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Evaluation of the DNA Replication licensing machinery as an anti-proliferative target

Sarah Rosemary Kingsbury

A thesis submitted for the degree of Doctor of Philosophy of the University of London

Wolfson Institute for Biomedical Research
University College London
Gower Street
London
WC1E 6BT

August 2006
DEDICATION

*

This thesis is dedicated to the memory of my grandparents, Ronald and Rose Beckett and Eileen Glassborow.

*
Acknowledgements

This work for the award of a degree for Doctor of Philosophy is funded by the Medical Research Council, to whom I am most grateful. I am also grateful to my supervisors Gareth Williams and Kai Stoeber for their support and guidance throughout this work, and for their review and constructive criticism of this manuscript.

I am grateful to Marco Loddo and Ellen Obermann for their guidance on the analysis of histological sections, to Thomas Fanshawe for his help on the statistical analysis of data and to Dick Campbell for advice and guidance on protein purification and peptide synthesis. I am also grateful to Andrei Okorokov, Renos Savva, Sally Roberts and Dave Selwood for helpful discussions and for kind gifts of antibodies, constructs and other research reagents.

I would like to thank past and present members of the Williams group for making the lab an enjoyable place to work. I would also like to thank all those who over the course of this work have provided ideas and suggestions, support and guidance and many fruitful discussions.

Finally, special thanks go to my family and to Richard for their unwavering support and encouragement.
ABSTRACT

Cancer is the second leading cause of death in the developed world. However despite significant advances in chemotherapy during the latter half of the 20th Century, the impact on mortality has been modest. There is therefore a profound need for the identification of novel strategies to treat cancer. The DNA replication licensing pathway, which co-ordinates the decision to initiate DNA replication, has recently emerged as a potential anti-cancer target. The early dysregulation of the replication licensing machinery during tumourigenesis suggests that agents targeting this pathway may have high efficacy in tumour cells. However, the response of normal cells to such agents must also be considered.

Here I show that withdrawal of cells from the mitotic cell cycle into the out-of-cycle states of quiescence and differentiation is tightly coupled to down-regulation of the DNA replication licensing machinery. Importantly, stem/progenitor cells of self-renewing tissues display an unlicensed replication phenotype. These results indicate that normal out-of-cycle functional, differentiated and stem/progenitor cell populations will be refractory to agents targeting the replication licensing machinery. Rapidly proliferating normal cell populations can suffer severe genotoxic and cytotoxic damage in response to chemotherapy. Here I show that inhibition of origin licensing in normal proliferating cells invokes the reversible activation of a putative origin licensing checkpoint which stalls cells in G1 until the block to origin licensing is removed, thereby protecting cells from damage. In contrast, transformed cells respond to inhibition of origin licensing by inducing apoptosis, suggesting that origin licensing inhibitors may represent highly specific cancer killing agents.

Finally, I have utilised a cell-free DNA replication and chromatin-binding assay to analyse the biochemical properties of two potential lead compounds: the endogenous origin licensing repressor geminin and a viral pathogen HPV1 E4. Collectively, these studies reinforce the concept that the inhibition of DNA replication licensing represents a novel chemotherapeutic strategy to combat cancer.
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ABBREVIATIONS

3D Three-dimensional
6-MP 6-mercaptopurine
ACS Autonomously replicating sequence consensus sequence
APC Anaphase-promoting complex
ARS Autonomously replicating sequence
ATM Ataxia telangiectasia mutated
ATR Ataxia telangiectasia related protein
BC Basal proliferating compartment
BrdU Bromodeoxyuridine
C Confluent
CDK Cyclin dependent kinase
CDKI Cyclin dependent kinase inhibitor
CBF Chromatin-bound fraction
CF Cytosolic fraction
CI Confidence interval
CIN Cervical intraepithelial neoplasia
CLL Chronic lymphocytic leukaemia
CRM1 Chromosomal region maintenance 1
CSF Cerebrospinal fluid
DAB 3’3’-diaminobenzidine tetrahydrochloride
DAPI 4’, 6’-diamidino-2-phenylindole
DC Differentiated compartment
DMF N,N,dimethylformamide
DHFR Dihydrofolate reductase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>DDK</td>
<td>Dbf4&lt;sup&gt;ASK&lt;/sup&gt;-dependent Cdc7 kinase</td>
</tr>
<tr>
<td>DSB</td>
<td>Double-strand breaks</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>G0</td>
<td>Quiescence/quiescent</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HER-2</td>
<td>Human epidermal growth factor receptor-2</td>
</tr>
<tr>
<td>HNSCC</td>
<td>Head and neck squamous cell cancer</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papilloma virus</td>
</tr>
<tr>
<td>Hsp90</td>
<td>Heat shock protein 90</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>MBF</td>
<td>Mlu1 binding factor</td>
</tr>
<tr>
<td>MCM</td>
<td>Minichromosome maintenance</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi-drug resistance</td>
</tr>
<tr>
<td>MTX</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch repair</td>
</tr>
<tr>
<td>NF</td>
<td>Nucleosolic fraction</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung carcinoma</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localisation sequence</td>
</tr>
<tr>
<td>ORC</td>
<td>Origin recognition complex</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>Pgp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PLM</td>
<td>Percent labelled mitosis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
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<tr>
<td>pRb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>Pre-IC</td>
<td>Pre-initiation complex</td>
</tr>
<tr>
<td>Pre-RC</td>
<td>Pre-replicative complex</td>
</tr>
<tr>
<td>PTD</td>
<td>Protein transduction domain</td>
</tr>
<tr>
<td>RLF</td>
<td>Replication licensing factor</td>
</tr>
<tr>
<td>RPA</td>
<td>Replication protein A</td>
</tr>
<tr>
<td>SCC</td>
<td>Stem cell compartment</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SMoC</td>
<td>Small molecule carrier</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>TAC</td>
<td>Transit amplifying compartment</td>
</tr>
<tr>
<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid stimulating hormone</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TPMT</td>
<td>Thiopurine methyltransferase</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Tdt-mediated dUTP-x nick end labelling</td>
</tr>
<tr>
<td>VCA</td>
<td>Vincristine mitotic accumulation</td>
</tr>
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CHAPTER ONE

INTRODUCTION

Over the past decade our understanding of the biological, genetic and molecular aspects of neoplastic disease has vastly increased. A plethora of studies have established that the underlying event in tumour formation and progression, the acquisition of the capacity for uncontrolled growth, results from multiple activating and inactivating mutations which uncouple the checkpoint pathways regulating transition through the cell cycle and the signalling transduction pathways coordinating mitogenic and anti-proliferative signals. However these advances arguably so far have not translated into greatly improved therapeutic strategies for the treatment of cancer and although some progress has been made, the impact on mortality rates has been modest and the 'magic bullet' of chemotherapy still remains elusive.

The complex, branched and often redundant nature of signalling pathways regulating transition through the cell cycle ensures that dysregulation can occur through a variety of different mutations at all levels of signal transduction and that the genetic status of malignant cells covers a very broad spectrum, with subgroups of single tumour types often having widely different genetic backgrounds. For this reason therapies which target upstream signalling pathways are often effective in only a small subset of patients, and are highly susceptible to the evolution of drug-resistant tumour clones. At the convergence point of these signalling pathways is the commitment to initiate DNA replication, a critical step in growth control. Unlike the branched, parallel and redundant signal transduction pathways, there appears to be only a single mechanism of initiation of DNA replication that is highly conserved in
all eukaryotic species studied, making this pathway a potentially attractive target for therapeutic intervention.

In the work of this thesis I have analysed the regulation of DNA replication initiation in examples of normal human tissues to predict how these tissues might respond to therapeutic agents targeting this pathway. In addition I have investigated mechanisms by which DNA replication initiation could be inhibited and, using an in vitro DNA replication assay, I have biochemically characterised two potential protein candidates for the development of lead compounds: an endogenous inhibitor of DNA replication, geminin, and a pathogenic viral inhibitor, HPV1 E4. Finally, I have exploited a novel delivery system to investigate the differential effect of inhibiting DNA replication initiation in normal and transformed mouse and human cell lines. Taken together these studies provide compelling evidence for the concept that inhibition of the DNA replication initiation pathway provides a potent anti-proliferative strategy. In this Chapter the literature relevant to this work is reviewed, focussing in the first part on current strategies for chemotherapy and in the second part on the DNA replication initiation pathway and its potential as a therapeutic target.

1.1. Treatment of cancer

In the developed world about one in three people will develop cancer over their lifetime and approximately one in four will die from the disease (Jemal et al., 2006). The mainstays of cancer treatment are surgery, radiotherapy and chemotherapy, with successful treatment generally involving a multidisciplinary approach, integrating these treatments to take advantage of the strengths of each one. Surgery is the longest
established treatment for cancer, and the principle hope of cure for most solid
tumours. It is primarily used when cancer is localised but, in combination with other
therapies, can also play an important role in treatment of tumours which show
metastasis on presentation. Radiotherapy, like surgery, is most successful for
treatment of localised tumours. Chemotherapy is a systemic treatment which can be
used to kill tumour cells throughout the body. It is therefore an essential mode of
treatment for metastatic disease. The success of chemotherapy is inversely related to
the tumour cell burden, and it therefore can prove most effective when used as an
adjuvant therapy after eradication of bulk tumour masses with surgery or
radiotherapy. Chemotherapeutic treatments have developed significantly since their
first introduction into clinical use in the 1940’s. However since 90% of cancer deaths
are a result of metastases (Sporn, 1996), the continued enhancement of
chemotherapeutic regimes is critical to improving cancer mortality rates.

1.2. The origins of chemotherapy

While the modern era of cancer chemotherapy began following World War II, the
origins of cancer treatment can be traced back to ancient times. Both the Ebers
papyrus and the Edwin Smith papyrus describe malignant diseases and their
treatments, whilst Celsus and Leonides reported the use of mastectomy in Roman
times. In the first century A.D. Dioscorides compiled a list of herbs and botanicals
for treatment of tumours and carcinomas, many of which remained in use until the
16th century (Reviewed in Papac, 2001). The first demonstration of effective
chemotherapy for malignant disease is considered to be the use of potassium arsenite
to treat chronic myelogenous leukaemia in 1865 by Lissauer (Papac, 2001), a
strategy which remained in use until the 1930s (Forkner, 1938) and, more recently,
was established as an effective treatment for acute promyelocytic leukaemia (Shen et al., 1997; Soignet et al., 1998). The beginnings of the modern era of chemotherapy can be traced directly to the discovery of nitrogen mustard as an effective treatment for cancer in 1942 (Gilman, 1963). The effects of nitrogen mustard were first noted in autopsy findings from soldiers dying of exposure to sulphur mustard gas during World War I (Krumbhaar, 1919), with subsequent studies on animal models indicating the potential use of nitrogen mustard for cancer therapy (Adair and Bagg, 1931). However, it wasn’t until May 1942 that the first chemotherapy clinical trial began with nitrogen mustard treatment of a patient suffering from non-Hodgkin’s lymphoma. The response of the patient was striking and although the remission only lasted a few weeks before disease again progressed, the principle was established that drugs could be administered systemically to induce tumour regression (Gilman, 1963). The pioneering work of Alfred Gilman with nitrogen mustard led to the clinical use of the first chemotherapeutic alkylating agent and ultimately to the production of a range of compounds with similar modes of action. This breakthrough catalysed the search for other strategies to treat cancer and over the following 50 years many new ‘classical’ chemotherapeutic strategies were developed, the majority acting by inhibiting nucleic acid synthesis or function. More recently, as our understanding of the biological, molecular and genetic aspects of neoplastic disease has increased, a new chemotherapeutic strategy has emerged in the form of targeted therapy.

1.3. Classical chemotherapeutic strategies

‘Classical’ chemotherapeutic agents have provided the mainstay of cancer treatment during the last 50 years (Table 1.1). In general, these agents act by targeting
<table>
<thead>
<tr>
<th>Class of compound</th>
<th>Examples of compounds in clinical use</th>
<th>Mode of action</th>
<th>Principle toxicities</th>
<th>Mechanisms of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkylating agents</td>
<td>Nitrogen mustards e.g. Mechlorethamine Nitrosoureas e.g. Carmustine Aziridines e.g. Thiotepa</td>
<td>DNA alkylation leading to DNA interstrand cross-links</td>
<td>Nausea, vomiting, alopecia, bone marrow depression, renal damage, development of secondary malignancies</td>
<td>Increased drug inactivation, enhanced repair of drug-induced defect</td>
</tr>
<tr>
<td>Anti-metabolites</td>
<td>Anti-folates e.g. Methotrexate Anti-purines e.g. 6-mercaptopurine Anti-pyrimidines e.g. Floraflur, cytarabine</td>
<td>Inhibition of nucleotide synthesis, incorporation into DNA and RNA</td>
<td>Neurotoxicity, bone-marrow depression, renal failure</td>
<td>Overexpression/mutation of target enzymes (e.g. DHFR, thymidylate synthase), altered drug transport</td>
</tr>
<tr>
<td>Anti-tumour antibiotics</td>
<td>Actinomycins e.g. Dactinomycin Anthracyclins e.g. Doxorubicin, Bleomycin Anthranediones e.g. Mitoxantrone</td>
<td>DNA intercalation, inhibition of topoisomerase II, generation of free radicals</td>
<td>Cardiotoxicity, myelosuppression, immunosuppression, nausea, vomiting, secondary neoplasms</td>
<td>P-glycoprotein amplification, decreased topoisomerase activity</td>
</tr>
<tr>
<td>Topoisomerase inhibitors</td>
<td>Topoisomerase I inhibitors e.g. topotecan Topoisomerase II inhibitors e.g. etoposide</td>
<td>Inhibition of topoisomerase I/II</td>
<td>Myelosuppression, nausea and diarrhoea, mucositis</td>
<td>Decreased intracellular drug accumulation, overexpression or mutation of topoisomerase</td>
</tr>
<tr>
<td>Platinum agents</td>
<td>Cisplatin, carboplatin</td>
<td>Formation of intrastrand crosslinks</td>
<td>Nephrotoxicity, peripheral neuropathy, ototoxicity, myelosuppression</td>
<td>Increased drug inactivation, enhanced repair of drug-induced defect</td>
</tr>
<tr>
<td>Microtubule inhibitors</td>
<td>Vinca alkaloids e.g. vinblastine, vincristine Taxanes e.g. paclitaxel, docetaxel</td>
<td>Destabilisation/stabilisation of microtubules</td>
<td>Neurotoxicity, myelosuppression</td>
<td>P-glycoprotein amplification, alterations in microtubule-polymer levels and dynamics, dysregulation of the mitotic spindle checkpoint</td>
</tr>
<tr>
<td>Endocrine therapy</td>
<td>Anti-oestrogens e.g. Tamoxifen Progestins e.g. Medroxyprogesterone acetate Glucocorticoids e.g. Cortisol</td>
<td>Stimulation/inhibition of hormone-mediated cellular activities</td>
<td>Tumour flare, impotence, gynecomastia, secondary neoplasms</td>
<td>Reduced intra-cellular drug levels, induction of growth factor receptors and second messenger systems allowing transcription in the presence of low level hormones</td>
</tr>
<tr>
<td>Immunotherapy</td>
<td>Microorganisms e.g. Bacillus Calmette-Guérin (BCG) Interferons, Interleukins, Tumour necrosis factor</td>
<td>Activation of immune responses</td>
<td>Sepsis, leukopenia, respiratory failure, hypertension, toxic fatalities in up to 10% of patients, angina, multi-organ malfunction, thrombocytopenia, CNS toxicity, impairment of renal function,</td>
<td>Impairment of adaptive immune response, for example by blocking the maturation and function of APCs and causing alterations in T-cell signal transduction and function. Expression of membrane complement regulatory proteins</td>
</tr>
</tbody>
</table>
physiological processes that are essential for cell proliferation, in particular DNA synthesis. For example, the anti-cancer activity of nitrogen mustards and other alkylating agents is coupled to the ability of these compounds to covalently interact with DNA and induce DNA damage (Roberts et al., 1968; Thomas et al., 1978; Erikson et al., 1980; Garcia et al., 1988; see also Appendix A, Table A.1). Other agents which inhibit tumour growth by interfering with nucleotide synthesis or function include anti-metabolites (Farber et al., 1948; Weiss and Pitot, 1974; Ahluwalia et al., 1990; Allegra et al., 1990; Chu et al., 1990; Schweitzer et al., 1990; Iwaniec et al., 1991; Jonsson and Kamen, 1991; Montford and Weichsel, 1997; Szekeres et al., 1997; Uribe-Luna et al., 1997; Danenberg et al., 1999; see also Appendix A, Table A.2), anti-tumour antibiotics (Reich et al., 1962; Lown et al., 1984; Tewey et al., 1984; Twentyman, 1984; Bowden et al., 1985; Glisson et al., 1986; Wang et al., 1987; see also Appendix A, Table A.3), topoisomerase inhibitors (Liu, 1989; Schneider et al., 1990; Lorence and Nessler, 2004), and platinum compounds (Rosenberg et al., 1965; Pinto and Lippard, 1985; Ozols and Young, 1991; Rice et al., 1988). In addition, further strategies for inhibiting tumour growth were developed in the form of microtubule inhibitors (Jordan et al., 1996; Jordan and Wilson, 2004), endocrine therapies (MacGregor and Jordan, 1998; Smith and Dowsett, 2003; Swinnen et al., 2004; Dowsett et al., 2005; Nicholson and Johnston, 2005; see also Appendix A, Table A.4) and immunotherapies (Masui et al., 1984; Vollmers et al., 1985; Roth and Weller, 1999; Tagawa et al., 2000; see also Appendix A, Table A.5). However despite these advances in chemotherapy, the impact on mortality has been modest (Landis et al., 1998). This failure to dramatically improve survival rates is linked to two common problems associated with classical chemotherapeutic strategies: lack of specificity and development of
The objective of cancer chemotherapy is the selective killing or inhibition of growth of neoplastic cells, leaving normal cells undamaged. In general, however, anti-cancer drugs lack specificity due to the dearth of consistent biochemical differences between normal cells and cancer cells that can be exploited for therapeutic intervention. The majority of classical chemotherapeutic strategies act by damaging DNA, based on the concept that sufficient amounts of DNA damage will inevitably kill cancer cells (Haince et al., 2005). Treatment with these agents relies on two assumptions; firstly that tumour cells proliferate more rapidly than normal cells and therefore incur higher levels of DNA damage, and secondly that tumours have defective checkpoint pathways and are thereby less able to respond to and repair DNA damage. However the assumption that all tumour cell populations proliferate more rapidly than normal cells does not always hold true. Tumours characterised by low growth fractions, for example chronic lymphocytic leukaemia, cycle considerably more slowly than many normal cell populations, particularly those in the gastrointestinal and haemopoietic systems which proliferate very rapidly (Keyomarsi and Pardee, 2003). These normal cell populations are therefore more sensitive to treatment than the tumour cell population, leading to loss of the therapeutic index. This results in debilitating side effects which may escalate to dose-limiting toxicities, including gastrointestinal toxicity, nausea, vomiting, and diarrhoea when the epithelial lining of the intestine is affected, alopecia when the hair follicles are affected, and marrow suppression and neutropenia when the haematopoietic precursors are affected (Ramirez et al., 1972; Legha et al., 1976; Wilson, 1978; Smith, 1989). In addition, these agents are frequently associated with the development of secondary neoplasms due to sublethal
DNA damage in healthy tissue (Haince et al., 2005). Limiting the dose of chemotherapeutic agents to reduce these side-effects can allow tumour cells to escape treatment and develop drug resistance, for example by the upregulation of DNA repair mechanisms (Cleton, 1995). The problems of toxicity and resistance can be illustrated by examining the activity of some classical chemotherapeutic agents.

