 2 h at RT. 5 µl of the reaction mix was transformed into chemically competent *E. coli.*

2.2.7. Polymerase chain reaction

For cloning PCR products, the 3'-5' exonuclease proof-reading *Pfu*-ultra DNA polymerase (Stratagene, La Jolla, CA, USA) was used to amplify the template. 1-10 ng of template DNA was mixed in a final reaction volume of 50 µl containing 180 ng/µl of each oligonucleotide primer, 200 µM dNTPs, 10% 10x DNA polymerase buffer and 2.5 U DNA polymerase (2.5 U/µl). Thermocycling was initiated by

denaturation of the template at 95°C for 2 min, then 30 cycles of template denaturation at 95°C for 1 min, primer annealing at 55°C for 2 min and DNA extension at 72°C for 3 min. Cycling was completed by a final extension at 72°C for 7 min. Blunt-end PCR products were cloned into a pGEM-T Easy vector (Invitrogen, Carlsbad, CA, USA), amplified, and subcloned into a pFastBac baculovirus expression vector (Invitrogen). Primer sequences for amplification of His-Cdc7 and His-ASK are 5'-GCTAGCATGGCAGATAATAAAGCTCCC-3' (forward nhe1 primer) and 3'-CAATTGTTACACAGACCACGGGTGG-5' (reverse mfe1 primer).

2.2.8. Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol. Briefly, reactions were set up containing 5 µl 10x reaction buffer, 100 ng dsDNA template, 125 ng of appropriate forward and reverse primers, 1 µl dNTP mix, 3 µl QuikSolution and DNase/RNase free dH_20 to a final volume of 50 µl. One µl *PfuTurbo* DNA polymerase (2.5 U/µL) was added to each tube and the reactions cycled using the parameters outlined in Table 2.2. Following temperature cycling, the reactions were cooled on ice for 2 min and parental supercoiled dsDNA digested by incubation with 1 µl *Dpn I* (10 U/µl) for 1 h at 37°C.

Table 2.2. Cycling parameters for QuikChange Site-Directed Mutagenesis

Segment	Cycles	Temperature	Time
1	1	95°C	1 min
2	25	95°C	50 s
		60°C	55 s
		68°C	7 min
3	1	68°C	7 min

To generate the Cdc7 kinase negative mutants, including pFastBac-D196A (Asp-196 replaced by Ala), pFastBac- D177A (Asp-177 replaced by Ala) and pFastBac-K90A (Lys-90 replaced by Ala), mutagenesis was conducted on the Cdc7 sequences in the pFastBac vector. Oligonucleotides using the mutagenesis outlined in Table 2.3.

Table 2.3. Oligonucleotides for Cdc7 kinase negative mutants

template	oligonucleotides
pFastBac-D196A	5'-GAAAAAGTATGCCTTGGTAGCCTTTGGTTTGGCCCAAGG-3' 5'-CCTTGGGCCAAACCAAAGGCTACCAAGGCATACTTTTTC-3'
pFastBac-D177A	5'-GGTATTGTTCACCGTGCTGTTAAGCCCAGCAAT-3' 5'-ATTGCTGGGCTTAACAGCACGGTGAACAATACC-3'
pFastBac-K90A	5'-CCTGAAGAGAAAATTGCTCTAGCACACTTGATTCCAACAAGTC-3' 5'-GACTTGTTGGAATCAAGTGTGCTAGAGCAATTTTCTCTTCAGG-3'

2.2.9. Agarose gel electrophoresis

Horizontal gels were cast with agarose concentrations ranging from 0.8 to 1.0% (w/v). Solid agarose was mixed with 1 x TAE buffer and heated in a microwave until the agarose had fully dissolved. The solution was cooled to about 50ºC and 0.075% (v/v) gel star (Cambrex Bio Science Rockland Inc., Maine, ME, USA), was added. The gel was poured into a gel try, with combs inserted for casting wells, and allowed to set for 30 min at RT. Samples were prepared for electrophoresis by mixing with 6 x gel loading buffer. Electrophoresis was carried out at 1 – 4 V/cm for 30 min. After electrophoresis DNA was visualised at 312 nm.

2.2.10. Recovery of DNA from agarose gels

DNA fragments were cut from agarose gels using a sterile scalpel. The DNA was recovered from the excised gel using the QiaEXII gel extraction kit (Qiagen GmbH) following the manufacturer's protocol.

2.2.11. DNA sequencing

DNA sequencing was carried out by the Wolfson Institute for Biomedical Research Scientific Support Services, UCL, London, UK (http://www.ucl.ac.uk/wibr/services/dna/index.html). DNA was sequenced at a concentration of 500 ng/µl and purity of ≥ 1.8 OD_{260}/OD_{280} ratio on a Beckman Coulter CEG 8000 DNA Sequencer.

2.3. Recombinant baculovirus methods

2.3.1. Cell lines and viruses

Sf9 insect cells (ATCC, Manassas, Va.) were maintained at 27ºC in serum-supplemented TMN-FH medium (BD Biosciences, San Jose, CA, USA) containing 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco-BRL, Invitrogen). Routine cell culture maintenance and virus infection procedures were carried out as described previously (Jarvis 2009).

2.3.2. Viral DNA isolation

Viral DNA was isolated from supernatants of virus infected cells. 25ml of cell supernatants were centrifuged at 15000 r.p.m. for 5 min to pellet viral particles. Particles were resuspended in DNA extract buffer (0.1M Na_2CO_3, 0.17M NaCl, 0.01M EDTA pH 10.9), and incubated overnight at 37°C with proteinase K and 1% SDS. DNA was isolated after a further extraction with phenol and chloroform: isoamyl alcohol and ethanol precipitation and stored in TE buffer at 4°C.

2.3.3. Generation of recombinant baculovirus

Baculoviruses expressing Cdc7, ASK and mutant Cdc7 were generated according to the Bac-to-Bac system (Invitrogen). As described above the *CDC7* and *ASK* coding

sequences were amplified and were separately cloned into the pFasbac vector following the manufacturer's protocol (Invitrogen). Bacmids containing the *CDC7* and *ASK* open reading frames were generated in DH10Bac *E. coli* cells and transfected into Sf9 cells according to the manufacturer's protocol (Invitrogen). After confirmation of high-level Cdc7 and ASK protein expression, the supernatant containing the recombinant virus was amplified over three rounds, and used for protein production.

2.3.4. Plaque Assay

Plaque assay (Jarvis 2009) was used to visualize and purify the recombinant virus. Dilutions of virus containing supernatant were incubated with Sf9 cells (2×10^6) attached to a cell culture dish for an hour and replaced with medium containing 0.5% agarose (Invitrogen) that forms a semisolid overlay. After incubation for 5-7 days at 27°C, the plates were examined for plaques under an inverted microscope.

2.3.5. Virus titeration

Dilution of 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} of the virus stock are infected with Sf9 cells seeded into a 96 well plate. After incubation for 5 days at 27°C, virus stocks were tittered by the number of infected and uninfected wells at all dilution. The titer was represented as plaque forming units (PFU) per millilitre.

2.4. Protein chemistry methods

2.4.1. Protein expression constructs

The following constructs were used for protein expression (Table 2.4):

Table 2.4. Protein expression constructs

Protein	Construct	Insert Size	Cloning fragment	Expression system
His-Cdc7	pFastBacB-Cdc7	1.7kbp	*Nco1-XhoI*	Baculoviral
His-Cdc7(D196A)	pFastBacB-Cdc7(D196A)	1.7kbp	*Nco1-XhoI*	Baculoviral
His-Cdc7(D177A)	pFastBacB-Cdc7(D177A)	1.7kbp	*Nco1-XhoI*	Baculoviral
His-Cdc7(K90A)	pFastBacB-Cdc7(K90A)	1.7kbp	*Nco1-XhoI*	Baculoviral
His-ASK	pFastBacA-ASK	2.0kbp	*EcoRI – XhoI*	Baculoviral
His-ASK (271-674)	pProEX-HTa-ASK(271-674)	1.2kbp	*XhoI– EcoRI*	*E. coli*
His-*hs*Mcm2 (1-154)	PProEX-HT2-Mcm2(1-154)	0.5kbp	*BamHI–HindIII*	*E. coli*

2.4.2. Expression of recombinant human Cdc7, ASK and mutant Cdc7 in Sf9 insect cells

Sf9 insect cells were grown in 400 ml of serum-supplemented TMN-FH medium containing 100 U/ml penicillin and 100 µg/ml streptomycin in square dish (245 x 245 x 25 mm, Corning Costar) at 27ºC up to a density of 1.5 x 10^6 cells/ml. cells were infected with the appropriate recombinant baculovirus at 5 M.O.I., and incubated for 72 h at 27ºC. Insect cells were harvested by centrifugation at 5,000 g for 5 min at 4ºC, washed in PBS and stored at -80ºC.

2.4.3. Expression of recombinant human Cdc7/ASK kinase complex in Sf9 insect cells

Sf9 insect cells were grown in 400 ml of serum-supplemented TMN-FH medium containing 100 U/ml penicillin and 100 µg/ml streptomycin in square dish (245 x 245 x 25 mm, Corning Costar) at 27ºC. Cells were co-infected with the Cdc7 and ASK recombinant baculoviruses at a multiplicity of infection of 5 and 10 for each recombinant baculovirus respectively and incubated for 72 h at 27ºC with gentle mixing. Infected cells were harvested by centrifugation at 5,000 g for 5 min at 4ºC,

washed in PBS and stored at -80ºC.

2.4.4. Purification of baculoviral-expressed human Cdc7, ASK, mutant Cdc7 and Cdc7/ASK kinase complex

Infected cells were harvested 72h post-infection, washed in PBS and lysed in lysis buffer (25 mM Tris-HCl pH 9, 250 mM KCl, 0.5% NP 40, 10% glycerol, 10 mM $MgCl_2$ and 0.02 volumes of complete EDTA-free Protease inhibitor cocktail) on ice for 30 min. Lysates were cleared by spinning at 13,000rpm for 1h at 4°C and loaded on to a 5 ml HiTrap chelating HP column (Amersham Biosciences AB, Uppsala, Sweden). The column was washed with five column volumes of wash buffer (25 mM Tris-HCl pH 9, 250 mM KCl, 0.5% NP 40, 10% glycerol, 10 mM $MgCl_2$ and 5 mM Imidazole) prior to elution. Recombinant proteins were eluted with a step gradient of 10%, 20%, 40% and 100% elution buffer (25 mM Tris-HCl pH 9, 250 mM KCl, 0.5% NP 40, 10% glycerol, 10 mM $MgCl_2$ and 250 mM Imidazole) using an Acta FPLC system (Amersham Biosciences AB, Uppsala, Sweden) (Figure 3.4, 3.5, and 3.6). Proteins were dialysed by passing through a HiPrep 26/10 desalting column (Amersham Biosciences AB), and concentrated using a Vivaspin concentrator (Sartorius AG, Goettingen, Germany).

2.4.5. Expression of recombinant ASK (271-674) fragment in *E. coli*

ASK (C-terminal 403 amino acids) fragment was amplified from pFastBacA-ASK vector by PCR using a primer complementary to the 5' region of 271 amino acid residues of ASK: 5'-AGAATCCAAACAGATGGC-3' and a primer complementary to the 3' region of 674 amino acid residues of ASK: 5'-ACATTTACTGGCTTTTAG-3'. The PCR product was cloned into pProEX-HTa expression vector. ASK fragment

(271-674) was expressed in Rosetta2 (DE3) plysS cells (Novagen, Madison, WI, USA) according to standard protocols. Briefly, cells were transformed with the appropriate expression construct and grown overnight at 37ºC with vigorous shaking in 20 ml of 2 x TY medium containing 100 µg/ml ampicillin, 34 µg/ml chloramphenicol and 1% glucose. The overnight culture was used to inoculate 4 x 500 ml of fresh 2 x TY medium, supplemented with 100 µg/ml ampicillin, 34 µg/ml chloramphenicol and 0.25% glucose. Cultures were grown at 30ºC with vigorous shaking until the OD_{600} reached 0.5.cells and collected from the incubation for 3 hrs at 30ºC after 1mM IPTG induction. Cells were harvested by centrifugation at 5,000 g for 20 min, washed in ice cold PBS, the wet weight of cells determined and the pellet frozen at -80ºC.

2.4.6. Purification of bacterial-expressed ASK (271-674) fragment

Bacterial cell pellets were resuspended in 5 ml of lysis buffer (25 mM Tris-HCl pH 7.5, 250 mM NaCl, 10% glycerol and 0.02 volumes of complete EDTA-free Protease inhibitor cocktail) per gram wet weight, containing bensonase (1 µl/ml), and incubated for 30 min at 4ºC. The cells were broken by sonication and the lysate cleared by centrifugation at 48,000 g at 4ºC for 1 h. The insoluble fractions were resuspended in denature beffer (25 mM Tris-HCl pH 7.5, 500 mM KCl, 2% Triton X-100 and 6M urea), and incubated with gentle end-over-end inversion for 20 mins at room temperature. The suspension was centrifuged at 13000 x g for 10 mins, and the supernatants containing solubilised proteins in denature buffer were loaded onto HiTRAP chelating IMAC column (Amersham Biosciences). The protein bound to the column was renatured for 3 hours by a linear gradient to renature buffer (25 mM Tris-HCl pH 7.5, 250 mM NaCl, 10% glycerol, and 20Mm imidazole). After renaturation,

protein was eluted from the column using a step gradient of 10%, 20%, 40% and 100% elution buffer (25 mM Tris-HCl pH 7.5, 250 mM NaCl, 10% glycerol, and 250 mM imidazole) (Figure 3.8B). Protein was dialysed by passing through a HiPrep 26/10 desalting column (Amersham Biosciences AB), and concentrated using a Vivaspin concentrator (Sartorius AG, Goettingen, Germany).

2.4.7. Expression of recombinant human Mcm2 (1-154) fragment in *E. coli*

The human Mcm2 (N-terminal 154 amino acids) fragment was amplified from a HeLa cDNA library by PCR using a primer complementary to the 5' region of Human Mcm2 containing a restriction site for HindIII: 5'-TGCCCGCAAGCGCCGCCA GGTGTAAGCTT-3' and a primer complementary to the 3' region of 154 amino acid residues of Mcm2: 5'-AAGCTTACACCTGGCGGCGCTTGCGGGCA-3'. The PCR product was cloned into the pET100d TOPO cloning vector (Invitrogen, Carlsbad, CA, USA) and subcloned into BamHI-HindIII sites of pProEX-HT2 expression vector. Mcm2 fragment was expressed in Rosetta (DE3) plysS cells (Stratagene, La Jolla, CA, USA) according to standard protocols. Cells were transformed with the appropriate expression construct and grown overnight at 37ºC with vigorous shaking in 20 ml of 2 x TY medium containing 100 µg/ml ampicillin, 34 µg/ml chloramphenicol and 1% glucose. The overnight culture was used to inoculate 4 x 500 ml of fresh 2 x TY medium, supplemented with 100 µg/ml ampicillin, 34 µg/ml chloramphenicol and 0.25% glucose. Cultures were grown at 30ºC with vigorous shaking until the OD_{600} reached 0.5.cells and collected from the incubation for 3 hrs at 30ºC after 1mM IPTG induction. Cells were harvested by centrifugation at 5,000 g for 20 min, washed in ice cold PBS, the wet weight of cells determined and the pellet frozen at -80ºC.

2.4.8. Purification of bacterial-expressed human Mcm2 (1-154) fragment

For purification, cell pellets were resuspended in 5 ml Bugbuster protein extraction buffer (Novagen, Madison, WI, USA) per gram wet weight, containing bensonase (1 µl/ml) and 0.02 volumes of complete EDTA-free Protease Inhibitor Cocktail (F. Hoffmann-La Roche, Basel, Switzerland), and incubated for 30 min at 4ºC. The cells were broken by sonication and the cell lysate was clarified by centrifugation at 48,000 g at 4ºC for 1 h, and the supernatant filtered through a 0.45 µm syringe filter. Cell lysate was loaded via a peristaltic pump on to a 5 ml HiTrap HP column (Amersham Biosciences) and protein was eluted with step gradients of 10%, 20% and 100% elution buffer (25 mM Tris-HCl pH 7.5, 250 mM NaCl, 10 % glycerol and 250 mM Imidazole). Protein was further purified using a Seperdex 200 Gel filtration column (Amersham Biosciences AB), and concentrated to 1 mg/ml using a Vivaspin concentrator (Sartorius AG, Goettingen, Germany).

2.4.9. SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using pre-cast Novex 4-20% Tris-glycine gels and the XCell *SureLock*^{TM} Mini-Cell Electrophoresis System (Invitrogen). Prior to loading, protein samples were mixed with 4x laemlli buffer, heated to 90°C for 3 min, and pulse centrifuged. Samples were resolved in 1x Tris-glycine running buffer at 125 mV for approximately 90 min. After electrophoresis, gels were stained with Coomassie Blue R-250 or immunoblotted.

2.4.10. Coomassie Blue staining of SDS-PAGE gels

For visualisation of recombinant protein, SDS-PAGE gels were stained with Coomassie Blue R-250 for 1-2 h with gentle shaking. The gels were destained with

Coomassie gel destain solution, with gentle shaking, until the background was colourless.

2.5 Immunological techniques

2.5.1. Antibodies

Affinity-purified rabbit polyclonal antibodies against *hs*geminin (Wharton *et al*., 2004), *hs*Cdt1 (Kingsbury 2005), *hs*Cdc45 and ASK (#SK3681) were generated in-house. The following commercially supplied antibodies were used: mouse anti-*hs*Orc4 (BD Transduction Laboratories, Lexington, KY, USA), mouse anti-*hs*Mcm2/BM28 (BD Transduction Laboratories), mouse anti-*hs*Cdc6 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-Cdc7 (MBL, Nagoya Japan), rabbit phospho-Mcm2 S40/41 (Bethyl, Montgomery, USA), rabbit phospho-Mcm2 S53 (Bethyl), rabbit anti-Cyclin A (Santa Cruz Biotechnology), mouse anti-Cyclin E (Oncogen), mouse anti-His_6 (BD Transduction Laboratories), mouse anti-β Actin (Sigma), HRP-conjugated goat anti-mouse/rabbit IgG (Dako), FITC-conjugated mouse anti-CD11B (Beckman Coulter, Miami, FL), RD1-conjugated mouse anti-CD14 (Beckman Coulter), FITC-conjugated mouse anti-BrdU (Alexis Biochemicals, Lausen, Switzerland).

2.5.2. Preparation of whole cell extracts

To prepare whole cell extracts, cells were harvested by treatment with trypsin, washed in PBS, and resuspended in RIPA buffer (50mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40, 1% Sodium deoxycholate, 0.1% SDS, 1mM EDTA) containing EDTA-free protease inhibitor cocktail (Roche Diagnostics GmbH). After incubation on ice for 30

min, the lysate was clarified by centrifugation at 13.000 x g for 15 min at 4°C, and stored at -80ºC.

2.5.3. Immunoblotting

For immunoblotting, protein was transferred from polyacrylamide gels onto nitrocellulose membranes (Amersham Biosciences) by semi-dry electroblotting in transfer buffer. Non-specific binding was blocked by incubating the membrane in PBS, 5% skimmed milk powder, 0.1% Tween-20 overnight at 4ºC. Membranes were incubated in primary antibody in PBS, 5% skimmed milk powder, 0.1% Tween-20 for 2 h at room temperature, except for some polyclonal antibodies for which PBS, 10% skimmed milk powder, 1% Tween-20 was used, followed by six 5 min washes in PBS, 0.1% Tween-20. Membranes were then incubated with the appropriate secondary antibody in PBS/milk/Tween-20 as before for 1 h at room temperature followed by six 5 min washes prior to visualisation. Immunoreactive bands were visualised on Hyperfilm ECL (Amersham Biosciences) by enhanced chemiluminescence (ECL, Amersham Biosciences).

2.5.4. Immunoprecipitation

Total cell lysate was immunoprecipitated with Cdc7 antibody in IP buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% NP40) containing EDTA-free protease inhibitor cocktail for 3h at 4°C. Protein A-Sepharose (Amersham Biosciences) was added to cell extract and incubated for 30 min at 4°C. The beads were extensively washed three times with IP buffer, incubate with 3 x SDS sample buffer and resolved by electrophresis.

2.5.5. Immunohistochemistry

Three μm sections of formalin-fixed, paraffin-embedded tissues were cut onto DAKO Techmate™ S2024 slides, baked in a 60°C oven overnight to maximise section adhesion, dewaxed in xylene and rehydrated through a series of alcohol to water. For antigen retrieval, tissues were pressure-cooked for 2 min in 0.1 M Citrate Buffer pH 6.0. Immunostaining was performed manually using a standard protocol. After antigen retrieval, slides were washed twice in TBS, 0.1% Tween and endogenous peroxidase activity was quenched with peroxidase blocking solution (DAKO) for 10 min. Primary antibodies were applied for 1 h at RT, and detected with a biotin-free polymer-based detection system (ChemMate DAKO EnVision). Sections were incubated with peroxidase-labelled secondary antibody for 1 h at RT, and the immunostain developed with 3,3-diaminobenzidine tetrahydrochloride (DAB) for 10 min. Sections were counterstained in Mayer's Haematoxylin, differentiated in 1% acid alcohol, dehydrated, cleared in xylene and coverslips applied using Leica CV Mount (Leica, Nussloch, Germany). Primary antibodies were omitted in negative controls and appropriate tissue sections were used as positive and negative controls.

2.6. Cell biology techniques

2.6.1. Cell culture and synchronisation

Hela S3 (CCL-2.2, ATCC®) and WI-38 (CCL-75, ATCC®, LGC Promochem, Middlesex, UK) cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% foetal calf serum (FCS, Gibco-BRL), 100 U/ml penicillin and 0.1 mg/ml streptomycin. HL60 (CCL-240, ATCC®) cells were grown in RPMI 1640 medium (Gibco-BRL, Paisley, Scotland) supplemented with 10% fetal

calf serum (FCS; Gibco-BRL), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco-BRL). Cell cycle synchronisation was performed essentially as described (Krude *et al.*, 1997). Briefly, for preparation of cycling cells, HeLa S3 cells were synchronised in very early S phase by two sequential 25 h blocks in 2.5 mM thymidine separated by a 12 h interval without thymidine (Rao and Johnson, 1970), followed by release into fresh culture medium for 2 h. For preparation of cells in G2/M phase, cells were released from the second thymidine block for 3 h, followed by adding 40 ng/ml nocodazole for an additional 12 h to arrest them in mitosis (Johnson et al., 1993), followed by release into fresh culture medium for 2 h. To prepare cells in quiescent state, WI-38 cells were driven into quiescence by density-dependent growth arrest and after five days were released back into cycle by subculturing 1 in 4 into fresh growth medium. For differentiation experiments, HL60 cells were seeded at 1-1.5 x 10^5 cells/ml 24 h before addition of 1,25 dihydroxyvitamin D_3 (1,25 Vit D_3) (Sigma-Aldrich, Dorset, UK). 1,25 Vit D_3 was diluted in medium from an ethanol stock at 1 mM to a final concentration of 100 nM. In experiments the medium containing 1,25 Vit D_3 or ethanol was changed every 48 h.

2.6.2. Bivariate flow cytometric analysis

For cell cycle analysis of DNA content, DNA was stained by incubating cells in PBS containing 20 µg/ml propidium iodide and 50 µg/ml RNase A for 20 min at RT in the dark. For cell cycle analysis of bromodeoxyuridine (BrdU) incorporation, cells were pulse labelled with BrdU for 1 h and cell samples fixed in 80% ethanol for a minimum of 12 h. After fixation, cells were washed in PBS and DNA was denatured by incubation in 4 N HCl for 20 min. Subsequently cells were washed twice in PBS, incubated for 1 h with a FITC-conjugated BrdU antibody (1:50 dilution; Alexis

Biochemicals) in the dark at 4ºC and cells were resuspened in 0.5 ml PBS at room temperature. To prevent clumping of cells and loss of sample, 100 µl of 1% BSA was added at each step and unautoclaved pipette tips were used. Analyses of light-scatter properties and DNA/BrdU incorporation were performed using a FACSCalibur flow cytometer (BD Biosciences) (Figure 4.2, 4.4, 4.7 and 6.7). Cell doublets were excluded by gating on a dot plot of the width vs the area of DNA fluorescence intensity (Erlanson and Landberg, 1998). In most samples, 10^4 cells were examined and data were analysed using CellQuest™ software (BD Biosciences) and WinMDI (V 2.8).

2.6.3. Monitoring differentiation

HL60 cell differentiation was monitored as described (Barkley et al., 2007). During differentiation time courses, 1 x 10^6 HL60 cells were harvested at the indicated times, washed twice with PBS, and incubated for 45 min at room temperature in the dark in 0.5 µg of FITC-conjugated mouse anti-CD11B and/or 0.5 µg of RD1-conjugated mouse anti-CD14 (Beckman Coulter, Miami, FL). Cells were washed three times with PBS and re-suspended in 0.5 ml PBS. Flow cytometric analyses were performed on cells which were singly stained (CD11b-FITC or CD14-RD1) and on cells dually stained (CD11b-FITC and CD14-RD1) (Figure 4.6).

2.6.4. Assaying for apoptosis

Apoptosis was quantified using the Annexin V-FITC Apoptosis Detection Kit (BioVision, USA), following the manufacturer's protocol. 1 x 10^6 cells were stained with 5 µl of propidium iodide and/or 5 µl of Annexin-V FITC in 1 x binding buffer, and analysed by flow cytometry (Figure 6.8).

2.6.5. Tissue specimens

Formalin-fixed, paraffin-embedded archival human oral squamous epithelium and liver tissue blocks from diagnostic biopsy or resection specimens were retrieved from the archives of the Department of Pathology, University College London, U.K. Slides were examined with an Olympus BX51 microscope/CCD camera setup and scanned at low magnification to identify areas of high expression for each marker. Three to five fields were captured from selected areas at x400 magnification and processed using ANAlysis software (SIS, Münster, Germany). Captured images were printed and within fields a labelling index was determined for a minimum of 300 cells.

2.7. Kinase Assay Development

2.7.1. *In vitro* kinase assay

The standard reaction mixtures (25 μl) for Cdc7/ASK *in vitro* kinase assays contained 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM ATP, 1 μg of Mcm2 N-terminal fragment and 50 ng of Cdc7/ASK kinase complex. The reaction mixtures were incubated at 30°C for 40 min. After addition of SDS-PAGE sample buffer, the reaction mixtures were incubated at 90°C for 3 minutes and loaded on 4-20% gradient SDS-PAGE. The reaction products were immunoblotted as described.

2.7.2. Antibody-based Kinase assay (96-well format)

HisGrab 96-well plates (Pierce, Rockford, IL, USA) were coated with 1 μg of His-Mcm2 N-terminal fragment in 100 μl TBS (Pierce, Rockford, IL, USA) at room temperature for 1h with shaking. The plates were washed three times with wash buffer

(TBS contained 0.05 % Tween20), blocked with blocking solution (Pierce, Rockford, IL, USA) for 1h and washed three times with wash buffer. The kinase reaction was started by adding Cdc7/ASK complex in a total volume of 50 µl that contained 50 mM Tris-Hcl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT and 0.1 mM ATP. After incubation for 45 minutes at room temperature, the plates were washed three times with wash buffer and incubated with 100 µl of Phospho Mcm2 antibody (A300-117A, Bethyl, Montgomery, TX, USA) in TBS for 1h. Plates were then washed three times with wash buffer and incubated with a anti-rabbit immuno-globulins/AP (Dako, Glostrup, Denmark) for 1h. P-nitrophenyl phosphate (PNPP) substrate (Sigma, St. Louis, MO, USA) solution was added and incubated for 20 min at room temperature for colour development. The reaction was stopped by adding 2N NaOH solution and the signal was measured at 405nm by a Spectramax plus (PO2877, Molecular Devices).

2.7.3. High-Throughput Screen for Cdc7 inhibitors (384-well format)

Assays were performed in a final volume of 20 µl/well in 384-well Immobilizer Nikel-chelate microplates (Nunc) using automated HTS workstation (Figure 6.1). Plates were coated with 250 ng of His-Mcm2 N-terminal fragment in 20µl TBS at room temperature for 1h with shaking. The plates were washed three times with 80 µl wash buffer (25 mM Tris-Hcl pH 8, 150 mM NaCl and 0.05 % Tween20). Prior to kinase reaction, 2 µl of test compounds were added to wells to give 30 µM. The kinase reaction was started by adding Cdc7/ASK complex in reaction buffer (50 mM Tris-Hcl pH 8.5, 10 mM $MgCl_2$, 1 mM DTT) with 0.2 µM of ATP. After incubation for 60 min at room temperature, the plates were washed, and incubated with 20 µl of Phospho Mcm2 antibody (A300-117A, Bethyl, Montgomery, TX, USA) and a anti-

rabbit immuno-globulins/AP (Dako, Glostrup, Denmark) in TBS for 30 min. PNPP development (Sigma, St. Louis, MO, USA) buffer was added for colour development, and the reaction was stopped by adding 2N NaOH solution. Absorbance A405 was read on the Analyst plate reader (Molecular Devices, USA, Serial No. AN0166).

2.8. Computing

Unless stated otherwise, all image processing and quantitation were performed with Adobe Photoshop Version 7.0 (Adobe Systems Inc., USA) using only standard contrast and brightness adjustment functions. Graphs were produced in MSExcel, (Microsoft Corporation, USA). The text of this thesis was written in MSWord, (Microsoft Corporation, USA) and figures were assembled in Adobe Illustrator Version 10.0 (Adobe Systems Inc., USA).

2.8.1. Bioinformatic and database resources

DNA sequences

Entrez nucleotide: http://www.ncb.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide

Protein sequences

Entrez protein: http://www.ncb.nlm.nih.gov/entrez/query.fcgi?db=Protein

DNA and protein sequence homology

BLAST: http://www.ncbi.mln.nih.gov/BLAST/

Multiple sequence alignments

ClustalW: http://www.ebi.ac.uk/clustalw.html

Restriction enzyme site identification

NebCutter 2: http://www.neb.com/nebcutter.html

Oligonucleotide primer design

Netprimer: http://www.premierbiosoft.com/netprimer.html

Protein molecular weight, pI, and extinction coefficient

ProtParam: http://ca.expasdy.org/tols/protparam.html

Protein structure visualisation

Pymol: http://pymol.sourceforge.net/

CHAPTER THREE

Purification of active, recombinant Cdc7/ASK complex and generation of rabbit polyclonal antibodies to Cdc7 and ASK

3.1. Introduction

Cdc7 kinase, first identified in *Saccharomyces cerevisiae* (Hartwell 1973; Patterson et al. 1986), is required for at least three aspects of DNA metabolism: initiation of DNA replication, cohesin loading, meiotic recombination and DNA repair (Hollingsworth, Jr. & Sclafani 1993; reviewed in Swords et al. 2010). Homologues of Cdc7 have been identified in *Schizosaccharomyes pombe* (Masai et al. 1995), mouse (Kim et al. 1998), *Xenopus* and *H. sapiens* (Jiang et al. 1997; Sato et al. 1997; Hess et al. 1998). Cdc7 is a serine/threonine kinase (Figure 3.1A) which is activated by the regulatory protein ASK (Activator for S phase Kinase in *H. sapiens*, also called Dbf4 in *S. cerevisiae* and Dfp1/Him1 in *S. pombe*) during the cell cycle (Diffley et al. 1995; Masai & Arai 2002; Sclafani et al. 2002; Stillman 1996) (Table 3.1). A second ASK/Dbf4-related molecule in *H. sapiens*, named Drf1, and its possible homologue in *Xenopus* have been reported (Montagonoli et al. 2002; Yanow et al. 2003).

Cdc7 protein contains conserved kinase domains that are common to all serine/threonine protein kinases (Patterson et al. 1986; Hanks et al. 1988; reviewed in Sawa & Masai 2008). Sequence analysis of full-length Cdc7 shows that the kinase domains of human Cdc7 have 44% and 42% overall identity in amino acid sequence with those of Cdc7p and Hsk1, suggesting that human Cdc7 is structurally related to

Table 3.1 Cdc7/ASK related Kinase complexes

	Catalytic subunit	Regulatory subunit	Substrates
Human	hCdc7	ASK, Drf1	hMcm2-7
Mouse	muCdc7	muASK	Mcm2
Xenopus	XeCdc7	XeDbf4, Drf1	Mcm2
S. cerevisiae	Cdc7	Dbf4	Mcm2,3,4,6
S. pombe	Hsk1	Dfp1/Him1	SpMcm2,4,6,7

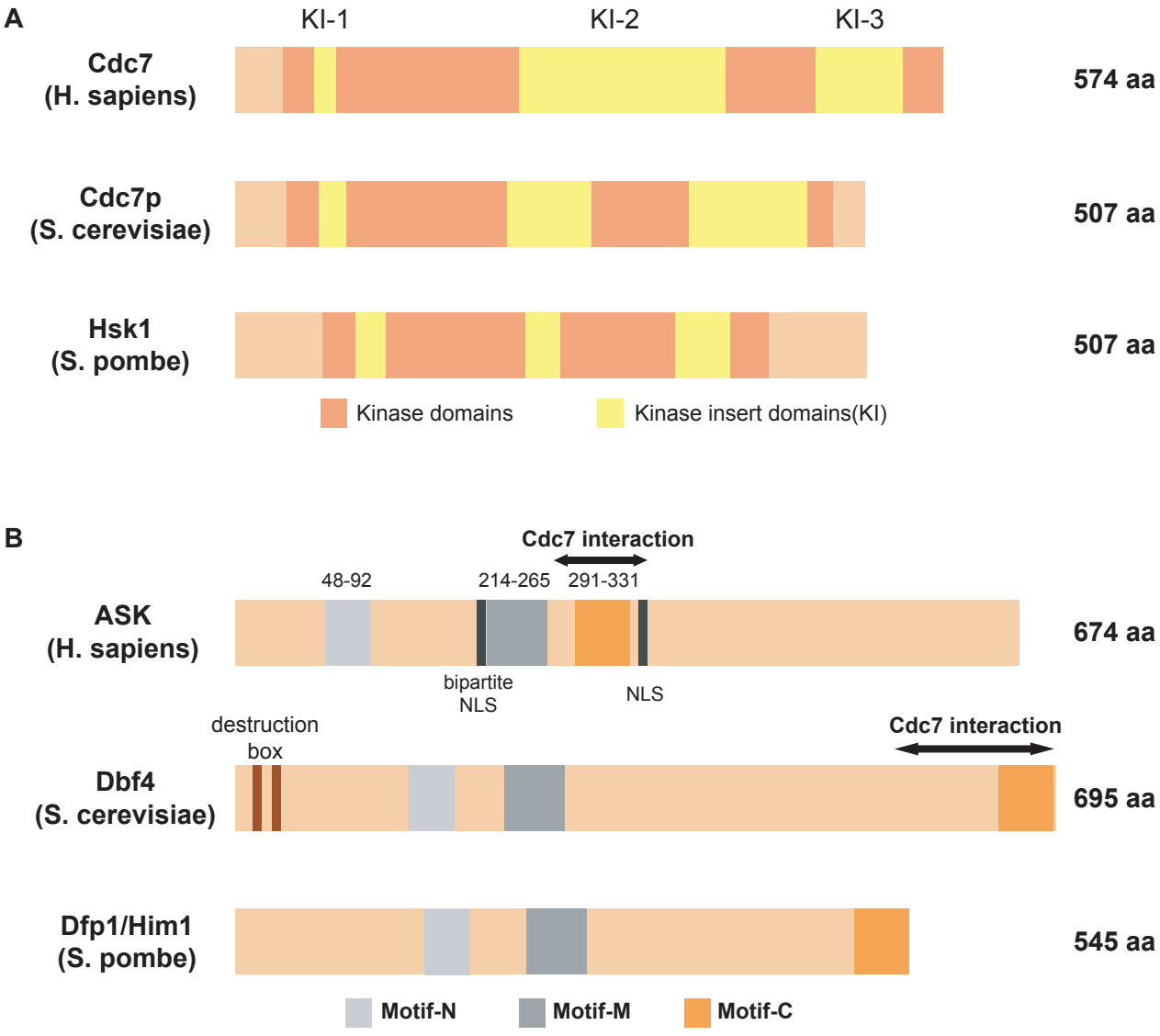


Figure 3.1: Schematic drawing and comparison of Cdc7 and Dbf4-related subunits from H. sapiens, S. cerevisiae and S. pombe. (A) Comparison of Cdc7-related subunits. Red and yellow segments represent conserved kinase domains and less conserved kinase insert sequences respectively. (B) Comparison of Dbf4-related subunits. Destruction box indicates a "RXXL" motif, known to be targeted by the APC-dependent degradation pathway. Two putative nuclear localization signals (NLS) in ASK are shown. Motif-N: related to BRCT, Motif-M: Proline-rich motif and Motif-C: CCHH-Type Zinc finger-like motif.

yeast Cdc7p and Hsk1 (Figure 3.1A) (Jiang & Hunter 1997). All Cdc7 homologues contain three kinase insert domains (KI) between the kinase domains, however the sequences and lengths of these kinase inserts are not conserved among Cdc7, Cdc7p and Hsk1. Sequence analysis has revealed that the degree of conservation of amino acid sequences between different species is much lower in the Dbf4-related protein family (reviewed in Masai et al. 1999; Kumagai et al. 1999). The overall homology identity between Dbf4 and Dfp1/Him1 is less than 25%, and similarity is 46%. Almost no similarity was observed between Dbf4 and human ASK, except for three short stretches of amino acids - motif-N, motif-M and motif-C - which are well conserved in Cdc7 regulatory subunits (Figure 3.1B). Drf1, a second human ASK/Dbf4-related protein, contains the three conserved Dbf4 motifs in the N-terminal region (35% identity with ASK) and a long C-terminal tail that is much less conserved (Yoshizawa-Sugata et al. 2005). Direct interaction between Cdc7 and Dbf4 has been demonstrated by the requirement of association with motif-M and motif-C for Cdc7 kinase activity (Dowell *et al.* 1994), whilst motif-N is essential for association with the replication machinery at origins (Ogino *et al.* 2001) (Figure 3.1B). Although ASK and its functional homologues in other eukaryotes are functionally similar to cyclins in terms of kinase activation of the catalytic subunit, their primary structures bear no similarity to known cyclins (Masai et al. 1999; Johnston et al 2000). It is well known that S-phase specific kinases, CDK and Cdc7 kinases, are essential activators of initiation of DNA replication through the recruitment of several replication factors (Bell & Dutta 2002; Takeda & Dutta 2005; Sclafani & Holzen 2007; Swords et al 2010). The mechanisms of these kinases activation of DNA replication in *S. cerevisiae* has been recently reported that CDK phosphorylate Sld2 and Sld3 to activate DNA replication initiation, whereas Cdc7 kinase phosphorylate the amino

terminal serine/threonine rich domain (NSD) of Mcm4 relieving an inhibitory activity for S phase progression (Masai et al 2006; Zegerman & Diffley 2007; Sheu & Stillman 2010).

Conservation between yeast and higher eukaryotes of the primary structure of Cdc7 and its regulatory subunit strongly suggests the functional conservation of these kinases in DNA replication (Masai et al. 1995; Sato et al. 1997; Kim et al. 1998; Jiang & Hunter 1997; Robert et al. 1999; Masai et al 1999; Masai & Arai 2002; reviewed in Sawa & Masai 2008; Figure 3.1). Previous genetic and biochemical studies indicate that *S. cerevisiae* Cdc7p depends on association with Dbf4 for its kinase activity, which is required for the activation of individual origins throughout S phase (Bousset & Diffley 1998; Donaldson et al. 1998). Hsk1 is also essential for cell viability and S phase initiation in *S. pombe*, suggesting that the function of Cdc7 kinase in eukaryotic DNA replication is evolutionarily conserved between the two yeasts (Masai et al. 1995). Studies in *Xenopus* egg extracts showed that either addition of XeCdc7 antibody or depletion of Cdc7 inhibits DNA replication, demonstrating an essential role of Cdc7 kinase for efficient DNA replication also in vertebrates (Roberts et al. 1999; Jares & Blow 2000; Walter 2000). Conditional knockout of the *CDC7* gene in mouse ES (embryonic stem) cells resulted in a block to DNA replication and subsequent cell death (Kim et al. 2002). An essential role of human Cdc7/ASK kinase for mammalian DNA replication was demonstrated by the inhibition of DNA replication after micro-injection of ASK or Cdc7 antibodies into human fibroblast cells (Kumagai et al. 1999; Jiang & Hunter 1997). Furthermore, inhibition of Cdc7 kinase activity by either RNA interference or small molecule inhibitors in various cancer cells caused an abortive S phase progression, followed by growth arrest and

apoptotic cell death, highlighting the potential of Cdc7 kinase as a novel target for anti-cancer drug development (Montagnoli et al. 2004; Montagnoli et al. 2008; Shafer et al.2008).

The initiation of DNA replication, a convergence point for mitogenic and signal transduction growth regulatory pathways, is a critical and tightly controlled step in cell proliferation, restricting origin activation to once per cell cycle. Cdc7/ASK has been shown to be a key regulator of origin activation through phosphorylation of key components of the pre-replicative complex (pre-RC) during the initiation of DNA replication (see also Chapter One - Introduction). To provide further insights into the molecular mechanisms that regulate DNA replication competence during the human mitotic cell division cycle and in out-of-cycle states, additional research into Cdc7/ASK function is required. Biochemical and functional studies of the human Cdc7/ASK kinase complex have been limited in the past by the lack of molecular tools, in particular functional recombinant kinase complex and specific antibodies. The aim of this part of the work described in this thesis was to generate these molecular tools, which were subsequently used to study cell cycle regulation of Cdc7/ASK kinase activity in human cells.