Alkylating agents are so-called due to their ability to react in a manner such that an alkyl group or a substituted alkyl group becomes covalently linked to cellular constituents, a process called alkylation (Price, 1975; see also Appendix A, Table A.1). The most common sites for alkylation are the N7 and O6 positions of guanine, often leading to the formation of DNA interstrand cross-links. Although alkylation of DNA decreases its ability to act as a template for DNA synthesis (Ruddon and Johnson, 1968; Roberts et al., 1971), the cytotoxicity of alkylating agents directly correlates with the ability of the agent to form interstrand crosslinks (Roberts et al., 1968; Thomas et al., 1978; Erikson et al., 1980; Garcia et al., 1988). Alkylating agents can react with the DNA of both proliferating and resting cells, but they are most cytotoxic to rapidly proliferating cells (van Putten and Lelieveld, 1971), presumably because resting cells have an extended period in which to repair the DNA damage. They are generally considered to be cell-cycle-phase non-specific although there is some evidence to suggest that the cytotoxic effects are more severe when exposure occurs during S phase (Roberts et al., 1968). As a result, alkylating agents are extremely toxic to rapidly proliferating normal tissues, invoking a number of side effects including nausea, vomiting, bone marrow depression and alopecia (Ramirez et al., 1972; Legha et al., 1976; Wilson, 1978; Smith, 1989). Alkylating agents are also associated with the production of secondary malignancies, most
commonly acute leukaemia (Penn, 1976; Tucker et al., 1988), due to errors in the repair of DNA damage (Allan and Travis, 2005).

A second class of classical chemotherapeutic compounds which show severe cytotoxic effects is the anti-tumour antibiotics (see also Appendix A, Table A.3). As with alkylating agents, anti-tumour antibiotics exert their effects by interacting with DNA, with reduction in DNA binding linked to a loss or reduction of anti-tumour activity. Since anti-tumour antibiotics can interfere with the DNA strand breakage-reunion reaction of topoisomerase II (Tewey et al., 1984), cytotoxicity is also directly linked to topoisomerase II activity (Glisson et al., 1986; Pommier et al., 1986). In addition some anti-tumour antibiotics, in particular the anthracylines, can precipitate the formation of active oxygen species that can cause single-strand DNA breakages (Muindi et al., 1985). Unlike alkylating agents, anti-tumour antibiotics are generally cytotoxic to cells in any phase of the cell cycle, and are equally toxic to cycling and resting cells (Bhuyan et al., 1977). Consequently extremely severe adverse effects are associated with their use, including nausea, vomiting, ulceration of the oral mucosa and gastrointestinal tract, alopecia and severe acneiform skin lesions (O'Bryan et al., 1977). The major and dose-limiting toxicity is usually myelosuppression which is manifested as leukopenia (Frei III, 1974). Anti-tumour antibiotics can also potentiate the effects of irradiation (Cassady et al., 1975), and are immunosuppressive, carcinogenic and mutagenic agents (Svoboda et al., 1970; Marquardt et al., 1976; Vecchi et al., 1976). Both the cytotoxicity and carcinogenicity of anti-tumour antibiotics can be attributed to the generation of DNA double-strand breaks (DSBs). These adducts are particularly difficult to process and can be repaired incorrectly, resulting in mutational events that can contribute to
therapy-induced carcinogenesis (Allan and Travis, 2005). In addition, the inhibition of topoisomerase activity often creates DSBs that are capable of participating in gene translocation (Richardson and Jasin, 2000). A further side effect associated with some anti-tumour antibiotics, particularly anthracyclines, is severe cardiotoxicity (Lenaz and Page, 1976). This heart failure has been linked to myocardial cell degeneration, which is probably a result of the drug-induced production of reactive oxygen species (Lefrak et al., 1973).

The reaction of cancer patients to chemotherapy can be highly influenced by variable genetic backgrounds, with approximately 7% of patients affected by extreme adverse drug reactions (Marsh and McLoed, 2004; Candelaria et al., 2005). For example, tolerance of the anti-purine agents azathioprine, methotrexate and other mercaptopurines, which are mainly used for the treatment of childhood leukaemia (Relling et al., 1999), is directly linked to the genetic background of the patient. Anti-purines, a sub-class of the anti-metabolite compounds, block the synthesis of ribo- and deoxyribonucleotides through inhibition of de novo purine synthesis, and can be incorporated into both RNA and DNA (Weiss and Pitot, 1974; Maybaum et al., 1987; Iwaniec et al., 1991; Uribe-Luna et al., 1997). Thiopurines are sometimes referred to as “self-limiting” drugs, since incorporation into DNA is decreased when total DNA synthesis is inhibited by a reduction in purine synthesis (Matsumura et al., 1983). Due to the increased activity of the purine synthesis pathway in cycling cells (Jackson et al., 1975), thiopurines demonstrate toxicity in normal proliferating tissues and are typically associated with nausea, vomiting and bone-marrow depression. Severe liver damage, haematuria and crystalluria (Shorey et al., 1968; Duttera et al., 1972), and the development of secondary malignancies due to loss of
DNA-mismatch repair (DNA-MMR) (Sobulo et al., 1997) have also been reported. Interestingly the expansion or retraction of microsatellite sequences (microsatellite instability), which is diagnostic of abrogated DNA-MMR, is detected in up to 90% of therapy-related leukaemias, but is very rare in de novo leukaemias (Ben Yehuda et al., 1996; Zhu et al., 1999; Sheikhha et al., 2002; Worrilow et al., 2003). Severe adverse reaction to thiopurines is governed by the activity of the thiopurine methyltransferase enzyme (TPMT), which inactivates the drug (McLeod et al., 2000). TPMT activity is highly variable and polymorphic in all large populations studied to date: approximately 90% of individuals have high activity, 10% have medium activity and 0.3% have low or no detectable enzyme activity (Weinshilboum and Sladek, 1980; McLeod et al., 1995). Patients with TPMT deficiency have higher intracellular levels of thiopurine nucleotides, leading to the development of severe and often fatal haematopoietic toxicity when treated with conventional doses of 6-mercaptopurine (6-MP; Lennard et al., 1989; Evans et al., 1991; McLeod, 1995; Black et al., 1998; Relling et al., 1999). However, these patients can be safely treated with lower doses of 6-MP. In contrast, patients with very high levels of TPMT form suboptimal levels of intracellular thioguanine nucleotides and hence the therapeutic effects are compromised. The introduction of pharmacogenetic screening has provided a strategy for overcoming this problem (Roses, 2001; McLeod and Siva, 2002; Marsh and McLeod, 2004; Lee et al., 2005). The reaction of patients to thiopurine treatment can be predicted by either assaying TPMT activity in erythrocytes (Weinshilboum et al., 1978) or by determining the TPMT genotype using genomic DNA (Ottersoness et al., 1997), thereby allowing safe starting doses to be established. There is however still some debate as to the logistical and economical viability of routine testing. Only one in 300 individuals are homozygous for TPMT
deficiency and thus genotyping of 300 patients is needed for the detection of a single null carrier (Sadee and Dai, 2005).

Although the toxic side effects of classical chemotherapeutics can potentially be managed by lowering the dosage of the drug that is used for treatment, in reality this only serves to exacerbate the second problem associated with these agents, namely the evolution of drug-resistant tumour clones (Cleton, 1995). Drug resistance can be defined as a state of insensitivity, or decreased sensitivity, of a population of neoplastic cells to drugs that ordinarily cause cell death (Wooley and Tew, 1988). This resistance can be either intrinsic, i.e. the tumour is insensitive to the drug at first presentation, or acquired. It is generally accepted that acquired drug resistance is the most common reason for the failure of drug treatment in cancer patients with initially sensitive tumours (Skipper and Schabel, 1978; Kim and Tannock, 2005). Resistance to chemotherapeutic agents may occur either by mutational events or by gene amplification, and can confer resistance to a single agent or to multiple drugs.

Multi-drug resistance (MDR) is normally the result of amplification of the $MDRI$ gene, which encodes the membrane transporter protein P-glycoprotein (Pgp) (Gros et al., 1986a,b; Ueda et al., 1987). Pgp is expressed at low levels in a variety of tissues, and at higher levels in kidney, liver, pancreas, small intestine, colon and adrenal gland, with these latter tissues displaying an intrinsic resistance to some classical chemotherapeutics (Cordon-Cardo et al., 1990; Thiebaut et al., 1987; Chaudhury and Roninson, 1991). Cancers derived from tissues which normally show low expression of Pgp can acquire increased levels of the protein following treatment (Kohno et al., 1989), particularly when treatment involves anti-tumour antibiotics (Diddens et al.,
1987), topoisomerase inhibitors (Rasheed and Rubin, 2003) and microtubule inhibitors (Geney et al., 2002). Overexpression of Pgp results in increased drug efflux, thereby lowering the intracellular concentration of the drug and reducing sensitivity to treatment (Endicott and Ling, 1989; Gottesman and Pastan, 1993).

Multi-drug resistance can also occur if different drugs, through perhaps different mechanisms, influence a common enzymatic reaction that is required for their killing effect. In this instance, alterations in the activity or level of that enzyme may yield resistance to multiple drugs. For example, a number of anti-cancer drugs rely on topoisomerase-II activity for their killing effects. Anti-tumour antibiotics (e.g. doxorubicin, daunorubicin) interfere with the DNA strand breakage-reunion reaction of topoisomerase II (Tewey et al., 1984), whilst topoisomerase-II inhibitors [e.g. etoposide (VP-16), teniposide (VM-28) and amsacrine (m-AMSA)] act by inhibiting the relegation of cleaved DNA complexes, leading to DNA double-strand breaks, illegitimate recombination and enhanced mutational rates (Schneider et al., 1990). Resistance to both classes of drugs can therefore be caused by decreased topoisomerase activity (Pommier et al., 1986) or expression of topoisomerase isoforms with altered catalytic activities (Sullivan et al., 1989).

Similarly, the cytotoxicity of several classes of drugs (e.g. nitrosoureas, nitrogen mustards, platinum compounds) is coupled to their ability to form covalent adducts with DNA (Roberts et al., 1968, 1971; Ruddon and Johnson, 1968; Thomas et al., 1978; Erikson et al., 1980; Pinto and Lippard, 1985; Garcia et al., 1988; Rice et al., 1988). Mammalian cells repair these adducts using the excision repair pathways (Regan and Setlow, 1974), the activities of which may be a significant factor in the
development of resistance to these agents (Ewig and Kohn, 1977; Eastman and Schulte, 1988; Hampson et al., 1997). In addition to increased excision repair activity, tumour cells may also develop resistance to these agents through increased drug inactivation due to reaction with cellular thiols (Meister, 1988). Glutathione reacts with the electrophilic derivatives of these agents and detoxifies them (Behrens et al., 1987), thereby preventing the delayed formation of DNA cross-links from drug:DNA monoadducts (Micetich et al., 1983). Consequently increased levels of glutathione-S-transferase, which catalyses the conjugation of electrophilic compounds with glutathione (Arrick and Nathan, 1984), or increased glutathione content (Behrens et al., 1987), confers resistance to these agents.

In addition to the mechanisms of resistance described above, reduced sensitivity to chemotherapy can occur in a variety of ways, including decreased conversion of drug to an active form, decreased affinity of target enzyme or receptor for drug, increased drug inactivation, decreased intracellular drug levels, and failure to apoptose (Table 1.2; Kessel et al., 1965; Wolpert et al., 1971; Ewig and Kohn, 1977; Sirotnak et al., 1981; Washtien, 1982; Micetich et al., 1983; Arrick and Nathan, 1984; Goldenberg and Begleiter, 1984; Pommier et al., 1986; Behrens et al., 1987; Berger et al., 1988; Eastman and Schulte, 1988; Meister, 1988; Sinha et al., 1989; Srimatkandada et al., 1989; Dicker et al., 1990; Grem, 1990; Rasheed and Rubin, 2003). One class of compounds which is associated with the development of many different types of drug resistance, and for which resistance has emerged as a particular problem, are the anti-metabolites (Banerjee et al., 1995). Anti-metabolites act by interfering with the normal cellular metabolism of nucleic acids and fall into three categories: anti-folates, anti-purines and anti-pyrimidines (Allegra et al., 1990). Resistance to anti-
Table 1.2. Mechanisms of drug resistance to classical chemotherapeutic agents. Summary of the mechanisms by which resistance to classical chemotherapeutic agents can develop, and examples of agents for which these mechanisms have been demonstrated.

<table>
<thead>
<tr>
<th>Mechanism of resistance</th>
<th>Examples of classes of drug for which mechanism has been demonstrated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decreased intracellular drug level due to increased drug efflux</td>
<td>Anti-tumour antibiotics, microtubule inhibitors, topoisomerase inhibitors</td>
</tr>
<tr>
<td></td>
<td>Amplification of P-glycoprotein (Diddens et al., 1987; Geney et al., 2002; Rasheed and Rubin, 2003)</td>
</tr>
<tr>
<td>Decreased intracellular drug level due to decreased inward transport</td>
<td>Anti-metabolites</td>
</tr>
<tr>
<td></td>
<td>Increased levels of glutathione-S-transferase (Arrick and Nathan, 1984; Meister, 1988)</td>
</tr>
<tr>
<td></td>
<td>Alkylating agents</td>
</tr>
<tr>
<td></td>
<td>Increased glutathione activity (Sinha et al., 1989)</td>
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<tr>
<td></td>
<td>Platinum compounds</td>
</tr>
<tr>
<td></td>
<td>Increased glutathione content (Micetich et al., 1983; Behrens et al., 1987)</td>
</tr>
<tr>
<td>Increased drug inactivation</td>
<td>Alkylating agents</td>
</tr>
<tr>
<td></td>
<td>Increased levels of glutathione-S-transferase (Arrick and Nathan, 1984; Meister, 1988)</td>
</tr>
<tr>
<td></td>
<td>Anti-tumour antibiotics</td>
</tr>
<tr>
<td></td>
<td>Increased glutathione activity (Sinha et al., 1989)</td>
</tr>
<tr>
<td>Decreased conversion of drug to an active form</td>
<td>Anti-metabolites</td>
</tr>
<tr>
<td></td>
<td>Deficiency of thymidine kinase (Grem, 1990)</td>
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<tr>
<td>Altered amount of target enzyme or receptor</td>
<td>Anti-pyrimidines</td>
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<tr>
<td></td>
<td>Increased expression (Washtien, 1982) or structural alterations of thymidylate synthase</td>
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<tr>
<td></td>
<td>Anti-purines</td>
</tr>
<tr>
<td></td>
<td>Deletion of HPRTase (Wolpert et al., 1971)</td>
</tr>
<tr>
<td>Decreased affinity of target enzyme or receptor for drug</td>
<td>Anti-metabolites, topoisomerase inhibitors</td>
</tr>
<tr>
<td></td>
<td>Structural mutations that diminish drug binding (Srinivaskandada et al., 1989; Dicker et al., 2000; Rasheed and Rubin, 2003)</td>
</tr>
<tr>
<td>Enhanced repair of drug-induced defect</td>
<td>Alkylating agents, platinum compounds</td>
</tr>
<tr>
<td></td>
<td>Increased excision repair activity (Ewig and Kohn, 1977; Eastman and Schulte, 1988)</td>
</tr>
<tr>
<td>Decreased activity of enzyme required for killing effect</td>
<td>Anthracyclines, microtubule inhibitors</td>
</tr>
<tr>
<td></td>
<td>Decreased topoisomerase activity (Pommier et al., 1986)</td>
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</tbody>
</table>
metabolites can take the form of either inherent or intrinsic cellular resistance, with
tumours presenting with low drug sensitivity at the start of treatment or acquired
resistance, with both having a major impact on treatment failure (Moscow, 1998;
Mader et al., 1998). One of the first anti-metabolites to be developed for clinical use
was methotrexate, (4-amino-10-methyl-folic acid, MTX), an anti-folate compound
which is used for the treatment of a range of neoplastic diseases including breast
cancer, osteogenic sarcoma and leukaemias (Jonsson and Kamen, 1991; Stoller et al.,
1977). MTX is a potent inhibitor of dihydrofolate reductase (DHFR), a key enzyme
for intracellular folate metabolism. Inhibition of DHFR results in depletion of the
tetrahydrofolate pool and cessation of de novo purine and thymidine nucleotide
production, ultimately causing impairment of DNA synthesis and repair, and the
accumulation of DNA strand breaks (Allegra et al., 1990; Schweitzer et al., 1990).
The effectiveness of MTX treatment is dependent upon decreased intracellular
accumulation and alterations to DHFR, with at least four different resistance
mechanisms known to occur (Bertino et al., 1996; Gorlick et al., 1997). MTX is
primarily transported into cells via the same specific, saturable system as natural
reduced folates (Goldman et al., 1968), a pathway which is often altered in MTX
resistant cells both in vitro (Kessel et al., 1965) and in in vivo animal tumour models
(Sirotnak et al., 1981). Moreover, alterations to this pathway have been identified in
acute lymphocytic leukaemia patients, indicating that decreased transport of MTX
into cells is a common resistance mechanism (Trippett et al., 1992; Gorlick et al.,
1997). Reduced intracellular accumulation of MTX has also been coupled to
impaired polyglutamylation. Long-chain polyglutamates of MTX have an equal
affinity for DHFR, but are transported out of the cell more slowly than MTX
monoglutamates (Goldman and Matherly, 1985). Tumours displaying an intrinsic
resistance to MTX, including acute nonlymphoblastic leukaemia (Gorlick et al., 1996), commonly show reduced levels of MTX polyglutamates compared to sensitive tumours (e.g. acute lymphoblastic leukaemia), suggesting that impairment of polyglutamylation is a significant factor in the development of clinical MTX resistance (Lin et al., 1991; Goker et al., 1993; Goker et al., 1995). Aside from reduced intracellular drug concentration, resistance to MTX has also been linked to alterations to the target enzyme DHFR. Amplification of the DHFR gene, which may be catalysed by loss of wild-type p53 function (Livingstone et al., 1992; Yin et al., 1992; Goker et al., 1995) has been linked to MTX resistance both in vitro (Carman et al., 1984; Ozols and Cowan, 1986; Schimike, 1988) and in relapsed acute lymphoblastic leukaemia (Goker et al., 1995). In addition, structural mutations that diminish methotrexate binding to DHFR have been identified in vitro (Srimatkandada et al., 1989; Dicker et al., 1990).

The lack of specificity of classical chemotherapeutic agents and the evolution of drug resistance have prevented these compounds from having a high impact on cancer survival rates. Indeed, although a large percentage of patients with disseminated disease experience at least relief from symptoms, classical cancer chemotherapies are only curative for a small subset of human cancers. Furthermore, despite the advent of supportive measures, such as the use of antibiotics to prevent infections in neutropaecic patients (Pizzo, 1984) and platelet transfusion to ameliorate the lethality of bone-marrow suppression (Freireich et al., 1959; Gaydos et al., 1962), the potential of some cytotoxic drugs to cause leukaemia, in addition to long-term effects on the lungs, heart and reproductive organs, remain formidable barriers and have become increasingly important as patients are cured of their primary tumours.
Evaluation of the DNA replication licensing machinery as an anti-proliferative target. Sarah Kingsbury

(Burstein and Winer, 2000). There is therefore a profound need to identify novel targets or strategies to treat cancer whilst protecting the host. Two approaches to protect normal cells against the toxic effects of chemotherapeutics can be envisioned. Firstly, normal proliferating cells can be targeted by cytostatic agents to inhibit their proliferation. Tumour cells with defective checkpoint pathways should be refractory to these agents and will continue cycling, leaving them susceptible to other agents (Blagosklonny and Pardee, 2001; Keyomarsi and Pardee, 2003). Secondly, tumour cells can be targeted differentially by the identification of novel targets that are specific to the cancer cell population.

1.4. Targeted therapy

Over the last decade our understanding of the biological, genetic and molecular aspects of neoplastic disease has vastly increased, leading to the establishment of targeted therapy (Ross et al., 2004; Bell and Ryan, 2005; Thiery-Vuillemin et al., 2005). However this is a far from straightforward concept. As summarised recently by Hahn and Weinberg: “For those who believe in the simplification and rationalization of the cancer process, the actual course of research on the molecular basis of cancer has been largely disappointing. Rather than revealing a small number of genetic and biochemical determinants operating within cancer cells, molecular analyses of human cancers have revealed a bewilderingly complex array of such factors” (Hahn and Weinberg, 2002). As a result, identifying novel targets for chemotherapy is a complicated process, and these targets must fulfil a number of criteria. The ideal cancer target can be defined as a macromolecule that is crucial to the malignant cancer phenotype but is not expressed significantly in vital organs and tissues (Ross et al., 2004). In addition the target should have biological relevance
that can be measured reproducibly in clinical samples, be definably correlated with clinical outcome, and when therapeutically targeted yield a significant clinical response in patients expressing the target but not in those patients whose tumours do not express the target (Ross et al., 2004).

Despite the vast array of mutations which can result in tumour progression, ultimately the successive accumulation of mutations provides cells with six characteristics that collectively dictate malignant growth and have been defined as the 'hallmarks' of neoplastic disease: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue evasion and metastasis (Hanahan and Weinberg, 2000). Consequently, the majority of targeted therapies have been developed to interfere with these processes and thereby selectively inhibit the growth of cancer cells (Table 1.3; Ross et al., 2004; Bell and Ryan, 2005; Thiery-Vuillemin et al., 2005). However despite the development of a large number of targeted therapies, the impact on cancer mortality has been modest, and many of these agents have proved disappointing due to poor efficacy, limited response, toxic side effects, and the development of resistance.

A limitation of many targeted therapies is the requirement for a particular tumour genotype for toxicity. Cancer is a highly complex disease with the genetic status of malignant cells covering a very broad spectrum, and human tumours of any given histological type having great genetic diversity. Therefore, although directly targeting physiological processes that are dysregulated in tumour cells (e.g. cell cycle regulation, growth signalling pathways, apoptosis) appears to overcome the problem
### Table 13. Targeted chemotherapeutic strategies.

Table showing the six main classes of targeted chemotherapeutic strategies, designed to interfere with the six hallmarks of neoplastic growth.

<table>
<thead>
<tr>
<th>Class of compound</th>
<th>Example of Target</th>
<th>Drug</th>
<th>Drawbacks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell cycle inhibitors</td>
<td>CDK</td>
<td>Flavopiridol</td>
<td>Low anti-tumour activity, severe side effects (Losiewicz et al., 1994; Senderowicz, 2000; Knackaert et al., 2002)</td>
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<tr>
<td></td>
<td>Kinesin-like spindle protein</td>
<td>SB-715992</td>
<td>Poor selectivity (Burris et al., 2004; Chu et al., 2004)</td>
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<td></td>
<td>Aurora A</td>
<td>VX-680, hesperidin</td>
<td>Loss of function mutations in Aurora A can cause centrosome amplification and tetraploidisation and therefore risk of secondary neoplasms (Anand et al., 2003; Hauf et al., 2003; Harrington et al., 2004)</td>
</tr>
<tr>
<td>Signal transduction inhibitors</td>
<td>EGFR</td>
<td>Gefitinib, erlotinib</td>
<td>Narrow efficacy, high incidence of resistance (Lynch et al., 2004; Paez et al., 2004; Pao and Miller, 2005)</td>
</tr>
<tr>
<td></td>
<td>ERBB2</td>
<td>Herceptin</td>
<td>Narrow efficacy, high incidence of resistance, severe cardiac toxicity (Hudelist et al., 2004; Emens, 2005)</td>
</tr>
<tr>
<td></td>
<td>Bcr-Abl</td>
<td>Gleevac</td>
<td>Limited response, high incidence of resistance (Kantarjian et al., 2000; Sawyers et al., 2000; Talpaz et al., 2000; Drucker, 2001; Drucker et al., 2001a,b; Gorre et al., 2001)</td>
</tr>
<tr>
<td>Apoptosis modulators</td>
<td>Bcl-2</td>
<td>Oblimersen sodium (Genasense)</td>
<td>Modest responses to treatment, major relapses, severe toxicities (Chapman et al., 1999; O'Brian et al., 2001; Rai et al., 2004; Chanan-Khan et al., 2004; Gleave and Monia, 2005)</td>
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<td></td>
<td>p53</td>
<td>ONYX-015, Ad5CMV-p53</td>
<td>Poor clinical response (Reid et al., 2001; Vassey et al., 2002; Hamid et al., 2003; Hecht et al., 2003; Zeimet and Marth, 2003)</td>
</tr>
<tr>
<td>Angiogenesis inhibitors</td>
<td>VEGF/VEGFR</td>
<td>Bevacizumab, DC101</td>
<td>Severe toxicity, high incidence of resistance due to expression of multiple angiogenic proteins (Reif et al., 1997; Egget al., 2000; Kerbel and Folkman, 2002; Uehara, 2003; Abdollahi et al., 2005; Folkman, 2005)</td>
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<tr>
<td></td>
<td>Integrin</td>
<td>Vitaxine</td>
<td></td>
</tr>
<tr>
<td>Anti-invasive agents</td>
<td>MMP</td>
<td>Marimastat, col-3, batimastat</td>
<td>Poor efficacy, dose-limiting toxicities (Coussens et al., 2002; Elvin and Garner, 2005)</td>
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<td>Inhibitors of immortalisation</td>
<td>hTERT/hTR</td>
<td>BIBR1532, GRN163L</td>
<td>Poor specificity (Kim et al., 1994; Rezler et al., 2002; Masutomi et al., 2003; Kelland, 2005)</td>
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which may account for the differential response in patients. As a result of the response in this subset of patients, gefitinib has been approved for the treatment of NSCLC patients, whilst trials are ongoing for treatment of head and neck small cell carcinoma (HNSCC), gastrointestinal cancer and breast cancer. However, even with the correct genetic background many patients still prove unresponsive. Further targeted therapies which show a limited spectrum of action include Gleevac™ (also known as Glivec, imatinib mesylate and ST1571), an inhibitor of the activated tyrosine kinase activity of the Bcr-Abl protein (Druker et al., 1996; Druker and Lydon, 2000), and Herceptin (trastuzamab), a recombinant monoclonal antibody that targets the human epidermal growth factor receptor-2 (HER-2) and which relies on overexpression of HER-2/neu (Slamon et al., 2001; Emens and Davidson, 2004; Hudelist et al., 2004; Emens, 2005; Neyt et al., 2005).

In addition to illustrating the problem associated with the narrow range of efficacy of many targeted therapies, tyrosine kinase inhibitors are also a prime example for the development of drug resistance which has emerged as a major clinical problem for targeted therapies (Hynes and Lane, 2005). As discussed for classical chemotherapeutic strategies, drug resistance can occur through a variety of different mechanisms. However, a particular problem with many targeted therapies is the position of these targets on complex signalling pathways. The signal transduction network that drives cellular growth and proliferation is extremely complex with many branched, redundant, and parallel pathways. Consequently drug resistance can rapidly develop, allowing the circumnavigation of blocked pathways by the activation of redundant, parallel signalling pathways (Dai et al., 2004). Moreover, just as sensitivity to some kinase inhibitors requires a mutation within the kinase
domain (Pao et al., 2005b), resistance to kinase inhibitors can be incurred by a single mutation in the active site of the kinase which prevents the drug from inhibiting kinase activity. For example, acquired resistance of lung adenocarcinomas to gefitinib is associated with a second mutation in the EGFR kinase domain (Pao et al., 2005a). Therefore in addition to the small proportion of patients who are initially responsive to these therapies, there is also a significant clinical problem with the rapid development of resistance to tyrosine kinase inhibitors, with nearly all patients who initially show remission following treatment eventually suffering progression of the disease (Kantarjian et al., 2000; Talpaz et al., 2000; Drucker, 2001; Drucker et al., 2001a,b; Gorre et al., 2001; Kute et al., 2004; Lan et al., 2005; Pao and Miller, 2005).

Cyclin Dependent Kinase inhibitors (CDKIs) are another example of a targeted therapy which was expected to have a huge impact on cancer treatment, but which has shown disappointing results in clinical development. Aberrant expression of cyclins has been causally linked to the development of a variety of tumours (Schauer et al., 1994; Lonardo et al., 1999; Buolamwini, 2000), suggesting that agents targeting these proteins may give tumour specificity (Cohen, 1999; Sausville, 2000; Knockaert et al., 2002; Senderowicz, 2003; Vermeulen et al., 2003; Schwartz and Shah, 2005). However since CDKs are involved in many physiological processes with many different substrates (Sánchez and Dynlacht, 2005), CDKIs lack specificity and invoke severe toxic side effects (Sausville et al., 1998, 2001; Fuse et al., 1998). This is further exacerbated by the significant cross-talk that occurs between signal transduction pathways, so that targeting one cascade may block or stimulate an unrelated cascade and therefore have multiple and unpredictable effects on cell cycle
modulation (Senderowicz, 2004). A CDK inhibitor that is currently undergoing clinical trial is flavopiridol, a flavonoid that interacts with the ATP-binding pocket of CDKs (Losiewicz et al., 1994). Although flavopiridol inhibits the proliferative potential of a broad range of different cell lines, human tumours, leukaemias and lymphomas (Drees et al., 1997; Parker et al., 1998), and showed some activity in phase I trials of patients with Hodgkin’s lymphoma, renal, colon and gastric malignancies (Senderowicz et al., 1998, 1999; Tan et al., 2002), it did not show significant anti-tumour activity as monotherapy in phase II trials in several epithelial tumours (Senderowicz, 2000). In addition, the specificity of flavopiridol, and other synthetic CDKIs (e.g. UCN-01, CYC202, BMS-387032, roscovitine) remains a limiting factor, with patients in phase I and II clinical trials experiencing severe side-effects (Sausville et al., 1998, 2001; Fuse et al., 1998). Other questions have been raised about the validity of targeting CDKs as a therapeutic strategy. For example, the activity of several of these agents depends on the expression of critical downstream regulators that must be present to induce cell cycle arrest (e.g. an intact pRb pathway is required in the case of a CDK4 inhibitor; Fry et al., 2001), whilst recent studies have suggested that inhibition of single CDKs may not be sufficient to induce cell cycle arrest (Tetsu and McCormick, 2003). So on the one hand decreased specificity may be important for improving cytotoxicity, but on the other hand this decreased specificity is likely to cause increased side-effects and lower the dose-limiting toxicity threshold.

The success of chemotherapy relies on the ability of cytotoxic agents to induce apoptosis in target cells. Alteration of the components of the apoptotic machinery can dramatically affect the dynamics of tumour progression (Kerr et al., 1972), whilst
aberrant cell death is a presumptive mechanism of resistance to current neoplastic therapy where the desired result, apoptosis of malignant cells, does not occur (Hickman, 1996). Strategies for restoring the apoptotic machinery may therefore provide a mechanism to induce tumour cell apoptosis and tumour regression, and to overcome resistance to current cytotoxic therapies (Senderowicz, 2004; Ghobrial et al., 2005). The pro-apoptotic regulator p53 is the most frequently inactivated gene in human cancers (Olivier et al., 2002), with mutations present in more than 50% of human cancers (Hollstein et al., 1994). Since loss of p53 has been linked to the development of resistance, restoration of p53 function may provide a mechanism for overcoming drug resistance (Perona and Sánchez-Pérez, 2003). Currently a number of p53-gene therapies are under clinical development including ONYX-015 and Ad5CMV-p53. ONYX-015 (dl1520), a replication competent adenovirus that lacks the ELB 55K gene which neutralises p53, is only able to replicate in cells with deficient p53 activity. Interactions between ONYX-015 and the p53 pathway cause cancer cells lacking p53 to induce viral replication and apoptose (Heise et al., 1997). However the specificity of ONYX-015 for p53-deficient cells remains controversial (Goodrum and Ornellas, 1998; Rothman et al., 1998; Harada and Berk, 1999; Turnell et al., 1999; Dix et al., 2001), whilst phase I/II trials in a range of tumours have proved unsuccessful, with no objective clinical response seen in any patient treated with ONYX-015 as a single agent (Khuri et al., 2000; Nemunaitis et al., 2001; Reid et al., 2001; Vasey et al., 2002; Hamid et al., 2003; Hecht et al., 2003). Similarly disappointing results were obtained in phase I/II clinical trials of Ad5CMV-p53, with no improvement in effectiveness and increased toxicity compared to traditional treatments (Zeimet and Marth, 2003). Failure of p53 gene therapy may occur for a number of reasons, including inoptimal gene transfer, insufficient p53 expression
leading to cell cycle arrest as opposed to induction of apoptosis (Vousden and Lu, 2002), requirement for downstream factors (e.g. Bax, Fas, PTEN) (Wolf et al., 2001) and inhibition of exogenous wild-type p53 by endogenous mutant p53 (de Vries et al., 2002).