3.2. Material and Methods

For transformation of *E. coli* (2.2.2.), plasmid isolation (2.2.3.), restriction endonuclease digestion (2.2.4), dephosphorylation of linearised vector DNA (2.2.5.), ligation of DNA (2.2.6.), polymerase chain reaction (2.2.7.), agarose gel electrophoresis (2.2.9.), recovery of DNA from agarose gels (2.2.10.), DNA sequencing (2.2.11.), preparation of recombinant baculoviruses (2.3.1), viral DNA

isolation (2.3.2.), generation of recombinant baculovirus (2.3.3.), plaque assay (2.3.4.), virus titeration (2.3.5.), expression of recombinant His-tagged human Cdc7, ASK and mutant Cdc7 in Sf9 insect cells (2.4.2.), expression of recombinant human Cdc7/ASK kinase complex in Sf9 insect cells (2.4.3.) purification of Cdc7, ASK, mutant Cdc7 and Cdc7/ASK complex (2.4.4.), expression and purification of ASK (271-674) fragment (2.4.5., 2.4.6.), expression and purification of Mcm2 (1-154) fragment (2.4.7., 2.4.8.), SDS-polyacrylamide gel electrophoresis (2.4.9.), Coomassie blue staining of SDS-PAGE gels (2.4.10.), antibodies (2.5.1.), preparation of whole cell extracts (2.5.2.), immunoblotting (2.5.3.) and immunoprecipitation (2.5.4.) please refer to Chapter Two (Materials and Methods).

3.3. Results

3.3.1. Generation of CDC7 and ASK recombinant baculoviruses

To characterise in human cells Cdc7/ASK kinase and its role in the initiation of DNA replication and cell cycle regulation, functional recombinant Cdc7 and ASK proteins were required. Baculoviruses expressing Cdc7, ASK and a kinase-dead mutant Cdc7 were kindly provided by Dr Wei Jiang (The Burnham Institute, La Jolla, California; Jiang et al. 1999). However, the virus stocks received did not express high levels of the recombinant proteins. Recombinant CDC7 and ASK baculoviruses were therefore generated *de novo* in the work of this thesis to express high levels of Cdc7 and ASK in insect cells, both as individual proteins and together as an active kinase complex.

To generate recombinant CDC7 and ASK baculoviruses, viral DNA was isolated from the provided budded viruses and PCR analysis was performed using CDC7 or ASK specific primers to amplify the *CDC7* and *ASK* coding genes (Figure 3.2A). DNA

sequence analysis confirmed that the PCR products were identical to the reported *CDC7* and *ASK* gene sequences. The amplified *CDC7* and *ASK* genes were separately cloned into pFastBac baculovirus expression vector containing a six-histidine tag on the N-terminus for protein purification (Figure 3.2B). The plasmids were transformed into DH10Bac competent *E. coli* cells to generate a recombinant bacmid by site-specific transposition. Recombinant bacmids were transfected into Sf9 insect cells to generate recombinant baculoviruses that express Cdc7 and ASK proteins (Figure 3.2C). The recombinant baculoviruses were amplified and titered. High-titer (3×10^9 pfu/ml: plaque forming units per ml) stock viruses were used for Cdc7 and ASK expression.

3.3.2. Expression and purification of Cdc7, ASK and active Cdc7/ASK kinase complex in insect cells

To optimise the expression of Cdc7 and ASK recombinant proteins, Sf9 insect cells were infected with recombinant baculoviruses either expressing His-tagged Cdc7 or His-tagged ASK proteins. Human *CDC7* encodes a protein of 574 amino acids with a predicted molecular weight of 58 kDa (Masai & Arai 2002). The gene for its regulatory subunit *ASK* encodes a protein of 674 amino acids with a predicted molecular weight of 77 kDa (Jiang et al. 1999). Western blot analysis shows high level expression of the 64 kDa His-tagged Cdc7 and the 77 kDa His-ASK proteins in insect cells 72 hours after infection (Figure 3.3A). Although both Cdc7 and ASK proteins were mainly expressed at 72 hours post-infection, both proteins were found

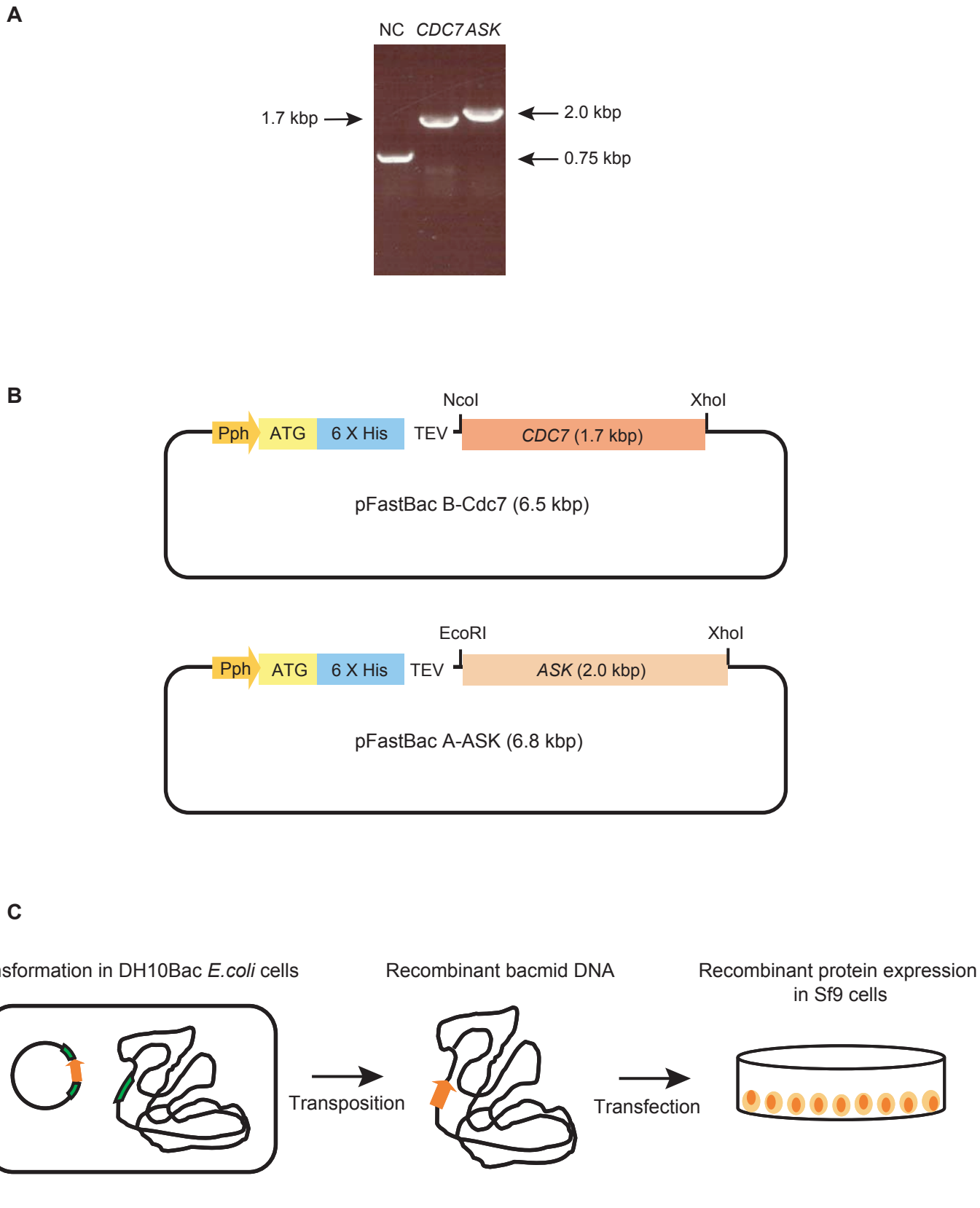


Figure 3.2: Generation of Cdc7 and ASK recombinant baculoviruses. (A) PCR amplification of *CDC7* and *ASK* genes from baculoviral template DNA (NC-Negative control). (B) Both *CDC7* (1.7kbp) and *ASK* (2.0kbp) PCR products were cloned into pFastBacHT vector to generate pFastBacHT B-Cdc7 and pFastBacHT A-ASK. (C) Vector constructs were transformed into DH10Bac competent *E.coli* to generate recombinant CDC7 or ASK bacmids. Recombinant bacmids were transfected into insect cells to generate CDC7 or ASK baculoviruses which overexpress Cdc7 and ASK recombinant protein.

in both soluble and insoluble fractions prepared from infected Sf9 cells. As shown in Figure 3.3A approximately 60% of total ASK protein was insoluble, whereas total Cdc7 protein was more soluble (Figure 3.3A).

Previous studies reported that the full-length proteins of Cdc7 and ASK were highly aggregated in solution and were too low to be purified for enzymatic characterisation (Roberts et al. 1999; Jiang et al. 1999; Kihara et al. 2000). Overexpressed proteins often appear to be insoluble when made in a heterologous host (Ouellette et al. 2003). To achieve good solubility of overexpressed protein, the most important variable conditions are pH and salts in the buffer (Minton & Wilf 1981). The lysis buffer conditions described by *Lindwall et al*. have been developed to encompass many conditions known to preserve protein solubility without repetition (Spare matrix) (Lindwall et al. 2000). The theoretical pIs of Cdc7 and ASK are 8.96 and 8.03, as determined by analysis of the amino acid sequence (http://www.expasy.org/tools/protparam). The high pIs of these proteins suit high pH buffer conditions, which can result in enhanced yields of active, soluble protein. Figure 3.3B shows the solubility of Cdc7 and ASK in buffers at different pH levels. Cdc7 was soluble in buffers at pH 6, 7.2, 8 and 9 (NaCl or KCl), however ASK showed only low solubility at pH 8 and 9. A lower molecular weight band was seen in the Cdc7 soluble fractions in buffers at pH 6, 7.2, 8 and 9 with KCl, but this band disappeared in buffer at pH 9 with NaCl (Figure 3.3B). Cdc7 requires ASK for kinase activity. Hence Cdc7 and ASK were co-expressed in insect cells with different MOIs (multiplicity of infection) to produce the active complex. The pH of the lysis buffers (25 mM Tris-HCl, 250 mM NaCl, 0.5% NP 40, 10% glycerol, 10 mM $MgCl_2$ and 0.02 volumes of complete EDTA-free Protease inhibitor cocktail) was kept constant at

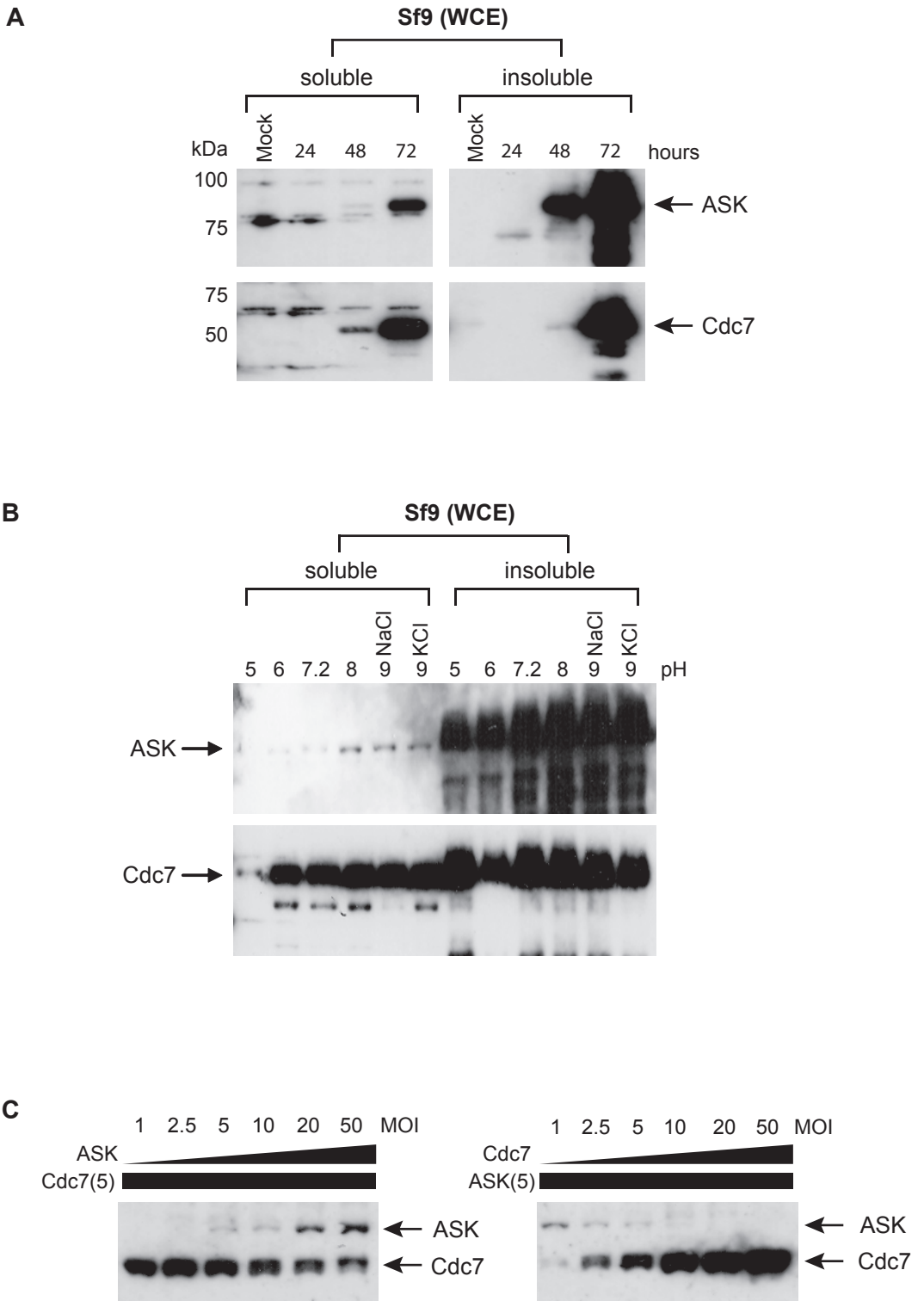


Figure 3.3: Optimisation of recombinant Cdc7 and ASK expression. Sf9 cells were infected with recombinant viruses expressing Cdc7 or ASK and cells were lysed at 24, 48 and 72 hours after infection. (A) Immunoblot analysis of soluble and insoluble fractions from the time course with anti-histidine tag antibody. (B) Immunoblot analysis of soluble and insoluble fractions from lysates prepared at 72 hours with lysis buffer of varying pH with Cdc7 and ASK antibodies. (C) Immunoblot analysis of Cdc7 and ASK co-expressing lysates at various MOIs with anti-histidine tag antibody. (WCE - Whole cell extract; Mock - Mock-infected cell; MOI-Multiplicities of infection)

pH 9.0. Similar amounts of soluble Cdc7/ASK complex were produced using Cdc7 at MOI 5 and ASK at MOIs of 20 and 50 (Figure 3.3C).

After optimisation of small-scale Cdc7 and ASK protein expression, the next stage was large-scale protein expression of recombinant Cdc7/ASK kinase complex. Cdc7 and ASK proteins containing six-histidine tags at the N-terminus were purified using immobilized metal affinity chromatography (IMAC) as shown in Figures 3.4 and 3.5. Weakly bound proteins were eluted at low imidazole concentrations, whereas both Cdc7 and ASK were eluted at imidazole concentrations between 60 mM and 120 mM (20%-40%). After nickel affinity purification, Coomassie blue staining and western blot analysis of the eluted protein fractions showed single bands with apparent molecular weights of ~64 kDa for His-tagged Cdc7 (Figure 3.4B and C) and ~77 kDa for His-tagged ASK (Figure 3.5B and C).

Cdc7/ASK kinase complex was purified as described for Cdc7 and ASK proteins. The complex was eluted by increasing the concentration of imidazole in lysis buffer (25 mM Tris-HCl pH 9, 250 mM NaCl, 0.5% NP 40, 10% glycerol, 10 mM $MgCl_2$) (Figure 3.6A). Coomassie blue stained gels show the 64 kDa His-tagged Cdc7 and the 77 kDa His-ASK proteins after nickel-affinity purification (Figure 3.6B). Western blot analysis shows that the Cdc7 and ASK protein bands from co-infected cells have lower electrophoretic mobility than those from separate infections. To determine whether this difference was due to phosphorylation, the Cdc7/ASK complex was treated with λ-phosphatase prior to loading onto the gel. After treatment, the bands corresponding to Cdc7 and ASK were detected at similar sizes compared to when Cdc7 and ASK proteins were expressed alone (Figure 3.6C). Interestingly, a stronger

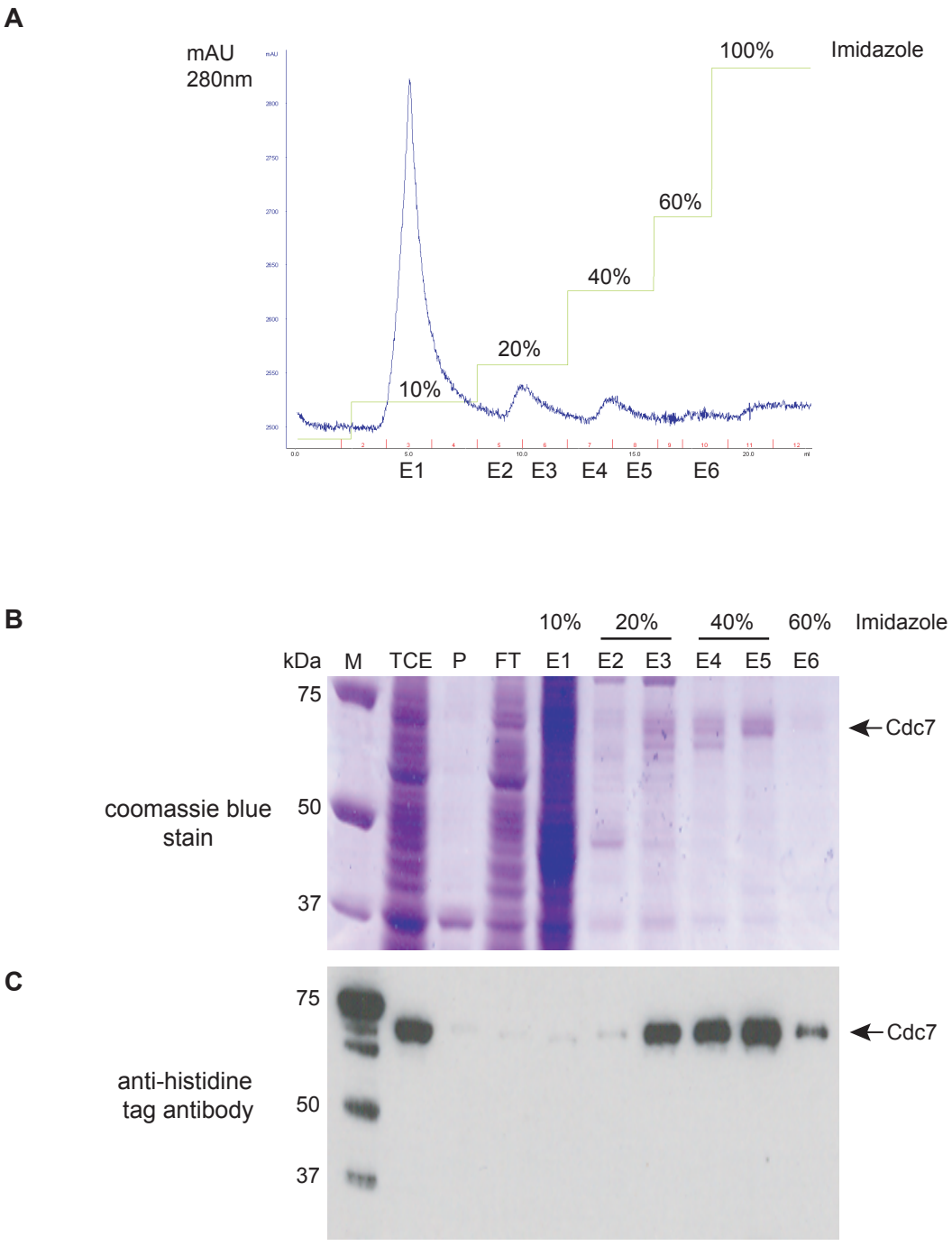


Figure 3.4: Expression and purification of His-tagged human Cdc7. (A) Elution profile obtained from nickel-affinity chromatography and (B) coomassie stained gel showing recombinant Cdc7 protein elution fractions. (C) Purified Cdc7 protein was immunoblotted with a anti-histidine tag antibody. (WCE - Whole cell extract; P - Cell pellet; E1/2/3/4/5/6 - Elution fractions).

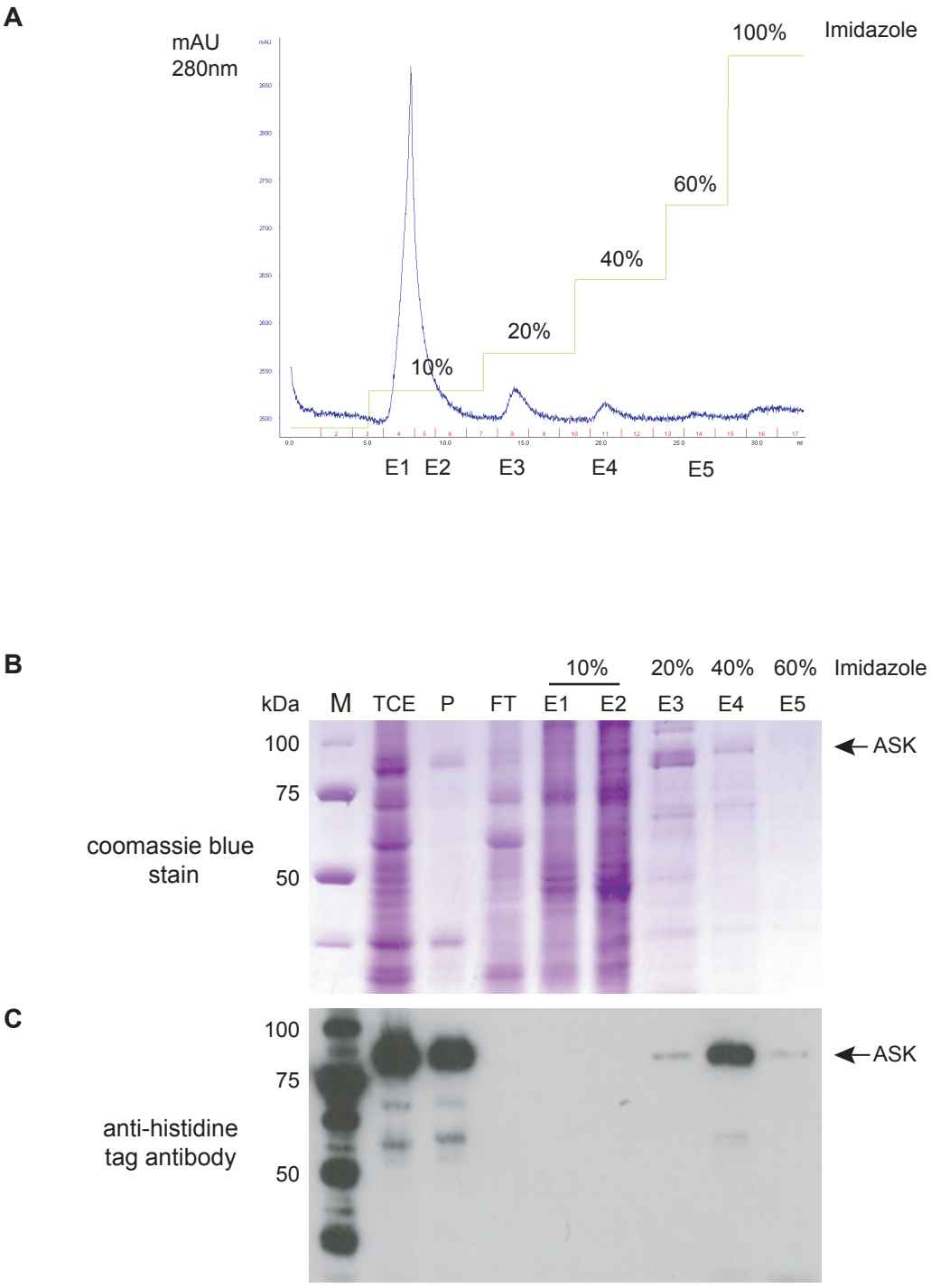


Figure 3.5: Expression and purification of His-tagged human ASK. (A) Elution profile obtained from nickel-affinity chromatography and (B) coomassie stained gel showing recombinant ASK protein elution fractions. (C) Purified ASK protein was immunoblotted with a anti-histidine tag antibody. (WCE - Whole cell extract; P - Cell pellet; E1/2/3/4/5/6 - Elution fractions).

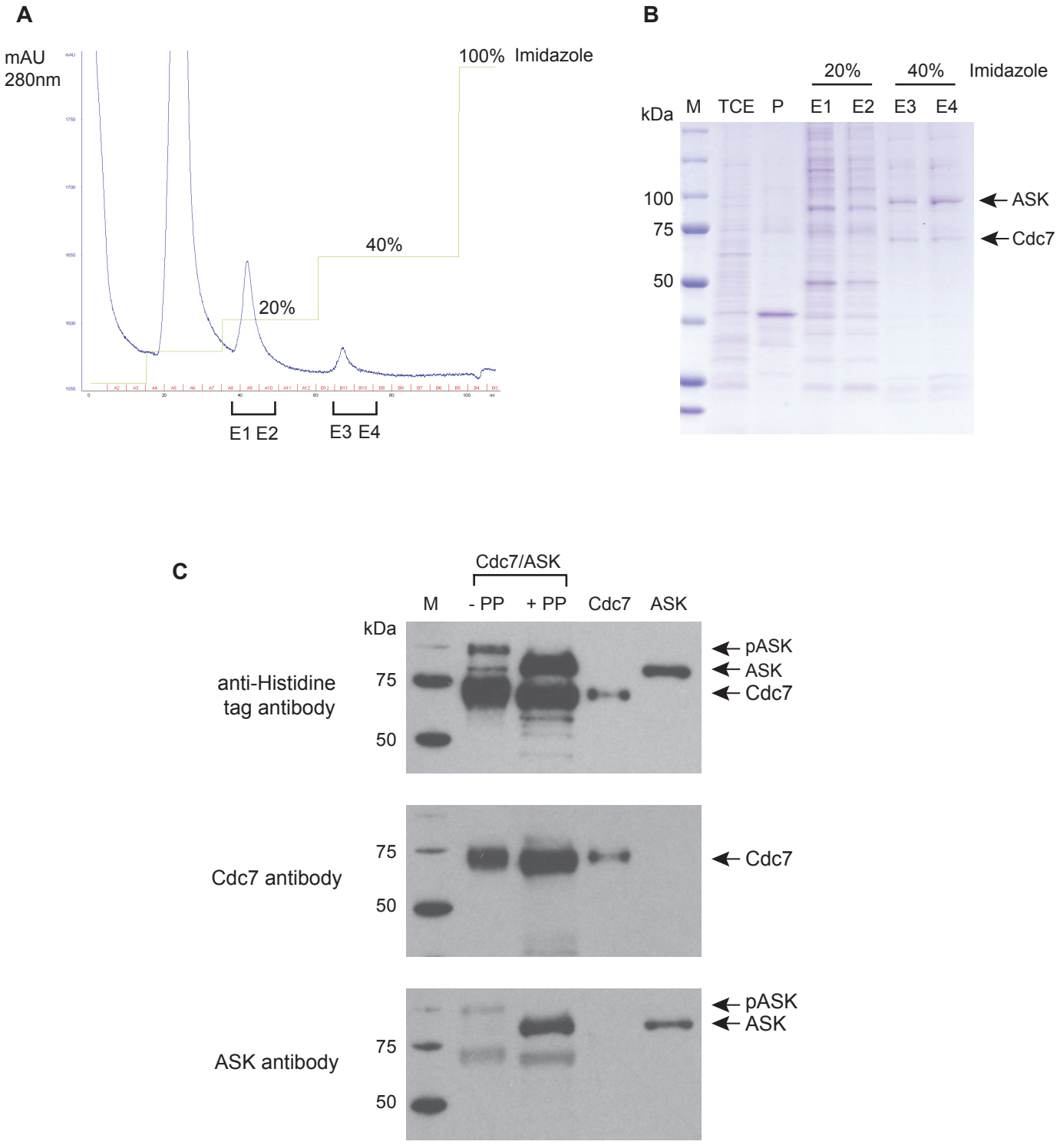


Figure 3.6: Expression and purification of His-tagged human Cdc7/ASK kinase complex. (A) Elution profile obtained from nickel-affinity chromatography and (B) coomassie stained gel showing Cdc7/ASK kinase elution fractions (WCE - Whole cell extract; P - Cell pellet; E1/2/3/4 - Elution fractions). (C) Purified Cdc7/ASK complex, Cdc7 and ASK were immunoblotted with histidine tag, Cdc7 and ASK antibodies. The Cdc7/ASK complex was treated with λ-phosphatase prior to electrophoresis to reverse phosphorylation, which causes a change in the migration of the protein band on the gel (PP - λ-phosphatase; pASK - phosphorylated ASK).

immunoreaction with the ASK band was observed with an anti-histidine tag antibody after dephosphorylation by λ-phosphatase treatment compared with no treatment. Notably, as observed by others for Cdc7/ASK co-expression (Jiang et al., 1999; Masai et al., 2000), these phosphorylation events are dependent on the presence of ASK, which is required for the kinase activity of Cdc7 in mammalian cells (Kumagai et al., 1999). Protein assays after purification showed concentrations of 0.6 mg/ml Cdc7 for infection with single virus expressing His-tagged Cdc7, 0.5 mg/ml ASK for infection with single virus expressing His-tagged ASK, and 1.1 mg/ml Cdc7/ASK kinase complex for co-infection with CDC7 and ASK viruses.

3.3.3. Generation of Cdc7 and ASK antibodies

A range of antibodies raised against synthetic polypeptides derived from the Cdc7 kinase catalytic and ASK regulatory subunit have been reported (Jiang & Hunter 1997; Jiang et al. 1999; Kumagai et al. 1999). In the work of this thesis, synthetic polypeptides corresponding to the C-terminal residues of Cdc7 and ASK (amino acids 557-574 and amino acids 644-663, respectively), which have been published (Jiang & Hunter 1997; Kumagai et al. 1999) , and additional synthetic polypeptides derived from Cdc7 and ASK (amino acids 284-298 and amino acids 112-129, respectively) were used for generation of Cdc7 and ASK antibodies following a standard immunisation protocol (Eurogentech, Seraing, Belgium) (Figure 3.7A). In western blot analysis of Cdc7 and ASK recombinant proteins, Cdc7 antibodies (#33576 and #33577) detected a single band of the reported molecular weight for Cdc7 (~ 64 kDa) (Figure 3.7B). ASK antibodies (#33578 and #33579) also detected a band of the reported molecular weights of ASK (~ 77 kDa), however antibody #33579 showed a stronger reaction than #33578 when applied at the same dilution (1:200 dilution)

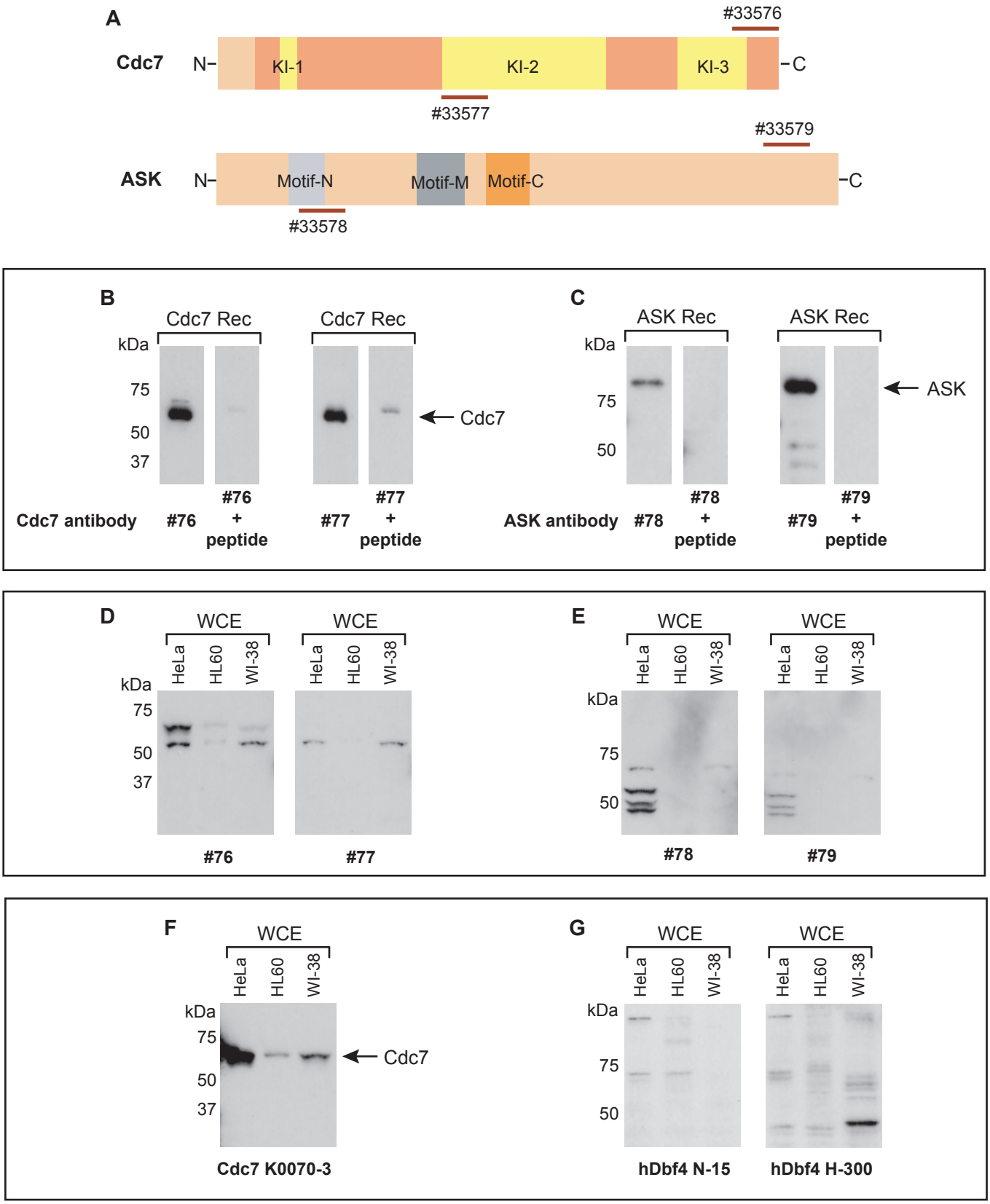


Figure 3.7: Generation of Cdc7 and ASK antibodies. (A) Cdc7 and ASK antibodies were raised in rabbits following immunisation with synthetic peptides #33576 (RITAEEALLHPFFKDMSL, aa557-574), #33577 (RSVFGERNFNIHSSI, aa284-298), #33578 (NVLDIWEEENSDNLLTAFF, aa112-129) and #33579 (YTAETTSPHPSHDGSSF, aa644-663). Immunoblot analysis of recombinant Cdc7 and ASK protein with (B) Cdc7 and (C) ASK antibodies. Pre-incubation of Cdc7 and ASK antibodies with peptides blocks detection of Cdc7 and ASK recombinant protein. Immunoblot analysis of asynchronous HeLa, HL60 and WI-38 whole cell lysates with affinity-purified (D) Cdc7 antibodies (#76 and #77), (E) ASK antibodies (#78 and #79), (F) Cdc7 K0070-3 antibody from MBL and (G) human Dbf4 antibodies (hDbf4 N-15 and hDbf4 H-300) from Sata Cruz Biothechnology. (Rec - Recombinant protein; WCE - Whole cell extract)

(Figure 3.7C). Detection of these bands was blocked when the antibodies were pre-incubated with the peptides used for immunisation (Figure 3.7B and C). Although both Cdc7 antibodies specifically recognised recombinant protein, antibody #33576 detected two bands in HeLa, HL60 and WI-38 whole cell extracts, whereas #33577 detected only a faster-migrating band in HeLa and WI-38 cell extracts. The ASK antibodies (#33578 and #33579) did not recognise a band corresponding to the molecular weight of the ASK protein in whole cell extracts (Figure 3.7C).

At the time when these experiments were conducted, a mouse monoclonal Cdc7 antibody that recognises mammalian Cdc7 became available through MBL (Nagoya, Japan). This antibody detects a 64 kDa single band corresponding to Cdc7 protein in HeLa, HL60 and WI-38 cell extracts (Figure 3.7 F). However, commercially available ASK antibodies that specifically detect ASK protein in human cell extracts were not identified (Figure 3.7 G). Thus additional immunizations with a different ASK-derived antigen were required. The characterisation of various mutant derivatives of ASK (Sato et al. 2003) enabled me to divide the ASK protein into functional domains. In order to express the fragments of ASK protein for immunization, I constructed a pair of N-terminal and C-terminal truncation fragments of ASK (Figure 3.8A) and expressed them in *E.coli* (Rosetta2 pLysS). However, both ASK truncated fragments were found in the insoluble fraction (data not shown). Experiments were undertaken to purify the ASK fragments by denaturation and renaturation (see Material and Methods). Coomassie blue staining showed the eluted fractions of both ASK fragments which recovered in the soluble fraction and were renatured using an on-column refolding technique (Figure 3.8B and C). To generate a rabbit polyclonal antibody against ASK, both fragments were used separately as antigens. However, on-

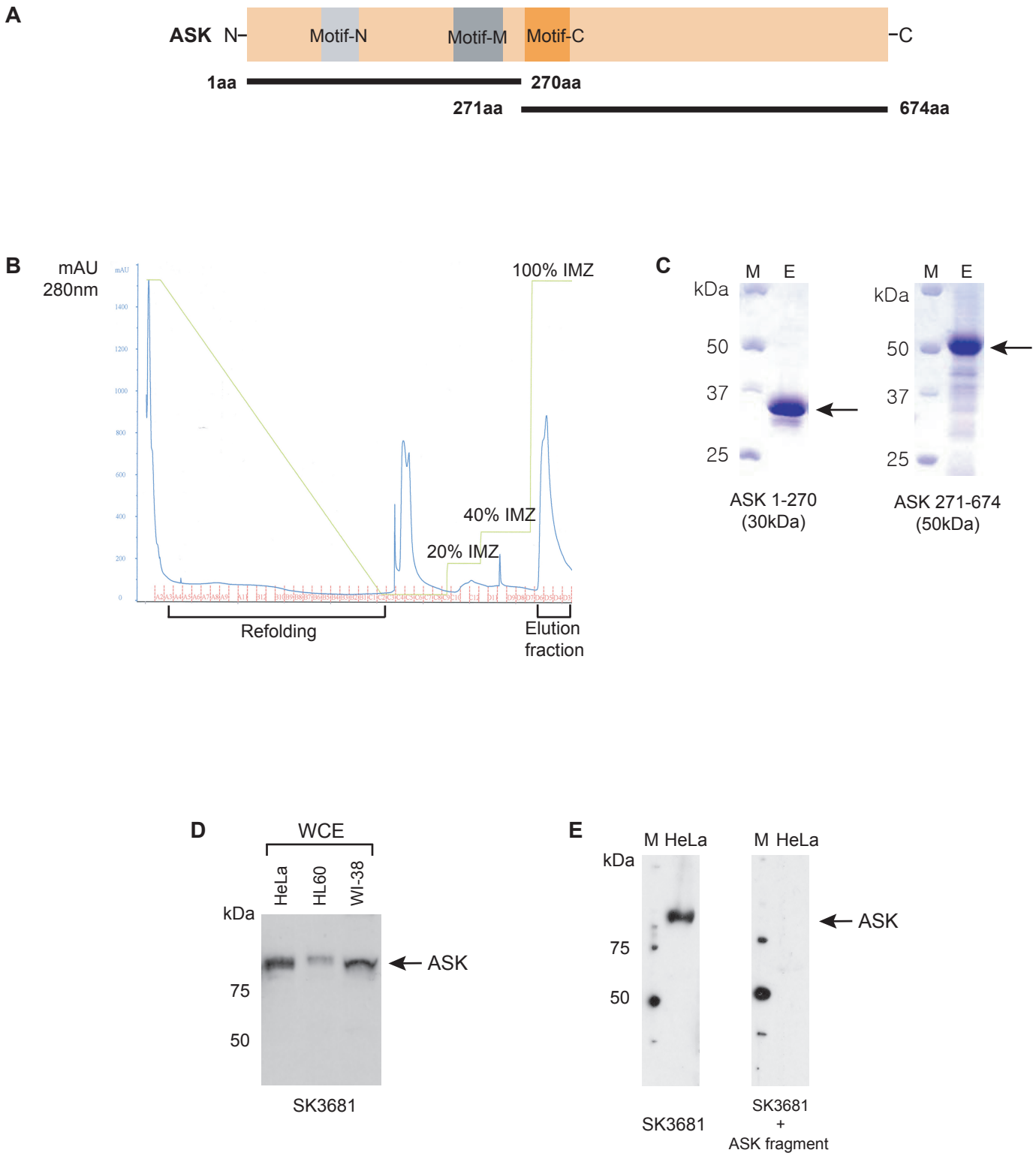


Figure 3.8: Generation of ASK antibody. (A) Schematic drawing of ASK fragments. (B) Chromatogram obtained from on-column refolding and nickel-affinity purification. (C) Coomassie stained gel showing ASK fragments (ASK 1-270 and ASK 271-674) from 100%IMZ elution fractions. (D) Immunoblot analysis of asynchronous HeLa, HL60 and WI-38 whole cell lysates with affinity-purified ASK antibody (SK3681). (E) Preincubation of ASK antibody (SK3681) with recombinant ASK fragment blocks detection of endogenous ASK from HeLa total cell lysates. (IMZ - Imidazole; M - Marker; E - Elution fraction; WCE - Whole cell extract).

ly polyclonal antibody (#SK3681) raised against the C-terminal 403 amino acids of the ASK fragment which contains motif-C (aa 271-674) detected a single band corresponding to ASK protein. In western blot analysis of whole cell extracts from asynchronously proliferating HeLa, HL60 and WI-38 cells, affinity-purified anti-ASK antibody (#SK3681) detected a single band of the reported molecular weight for ASK (~77 kDa) (Figure 3.8D). This band was not detected when the ASK antibody was pre-incubated with the recombinant C-terminal ASK fragment (1:25 dilution) (Figure 3.8E).

ASK antibody #SK3681 was reciprocally tested for its ability to co-immunoprecipitate Cdc7/ASK complex from HeLa whole cell extracts with the commercially available Cdc7 mouse monoclonal antibody. The ASK antibody co-precipitated Cdc7, as detected by immunoblotting with the Cdc7 antibody, and conversely the Cdc7 antibody co-precipitated ASK protein (Figure 3.9A). These data demonstrate that Cdc7 and ASK form a stable complex in human cells and that the newly generated ASK antibody can be used to precipitate the endogenous Cdc7/ASK complex from HeLa cells. In addition, the immunoprecipitates were treated with λ-phosphatase prior to western blotting. Immunoblot analysis shows that ASK is highly phosphorylated when associated with Cdc7 as a complex in HeLa cells (Figure 3.9B), consistent with Cdc7/ASK complexes co-expressed in insect cells (Figure 3.6C).

3.4. Discussion and Conclusions

The initiation of DNA replication is highly regulated in eukaryotic cells and involves a large set of proteins. Identification and characterization of DNA replication factors that assemble as complexes at replication origins in yeast and *Xenopus* have advanced

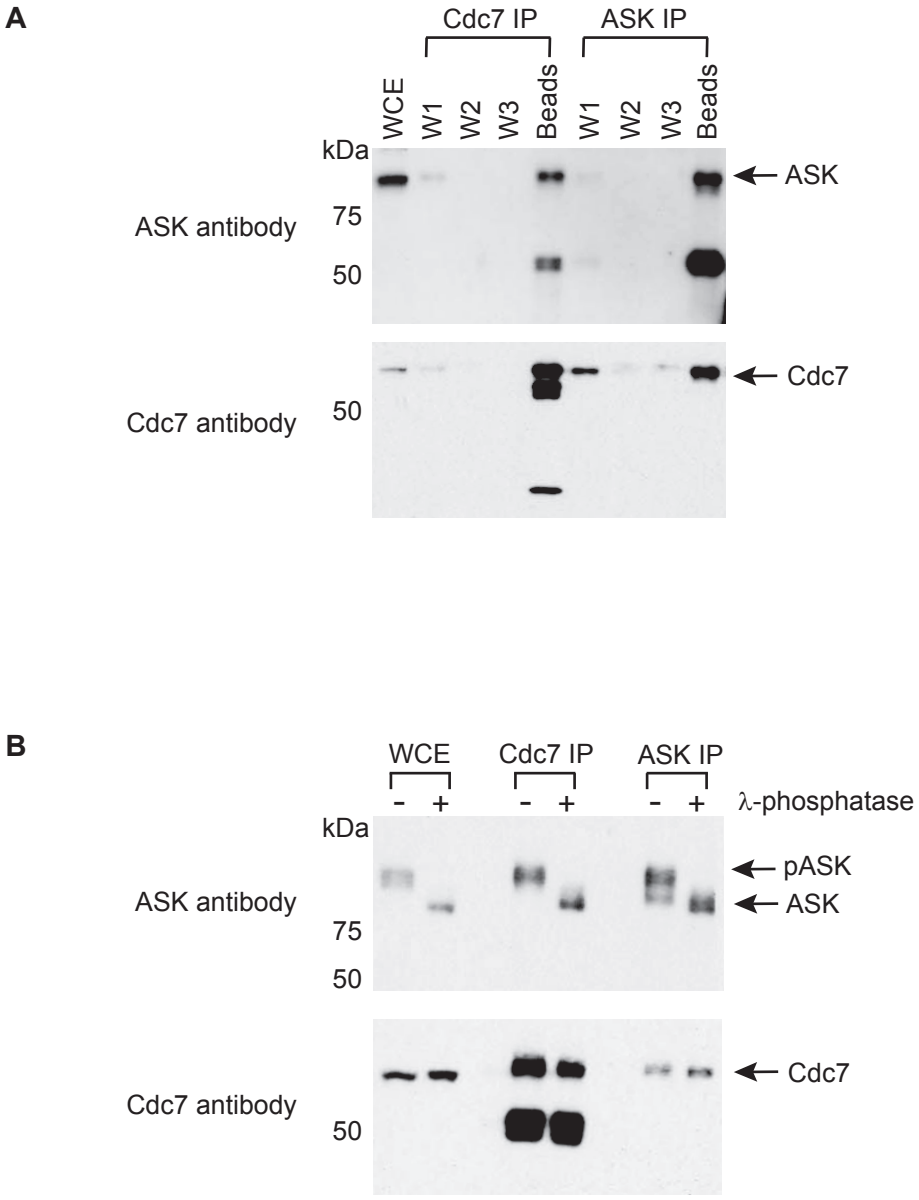


Figure 3.9: Characterisation of ASK antibody (SK3681). (A) Immunoprecipitation (IP) of HeLa cell lysates with ASK and Cdc7 antibodies followed by western blot analysis with ASK antibody (upper panel) or Cdc7 antibody (lower panel) (WCE - Whole cell extract; W1/2/3 - Wash fractions; Beads - Protein A-sepharose complex with antibody-antigen). (B) λ-phosphatase treatment of immunoprecipitates followed by immunoblot analysis with ASK antibody (upper panel) or Cdc7 antibody (lower panel) (pASK - phospholyated ASK).

our understanding of the biochemical mechanisms of the DNA replication initiation process. Despite characterisation of the biochemical and functional role of the Cdc7/Dbf4 kinase in yeast and *Xenopus* systems, our understanding of this important cell cycle regulator in mammalian cells is still relatively limited. Therefore, in the work described in this chapter, I have focused on generating the molecular tools essential for a biochemical and functional characterization of Cdc7/ASK kinase complex in mammalian cells which is described in the following chapters.

To this end, I produced recombinant human Cdc7/ASK kinase complex by co-expression in insect cells. Analysis of purified Cdc7/ASK kinase complex allowed a characterisation of the phosphorylation state when the two proteins form a complex and an investigation of the interaction between the catalytic subunit Cdc7 and its regulatory subunit ASK (Figure 3.6). It has been reported that co-expression of ASK is required for kinase activity of Cdc7 in transient transfection assays performed in mammalian cells (Kumagai et al., 1999). These observations are consistent with my *in vitro* findings (Figure 3.6C) using purified Cdc7/ASK kinase complex discussed in this chapter. The active Cdc7/ASK kinase complex was essential for the development of a primary screening assay for Cdc7 small molecule inhibitors, which is discussed in Chapter 5. Affinity-purified rabbit polyclonal antibodies for Cdc7 and ASK protein were raised against synthetic polypeptides (Figure 3.7A) derived from Cdc7 and ASK. However, the antibodies did not recognise specific bands for Cdc7 and ASK in human cell extracts. A commercial Cdc7-specific monoclonal antibody was thereafter identified and used for further experiments. A polyclonal ASK antibody (#SK3681) was raised against a C-terminal fragment of ASK (Figure 3.8A). The data discussed in this chapter show that the ASK antibody (#SK3681) is specific for endogenous ASK

in human cell extracts, detecting a protein with a molecular weight corresponding to the reported size of human ASK. These antibodies were essential for investigations of the causal molecular events in cell-cycle-dependent regulation of DNA replication in human cells.

Taken together, the data presented in this chapter demonstrate that the molecular tools required for the work described in the following chapters, including affinity-purified ASK antibody and kinase active Cdc7/ASK complex, were successfully generated. These molecular tools were utilised for an analysis of the functional role of Cdc7/ASK in human cells and the development of a sensitive screening assay for Cdc7 kinase activity, which is essential at an early stage in the drug development process. Furthermore, these tools were utilised to gain a better understanding of the role of Cdc7/ASK in human cell proliferation, thereby providing important target validation for exploitation of this kinase as a potential therapeutic target. In the following chapter, I discuss the application of these antibodies to *in vitro* tissue culture model systems of proliferation, quiescence and terminal differentiation established in our laboratory (Stoeber et al. 2001; Kingsbury et al. 2005), and to normal human tissues (self-renewing, stable and permanent) and tumours. By analyzing the regulation and activity of Cdc7 kinase *in vitro* and *in vivo*, I studied the causal molecular events that underlie replication competence during the cell cycle and loss of replicative capacity in out-of-cycle states.

CHAPTER FOUR

Cell cycle dependent regulation of Cdc7/ASK kinase complex in proliferating cells and out-of-cycle states

4.1. Introduction

The eukaryotic DNA replication initiation pathway is tightly controlled through the assembly of pre-replicative complexes (pre-RCs) and the activation of replication origins to ensure correct duplication of the genome during cell division (see also Chapter 1, Section 1.5). Cdc7/ASK is an essential kinase required for origin activation through initiating by phosphorylation a cascade of protein associations that activate the Mcm2-7 replicative helicase (reviewed in Sclafani & Holzen 2007; Swords et al. 2010). Similar to cyclin-dependent kinase activation, Cdc7 kinase is activated by the binding of two regulatory subunits, ASK/Dbf4 which is well understood, and Drf1 that comparatively lesser known (Jiang et al. 1999; Kumagai et al. 1999; Montagnoli et al. 2002; Takahashi & Walter 2005; Yoshizawa-Sugata et al. 2005).

The levels of Cdc7 mRNA and protein are relatively constant during the cell cycle, however expression of the regulatory subunits are cell cycle dependent (reviewed in Swords et al. 2010) (Figure 4.1A). The ASK mRNA and protein levels are very low in early G1 phase and high at G1/S boundary throughout S phase, whereas Drf1 transcription and protein levels increase at late S phase to G2/M phase (Jiang et al. 1999; Kumagai et al. 1999; Ferreira et al. 2000; Yoshizawa-Sugata et al. 2005). Since Drf1 has been identified only in *Xenopus* and human cells, the presence of two regulatory subunits, ASK/Dbf4 and Drf1, suggest a new level of complexity in the re-

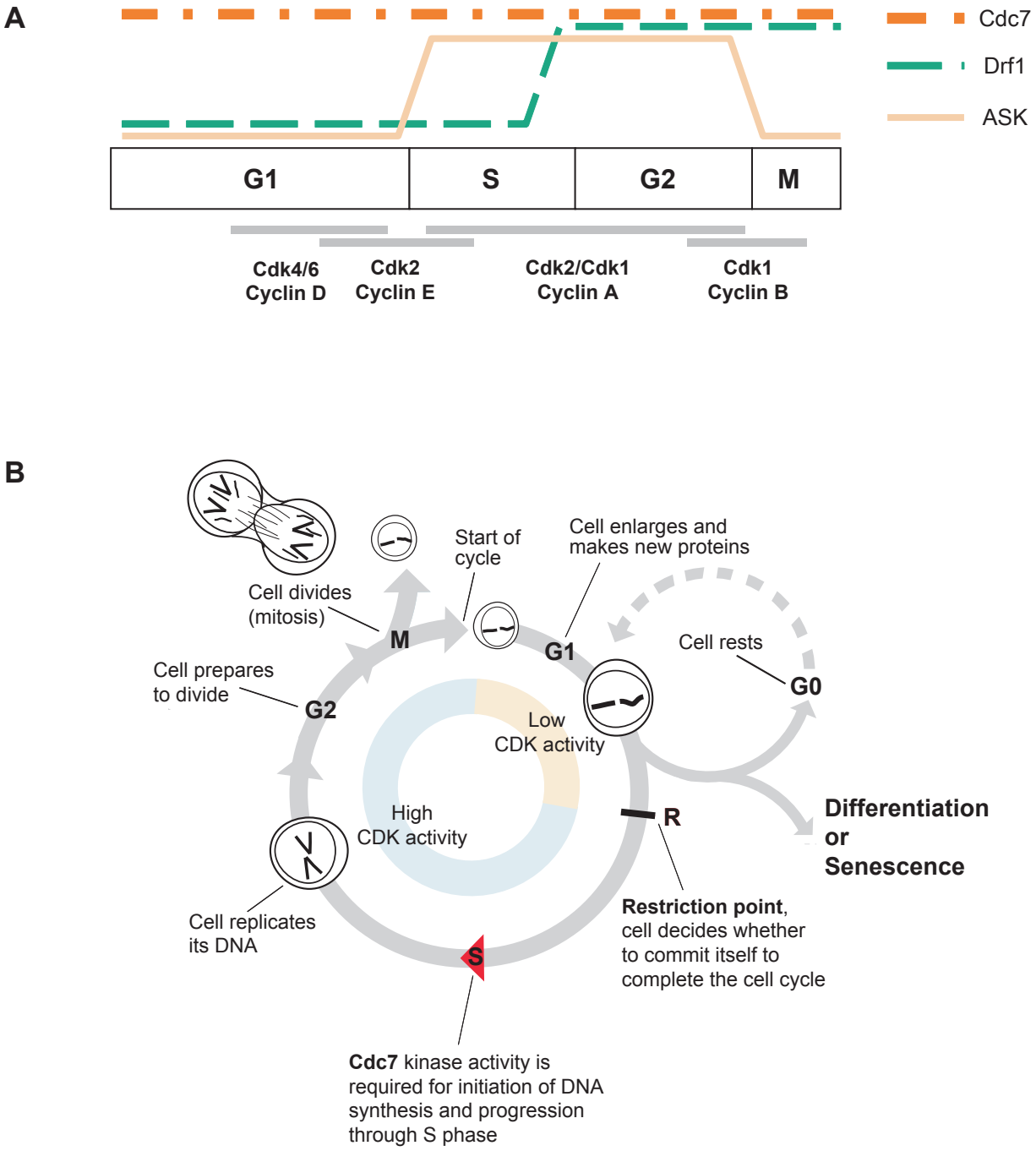


Figure 4.1: (A) Cell cycle regulation of Cdc7, ASK and Drf1 protein levels. Cdc7 protein levels do not change during the cell cycle. Levels of ASK increase in late G1, staying high during S and G2 phases and decrease in M phase. Drf1 protein levels increase in late S, staying high at G2 and M phases. As cells proceed through the cycle, four major cyclins are produced sequentially (cyclins D, E, A and B), that activate CDKs. Cyclin D starts accumulating at mid G1, whereas cyclin E appears at the G1/S transition. Progression through S phase requires cyclin A. Cyclin B associates with Cdk1 to trigger mitosis. **(B) Diagrammatic representation of the mitotic cell division cycle.** In passing through the cell cycle a newborn eukaryotic cell first passes through G1 phase, then S phase (period of DNA synthesis), then G2 phase (period of preparing to divide), with division occurring at M (Mitosis). G0 is the quiescent state wherein cells are resting. Upon stimulation with growth factors, cells leave G0 and enter the G1 phase. Cells can withdraw irreversibly from the cell cycle through the process of terminal differentiation or replicative senescence. (G0 - quiescent cells; G1, G2 - gap phase 1, 2; S - DNA synthesis; M - mitosis)

gulation of DNA replication in vertebrates (Masai et al 2002; Yanow et al. 2003; Yoshizawa-Sugata et al. 2005). In other words, different Cdc7 kinase complexes might be responsible for the temporal activation of origins through early and late firing (Masai et al. 2002; Yoshizawa-Sugata et al. 2005).

Previous studies have shown that the expression of CDC7 and ASK mRNA is generally at a high level in most cancer cell lines, except for lung cancer cell line A549 (Hess et al. 1998; Kumagai et al.1999). Cdc7 kinase activity is upregulated in melanoma cell lines with defects in the DNA damage G1 checkpoint (Kaufmann et al. 2008). Furthermore, *Nambiar et al*. identified ASK/Dbf4 as a key factor in melanoma development using oligonucleotide microarray-based screening (Nambiar et al. 2007), suggesting that alterations in Cdc7/ASK protein abundance or activity may occur during tumorigenesis. A recent study showed that expression levels of Cdc7 and ASK proteins increased in 50% of the NCI-60 cell lines and additional leukemia cell lines (Bonte et al. 2008). It also showed a high correlation between mutated p53 and increased Cdc7 and ASK expression levels in primary breast cancer cell lines, suggesting that p53 may directly represses the *CDC7* and *ASK* gene, or that p53 mutations activate *CDC7* and *ASK* gene expression (Bonte et al. 2008). However, on the whole the role of Cdc7/ASK kinase and the regulation of its activity in tumorigenesis is not fully understood.

Given the essential role of Cdc7/ASK kinase in DNA metabolism, including replication initiation, chromosomal segregation and checkpoint pathways, and increased expression levels in most cancer cells, Cdc7 kinase has been pursued as an attractive target for the therapeutic intervention. Particularly, several reports studied

the cellular responses of human cells to loss or inhibition of Cdc7 kinase and showed that inhibition of Cdc7 kinase may provoke a differential response in normal and transformed cell lines, causing cancer-cell-specific killing (Montagnoli et al. 2004; Yoshizaw-Sugata et al. 2005; Im & Lee 2008; Kim et al. 2008; Montagnoli et al 2008). These *in vitro* findings so far have provided a strong rationale for Cdc7/ASK kinase as an attractive anti-cancer target.

In mammalian tissues the majority of cells have exited the cell cycle and have arrested in out-of-cycle states, either temporally withdrawing into the quiescent state or permanently withdrawing from the cell cycle through the processes of terminal differentiation or replicative senescence (Pardee 1989; Hayflick & Moorhead 1961: Campisi 1996; Myster & Duronio 2000). In order to evaluate Cdc7/ASK as a novel target for anti-cancer drug development, it is therefore essential to understand regulation of the Cdc7/ASK kinase complex during establishment of the quiescent (G0) and differentiated out-of-cycle states, and during re-entry from quiescence into the cell cycle. To address these important issues, I used *in vitro* tissue culture model systems that were established to enable generation of a detailed molecular map of the replication initiation machinery in cycling cells, in quiescent or differentiated out-of-cycle states, and during re-entry from quiescence into the cell cycle (Figure 4.1 B).

In vitro and *in vivo* studies have shown that the Mcm2-7 subunits are a major substrate of Cdc7/ASK, and that Cdc7/ASK can phosphorylate several MCM subunits with some preference for the Mcm2 subunit (Lei et al., 1997; Sato et al., 1997; Montagnoli et al. 2006; Tsuji et al. 2006; Cho et al., 2006; Chuang et al. 2009). High resolution mass-spectrometric analysis of the N-terminal region of Mcm2 revealed six

phosphorylation sites for Cdc7/ASK, and these phosphorylated sites were confirmed using phospho-specific antibodies in cancer cells (Montagnoli et al. 2006; Charych et al. 2008). Furthermore, depletion of Cdc7 caused a decrease in the levels of Mcm2 phosphorylation at serine 40 and serine 53, suggesting that Mcm2 phosphorylated at serine 40 and serine 53 is completely dependent on Cdc7/ASK activity in human cells (Montagnoli et al. 2006; Tenca et al. 2007). Therefore, to determine the role of the Cdc7/ASK kinase complex in activating DNA replication origins in human tissues, in this chapter I discuss the study of Cdc7 expression dynamics and its activity as determined by Mcm2 phosphorylation in self-renewing and conditional renewal tissues to investigate the growth regulatory strategies in normal and cancerous human tissues. The aim of these studies was to provide further target validation for Cdc7 kinase as a novel theranostics target.

4.2. Material and Methods

For antibodies (2.5.1.), preparation of whole cell extracts (2.5.2.), immunoblotting (2.5.3.), immunoprecipitation (2.5.4.), immunohistochemistry (2.5.5.), cell culture and synchronisation (2.6.1.), bivariate flow cytometric analysis (2.6.2), monitoring differentiation (2.6.3.), tissue specimens (2.6.5.) and assaying for cell differentiation please refer to Chapter Two (Materials and Methods).

4.3. Results

Characterization of pre-RC/pre-IC regulation, and in particular the role of Cdc7/ASK kinase, in an *in vitro* tissue culture model system is essential before progressing to human tissue studies to understand growth regulatory mechanisms that often operate

strictly in normal cells but are defective in tumour cells. To generate a molecular picture of pre-RC assembly during the cell cycle, exit into G0, re-entry from quiescence, and in the differentiated state, and to study the role of Cdc7/ASK kinase in activating assembled pre-RC to form pre-initiation complex (pre-IC) in these processes, I prepared whole cell extracts at various time points following cell cycle arrest, contact inhibition, release from contact inhibition and during 1,25 dihydroxyvitamin D_3 (1,25 Vit D_3) induced differentiation of HL60 cells.

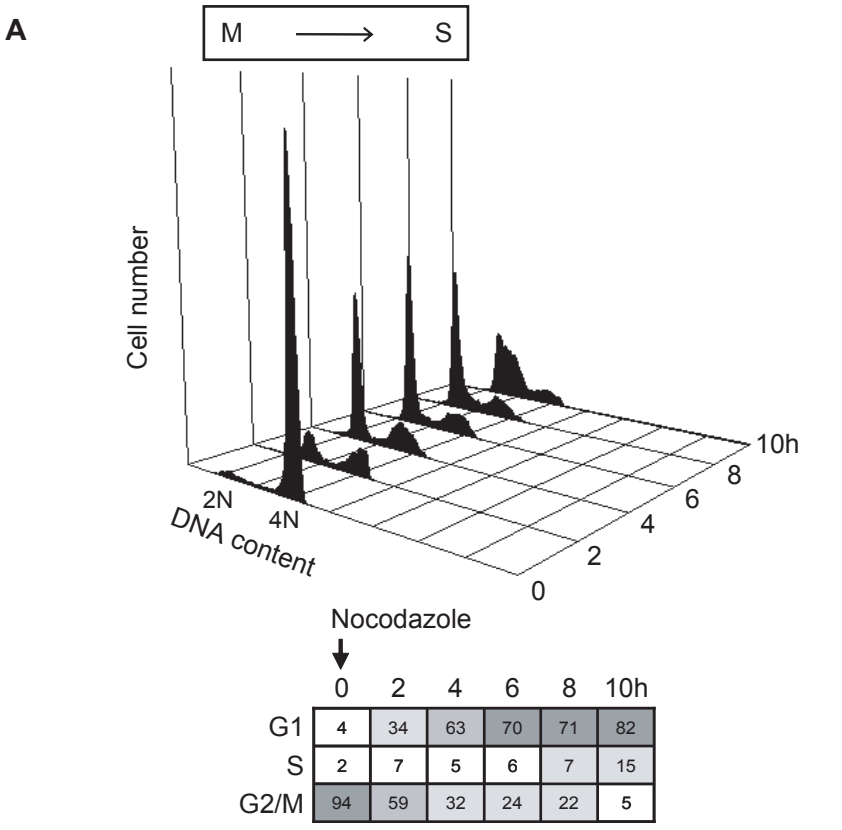
4.3.1. Expression dynamics and regulation of Cdc7/ASK kinase in cycling cells

Chemical cell synchronization methods such as double-thymidine block or hydroxyurea treatment affect DNA synthesis and arrest cells at the G1/S border (Puck 1964; Stubblefield et al 1967). Thymidine is an inhibitor of the deoxyribonucleotide triphosphates. Excess thymidine in the medium results in the inhibition of DNA synthesis. Cells in the S phase remain blocked in this phase, while cells that are outside at S phase continue their route until being blocked at the G1/S boundary. The thymidine excess is then removed, and the cells are released. A second thymidine blockade then gives final cell synchronization at the end of G1. These methods generally do not work for cell lines with intact p53 apoptotic responses, but can be used to produce highly synchronized populations of p53 deficient cell lines such as HeLa and U2-OS (Pederson & Robbins 1971). Nocodazole, a microtubule inhibitor, is commonly used to arrest cells at mitosis (Sentein 1977). In the work of this thesis, I chose HeLa S3 cells to perform analysis of Cdc7/ASK regulation in cycling cells. Cells at various stages of S and G2/M phases were obtained by releasing cells arrested at the G1/S boundary by a double thymidine block (see Material and Methods). For synchronization in mitosis, thymidine arrested cells were released and treated with

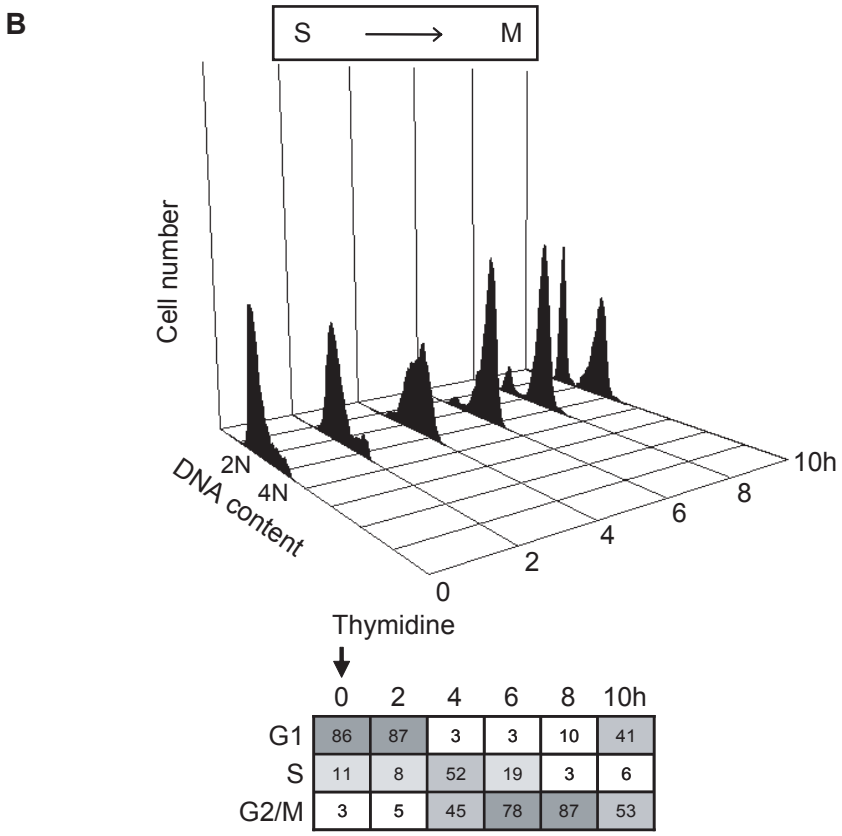
nocodazole to arrest them in mitosis (see Material and Methods). Following release, these cells divided synchronously and progressed as a cohort through the sequential stages of the cell cycle.

Cell cycle distribution of synchronized HeLa cell populations was determined by flow cytometry (Figure 4.2). Flow cytometric analysis of DNA content shows that most of cells (94%) were arrested in mitosis by blocking in nocodazole after thymidine arrest. After release from the nocodazole block, cells were divided synchronously within 2 hours of release from the arrest, more than half of the original M phase cells (63%) moved into G1 phase 4 hours after release and cells began to enter S phase by 10 hours after release. Upon release from thymidine block, cells progressed into S phase 4 hours after release, the majority of cells (78%) reached G2/M phase by 6 hours, and 41% of cells entered G1 phase 10 hours after release from thymidine block (Figure 4.2).

To generate detailed molecular maps of pre-RC constituents and to study the role of Cdc7/ASK kinase in regulating pre-RC to pre-IC transition during the mitotic division cell cycle, whole cell extracts from synchronised HeLa cells were immunoblotted with antibodies against human Orc4, Cdc6, Cdt1, Mcm2, Mcm2 phosphorylated at serine 40/41 (pMcm2 S40/41), Mcm2 phosphorylated at serine 53 (pMcm2 S53), Cdc45, Mcm10, Cdc7, ASK, cyclin A, cyclin E and the cytoskeletal filamentous protein β-actin to control for protein level (Figure 4.3A, also see Material and Methods). Orc4 and Mcm2 were detectable throughout the cell cycle. Notably, levels of pMcm2 S40/41 was present as a fast-migrating form at G2/M, but on entry into G1, the protein was converted into a slow-migrating form. With the onset of S phase, the



	0	2	4	6	8	10h
G1	4	34	63	70	71	82
S	2	7	5	6	7	15
G2/M	94	59	32	24	22	5



	0	2	4	6	8	10h
G1	86	87	3	3	10	41
S	11	8	52	19	3	6
G2/M	3	5	45	78	87	53

Figure 4.2: Flow cytometric DNA profiles of cycling HeLa cells. (A) Cells were arrested in mitosis by blocking with nocodazole after thymidine arrest. After release from the nocodazole block, cells entered G1 2 hours after release. (B) Cells were arrested at G1/S by a double thymidine block. After release from thymidine arrest, cells started to progress through S phase between 2 and 4 hours, and reached G2/M 6 hours after the release. Tables show the percentage of cells with G1, S, and G2/M DNA content.

slow-migrating form was progressively converted into the fast-migrating form (Figure 4.3A). Phosphorylation analysis of Mcm2 (BM28, the human homologue of the *S. cerevisiae MCM2*) shows both fast and slow-migrating forms of the protein are phosphorylated, and the fast-migrating form of the protein appears to be hyperphosphorylated than the slow-migrating form (Todorov et al. 1995). Although this is opposite to the relationship usually found for phsphorylated proteins, it might be related to the abnormal mobility of Mcm2 in SDS-polyacrylamide gels (Todorov et al. 1994). Cdc6 levels were slightly increased as cells went through late G1 phase (10 hours after release from nocodazole arrest) and remained high throughout S phase. Protein levels of Cdt1 peaked between two and four hours after release from nocodazole arrest and rapidly disappeared at the onset of S phase (Figure 4.3A). As cells were released from the nocodazole block, Cdt1 migrated faster than in nocodazole arrested cells (Figure 4.3A), indicating that Cdt1 accumulates in a modified form in the mitotic block (Nishitani et al. 2001). The origin licensing repressor geminin, required for preventing re-replication through interaction with Cdt1, was present at low levels during G1 with levels increasing during S phase (Eward et al. 2004; Wharton et al. 2004). To indirectly confirm activation of the pre-RC through the phosphorylation status of endogenous Mcm2, I performed western blots with antibodies detecting phosphorylated Mcm2 at serine 40/41 and phosphorlyated Mcm2 at serine 53. The levels of pMcm2 S40/41, Cdc7- and CDK-dependent phosphorylation sites, showed a similar pattern as Mcm2, whereas levels of pMcm2 S53, known to be a target for Cdc7, increased as the cells moved through late G1 and S phase (Figure 4.3A).

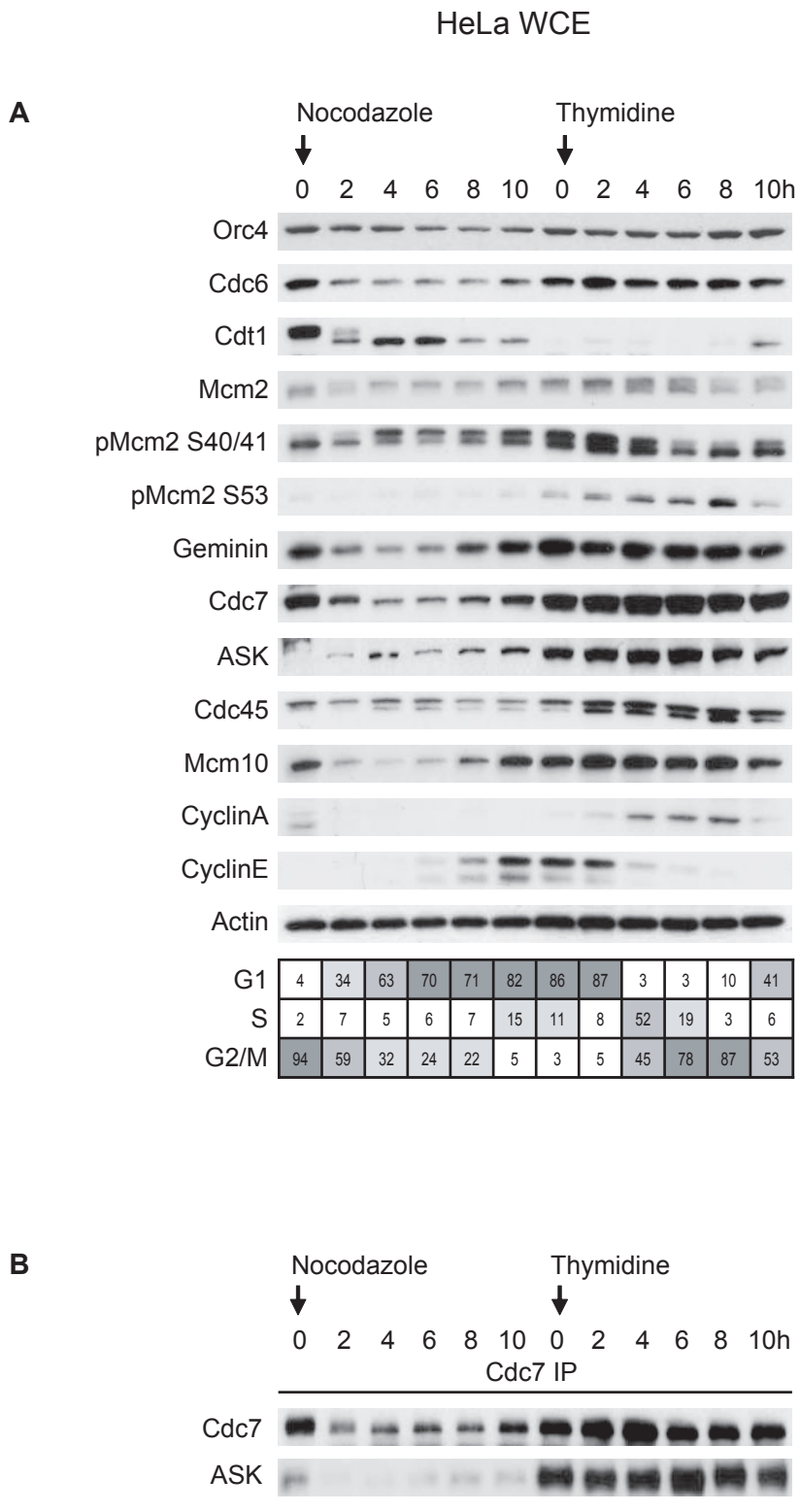


Figure 4.3: Expression dynamics and regulation of DNA replication initiation factors during the cell cycle. (A) Immunoblot analysis of DNA replication initiation factors and loading control (actin) in HeLa whole cell extracts at the indicated times. (B) Immunoblot analysis of Cdc7 and ASK proteins after immunoprecipitation using Cdc7 antibody from HeLa cell extracts. (IP - immunoprecipitation; WCE - Whole cell extract; pMcm2 S40/41 - Mcm2 phosphorylated at serine 40/41; pMcm2 S53 - Mcm2 phosphorylated at serine 53)

It has been reported that protein levels of human Cdc7 are nearly constant and appear to be stable during the cell cycle (Sato et al. 1997; Takeda et al. 1999). However, Figure 4.3A shows that in my experimental system Cdc7 protein levels are low in early G1 phase, increase towards the G1/S boundary and remain high throughout S to G2/M phase. Protein levels of ASK also increased in late G1 phase and stayed high during S phase (Figure 4.3A), suggesting that expression of ASK is regulated during the cell cycle, in keeping with a previous report (Jiang et al. 1999). To determine the formation of Cdc7/ASK complexes during the cell cycle, synchronized HeLa cells were lysed at various stages and the lysates immunoprecipitated with an antibody against Cdc7. Immunoprecipitates were resolved by gel electrophoresis and subsequently immunoblotted with Cdc7 and ASK antibodies (Figure 4.3B). The levels of endogenous Cdc7 and ASK proteins detected in Cdc7 immunoprecipitates were si milar to those detected by western blotting of lysates prepared from cells progressing synchronously though the cell cycle. These results show that levels of Cdc7 and ASK proteins vary during the cell cycle, thus regulating the formation of Cdc7/ASK kinase complex (Figure 4.3B). Consistent with this, Mcm2 phosphorylation on serine 53, which is a Cdc7-dependent phosphorylation site, showed a similar cell cycle dependent variation (Figure 4.3A), indicating that Cdc7 kinase activity during the cell cycle correlates with Cdc7-bound ASK complex levels.

Levels of Cdc45, required for loading of DNA polymerase-α onto chromatin (Mimura & Takisawa 1998; Mimura et al. 2000; Walter & Newport 2000), increased two hours after release from thymidine arrest (Figure 4.3A). Protein levels of Mcm10 were also low in early G1 and increased as cells reached late G1 and progressed though S phase (Figure 4.3A). Cyclin A became detectable in S phase, whereas cyclin E levels peaked

during the G1/S transition and decreased during early S phase (Dulic et al. 1992; Dou et al. 1993; Desdouets et al. 1995). Taken together, these data show that the pre-RC constituents ORC, Cdc6 and Mcm2-7 are present throughout the human proliferative cell cycle, and that the MCM loading factor Cdt1 is regulated at least in part through the controlled expression of geminin. The data also show that cell cycle dependent regulation of Cdc7 activity occurs through association with its regulatory subunit ASK, levels of which increase at the onset of S phase. Moreover, phosphorylation of Mcm2 on serine 53 is dependent on Cdc7/ASK activity and correlates with increased levels of Cdc45 and Mcm10 proteins during S phase progression, demonstrating that Cdc7/ASK kinase activity is essential for S phase progression.

4.3.2. Expression dynamics and regulation of Cdc7/ASK kinase during exit into G0 and re-entry into the cell cycle

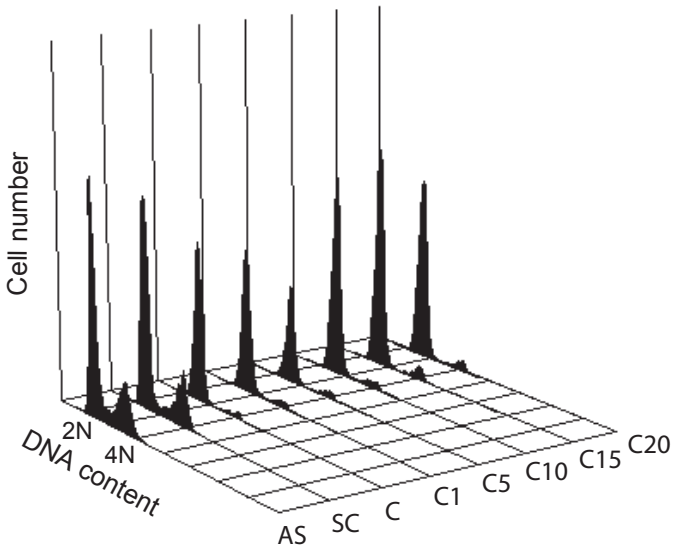
Density dependent growth arrest and serum starvation have been widely used as a synchronization method to arrest cells in the resting, quiescent G0 state. Cultures are allowed to reach confluency, and cells are incubated without medium change. Some cell types, for example several murine fibroblast cell lines (NIH3T3, Balb/c3T3, Swiss3T3) and primary fibroblast cultures (MEF-mouse embryonic fibroblast, HDF-human diploid fibroblast, WI-38 human embryonic fibroblast) can be synchronized in G0 in this way. Arrested cells can be stimulated to re-enter the cell cycle by subculturing with medium containing fresh serum (Pardee 1974). Transformed cell lines typically cannot be synchronized in G0 by these methods, because they have acquired mutations that allow them to grow independent of growth factors (Schorl & Sedivy 2006). To generate a molecular picture of the levels of pre-RC/pre-IC constituents, and to study the regulation of Cdc7/ASK kinase during exit into G0 and

re-entry into cycle, in the work of this thesis I synchronised WI-38 human diploid fibroblasts in G0 by contact-inhibition and subsequently released the cells from quiescence by subculturing at lower density. In the experimental model, cells begin to enter S phase approximately 21 hours after release, as determined by flow cytometry (Stoeber et al. 2001; Kingsbury et al. 2005).

To monitor exit of WI-38 fibroblasts from cycle into quiescence, flow cytometric analysis of DNA content was performed on asynchronously proliferating cells and cells progressively becoming contact-inhibited (Figure 4.4A). Cells began to arrest when contact-inhibited (C) (3% of cells in S phase), and the majority of cells entered a viable state of growth arrest five days after contact-inhibition, confirming that WI-38 fibroblasts were synchronized in quiescence by density-dependent growth arrest. But there is a subpopulation (14-17%) of cells in G2/M phase which might have been introduced to quiescence population as contaminants. Re-entry of WI-38 fibroblasts into the cell cycle was continuously monitored following subculturing of cells at lower density five days after contact-inhibition (Figure 4.4B). The percentage of cells entering S phase increased from 2 % at 0 hours to 5 % at 18 hours and 12 % at 21 hours after release from contact-inhibition, confirming that WI-38 fibroblasts synchronously re-enter the cell cycle from quiescence approximately 21 hours after release from contact-inhibition.

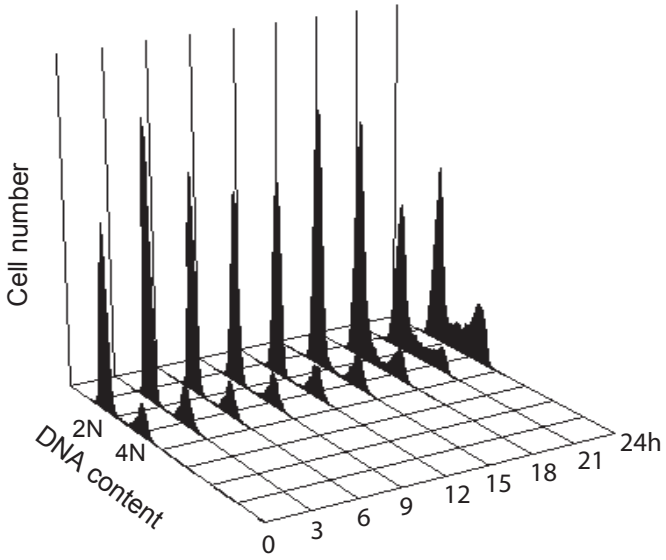
Orc4 protein levels did not change during density-dependent growth arrest and remained relatively constant during re-entry into the cell cycle (Figure 4.5A). Mcm2 protein levels began to decrease within 48 hours of exit from the cell cycle into quiescence and increased 18 hours after release from contact inhibition (Figure 4.5A).