Despite the development of a large number of novel chemotherapeutic strategies during the last decade, there are still many hurdles in cancer treatment that have yet to be overcome. The agents presented here represent some of the more successful and more highly developed chemotherapeutic strategies currently in preclinical/clinical development and in clinical use. In addition many other chemotherapeutic strategies are currently undergoing investigation and evaluation, and new targets are continually sought. Examples of such strategies include angiogenesis inhibitors (Relf et al., 1997; Eggert et al., 2000; Kerbel and Folkman, 2002; Uehara, 2003; Abdollahi et al., 2005; Folkman, 2005), anti-invasive agents (Coussens et al., 2002; Nakajima et al., 2003; Sattler et al., 2003; Ertongur et al., 2004; Lombardo et al., 2004; Elvin and Garner, 2005), inhibitors of immortalisation (Kim et al., 1994; Neidle and Parkinson, 2002; Rezler et al., 2002; Shay and Wright, 2002; Masutomi and Hahn, 2003; Hsu and Lin, 2005; Kelland, 2005), DNA damage response modifiers (e.g. PARP inhibitors; Bryant et al., 2005; Farmer et al., 2005; Graziani and Szabo, 2005; Haince et al., 2005; Madhusen and Middleton, 2005) heat shock protein 90 (Hsp90) inhibitors (Neckers, 2002; Sreedhar et al., 2004), proteasome inhibitors (Adams, 2002a,b), histone deacetylase inhibitors (Das and Singal, 2004; Thiery-Vuillemin et al., 2005), statins (Thiery-Vuillemin et al., 2005) and carbohydrate ligands (e.g. modified pectin; Nangia-Makker et al., 2002). The majority of these strategies are currently only in very early stages of development.
and hence a more comprehensive discussion of these agents is beyond the scope of this introduction.

1.5. The DNA replication licensing pathway – a novel diagnostic and therapeutic target?

The initiation of DNA replication is a crucial decision point in cell proliferation that lies at the convergence point of all oncogenic and mitogenic signalling transduction pathways that drive proliferation, and hence is of fundamental importance in tumourigenesis. The DNA replication licensing pathway can therefore be regarded as a relay station providing a molecular switch to couple growth regulatory pathways with the initiation of chromosomal replication. Tumour progression can be catalysed by a vast array of activating and inactivating mutations occurring at all levels of these signal transduction pathways, presenting a major challenge for the development of effective diagnostic and therapeutic strategies due to the complexity of addressing multiple individual mutations for each tumour type and subtype. However the position of the DNA replication licensing pathway at the convergence point of growth transduction pathways may provide a reductionist solution to this problem.

At a diagnostic level it can be postulated that the initiation of DNA replication should provide a set of cancer biomarkers that are applicable to a broad range of tumours independent of genetic background. Similarly, the initiation of DNA replication may provide an attractive therapeutic anti-cancer target since the mutational status of upstream signalling components should have no implication on the action of agents targeting this highly evolutionary conserved process. Such agents therefore have the potential to provide an effective therapeutic strategy for a wide range of tumour types and subtypes with varying genetic backgrounds.
Moreover, targeting DNA replication initiation should prove less susceptible to the evolution of drug resistant clones that can arise due to the branched, parallel and redundant nature of growth regulatory pathways. Importantly, inhibiting the initiation of DNA synthesis should minimize collateral damage to the DNA, eliminating the risk of genomic damage and therefore circumventing the problems of infertility and further neoplastic transformation associated with the use of DNA damaging agents and drugs which interfere with elongation DNA synthesis. A further criterion which must also be considered is the specificity of therapeutic agents targeting DNA replication initiation, especially given that DNA replication is essential to all dividing cells and that agents targeting elongation DNA synthesis are known to invoke severe toxic effects in normal cells. However, prior to addressing this issue I will first provide an overview of the DNA replication licensing pathway.

1.5. Licensing of DNA

The precise duplication of DNA once, and only once, during each cycle of cell proliferation is central to maintaining eukaryotic genome integrity. The initiation of chromosomal replication is therefore tightly regulated by the DNA replication licensing pathway. During late M and early G1 phase DNA is ‘licensed’ for replication by the ordered assembly of DNA replication licensing factors (ORC, Cdc6, Cdt1 and Mcm2-7) into pre-replicative complexes (pre-RCs), so termed because they generate a characteristic footprint prior to replication initiation (Diffley et al., 1994), at origins of replication (Figure 1.1). These licensed origins are subsequently fired at different times during S phase by the concerted action of S-phase promoting CDKs and the Dbf4^{ASK}-dependent Cdc7 kinase (DDK), leading to the initiation of chromosomal replication (Figure 1.1).
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Figure 1.1. The DNA replication licensing pathway. During late M phase and early G1 phase pre-replicative complexes (pre-RCs) are assembled in a stepwise manner at origins of replication. The origin recognition complex (ORC) recruits Cdc6 and Cdt1, which in turn load multiple copies of the Mcm2-7 complex onto chromatin forming the pre-RC. During the G1-S phase transition the pre-RC is converted to a pre-initiation complex (pre-IC) allowing DNA replication to be initiated. Phosphorylation of MCM proteins by Cdc7-Dbf4 and S phase-promoting CDKs triggers a conformational change in the MCM complex that is required for unwinding of DNA and allows recruitment of further initiation factors Mcm10 and Cdc45. The MCM loading factors Cdt1 and Cdc6 are removed from the origin and the origin licensing repressor geminin, which interacts with Cdt1, is expressed to prevent re-loading of pre-RCs. Finally replication protein A (RPA) and DNA polymerase α-primase are recruited to the origin and DNA replication is initiated.
Replication origins are determined, at least in part, by the binding of the six subunit origin recognition complex (ORC), which is conserved in evolution from yeast to humans (Bell and Stillman, 1992; Diffley et al., 1994; Bell et al., 1995; Gavin et al., 1995; Gossen et al., 1995; Muzi-Falconi and Kelly, 1995; Carpenter et al., 1996; Leatherwood et al., 1996; Rowles et al., 1996). In budding yeast, ORC binds specifically to the \textit{S. cerevisiae} ACS (ARS [automonously replicating sequence] consensus sequence) (Bell and Stillman, 1992), an 11 bp AT-rich sequence element which together with two or three stimulatory elements (B1-B3) forms the core of the replicator (Marahrens and Stillman, 1992). ORC binding is also mediated by short AT-rich elements in \textit{S. pombe} (Okuno et al., 1999; Segurado et al., 2003), although no well-defined consensus sequence has been identified (Kim and Huberman, 1998). No consensus sequences for ORC binding have been identified in higher eukaryotes either, with human ORC capable of binding functionally to any DNA sequence (Vashee et al., 2003; Schaarschmidt et al., 2004). Instead origin selection in higher eukaryotes appears to be influenced by chromatin and nuclear structure, with no requirement for specific sequence information (DePamphilis, 1999; Lucas et al., 2000; Blow et al., 2001; Aggarwal and Calvi, 2004; Remus et al., 2004). In \textit{Xenopus} egg extracts, for example, initiation occurs at regular intervals of approximately 10 kb (Lucas et al., 2000; Blow et al., 2001) whilst in \textit{Drosophila} ORC binds preferentially to negatively coiled DNA (Remus et al., 2004). In mammalian cells replication can occur within replication 'zones' that vary in size from 6 to 55 kb (Dijkwel and 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 (DUE) (DePamphilis, 1999). This definition of origins by chromatin structure and epigenetic factors has been proposed to provide an
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evolutionary advantage since the genome can sustain genetic alterations without concern for losing origins in the process (Gilbert, 2001). Moreover, the epigenetic specificity of origins may provide a mechanism by which different programmes of gene expression can be established and controlled during development (Méchali, 2001).

Approximately 30,000 origins are postulated to exist in the mammalian genome (Todorovic et al., 1999); however the number of licensed origins exceeds the number of origins activated in each cell cycle due to the assembly of pre-RCs at both active and silent origins (Santocanale and Diffley, 1996; Walter and Newport, 1997). This acts to ensure that the entire genome is replicated even if some origins fail to initiate, with silent origins activated if neighbouring origins fail to fire (Santocanale et al., 1999; Vujcic et al., 1999). In the absence of these secondary origins failed initiation events would likely result in replication errors, in particular replication fork stalling due to forks having to traverse much larger distances. In *S. cerevisiae*, for example, removal of ARS elements eventually results in chromosome loss, presumably because replication is not completed during S phase (Dershowitz and Newlon, 1993). The mechanism that determines which origins fire is not clear, and is likely influenced by a combination of factors including chromosomal context and epigenetic elements (Gilbert, 2001).

Although the process of origin selection is vastly different amongst eukaryotes, the process of licensing is highly conserved. During G1 ORC acts as a landing pad for the recruitment of Cdc6 and Cdt1 to origins of replication, which in turn load the Mcm2-7 heterohexameric complex onto chromatin. The helical structure of Mcm2-7,
and its location around the DNA has led to speculation that ORC and Cdc6 might act as an ATP-dependent clamp-loader. This is supported by the presence of ATP binding sites (Walker A motifs) in Orc1, Orc4, Orc5 and Cdc6, and ATP hydrolysis sites (Walker B motifs) in Orc1, Orc4 and Cdc6 (Bell et al., 1995; Loo et al., 1995; Klemm et al., 1997; Perkins and Difflley, 1998; Weinreich et al., 1999). Mutagenesis studies indicate that ATP binding is required for the association of ORC and Cdc6 with DNA (Chesnokov et al., 2001; Klemm and Bell, 2001), whilst ATP hydrolysis is essential for loading of the Mcm2-7 putative helicase (Gillespie et al., 2001; Harvey and Newport, 2003).

Several lines of evidence suggest that Mcm2-7 acts as the replicative helicase during S phase. Studies in *Xenopus* and mammalian cells have established that MCMs are present on chromatin during G1, are displaced during DNA replication and are absent from chromatin during G2 (Chong et al., 1995; Kubota et al., 1995; Madine et al., 1995a,b). Although recruited by ORC, Cdc6 and Cdt1, MCM proteins do not remain associated with ORC once bound to the chromatin (Aparicio et al., 1997) and probably move away from the origin after firing (Ritzi et al., 1998; Labib et al., 2000). Indeed once the Mcm2-7 complex has been loaded at origins of replication ORC, Cdc6 and Cdt1 are no longer required for the initiation of DNA replication (Donovan et al., 1997; Hua and Newport, 1998; Rowles et al., 1999; Maiorano et al., 2000; Harvey and Newport, 2003). This indicates that as well as being involved in the initial binding and melting of replication origins (Borowiec and Hurwitz, 1988), Mcm2-7 may also act as a helicase at the elongation forks (Stahl et al., 1986). Further evidence supporting this hypothesis is provided by the conserved NTPase motif shared by all MCM proteins, which has sequence similarity to members of the...
DNA helicase family (Koonin, 1993), the weak in vitro helicase activity identified in a complex comprised of Mcm4, 6 and 7 (Ishimi et al., 1997; You et al., 1999; Lee and Hurwitz, 2000), and the helicase activity observed for the single MCM of the archeon Methanobacterium thermoautotrophicum (Kelman et al., 1999; Chong et al., 2000; Shecter et al., 2000). Initial models for Mcm2-7 activity suggested that two complexes were loaded at each origin for bi-directional replication. However, recent findings in Xenopus have proposed that multiple MCM complexes are loaded at each origin and spread out over the DNA during S phase (Edwards et al., 2002), consistent with the observation that the majority of MCM proteins are associated with unreplicated DNA (Madine et al., 1995; Krude et al., 1996; Dimitrova et al., 1999).

Precise regulation of pre-RC assembly is ensured through a lack of redundancy between pre-RC components and through the strict cell cycle dependent regulation of each replication licensing factor. Each pre-RC constituent is essential for the initiation of DNA replication, with reduced levels of any component leading to loss of replicative capacity and an inability to progress into S phase (Sinha et al., 1986; Gibson et al., 1990; Chen et al., 1992; Yan et al., 1993; Kimura et al., 1994; Ray et al., 1994; Todorov et al., 1994; Fox et al., 1995; Liang et al., 1995; Madine et al., 1995; Carpenter et al., 1996; Coleman et al., 1996; Fujita et al., 1996; Rowles et al., 1996; Detweiler and Li, 1997; Kubota et al., 1997; Lei et al., 1997; Thommes et al., 1997; Perkins and Diffley, 1998; Yan et al., 1998; Madine et al., 2000; Maiorano et al., 2000; Nishitani et al., 2000; Petersen et al., 2000; Tada et al., 2001; Riall et al., 2002; Higa et al., 2003; Cook et al., 2004). Although the process of replication licensing is highly conserved from yeast to vertebrates, the regulation of replication licensing factors throughout the cell cycle is species dependent. In yeast, for
example, all ORC subunits remain bound to chromatin through all phases of the cell cycle with some subunits undergoing CDK-dependent phosphorylation in S phase (Weinreich et al., 2001). In contrast, ORC-chromatin association in mammalian cells is highly dynamic. Orc1, for example, undergoes ubiquitin-mediated degradation during S phase in human cells (Li and DePamphilis, 2002; Mendez et al., 2002; Li et al., 2004) whilst Orc2 relocates to centromeric heterochromatin in mitosis (Prasanth et al., 2004). Both Cdc6 and Cdt1 accumulate during late mitosis and early G1, producing a peak in nuclear Cdc6 and Cdt1 levels prior to pre-RC assembly, (Muzi-Falconi et al., 1996; Stoeber et al., 1998; Coverley et al., 2000; Nishitani et al., 2001; Rialland et al., 2002). However as with ORC the precise regulation of these proteins is species dependent, with protein levels controlled by degradation, inactivation or nuclear exclusion (Drury et al., 1997, 2000; Jallepalli et al., 1997; Saha et al., 1998; Elsasser et al., 1999; Fujita et al., 1999; Petersen et al., 1999; Pelizon et al., 2000; Coverley et al., 2000, 2002; Mendez and Stillman, 2000; Okuno et al., 2001; Biermann et al., 2002; Tanaka and Diffley, 2002; Alexandrow and Hamlin, 2004).

Interestingly in addition to their role in co-ordinating the initiation of DNA replication, a number of pre-RC components appear to be involved in other processes throughout the cell cycle. ORC proteins have been implicated in the silencing of gene expression in budding yeast, drosophila and vertebrates (Bell et al., 1993; Fox et al., 1995; Huang et al., 1998; Iizuka and Stillman, 1999; Pak et al., 1997) and in the co-ordination of chromosome segregation and cytokinesis (Prasanth et al., 2002), whilst Cdt1 has been implicated in a G1 checkpoint which induces Cdt1 proteolysis in response to DNA damage (Higa et al., 2003) and MCM proteins have been suggested to be important for damage response, transcription and chromatin
remodelling (Forsburg, 2004).

In addition to the core pre-RC components, ORC, Cdc6, Cdt1 and Mcm2-7, a further MCM protein, Mcm10, is also essential for the initiation of DNA replication (Solomon et al., 1992; Merchant et al., 1997; Aves et al., 1998; Izumi et al., 2000; Wohlschlegal et al., 2002; Christensen and Tye, 2003). Mcm10 interacts with Mcm2-7 (Merchant et al., 1997; Homesley et al., 2000; Izumi et al., 2000; Kawasaki et al., 2000), as well as Orc2 and Cdc7-Dbf4, and has been suggested to facilitate phosphorylation of Mcm2-7 by Cdc7-Dbf4 (Lee et al., 2003). In addition, Mcm10 is required for the loading of Cdc45 onto chromatin (Wohlschlegal et al., 2002b; Gregan et al., 2003; Sawyer et al., 2004) and, like Mcm2-7, is essential for elongation DNA synthesis (Kawasaki et al., 2000).

1.5.2. Initiation of DNA replication

Once the components of the pre-RC have been assembled, activation is mediated by the concerted action of S-phase specific CDKs and DDK. CDKs act as global S-phase promoting factors whilst DDK acts more specifically and has been shown to phosphorylate various members of the MCM family in vitro (Lei et al., 1997; Sato et al., 1997; Brown and Kelly, 1998; Jiang et al., 1999; Kumagai et al., 1999; Takeda et al., 1999, 2001; Kihara et al., 2000; Masai et al., 2000; Ishima et al., 2001), a process that may alter the conformation of the MCM complex and allow unwinding of the origin DNA structure. Like the pre-RC components, Cdc7 and Dbf4 are essential for the initiation of DNA replication (Jares and Blow, 2000; Walter, 2000; Kim et al., 2002) and are subject to precise cell cycle regulation. Cdc7 is expressed at constant levels throughout the cell cycle, whilst Dbf4 is expressed during the G1/S
transition under the control of the MBF transcription factor (Moll et al., 1992; Iyer et al., 2001) and undergoes anaphase-promoting complex (APC)-dependent degradation (Oshiro et al., 1999; Weinreich and Stillman, 1999; Ferreira et al., 2000). Protein synthesis is no longer required for completing S phase once Cdc7 function has been executed, suggesting that it is the ultimate factor required for the initiation of DNA replication.