A **Exit from the cell cycle into quiescence**



	AS	SC	C	C1	C5	C10	C15	C20
G1	68	72	83	82	81	82	83	83
S	9	8	3	3	2	2	2	2
G2/M	23	20	14	15	17	16	15	15

B **Re-entry into the cell cycle from quiesence**



	0	3	6	9	12	15	18	21	24h
G1	83	83	83	81	83	84	84	72	60
S	2	2	2	2	1	3	5	12	17
G2/M	15	15	15	17	16	13	11	16	23

Figure 4.4: Flow cytometric DNA profiles during exit into quiescence and re-entry into the cell cycle. (A) Exit of WI-38 fibroblasts into quiescence by density-dependent growth arrest was monitored by flow cytometric analysis of propidium iodide stained cells. An asynchronously proliferating population (AS) showed a normal DNA content distribution. Cells began to arrest in G1 when contact inhibited. (B) Re-entry of WI-38 fibroblasts into the cell cycle from quiescence was also monitored by flow cytometric analysis. After release from quiescence, cells progressed into S phase between 18 and 21 hours after release. Tables show the percentages of cells with G1, S, and G2/M DNA content. (AS - asynchronous; SC - semi-confluent; C - confluent; C1 - 15-days in density dependent growth arrest)

Phosphorylation of Mcm2 on serine 40/41 decreased with the onset of quiescence, and increased in a similar pattern to that observed for Mcm2 during re-entry into the cell cycle. Phosphorylation of Mcm2 on serine 53 decreased more rapidly, becoming undetectable 48 hours after exit from cycle and detectable again 24 hours after release from quiescence (Figure 4.5A). Cdc6 protein levels were downregulated 48 hours after exit from cycle, in keeping with previous reports (Stoeber et al. 2001; Kingsbury et al. 2005) and started to reappear 12 hours after re-entry into cycle. Cdt1 and geminin proteins were also downregulated within 48 hours of density-dependent growth arrest. During re-entry into cycle, Cdt1 was detectable at 12 hours and started to decrease by 21 hours. Geminin was also detectable at 12 hours, with levels peaking 18 hours after release from quiescence (Figure 4.5A). Cdc45 and Mcm10 were down-regulated with a similar pattern to that observed for Cdt1 and geminin, however their *de novo* expression during re-entry into the cell cycle occurred later than Cdt1 and geminin (Figure 4.5A). Cyclin A was downregulated in G0 and became detectable again 24 hours after cells re-entered the cell cycle (Figure 4.5A).

Cdc7 and ASK protein levels rapidly decreased 48 hours after exit from cycle into quiescence. During re-entry into cycle, Cdc7 and ASK levels were detectable by 15 hours and were significantly increased 24 hours after the release (Figure 4.5A). The formation of the Cdc7/ASK complex in quiescent cells and during re-entry into the cell cycle was determined by coimmunoprecipitation with a Cdc7 antibody (Figure 4.5B). Endogenous Cdc7 was bound to ASK in asynchronously proliferating and semi-confluent cells, with both proteins being downregulated rapidly following the onset of quiescence.

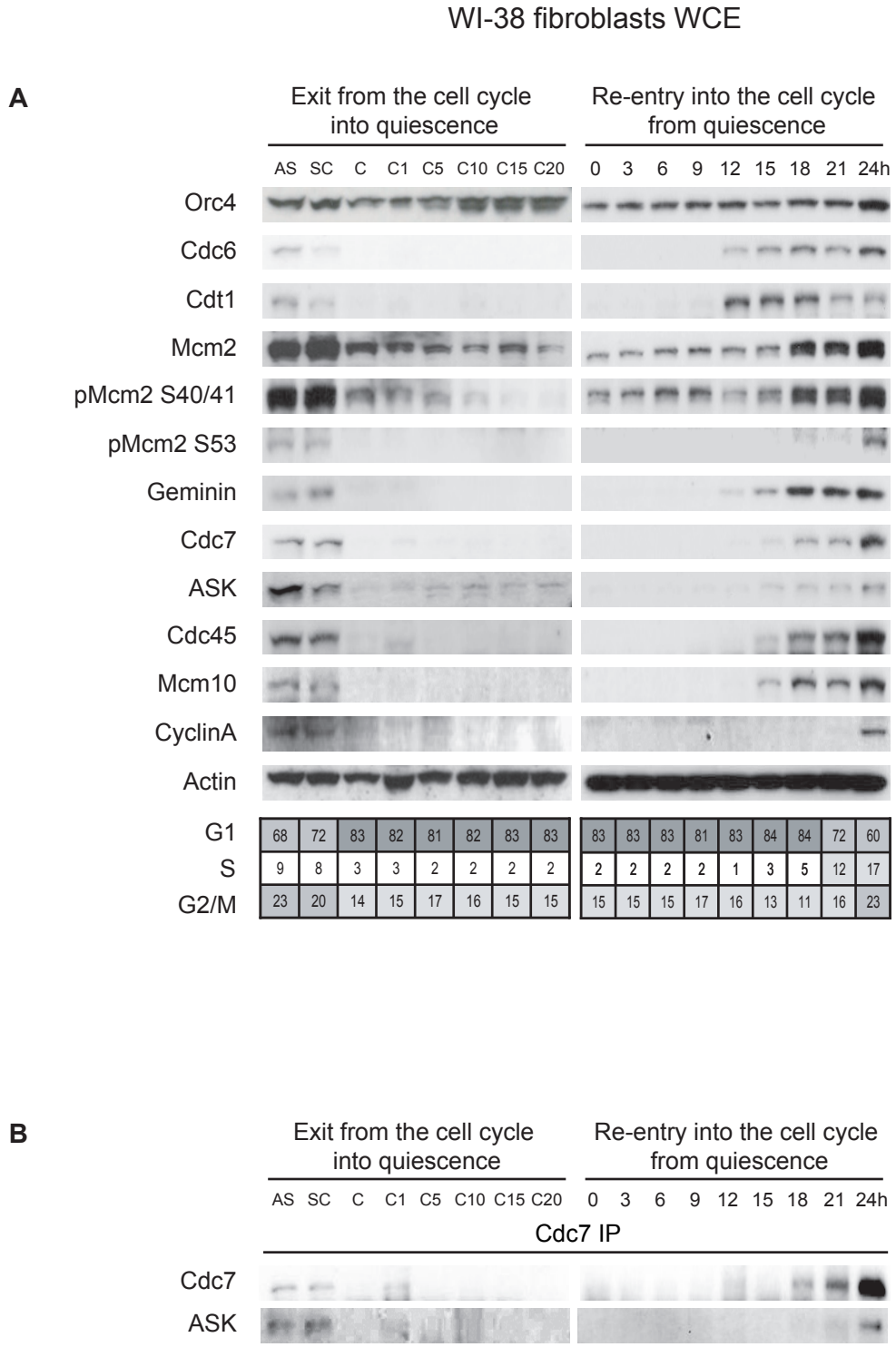


Figure 4.5: Expression dynamics and regulation of replication initiation factors during exit into G0 and re-entry into cycle. (A) Immunoblot analysis of replication initiation factors and loading control (actin) in WI-38 fibroblasts whole cell extracts at the indicated times. (B) Immunoblot analysis of Cdc7 and ASK proteins after immunoprecipitation using Cdc7 antibody from WI-38 cell extracts. (IP - immunoprecipitation; WCE - whole cell extract; pMcm2 S40/41 - Mcm2 phosphorylated on serine 40/41; pMcm2 S53 - Mcm2 phosphorylated on serine 53)

Although Cdc7 and ASK proteins were detectable 15 hours after release from G0, Cdc7 association with ASK did not occur until 21 hours (Figure 4.5B), coinciding with phosphorylation of Mcm2 on serine 53 (Figure 4.5A). Taken together, these data show that downregulation of replication initiation factors, except Orc4, results in loss of DNA replication competence which contributes to the suppression of proliferative capacity in quiescence. Notably, Cdc7/ASK kinase activity is not only dependent on expression of Cdc7 and ASK proteins, but also their ability to associate during re-entry into the cell cycle.

4.3.3. Expression dynamics and regulation of Cdc7/ASK kinase in terminally differentiated cells

Differentiating cells normally mature over the passage of time to form functionally competent cells for a particular tissue, culminating in terminal maturation or differentiation (Lajtha 1979). A wide range of cellular regulators are involved in coordinating the proliferation-differentiation switch in mammalian cells, including $p21^{cip1}$, $p27^{kip1}$, pRB and p53 (Coffman & Studzinski, 1999). Induction of the differentiation pathway in cultured HL60 cells, derived from a patient with acute promyelocytic leukaemia, with agents such as the phorbol ester phorbol 12-myristate 13-acetate (PMA) and 1,25 dihydroxyvitamin D_3 (1,25 Vit D_3) is a well characterised *in vitro* model system for studying the relationship of cell proliferation and differentiation (Collins 1987). To investigate how regulation of the Cdc7/ASK kinase complex is linked to the molecular mechanisms that control DNA replication initiation during differentiation, in the work of this thesis I induced differentiation of promyeloblastic HL60 cells down the macrophage lineage. HL60 cells can be induced

to differentiate *in vitro* into monocyte-, granulocyte-, or macrophage-like cells by a number of physiological and non-physiological agents (Collins 1987). Exposure of HL60 cells to 1,25 dihydroxyvitamin D_3 (1,25 Vit D_3) results in cellular differentiation indicated by morphological changes, acquisition of markers characteristic of mature monocytes, and accumulateion of cells in G1 phase (Wang & Studzinski 2001). To generate a molecular picture of pre-RC/pre-IC constituents and to study the regulation of Cdc7/ASK kinase during differentiation, HL60 total cell extracts were prepared at 6 hour-intervals after administration of 1,25 Vit D_3 as described (Barkley et al. 2007).

To monitor the differentiation process, I measured expression of the cell surface markers CD11b and CD14 by bivariate flow cytometry (Figure 4.6A). Sixty percent of HL60 cells expressed CD11b by 24 hours, whereas 40% of cells expressed CD14. Maximum expression of both markers was detected by 72 hours after 1,25 Vit D_3 trea tment (Figure 4.6B). In addition, flow cytometric analysis of DNA content shows that cells began to arrest in G1 by 36 hours, with 86% of cells accumulated in G1 phase 120 hours after 1,25 Vit D_3 treatment (Figure 4.7A). These data are in keeping with bromodeoxyuridine (BrdU) cell proliferation assays, which indicate that 45% of asynchronously proliferating HL60 cells incorporated BrdU during a one hour pulse. In contrast, the BrdU labelling index decreased to 20% at 48 hours and to less than 10% 72 hours after the addition of 1,25 Vit D_3 (Figure 4.7B), confirming 1,25 Vit D_3 induced accumulation of HL60 cells in G1 phase by 48 hours together with a decrease in DNA synthesis.

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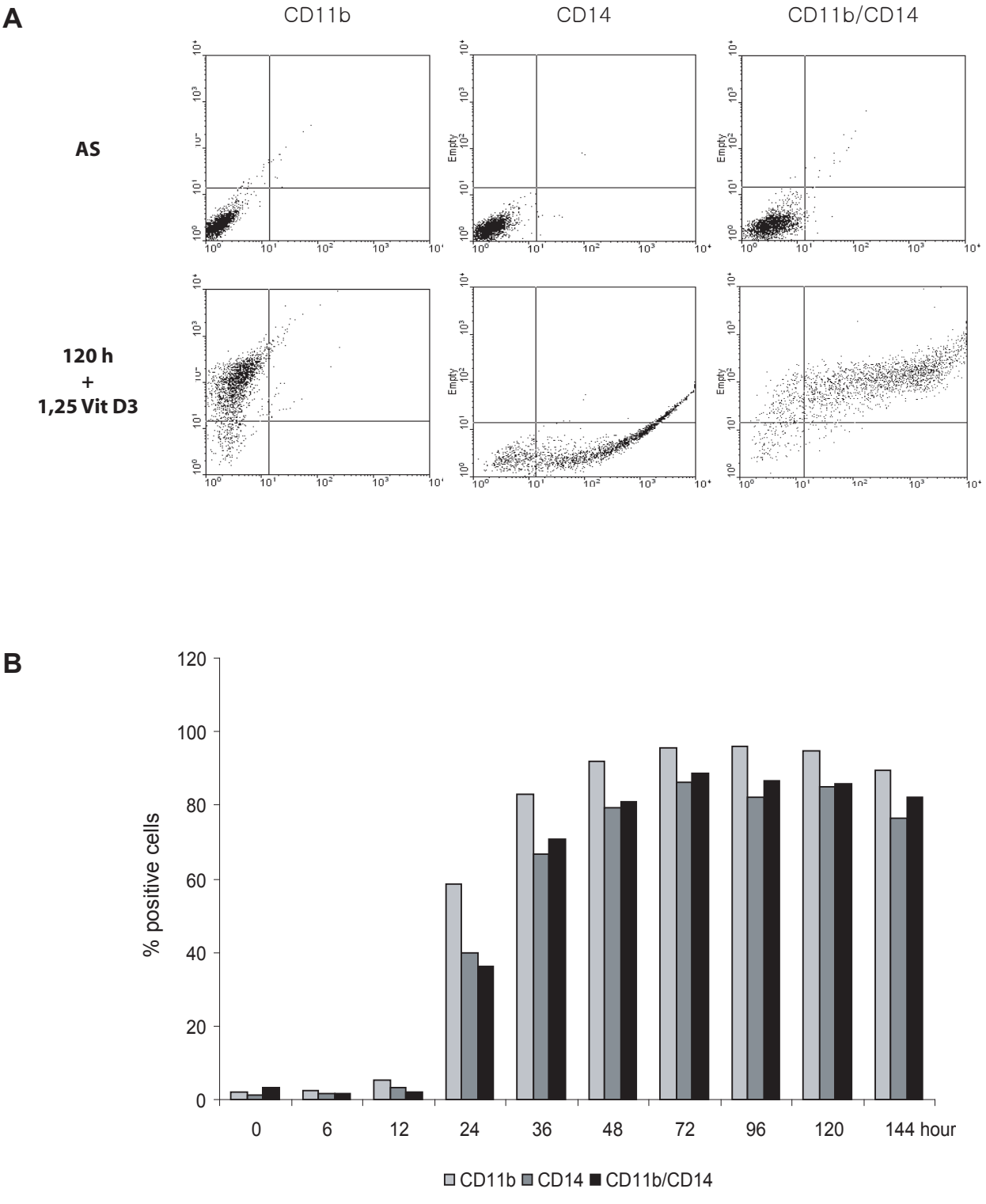
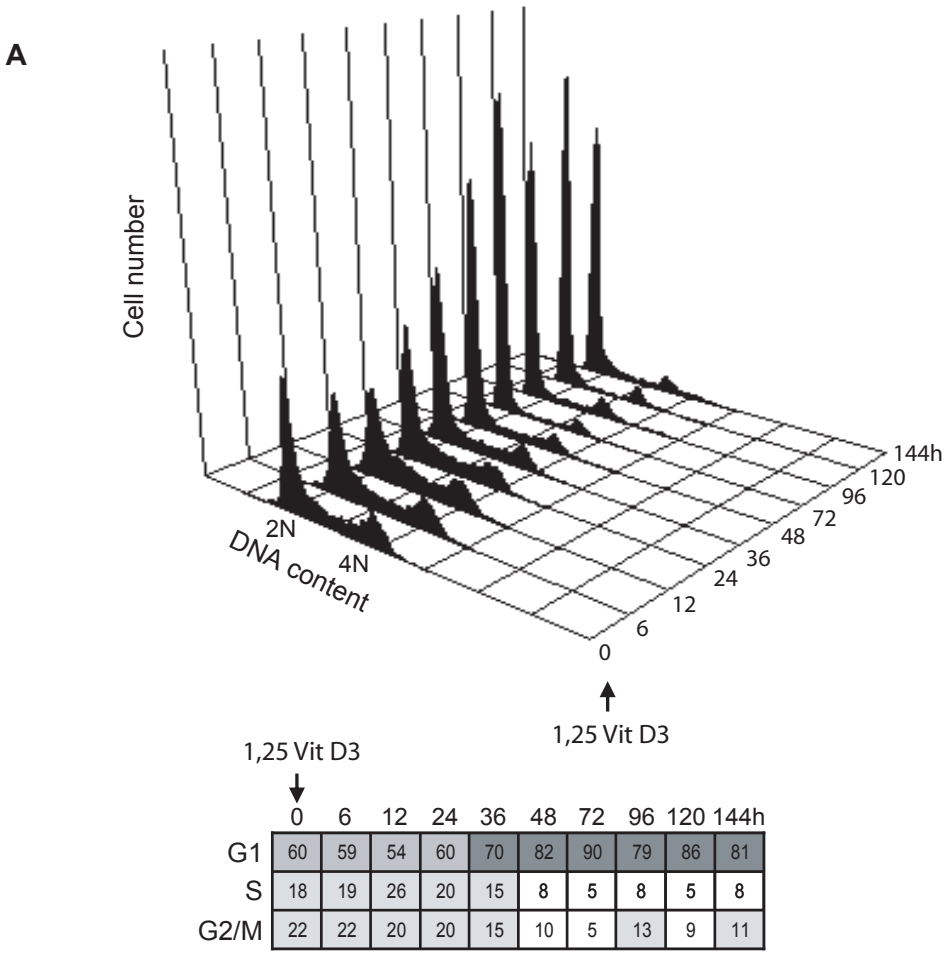


Figure 4.6: Expression of the monocyte/macrophage differentiation markers CD11b and CD14 in HL60 cells after addition of 1,25 Vit D3. (A) Cytogram of CD11b and CD14 expression in HL60 cells induced by 1,25 Vit D3 for 120 hours compared to asynchronously proliferating cells (AS). (B) HL60 cells were incubated with 1,25 Vit D3 for the indicated times to determine CD11b and CD14 positive cells by flow cytometry. (AS - asynchronous)

Protein levels of the Orc4 subunit were constant during differentiation. Mcm2 protein levels were downregulated during differentiation after the addition of 1,25 Vit D_3 (Figure 4.8A). Phosphorylation of Mcm2 on serine 40/41, which is indicative of phosphorylation by Cdc7 and CDK, started to decrease by 72 hours, whereas phosphorylation of Mcm2 on serine 53 which is Cdc7-dependent phosphorylation site decreased more rapidly within 48 hours (Figure 4.8A). Cdc6 and Cdt1 proteins were undetectable 72 hours after 1,25 Vit D_3 addition. Geminin protein levels started to decrease slightly by 72 hours. Mcm10 became undetectable 36 hours after the addition of 1,25 Vit D_3 (Figure 4.8A). PMA (4β-phorbol 12-myristate 13-acetate)-induced differentiation along the megakaryocytic phenotype is accompanied by down-regulation of cyclin E (Bermejo et al. 2002) and cyclin A, indicating that cells exit from cell cycle (Pollok et al. 2007). Similarly, data generated in my experimental differentiation model show that cyclins A and E became undetectable 72 hours after 1,25 Vit D_3 addition (Figure 4.8A).

During HL60 differentiation, protein levels of Cdc7 and ASK were found to be downregulated by 72 hours after 1,25 Vit D_3 addition (Figure 4.8A). The formation of Cdc7/ASK kinase complex during differentiation was determined by coimmunoprecipitation with a Cdc7 antibody. Endogenous Cdc7-bound ASK complex was undetectable 72 hours after 1,25 Vit D_3 addition (Figure 4.8B). Notably, phosphorylation of Mcm2 on serine 53 was undetectable by 72 hours, coinciding with down-regulation of Cdc7 and ASK protein and the loss of Cdc7-bound ASK complex at this time point, indicating that phosphorylation of Mcm2 is dependent on active Cdc7/ASK kinase complex (Figure 4.8B).



	0	6	12	24	36	48	72	96	120	144h
G1	60	59	54	60	70	82	90	79	86	81
S	18	19	26	20	15	8	5	8	5	8
G2/M	22	22	20	20	15	10	5	13	9	11

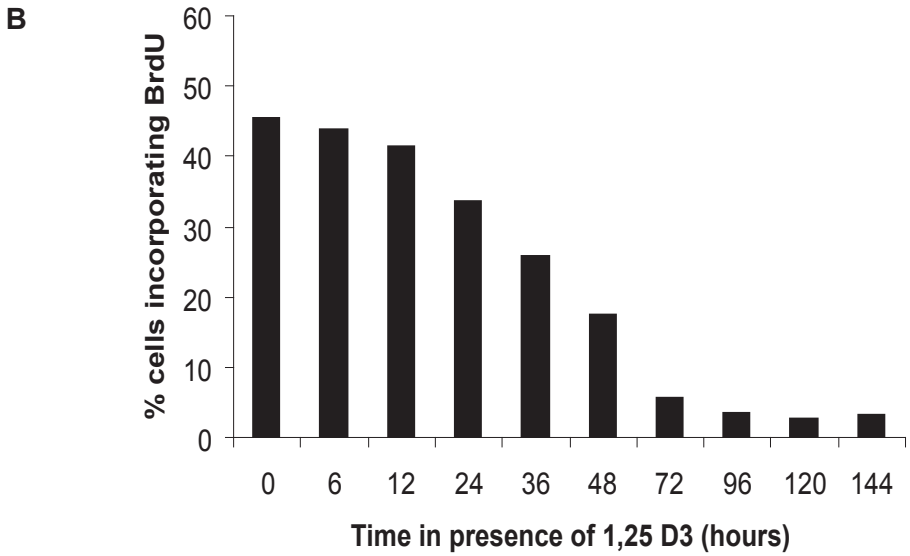


Figure 4.7: Flow cytometric DNA profiles of HL60 cells undergoing differentiation. Differentiation of HL60 cells after 1.25 Vit D3 treatment was monitored by bivariate flow cytometric analysis of (A) DNA content and (B) BrdU incorporation. Cells began to arrest in G1 at 36 hours and 85% of cells accumulated in G1 phase 120 hours after 1,25 Vit D3 treatment. In BrdU assays, 45% of asynchronously proliferating HL60 cells incorporated BrdU. The BrdU labelling index decreased to 20% by 48 hours and to less than 10% 72 hours after the addition of 1,25 Vit D3.

Taken together, these data show that loss of proliferative capacity as cells undergo differentiation is associated with down-regulation of replication initiation factors, including Cdc7 and ASK. Importantly, the essential role of Cdc7/ASK kinase in activating pre-RCs in proliferating cells its tight downregulation in out-of-cycle states, if confirmed for human tissues, raises the possibility that inhibition of this kinase might represent a powerful approach for the development of anti-cancer agents.

4.3.4. Cdc7 and Mcm2 protein expression and Cdc7-dependent phosphorylation of Mcm2 on serine 53 profile analysis in human tissues

As discussed above, the studies in tissue culture model systems show that the replication initiation machinery is assembled in a cell cycle dependent manner in proliferating humans cells, and that downregulation of initiation factors is linked to loss of proliferative capacity as cells exit into quiescent (G0) and differentiated states (Figures 4.3, 4.5, 4.8). Previous work has shown repression of the DNA replication initiation pathway through downregulation of Cdc6 and MCM proteins in a wide range of human organ systems, and that Cdc6, Cdt1 and Mcm2-7 expression is dysre gulated in human cancers (Williams et al. 1998; Freeman et al. 1999; Stoeber et al. 1999; Going et al. 2002; Stoeber et al. 2001; Stoeber et al. 2002; Karakaidos et al. 2004; Korkolopoulou et al. 2005). Cdc7 expression also appears altered in some cancer cell lines and primary tumours (Hess et al. 1998; Bonte et al. 2008), and in a wide range of melanocytic lesions (Clarke et al. 2009). Moreoever, a recent study showed that Cdc7 and CDK-dependent phosphorylation of Mcm2 at Ser40/41 is an indicator of S phase progression during the cell cycle re-entry of neurons in Alzheimer Disease (Bonda et al. 2009).

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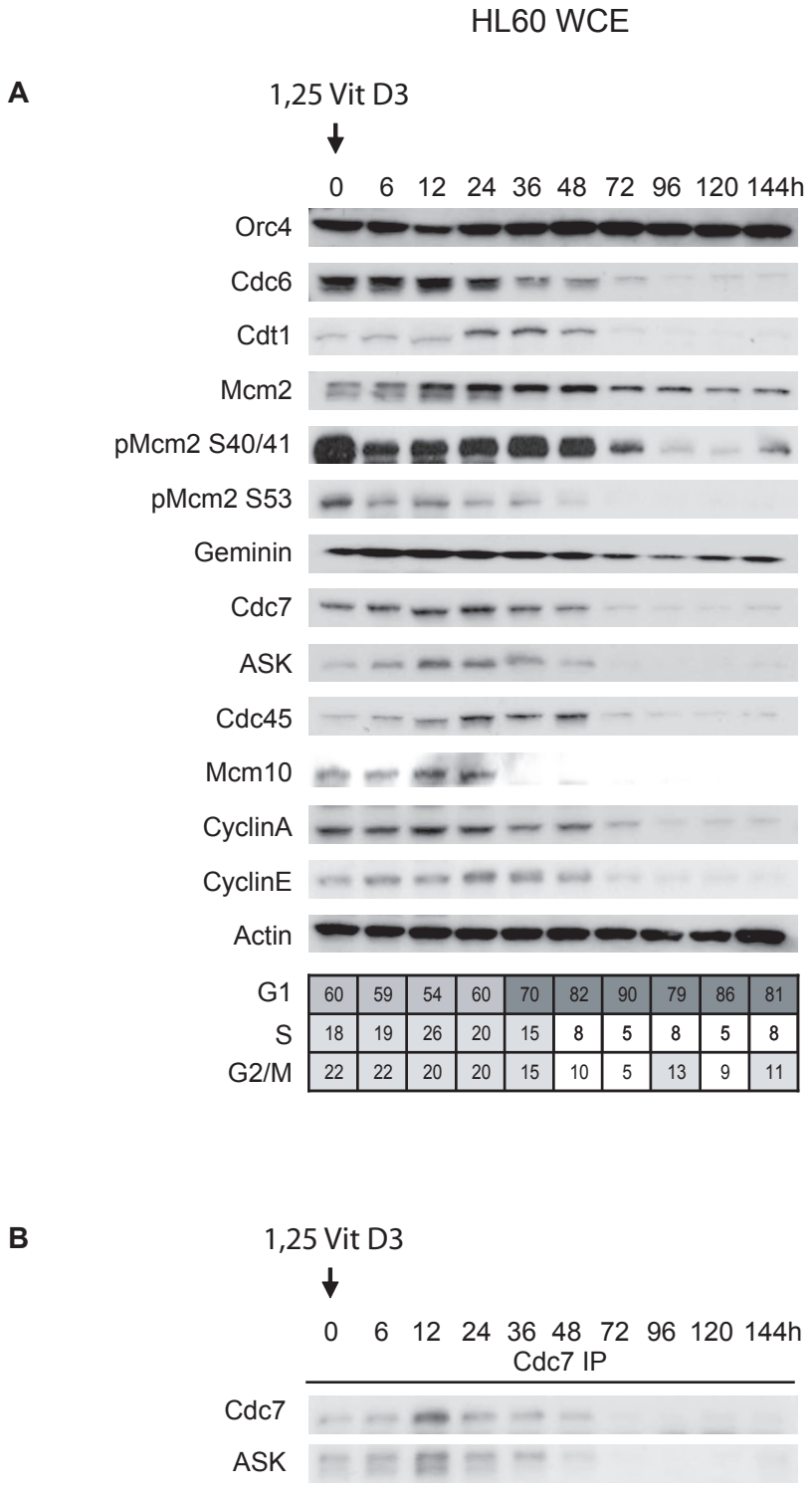

Figure 4.8: Expression dynamics and regulation of replication initiation factors during 1,25 Vit D3 induced differentiation in HL60 cells. (A) Immunoblot analysis of replication initiation factors and loasing control (actin) in HL60 whole cell extracts at the indicated times. Table shows the percentage of cells with G1, S, and G2/M DNA content. (B) Immunoblot analysis of Cdc7 and ASK proteins after immunoprecipitation using Cdc7 antibody from HL60 cell extracts. (IP - immunoprecipitation; WCE - whole cell extract; pMcm2 S40/41 - Mcm2 phosphorylated on serine 40/41; pMcm2 S53 - Mcm2 phosphorylated on serine 53)

However, while the above studies have shed some light on Cdc7 regulation *in vivo*, I sought to add systematically to our understanding of Cdc7/ASK kinase activity (indirectly determined by Mcm2 phosphorylation at Ser 53) in different types of normal and cancerous human tissues.

Human tissues can be divided into three classes: self-renewing (e.g. skin, gastrointestinal mucosa and the haemopoetic system), conditional renewal (e.g. liver or thyroid) and permanent (e.g. nerve or skeletal muscle) (Leblond, 1963). Tissues in adults with the most rapid turnover are self-renewing stem cell systems such as skin, colon or uterine cervix, typically showing a grading of cellular development from stem cells to terminally-differentiated mature cells via transient amplifying cells (reviewed by Watt & Hogan 2000). In the work of this thesis, I sought to define the regulation of Cdc7/ASK activity during tissue maintenance in the adult, especially in self-renewing system and conditional renewal tissue, and to determine the likely response of these tissues to pharmacological Cdc7 inhibitors. I analyzed Cdc7 and Mcm2 expression, as well as phosphorylation of Mcm2 on serine 53 (readout of Cdc7/ASK activity *in vivo*), in a typical self-renewing system, oral squamous epithelium (Figure 4.9), and in a typical conditional renewal tissue, liver (Figure 4.10).

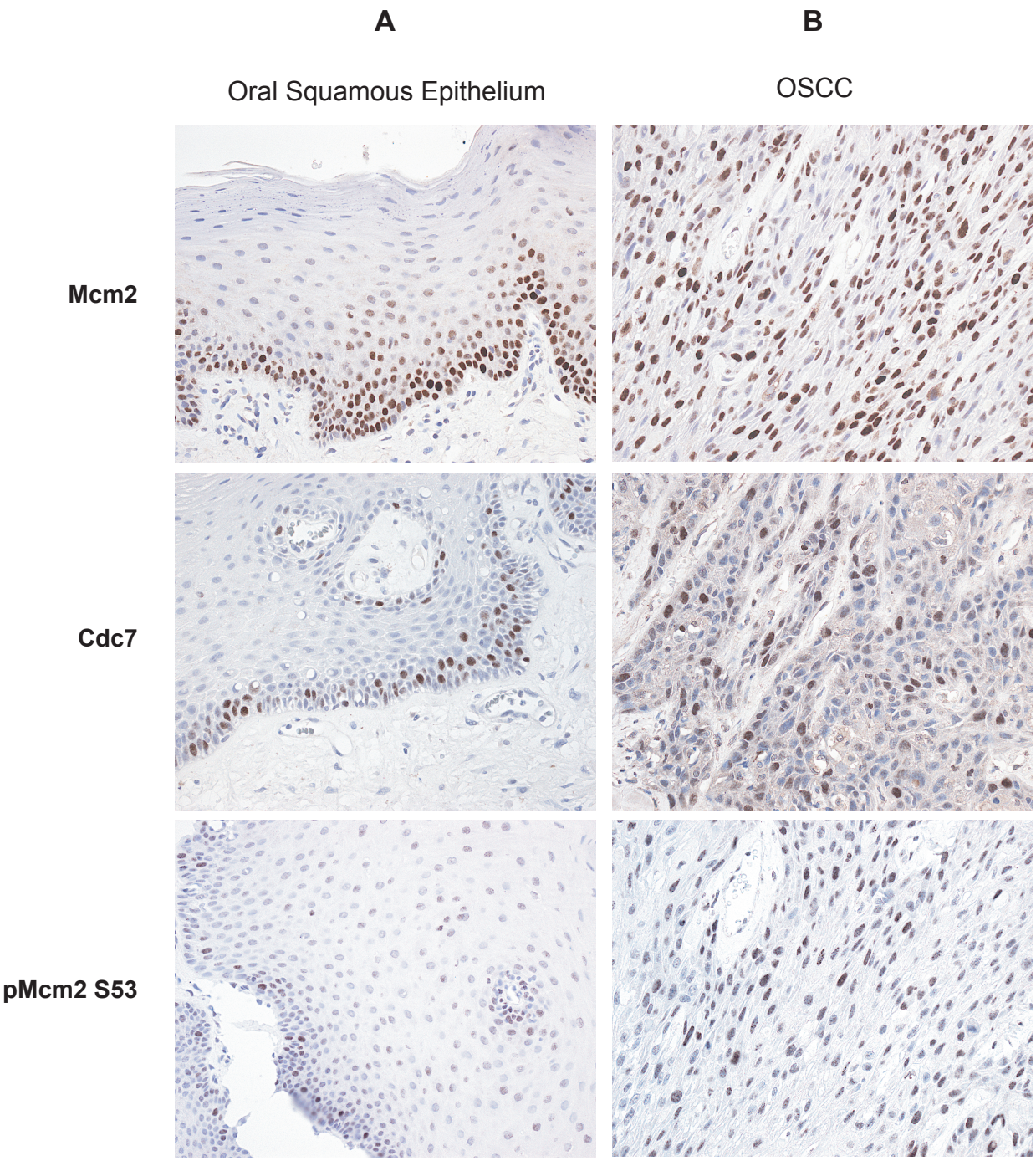

Figure 4.9: Detection of Mcm2, Cdc7 and phospho Mcm2 in oral squamous epithelium and oral squamous cell carcinoma (OSCC). Photomicrographs of formalin fixed paraffin-embedded tissue sections of normal oral squamous epithelium and OSCC immunostained with antibodies against Mcm2, Cdc7 and pMcm2 S53. Original magnification, x400. (A) Immunostaining of normal oral squamous epithelium shows that expression of Mcm2, Cdc7 and phosphorylation of Mcm2 on serine 53 are restricted to the basal compartment of oral squamous epithelium, which corresponds to the proliferative zone. Mcm2, Cdc7 and pMcm2 were not detected in epithelial cells during the transition to a terminally differentiated phenotype. (B) Immunostaining of OSCC shows a high proportion of Cdc7 and Mcm2 expressing cells, and phospho Mcm2 on serine 53, indicating that a large proportion of tumour cells are engaged in the mitotic cell division cycle. (pMcm2 S53 - Mcm2 phosphorylated on serine 53)

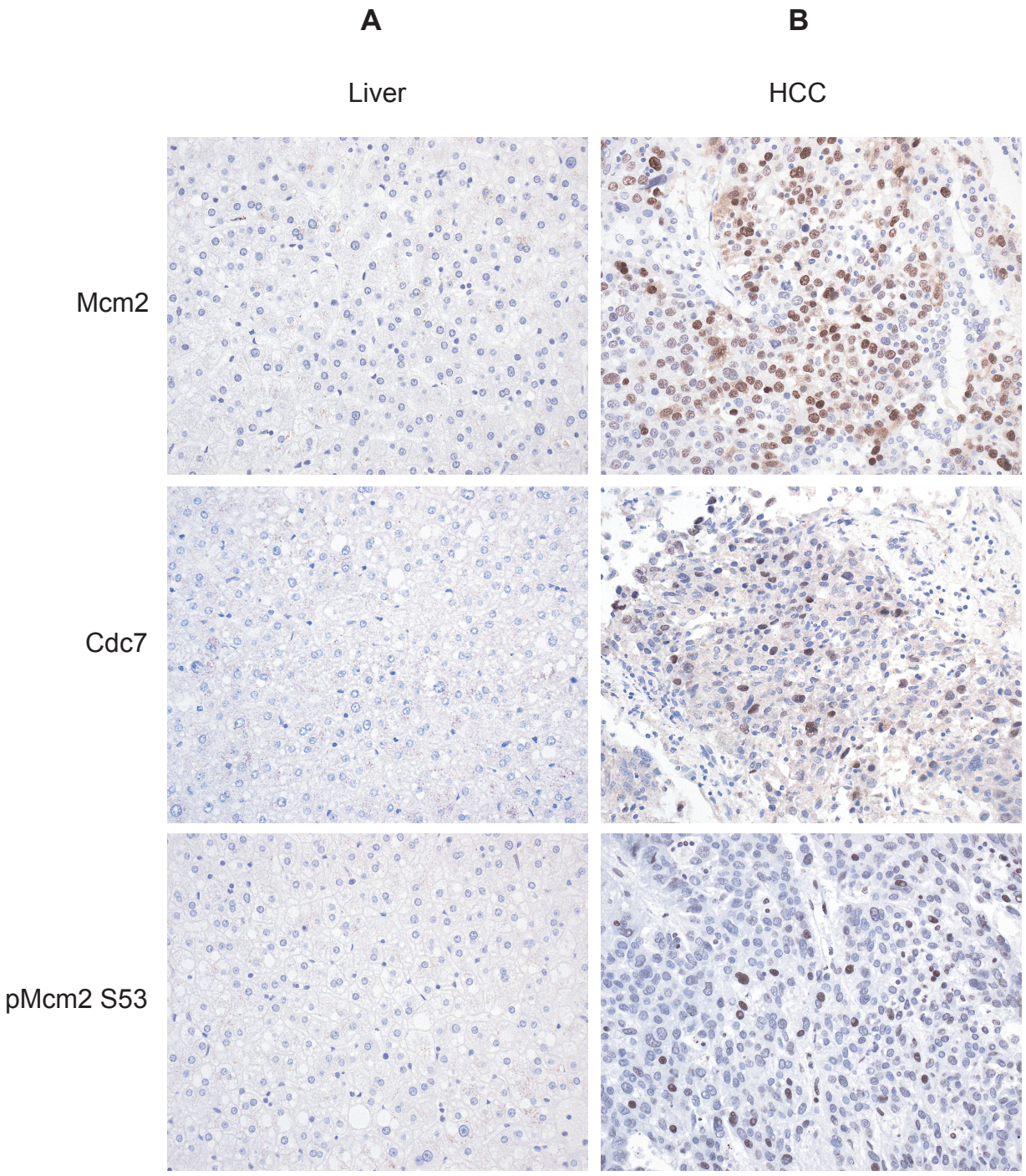

Figure 4.10: Detection of Mcm2, Cdc7 and phospho Mcm2 in human liver and hepatocellular carcinoma (HCC). Photomicrographs of formalin fixed paraffin-embedded tissue sections of normal liver and hepatocellular carcinoma (HCC) immunostained with antibodies against Mcm2, Cdc7 and pMcm2 S53. Original magnification, x400. (A) Immunostaining of normal liver is for Mcm2, Cdc7 and phospho Mcm2 consistent with withdrawal of hepatocytes from the cell cycle into a replication-incompetent quiescent state. (B) Immunostaining of HCC shows a high proportion of Mcm2 and Cdc7 expressing cells as well as phospho Mcm2 on serine 53 coinciding with re-entry into the proliferative cycle. (pMcm2 S53 - Mcm2 phosphorylated on serine 53)

For Mcm2, Cdc7 and phosphorylated Mcm2 protein expression profiling, five cases of oral squamous cell carcinoma (OSCC) and hepatocellular carcinoma (HCC) were selected and analysed using immunohistochemistry[1]. Three to five fields were captured from selected areas and representative immunostained tissue sections are illustrated in Figure 4.9 and 4.10. Semi-quantitatve labelling indices for each proteins are shown in Table 4.1, and images with amounts of labelling indices spanning ranges of >0% to 25%, >25% to 75%, and >75% were assigned scores of +1, +2, and +3, respectively (Table 4.1). Immunostaining of oral squamous epithelium showed that Mcm2 and Cdc7 expression and Mcm2 phosphorylation is restricted to the proliferative, basal compartment. Mcm2, Cdc7 and phosphorylated Mcm2 were not detected in epithelial cells during the transition to a mature differentiated phenotype (Figure 4.9A and Table 4.1). These findings are in keeping with the *in vitro* data, which show that the Mcm2 and Cdc7 replication initiation factors are present in proliferating cells and downregulated during differentiation. In contrast, immunostaining of oral squamous cell carcinoma (OSCC) shows a high proportion of Mcm2 and Cdc7 expressing cells as well as phosphorylation of Mcm2, indicating a greater number of cells engaged in the cell cycle due to deregulation of normal controls over cell proliferation (Figure 4.9B and Table 4.1). In normal oral squamous epithelium, Mcm2 expression levels are highest in the anatomical zone corresponding to the proliferative compartment. Compared to Mcm2 staining, a smaller population of cells in the proliferative compartment are positive for Cdc7 and phosphorylated Mcm2, indicating that licensed replication origins are not simultaneously fired by Cdc7/ASK dependent phosphorylation.

[1] Technical assistance in the immunostaining and analysis of histological sections was provided by Marco Loddo and Mohammed Rashid (Research Department of Pathology, UCL).

Table 4.1 Mcm2, Cdc7 and phosphorylated Mcm2 expression profile in oral squamous eipthelium and liver tissue

Tissue Name		Case No	Mcm2	Cdc7	pMcm2 S53
		U07-1678	++	+	+
		U07-6589	++	+	+
	Normal *	U06-18395	++	+	+
		U06-16463	++	+	+
Oral squamous epithelium		U06-14973	++	+	+
		U07-1543	+++	+++	++
		U06-14206	+++	++	+
	OSCC	U06-17635	+++	++	++
		U06-16421	+++	+++	++
		U06-16463	+++	+++	++
		U07-7402	-	-	-
		U08-14363	-	-	-
	Normal	U07-4805	-	-	-
		U08-10970	-	-	-
Liver		U07-15351	-	-	-
		U03-5003	+++	++	++
		U05-2634	+++	++	++
	HCC	U06-8092	+++	++	++
		U08-14222	+++	++	++
		U04-19197	+++	++	++

* Expression restricted to the basal proliferative compartment of normal oral squamous epithelium.
OSCC - Oral squamous cell carcinoma; HCC - Hepatocellular carcinoma

To determine whether downregulation of Cdc7/ASK kinase activity during withdrawal from cycle into the quiescent state *in vitro* extends to conditional renewal tissues systems, the expression of Mcm2 and Cdc7 proteins and Mcm2 phosphorylation were examined in normal and cancerous liver tissues. Normal liver shows little growth in adult life but retains proliferative capacity for further growth (Leblond et al. 1963). After hepatocyte loss as a result of surgery/transplantation, viruses or toxins, quiescent hepatocytes re-enter the mitotic cell cycle and proliferate to restore liver function and mass. In keeping with liver physiology, in normal liver, staining of Mcm2, Cdc7 and phosphorylated Mcm2 was observed in only a small percentage (<1%) of cells (Figure 4.10A and Table 4.1). Positive immunostaining, however, was evident in hepatocellular carcinoma, indicative of cell cycle re-entry and increased proliferation (Figure 4.10B and Table 4.1). These *in vivo* findings are consistent with the *in vitro* data generated in the fibroblast quiescence model system, which show that downregulation of some replication initiation factors and Cdc7/ASK kinase activity is coupled to reversible loss of proliferative capacity (Figure 4.5). Furthermore, the acquisition of proliferative capacity following re-entry into cycle is linked to induction of replication licensing and origin firing. Taken together, the data presented in this chapter show that Mcm2 and Cdc7 are present in actively proliferating cells and downregulated in resting and differentiated cells, both *in vitro* and *in vivo*. Importantly, Cdc7 immunostaining revealed a positive correlation with phosphorylation of Mcm2 on serine 53, confirming the activation of Cdc7/ASK kinase in preparation for origin firing and cell proliferation *in vivo*. The high proportion in human cancers of Cdc7 expressing cells with phosphorylated Mcm2 has

important implications for the potential use of Cdc7 as a diagnostic and prognostic biomarker and as a therapeutic target.

4.4. Discussion and Conclusions

An understanding of cell cycle regulation of chromosomal replication in human cells is fundamental to the understanding of diseases characterized by disorder in cell growth and division (reviewed in Negrini et al. 2010). Loss or alterations of cell cycle controls, for example, have been implicated in the development of certain cancers (Deshpande et al. 2005). In mammalian tissues the majority of cells exit the cell cycle reversibly into an out-of-cycle, quiescent state (G0), or alternatively withdraw irreversibly from the cell division cycle through the processes of terminal differentiation or replicative senescence (Pardee 1989; Myster & Duronio 2000; Hayflick & Moorhed 1961; Campisi 1996). Downregulation of DNA replication initiation factors has been previously linked to exit from the cell cycle into quiescence, differentiation and senescence (Williams et al. 1998; Stoeber et al. 2001; Kingsbury et al. 2005). Although the role of Cdc7/ASK kinase the DNA replication initiation pathway has been well characterised in yeast and *Xenopus,* and the increased Cdc7/ASK expression has been shown in some cancer cell lines and tumour specimens, its potentially important role in growth regulation of human tissues and their respective tumours warrants further investigation. In the work of this thesis, I used tissue culture model systems to characterise the expression dynamics of replication initiation factors, including Cdc7/ASK kinase, during the cell cycle, and during exit into the quiescent and differentiated out-of-cycle states. A second aim was to extrapolate Cdc7/ASK expression dynamics to an investigation of Cdc7 kinase

activity in selected normal and cancerous human tissues, to test its diagnostic utility and to provide target validation of Cdc7 for cancer therapy.

Previous reports have shown that the amount of human Orc2 and Mcm2-7 (Stoeber et al. 2001; Tsuruga et al. 1997) and Cdc45 (Pollok et al. 2007) remain constant during the cell cycle, whereas levels of Cdc6 (Stoeber et al. 2001; Petersen et al. 2006), Cdt1 (Wohlschlegel et al. 2000), geminin (Eward et al. 2004; Wharton et al. 2004) and ASK (Jiang et al. 1999; Yamada et al. 2002) appear to fluctuate. In the work of this thesis, the protein levels of pre-RC/pre-IC constituents are consistent with a model in which a core set of replication initiation factors sequentially assemble at origins during G1 phase (Figure 4.2A). As the replication initiation factors assemble onto the origin and form the pre-RC, activation of S-phase specific kinases, CDKs and Cdc7, triggers the transition from pre-RC to pre-IC (reviewed in Sclafani & Holzen 2007). Phosphorylation of Mcm2 at multiple sites correlates with pre-RCs being activated by S-phase specific kinases (Montagnoli et al. 2006; Cho et al. 2006; Tsuji et al. 2006; Charych et al. 2008). The data discussed in this chapter show that expression levels of Cdc7 and ASK were found to be upregulated as cells progressed into S phase coinciding with increased levels of Mcm2 phosphorylation on serine 53. This suggests that Cdc7/ASK kinase activity is required for pre-RC activation through phosphorylation of Mcm2 which is essential for S phase entry and progression (Figure 4.2A). The data also show that downregulation of some replication initiation factors, including Cdc7 and ASK results in loss of DNA replication competence which contributes to the suppression of proliferative capacity in quiescent and differentiating human cells (Figure 4.5A and 4.8A).

Cdc7 requires association with ASK, forming a complex to generate its kinase activity which is essential for pre-RC activation (Jiang et al.1999; Kumagai et al. 1999; reviewed in Swords et al. 2010). Immunoprecipitation experiments show that levels of Cdc7-bound ASK fluctuate with the same temporal sequence as Cdc7 and ASK proteins (Figure 4.3). Interestingly, the association of Cdc7 with ASK is not detectable until 21 hours after release from quiescence, correlating with phosphorylation of Mcm2 on serine 53, while the individual proteins are re-expressed already 15 hours after the release (Figure 4.5B). These data argue that Cdc7/ASK kinase activity is not only dependent on expression of the two subunits but also their ability to associate and form a complex. Indeed, phosphorylation of Mcm2 on serine 53 was detectable in late G1 and S phase with the same kinetics as Cdc7-bound ASK complex (Figure 4.3A and B), suggesting that phosphorylation of Mcm2 at this residue is dependent on Cdc7/ASK kinase complex activity.

Most cells in human out-of-cycle tissues reside in differentiated and quiescent states, and start to proliferate in response to external growth stimulation (Masai et al. 2005). Immediate responses of resting cells to growth factors include induction of a set of immediate early genes, including Fos, Jun, Ets and Myc, which induce cyclin D expression (Masai et al. 2005). Cyclin D-Cdk4 phosphorylates retinoblastoma (Rb) protein, releasing members of the E2F family of transcription factors for activation of their target genes. It is well known that the E2F family is essential for coordinating transcription during the mammalian cell cycle (Harbour & Dean, 2000). A number of factors involved in the DNA replication initiation machinery and regulation of its assembly and functions are targets of E2F. These include Orc1 (Ohtani et al. 1996), Mcm4 and 7, Cdc6, Cdc45 (Arata et al. 2000), geminin (Markey et al. 2004), Cdc7

(Kim et al. 1998) and ASK (Yamada et al. 2002). It has been reported that E2F-regulated promoters are transcriptionally silenced in quiescent and senescent cells (Ogawa et al. 2002; Narita et al. 2003). E2F-binding sites were identified in the promoter regions of genes coding for Mcm5 (Tsuruga et al.1997), Cdc6 (Ohtani et al. 1998), DNA polymerase-α (Pearson et al. 1991), Cdc45 (Arata et al. 2000), Geminin (Markey et al. 2004), Cdc7 (Kim et al. 1998) and ASK (Yamada et al. 2002). The presence of E2F binding sites in the CDC7 and ASK genes and their observed downregulation in non-cycling cells suggests that Cdc7 and ASK could be regulated via the Rb-E2F pathway (Kim et al.1998; Kumagai et al. 1999; reviewed in Masai & Arai 2002).

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Previous studies have shown that Cdc7 and ASK are often overexpressed in various tumour specimens, but not in matched normal tissues (Hess et al. 1998; Bonte et al. 2008). Mcm2-7 expression is restricted to cycling cells and has been identified as a powerful diagnostic and prognostic marker in a wide range of malignancies (Williams & Stoeber 2007). Since phosphorylation of Mcm2 is regulated by Cdc7/ASK activity, the labelling index for Mcm2 phosphorylated on serine 53 could also be a potential biomarker downstream of Mcm2-7, however it has yet to be shown in human tissues. In the work of this thesis, expression profiling in oral squamous epithelium showed that cells of the transient amplifying compartment display a replication phenotype indicative of cycling cells, with positive immunostaining for Mcm2, Cdc7 and phosphorylated Mcm2. Progression into the terminally-differentiated compartment, where cells obtain a functional phenotype and lose their proliferative capacity, is associated with downregulation of these replication initiation factors and loss of Mcm2 phosphorylation (Figure 4.9A). These results are consistent with previous

reports that differentiation and DNA replication licensing are mutually exclusive processes in self-renewing human tissues (Stoeber et al. 2001), and are in keeping with the concept of antagonism between the cellular circuits that control proliferation and differentiation (reviewed by Olson & Spiegelman 1999). Notably, in the proliferative compartment the labelling index for Mcm2 is higher than those for Cdc7 and phosphorylated Mcm2, indicating that replication origins in most cells are licensed, as determined by Mcm2 protein expression, but origins are not fired in every cell. This suggests that *in vivo* in addition to out of cycle cells and actively cycling cells there is a third population of cells that has the potential to proliferate (licensed origins) but is not actively cycling.

Expression profiling in liver shows that reversible loss of proliferative capacity is also coupled to repression of the replication initiation machinery, as defined by downregulation of Mcm2 and Cdc7 expression and loss of Mcm2 phosphorylation on serine 53 (Figure 4.10A). By contrast, hepatocellular carcinoma showed high levels of cells positively stained for Mcm2, Cdc7 and phosphorylated Mcm2 (Figure 4.10B), indicative of cell cycle re-entry and proliferation. Importantly, the downregulation of Cdc7 during withdrawal of cells from cycle into quiescence and the fact that the majority of cells in the stem-progenitor compartment are unlicensed (Williams & Stoeber, 2007) suggests that conditionally renewing and self-renewing tissues should be largely refractory to therapeutic Cdc7 inhibitors. Moreover, the data generated for Cdc7 suggest that determination of its labelling index in human tumours may yield important prognostic information. Indeed this concept has already been proven for epithelial ovarian cancer (Kulkarni et al. 2009).

Several conclusions can be drawn from the *in vitro* and *in vivo* data discussed in this chapter. Firstly, Cdc7 and ASK are required for S phase entry and progression and phosphorylation of Mcm2 on serine 53 is dependent on Cdc7/ASK activity. Secondly, downregulation of replication initiation factors, including Cdc7/ASK, leads to loss of replication competence, which contributes to the suppression of proliferative capacity in quiescent and differentiated out-of-cycle states. Thirdly, in self-renewing systems such as oral squamous epithelium, Cdc7 is expressed in the transit amplifying compartment and downregulated in the terminally-differentiated compartment, in keeping with data generated in the HL60 *in vitro* differentiation system. Fourthly, the presence of Cdc7 and phosphorylation of Mcm2 on serine 53 in proliferating cancer cells, while the kinase in downregulated in normal liver (quiescence) and squamous epithelial cells (terminally-differentiated state), argues for Cdc7 as a potential diagnostic and prognostic target. Finally, the data presented in this chapter can be used to predict how normal cell populations may respond to pharmacological Cdc7 inhibitors.

Most functional cells reside in a reversible quiescent or terminally differentiated state with an unlicensed replication phenotype. Cancer cells generally show uncontrolled cell growth and a high proportion of cycling cells. Therefore, agents that specifically inhibit the replication initiation machinery are promising candidates for effective anti-cancer drugs. This contrasts with normal cycling cell populations which have a licensed phenotype and thus could be targeted by pharmacological Cdc7 inhibitors. Experimental evidence, however, has shown that RNAi against Cdc7 causes tumour cell lines to progress through a defective S phase leading to p53-independent apoptotic cell death, while primary fibroblasts avoid this lethal event by activating a

p53-dependent cell cycle checkpoint at G1/S (Montagnoli et al. 2004). Thus, Cdc7/ASK inhibitors may serve as powerful agents to selectively kill tumour cells, either alone or in combination with standard genotoxic drugs.

In the work of this thesis, I developed a sensitive assay for Cdc7 activity to identify small molecule inhibitors. In the following chapter, I describe the development of this antibody-based kinase assay, in conjunction with the generation of a homology model for Cdc7 and its active site, which provided the groundwork for the design of selective inhibitors of Cdc7.

CHAPTER FIVE

Development of a Cdc7 kinase assay

5.1. Introduction

The *in vitro* data discussed in the previous chapter indicate that Cdc7/ASK kinase is required for S phase progression, and that downregulation of this kinase is coupled to repression of origin licensing in quiescent and differentiated human cells. In keeping with the *in vitro* data, tissue specimen show the presence of Cdc7 and Mcm2 phosphorylated on serine 53 in a high proportion of proliferating cancer cells, and their absence in normal quiescent liver and differentiated oral squamous epithelial cells. It follows from this that Cdc7 may constitute a potential diagnostic and therapeutic target. Cdc7 inhibitors should not affect non-cycling cell populations in human tissues, thus ensuring that the majority of normal cells within the body are protected from potential cytotoxic effects.

Several steps in the DNA replication initiation pathway have been identified as potential targets for the discovery of new anti-cancer agents (see Chapter 1). Among them, small molecule inhibitors of CDKs have already shown some success in clinical development, but are compromised by the wide spectrum of action of these global cell cycle regulators and the rapid development of resistance (Blagden & de Bono, 2005). Cdc7/ASK kinase, which like CDKs is required for origin activation, has very few protein substrates (reviewed in Takeda & Dutta, 2005) and therefore could be a novel attractive target for anti-cancer therapy. *Montagnoli et al.* have demonstrated that Cdc7 knockdown by siRNA causes tumour cell lines to progress through a defective S phase, leading to p53-independent apoptotic cell death. In contrast, primary normal

human dermal fibroblasts avoid this lethal event by activating a putative p53-dependent cell cycle checkpoint leading to cell-cycle arrest (Montagnoli et al. 2004). Based on these findings, Cdc7/ASK kinase has been actively pursued as a target for anti-cancer drug discovery and development.

Recently, a series of small molecule Cdc7 inhibitors have been reported by Nerviano Medical Sciences (Vanotti et al. 2008) and one compound (PHA-767491) has been characterized *in vitro* and *in vivo* (Montagnoli et al. 2008). PHA-767491 affects the Cdc7/ASK dependent phosphorylation sites, on Mcm2, blocks DNA synthesis, and induces cell death in multiple cancer cell lines. PHA-767491 anti-cancer activity was confirmed in AML (Acute myeloid leukaemia), colon and breast cancer xenograft models (Montagnoli et al. 2008), and also in a rodents with induced mammary tumours (Benakanakere et al. 2006). However, it also shows off target effects on Cdk9 and subsequent RNA polymerase II phosphorylation. NMS-354, another Neviano compound, was reported as an orally available Cdc7 inhibitor (Montagnoli et al. 2008). This compound demonstrated p53-dependent anti-proliferative activity and induction of apoptosis in a broad panel of cancer cell lines, and tumour regression of up to 80% in different xenograft tumour models including ovarian, colon, mammary and leukemia and also in rat mammary carcinogenic-induced tumour models (DMBA model) (reviewed in Sawa & Masai 2008; Swords et al. 2010). In addition to the Nerviano compounds, several other compounds are in development and various potent Cdc7 inhibitors have been reported in the patent literatures (reviewed in Sawa & Masai 2008; Swords et al. 2010). Roche (Zhao et al. 2005), Novartis (Shafer et al. 2008), Pfizer (Tonani et al. 2004) and Sanofi-Aventis (Leroy et al. 2008) have all generated potent compounds capable of inhibiting Cdc7 kinase with low-nanomolar

activity. However, more detailed evaluations of these compounds at the cellular level or in animal models are required for further Cdc7 inhibitor development. Interestingly, Exelixis/Bristol Myer Squibb (BMS) has developed a potent Cdc7 inhibitor (BMS-863233) that is currently under clinical evaluation (reviewed in Swords et al. 2010).

The drug discovery and development process involves several steps, ranging from target identification/validation, assay development, high throughput screening (HTS), hit validation, hit to lead (lead generation/optimization), preclinical and clinical studies to final registration of a drug (Bleicher et al. 2003, also see Chapter 1, section 1.4). Recent drug development has focused on small molecule compounds that specifically inhibit and/or modify tumour-specific molecular biological changes (Saijo et al. 2003). To identify a specific small molecule inhibitor of Cdc7, a well-characterized, functional primary assay is essential at an early stage in the drug development process. An advantage in the development of a primary assay for Cdc7 inhibitors is that Cdc7/ASK has few downstream targets and that specific Cdc7-dependent phosphorylation sites on Mcm2 have been identified *in vitro* and *in vivo*. Cdc7/ASK kinase activity is required for initiation of DNA replication through phosphorylation of the Mcm2-7 complex (Jares et al. 2000; Zou & Stillman 2000; Walter 2000). An abundance of biochemical data show that the Mcm2-7 complex is a downstream target of the Cdc7/ASK kinase complex (Lei et al. 1997; Sato et al. 1997; Jares et al. 2000). Among the six subunits that form the Mcm2-7 complex, Mcm2 is a good substrate for Cdc7/ASK kinase (Cortez et al. 2004; reviewed in Charych et al 2008). Several reports have demonstrated Cdc7/ASK-dependent phosphorylation sites within the N-terminal region of Mcm2 (Montagnoli et al. 2006; Cho et al. 2006; Tsuji

et al. 2006; Charych et al. 2008). These phosphorylated sites can be recognized by serine phosphorylated antibodies, which provides a useful readout for Cdc7/ASK kinase activity *in vitro* and *in vivo* (reviewed in Charych et al. 2008). Exploiting these reagents in the work of this thesis, I sought to develop an antibody-based primary screening assay for identification and characterisation of Cdc7 inhibitors.

In recent years a number of kinases have been implicated in the development and progression of human diseases and, thus, modulation of kinase activity by small molecules has been proposed as an attractive strategy for drug discovery programmes (Cohen 1999; reviewed in Malumbres & Barbacid 2007). Protein kinases share a relatively high degree of sequence similarity, with conserved, regulatory elements, a similar three-dimensional structure, and the same catalytic mechanism of gamma phosphate transfer from ATP to a serine, threonine or tyrosine residues on a protein substrate (Fedorov et al. 2007). These established kinase structural aspects and the binding modes of various known CDK inhibitors can be used to improve potency and specificity in drug discovery and design (Sridhar et al., 2006). High-resolution structural information of the catalytic domain of the kinase family is important for the rational design of selective inhibitors. More than 80 high-resolution structures of Cyclin dependent kinase (Cdk) 2 (De Bondt et al. 1993), Cdk5 (Lew et al. 1995) and Cdk6 (Russo et al. 1998), both alone and in complex with inhibitors, have been published. The kinase domain of Cdc7, a member of the serine/theronine kinase family, has sequence similarities with other kinases, but, the three-dimensional structure of this domain has not yet been reported. The available structural information for other kinases may therefore provide a useful starting point for building a structural homology model of Cdc7 which could be used to develop more

specific Cdc7 inhibitors. Such a model could be validated using a biochemical kinase activity assay which allows high-throughput screening of compound libraries (Gaarde et al. 1997; Mallon et al. 2001).

In this chapter, I describe the development of an antibody-based kinase assay for Cdc7/ASK kinase activity which allows high-throughput screening of compound libraries to identify Cdc7 inhibitors. Furthermore, applying a homology model for Cdc7 based on available crystal structures, analysis of the Cdc7 active sites which are essential for mediating its activity and mutagenesis of key residues in the Cdc7 active site to produce kinase-dead Cdc7 mutants are described.

5.2. Material and Methods

For preparation of recombinant baculoviruses (2.3.1), viral DNA isolation (2.3.2.), generation of recombinant baculovirus (2.3.3.), plaque assay (2.3.4.), virus titeration (2.3.5.), expression of recombinant His-tagged human Cdc7, ASK and mutant Cdc7 in Sf9 insect cells (2.4.2.), expression of recombinant human Cdc7/ASK kinase complex in Sf9 insect cells (2.4.3.) purification of Cdc7, ASK, mutant Cdc7 and Cdc7/ASK complex (2.4.4.), expression and purification of ASK (271-674) fragment (2.4.5., 2.4.6.), expression and purification of Mcm2 (1-154) fragment (2.4.7., 2.4.8.), SDS-polyacrylamide gel electrophoresis (2.4.9.), coomassie blue staining of SDS-PAGE gels (2.4.10.), antibodies (2.5.1.), preparation of whole cell extracts (2.5.2.), immunoblotting (2.5.3.), immunoprecipitation (2.5.4.), *in vitro* kinase assay (2.7.1.) and antibody-based kinase assay for 96-well format (2.7.2.) please refer to Chapter Two (Materials and Methods).

5.3. Results

5.3.1. Development of an antibody-based Cdc7 kinase assay

To identify small molecule inhibitors of Cdc7/ASK kinase activity as potential anti-cancer agents, the development of a primary screening assay is essential. Such an assay should measure the ability of small molecules to inhibit phosphorylation of a substrate peptide. Since Mcm2 is the major physiological substrate of Cdc7/ASK kinase and phosphorylation of Mcm2 at Ser-53 by Cdc7/ASK can be specifically detected by a pMcm2 S53 antibody, recombinant human Mcm2 protein containing the N-terminal 154 amino acids was expressed in *E. coli* and purified using immobilized metal affinity chromatography (IMAC). Coomassie blue stained gels show the 30 kDa N-terminal fragment of Mcm2 and a small amount of a ~29 kDa Mcm2 degradation product after purification (Figure 5.1A). Protein assays after purification showed concentration of 1 mg/ml Mcm2 protein. To establish whether the N-terminal recombinant fragment of Mcm2 can serve as a substrate for Cdc7/ASK in an *in vitro* kinase assay, the fragment was incubated with purified Cdc7/ASK kinase complex in the presence of ATP (Figure 5.1B). Recombinant Cdc7/ASK kinase complex which was used in this *in vitro* kinase assay had been generated in the work described in Chapter Three (Figure 3.6). Using this assay, I was able to demonstrate that the N-terminal region of Mcm2 protein is phosphorylated by purified Cdc7/ASK in the presence of ATP, which is detectable with a pMcm2 S53 antibody (Bethyl, Montgomery, USA). In the absence of ATP, no phosphorylation of Mcm2 was observed (Figure 5.1C). The data discussed in Chapter Four show that the level of

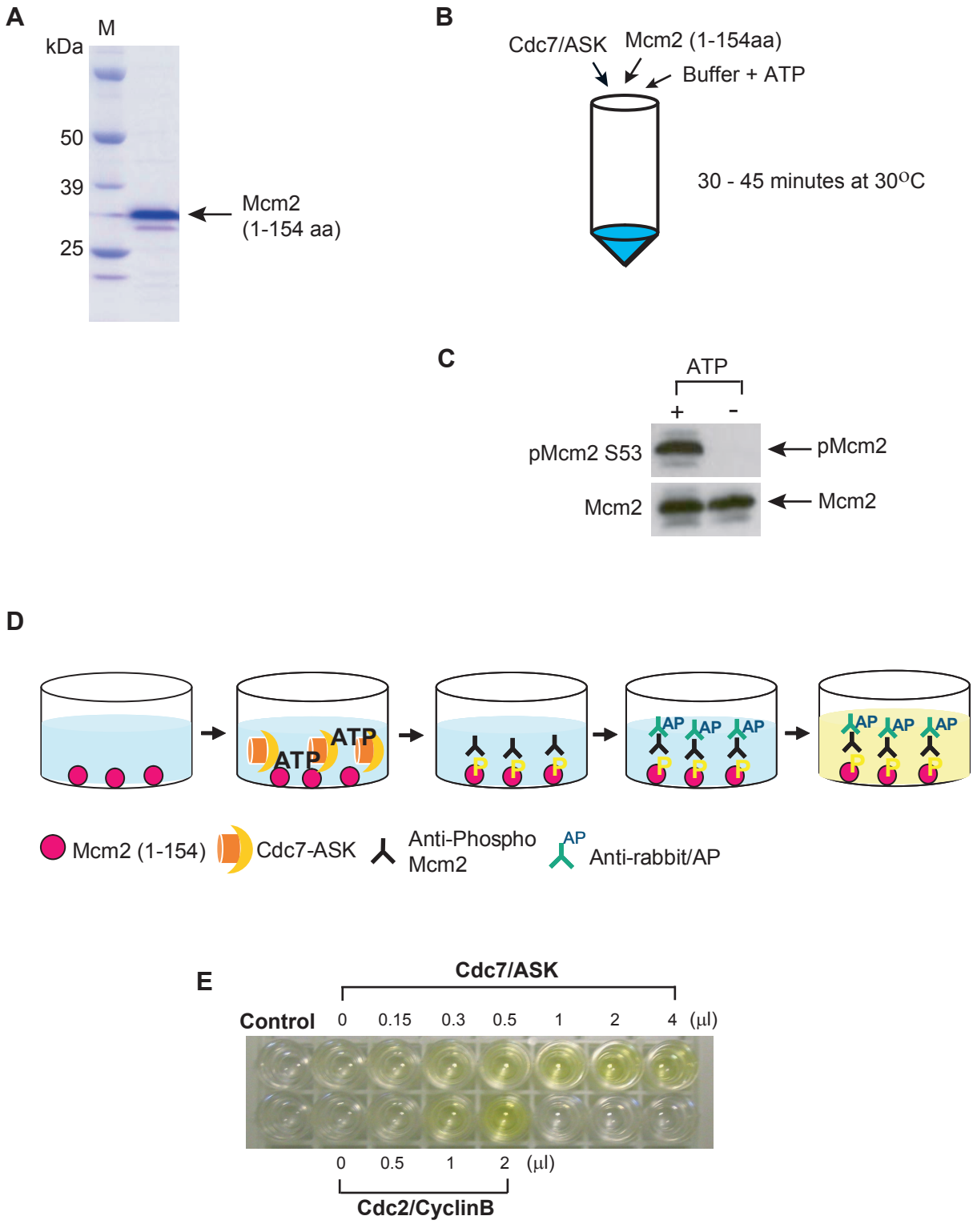


Figure 5.1: Kinase assay with purified human Cdc7/ASK kinase complex and Mcm2 fragment. (A) Coomassie stained gel showing N-terminal fragment of human Mcm2 expressed and purified in *E.coli.* (B) *In vitro* kinase assays were conducted with the purified Cdc7/ASK complex in the presence of Mcm2 fragment as a substrate. (C) After incubation the reaction products were separated by SDS-PAGE and immunoblotted with Mcm2 phosphorylated on serine 53 and Mcm2 antibodies (pMcm2- phosphorylated Mcm2). (D) Schematic of the antibody-based kinase assay which was developed using pMcm2 S53 antibody in 96 well plates. (E) Intensity in colourimetric assay is dependent on kinase concentration (absorbance at 405nm). Cdc2/CyclinB kinase was used as a positive control for the kinase reaction.

pMcm2 S53 oscillates in parallel to the level of Cdc7-bound ASK complex (Figures 4.3, 4.5, and 4.8), reinforcing the notion that phosphorylation of Mcm2 is dependent on Cdc7/ASK kinase activity. Consistent with this finding, pMcm2 S53 antibody recognised phosphorylated Mcm2 after *in vitro* kinase reactions with recombinant Cdc7/ASK (Figure 5.1C and 5.2B).

The activity of the Cdc7/ASK complex can be determined using a ^{32}P-ATP assay in which the transfer of γ^{32}P is measured. Such assays have been routinely employed in the past, for example for cyclin-dependent kinases, and generic peptide substrates have been identified for several classes of kinases (Xu et al., 2003). However, due to several limitations, such as radioactive waste management and the short half-life of reagents, there is a need for more robust non-radioactive assays. With this in mind, I sought to develop a non-radioactive antibody-based assay to measure Cdc7/ASK kinase activity. To allow for high-throughout screening, the original kinase assay was further modified in that purified recombinant Mcm2 fragment was coated onto HisGrab 96-well plates before the kinase reaction. The degree of phosphorylation was determined with a colorimetric method by measuring absorbance at 405nm after the kinase reaction as described in Materials and Methods (Figure 5.1D and E). Taken together, these data indicate that recombinant Cdc7/ASK kinase and its protein substrate Mcm2 can be used for an antibody-based kinase assay system that allows discrimination of specific phosphorylation of the Mcm2 fragment by Cdc7/ASK without using radioactive phosphate.

5.3.2. Optimisation of antibody-based kinase assay for primary screening

To determine the concentration of Mcm2 fragment for maximum coating in each well, varying concentrations of Mcm2 protein were added to 96-well plates. Plates were washed and incubated with reaction buffer and Cdc7/ASK complex to allow phosphorylation of Mcm2. The phosphorylated Mcm2 was then detected using the pMcm2 S53 antibody. Half maximal absorbance was obtained with 500 ng/well of Mcm2 protein, and maximal absorbance was from 1μg/well where binding to the surface reached a plateau (Figure 5.2A). Hence this Mcm2 concentration was used in all further experiments. To determine the dependence of the assay on enzyme concentrations, the assay was performed with increasing amounts of Cdc7/ASK kinase. A titration curve of Cdc7/ASK shows that the Mcm2 phosphorylation signal increases with increasing Cdc7/ASK concentration of up to 15.6μM of Cdc7/ASK kinase complex, indicating that the signal is dependent on enzyme concentration and reflects the phosphorylation status of Mcm2 (Figure 5.2B). The assay was also performed with increasing concentrations of ATP (0-10 μM) to determine an ATP concentration sufficient to obtain reasonable reaction kinetics and still allowing the detection of potential ATP competitive inhibition. Figure 5.3A shows that the *Km* for ATP in the phosphorylation of Mcm2 by Cdc7/ASK is 0.3 μM, indicating that the Cdc7/ASK kinase complex has a high affinity for ATP in this assay (Figure 5.3A).

To establish a robust automated high-throughput screening (HTS) platform, I examined additional parameters such as enzyme stability and pH optimum. The purified Cdc7/ASK kinase aliquots were stored at -80°C for a total duration of up to six months. Cdc7/ASK activity was found to be stable for up to four months (Figure 5.3B). Moreover, the activities of kinase complex purified from different batches of

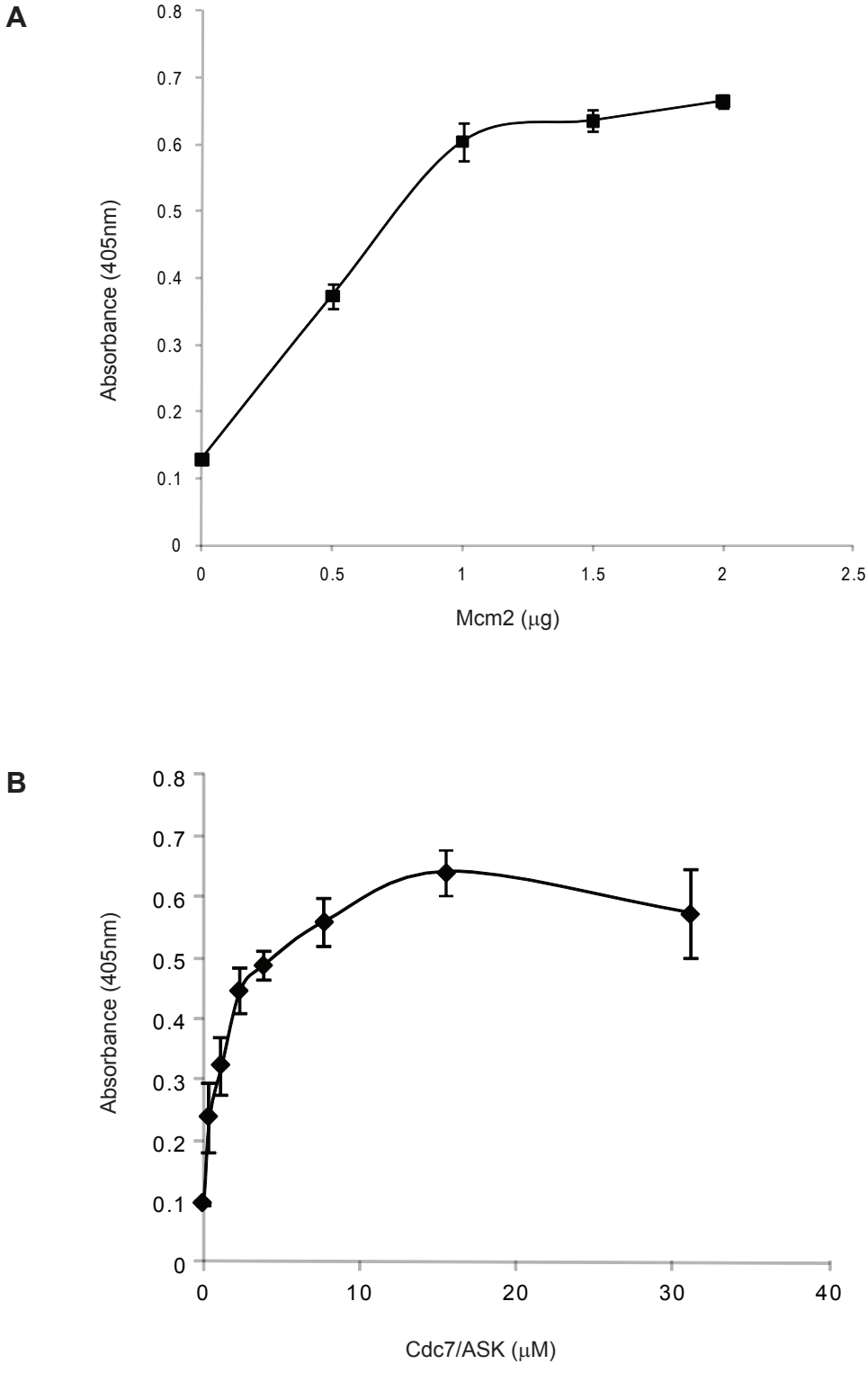


Figure 5.2: Optimisation of antibody-based kinase assay for Cdc7/ASK kinase activity. (A)Titration of Mcm2 protein binding capacity on the 96-well plates. Coating step with various concentrations of Mcm2 fragment was followed by a one hour incubation at room temperature with Cdc7/ASK kinase complex in the presence of ATP. (B)Titration of Cdc7/ASK kinase. Various concentrations of Cdc7/ASK kinase complex were incubated with Mcm2 fragment in the presence of ATP at room temperature for one hour. pMcm2 S53 antibody and secondary antibody incubations for one hour were followed by the addition of a development reagent. The colorimetric signal was detected as absorbance units at 405nm.

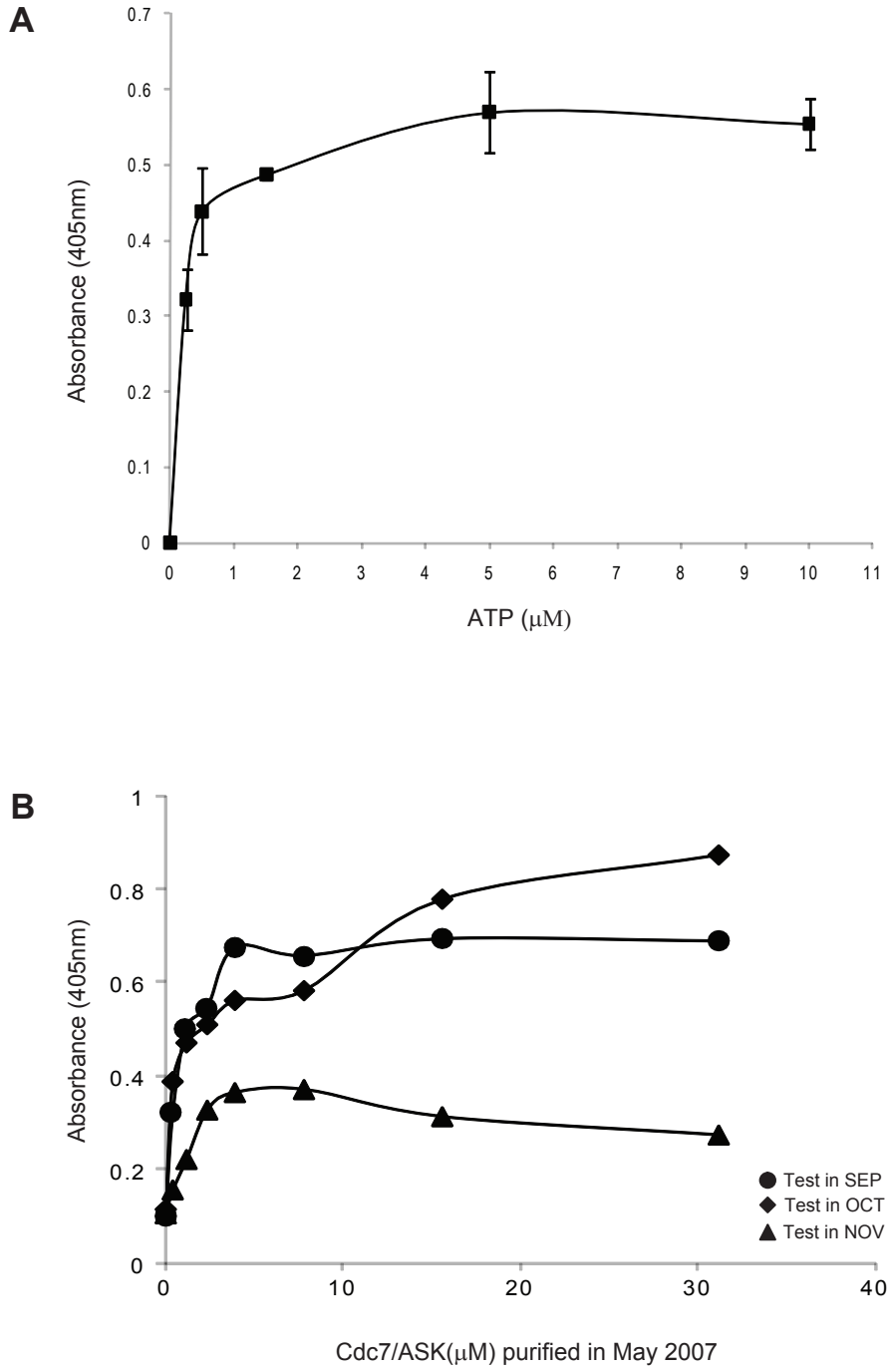


Figure 5.3: Optimisation of antibody-based kinase assay for Cdc7/ASK kinase activity. (A)*Km* for ATP of Cdc7/ASK. ATP was titrated in serial dilutions in the presence of Cdc7/ASK and Mcm2 fragment. (B)Stability test of Cdc7/ASK. The activity of Cdc7/ASK was tested after storage of the purified protein for the indicated periods.

insect cell pellets were consistently high (Figure 5.4A). The kinase reaction reaches a maximum signal at pH 8.5 in reaction buffer and slightly decreases at pH 9.0 (Figure 5.4B). These data suggest that this non-radioactive antibody-based kinase assay can be adapted for high-throughput screening of compound libraries. In Chapter Six, I will be discussing how I have adapted this assay to an automated assay platform for high-throughput screening with guidance from the Cancer Research UK Technology (CRT) assay development team.

5.3.3. Cdc7 homology modelling and structural analysis of Cdc7 active site

In order to generate an effective screening library, it is necessary to identify compounds with a high probability of being hits in a given assay. When crystal structures or homology models of the target protein are available, a screening library can be constructed from a structure-based perspective (Sridhar et al. 2006).

I wish to clearly state that the Cdc7 homology modelling and structural analysis of the Cdc7 active site described in this section are entirely the work of Dr. A. Okorokov (Wolfson Institute for Biomedical Research, UCL, UK). These data are included here as part of the connected argument because they provide the rationale for the mutational analysis described in 5.3.4., performed entirely by myself in the work of this thesis.