There is some confusion as to whether DDK or CDK is the first to act during the G1–S transition with experiments in different organisms giving conflicting data and some even suggesting that CDK may in fact be required to function twice, first to generate a pre-activated pre-RC for Cdc7 action and second to activate the replication machinery (Jares and Blow, 2000; Masai et al., 2000; Nougerade et al., 2000; Walter, 2000). The mechanism by which Cdc7-Dbf4 activates the pre-RC has been postulated from the results of two mutations. The mcm5-bobl (Hardy et al., 1997) mutation bypasses Cdc7 function whilst a mutation in mcm2 (Lei et al., 1997) which prevents its interaction with Dbf4, is suppressed by the dbf4-6 mutation. These results suggest that the only essential function of Cdc7-Dbf4 in initiating DNA replication is mediated through the MCM complex and that Mcm2 may be the only essential target of regulation for Cdc7-Dbf4. It is likely that the MCM complex is inactive until it comes into contact with Cdc7-Dbf4, and most probably until Mcm2 is phosphorylated, and that this phosphorylation causes a conformational change that leads to the activation of the MCM complex. The mcm5-bobl mutation may therefore alter the structure of Mcm5 in such a way that it constitutively activates MCM without the need for Cdc7-Dbf4.
Phosphorylation of the MCM complex by CDK and DDK results in the recruitment of Cdc45 to the origin. Cdc45 is essential for the unwinding of DNA (Walter and Newport, 2000; Pacek and Walter, 2004), potentially by inducing a conformational change in the MCM complex which leads to activation of its DNA helicase activity (Masuda et al., 2003). Cdc45 has also been implicated in the recruitment of DNA replication proteins RPA and DNA polymerase α (Mimura and Takisawa, 1998; Uchiyama et al., 2001) to origins and appears to associate with moving forks suggesting that, like MCM, Cdc45 may be essential for elongation as well as initiation of DNA replication (Aparicio et al., 1997; Tercero et al., 2000).

The final step in replication initiation is the loading of the replicative polymerases DNA pol α, DNA pol δ and DNA pol ε (Waga and Stillman, 1998; Kawasaki and Sugino, 2001; Hubscher et al., 2002), which occurs after origin unwinding has been stimulated by Cdc45 and RPA binding (Mimura et al., 2000; Walter and Newport, 2000). A number of factors are necessary for the recruitment of the replicative polymerases to replication origins including Mcm10 (Kawasaki et al., 2000), Cdc45 (Mimura and Takisawa, 1998; Uchiyama et al., 2001), Sld3 (Kamimura et al., 2001), Dpb11 (Masumoto et al., 2000; Van Hatten et al., 2002; Hashimoto and Takisawa, 2003) and GINS (Kubota et al., 2003; Takayama et al., 2003). Once these factors have been assembled at replication origins the conversion of pre-RCs to active replication forks is complete and DNA replication can commence.

1.5.3. Prevention of re-replication and genomic instability

In addition to the regulation of DNA replication initiation, tight controls exist to ensure that each origin is replicated only once during each cycle of cell proliferation,
with CDKs central to this process (Nguyen et al., 2001). Multiple, redundant mechanisms are utilised to achieve the block to origin re-licensing, with many components of the pre-RC subject to negative regulation by CDKs. However, the precise strategy by which this block is achieved varies between different organisms (Feng and Kipreos, 2003). In yeast, for example, Cdc6 undergoes ubiquitin-dependent degradation through the SCF complex following CDK-dependent phosphorylation (Drury et al., 1997, 2000; Jallepalli et al., 1997; Elsasser et al., 1999), whilst phosphorylation of MCMs (Labib et al., 1999; Nguyen et al., 2000) and Cdt1 (Tanaka and Diffley, 2002) leads to their exclusion from the nucleus and phosphorylation of Orc2 prevents it from interacting with Cdc6 (Seki and Diffley, 2000). In metazoans phosphorylated Cdc6 is either transported out of the nucleus after S-phase onset (Saha et al., 1998; Fujita et al., 1999; Petersen et al., 1999; Pelizon et al., 2000) or remains associated with chromatin in an inactive state (Coverley et al., 2000, 2002; Mendez and Stillman, 2000; Okuno et al., 2001; Biermann et al., 2002; Alexandrow and Hamlin, 2004). Both Cdt1 and Orc1 are subject to phosphorylation dependent proteolysis in mammalian cells (Kreitz et al., 2001; Li and DePamphilis, 2002; Liu et al., 2004; Sugimoto et al., 2004), whilst phosphorylation of MCMs decreases their affinity for chromatin (Coue et al., 1996; Hendrickson et al., 1996; Fujita et al., 1998) and inhibits helicase activity (Ishimi and Komamura-Kohno, 2001). In addition, metazoans have a further mechanism of defence in a recently identified endogenous negative regulator of DNA replication, geminin (McGarry and Kirschner, 1998).

Geminin is a dual function 23.5 kDa protein that was identified almost simultaneously in two separate expression screens; the first for substrates of the APC
that degraded in mitotic *Xenopus* egg extracts (McGarry and Kirschner, 1998) and the second for proteins involved in neuralisation of *Xenopus* embryos (Kroll *et al*., 1998). Geminin is highly conserved in metazoans with homologs identified in *Drosophila*, mouse, humans (Quinn *et al*., 2001; Yanagi *et al*., 2002) and *Caenorhabditis elegans* (Yanagi *et al*., 2005) but none identified so far in yeast. Geminin is expressed during S, G2 and M phases of the cell cycle during which time it functions to prevent re-replication from fired origins by interacting with Cdt1 and thereby preventing re-assembly of pre-RCs (Wohlschlegal *et al*., 2000; Tada *et al*., 2001; Yanagi *et al*., 2002; Cook *et al*., 2004). At the end of mitosis, geminin undergoes CDK-dependent ubiquitination resulting in proteolysis by the 26S proteosome and providing a critical geminin-free window in G1 where pre-RC assembly can occur (McGarry and Kirschner, 1998) and the next round of replication can be initiated (Benjamin *et al*., 2004).

1.5.4. The DNA replication licensing pathway as an anti-cancer target

As discussed in Section 1.5.1., the constituents of the pre-RC can be regarded as relay stations coupling growth regulatory pathways to the initiation of DNA replication. The hypothesis that replication licensing factors may be of major diagnostic utility is currently being confirmed in a wide range of tumour systems. Importantly the DNA replication licensing pathway appears to become dysregulated early in multi-step tumour progression. For example, dysregulation of the MCM helicase proteins is observed in precursor dysplastic (pre-invasive) lesions of many epithelial organ systems including cervix, colon, prostate, oesophagus and bladder (Freeman *et al*., 1999). This is exemplified in Figure 1.2. which shows the expression of Mcm2 in normal oesophageal squamous mucosa, oesophageal precursor lesions
Evaluation of the DNA replication licensing machinery as an anti-proliferative target. Sarah Kingsbury

Dysregulation of DNA replication initiation pathway during oesophageal tumourigenesis. Oesophageal cancer is divided into two main histological subtypes, squamous cell carcinoma and adenocarcinoma, with very different risk factors, incidence and molecular biology. Both subtypes of oesophageal cancer have precursor lesions. Squamous dysplasia is recognised as a premalignant precursor to squamous cell carcinoma and Barrett's glandular metaplasia a precursor for adenocarcinoma. The figure shows indirect immunoperoxidase staining of biopsy material from normal oesophageal squamous mucosa, oesophageal precursor lesions and the two histological variants of oesophageal cancer with an anti-Mcm2 MAb. In normal oesophageal mucosa and metaplastic Barrett's oesophagus, MCM expression is restricted to the proliferative compartment with down-regulation occurring as epithelial cells engage the differentiation programme and migrate to the surface layers. In contrast the dysplastic epithelium characterised by maturation arrest and loss of epithelial polarity shows dysregulation of the DNA replication initiation pathway with failure to down-regulate the MCM proteins as cells migrate to the superficial differentiated compartment. Progression to squamous cancer and adenocarcinoma is characterised by strikingly high expression levels of the MCM proteins coupled to development of marked nuclear pleomorphism and nuclear megalogy, the phenotype associated with severe aneuploidy.
and two histological variants of oesophageal cancer. In normal oesophageal mucosa and metaplastic Barrett’s oesophagus MCM expression is restricted to the proliferative compartment. In contrast the dysplastic epithelium shows dysregulated MCM expression, whilst squamous cancer and adenocarcinoma show strikingly high expression levels of Mcm2. Significantly MCM licensing factors are detected in a far greater proportion of tumour cells than conventional proliferation markers such as Ki67 or BrdU S phase labelling index, due to the fact that licensing factors can be detected in non-cycling cells with proliferative potential (Stoeber et al., 1999, 2001; Eward et al., 2004). These unique biomarkers of growth have now been exploited in a wide range of tumours, including colorectal cancer (Davies et al., 2002; Scott et al., 2003), oligodendroglioma (Wharton et al., 2001, 2004), oesophageal squamous cell carcinoma (Going et al., 2002; Siriex et al., 2003; Kato et al., 2003; Williams et al., 2004; Huang et al., 2005), non-small cell lung cancer (Ramnath et al., 2001), renal cell carcinoma (Rodins et al., 2002; Dudderidge et al., 2005), cervical carcinoma (Williams et al., 1998; Mukherjee et al., 2001), prostate cancer (Meng et al., 2001; Ren et al., 2005), genito-urinary tract cancer (Stoeber et al., 1999, 2002), bladder cancer (Kruger et al., 2003), ovarian cancer (Scott et al., 2004) and breast cancer (Bukholm et al., 2003; Gonzalez et al., 2003; Shetty et al., 2005), as predictive and prognostic markers and as molecular tools for cancer screening and surveillance. TriPath Imaging®, the leading company in liquid-based cervical screening, is currently seeking FDA approval for the use of MCM proteins for automation of cervical screening (ProEx™ C) whilst a large CR-UK funded multi-centre trial (UK National Cancer Research Network [NCRN] Trial ID 1279) for bladder and prostate cancer is currently ongoing.
Further to their exciting potential as cancer markers, the abundant expression of MCM proteins in cancer cells and dysregulation early in tumourigenesis indicates that agents targeting the replication licensing machinery may have high efficacy as anti-cancer agents. Importantly since aberrant expression of MCM proteins is common to a wide range of tumours such agents should provide effective treatment for multiple tumour types and subtypes, thus recapitulating the observed extensive diagnostic application of MCM proteins. In addition to the potential broad application and sensitivity of agents targeting the replication licensing machinery these agents should also be non-genotoxic and less susceptible to the evolution of resistance than many conventional agents, as discussed in Sections 1.3 and 1.4.

The potential exploitation of the DNA replication licensing pathway as an anti-cancer target has gained further impetus through a recent study by Shreeram et al., which intriguingly suggests that inhibitors of origin licensing may provoke a differential response in normal and transformed cell lines (Shreeram and Blow, 2003). In untransformed IMR-90 fibroblast cells, inhibition of origin licensing causes a cell cycle arrest associated with elevated p21\(^{Cip1}\) levels, low levels of cyclins A and E and markedly reduced levels of phosphorylated pRb consistent with cells arresting in G1 (Shreeram et al., 2002). In contrast, inhibition of origin licensing in transformed Saos2 and U2OS cells results in progression into S or G2 phase after which cells undergo apoptosis. Transformed U2OS cells with intact pRb and p53 tumour suppressor growth inhibitory pathways undergo early S phase arrest with down-regulation of cyclin A and up-regulation of p53 and p21\(^{Cip1}\), consistent with activation of an intra-S phase checkpoint. Notably, in a separate study Cdc7 inhibition by small interfering RNA also revealed a p53-dependent checkpoint that
appears to be defective in cancer cells (Montagnoli et al., 2004). In contrast, transformed Saos2 cells deficient in p53 and pRb undergo a late S and G2/M phase arrest with detectable cyclin A, consistent with loss of the intra-S phase checkpoint (Shreeram et al., 2002). The differential response to blocking origin licensing in these cell lines has raised the hypothesis that a checkpoint may exist in G1 to monitor the assembly of pre-RCs at origins of replication. Up to 30,000 origins are postulated to exist in the genome (Todorovic et al., 1999), and it can be envisioned that a critical number of these must be licensed before cells can proceed into S phase and initiate DNA replication (Figure 1.3). Defects in this putative 'origin licensing checkpoint' may allow the initiation of DNA replication to commence with only a subset of these replication origins licensed. In the presence of an origin licensing inhibitor cells with a defective checkpoint may move into S phase and attempt to replicate their DNA from the few origins which have been licensed (Figure 1.3). In these circumstances DNA replication is likely to break down, leading to stalled replication forks and in cells with a complete intra-S phase checkpoint inducing an apoptotic response, as was observed in U20S cells (Shreeram et al., 2002). However, defects in the intra-S phase checkpoint will allow cells to continue through the cell cycle, reaching M phase with incompletely replicated DNA. In this circumstance the attempt to segregate chromosomes during mitosis will result in the generation of DNA double-strand breaks, causing a mitotic catastrophe and the induction of apoptosis, as was observed for Saos2 cells (Shreeram et al., 2002). Whether blocking origin licensing will generate a similar response in transformed cells with vastly different genetic spectrums is currently unknown.
Figure 1.3. A putative origin licensing checkpoint. (a) Under physiological conditions cells are able to assemble a sufficient number of pre-RCs at replication origins during G1, enabling DNA replication to be initiated in S phase. (b) When origin licensing is blocked in normal cells the initiation of DNA replication is impeded by activation of a putative 'origin licensing checkpoint' and cells arrest in G1. (c) Transformed cells do not arrest in G1 due to a defective 'origin licensing checkpoint'. Instead DNA replication is initiated from the few licensed origins, leading to DNA damage (e.g. stalled replication forks and double strand breaks [DSB]) and apoptosis.
inhibitors, in order to fully understand how a patient may respond to treatment it is of critical importance to ascertain the response of normal cell populations to these agents. To this end it is therefore essential to analyse the normal physiological regulation of the DNA replication licensing machinery in all normal cell populations, including stem/progenitor cell populations, transit amplifying cell populations and functional differentiated and quiescent cell populations. Moreover, a further question which remains to be answered is whether the G1 arrest observed in normal cells following a block to origin licensing is reversible. If normal cells were able to recommence proliferation following removal of an origin licensing inhibitor cytotoxicity would be significantly reduced and such an agent could be used at much higher doses, allowing a greater insult on tumour cells.

Taken together, these combined findings allow the hypothesis to be put forward that inhibitors of the replication licensing pathway could potentially provide effective non-genotoxic anti-cancer agents for treatment of a wide range of tumours. Notably, the existence of a putative origin licensing checkpoint suggests that such agents may provide cancer specific cell killing effects.

1.6. Summary and aims of thesis

The DNA replication licensing machinery has recently emerged as a potential target for novel therapeutic anti-cancer agents. Dysregulation of the replication licensing pathway is an early event in carcinogenesis. However, little is known about the regulation of the replication licensing pathway, and in particular the endogenous origin licensing repressor geminin, in normal human tissues. In the first part of this thesis, I describe a detailed expression analysis of the replication licensing machinery...
in the out-of-cycle states of quiescence and differentiation, which constitute a large proportion of normal cells within the body, and in stem cell populations. These studies show that withdrawal of cells from cycle into the differentiated and quiescent states is tightly coupled to down-regulation of the DNA replication licensing pathway, indicating that these cell populations should be refractory to origin licensing inhibitors. Extending on these studies, I have carried out early proof-of-principle studies to further define the replication licensing machinery as an anti-proliferative and anti-cancer target. In Chapters Four and Five I describe an \textit{in vitro} biochemical analysis of two potential future lead compounds, mimicking the endogenous origin licensing repressor geminin and a pathogenic inhibitor of origin licensing, HPV1 E4. Finally in Chapter Six, I have utilised a non-degradable fragment of geminin and a novel cell delivery system to investigate the differential response of normal and transformed cell lines to inhibition of origin licensing. These studies show that normal cells respond to origin licensing by reversibly arresting in G1 whilst transformed cells proceed through the cell cycle and undergo apoptosis, indicating that origin licensing inhibitors should be highly specific anti-cancer agents and show little or no toxicity to normal cells. Collectively, the work presented in this thesis provides compelling evidence to support the concept that targeting the origin licensing machinery provides a potential anti-cancer strategy applicable to a wide range of tumour types regardless of their genetic background.
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 Laemmli Sample Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-Cl pH 6.8</td>
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<tr>
<td>SDS</td>
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<tr>
<td>Glycerol</td>
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<tr>
<td>β-mercaptoethanol</td>
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<tr>
<td>Bromophenol blue</td>
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</table>

5 x Tris-Glycine Electrophoresis Buffer

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</thead>
<tbody>
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<td>Glycine</td>
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<tr>
<td>SDS</td>
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Coomassie Blue R-250 Staining Solution

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<th>Concentration</th>
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</thead>
<tbody>
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<tr>
<td>Glacial acetic acid</td>
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</tr>
<tr>
<td>Coomassie Blue R-250</td>
<td>0.25% (w/v)</td>
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## COOMASSIE GEL DESTAIN SOLUTION

<table>
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<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
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<td>10% (v/v)</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>10% (v/v)</td>
</tr>
</tbody>
</table>

## TRANSFER BUFFER

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-base</td>
<td>0.3 M</td>
</tr>
<tr>
<td>CAPS</td>
<td>10 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>0.02% (w/v)</td>
</tr>
<tr>
<td>Methanol</td>
<td>10% (v/v)</td>
</tr>
</tbody>
</table>

## GEL LOADING BUFFER

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
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<tr>
<td>Bromophenol blue</td>
<td>0.025% (w/v)</td>
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## TE BUFFER

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<th>Ingredient</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl pH 8.0</td>
<td>10 mM</td>
</tr>
<tr>
<td>EDTA pH 8.0</td>
<td>1 mM</td>
</tr>
</tbody>
</table>

## LS BUFFER

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-HEPES pH 7.8</td>
<td>20 mM</td>
</tr>
<tr>
<td>KAc</td>
<td>5 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>0.5 mM</td>
</tr>
</tbody>
</table>

## SUNASP

<table>
<thead>
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<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
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</tr>
<tr>
<td>NaCl</td>
<td>75 mM</td>
</tr>
<tr>
<td>Spermine trihydrochloride</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>Spermidine tetrahydrochloride</td>
<td>0.15 mM</td>
</tr>
</tbody>
</table>

## 20 x NPS

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<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH₄)₂SO₄</td>
<td>0.5 M</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1 M</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>1 M</td>
</tr>
</tbody>
</table>
50 x 5052

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>25% (w/v)</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.5% (w/v)</td>
</tr>
<tr>
<td>( \alpha )-lactose</td>
<td>10% (w/v)</td>
</tr>
</tbody>
</table>

**Fractionation/Chromatin-Binding Buffer A**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES, pH 7.9</td>
<td>10 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>10 mM</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.34 M</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10% (v/v)</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>0.1% (v/v)</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>1 mM</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>5 ( \mu )g/ml</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>5 ( \mu )g/ml</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>5 ( \mu )g/ml</td>
</tr>
<tr>
<td>PMSF</td>
<td>0.1 mM</td>
</tr>
</tbody>
</table>

**Fractionation/Chromatin-Binding Buffer B**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>3 mM</td>
</tr>
<tr>
<td>EGTA</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>1 mM</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>5 ( \mu )g/ml</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>5 ( \mu )g/ml</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>5 ( \mu )g/ml</td>
</tr>
<tr>
<td>PMSF</td>
<td>0.1 mM</td>
</tr>
</tbody>
</table>

**Elongation Buffer A**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>60 mM</td>
</tr>
<tr>
<td>Tris-HCl pH 7.4</td>
<td>15 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>15 mM</td>
</tr>
<tr>
<td>( \beta )-mercaptoethanol</td>
<td>1 mM</td>
</tr>
<tr>
<td>Spermidine tetrahydrochloride</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>Spermine trihydrochloride</td>
<td>0.5 mM</td>
</tr>
</tbody>
</table>
Evaluation of the DNA replication licensing machinery as an anti-proliferative target. 

Sarah Kingsbury

\textbf{\textit{In Vitro DNA Replication Premix Buffer}}

- K-HEPES pH 7.8: 160 mM
- MgCl$_2$: 28 mM
- ATP: 12 mM
- GTP: 0.4 mM
- CTP: 0.4 mM
- UTP: 0.4 mM
- dATP: 0.4 mM
- dGTP: 0.4 mM
- dCTP: 0.4 mM
- Biotin-16-dUTP: 1 µM
- Dithiothreitol: 2 mM
- Creatine phosphate: 160 mM
- Phosphocreatine kinase: 20 µg

\textbf{Total Cell Extract Buffer}

- Tris-Cl pH 7.5: 50 mM
- NaCl: 150 mM
- EDTA: 20 mM
- NP40: 0.5% (v/v)

\subsection*{2.1.3 Culture media}

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

\textbf{LB Broth [Agar]}

- Tryptone: 10 g
- Yeast extract: 5 g
- NaCl: 10 g
- [Bacteriological (No1) agar]: 15 g
- pH 7.0
**2x TY BROTH [AGAR]**

- Tryptone: 16 g
- Yeast extract: 10 g
- NaCl: 5 g
- [Bacteriological (No1) agar]: 15 g
- pH 7.0

**SOC MEDIUM**

- Tryptone: 2% (w/v)
- Yeast extract: 0.5% (w/v)
- NaCl: 10 mM
- KCl: 2.5 mM
- MgCl₂·6H₂O: 10 mM
- MgSO₄: 10 mM
- Glucose: 20 mM
- pH 7.4

**P-0.5G**

- MgSO₄: 1 mM
- Glucose: 40% (w/v)
- 20 x NPS: 5 ml

**ZY**

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

**ZYP-5052**

- ZY: 928 ml
- MgSO₄: 1 mM
- 50 x 5052: 10 ml
- 20 x NPS: 25 ml
2.1.4. Tissue specimens

Formalin-fixed, paraffin-embedded archival human colon and thyroid tissue blocks from diagnostic biopsy or resection specimens were retrieved from the archives of the Department of Pathology, University College London, U.K. Ethical approval was obtained from the Joint UCL/UCLH Committees on the Ethics of Human Research.

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>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Antibiotic resistance</th>
<th>Partial genotype</th>
<th>Application</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top10</td>
<td>F-</td>
<td>F-rectA1 endA1</td>
<td>DNA cloning</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>Tetracycline</td>
<td>FproAB lacI*ZAM15 Tn10 (Tet^k^)</td>
<td>Site-directed mutagenesis</td>
<td>Stratagene</td>
</tr>
<tr>
<td>BL21 (DE3) pLysS</td>
<td>Chloramphenicol</td>
<td>F-ompTdcC (DE3) pLysS (Cam^k^)</td>
<td>Expression of His6-El, E1, E4, His6-E1, E4R45A, His6-E1, E4A4-48</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Rosetta (DE3) pLysS</td>
<td>Chloramphenicol</td>
<td>F-ompTdcC lacY1 (DE3) pLysS pRARE (Cam^k^)</td>
<td>Expression of His6-hsgeminin, His6-hsgemininΔN, His6-hsgemininFF</td>
<td>Novagen</td>
</tr>
</tbody>
</table>

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 30 s 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 10 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 3 min, then 30 cycles of template denaturation at 95°C for 40 s, primer annealing at 58°C for 40 s and DNA extension at 72°C for 25 s. Cycling was completed by a final extension at 72°C for 3 min. Blunt-end PCR products were cloned into a pCR-BLUNT II-TOPO vector (Invitrogen, Carlsbad, CA, USA), amplified, and subcloned into a pRSETc expression vector (Invitrogen). Primer sequences for amplification of His6-E1^E4R45A and His6-E1^E4A44-48 are 5'-gctagcatggcagataataaagctccc-3' (forward nhel primer) and 3'-caattgttacacagaccagataaaagctccc-3' (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 dH20 to a final volume of 50 μl. Control reactions were performed using 10 ng pWhitescript 4.5 kb control template and control oligonucleotide primers. 1 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. Mutagenesis products were transformed into XL1-Blue competent cells. Primer sequences for generation of His_6^-hsgemininFF are 5’-gattaaagaaaatccatcctctcagttttttaaggaagtgcagaaaaacggag-3’ (forward primer; mutated nucleotides) and 3’-ctaatttcttttagtaggagtcaaaaaattctctaccgcttctttgcctc-5’ (reverse primer).

**Table 2.2. Cycling parameters for QuikChange Site-Directed Mutagenesis**

<table>
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<tr>
<th>Segment</th>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>1</td>
<td>95°C</td>
<td>1 min</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>95°C</td>
<td>50 s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60°C</td>
<td>50 s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68°C</td>
<td>7 min</td>
</tr>
</tbody>
</table>

**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 50°C 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.
Evaluation of the DNA replication licensing machinery as an anti-proliferative target. Sarah Kingsbury

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. Protein chemistry methods

2.3.1. Antibodies

Affinity-purified rabbit polyclonal antibodies against hsgeminin (Wharton et al., 2004) and hsCdt1 (Figure 2.1) were generated in-house. Mouse monoclonal antibody 4.37 against HPV1 E4 proteins (Doorbar et al., 1988) was a kind gift from S. Roberts (CR-UK Institute for Cancer Studies, University of Birmingham, UK). The following commercially supplied antibodies were used: mouse anti-hsOrc4 (BD Transduction Laboratories, Lexington, KY, USA), mouse anti-hsMcm2/BM28 (BD Transduction Laboratories), mouse anti-hsMcm3 (MBL, Nagoya, Japan), mouse anti-hsMcm5 (Stoeber et al., 2002), mouse anti-hsCdc47/Mcm7 (Neomarkers, Lab Vision, Fremont, CA, USA), mouse anti-hsCdc6 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-hsKi67 (Dako, Glostrup, Denmark), mouse anti-hsPCNA (BD Transduction Laboratories), mouse anti-mmKip1/p27 (BD Transduction Laboratories), mouse anti-mmMek2 (BD Transduction Laboratories), rabbit anti-hsHistone H1 (Santa Cruz Biotechnology), rabbit anti-hsCrm1 (Santa Cruz Biotechnology), mouse anti-His6 (BD Transduction Laboratories), HRP-conjugated goat anti-mouse/rabbit IgG (Dako), fluorescein-conjugated goat anti-mouse IgG (Dako), fluorescein-conjugated donkey anti-rabbit IgG (Abcam, Cambridge, MA, USA), fluorescein-conjugated mouse anti-BrdU (Alexis Biochemicals, Lausen,
Figure 2.1. Characterisation of a polyclonal Cdt1 antibody. (a) Polyclonal antibody Cdt1_2660 was raised following inoculation of a rabbit with a fragment of Cdt1 corresponding to amino acids 237-564. (b) Immunoblot analysis of NIH/3T3 asynchronous total cell extracts (TCE) and recombinant hsCdt1 with ab Cdt1_2660. Pre-incubation of the Cdt1_2660 antibody with recombinant Cdt1 blocks detection of Cdt1 in NIH/3T3 and reduces detection of recombinant Cdt1 (right panel).
Evaluation of the DNA replication licensing machinery as an anti-proliferative target. Sarah Kingsbury

Switzerland), biotin-conjugated anti-mouse/rabbit ChemMate™ detection kit (Dako).

2.3.2. Protein expression constructs

The following constructs were used for protein expression (Table 2.3):

Table 2.3. Protein expression constructs

<table>
<thead>
<tr>
<th>Protein Construct</th>
<th>Insert Size</th>
<th>Cloning fragment</th>
<th>Expression system</th>
</tr>
</thead>
<tbody>
<tr>
<td>His6-/tgeminin pET14b:geminin</td>
<td>1-212</td>
<td>Nhel-BamHI</td>
<td>E. coli</td>
</tr>
<tr>
<td>His6-/wgemininANtpET 15bTEF: gemininANt280-212</td>
<td>80-212</td>
<td>Nhel-BamHI</td>
<td>E. coli</td>
</tr>
<tr>
<td>His6-/wgemininFF pET 14b:gemininFF3</td>
<td>1-212</td>
<td>Nhel-BamHI</td>
<td>E. coli</td>
</tr>
<tr>
<td>His6-E1^E4 pPRSETc:E1^E441-125</td>
<td>BamHI - EcoRI</td>
<td>E. coli</td>
<td></td>
</tr>
<tr>
<td>His6-E1^E4 R45A pRSETc:E1^E4R45A3</td>
<td>1-125</td>
<td>BamHI - EcoRI</td>
<td>E. coli</td>
</tr>
<tr>
<td>His6-E1^E4 A44 pRSETc:E1^E4A44-485</td>
<td>1-121</td>
<td>BamHI - EcoRI</td>
<td>E. coli</td>
</tr>
<tr>
<td>His6-XeCdc6 pVL1393L-His6</td>
<td>1-554</td>
<td>-</td>
<td>Baculoviral</td>
</tr>
</tbody>
</table>

2.3.3. Expression of recombinant hsgeminin, hsgemininΔNt and hsgemininFF in E. coli

Rosetta (DE3) pLysS bacteria were transformed with the appropriate expression construct and grown overnight at 37°C with vigorous shaking in 20 ml of 2 x TY medium containing 100 μg/ml ampicillin, 34 μg/ml chloramphenical 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 chloramphenical and 0.25% glucose. Cultures were grown at 30°C with vigorous shaking until the OD<sub>600</sub> reached 0.5. Protein expression was induced with 1 mM IPTG at 37°C for 90 min.

1 A kind gift from A. Dutta, University of Virginia, VA, USA
2 A kind gift from A. Okorokov, Department of Pathology, UCL, UK
3 Generated by site-directed mutagenesis
4 A kind gift from R. Savva, School of Crystallography, Birkbeck College, London, UK
5 Subcloned from baculovirus expression vectors (kind gifts from S. Roberts, CR-UK Institute for Cancer Studies, University of Birmingham, UK)
6 A kind gift from T. Coleman and W. Dunphy (Coleman et al., 1996)
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 -80°C.

2.3.4. Expression of recombinant HPV1 E1^E4, E1^E4R45A and E1^E4Δ44-48 in E. coli

BL21 (DE3) pLysS bacteria were transformed with the appropriate expression construct and grown overnight at 37°C with vigorous shaking in 20 ml of P-0.5G medium containing 100 μg/ml ampicillin and 1% glucose. The overnight culture was used to inoculate 2 x 500 ml of fresh ZYP-5052 auto-induction medium, supplemented with 100 μg/ml ampicillin. Cultures were grown at 37°C for 8-10 h with vigorous shaking. 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 -80°C.

Recombinant baculovirus-expressed and purified E1^E4 and ΔE4 proteins (see Chapter 5) were kind gifts from S. Roberts, CR-UK Institute for Cancer Studies, University of Birmingham, UK.

2.3.5. Expression of recombinant XeCdc6 in Sf9 insect cells

Sf9 insect cells were grown in 400 ml of serum-supplemented TMN-FH medium (BD Biosciences, San Jose, CA, USA) containing 5 μg/ml gentamycin (Gibco-BRL, Invitrogen) in spinner flasks (spin rate 80 to 90 r.p.m.) at 27°C up to a density of 1.5 x 10^6 cells/ml. Cells were harvested by centrifugation at 800 g for 5 min, resuspended in 50 ml of serum-free Grace's medium containing 5 μg/ml gentamycin and appropriate recombinant baculovirus at 5 M.O.I., and incubated for 2 h at 27°C
with gentle mixing. The culture was diluted to 400 ml with fresh TMN-FH medium and incubated for 48 h at 27°C. Insect cells were harvested by centrifugation at 5,000 g for 5 min at 4°C, washed in PBS and stored at -80°C.

2.3.6. Purification of His₆-hsgeminin, His₆-hsgemininANt and His₆-hsgemininFF

Bacterial cell pellets were thawed, 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 4°C. The lysate was clarified by centrifugation at 48,000 g at 4°C for 1 h and the supernatant filtered through a 0.45 μm syringe filter and loaded via a peristaltic pump onto 2 x 5 ml NiCl₂ charged HiTRAP chelating columns (Amersham Biosciences AB, Uppsala, Sweden). The columns were washed with 5 c.v. of wash buffer (25 mM Tris-Cl pH 7.5, 250 mM NaCl) to remove unbound protein. Non-specifically bound proteins were removed with 4 c.v. of 10% elution buffer (25 mM Tris-Cl pH 7.5, 250 mM NaCl, 250 mM imidazole) and 8 c.v. of 20% elution buffer. Geminin was eluted with 2 c.v of 100% elution buffer. Protein containing fractions were put through a Superose 6 gel filtration column and final protein-containing fractions pooled and desalted into 25 mM Tris-Cl pH 7.5, 250 mM NaCl, 10% glycerol (v/v) and stored at -80°C (Figures 2.2, 2.3, 2.4).