Cdc7 is a member of the serine/threonine protein kinase family. Most of the kinases belonging to this family share a similar structure in their catalytic domain which consists of two lobes, a smaller N-terminal lobe and a larger C-terminal lobe (Hanks & Hunter 1995). The N-terminal lobe consists of mostly antiparallel β-sheets and one

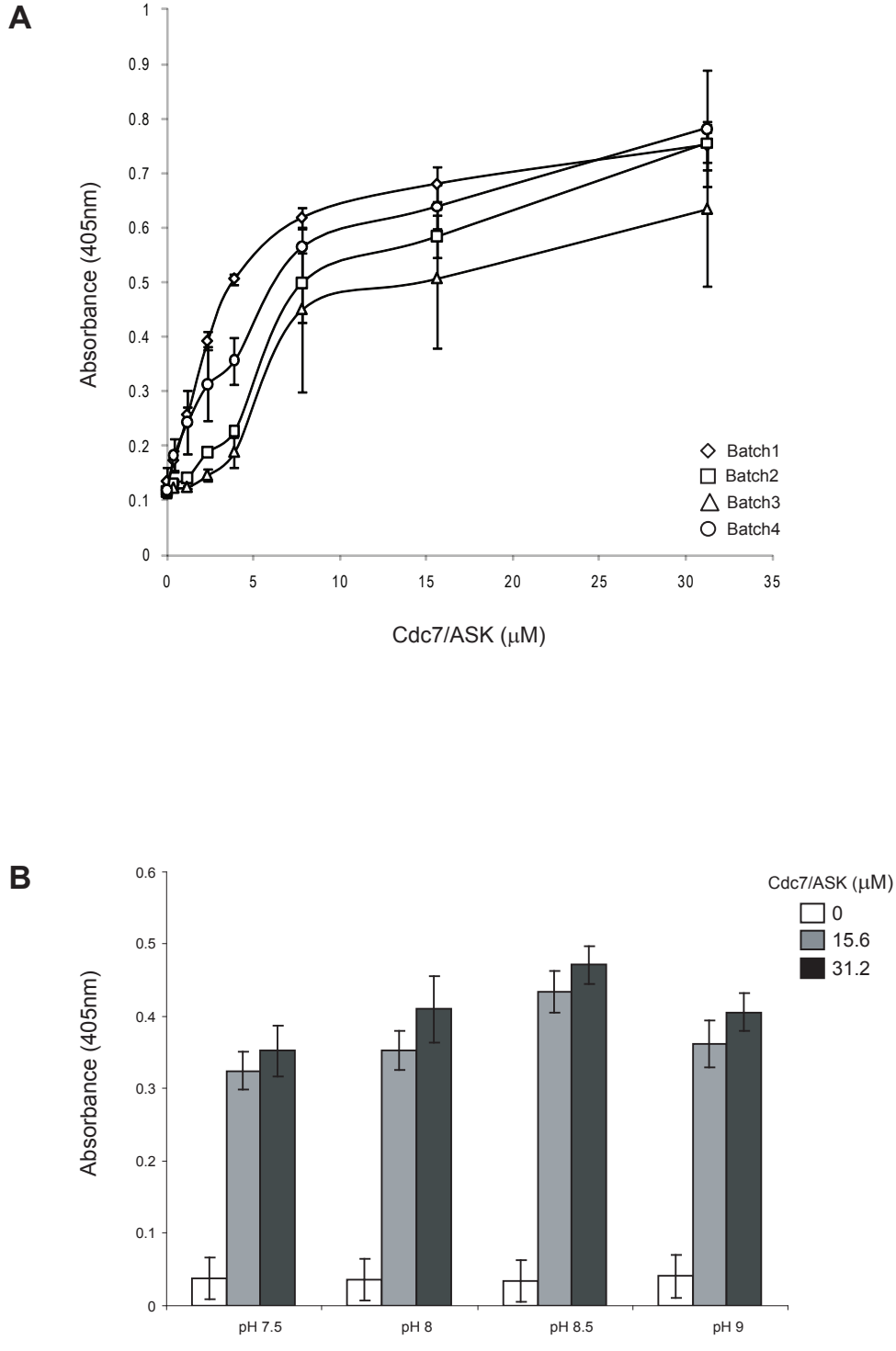


Figure 5.4: Optimisation of antibody-based kinase assay for Cdc7/ASK kinase activity. (A) Assays were performed with Cdc7/ASK purified from different batches of insect cell pellets. (B) pH optimisation of kinase buffer for Cdc7/ASK.

α-helix, whilst the C-terminal lobe is mainly helical. ATP binds in a pocket formed between the two lobes which are linked by a segment referred to as the hinge loop (Hanks & Hunter 1995). In order to identify the most suitable structure for a modelling template, human Cdc7 kinase protein sequence (aa 54-208) was used to predict secondary structure and compared with the known atomic structures in the PDB (Protein data bank) by 3D Threader and Protein Homology/analogy Recognition software packages (Imperial College, London). A computer model of the Cdc7 kinase subdomain based on the crystal structures of human Cdk2 and rat Erk2 is shown in Figure 5.5. The predicted structural homologues with highest accuracy (>90%) were used to derive a 3D structure model of the human Cdc7 kinase core domain which is the most structurally conserved domain within the Cdc7 protein. The highest score (95% accuracy) identified rat Erk2 kinase (PDB 1D 1gol) as the closest structural homologue of human Cdc7 (Figure 5.5B and C). The resulting 3D model of the Cdc7 kinase domain was aligned onto the corresponding domain in Erk2 from structures available at PDB to fine tune the model in PyMOL and SwissModel software packages.

The model allows the location and comparison of amino acid residues of Cdc7 forming the ATP-binding pocket and the catalytic site (Figure 5.6A). In this model, aspartic acid (D) residues 177 and 196 are predicted to be involved in direct catalysis, lysine (K) residues 90 and 179 appear to be important for phosphoryl transfer reactions, whilst threonine (T) 68, known as the "gatekeeper", is involved in the H_2O-Mg_2 activity in the Cdc7 active site (Figure 5.6A). These residues are highly conserved among the Cdc7 kinase family and other serine/theonine kinases. Structure-based library design is typically carried out by computationally docking sets of

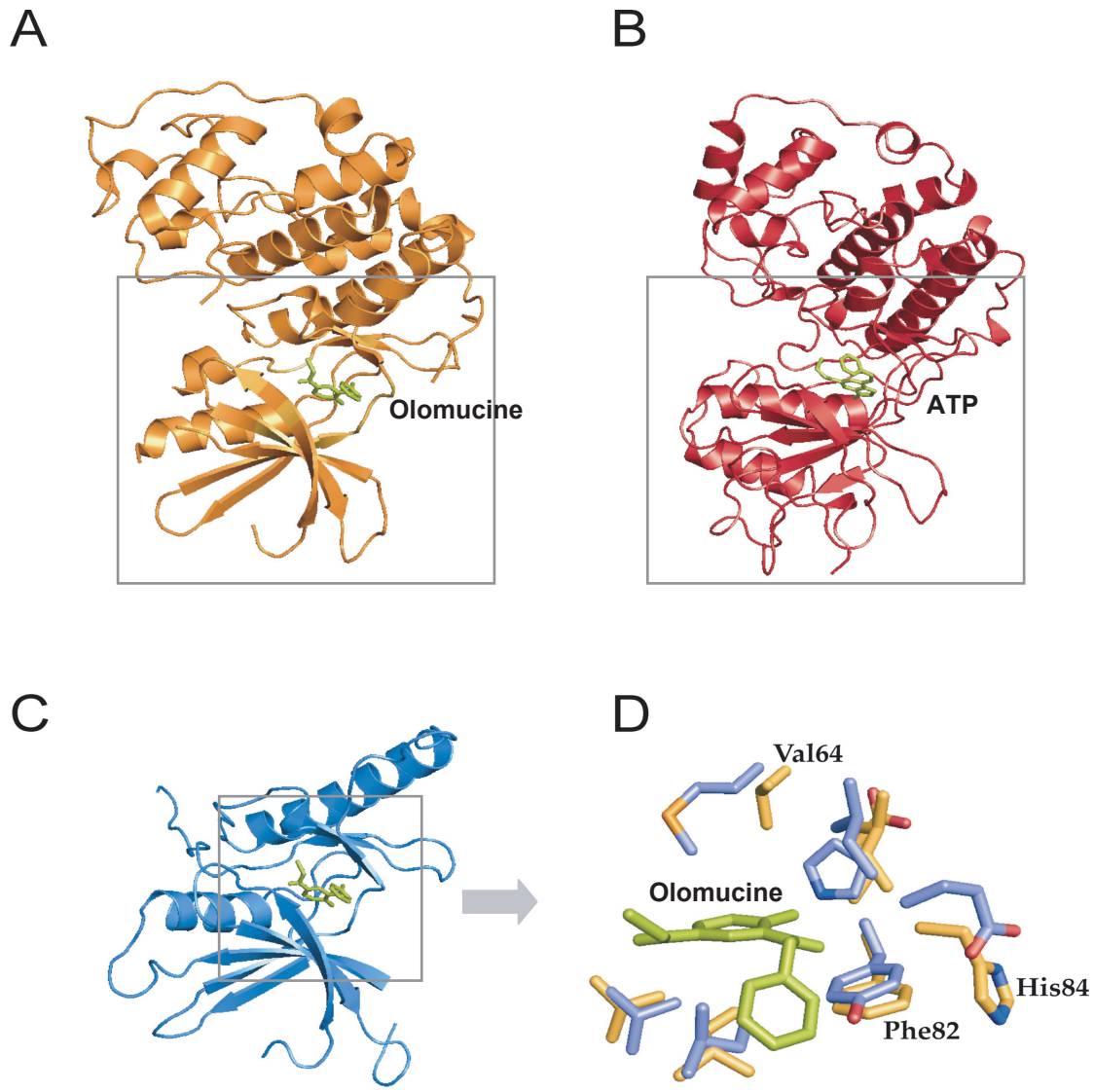


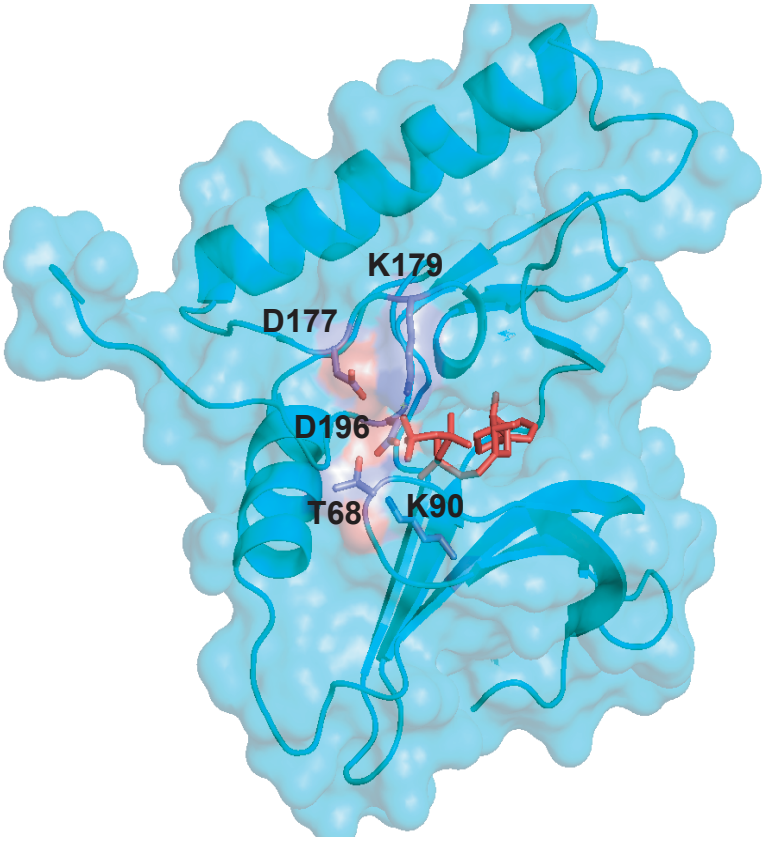
Figure 5.5: High-degree of conservation at molecular level between Cdk2, Erk2 and Cdc7 demonstrated by comparison of kinase structures. Structures of Cdk2 (A), Erk2 (B) and a 3D model of the Cdc7 kinase domain (C) (PDB entries 1 W0X and 1 GOL; Andrei Okorokov, unpublished data). (D)Super-imposed kinase active sites of Cdk2 (shown in orange) and Cdc7 (shown in blue) demonstrate subtle differences in amino acid residues around the ATP binding site, with main alterations corresponding to Cdk2 positions Val 64, Phe 82 and His 84 residues. The active site is occupied by an inhibitor molecule, olomucine (shown in green).

compounds into a rigid model of the protein active site. In the past, docking methods were used to identify small sets of molecules which would be active against a single target. More recently, docking methods have been used to create screening libraries focused on specific gene families such as kinases (reviewed in Walters & Nmchuk 2003). Most existing small molecule inhibitors target the ATP-binding pocket of kinases and compete with ATP. Figure 5.6B shows a comparison of amino acid residues that are found to be important in the ATP-binding pocket of Cdc7 and several related kinases. Importantly, residues F15, S16, I29, A50, Q60, Y68, E86, H87, V104, N110, F112, A114, V128 and A142 are not conserved between Cdc7 and other kinases, thereby providing a starting point for the design of selective inhibitors of Cdc7 (Figure 5.6B).

5.3.4. Biochemical analysis of wild-type and mutant Cdc7 kinase activity

Mutation of the conserved lysine in the kinase subdomain has become a standard method for inactivating protein kinases. It has previously been demonstrated that the mutation of lysine (K) 90 to arginine in the Cdc7 kinase subdomain II inactivates the catalytic activity of Cdc7/Dbf4 complex (Jiang & Hunter 1997; Jiang et al 1999; Masai et al. 2000). To validate the activity prediction of the Cdc7 three-dimensional model, K90, D177 and D196 were replaced with alanines (A) to negate catalytic activity using site-directed mutagenesis (see Material and Methods). The presence of the site-specific mutations was confirmed by DNA sequencing. Three mutants (K90A, D177A and D196A) and wild-type Cdc7 were individually co-expressed with ASK protein and purified from insect cells as described in Chapter Three.

A



B

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                                                    T68                                 K90
CDK2_HUMAN      -----------------MEN--FQKVEKIGEGTYGVVYKARNKLTG---EVVALKKIRX- 37
CDK4_HUMAN      -----------------MATSRYEPVAEIGVGAYGTVYKARDPHSG---HFVALKSVRVP 40
ERK2_RAT        MAAAAAAGPEMVRGQVFDVGPRYTNLSYIGEGAYGMVCSAYDNLNK---VRVAIKKISP- 56
CDC7_HUMAN      ------------------LSNVFKIEDKIGEGTFSSVYLATAQLQVGPEEKIALKHLIP- 41
                                                                        A

CDK2_HUMAN      --DTETEGVPSTAIREISLLKELN---HPNIVKLLDVIHT-----ENKLYLVFEFLHQDL 87
CDK4_HUMAN      NGGGGGGGLPISTVREVALLRRLEAFEHPNVVRLMDVCATSRTDREIKVTLVFEHVDQDL 100
ERK2_RAT        ---FEHQTYCQRTLREIKILLRFR---HENIIGINDIIRAPTIEQMKDVYIVQDLMETDL 110
CDC7_HUMAN      ------TSHPIRIAAELQCLTVAGG--QDNVMGVKYCFRK-------NDHVVIAMPYLEH 86

                                                    D177 K179                   D196
CDK2_HUMAN      KKFMDASALTGIPLPLIKSYLFQLLQGLAFCHSHRVLHRDLKPQNLLIN-TEGAIKLADF 146
CDK4_HUMAN      RTYLDKAPPPGLPAETIKDLMRQFLRGLDFLHANCIVHRDLKPENILVT-SGGTVKLADF 159
ERK2_RAT        YKLLKTQHLS---NDHICYFLYQILRGLKYIHSANVLHRDLKPSNLLLN-TTCDLK---- 162
CDC7_HUMAN      ESFLDILNSLS--FQEVREYMLNLFKALKRIHQFGIVHRDVKPSNFLYNRRLKKYALVDF 144
                                                      A                     A
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Figure 5.6: Modelling of the Cdc7 ATP-binding pocket. (A) Structure model of the Cdc7 ATP-binding pocket with bound ATP (shown in red). Five residues (T68, K90, D177, K179 and D196) around the ATP-binding pocket are indicated (Andrei Okorokov, unpublished data). (B) Sequence alignment of the conserved motifs from human Cdc7 and selected kinases (human Cdk2, Cdk4 and rat Erk2), showing 14 unique residues (shown in yellow) of the Cdc7 domain and five key residues (shown in gray) of the Cdc7 active site. Three residues (K90, D177 and D196) were targeted for mutagenesis in the work of this thesis.

To determine the kinase activity of these proteins, activity was measured in the antibody-based kinase assay under optimized conditions established for the wild-type protein. As shown in Figure 5.7A, kinase activity was only detected for wild-type Cdc7/ASK complex but not for the three mutants. An ADP accumulation assay is a biochemical assay to measure ADP generation resulting from kinase phosphorylation of a substrate. This assay is useful for determining substrate Michaelis-Menten kinetics and for profiling inhibitor potency and mode of action. To confirm the importance of the three residues (K90, D177 and D196) in the catalytic site for kinase activity, the amount of ADP produced as a result of kinase activity was measured after *in vitro* kinase reactions with wild-type and mutant Cdc7/ASK complexes (Figure 5.7B). The amount of ADP increased linearly with wild-type Cdc7/ASK over time. However, ADP accumulation with the mutant D177A reached only 50% level relative to wild type Cdc7/ASK and K90A and D196A mutants showed only 30% level of ADP accumulation (Figure 5.7B). These data indicate that three residues, K90, D177 and D196 form part of the catalytic site of Cdc7, and that mutation of K90 and D196 has created a catalytically inactive Cdc7 kinase as predicted by the structural model.

5.4. Discussion and Conclusions

The initiation of DNA replication, which lies downstream of oncogenic and mitogenic signalling transduction pathways, is a critical step in cell proliferation. Because Cdc7/ASK is an essential kinase required for the regulation of DNA replication initiation, a specific inhibitor of Cdc7/ASK activity could be an attractive anti-cancer agent. The anti-cancer potential of targeting Cdc7 kinase has led to an increasing interest in selective inhibitors that could represent lead compounds for drug development. In an effort to generate Cdc7 inhibitors, various compounds were

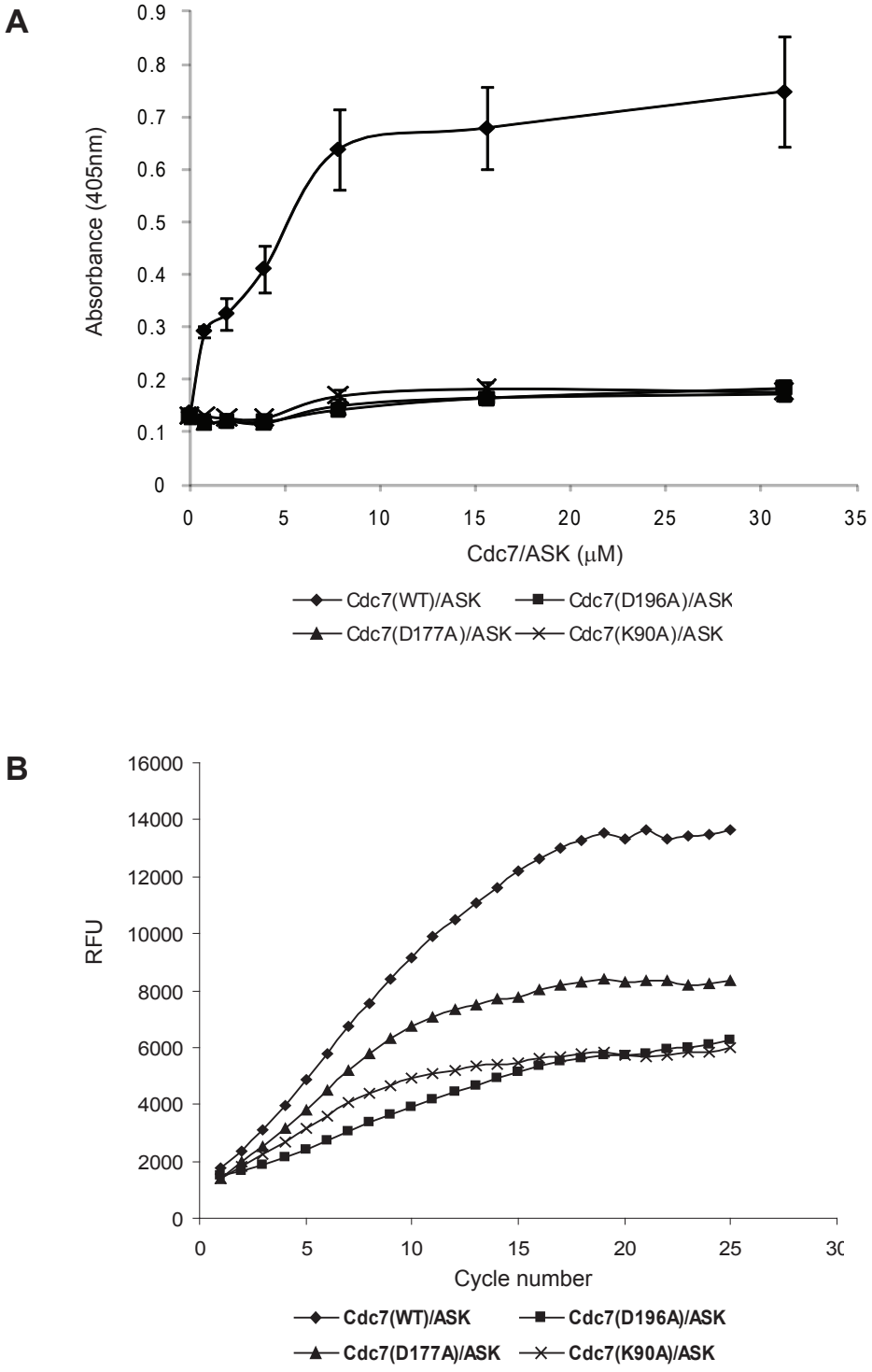


Figure 5.7: Kinase activity analysis of wild-type and Cdc7 mutants. Using site-directed mutagenesis, K90, D177 and D196 were replaced with alanines to negate catalytic activity. Three mutants (K90A, D177A and D196A) and wild-type Cdc7 were co-expressed with ASK and purified from insect cells. Increasing concentrations of wild-type and mutant Cdc7 and ASK kinase complex were incubated with Mcm2 fragment in the presence of ATP. Kinase activity was measured in the antibody-based kinase assay (A) and in the ADP Quest assay with kinetic mode (B).

recently reported as potent Cdc7 inhibitors, some of which were shown to potently induce apoptotic cell death in multiple cancer cell lines and to cause tumour growth inhibition in animal models (reviewed in Sawa & Masai 2008; Swords et al. 2010). As a result, several compounds are currently undergoing pre-clinical and/or clinical evaluation, and the search for more specific small molecule Cdc7 inhibitors continues.

The development of a primary screening assay is essential for the identification and development of a potent small molecule inhibitor of Cdc7 kinase. In the case of kinase assays, the expression and purification of active protein is often a limiting step and restricts the targets pursued or the methods used for screening (Walters & Namchuk 2003). In the antibody-based kinase assay established during the work of this thesis, active baculovirus-expressed human Cdc7/ASK complex (Figure 3.6) and human Mcm2 protein (Figure 5.1A) as a substrate have been used to measure the phosphorylation of Mcm2 by Cdc7/ASK kinase (Figure 5.1D and E). Figure 5.2B shows that the effect of kinase concentration on Mcm2 phosphorylation is dose dependent and only low amounts (2.34μM) of Cdc7/ASK kinase are required for a sufficient phosphorylation signal, indicating the high sensitivity of this assay for Cdc7/ASK activity (Figure 5.2B). Furthermore, the kinase activity is stable for up to four months (Figure 5.3B) and kinase activity from different batches of cell pellets is consistent (Figure 5.4A). These data show that the availability of active protein such as recombinant Cdc7/ASK kinase and its substrate Mcm2 provided significant advantages for the development of a specific primary assay suitable for high-throughput screening (HTS) of compound libraries.

In the past, kinase activity tests mostly relied on the incorporation of radioactive phosphate into proteins, but for large-scale investigations radioactive waste products should and can be avoided. Human Mcm2 has been shown to be phosphorylated by Cdc7/ASK kinase and distinct phosphorylation sites in the Mcm2 N-terminal region were identified *in vitro* and *in vivo* (Montagnoli et al. 2006; Cho et al. 2006; Tsuji et al 2006; Charych et al. 2008). Furthermore, phospho-specific Mcm2 antibody recognising serine 53 phosphorylation site was generated and have been used as a marker of Cdc7/ASK kinase activity in cells (Montagnoli et al. 2006; Charych et al. 2008). By using the pMcm2 S53 antibody recognised Cdc7-dependent-phosphorylated Mcm2 I was able to demonstrate that the antibody-based assay described in this chapter is a reliable and a convenient kinase assay suitable to screening of large compound libraries.

Biochemical assays can generally be classified into separation-based assays and homogeneous assays (reviewed in Walters & Namchuk 2003). Homogeneous assays in which the detection of the product does not require a separation step can have assay artefacts from the reaction product. Separation-based assays in which the reaction product is detected after its separation from the starting material on the other hand have the advantage of minimal interference from the reaction product (reviewed in Walters & Namchuk 2003). In keeping with this rationale, the antibody-based assay was designed to include wash steps, and therefore interference from the compound library should be minimal. However, it needs to be considered that the requirement for additional steps such as pre-wash and blocking steps may increase total screening time and assay complexity.

In the absence of the crystallographic structure of the Cdc7 catalytic subunit, a three-dimensional model of Cdc7 was built based upon homology with other kinases (Figure 5.5). It is evident from the model that the structural scaffold of the Cdc7 kinase subdomain is very similar to those of human Cdk2 and MAP kinase Erk2 (Figure 5.5A, B and C). The model allowed the location and comparison of amino acid residues of Cdc7 (Figure 5.6) that are known to be important for catalysis, and are highly conserved among the Cdc7 families, suggesting the model can be used for recognition of functionally important elements for a structure-based perspective. Using site-directed mutagenesis, three conserved residues located in the Cdc7 active site were mutated to alanine to examine their role in kinase activity. Figure 5.7A shows that the three mutants produce inactive kinases, confirmed by the specificity of Cdc7-dependent phosphorylation of Mcm2 in the antibody-based assay (Figure 5.7A). In addition, the ADP accumulation assays clearly show that phosphorylation activity is impaired in the K90, D177 and D196 mutants (Figure 5.7B), suggesting that these residues are involved in creating catalytically inactive Cdc7, thus drastically negating kinase activity as predicted by the structural model.

Interestingly, disruption of the ATP binding mode is a mechanism of regulation of protein kinase, for example the reduced activity of Cdk2 associated with the K52 position mutation is due to non-productive binding of ATP (De Bondt et al. 1993). The exquisite sensitivity to the position and conformation of ATP may serve a regulatory role in preventing kinases from hydrolyzing ATP in the absence of protein substrates (Robinson et al. 1996). An *in silico* approach has been used with great success in the design of selective inhibitors of Cdk4 (Ikuta et al 2001). Initial prospective inhibitors were improved by 1.5 log units by analyzing the sequence

differences around the ATP-binding site and incorporating this information into the rational design of compounds optimized for the kinase of interest. Importantly, Figure 5.6B shows that the 14 amino acid residues of the ATP binding pocket were found to be less conserved within the Cdc7 kinase family and among other kinases providing a structural rationale for medicinal chemistry to design selective Cdc7 inhibitors.

In summary, the data presented in this chapter show the development of an assay for Cdc7 kinase activity based on measuring Cdc7-dependent phosphorylation of Mcm2 using a phospho-specific antibody. The assay was optimized for parameters including substrate binding capability, optimum pH for kinase buffer and kinase stability, which is essential for robust, automated high-throughput screening of compound libraries identifying Cdc7 inhibitors. In addition, a computer model of Cdc7 kinase showed highly conserved amino acid residues which form the catalytic site of Cdc7 kinase. Furthermore, a comparison of amino acid residues in the ATP-binding pocket revealed 14 unique residues of Cdc7 kinase, suggesting that this difference in the composition of the ATP-binding pocket may provide a platform for future design of selective Cdc7 inhibitors. In the following chapter, I discuss how, after further optimisation, the screening assay was successfully transferred into a 384-well format for HTS. Automation of the assay has been accomplished by employing a batch-mode operation using automated workstations for buffer handling and plate washing, which are in principle amenable to fully automated operation. Using the HTS platform, Cdc7/ASK kinase was screened against the Biomol Kinase Inhibitor Plates (Biomol Research Laboratories, USA) and the main CRT DL chemical libraries (60000 compounds). As discussed in Chapter Six, one of the “hits” that emerged from

screening was validated and exploited to further study the response of transformed cells to origin activation repression.

CHAPTER SIX

Compound screening and characterization of small molecule Cdc7 inhibitors

6.1. Introduction

The rapidly growing interest in kinases as drug targets has prompted the development of biochemical assays which can be adapted to high-throughput screening (HTS), wherein large libraries of compounds are tested for the identification of kinase inhibitors. Accordingly, HTS has become an important component of modern drug discovery research. This screening is very much influenced by advances in automation and miniaturization technology and is under constant development (Fox et al. 2002; Bajorath 2001). The interference of compounds on the detection system is an important consideration in the development of suitable HTS for large compound library screening. Homogeneous HTS assays that allow the addition of all reaction reagents in one well without separation steps could be compromised by compounds affecting the reaction product. Separation-based HTS assays in which the reaction product is detected after its separation from the starting material, on the other hand, have the advantage of minimal compound interference on the reaction product (reviewed in Walters & Namchuk 2003). The data discussed in the previous chapter show that the establishment of a primary assay based on measuring Cdc7-dependent phosphorylation of Mcm2 using a phospho-specific antibody offers significant advantages for the development of a specific Cdc7 kinase assay suitable for HTS of compound libraries. Furthermore, this assay contains wash steps and therefore expected interference from compounds should be minimal.

Over the past few years, diverse computational concepts and methods have been introduced to analyse screening data, extract information from HTS experiments and derive predictive models of activity, thereby attempting to exploit the complementary nature of HTS and combinatorial chemistry (Bajorath 2001). These computational approaches that 'dock' small molecules into the structures of targets and 'score' their potential complementarily to binding sites are widely used in hit identification and lead optimization (Kitchen et al. 2004). The data presented in the previous chapter indicate that a homology model of the Cdc7 active site based on published crystal structure data of other kinases which highlight the unique residues of the Cdc7 ATP-binding pocket, may provide a structural-chemical basis for designing selective Cdc7 inhibitors.

In this chapter, I describe how an antibody-based primary assay was successfully transferred after further optimisation into an automated 384-well format HTS to identify Cdc7 inhibitors alongside the Cancer Research UK Technology (CRT) assay development team (based at Wolfson Institute for Biomedical Research, UCL, UK). CRT has a large collection of diverse molecules (approximately 60,000 compounds) which is being screened in this automated HTS primary assay to search for Cdc7 kinase inhibitors. Through a series of screening, 16 compounds showed inhibition of Cdc7/ASK kinase and were selected for further hit validation studies. In order to identify a reference compound for screening, Cdc7/ASK kinase was screened against the Biomol Kinase Inhibitor Plates (Biomol Research Laboratories, USA). Compound GW5074 was identified from Biomol Kinase Inhibitor Plates and used as a standard compound for screening. Furthermore, I describe a second three-dimensional model

of the Cdc7 active site based on sequence identity over the kinase domain in this chapter to validate initial hit compounds which are currently being taken forward into lead identification/optimization by CRT for the development of selective Cdc7 inhibitors. In order to develop a cell-based assay system for assessing the cellular activity of compounds, I used the Nerviano compound PHA-767491 (referenced to as CRT100115) as a reference compound in SK-OV3 ovarian cancer cells and BT549 breast cancer cells to study cellular effects in this Chapter.

6.2. Material and Methods

For preparation of recombinant baculoviruses (2.3.1), expression of recombinant human Cdc7/ASK kinase complex in Sf9 insect cells (2.4.3.) purification of Cdc7, ASK, mutant Cdc7 and Cdc7/ASK complex (2.4.4.), expression and purification of Mcm2 (1-154) fragment (2.4.7., 2.4.8.), SDS-polyacrylamide gel electrophoresis (2.4.9.), antibodies (2.5.1.), preparation of whole cell extracts (2.5.2.), immunoblotting (2.5.3.), bivariate flow cytometric analysis (2.6.2), assaying for apoptosis (2.6.4.) and high-throughput screen for Cdc7 inhibitors (2.7.3.) please refer to Chapter Two (Materials and Methods). For synthesis of PHA-767491 compound please see Appendix B.

6.2.1. HTS automation equipment

The assay was performed in clear, 348-well, Nunc Immobilizer Nickel-chelate microplates (ThermoFisher Scientific, USA). For HTS procedures, each assay component was added to individual wells using the Matrix Plate Mate (ThermoFisher Scientific, USA, Serial No.501-1-05214), Matrix Well Mate (ThermoFisher Scientific, USA, Serial No.201-2-0170) and Perkin Elmer FlexDrop (Perkin Elmer, USA, Serial

No.560301). All wash steps were performed using the Bio-tek ELx405 Select CW Microplate Washer (Bio-tek, USA, Serial No. 202242). To measure absorbance, the Analyst plate reader (Molecular Devices, USA, Serial No. AN0166) was used (Figure 6.1).

6.2.2. HTS reagents and supplies

TBS, wash buffer (TBS containing 0.05% Tween-20), kinase reaction buffer (50 mM Tris-HCl pH 8.5, 10 mM $MgCl_2$, 1 mM DTT), diethanolamine buffer (1 M diethanolamine pH 9.8, 0.5 mM $MgCl_2$), development reagent (one P-nitrophenyl phosphate (PNPP) tablet was dissolved in 20 ml diethanolamine buffer) and stop solution (1 M NaOH) were used as described in Chapter Two (Material and Methods). I made his-tagged Cdc7/ASK kinase and N-terminal fragment of Mcm2 as described in Chapter Two (Material and Methods). Rabbit anti-phospho Mcm2 S53 (BL3353) was purchased from Bethyl (Universal Biologics, USA) and anti-rabbit/AP secondary antibody was obtained from Dako (Glostrup, Denmark). P-nitrophenyl phosphate (PNPP) substrate was purchased from Sigma-Aldrich (St. Louis, MO, USA). The Biomol kinase inhibitor plate (Catalogue No. 2832-0001) was supplied by Biomol Research Laboratories (USA) and the main CRT DL chemical libraries were made available by the CRT chemistry department.

6.2.3. Antibody-based assay protocol for HTS

384-wells of a clear Nickel-chelate micro-plate were pre-coated in one hour incubation with 250 ng of Mcm2 fragment (substrate). Non-bound protein was removed by washing the plate three times with wash buffer. Cdc7/ASK kinase

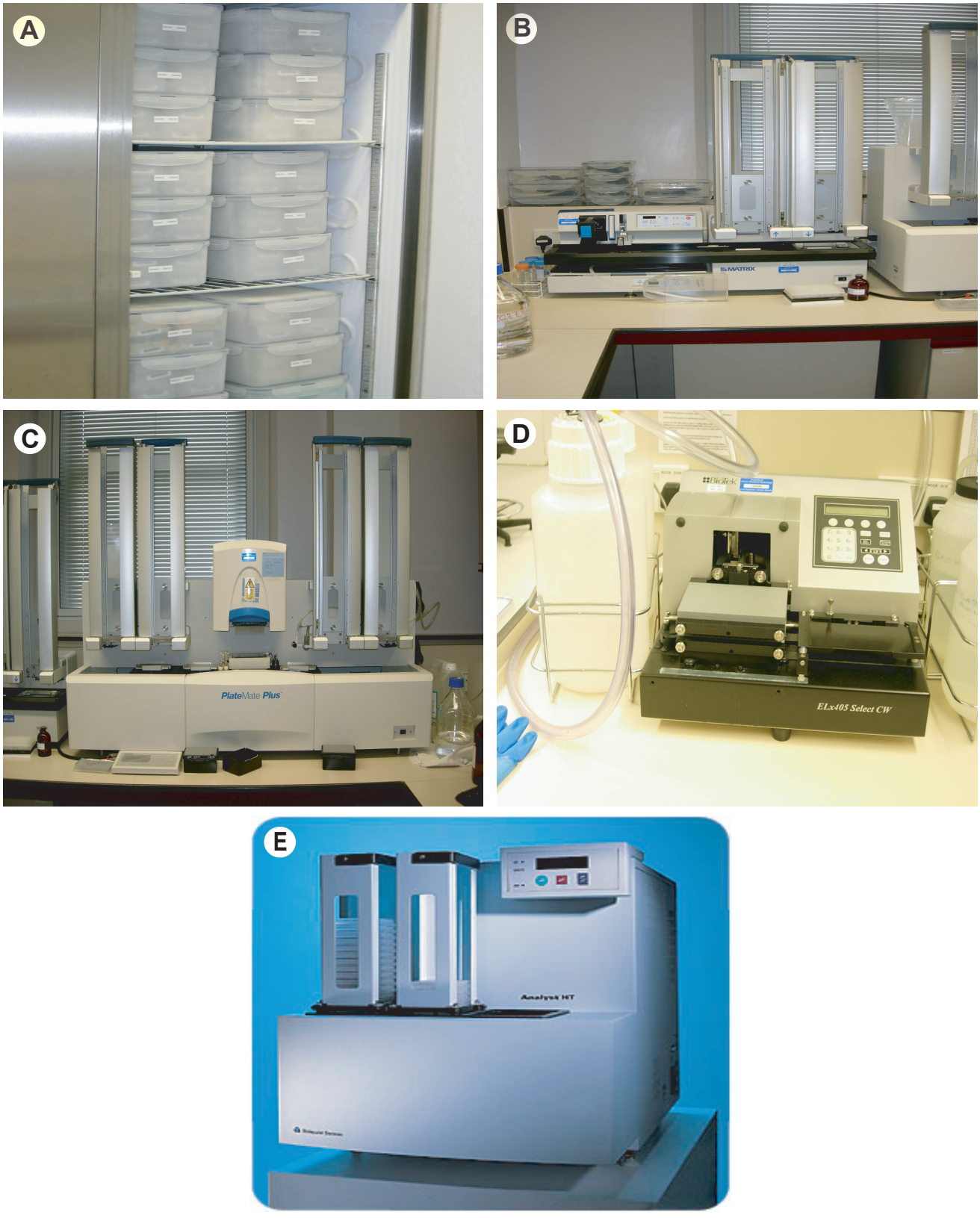


Figure 6.1: HTS automation equipment. (A) CRT DL chemical libraries (60,000 compounds). (B) Matrix well Mate and Perkin Elmer Flex Drop. (C) Matrix Plate Mate (D) Bio-tek ELx405 Select CW Microplate Washer. (E) Analyst Plate Reader.

reactions were assembled by stepwise addition of compound in 4% DMSO, Cdc7/ASK kinase and ATP in kinase reaction buffer. The final concentrations of Cdc7/ASK, compounds and ATP were 5 ng/well, 30 μM and 0.2 μM respectively in 20 μl of final assay volume. After one hour incubation for the kinase reaction, the plate was washed and incubated for 30 min with a primary pMcm2 S53 antibody. Non-bound antibody was removed by washing with wash buffer, and the plate was incubated with secondary antibody for 30 min, followed by three washes and the addition of a development reagent. Signal development was stopped after one hour by addition of sodium hydroxide. Raw data were collected as absorbance units at 405 nm using the Molecular Devices Analyst (Molecular Devices, USA, Serial No. AN0166) and were normalized as percentage inhibition of Cdc7/ASK kinase relative to the percentage control values. I used GW5074 as a standard inhibitor compound, which was identified from the Biomol kinase inhibitor plate (Biomol Research Laboratories, USA) and included on every plate at 100 nM as a positive control.

6.3. Results

6.3.1. Optimization of automated HTS

Initial experiments were focused on transferring the antibody-based assay to the 384-well format for automated HTS[2]. In preliminary experiments several automation issues were encountered. Firstly, due to the high number of washing steps in the assay system and the limitations of the micro-plate washer, a daily throughput of only 10 plates could be achieved. In order to achieve this throughput, pre-wash and blocking

[2] Experiments were done with guidance from the CRT assay development team. CRT operates laboratory automated HTS workstations that are robust enough to support the screening of CRT DL chemical libraries. I established the antibody-based kinase assay, and provided active baculovirus-expressed human Cdc7/ASK kinase complex and human Mcm2 protein as substrate.

steps were eliminated and antibody incubation times were also reduced. Secondly, Cdc7/ASK kinase was added using the Perkin Elmer Flex Drop (Perkin Elmer, USA, Serial No.560301). Patterns of low signal in the first wells dispensed were observed in early experiments, indicating low enzyme levels in these wells. However, this issue was eliminated by the inclusion of a prime plate at the start of the run. Thirdly, problems were encountered with the Matrix Well Mate (ThermoFisher Scientific, USA, Serial No.201-2-0170). This system was used to add 20 µl of primary and secondary antibodies. The tubing became blocked after 4-5 days of screening, even with vigorous washing with 0.1 M NaOH and dispensing accuracy/precision errors were noticeable. However, when dispensing steps were carried out using the Dynex Multidrop 384 plate dispenser (Magellan Bioscience, USA), the issue was resolved. Thus the antibody-based assay was eventually made suitable for automated HTS small molecule Cdc7 inhibitors.

6.3.2. HTS assay reproducibility

Assay quality has been traditionally judged by several metrics such as signal to noise ratio or signal to background ratio (Zhang et al. 1999). These metrics enable definition of significant results from a screen and are also useful for maintaining the same standard for assay data over multiple days screening, or for comparison of data quality across screens. The main quality parameter in HTS is the Z factor (Z'), as described by equation (1):

$$Z' = 1 - (3\delta_+ + 3\delta_-)/(\mu_+ - \mu_-) \quad (1)$$

where δ_+ is the standard deviation for the positive control, δ_- is the standard deviation for the negative control for the assay. $\mu_+ - \mu_-$ defined the mean value for the positive

and negative control (Zhang et al. 1999). The Z factor has the advantage of expressing the noise in relation to the signal window and, thus, gives a more complete estimation of assay quality than signal to noise or signal to background alone would do. Z factors above 0.5 indicate a large separation band between the values for the positive (100% activity) and negative controls (0% activity). Therefore, in practice, assays with values Z'> 0.5 have acceptable characteristics for HTS. Figure 6.2 shows the scatter plots from all 198 plates over screening. Each plate contains up to 320 test compounds. The average of percentage error in controls and blanks across the entire screening were 3.6% and 5.7 % respectively. The Z factor of all accepted plates was greater than 0.5, with a Z factor of 0.41 in one data set and an overall average Z factor of 0.7 (Figure 6.2). These data confirm the reproducibility of the assay, indicating that the precision and robustness of the assay were high enough to support HTS campaigns.

6.3.3. Cdc7 primary screen of the Biomol kinase inhibitor plate and hit characterization

The Biomol Kinase Inhibitor Plate (Biomol Research Laboratories, USA) contains 80 known kinase inhibitors of well-defined activity. To demonstrate assay sensitivity and robustness of the overall screening process, I tested the inhibitor plate for inhibition of Cdc7/ASK in triplicate at two different ATP concentrations (0.2 μM and 5 μM) using automated HTS conditions. Each test compound was assayed at a single concentration of 10 μM in 2% DMSO, with 12 compounds showing inhibition of Cdc7/ASK kinase of greater than 50% at the ATP *Km* 0.2 μM (Table 6.2). Notably, two known compounds, Staurosporine and GW5074, were revealed to be potent inhibitors of Cdc7/ASK kinase, with IC_{50} values of 0.010 μM and 0.108 μM, respectively, in the

Table 6.1 HTS protocol

Step	Parameter	Value	Description
1	Substrate	20 ml	Incubated over night at 4°C
2	TBS/Tween wash	3 x 80 ml	
3	Library compounds	2 ml	
4	Cdc7/ASK kinase	13 ml	containing kinase buffer
5	ATP	5 ml	Incubated 60 min at RT
6	TBS/Tween wash	3 x 80 ml	
7	Primary antibody	20 ml	Incubated 30 min at RT
8	TBS/Tween wash	3 x 80 ml	
9	Secondary antibody	20 ml	Incubated 30 min at RT
10	TBS/Tween wash	3 x 80 ml	
11	Colour Development	20 ml	Incubated 60 min at RT
12	Stop solution	20 ml	Absorbance read at 405nm

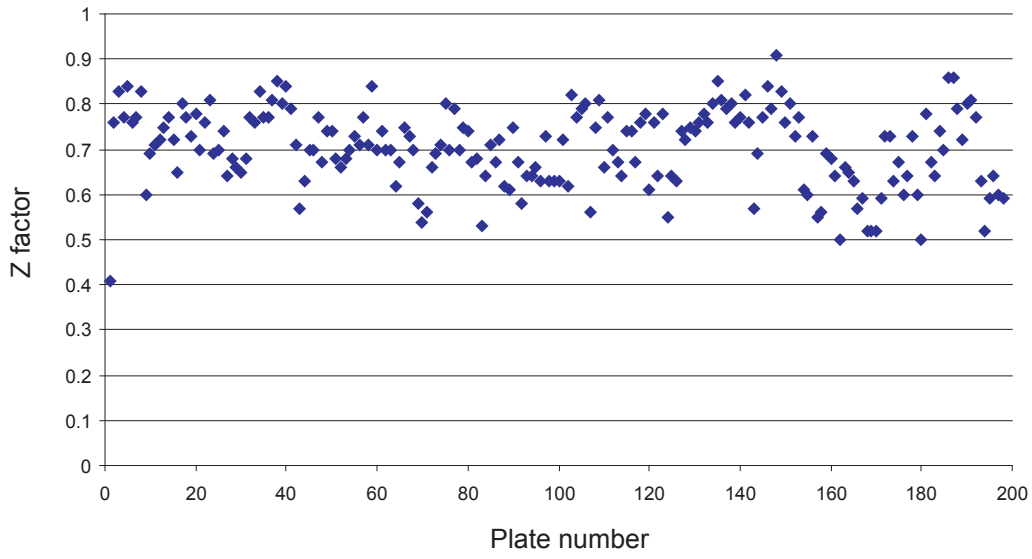


Figure 6.2: Z factor distribution for the CRT main library screen of 348-well microplates. 198 plates were screened, each plate containing up to 320 test compounds. All accepted plates had a Z factor of > 0.5 (except one set; Z factor 0.41), overall average Z factor is 0.7.

presence of 5 μM ATP (Figure 6.3A, B). GW5074 is a potent and selective cell permeable inhibitor of cRAF1 kinase (IC_{50} 9 nM), which is downstream of Ras in the MAPK signalling pathway (Lackey et al. 2000). It also shows 100-fold selectivity over Cdk1, Cdk2, c-src, ERK2, MEK, p38, Tie2, VEGFR2 and c-fm (Lackey et al. 2000). To assess Cdc7/ASK kinase inhibition by GW5074 in intact cells, I examined Mcm2 phosphorylation at serine 53 in GW5074 treated HeLa cells (Figure 6.3C). I used a CDK inhibitor olomucine as a comparison. Western blotting results showed that 10 μM of GW5074 inhibits phosphorylation of Mcm2 at serine 53 after 24 hours, compared with either non-treated or olomucine treated cells (Figure 6.3C). These results show that GW5074 significantly affects cellular Cdc7/ASK activity in HeLa cells, indicating the HTS assay described here is specific for the identification of small molecule Cdc7 inhibitors. For the kinase list of the Biomol Kinase Inhibitor Plate please refer to Appendix C.

6.3.4. CRT DL main library screening and Hit analysis

The CRT main library (approximately 60,000 compounds) was screened in the 384-well formatted HTS assay to identify small molecule inhibitors of Cdc7/ASK kinase using optimized assay conditions[3]. Due to the high number of washing steps and the limitations of the washer, the screen achieved a throughput of approximately 3200 compounds per day. Each compound was assayed at a single concentration of 30 μM, and any compound that resulted in greater than 50 % inhibition of Cdc7/ASK signal was considered a hit compound. The initial screens identified 787 compounds with

[3] Technical assistance in the iterative screening of the CRT main library was provided by the CRT assay development team. In the work of this thesis, I analysed and interpreted the screening data, which had been generated from main library screening.

Table 6.2 Screening results of Biomol kinase inhibitor plates against Cdc7/ASK kinase

Inhibitor	IC_{50} (μM) Cdc7/ASK	% Inhibition at 10 μM compound
STAUROSPORINE	0.01	95.7
GW 5074	0.108	111.3
KENPAULLONE	3.7	105.2
INDIRUBIN-3''-MONOOXIME	5	94.9
TYRPHOSTIN 51	7.0	82.0
5-IODOTUBERCIDIN	8.2	60.1
HYPERICIN	9.4	77.6
TYRPHOSTIN 47	9.5	70.6
SP 600125	25.2	61.7
QUERCETIN	52.7	59.8
ROTTLERIN	67.6	53.5
GF 109203X	>120	5.0
H-7	>120	59.5
H-9	>120	-0.6

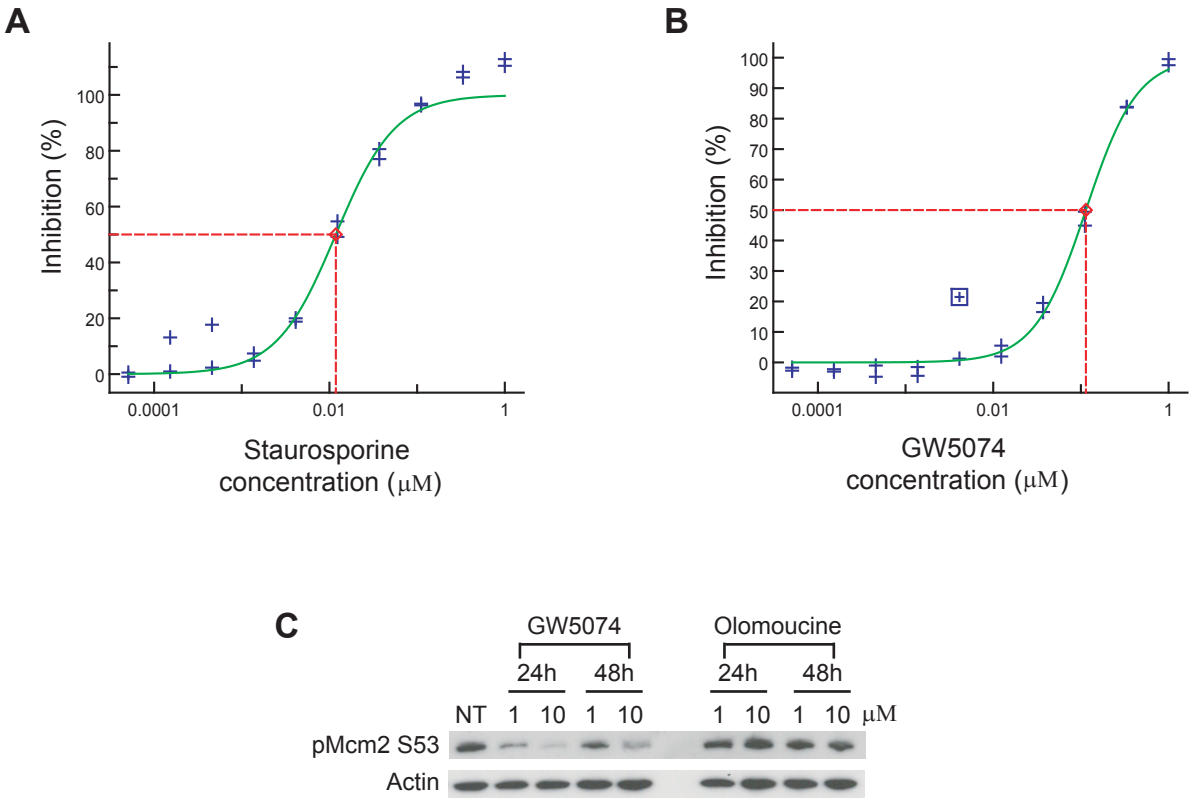


Figure 6.3: Cdc7/ASK primary screen of the Biomol Kinase Inhibitor Plate. IC_{50} determination of Staurosporine (A) and GW5074 (B). Cdc7/ASK kinase reactions were performed in triplicate using HTS optimized conditions and assayed in the presence of varying concentrations of each compound. (C) Immunoblot analysis of cellular effects of GW5074 and olomoucine on the phosphorylation of Mcm2 serine53 (actin - loading control) in HeLa cells at the indicated treatment times. (NT- non treated cells; pMcm2 S53 - Mcm2 phosphorylated at serine 53)

greater than 50 % Cdc7/ASK inhibition for retest, and 365 out of 787 compounds showing percent inhibition values greater than 50 % were selected as confirmed hits for a 0.8 % hit rate. Of the 365 hit compounds, 120 of the top compounds showing the highest percent inhibition were selected for IC_{50} determination and a compound purity and kinase specificity test. As shown in Table 6.3, 16 hits from 120 compounds showed an IC_{50} of ≤ 1 μM and Hill slope values were approximately one, except for two compounds with Hill slope values of 0.673 (CRT0059174) and 0.5 (CRT0003767) (Table 6.3). A Hill slope can be useful in describing the binding or kinetic behaviour of a target with compound (reviewed in Walters & Nmchuk 2003). If ATP and compound compete for a single binding site, the Hill slope for the titration of a compound should be approximately one. However, if Hill slope values are far from one, the interpretation is that multiple binding sites for interaction may exist and compounds have low potential as leads. Two of 60,000 compounds (CRT compound ID CRT0058701 and CRT0001660) showed inhibition against Cdc7/ASK kinase with an IC_{50} value of 0.018 μM and 0.349 μM respectively (Figure 6. 4 A, B). Hit compounds were also initially examined for their ability to inhibit the activity of a selected panel of 10 kinases by implementing an IMAP-base HTS assay (Millipore, USA) (Table 6.4)[4]. Four of the hit compounds (CRT0055760, CRT0001660, CRT0012241 and CRT0003767) were either inactive or lacked reproducible inhibition against this set of kinases, and were confirmed as Cdc7 inhibitors with IC_{50} values below 1 μM (Table 6.4). These identified hits were classified based on their chemical composition using a chemical classification program, which groups compounds based on structural fragments and overall molecule topological features.

Figure6.4

[4] Details of the kinases tested are commercially sensitive and are therefore not disclosed here.

Table 6.3 Screening results of CRT DL main library against Cdc7/ASK kinase

Compound ID	IC_{50} (μM)	Hillslope[a]	Maximum Inhibition	Purity[b]
CRT0058701	0.018	1.188	103.31	62
CRT0055760	0.221	1.04	100.97	100
CRT0001660	0.349	0.962	95.58	100
CRT0059174	0.639	0.673	88.68	0
CRT0064299	0.677	0.967	98.72	100
CRT0012241	0.729	1.049	99.35	94
CRT0003767	0.747	0.53	85.47	100
CRT0039235	0.773	0.785	84.57	100
CRT0016575	0.819	1.072	102.67	100
CRT0012536	0.823	0.797	93.12	66
CRT0005504	0.857	0.84	102.08	100
CRT0056182	0.897	0.943	103.1	87
CRT0058541	0.918	0.934	102.89	100
CRT0058541	0.952	1.058	103	100
CRT0039234	1.042	0.958	93.77	98
CRT0058545	1.066	0.817	102.5	100

a The variable Hillslope describes the steepness of the IC_{50} curve. This variable is called the Hillslope or the slope factor. When Hillslope is less than 1.0, the curve is more shallow. When Hillslope is greater than 1.0, the curve is steeper.

b A purity value of 70-90% based on UV detection is generally considered acceptable for compounds that will be used for early drug discovery. The common target purity of a compound for activity is between 90% and 95% pure.

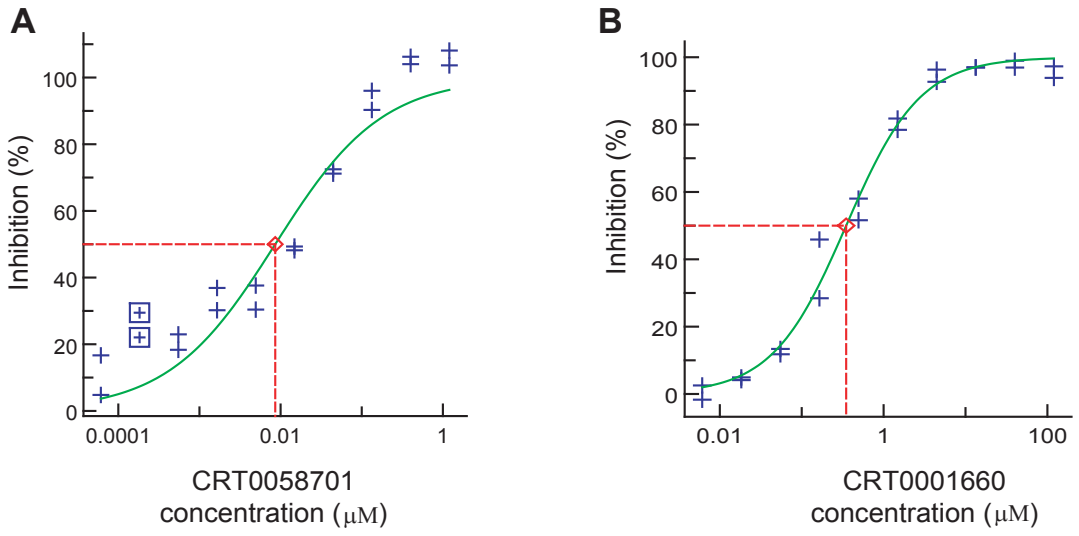


Figure 6.4: Cdc7/ASK primary screen of CRT DL main library. IC_{50} determination of CRT0058701 (A) and CRT0001660 (B). Cdc7/ASK kinase reactions were performed in triplicate using HTS optimized conditions and assayed in the presence of varying concentrations of each compound.

Table 6.4 Selectivity profile of hit compounds against kinases panel (% inhibition at *Km*)

	Cdc7	Kinase 1	Kinase 2	Kinase 3	Kinase 4	Kinase 5	Kinase 6	Kinase 7	Kinase 8	Kinase 9
CRT0058701	100	2	3	41	22	37	54	2	5	68
CRT0055760	99	0	35	14	13	2	8	2	0	48
CRT0001660	100	7	0	0	48	0	0.9	0	0	20
CRT0059174	86	29	21	86	0	94	55	7	4	28
CRT0064299	100	50	0	8	9	1.6				6
CRT0012241	100	12	0	0	0	0.5	0	3	10	1
CRT0003767	90	36	0	15	6	15	5		0	25
CRT0039235	95	8	0	0	15	0	0	85	0	0
CRT0016575	100	0	4	56	0	16	11	13	11	59
CRT0012536	90	1	0	0	9	0	0	51	0	0

The homogeneity and redundancy were adjusted to generate chemically relevant classes, resulting in three structural classes. Three structurally unrelated compounds at submicromolar concentrations were selected and have been the subject of a hit to lead programme over the last 18 months. For raw data of CRT main library screening please refer to Appendix C.

6.3.5. Three dimensional modelling of the Cdc7 active site

In most cases small molecules have been found to have poor selectivity in the first instance, and it is only following optimization for a specific kinase that selectivity is achieved (reviewed in Walters & Nmchuk 2003). The availability of sequence and structural information makes such an optimization of activity and selectivity a realistic objective. In the previous chapter, I discussed a homology model for Cdc7 based on structural information of the active site for Cdk2 and Erk2 (Figure 5.3). I identified several unique amino residues in the Cdc7 ATP-binding pocket (Table 5.1) and validated the Cdc7 homology model for activity prediction using site-direct mutagenesis (Figure 5.4 and 5.5).

A second homology model of Cdc7 was constructed using CK2 (1M2R.pdb) as a template by Dr Susan Boyd (CompChem Solutions) as part of the Cdc7 drug discovery programme steered by CRT Ltd (Figure 6.5)[5]. BLAST searching revealed CK2 as the closest homologue to CDC7 based on sequence identity over the whole kinase domain (residues 18-391), with 21% of residues being identical. In contrast to previous homology model described in Chapter 5 (Figure 5.5), Erk2 was found to be 17% identical, as was Cdk2. Additionally, on alignment of all four kinases, it can be

[5] Discussed here as part of the connected argument.

seen that the Gly-rich loop of Cdk2 is a relatively closed conformation compared with that of Erk2. The CK2 Gly-rich loop actually sits in an intermediate position between those of the other two structures (Figure 6.5). This second Cdc7 model was constructed and refined using the Molecular Operating Environment software from Chemical Computing Group Inc (Montreal, Canada). Refinement was carried out using the Amber 99 forcefield (UC San Francisco), which is a set of molecular mechanical force fields for the simulation of bio-molecules, with Gas Phase solvation (i.e. no solvation model applied) and On/Off cutoff parameters set to 10 and 12, respectively, to define limits of non-bonded interactions to be included in the computation. The active site underwent additional refinement to expand the volume accessible to ligand structures, by selection of appropriate rotamers of side-chains and by restrained minimisation to attempt to replicate the position of the CK2 hinge backbone atoms. As shown in Figure 6.5A, this new hinge position represents the greatest change over the initial model described, but it should be noted that the Gly-rich loop positions also vary significantly. Otherwise, the models show much similarity in the position of both the backbone and side chain atoms, with an overall RMSD (Root mean square deviation) of around 5A (Figure 6.5A). The second model covers the entire range of the kinase domain (residues 18-391), whilst the previous models only covered discrete regions of the kinase domain, with the Erk2 model only covering residues 54-207. Figures 6.5B and 6.5C show the second interaction model of ATP and reference compound (CRT100115) in the Cdc7 ATP-binding pocket. The adenine ring of ATP directly hydrogen bounds with Pro135 and Leu137 residues of the hinge region (Figure 6.5B). This model is currently being validated with early potent Cdc7 inhibitors and will be used for structure-activity relationship (SAR) studies to identify modifications and substituents that might increase potency against

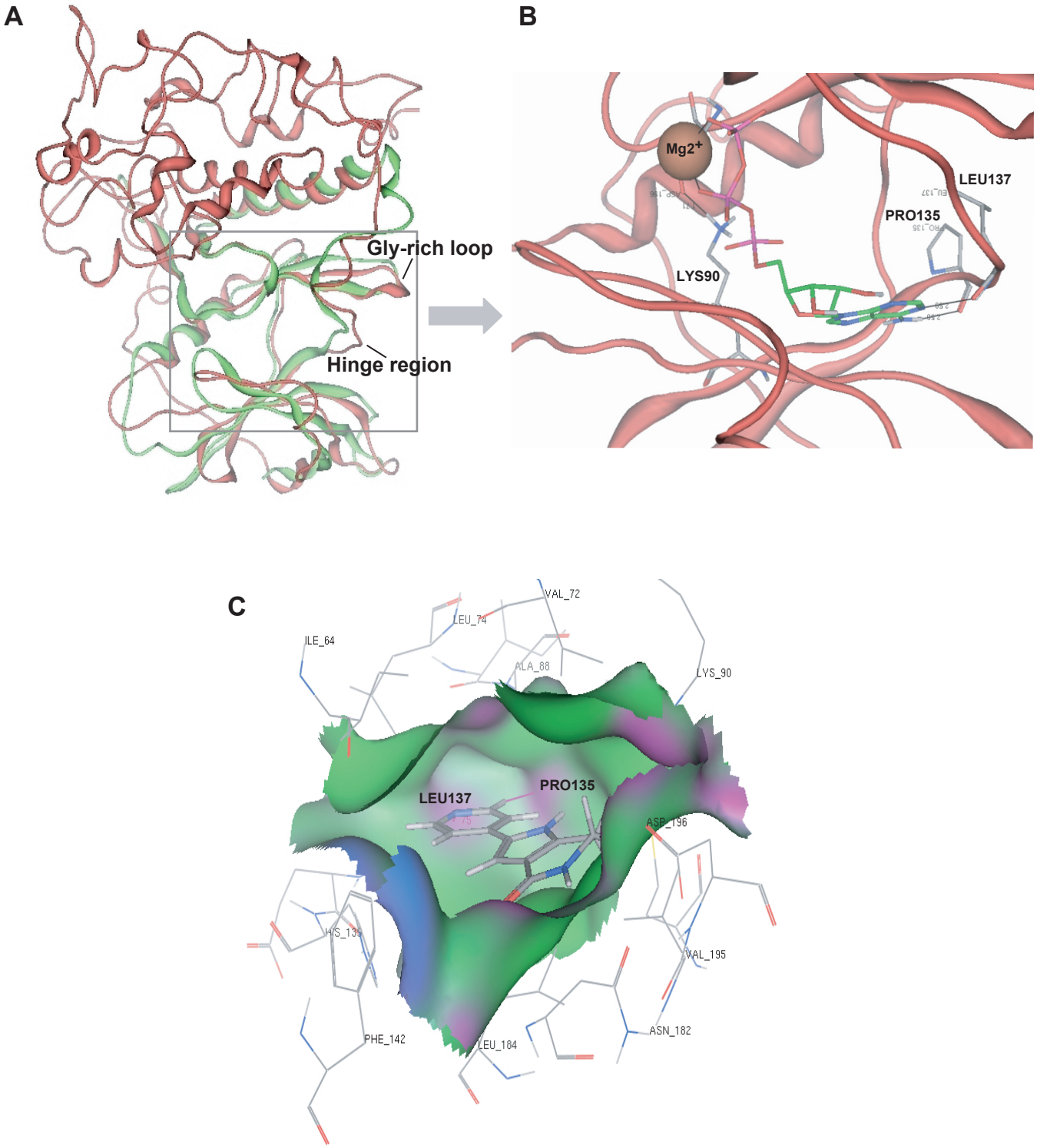


Figure 6.5: Three dimensional model of the Cdc7 active site. (A) High degree of conservation at molecular level demonstrated by comparison of the initial model (shown in green, constructed by Dr A Okorokov) and the second model (shown in red, constructed by Dr S Boyd). Second model of the Cdc7 active site is occupied by ATP (B) and CRT100115 compound (C).

Cdc7 kinase. This structural-chemical basis will enable rapid identification of those scaffolds with the best suitability for optimization as Cdc7 inhibitors.

6.3.6. Development of a cell-based assay for Cdc7 inhibitors

Although many compounds showed a good efficacy in the *in vitro* HTS assay, they not always elicit a cellular effect due to cell permeability and active export mechanisms in cells (Copeland 2005). In order to test for correlation between *in vitro* efficacy of compounds and their target-specific cellular activity, it is essential to take forward into a cell-based assay these hits that demonstrate potent activity in HTS. Cell-based assays are becoming a more frequent assay format in target validation, lead identification and optimization, and have been developed as a variety of assays that measure cell proliferation, cell cycle progression, toxicity, apoptosis induction, production of signal transduction and morphology. To develop a cell-based assay and to examine the effects of a reference Cdc7 inhibitor on cellular phenotype, I made use of PHA-767491 (CRT100115) which was synthesized by the CRT Chemistry department (Figure 6.6A; see Appendix B). Notably, PHA-767491 has been previously reported by Nerviano Medical Sciences as a potent Cdc7 inhibitor with an IC_{50} value of 10 nM in the presence of 1.5 μM ATP (Vanotti et al. 2008). Treatment with either 5 or 10 μM of PHA-767491 for 24 hours was sufficient to cause cell death in multiple cancer cell lines (Montagnoli et al. 2008).

In order to characterize the cellular response to CRT100115 as a reference Cdc7 inhibitor, in the work of this thesis I treated with either 1 or 10 μM of CRT100115 in multi-drug resistant SK-OV3 ovarian cancer cells and BT549 breast cancer cells for

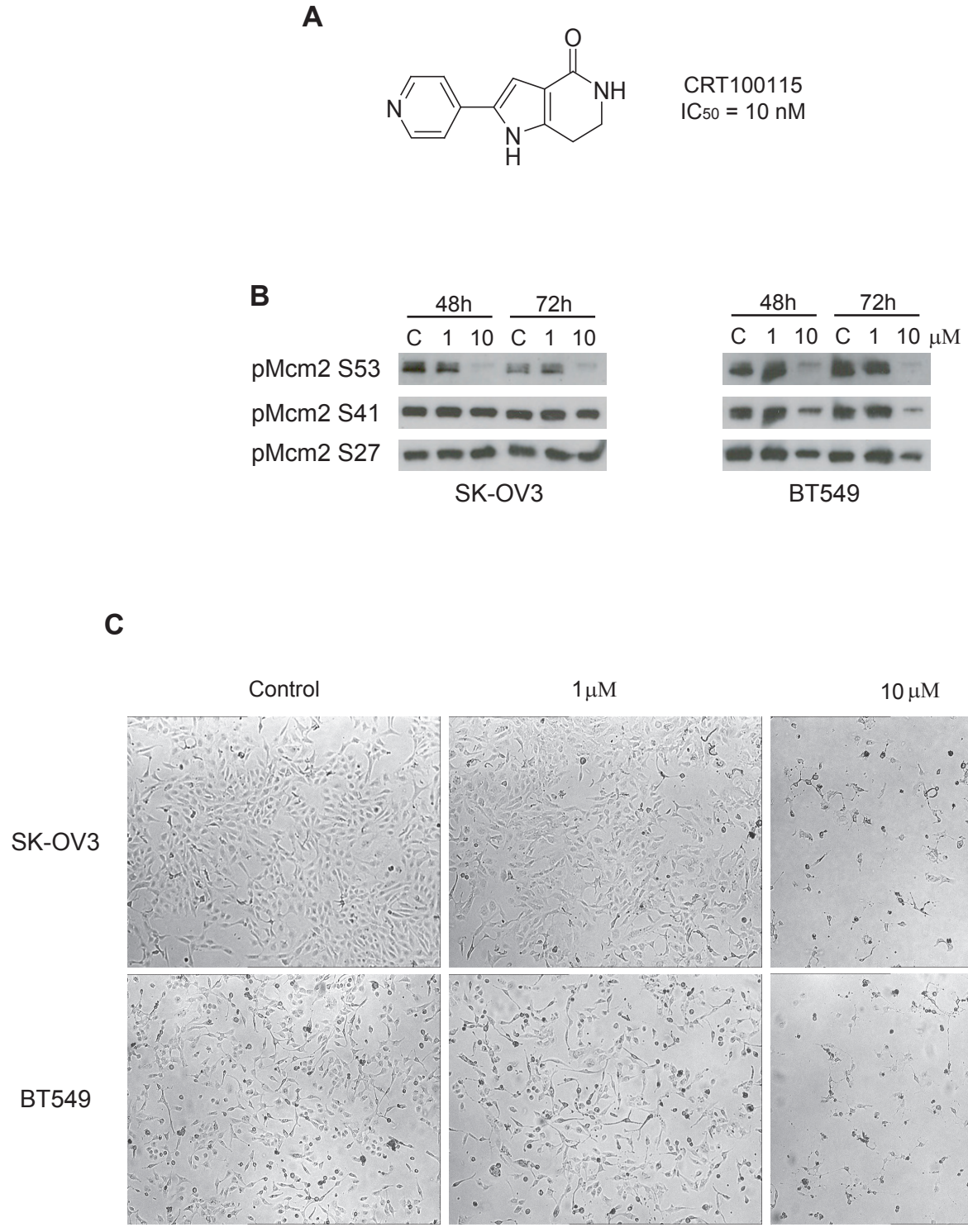
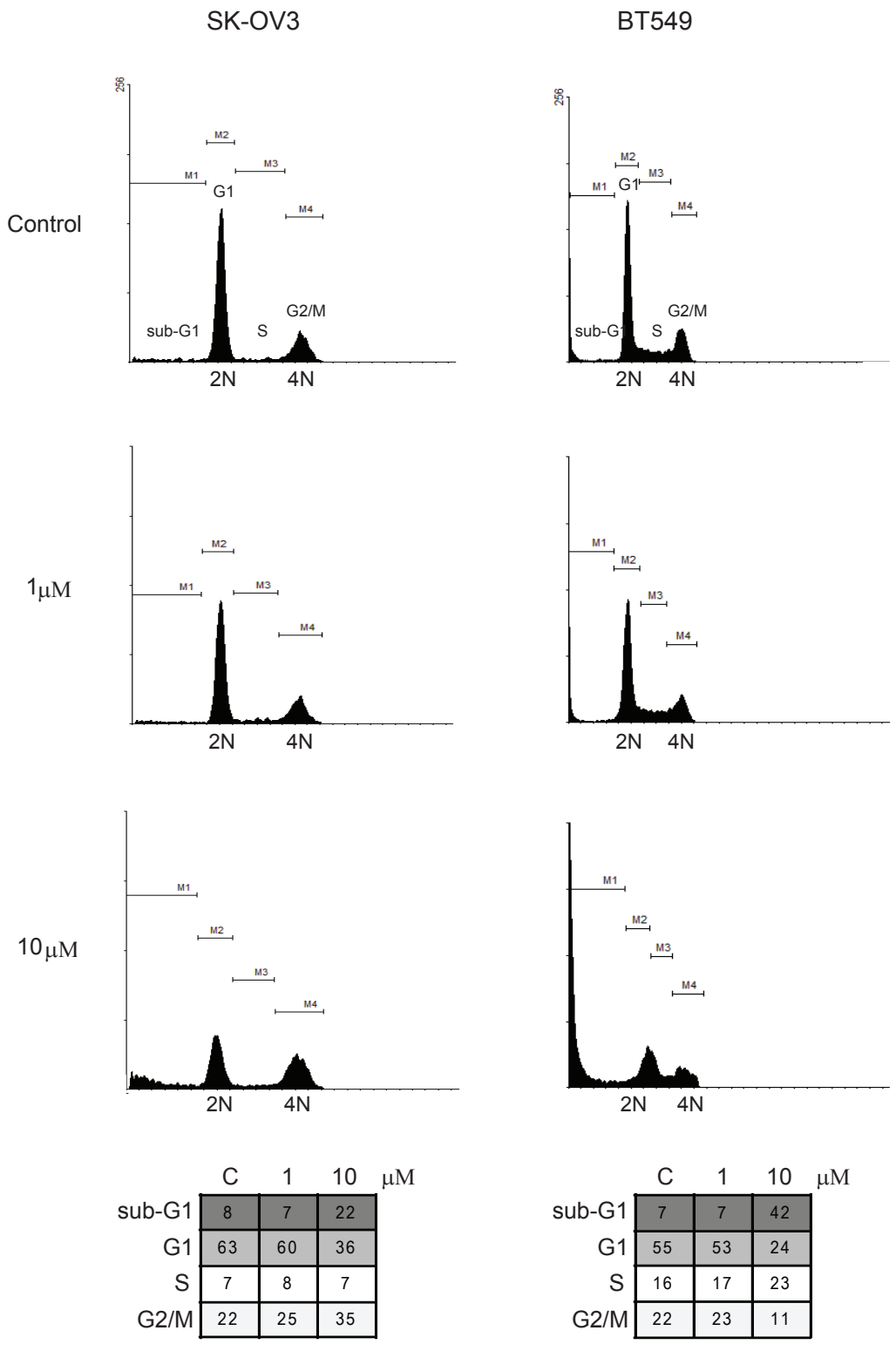


Figure 6.6: Cellular effects of CRT100115 on SK-OV3 and BT549 cells. (A) CRT100115 molecular structure. (B) Immunoblot analysis of CRT100115 treated cells. Cells were incubated with either 1 or 10 μM of CRT100115 for the indicated time. Total cell lysates were prepared and analyzed by immunoblot with pMcm2 S53 (target of Cdc7 kinase) and pMcm2 S27, S41 (target of CDKs) antibodies. (C - control; pMcm2 S53 - Mcm2 phosphorylated at serine 53; pMcm2 S41 - Mcm2 phosphorylated at serine 41; pMcm2 S27 - Mcm2 phosphorylated at serine 27) (C) Phase contrast micrographs of SK-OV3 and BT549 cells treated with either 1 or 10 μM of CRT100115 at 72 hours (original magnification 100x).

48 and 72 hours. Western blot data show that phosphorylation of Mcm2 at serine 53 was largely abolished with 10 μM treatment for 48 hours in both cell lines (Figure 6.6

B). Phosphorylation at serine 27 and 41, phosphorylation sites for CDKs (Ishimi et al. 2001; Montagnoli et al. 2006) were not affected in SK-OV3 cells, whereas the levels of phosphorylation of serine 27 and 41 were decreased by 48 hours in 10 μM treated BT549 cells (Figure 6.6B), suggesting that in BT549 cells CRT100115 also inhibits Cdc7/ASK kinase activity and possibly other kinases. Phase contrast microscopy showed a decline in both SK-OV3 and BT549 cell numbers following 10 μM of CRT100115 treatment, whereas control and 1 μM of CRT100115 treated cells remained viable (Figure 6.6C).

To examine whether CRT100115 interferes with cell cycle progression in SK-OV3 and BT549 cells, I treated with either 1 or 10 μM of CRT100115 for 72 hours. Flow cytometric analysis of DNA content showed that in both SK-OV3 and BT549 cells that CRT100115 caused an abnormal cell cycle profile. SK-OV3 cells treated with 10μM CRT100115 reveal two different subpopulations of cells in G1 (36%) and G2/M (35%) with a very small fraction of S phase (7%) cells, whereas BT549 cells mostly show a delayed/abortive S phase progression compared to control cells and those treated with 1 μM of CRT100115 (Figure 6.7). The data also show sub-G1 populations, indicative of cell death, in both cell lines (22% in SK-OV3 and 42% in BT549) with 10 μM CRT100115. To quantify the CRT100115-induced cell death, cells were analyzed using the annexin-V apoptosis assay after treatment with either 1 or 10 μM of CRT100115 over a 96 hours course (Figure 6.8A). In the presence of 10



	C	1	10 μM
sub-G1	8	7	22
G1	63	60	36
S	7	8	7
G2/M	22	25	35

	C	1	10 μM
sub-G1	7	7	42
G1	55	53	24
S	16	17	23
G2/M	22	23	11

Figure 6.7: Flow cytometric DNA profile of SK-OV3 and BT549 cells treated with CRT100115. Cells were incubated with either 1 or 10 μM of CRT100115 for 72 hours. DNA content of cell cycle populations was measured by flow cytometry. Tables show the percentage of cells with sub-G1, G1, S, and G2/M DNA content. (sub-G1- cell population with DNA content below 2N; G1- cell population in G1 phase (2N DNA content); S- cell population in S phase; G2/M- cell population in G2/M phase (4N DNA content)

μM CRT100115, 10% of the SK-OV3 population were annexin-V positive at the 24 hours and 60% of the population were positive 96 hours after treatment, whereas 60% of the BT549 population became annexin-V positive after 24 hours, and by 96 hours 90% of the population were positive (Figure 6.8A). No significant annexin-V positive fraction was induced by treatment with 1 μM CRT100115 or in control populations of both cells. Cell growth curves indicate that the cell growth inhibition effected by CRT100115 was dose and time dependent (Figure 6.8B). Taken together, the data presented in this chapter show that CRT100115 affects the phosphorylation of Mcm2 at a Cdc7-specific phosphorylation site as well as Cdk-dependent phosphorylation site in BT549 cells, and affects normal cell cycle distribution by inducing apoptotic cell death in SK-OV3 and BT549 cells, suggesting that the cellular phenotypes induced by Cdc7 inhibition can be applicable to development of a cell-based assay and Mcm2 phosphorylation at serine 53 is a good biomarker to verify the activity of Cdc7 inhibitors.

6.4. Discussion and Conclusions

Screening for protein kinase inhibitors usually requires a high-throughput kinase assay, which is a well-established process in lead discovery for pharmaceutical research. The development of a suitable assay system to test the target activity and to screen a large compound library is crucial for obtaining solid and reliable results from HTS. In the past, kinase activity determinations mostly relied on the incorporation of radioactive phosphate into proteins, but for large scale investigations radioactive waste products should be avoided (Rykx et al. 2007). In this chapter, I describe the development, validation, and implementation of a 384-well automated antibody-based

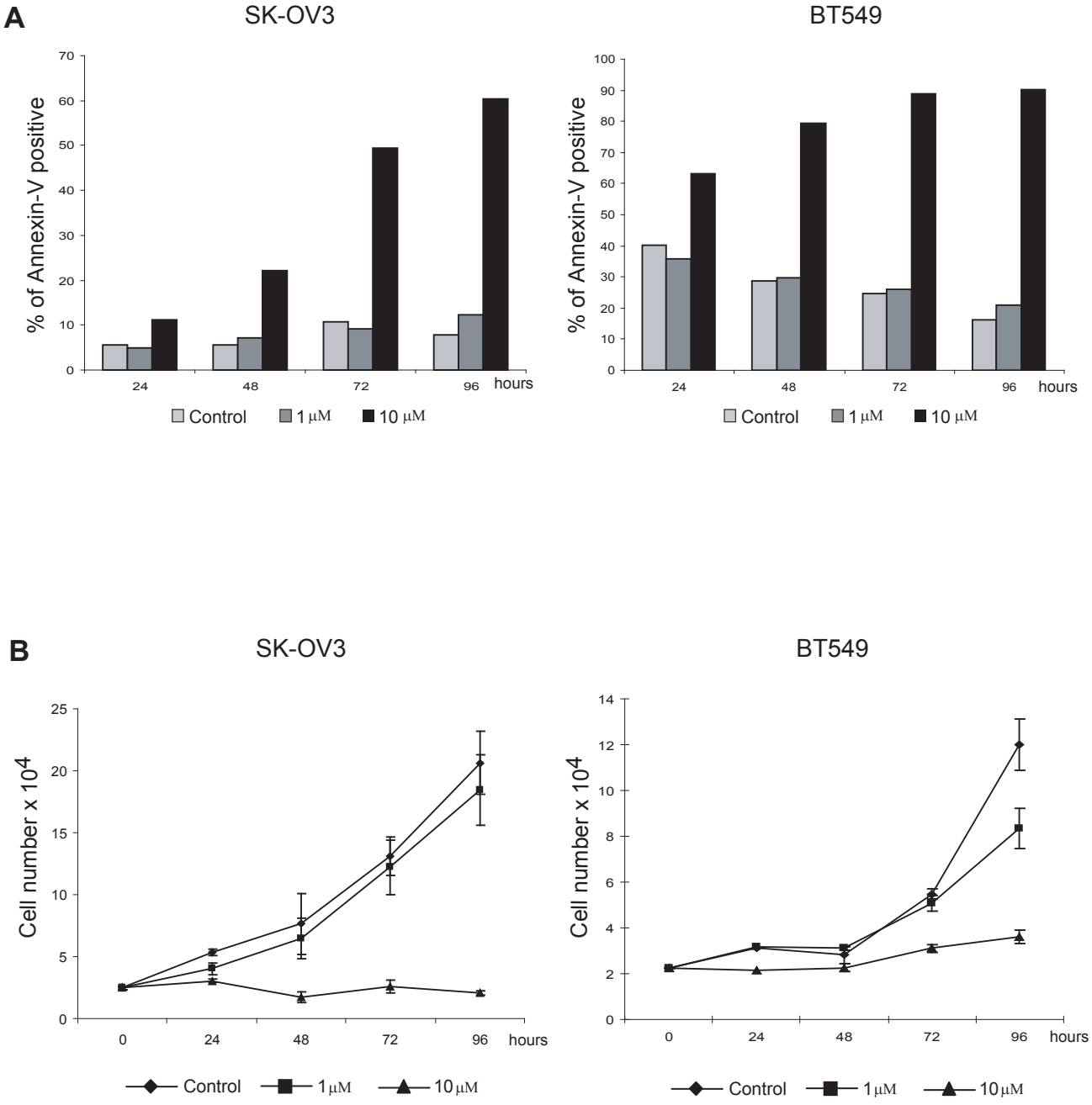


Figure 6.8: Cellular effects of CRT100115 on SK-OV3 and BT549 cells. Cells cultured in the presence of either 1 or 10 μM of CRT100115 were harvested at the indicated time, and apoptosis was assessed by flow cytometry after annexin-V and propidium iodide staining. (A) Annexin-V positive cells were indicated as the percentage of gated cells undergoing apoptosis. (B) Cell growth rate of control and CRT100115 treated cells of either 1 or 10 μM that is measured at the indicated time shown as fold increase in cell numbers.

HTS assay designed to identify Cdc7 inhibitors. The assay described in this chapter, although somewhat laborious due to the plate-coating with Mcm2 protein and several wash steps, is specific, sensitive, and in particular, free of radiolabeling. In preliminary experiments, several automation issues were encountered. However, through the introduction of the robotic liquid handler and automated plate washer to the antibody-based HTS platform, these limitations were overcome.