2.3.7. Purification of His₆-E1^E4, His₆-E1^E4R45A and His₆-E1^E4Δ44-48

Bacterial cell pellets were thawed, resuspended in 5 ml lysis buffer (30 mM Tris-Cl pH 8, 30 mM imidazole, 30 mM NaCl, 10% glycerol, 1 mM PMSF) per gram wet
Figure 2.2. Purification of His<sub>6</sub>-hsgeminin. (a) Elution profile and Coomassie Blue stained SDS-PAGE gel obtained from Ni-affinity purification of His<sub>6</sub>-hsgeminin. (b) Superose 6 gel filtration chromatography. (c) Immunoblot of purified recombinant His<sub>6</sub>-hsgeminin with rabbit polyclonal anti-geminin antibody. FT - flowthrough, Buffer B (B) contained 250 mM imidazole.
Figure 2.3. Purification of His$_{6}$-hsgeminin$\Delta$Nt. (a) Elution profile and Coomassie Blue stained SDS-PAGE gel obtained from Ni-affinity purification of His$_{6}$-hsgeminin$\Delta$Nt. (BI - before induction, AI - after induction, Buffer B [B] contained 250 mM imidazole). (b) Superose 6 gel filtration chromatography. (c) Immunoblot of purified recombinant His$_{6}$-hsgeminin$\Delta$Nt with rabbit polyclonal anti-geminin antibody.
Figure 2.4. Purification of His6-hsgemininFF. (a) Elution profile and Coomassie Blue stained SDS-PAGE gel obtained from Ni-affinity purification of His6-hsgemininFF. (S - soluble crude extract, I - insoluble crude extract, Buffer B [B] contained 250 mM imidazole). (b) Superose 6 gel filtration chromatography. (c) Immunoblot of purified recombinant His6-hsgemininFF with rabbit polyclonal anti-geminin antibody.
weight, containing benzonase (1 µl/ml) and 0.02 volumes of complete EDTA-free Protease Inhibitor Cocktail. The cells were broken by sonication and conditions adjusted according to lysate volume. Typically for a 25 ml suspension, sonication was carried out on ice for 6 x 30 s 6-Watt pulses with 30 s rest between each pulse. The lysate was clarified by centrifugation at 48,000 g at 4°C for 1 h and the supernatant filtered through a 0.45 µm syringe filter and loaded via a peristaltic pump onto 2 x 5 ml NiCl₂ charged HiTRAP chelating columns. The columns were washed with 5 c.v. of wash buffer (30 mM Tris-Cl pH 8, 30 mM imidazole, 300 mM NaCl, 0.1 mM PMSF) to remove unbound protein. Non-specifically bound proteins were removed with 4 c.v. of 10% elution buffer (30 mM Tris-Cl pH 8, 300 mM imidazole, 30 mM NaCl, 0.1 mM PMSF) and 8 c.v. of 20% elution buffer. E1^E4 was eluted with 2 c.v of 100% elution buffer and desalted into 20 mM Tris-Cl pH 8, 50 mM NaCl (Figure 2.5).

2.3.8. Purification of baculovirus-expressed His₆-XeCdc6

Insect cell pellets were resuspended in 10 ml of hypotonic buffer (20 mM HEPES pH 7.4, 10 mM NaCl, 0.5 mM EGTA, 5% glycerol, 5 µg/ml each of leupeptin, aprotinin, pepstatin A, and chymostatin) per ml. Cells were allowed to swell for 10 min and lysed by passing through a Dounce homogeniser (Wheaton, Millville, NJ, USA) using a tight-fitting pestle.Immediately after lysis, NaCl concentration was restored to 150 mM. The lysate was centrifuged at 5,000 g for 5 min at 4°C. The supernatant was clarified at 100,000 g for 1 h. The supernatant (cleared lysate) was transferred to a fresh tube and bound to 1 ml of Ni²⁺-NTA agarose (Qiagen), pre-equilibrated with binding buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 0.5 mM EGTA, 5% glycerol, 5 µg/ml of each leupeptin, aprotinin, pepstatin A, and chymostatin), for 90 min at
Figure 2.5. Purification of bacterially expressed wild-type HPV1 His6-E4 and mutant His6-E4 proteins. Coomassie Blue stained SDS-PAGE gels showing fractions eluted following Ni-affinity purification and immunoblots of purified protein with a monoclonal E4 antibody (4.37). (a and b) wild-type E4 (c and d) E4 R45A and (e and f) E4 Δ44-48. FT - flowthrough, Buffer B (B) contained 300 mM imidazole.
4°C with gentle shaking (200 r.p.m. on a rotary shaker). One wash and three elution steps were performed at 4°C with 20 mM HEPES pH 7.4, 150 mM NaCl, 0.5 mM EGTA, 5% glycerol, 5 µg/ml of each: leupeptin, aprotinin, pepstatin A, and chymostatin containing 10 mM, 20 mM, 50 mM, and 100 mM imidazole respectively (Figure 2.6).

2.3.9. Coupling of proteins to SMoC

Coupling of proteins to SMoC was carried out as described by Pierce protocol 21341 for EZ-Link™ Biotin-HPDP (Pierce, Rockford, IL, USA). Briefly, protein was dialysed into 0.1 M sodium phosphate buffer, 5 mM EDTA pH 6.0 and concentrated to 1 mg/ml using a Vivaspin concentrator (Sartorius AG, Goettingen, Germany). To reduce free cysteine residues, 2 mg protein was treated with 14 mg 2-mercaptoethylamine.HCl at 37°C for 90 min. Following reduction, excess 2-mercaptoethylamine.HCl was removed by buffer exchange through a PD-10 desalting column (Amersham Biosciences). Protein was eluted into PBS-EDTA (20 mM sodium phosphate pH 7.4, 150 mM NaCl, 1 mM EDTA) and concentrated to 1 mg/ml. Reduced protein was incubated with 0.1 mg SMoC for 90 min at RT and coupled protein was isolated by buffer exchange as before.

2.3.10. Assaying for efficiency of SMoC-protein coupling

Efficiency of coupling of SMoC to proteins was assayed by quantitation of sulphydryl groups using Ellman’s Reagent (Pierce) according to Pierce protocol 22582. Briefly, 250 µl of sample before and after coupling was mixed with 50 µl of Ellman’s Reagent Solution and 250 µl of reaction buffer (0.1 M sodium phosphate pH 8.0, 1 mM EDTA) and incubated at room temperature for 15 min. Absorptivity of
Figure 2.6. Purification of baculovirus-expressed His6-XeCdc6. (a) Coomassie Blue stained SDS-PAGE gels showing fractions eluted following Ni-affinity purification of His6-XeCdc6. Wash buffer contained 10 mM imidazole. (b) Immunoblot of purified recombinant His6-XeCdc6 with a monoclonal Cdc6 antibody.
each sample was measured at 412 nm.

2.3.11. Peptide synthesis

Peptides were synthesized on a Gilson Automated Multiple Synthesiser AMS 422 according to standard operating procedures. The AMS 422 employs standard Fmoc-chemistry with in situ activation of the amino acids by the hydroxybenzotriazole derivative PyBOP. Double coupling with N,N-Dimethylformamide (DMF) was employed to ensure complete elongation of the peptides at each step. Following synthesis, the peptides were cleaved from the resin by treatment with trifluoracetic acid (TFA) and analysed by reverse phase HPLC and mass spectroscopy. The peptides were purified using a C18 column and freeze dried. For use in in vitro DNA replication reactions, peptides were resuspended in water at a final concentration of 20 µM.

2.3.12. 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™ 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.

2.3.13. 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.

2.3.14. Concentrating protein solutions

Protein solutions were concentrated in ultrafiltration centrifugation cartridges with an appropriate molecular weight cut-off. Cartridges were centrifuged at 3,500 g at 4°C until the desired concentration was reached by the reduction in volume of the protein.

2.4. Immunological techniques

2.4.1. 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 4°C. Membranes were incubated in primary antibody in PBS, 5% skimmed milk powder, 0.1% Tween-20 for 2 h at RT, 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 RT followed by six 5 min washes prior to visualisation. Immunoreactive bands were visualised on Hyperfilm ECL (Amersham Biosciences) by enhanced chemiluminescence (ECL, Amersham Biosciences).

2.4.2. Immunofluorescence

For immunofluorescence analysis, cells were grown on sterile coverslips. Coverslips were briefly rinsed in PBS and cells fixed in 4% paraformaldehyde for 5 min at RT. Following fixation, cells were permeabilised with PBS, 0.1% Triton X-100, 0.02%
SDS for 5 min at RT. Coverslips were blocked for 10 min in PBS, 2% BSA and incubated with an appropriate dilution of primary antibody for 1 h at 37°C. Coverslips were then washed twice in PBS, 0.1% Triton X-100, 0.02% SDS and twice in PBS, 2% BSA and incubated with the appropriate fluorescein isothiocyanate (FITC)-conjugated secondary antibody, propidium iodide (PI; 50 ng/ml) and RNase A (50 ng/ml) for 1 h at 37°C. Coverslips were washed and slides were mounted in Vectorshield (Vector Laboratories, CA, USA). For detection of chromatin and/or nuclear matrix associated proteins, cells were treated with 0.1% Triton X-100 for 30 s prior to fixation with 4% paraformaldehyde. Confocal fluorescence microscopy was performed on a Leica TCS DMRE confocal microscope (Leica Microsystems AG, Wetzler, Germany). Merged pictures of the different channels were obtained using Adobe Photoshop Version 7.0 with standardised brightness and contrast enhanced operations. ImageJ (http://rsb.info.nih.gov/ij) was used for quantification of fluorescence intensity.

2.4.3. 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.

2.5. Cell biology techniques

2.5.1. Cell culture and synchronisation

NIH/3T3 (Stoeber et al., 1998), WI-38 (CCL-75, ATCC®, LGC Promochem, Middlesex, UK), Hela S3 (CCL-2.2, ATCC®) and U20S (HTB-96, ATCC®) tissue culture 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. Cell cycle synchronisation was performed essentially as described (Krude et al., 1997). Briefly, for preparation of cells in G1/S, 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). 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). For preparation of cells in mid G1 phase, these mitotic cells were released into fresh culture medium for 6 h prior to collection. To prepare nuclei from NIH/3T3 cells, 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. Progress through G1 into S phase
was monitored by BrdU incorporation and FACS (flow assisted cell sorting) analysis (Stoeber et al, 1998). To prepare S phase HeLa S3 cytosolic extracts, HeLa S3 cells were synchronised in S phase by a single block in culture medium containing 2.5 mM thymidine for 25 h (Rao and Johnson, 1970), followed by release into fresh culture medium for 2 h. Sf9 (ECACC, Wiltshire, UK) cells were cultured in TMN-FH media (BD Biosciences) supplemented with 5 μg/ml gentamycin for normal growth and protein expression and in Grace’s medium (Gibco-BRL) supplemented with 5 μg/ml gentamycin for baculovirus infection.

2.5.2. **Bivariate flow cytometric analysis**

For cell cycle analysis of DNA content and bromodeoxyuridine (BrdU) incorporation, NIH/3T3 fibroblasts 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 dPBS and DNA was denatured by incubation in 4 N HCl for 20 min. Subsequently cells were washed twice in dPBS, incubated for 1 h with a FITC-conjugated BrdU antibody (1:50 dilution; Alexis Biochemicals) (Dolbeare, 1983; Lacombe, 1988) in the dark at 4°C and for 20 min with 0.5 ml PI (20 μg/ml) and RNase A (50 μg/ml) (Helmstetter et al., 2003) and 0.5 ml dPBS at RT. 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). 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).
2.5.3. **Assaying for cell proliferation**

Cell proliferation was monitored by either continuous or pulse labelling with 50 μM BrdU and antibody staining. Cells on coverslips were washed in PBS, fixed for 5 min in 4% paraformaldehyde, and washed two times with PBS. Following fixation, cells were permeabilised for 5 min in 0.2% Triton X-100 and washed in PBS. DNA was denatured by incubation in 2 M HCl for 1 h followed by three washes with PBS. Coverslips were incubated for 1 h with FITC-conjugated mouse anti-BrdU MAb (1:20 dilution) and with the DNA stain PI/RNase A (both at 50 ng/ml), washed again and mounted in Vectorshield. Confocal fluorescence microscopy of random fields of nuclei was performed on a Leica TCS DMRE confocal microscope. Images were collected, and merged pictures of the PI channel (red) and FITC channel (green) were obtained using Adobe Photoshop Version 7.0 with standardised brightness and contrast enhanced operations for all samples. Images were printed and the number of cells incorporating BrdU (yellow) and non-replicating cells (red) were counted. Proliferating (BrdU-incorporating) cells were quantitated as percentages of the total number of cells.

2.5.4. **Assaying for apoptosis**

TdT (terminal deoxynucleotidyl transferase)-mediated dUTP-x nick end labelling (TUNEL) assay to detect apoptotic cells was performed using the ApopTag Fluorescein Direct *in Situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA, USA) according to the manufacturer's protocol. Briefly, cells were fixed in 1% paraformaldehyde for 10 min at RT, washed twice in PBS, post-fixed in ethanol:acetic acid 2:1 for 5 min at -20°C, and washed a further two times in PBS. Fixed, permeabilised cells were incubated with Equilibration buffer (Chemicon
International) for 10 s at RT, incubated with TdT enzyme for 1 h at 37°C and finally the reaction was stopped by incubation with Stop/wash buffer (Chemicon International) for 10 min. Coverslips were mounted in Vectorshield containing DAPI (4'6'-diamidino-2-phenylindole), and visualised by confocal fluorescence microscopy.

2.5.5. Preparation of total cell extracts

To prepare whole cell extracts, cells were harvested by treatment with trypsin, washed in PBS and resuspended in lysis buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 20 mM EDTA, 0.5% NP40) at 5 x 10⁶ cells/ml. After incubation on ice for 30 min the lysate was clarified by high speed centrifugation (13,000 g, 15 min, 4°C) and stored at -80°C.

2.5.6. Biochemical subcellular fractionation

Cells were pelleted and resuspended in fractionation buffer A at 4 x 10⁷ cells/ml, and incubated on ice for 10 min. Nuclei were pelleted by low speed centrifugation (1,300 g, 5 min, 4°C) and the supernatant (cytosolic fraction) further clarified by high speed centrifugation (14,000 g, 15 min, 4°C) to remove cell debris and insoluble aggregates. Nuclei were washed twice in fractionation buffer A, resuspended in fractionation buffer B, and incubated for 45 min at 4°C. Insoluble chromatin-bound proteins were obtained by centrifugation (1,500 g, 5 min, 4°C), and the supernatant (nucleosolic fraction) further clarified by high speed centrifugation (14,000 g, 15 min, 4°C). Chromatin was washed twice in fractionation buffer B, resuspended in fractionation buffer A plus 1000 U/ml DNase I (Invitrogen) and incubated for 30 min at RT, and a further 30 min at 4°C with 1 v/v 0.5 M NaCl. Solubilised chromatin-
bound proteins were obtained by high speed centrifugation (14,000 g, 5 min, 4°C).

2.6. **In vitro DNA replication reactions**

2.6.1. **Preparation of nuclei and cytosolic extracts**

Cytosolic extracts and nuclear preparations were produced by hypotonically swelling, scrape harvesting, and Douncing essentially as described (Heintz and Stillman, 1988; Krude *et al.*, 1997; Stoeber *et al.*, 1998). Briefly, cells were washed once with ice-cold LS buffer and all subsequent steps carried out at 4°C. Cells were allowed to swell for 10 min in 20 ml LS buffer per plate, and excess buffer was removed. Interphase cells attaching to the substratum were scraped off the plates and disrupted with 25 strokes in a Dounce homogeniser (Wheaton Science Products, Millville, NJ, USA) using a loose-fitting pestle. Nuclei were centrifuged at 1,500 g for 3 min, washed once in 1 ml of ice-cold SuNaSp/3% BSA, resuspended in an equal volume of SuNaSp/3% BSA and stored at -80°C. The cytosolic supernatant from the first separating spin was clarified at 18,000 g for 15 min. Supernatant fractions were aliquoted, snap-frozen, and stored in liquid N₂.

2.6.2. **Assaying for DNA synthesis in vitro**

*In vitro* DNA synthesis reactions were performed essentially as described (Krude *et al.*, 1997; Stoeber *et al.*, 1998). Briefly, reactions contained 30 μl of cytosolic extracts (250-300 μg of protein), 10 μl of premix buffer, 1 x 10⁵ nuclei and, where indicated, up to 10 μl of recombinant protein(s). When smaller volumes were used, the remaining volume was adjusted to the final 10 μl by addition of nuclear extraction buffer or of the relevant buffer for recombinant protein(s). Nuclei were incubated with recombinant protein, or control buffer, for 15 min prior to addition of
Evaluation of the DNA replication licensing machinery as an anti-proliferative target. Sarah Kingsbury

premix buffer and cytosolic extract. Reactions were then incubated for 3 h at 37°C. His6-XeCdc6 protein in 20 mM HEPES pH 7.4, 150 mM NaCl, 5% glycerol, 1 mM DTT was added to in vitro replication reactions at a final concentration of 0.65 μM, baculovirus-expressed E4/ΔE4 proteins (Roberts et al., 1994b) in 10 mM phosphate buffer pH 7.4, 0.1 mM DTT at 3 μM, bacterially-expressed His6-E1^E4 proteins in 40 mM Tris-HCl pH 7.6, 30 mM NaCl at 3 μM, and His6-hsgeminin proteins in 25 mM Tris-Cl pH 7.5, 250 mM NaCl, at 4 μM where indicated.

2.6.3. Analysis of in vitro DNA synthesis products by confocal fluorescence microscopy

For analysis of in vitro DNA synthesis reactions by confocal fluorescence microscopy, reactions were stopped by diluting with 500 μl of PBS and nuclei fixed for 5 min by adding 500 μl of 8% paraformaldehyde at RT. After fixation, nuclei were spun through a 30% sucrose/PBS cushion onto poly-L-lysine coated coverslips. All subsequent washing and staining steps were carried out in PBS, 0.2% Triton X-100, 0.04% SDS. Coverslips were washed, stained for incorporated biotin-16-dUTP with fluorescein-linked streptavidin (1:100 dilution, Amersham) and for DNA with propidium iodide/RNase A (both at 50 ng/ml), washed again, and mounted in Vectorshield. Confocal fluorescence microscopy of random fields of nuclei was performed on a Leica TCS DMRE confocal microscope and merged pictures of the PI channel (red) and FITC channel (green) were obtained using Adobe Photoshop Version 7.0 with standardised brightness and contrast enhanced operations for all samples. Images were printed and the number of nuclei incorporating biotin-16-dUTP in vitro (yellow) and non-replicating nuclei (red) were counted. Routinely 800-1000 nuclei were scored for each reaction and quantitated as percentages of the
total number of nuclei that synthesised DNA in vitro.