Using the Biomol Kinase Inhibitor Plate to evaluate high-throughput assay robustness and reproducibility, I found twelve known kinase inhibitors, including GW5074 which was selected as a standard inhibitor compound (Figure 6.3). The validated HTS assays identified 365 compounds showed greater than 50% of Cdc7/ASK inhibition from initial screening of the CRT DL main library (60,000 compounds), giving an overall 0.8 % hit rate. One hundred twenty of these top compounds which show the highest % inhibition were selected for IC_{50} determination, 16 of which showed an IC_{50} of $\leq$ 1 μM (Table 6.2) against Cdc7/ASK kinase activity and were selected for further hit validation studies. I also examined the inhibition ability of identified 16 compounds against a selected panel of other kinases, and found that four of the hit compounds were either inactive or lacked reproducible inhibition (Table 6.4). These compounds should be useful as hits compounds which will take forward to be a lead compound for the development of new selective Cdc7 inhibitors.

Targeting the catalytic site of kinase with ATP competitive inhibitors appears to be the most promising approach for drug intervention. Thus, protein crystallization and structure modelling are well established as important tools for drug design (Fabbro et al. 2002). Indeed, the recent progress made in the crystallization of Cdk2 kinase-

inhibitor complex and the structure modelling of Cdk4 ATP-binding pocket provides the great success in the design of selective inhibitors (Ikuta et al. 2001; Fabbro et al. 2002: Rossi et al. 2005). In the absence of the crystallographic structure of the Cdc7 catalytic subunit, in the work of this thesis, I sought to build a three-dimensional model of Cdc7 based on available structural information for other kinases. The model of Cdc7 active site discussed in this chapter showed the location and interaction model of ATP and reference compound in the Cdc7 ATP-binding pocket (Figure 6.5). This model is currently being validated with early potent Cdc7 inhibitors and an iterative cycle of chemistry and modelling will be applied in the search for potent and selective Cdc7 inhibitor.

Due to the interest in Cdc7 as an attractive target for cancer therapy (Montagnoli et al. 2004; Yoshizaw-Sugata et al. 2005; Im & Lee 2008; Kim et al. 2008; Montagnoli et al. 2008), several reports of first-in-class Cdc7 inhibitors have been published, and various lead compounds are being validated in order to identify available Cdc7 inhibitors for clinical trials (Sawa & Masai 2008; Swords et al 2010). Lack of selectivity for particular protein kinases is always encountered when developing inhibitors against kinases, all of which share highly conserved catalytic domains (Smyth & Collins 2009). PHA-767491 was identified as a potent inhibitor of Cdc7 (IC_{50} = 10 nM) from HTS and showed anti-cancer activity in several cancer cell lines and in a range of pre-clinical animal models (Vanotti et al. 2008; Montagnoli et al. 2008). However, it also showed off target effects on Cdk9 and RNA polymerase II which may be important for cellular mechanisms that cause unpredictable cellular responses to this compound. Notably, Figure 6.6B showed that the levels of Mcm2 phosphorylation at serine 27 and 41 (Cdk specific phosphosites) were decreased by

CRT100115 in BT549 cells, indicating that PHA-767491 not only affects Cdc7 kinase but also other kinases. During the work of this thesis, PHA-767491 was the only reported Cdc7 inhibitor. Although questions remain as to whether this compound could be a reference compound, I decided to make use of this molecule as a reference compound during work towards a cell-based assay development for evaluation of hits identified through antibody-based HTS of the CRT DL main library.

After initial screening for hits, the next step in the drug discovery process is generally to evaluate the efficacy of selected hits in intact cells to select lead compounds for further preclinical evaluation in animal models (Verkman 2004). Recently another series of phenol pyrimidin-2(1H)-ones (IC_{50} = 3 nM, 30 nM) have been identified as inhibitors of Cdc7 (Shafer et al 2008). However, although the phenol pyrimidin-2(1H)-one analogs showed marked affinity for Cdc7 in *in vitro* assays, these series of compounds showed weak cellular activity with low membrane permeability (Shafer et al. 2008). In fact, over the last year various lead compounds have been reported as potential Cdc7 inhibitors, and most of these compounds are currently being evaluated at the cellular level or in animal models prior to entering clinical trials (Sawa & Masai 2008; Swords et al 2010). Thus, using the cell-based screening assay as a more biologically relevant surrogate to validate the efficacy of hits is important during the hit to lead development stage in the drug discovery process.

Cell-based assays have been developed in various assay formats that measure cellular phenotypes, including cell proliferation, cell cycle progression, apoptosis induction, substrate phosphorylation and cell morphology. Several studies have shown that the cellular responses to inhibition of Cdc7 kinase by either siRNA or small molecule

inhibitors caused an impaired Cdc7-dependent phosphorylation of Mcm2, an abortive S phase progression, growth arrest and apoptotic cell death in a large number of tumour cell lines of different origins and irrespective of their p53 status (Montagnoli et al. 2004; Yoshizaw-Sugata et al. 2005; Im & Lee 2008; Kim et al. 2008; Montagnoli et al 2008). In keeping with previous reports, the data presented in this chapter show that treatment of two different cell lines, SK-OV3 (p53-null/Rb-negative) and BT549 (p53 mutated), with CRT100115 impairs cell cycle progression, leading to p53-independent apoptotic cell death (Figure 6.6B, 6.7 and 6.8). These results suggest that the cellular phenotype induced by Cdc7 inhibitors can be used as a biomarker for hit validation. Notably, the findings discussed in Chapter 4 indicate that phosphorylation of Mcm2 at serine 53 is a reliable marker of Cdc7/ASK kinase activity both in cells and tissue specimen, suggesting that measuring phosphorylation of Mcm2 at serine 53 is a specific biomarker in a cell-based assay system that allows discrimination of putative Cdc7 inhibitors.

In summary, the robust and sensitive HTS assay for Cdc7 activity in conjugation with the three dimensional model of the Cdc7 active site described in this chapter may help to overcome specificity concerns by enabling structural-chemical design of potent, selective Cdc7 inhibitors. Importantly, Table 6.4 shows that four compounds from 16 hits have a promising selectivity prolife against a selected panel of 10 kinases and are now progressing through a hit-to-lead development programme. Small molecule inhibitors identified in the screen will be subsequently tested for their effect(s) on the DNA replication initiation pathway and the cellular response of transformed and untransformed human cell lines. Thus, the development work discussed here provides a platform for further development of pharmacological inhibitors of Cdc7 kinase.

CHAPTER SEVEN

Conclusions and implications

A large number of conventional chemotherapeutic strategies act by targeting DNA synthetic pathways, based on the concept that sufficient amounts of DNA damage will inevitably kill cancer cells. As our knowledge of the biological and molecular basis of cancer has increased, a new chemotherapeutic strategy has emerged in the concept of targeted therapy, hitting crucial signalling pathways and transduction mechanisms in cell growth regulation. However, despite the development of a large number of new approach chemotherapies, the impact on cancer mortality so far has been modest, with many of these agents compromised by their lack of specificity, poor efficacy, limited response, toxic side effects, and the development of drug resistance (Widakowich et al. 2007).

To improve the rate of success of new anti-cancer drug development, three main stages involved in this process are essential to implementing such drug discovery projects. The first stage is target identification and validation, a process of biological hypothesis generation and testing in available experimental models, and the most difficult step associated with new drug discovery. The second stage is lead identification and optimization which requires assay development that can be applied to high-throughput screening and large compound collections that are screened against molecular targets on HTS platforms for lead finding. Finally, the third stage is preclinical and clinical studies that are concerned with whether the compound can be made into a drug that will treat cancer, has minimum toxic side effects, good efficacy,

stability and safety requirements for new drug development. The work of this thesis has generated data which provide a strong rationale for exploiting the replication initiation pathway as a novel anti-cancer target, and have resulted in the development of HTS and cell-based assays which are able to detect biological activity of the target to identify a lead compound from screening.

The initiation of DNA replication is a crucial decision point in cell proliferation that lies at the point of convergence of oncogenic signalling and transduction pathways. Unlike those branched, redundant and parallel pathways, there appears to be a single mechanism of DNA replication initiation and basic components of this mechanism are conserved in all eukaryotic species studied. Many studies have shown that DNA replication initiation factors are dysregulated in a wide range of premalignant dysplasia and cancers (reviewed in Lau et al. 2007; Blow & Gillespie 2008). Therefore, various points in the replication initiation pathway may be considered as potential therapeutic targets and drugs targeting this pathway should be of clinical benefit in a wide range of different tumour types, independent of genetic background. This is in stark contrast to drugs targeting upstream events where great heterogeneity exists between subgroups of even single tumour types.

Strong clues for the potential to exploit inhibition of the DNA replication initiation pathway as a therapeutic target have come from early studies, which showed that normal and cancer cells may respond differently to replication initiation inhibitors (Shreeram et al. 2002; Feng et al. 2003; Montagnolie et al. 2004). Cancer specific cell killing appears to be a distinctive feature of the inhibition of replication initiation by targeting either pre-RC assembly or origin activation. Although the exact mechanisms

accounting for the cancer cell specific cell killing are not yet clear, it has been suggested that an origin activation checkpoint may exist in normal cells to monitor insufficient replication-competence at origins of replication, whereas a defective checkpoint in cancer cells may allow cells to progress through S phase in spite of incomplete DNA replication which then results in stalled replication forks and the generation of DNA double-strand breaks, causing the induction of apoptosis (reviewed in Blow & Gillespie 2008; Sawa & Masai 2008; Swords et al. 2010).

Cdc7 kinase is essential for origin activation. Depletion of Cdc7 kinase by siRNA in various cancer cell lines induces defective S phase progression leading to p53-independent apoptotic cell death caused by genomic instability resulting from stalled replication forks, whereas primary fibroblasts avoid entering an abortive S phase by activating a p53-dependent cell cycle checkpoint (Im & Lee 2008; Kim et al. 2003; Montagnoli et al. 2004; Yoshizawa-sugata et al. 2005). Importantly, in contrast to replication fork blockade, Cdc7 depletion does not cause sustained activation of the S-phase checkpoint kinases (Montagnoli et al. 2004). Therefore, inhibition of Cdc7 kinase which should minimize collateral damage to the DNA could be a new strategy for anti-cancer therapy. The data shown in this thesis take the concept further and validate Cdc7 kinase as a novel and promising therapeutic target. The results discussed in Chapter Four indicate that Cdc7 kinase is required for S phase progression during the cell cycle and down-regulation of this kinase in response to reversible and irreversible loss of proliferation capacity is coupled to repression of origin licensing. In keeping with the *in vitro* studies, the finding that functional cells in self-regenerating, stable and permanent tissues including quiescent and

differentiated cell populations show a lack Cdc7 and its activity indicates that these cells should be refractory to a Cdc7 inhibitor. Consequently, the majority of normal cells within the human body do not present a target, minimising potential cytotoxic effects of Cdc7 inhibitors.

The critical importance of Cdc7 kinase in normal cell cycle progression, its absence in non-cycling cell populations, and the cancer cell specific death following Cdc7 depletion makes Cdc7 kinase an attractive novel target for new anti-cancer drug development. However, there is a possibility that Cdc7 kinase inhibitors may affect normal stem cell viability and germ line cell cycle progression. Inducible inactivation of Cdc7 in mouse ES (embryonic stem) cells results in the arrest of DNA synthesis and checkpoint responses, including recombination repair of replication forks, G2/M block and activation of p53, and cells undergo p53-dependent apoptosis (Kim et al. 2002; Kim et al. 2004). Furthermore, knockout Cdc7 genes in mice lead to early embryo death and sterility with testicular and ovary atrophy (Kim et al. 2002). In addition, Cdc7 kinase has been implicated in chromosomal segregation in mitosis through cohesion loading in *Xenopus* egg extracts models (Takahashi et al. 2008), and in homologous chromosomal segregation in meiosis by initiation of recombination and monoorientation in yeast models (Wan et al. 2006, 2008; Sasanuma et al. 2008; Matos et al. 2008; Marston 2008; Katis et al. 2010). Therefore, further Cdc7 inhibition studies in human stem cell models are required to investigate whether Cdc7 inhibition will adversely affect cells in stem-progenitor compartments and also germ cell development.

Cdc7 and its regulator protein ASK were observed to be up-regulated in both cancer cell lines and primary tumour tissues in comparison to their respective normal counterparts, suggesting that alterations in Cdc7/ASK protein abundance or activity may occur during tumorigenesis. (Hess et al. 1998; Nambiar et al. 2007; Bonte et al. 2008; Clarke et al. 2009). However, the role of Cdc7/ASK kinase in tumorigenesis and the prognostic significance of this kinase is not fully understood. Recently, the association between increased Cdc7 expression levels and the development of an aggressive tumour phenotype has been shown in epithelial ovarian carcinoma (Kulkarni et al. 2009). Mcm2-7 has been identified as powerful prognostic markers and as molecular tools for cancer screening and surveillance in a wide range of malignancies (Williams & Stoeber 2007). In the work of this thesis, expression profiling in human cancers shows positive immunostaining for Cdc7, Mcm2 and Mcm2 phosphorylated on serine 53. Importantly, the differences in labelling indices for phospho Mcm2 and Mcm2 in the proliferative compartment of human tissues suggest that Cdc7 and phosphorylation of Mcm2 on serine 53 can be use as a potential biomarker for proliferation and prognosis (see Chapter Four). Further studies on a wide range of malignancies will reveal whether the findings shown in this work can be generalised for cancers of different genetic background and histological type.

Many genes are likely to participate in cancer development by mechanisms that involve changes in expression levels or in their pattern of expression, but only a limited number of genes encode anti-cancer targets for which suitable drugs can be generated (reviewed in Malumbres & Barbacid 2007). Among them, protein kinases represent the most suitable targets for small molecule inhibitors. The drugable nature of kinases in general, along with the frequent misregulation of CDK activity in human

cancer has led to intensive efforts to develop selective CDK inhibitors (Malumbres & Barbacid 2007). However, none of them have yet successfully completed the clinical development, mainly due to toxic effects (Shapiro 2006). A number of Cdc7 inhibitors were recently reported, and were shown to have anti-cancer activity not only in cancer cell lines but also in animal xengraft tumour models (Montagnoli et al. 2008; reviewed in Sawa & Masai 2008; Swords et al. 2010). BMS-863233, a potent Cdc7 inhibitor developed by Exelixis and Bristol Myer Squibb (BMS), is the first compound which is in phase I clinical evaluation (Swords et al. 2010).

As a member of the family of serine/threonine kinase, Cdc7 kinase has conserved catalytic domains that form the ATP-binding site which is a prime target for the design and development of selective inhibitors. To identify a specific small molecule inhibitor of Cdc7, the development of a convenient, well-characterized and sensitive primary assay is crucial prior to initiating HTS at an early stage in the drug development process. In the work of this thesis I developed the antibody-based kinase assay, a non-radioisotope, phosphorylated Mcm2 quantitative detection assay for Cdc7 kinase activity (see Chapter Five). This assay has since been fully automated and become the primary assay operated by Cancer Research Technology Ltd, the technology transfer arm of Cancer Research UK, for determining Cdc7 kinase activity in HTS. Although the antibody-based HTS assay required additional steps which increase total screening time and assay complexity, the precision and robustness of the assay indicated that it is acceptable for HTS of Cdc7 inhibitors (see Chapter Six). The HTS assay identified 16 hits showing an IC_{50} of $\leq$ 1 μM (Table 6.2) from the CRT main library (60,000 compounds) against Cdc7/ASK kinase activity which have been the subject of further hit-to-lead development over the last 18 months. In addition,

analysis of key residues of the Cdc7 kinase active site based on a three-dimensional homology model indicates that 14 amino acid residues are unique in the Cdc7 ATP-binding pocket, providing a structural rationale for medicinal chemistry to design selective Cdc7 inhibitors. A further refined homology model of Cdc7 kinase is validated with early potent inhibitors from screening and used for structure-activity relationship (SAR) studies to identify modifications and substituents that might increase selectivity and potency against Cdc7 kinase. Importantly, hit compounds were either inactive or lacked reproducible inhibition against a selected panel of 10 kinases (Table 6.4). By exploiting the combination of extensive combinatorial chemistry with well-characterized HTS and a structural homology model for Cdc7, the efficiency of the lead identification and optimization stage is enhanced, thereby improving the quality of the output of hit finding, and offers a more economical route for hit identification during the drug discovery and development programme.

Promising drug candidates arising from lead identification and optimization will be subsequently subject to toxicology and other preclinical studies, before being tested further in clinical trials involving both healthy and sick patients. The process of selecting which hits to take forward into lead optimisation, based on those which offer the most suitable overall profile of potency, efficacy in animal models of proliferation, preliminary safety profile, predicted human ADME (Absorption, Distribution, Metabolism and Excretion) properties and transplant models of tumour growth (*Xenograft*) is beyond the scope of this thesis. However, the work presented in this thesis provides a strong biological rational for therapeutic targeting of the DNA replication initiation pathway and development of Cdc7 inhibitors as novel anti-cancer agents, which is essential for the process of drug discovery. Future studies of

the cellular response to replication initiation inhibitors will further advance our understanding of how origin activation is linked to the cellular circuits that control proliferation, DNA damage response and apoptosis. Such studies will also help to characterise the molecular nature of the origin activation checkpoint and its mechanism of action, and moreover comparative studies of normal and transformed human cells will help to understand loss of this checkpoint function in cancer cells. Therefore, identification and development of early stage Cdc7 inhibitors and further study of the response of cells to origin activation repression will be beneficial for the ultimate goal of developing a new class of non-genotoxic and specific anti-cancer agents.

CHAPTER EIGHT

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Apendix A : Summary of data from antibody-based Cdc7/ASK kinase assay (1)

Figure 5.2 A					
Mcm2 concentration (μg)	Set1	Set2	Set3	Mean of signal	St Dev
0	0.1316	0.1199	0.1349	0.1288	0.004551
0.5	0.3359	0.3922	0.3917	0.373266667	0.018684
1	0.6038	0.554	0.6545	0.6041	0.029012
1.5	0.6045	0.6439	0.6583	0.635566667	0.01608
2	0.6491	0.6811	0.6641	0.664766667	0.009244

Figure 5.2 B					
Cdc7/ASK concentration (μM)	Set1	Set2	Set3	Mean of Signal	St Dev
0	0.1009	0.0904	0.0984	0.096566667	0.003167
0.39	0.3362	0.136	0.2445	0.2389	0.057861
1.17	0.3806	0.2316	0.3599	0.324033333	0.046601
2.34	0.5084	0.3814	0.4486	0.446133333	0.036682
3.9	0.5177	0.5033	0.443	0.488	0.022881
7.8	0.4854	0.6121	0.581	0.5595	0.038122
15.6	0.6819	0.675	0.5645	0.640466667	0.038036
31.2	0.4279	0.6619	0.6277	0.5725	0.072971

Figure 5.3 A					
ATP concentration (μM)	Set1	Set2	Set3	Mean of Signal	St Dev
0	0	0	0	0	0
0.25	0.2412	0.36	0.365	0.322066667	0.040459
0.5	0.3267	0.513	0.476	0.438566667	0.056944
1.5	0.4992	0.482	0.481	0.4874	0.005907
5	0.4823	0.67	0.558	0.5701	0.054521
10	0.5006	0.618	0.546	0.554866667	0.034179

Figure 5.3 B			
Cdc7/ASK concentration (μM)	Test in Sep	Test in Oct	Test in Nov
0	0.113	0.099	0.1037
0.39	0.387	0.319	0.1553
1.17	0.47	0.502	0.2207
2.34	0.509	0.545	0.3272
3.9	0.561	0.673	0.3649
7.8	0.582	0.655	0.3714
15.6	0.778	0.691	0.313
31.2	0.873	0.686	0.2735

Apendix A : Summary of data from antibody-based Cdc7/ASK kinase assay (2)

Figure 5.4 A					
	Batch1				
Cdc7/ASK concentration (μM)	Set1	Set2	Set3	Mean of signal	St Dev
0	0.0904	0.1832	0.1311	0.1349	0.02686
0.39	0.136	0.198	0.1857	0.173233333	0.01895
1.17	0.2316	0.2846	0.2552	0.257133333	0.01533
2.34	0.3814	0.4248	0.3704	0.3922	0.01661
3.9	0.5033	0.5244	0.4918	0.5065	0.00955
7.8	0.6121	0.6536	0.5892	0.6183	0.01885
15.6	0.675	0.7355	0.6294	0.679966667	0.03073
31.2	0.6619	0.7941	0.7969	0.750966667	0.04454
	Batch2				
Cdc7/ASK concentration (μM)	Set1	Set2	Set3	Mean of signal	St Dev
0	0.1299	0.1265	0.0955	0.1173	0.01094
0.39	0.134	0.1422	0.1151	0.130433333	0.00802
1.17	0.1461	0.1427	0.1354	0.1414	0.00316
2.34	0.1789	0.1916	0.1949	0.188466667	0.00488
3.9	0.2352	0.22	0.2264	0.2272	0.00441
7.8	0.439	0.4165	0.6411	0.498866667	0.07141
15.6	0.5517	0.5378	0.662	0.583833333	0.03929
31.2	0.7271	0.7122	0.8259	0.755066667	0.03568
	Batch3				
Cdc7/ASK concentration (μM)	Set1	Set2	Set3	Mean of signal	St Dev
0	0.1221	0.1253	0.0993	0.115566667	0.00819
0.39	0.1355	0.1235	0.1098	0.122933333	0.00742
1.17	0.1215	0.1236	0.1284	0.1245	0.00204
2.34	0.1328	0.1374	0.1693	0.1465	0.01148
3.9	0.1591	0.1629	0.2448	0.188933333	0.02795
7.8	0.3065	0.2906	0.7542	0.450433333	0.15195
15.6	0.3702	0.3874	0.7638	0.507133333	0.12843
31.2	0.4819	0.5062	0.9144	0.634166667	0.14029
	Batch4				
Cdc7/ASK concentration (μM)	Set1	Set2	Set3	Mean of signal	St Dev
0	0.129	0.1296	0.0984	0.119	0.0103
0.39	0.1486	0.1577	0.2445	0.1836	0.03056
1.17	0.1762	0.196	0.3599	0.244033333	0.05821
2.34	0.2479	0.2439	0.4486	0.313466667	0.06758
3.9	0.3088	0.32	0.443	0.357266667	0.04299
7.8	0.545	0.5668	0.581	0.564266667	0.01047
15.6	0.6532	0.6989	0.5645	0.638866667	0.03945
31.2	0.7317	0.9867	0.6277	0.782033333	0.10665

Apendix A : Summary of data from antibody-based Cdc7/ASK kinase assay (3)

Figure 5.4 B					
	pH 7.5				
Cdc7/ASK concentration (μM)	Set1	Set2	Set3	Mean of signal	St Dev
0	0.008	0.01	0.096	0.038	0.029006
15.6	0.31	0.288	0.376	0.324666667	0.026441
31.2	0.284	0.401	0.372	0.352333333	0.035177
	pH 8				
Cdc7/ASK concentration (μM)	Set1	Set2		Mean of signal	St Dev
0	0.008	0.004	0.096	0.036	0.030022
15.6	0.332	0.32	0.408	0.353333333	0.027552
31.2	0.328	0.487	0.416	0.410333333	0.045987
	pH 8.5				
Cdc7/ASK concentration (μM)	Set1	Set2		Mean of signal	St Dev
0	0.003	0.007	0.091	0.033666667	0.02869
15.6	0.46	0.377	0.465	0.434	0.028537
31.2	0.42	0.486	0.508	0.471333333	0.026441
	pH 9				
Cdc7/ASK concentration (μM)	Set1	Set2		Mean of signal	St Dev
0	0.015	0.004	0.103	0.040666667	0.031328
15.6	0.322	0.339	0.427	0.362666667	0.032539
31.2	0.37	0.39	0.458	0.406	0.026633

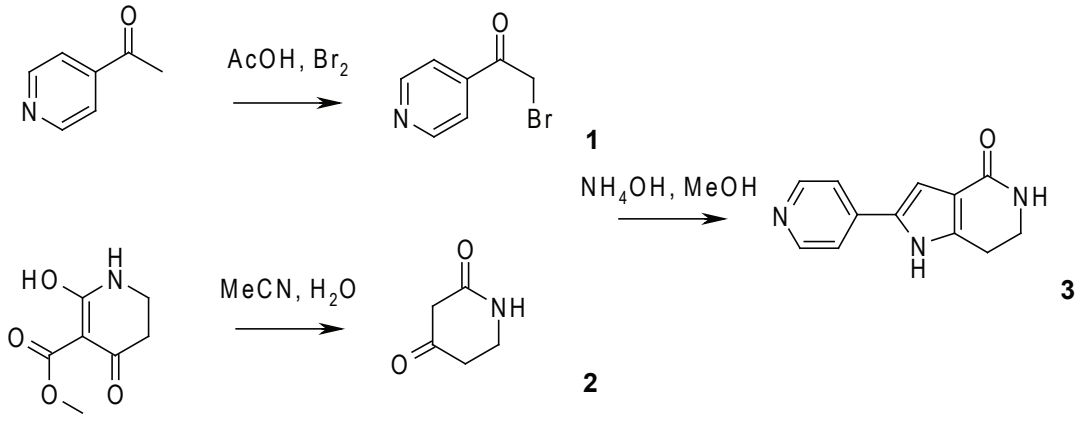
Apendix A : Summary of data from antibody-based Cdc7/ASK kinase assay (4)

Figure 5.7 A					
	Cdc7(WT)/ASK				
Kinase concentration (μM)	Set1	Set2	Set3	Mean of signal	St Dev
0	0.1385	0.1181	0.1537	0.136766667	0.01031331
0.78	0.3102	0.2726	0.2939	0.292233333	0.01088613
1.95	0.3514	0.2665	0.3586	0.3255	0.02957313
3.9	0.4789	0.3309	0.424	0.411266667	0.04319569
7.8	0.6802	0.4902	0.7456	0.638666667	0.07659646
15.6	0.7799	0.5253	0.732	0.679066667	0.07811689
31.2	0.8508	0.5408	0.8535	0.748366667	0.10378626
	Cdc7(D196A)/ASK				
Kinase concentration (μM)	Set1	Set2	Set3	Mean of signal	St Dev
0	0.121	0.1168	0.1572	0.131666667	0.01282411
0.78	0.1194	0.1131	0.1234	0.118633333	0.00299796
1.95	0.1207	0.1121	0.1302	0.121	0.00522717
3.9	0.1213	0.1129	0.1251	0.119766667	0.00360432
7.8	0.153	0.1302	0.1457	0.142966667	0.00672219
15.6	0.1775	0.1526	0.1668	0.165633333	0.00721164
31.2	0.2074	0.1593	0.1851	0.183933333	0.01389752
	Cdc7(D177A)/ASK				
Kinase concentration (μM)	Set1	Set2	Set3	Mean of signal	St Dev
0	0.1385	0.1269	0.1299	0.131766667	0.00347627
0.78	0.1195	0.1203	0.1121	0.1173	0.00261024
1.95	0.1251	0.1264	0.1139	0.1218	0.00396779
3.9	0.1179	0.1246	0.1104	0.117633333	0.00410135
7.8	0.146	0.1742	0.1277	0.1493	0.01352442
15.6	0.1733	0.1725	0.1455	0.163766667	0.00913625
31.2	0.1953	0.1555	0.1615	0.170766667	0.01238835
	Cdc7(K90A)/ASK				
Kinase concentration (μM)	Set1	Set2	Set3	Mean of signal	St Dev
0	0.1332	0.1238	0.1362	0.131066667	0.00373512
0.78	0.1411	0.1235	0.1297	0.131433333	0.00515407
1.95	0.1237	0.115	0.1433	0.127333333	0.00836906
3.9	0.1297	0.119	0.1345	0.127733333	0.00458124
7.8	0.1954	0.1569	0.1571	0.1698	0.01280013
15.6	0.2036	0.1606	0.1857	0.1833	0.0124709
31.2	0.1947	0.1563	0.1806	0.1772	0.01121472

Apendix A : Summary of data from antibody-based Cdc7/ASK kinase assay (5)

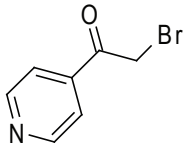
Figure 5.7 B				
	Cdc7(WT)/ASK	Cdc7(D196A)/ASK	Cdc7(D177A)/ASK	Cdc7(K90A)/ASK
Cycle number				
1C	1788	1506	1407	1386
2C	2360	1648	1980	1830
3C	3115	1864	2528	2243
4C	3953	2127	3139	2688
5C	4862	2424	3802	3140
6C	5782	2741	4496	3606
7C	6718	3056	5195	4045
8C	7548	3346	5773	4377
9C	8397	3650	6334	4671
10C	9173	3904	6739	4929
11C	9881	4194	7050	5094
12C	10507	4427	7343	5197
13C	11099	4656	7484	5334
14C	11633	4900	7679	5418
15C	12194	5111	7783	5475
16C	12624	5348	8035	5605
17C	12990	5489	8167	5681
18C	13279	5640	8321	5803
19C	13559	5721	8411	5814
20C	13329	5706	8309	5702
21C	13637	5785	8347	5688
22C	13346	5924	8350	5740
23C	13407	6007	8212	5826
24C	13473	6093	8247	5858
25 C	13653	6244	8330	5974

Appendix B: Synthesis of CRT100115 compound



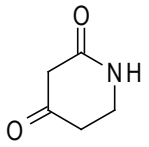
4-acetyl pyridine was brominated to prepared α-bromo ketone **1**. 2,4-dioxopiperidine **2** was prepared from commercially available starting material in one step by decarboxylation. Both compounds were condensed in the presence of ammonium acetate to deliver the desired compound **3**. NMR of compound **3** corresponded to the reported NMR and the purity was above the 99.5%, confirmed by LCMS.

2-Bromo-1-pyridine-4-ylethanone (**1**):



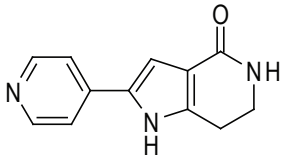
4-Acetyl pyridine (1.21 g, 10 mMol) was dissolved in glacial acetic acid (20 mL). Bromine in chloroform (1.59 g, 10 mMol in 10 mL of chloroform) was added. After 15 minutes a precipitate formed and the reaction was stopped after 30 minutes. The filtrate was collected by filtration and triturate with diethyl ether (150 mL). The compound (1.76g, 88 %) was used without further purification. 1H NMR (300 MHz, DMSO d6) δ 8.23 (2H, d, *J* 7.1 Hz), 7.98 (2H, d, *J* 7.1 Hz), 4.90 (2H, s); MS *m/z* (m+H) 200, 202.

2,4-Dioxopiperidine (**2**)



Sodium 3-9methoxycarbonyl-4-oxo-1,4,5,6-tetrahydropyridin-2-olate (5.0 g, 25.9 mMol) was portioned between 2N aqueous hydrogen chloride (50 mL) and dichloromethane (50 mL). The aqueous layer was extracted twice with dichloromethane (2 x 50 mL). The organic extract were dried over sodium sulphate, filtered and evaporated. The residue was suspended in acetonitrile (50 mL) and water (10 mL) and refluxed for 3hours. The reaction was cooled and evaporated. The residue was crystallised from 1:1 ethyl acetate: cyclohexane to provide the desired compound **2** (2.5g, 86%) as a white solid. 1H NMR (300 MHz, $CDCl_3$) δ 7.05 (s, 1H), 3.59 (m, 2H), 3.40 (s, 2H), 2.64 (t, 2H). MS *m/z* (M + H) 114.

2-Pyridin-4-yl-1,5,6,7-tetrahydro-pyrrolo[3,2-c]pyridin-4-one (**3**)



2-Bromo-1-pyridine-4-ylethanone **1** (1.0 g, 5.0 mMol) and 2,4-Dioxopiperidine **2** (725 mg, 6.0 mMol) were dissolved in methanol. Ammonium acetate (770 mg, 10 mMol) was added and the mixture was stirred at room temperature for 2 hours. The solvent was evaporated and the residue was purified by reverse column chromatography (water acetonitrile) and 250 (20 %) mg was recovered. 1H NMR (300 MHz, DMSO d6) δ 12.23 (bs, 1H), 8.66 (2H, d, *J* 7.2), 8.18 (d, 2H, *J* 7.2), 7.59 (s, 1H), 3.45(m, 2H), 2.94 (m, 2H). MS *m/z* 214.

Appendix C: Raw data of Cdc7 screening

C.1 Kinase list of the Biomol kinase inhibitor plate

Well Reference	Object Name	Mean Cdc7/ASK % Inhibition	(single point)	(single point)
K016	GW 5074	111.3	110.8	111.9
O020	KENPAULLONE	105.2	105.7	104.7
C012	STAUROSPORINE	95.7	94.4	97.0
M020	INDIRUBIN-3"-MONOOXIME	94.9	95.5	94.3
E006	TYRPHOSTIN 51	82.0	77.2	86.7
G016	HYPERICIN	77.6	70.9	84.4
E004	TYRPHOSTIN 47	70.6	74.7	66.6
M016	SP 600125	61.7	58.5	64.8
K002	5-IODOTUBERCIDIN	60.1	103.9	16.3
M004	QUERCETIN	59.8	69.7	50.0
C008	H-7	59.5	-17.6	136.6
K020	ROTTLERIN	53.5	50.8	56.3
M012	5 6-DICHLORO-1-beta-D-RIBOFURANOSYLBENZIMIDAZOLE	44.3	43.3	45.2
G018	Ro 31-8220	39.4	38.2	40.6
E020	DAMNACANTHAL	37.7	40.3	35.2
O018	Y-27632	29.2	24.4	33.9
I004	HA-1077	26.7	35.9	17.4
M014	22"33"44"-HEXAHYDROXY-11"-BIPHENYL-66"-DIMETHANOL DIMETHYL ETHER	26.2	24.8	27.7
G010	LY 294002	21.2	23.2	19.1
K008	PP2	20.3	36.6	3.9
K004	LFM-A13	17.1	24.0	10.3

O012	APIGENIN	16.6	13.6	19.6
A004	TYRPHOSTIN 25	15.1	36.5	-6.3
O008	SC-514	13.1	18.0	8.1
O016	RAPAMYCIN	12.6	9.7	15.5
O014	BML-265	12.1	12.6	11.5
O004	TRICIRIBINE	9.8	7.1	12.6
K018	PALMITOYL-DL-CARNITINE	8.8	8.5	9.1
M018	INDIRUBIN	7.4	4.9	10.0
K006	SB-202190	7.2	16.3	-2.0
C016	AG-825	6.9	1.5	12.4
K012	SU 4312	5.2	4.0	6.4
G014	GF 109203X	5.0	7.9	2.2
A012	H-8	4.0	7.8	0.3
E010	TYRPHOSTIN AG 1288	3.0	-3.7	9.8
I016	2-AMINOPURINE	1.1	1.4	0.8
K014	AG-1296	0.5	1.2	-0.1
M006	SU1498	-0.2	-4.4	4.1
C010	H-9	-0.6	0.5	-1.7
M010	BAY 11-7082	-0.6	4.1	-5.4
M008	ZM 449829	-0.7	1.0	-2.4
G020	SPHINGOSINE	-1.8	-2.3	-1.3
A002	TYRPHOSTIN 23	-1.8	-4.8	1.1
K010	ZM 336372	-2.1	3.3	-7.5
O006	BML-257	-2.4	-4.7	0.0
I018	N9-ISOPROPYL-OLOMOUCINE	-2.6	-5.5	0.3
M002	ERBSTATIN ANALOG	-3.3	-7.0	0.4
I006	2-HYDROXY-5-(2 5-DIHYDROXYBENZYLAMINO)BENZOIC ACID	-3.5	-6.2	-0.9
I020	OLOMOUCINE	-3.8	-2.6	-5.0
G008	AG-879	-4.3	-3.4	-5.2
G006	AG-370	-4.7	-3.7	-5.8

G012	WORTMANNIN	-5.5	-6.9	-4.2
C018	LAVENDUSTIN A	-6.1	-14.4	2.1
I010	KN-93	-6.3	-4.0	-8.6
I002	HA-1004	-6.4	-3.6	-9.2
G002	AG-490	-6.4	-4.2	-8.7
A016	ROSCOVITINE	-7.0	-4.4	-9.6
C014	AG-494	-7.2	-13.2	-1.2
O010	BML-259	-7.9	-7.2	-8.5
G004	AG-126	-7.9	-4.7	-11.2
O002	TERREIC ACID	-8.5	-5.8	-11.2
I008	KN-62	-9.0	-11.1	-6.8
A018	GENISTEIN	-9.4	-16.2	-2.6
C020	RG-14620	-9.4	-13.6	-5.3
I014	ML-9	-11.2	-10.9	-11.6
E002	TYRPHOSTIN 46	-11.4	-13.3	-9.5
E016	TYRPHOSTIN 9	-11.5	-13.2	-9.9
A008	PP1	-12.4	1.9	-26.8
A020	DAIDZEIN	-12.5	-8.8	-16.1
E018	HYDROXY-2-NAPHTHALENYLMETHYLPHOSPHONIC ACID	-13.2	-19.4	-7.0
C006	SB-203580	-14.2	-13.4	-15.0
A006	PICEATANNOL	-15.9	-12.1	-19.7
E012	TYRPHOSTIN AG 1478	-16.1	-17.0	-15.2
A014	ISO-OLOMOUCINE	-18.0	-16.2	-19.9
I012	ML-7	-18.1	-15.8	-20.4
E008	TYRPHOSTIN 1	-20.2	-22.0	-18.4
E014	TYRPHOSTIN AG 1295	-21.1	-20.9	-21.2
A010	H-89	-26.9	-22.6	-31.2
C004	U-0126	-30.8	-34.6	-27.1
C002	PD-98059	-33.5	-36.1	-30.9

C.2 Raw data of CRT DL main library screening

Table 6.3																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	0.57	0.51	0.39	0.26	0.51	0.45	0.74	0.88	1	0.98	1.17	1.14	1.24	1.21	1.26	1.19	1.25	1.21	1.28	1.16	1.26	1.09	0.04	0.07
B	0.14	0.11	0.1	0.09	0.26	0.22	0.51	0.61	0.85	0.88	1.04	1.05	1.12	1.11	1.07	1.17	1.17	1.14	1.14	1.16	1.11	1.17	0.02	0.02
C	0.18	0.15	0.25	0.23	0.25	0.27	0.43	0.38	0.66	0.57	0.91	0.79	1.07	0.97	1.13	1.08	1.21	1.11	1.18	1.12	1.17	1.11	0	0.02
D	0.33	0.24	0.15	0.14	0.3	0.28	0.7	0.72	0.96	1.01	1.03	1.13	1.13	1.13	1.17	1.15	1.2	1.2	1.16	1.2	1.15	1.15	0.01	0.03
E	0.08	0.01	0.08	0.08	0.2	0.17	0.43	0.48	0.8	0.71	1.01	0.94	1.15	1.04	1.1	1.11	1.19	1.1	1.18	1.13	1.18	1.13	0	0.05
F	0.12	0.08	0.12	0.1	0.35	0.41	0.57	0.65	0.8	0.86	0.98	0.96	1.03	0.98	1.13	1.12	1.18	1.13	1.16	1.15	1.09	1.22	0.02	0.04
G	0.06	0.03	0.07	0.08	0.14	0.16	0.41	0.37	0.73	0.73	0.83	0.91	1.07	1.04	1.14	1.11	1.2	1.03	1.06	1.14	1.16	1.15	0	0
H	0.04	0.01	0.03	0.04	0.2	0.15	0.42	0.37	0.71	0.67	0.92	0.9	1.07	1.05	1.14	1.07	1.2	1.15	1.23	1.09	1.22	1.21	0	0.01
I	0.1	0.06	0.04	0.06	0.06	0.06	0.11	0.07	0.27	0.24	0.58	0.51	0.64	0.84	1.03	1.01	1.11	1.12	1.18	1.14	1.14	1.1	0.04	0.04
J	0.17	0.13	0.1	0.1	0.16	0.21	0.5	0.43	0.61	0.73	0.99	0.96	1.01	1.08	1.11	1.1	1.06	1.13	1.12	1.15	1.2	1.21	0.01	0.05
K	0.07	0.05	0.11	0.1	0.21	0.23	0.41	0.45	0.74	0.66	1	0.88	1.13	1	1.12	1.06	1.16	1.17	1.13	1.16	1.18	1.11	0.04	0.06
L	0.08	0.09	0.17	0.16	0.32	0.35	0.56	0.57	0.78	0.79	1.02	1.06	1.04	1.07	1.11	1.14	1.17	1.14	1.13	1.2	1.14	1.22	0.02	0.06
M	0.18	0.14	0.2	0.13	0.29	0.34	0.56	0.53	0.87	0.74	0.92	0.97	1.11	1.03	1.17	1.06	1.12	1.15	1.16	1.14	1.24	1.16	0.03	0.05
N	0.2	0.23	0.16	0.17	0.33	0.4	0.6	0.66	0.92	0.89	0.86	1.02	1.1	1.08	1.01	1.13	1.17	1.13	1.11	1.16	1.17	1.18	0.02	0.07
O	0.26	0.45	0.18	0.18	0.35	0.28	0.58	0.59	0.68	0.85	0.75	1.03	1.13	1.09	1.13	1.12	1.24	0.96	1.12	1.15	1.13	1.08	0	0
P	0.07	0.05	0.06	0.03	0.06	0.07	0.11	0.09	0.17	0.16	0.44	0.33	0.79	0.68	0.91	0.91	1.1	1.11	1.18	1.17	1.23	1.19	0	0

A1:P20 – Test compounds (320 singletons at 30μM)

A21:N22 – Controls (28 replicates)

O21:P22 – Standard Inhibitor (4 replicates)

A23:N24 – Blanks (28 replicates)

O23:P24 – Absorbance Buffer Blank (4 replicates)

Apendix D : Kulkarni et al. 2009. Cdc7 kinase is a predictor of survivial and a novel theraputic target in epithelial ovarian carcinoma. Clin Cancer Res. 15:2417-2425

Apendix E : Okorokov et al. 2007. Hexameric ring structure of human MCM10 DNA replication factor. *EMBO Rep*. 8:925-930

Apendix F : Barkley et al. 2007. Cdc6 is a rate-limiting factor proliferative capacity during HL60 cell differentiation. *Exp Cell Res*. 15:3789-3799