2.6.4. Chromatin-binding assay

1 x 10^5 NIH/3T3 nuclei were incubated in 30 μl of cytosolic HeLa S phase extract (250–300 μg of protein), a buffered mix of NTPs, dNTPs, and an energy regeneration system as described in Section 2.6.2. After 3 h, nuclei were pelleted by low speed centrifugation (1,300 g, 5 min, 4°C), resuspended in fractionation buffer B and incubated for 45 min at 4°C. Insoluble chromatin was obtained by centrifugation (1,500 g, 5 min, 4°C), washed twice in buffer B, resuspended in fractionation buffer A and 1000 U/ml DNase 1 and incubated for 30 min at RT, and a further 30 min at 4°C with 1 v/v 0.5 M NaCl. Solubilised chromatin-bound proteins were obtained by high speed centrifugation (14,000 g, 5 min, 4°C).

2.7. 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).

2.7.1. Bioinformatic and database resources

DNA sequences


Protein sequences

DNA and protein sequence homology


Multiple sequence alignments

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

Restriction enzyme site identification


Oligonucleotide primer design

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

Protein molecular weight, pI, and extinction coefficient


Atomic co-ordinates of protein structures

RCSB Protein Data Bank:

http://www.rcsb.org/pdb/Welcome.do;jsessionid=fv2A2Av16oGwCepMGMt9GQ**

Protein structure visualisation

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

Densitometry analysis

CHAPTER THREE

REGULATION OF THE ORIGIN LICENSING REPRESSOR GEMININ DURING QUIESCENCE

3.1. Introduction

The response of normal tissue to chemotherapy regimes is a fundamental consideration in the development of novel therapeutic strategies. A major constraint of many classical chemotherapeutic agents is their toxicity to normal tissues; particularly the rapidly proliferating populations of the gastrointestinal and haemopoietic systems (see also Chapter 1, Section 1.3.). As a result, these agents cause debilitating side-effects in cancer patients that may escalate to dose-limiting toxicities. Lowering the dose of chemotherapeutic agents to reduce these side-effects can allow tumour cells to escape treatment and to develop drug resistance (Cleton, 1995). Therapeutic regimes which specifically destroy tumour cell populations are therefore continually sought after.

The putative existence of an origin licensing checkpoint implies that inhibiting the DNA replication licensing pathway may provide a strategy for cancer specific killing (see also Chapter 1, Section 1.5). Preliminary studies by Shreeram et al. suggest that transformed cells respond to inhibition of DNA replication licensing by committing apoptosis, whilst normal cycling cells respond by arresting in G1 (Shreeram et al., 2002). However in mammalian tissues the majority of cells reside in out-of-cycle states, having either temporally withdrawn into the quiescent state ready to re-enter the cell cycle upon the appropriate mitogenic stimulation, or permanently withdrawn into the terminally differentiated state. In order to predict how these different normal
cell populations may respond to agents targeting the DNA replication licensing pathway, the regulation of the replication licensing machinery in normal human tissues must be understood. During the mitotic cell cycle the components of the replication licensing pathway are tightly regulated, ensuring that replication can occur once and only once during each round of cell division (see also Chapter 1, Section 1.5.). However, little is known about the regulation of the replication licensing machinery, and in particular the origin licensing repressor geminin, upon withdrawal from the cell cycle into the out-of-cycle states of quiescence, differentiation and senescence.

Human tissues can be divided into three separate classes: self-renewing, conditional renewal, and permanent (Leblond, 1963). Self-renewing systems (e.g. skin, gastrointestinal mucosa and the haemopoetic system) are characterised by rapid and continuous turnover of cells and show a typical hierarchy of cellular development from stem cells to terminally-differentiated mature cells via transient amplifying cells. Separate from self-renewing systems are tissues with little or no replacement of lost cells; permanent tissues (e.g. nerve or skeletal muscle) are composed of static cell populations, whereas conditional renewal tissues (e.g. liver or thyroid), although normally showing little cell replacement, are capable of extensive proliferation in response to a stimulus (Leblond, 1963; Hall and Watt, 1989). In order to determine the likely response of normal tissues to agents targeting the replication licensing machinery the regulation of replication licensing factors (RLFs) was evaluated in a typical conditional renewal tissue, thyroid, and a typical self-renewing system, the colonic crypt. To provide support for these immunohistochemical analyses, an in vitro quiescence model system was established to allow the generation of a detailed
molecular map of the replication licensing machinery during exit from and re-entry into the mitotic cell cycle.

Of particular importance in these studies is an analysis of the regulation of the origin licensing repressor geminin. The ability of geminin to prevent pre-RC assembly and block the initiation of DNA replication suggests that compounds mimicking geminin activity could prove successful as anti-proliferative agents. In order to evaluate this hypothesis it is therefore essential to understand the regulation of geminin during tissue maintenance in the adult; specifically in the three classes of human tissues described above. The role of geminin as a negative regulator of DNA replication has led to speculation that geminin may act as a classic tumour suppressor protein and may therefore be mutated or suppressed in human cancers (Lygerou and Nurse, 2000). However, analyses of geminin expression in human cancers suggest that geminin expression is positively correlated with cell proliferation (Wohlschlegal et al., 2002; Wharton et al., 2004). These studies may therefore also provide further evidence as to whether geminin acts as a classic tumour suppressor protein or a marker of cell proliferation.

3.2. Materials and Methods

For Antibodies (2.3.1.), Immunoblotting (2.4.1.), Immunohistochemistry (2.4.3.), Cell culture and synchronisation (2.5.1.), Bivariate flow cytometric analysis (2.5.2.), Assaying for cell proliferation (2.5.3.), and Biochemical subcellular fractionation (2.5.6.) refer to Chapter Two (Materials and Methods).
3.2.1. **Protein expression profile analysis in colon and thyroid**

**Colonic crypts**

In this study protein expression analysis for Ki67, Mcm2 and geminin was performed on distal colonic tissue samples only. Haematoxylin and eosin (H&E) stained sections were carefully assessed to exclude the presence of paneth cells. Slides were examined and images captured with an Olympus BX51 microscope/CCD camera setup using ANAlysis software (SIS, Münster, Germany). Captured images were printed and cells expressing the protein of interest counted. Epithelial cells in the basal proliferative compartments (BC, lower third of colonic crypts) and differentiated compartments (DC, upper two thirds of colonic crypts) were evaluated separately. The basal proliferative compartments were further segregated into stem cell (SCC) and transit amplifying (TAC) compartments (Potten, 1986).

**Thyroid**

For Ki67, Mcm2 and geminin protein expression profiling, six cases of follicular adenoma and five cases of Graves Disease were selected. On histological examination cases of follicular adenoma also contained surrounding normal thyroid tissue. 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.
3.3. Results

3.3.1. Reversible growth arrest of NIH/3T3 fibroblasts in quiescence

In order to define the replication licensing phenotype of quiescent cell populations, the expression of the replication licensing machinery was first analysed in an *in vitro* quiescence model system. The aim of these detailed *in vitro* studies was to generate a molecular map describing the regulation of replication licensing factors during exit from and re-entry into the mitotic cell cycle, which can be extrapolated for the analysis of conditional renewal and self-renewing tissues.

NIH/3T3 fibroblasts are widely accepted as an immortalised but non-transformed cell line of highly contact-inhibited cells (Jainchill *et al.*, 1969), which can be used as an *in vitro* model for replicative quiescence (Holley and Kieman, 1968). NIH/3T3 diploid fibroblasts cease proliferating and enter a viable state of growth arrest, quiescence (G0), following contact-inhibition at high cell densities (density-dependent growth arrest). Cell cycle re-entry can be stimulated by sub-culturing at lower density, relieving the contact-inhibition and producing a tightly synchronised cohort of cells progressing into S phase.

To monitor exit of NIH/3T3 fibroblasts from cycle into quiescence, bromodeoxyuridine (BrdU) cell proliferation assays were performed on asynchronously proliferating cultures and on contact-inhibited cells. In an asynchronously proliferating population 45% of cells incorporated BrdU during a one hour pulse (Figure 3.1). In contrast, the BrdU labelling index dropped to 2.4% one day post contact-inhibition and to 0% five days post contact-inhibition, confirming that the NIH/3T3 fibroblasts used in this study can be synchronised in
Figure 3.1. NIH/3T3 fibroblasts undergo density-dependent growth arrest and enter the reversible resting state of quiescence. Cell proliferation of NIH/3T3 fibroblasts was monitored by pulse-labelling with BrdU during the transition from asynchronous growth (AS) into quiescence, by growth of cells to confluence and subsequent density-dependent growth arrest (SC - semi-confluent, C1/5- confluent day 1/5). Cells were stained with propidium iodide (red) for total DNA and fluorescein-AbBrdU (green) to detect BrdU incorporation. 5 days post confluency incorporation of BrdU had ceased indicating that cells were no longer progressing through the cell cycle.
quiescence by density-dependent growth arrest.

Re-entry of NIH/3T3 fibroblasts into the cell cycle was monitored by continuous BrdU labelling following sub-culturing at lower density five days after contact-inhibition. The percentage of cells incorporating BrdU remains low for approximately 16 hours after the release (Figure 3.2a), consistent with cells re-entering the cell cycle with a prolonged G1 period. The percentage of cells incorporating BrdU begins to increase between 16 and 18 hours after release with cells reaching S phase between 18 and 20 hours. These data correlate with flow cytometric analysis of propidium iodide stained NIH/3T3 nuclei, which indicates that at 15 hours cells are in G1 phase with a DNA content equivalent to the DNA ploidy index 1.0. Transition into S phase occurs between 18 and 20 hours after release (Figure 3.2b). Taken together these data confirm that NIH/3T3 fibroblasts synchronously re-enter the cell cycle from quiescence with a prolonged ‘G1’ phase following removal of contact-inhibition.

3.3.2. Repression of origin licensing in G0 ensures loss of proliferative capacity independently of the origin licensing inhibitor geminin

To generate a molecular picture of origin licensing during exit into and re-entry from G0, and to determine whether geminin is required for maintenance of the quiescent state as may be expected for a putative tumour suppressor protein, a published sub-cellular fractionation protocol (Mendez and Stillman, 2000) was followed to separate the total protein content into cytosolic, nucleosolic and chromatin-bound fractions (Figure 3.3a). To validate this protocol, distribution of several marker proteins was assessed following biochemical fractionation of asynchronous NIH/3T3 fibroblasts
Figure 3.2. NIH/3T3 fibroblasts synchronously re-enter the cell cycle following release from density-dependent growth arrest. (a) Re-entry of NIH/3T3 fibroblasts into the cell cycle by subculturing of cells five days post contact-inhibition was monitored by continuous BrdU labelling. Cells were stained with propidium iodide (red) for total DNA and fluorescein-AbBrdU (green) to detect BrdU incorporation. Cells become competent to replicate 16-18 hours after release. (b) FACS analysis of propidium iodide stained NIH/3T3 nuclei for DNA content indicates that cells enter S phase approximately 20 hours after release from quiescence.
into cytosolic, nucleosolic and chromatin-bound protein fractions (Figure 3.3b). Mek2, a cytosolic serine threonine kinase involved in the MAPK signal transduction pathway (Zheng and Guan, 1994), was recovered exclusively in the cytosolic fraction, indicating that extraction of soluble cytosolic proteins after cell lysis was complete. Chromosomal Region Maintenance 1 protein (Crm1) was recovered in both the cytosolic and nucleosolic fractions but not in the chromatin-bound fraction. This is consistent with its role as a nuclear export receptor for leucine-rich nuclear export signals (Fornerod et al., 1997) and confirms that full extraction of nuclear proteins was achieved after nuclear lysis. Histone H1 was detected exclusively in the chromatin-bound fraction, excluding release of chromatin-bound proteins into the nucleosolic fraction during nuclear lysis.

To investigate the regulation of origin licensing during exit into and re-entry from G0, and the role of geminin in this process, protein fractions were prepared during asynchronous growth, at various time points following density-dependent growth arrest (C), and during release back into the mitotic cell cycle by sub-culturing five days after the cells had undergone growth arrest (C5). Proteins in all three fractions were resolved by gel electrophoresis and probed for the RLFs Orc4, Cdc6, Cdt1, Mcm2, Mcm3, Mcm5 and Mcm7, the origin licensing repressor geminin, the CDK inhibitor p27 and the cytoskeletal filamentous protein actin to control for protein levels. Exit into G0 (Figure 3.4a and b) and re-entry into cycle (Figure 3.5a and b) were monitored by bi-variate flow cytometric analysis of DNA content and BrdU incorporation.

Fifty-one percent of asynchronously proliferating NIH/3T3 fibroblasts incorporated
**Figure 3.3. Subcellular biochemical fractionation.** (a) Scheme showing the biochemical fractionation protocol used to separate cytosolic (CF), nucleosolic (NF), and chromatin-bound (CBF) protein fractions. (b) In sub-cellular fractionations from asynchronously proliferating NIH/3T3 fibroblasts, Mek2, a cytosolic kinase involved in signal transduction, was recovered exclusively in the CF, indicating that cell lysis was complete. The nuclear transport factor Crm1 was recovered in both the CF and NF, but not in the CBF, indicating that nuclear lysis was complete and excluding spill-over into the CBF. Histone H1 was found exclusively in the CBF, excluding release of chromatin-bound proteins during nuclear lysis.
BrdU after a one hour pulse, as determined by FACS analysis. After one day of density-dependent growth arrest, BrdU incorporation had dropped to 4% and by day 10 less than one percent of cells were synthesising DNA (Figure 3.4b). The CDK inhibitor p27, required for establishment and maintenance of the quiescent state (Resnitzky et al., 1995), was up-regulated and found in all three fractions upon the onset of density-dependent growth arrest (SC) (Figure 3.4c). Up-regulation of p27 coincided with rapid down-regulation of the MCM loading factor Cdc6 within 48 hours and its complete removal from chromatin by the time cells were contact-inhibited (C). Protein levels of Cdt1 also began to drop at the onset of density-dependent growth arrest, but down-regulation was significantly slower than Cdc6 and protein could still be detected in all three fractions 15 days post-confluency. Interestingly, geminin protein levels were not up-regulated during entry into quiescence as might be expected for a putative tumour suppressor protein, but were down-regulated with similar kinetics to those observed for Cdc6. Protein levels of the DNA helicase subunits Mcm2-7 began to drop with the onset of density-dependent growth arrest in all three fractions, with complete removal from chromatin occurring by day 10 of contact inhibition (C10). The Orc4 subunit of the origin landing pad was also down-regulated as cells progressed into G0, although to a much lesser extent than Mcm2-7 and was still present in significant quantities in all three fractions by day 15 of contact inhibition (C15). In contrast, Orc1 and Orc2 were not down-regulated and remained chromatin-bound during entry into G0 (Madine et al., 2000; Stoeber et al., 2001), suggesting differential regulation of ORC subunits as cells exit from cycle. Taken together, these data show that loss of proliferative capacity upon exit into quiescence is a two-step process. The immediate response of fibroblasts to density-dependent growth arrest is a block to pre-RC assembly
Figure 3.4. Regulation of replication licensing factors during withdrawal from cycle into quiescence. Exit of NIH/3T3 fibroblasts into quiescence by density-dependent growth arrest was monitored by bi-variate flow cytometric analysis of (a) DNA content and (b) BrdU incorporation. An asynchronously proliferating population (AS) showed a normal DNA content distribution and 51% incorporation of BrdU. One day post contact-inhibition the DNA profile showed a strong G1 peak and the percentage of cells incorporating BrdU dropped to 4%. (c) Immunoblot analysis of Orc4, Cdc6, Cdt1, Mcm2, Mcm3, Mcm5, Mcm7, geminin (Gem), p27 and actin in cytosolic, nucleosolic, and chromatin-bound protein fractions prepared from NIH/3T3 cells during transition from asynchronous growth into G0 (AS - asynchronous, SC - semi-confluent, C - confluent, C1-15 - days in density-dependent growth arrest).
mediated through rapid down-regulation of the master regulator Cdc6 and induction of the global CDK inhibitor p27. The late response involves complete down-regulation and removal from chromatin of the replicative Mcm2-7 helicase (Figure 3.4c) (Kingsbury et al., 2005; see Appendix D).

After five days of density-dependent growth arrest, cells were released back into the proliferative cell cycle and DNA synthesis was monitored by bi-variate flow cytometric analysis of DNA content and BrdU incorporation (Figure 3.5a and b). The percentage of cells synthesising DNA increased to 11% at 15 hours, 30% at 18 hours and 51% at 21 hours after release from G0. Chromatin-bound levels of Orc4 increased two-fold by 9 hours and three-fold by 12 hours after release from contact inhibition (relative to protein levels in resting cells) (Figure 3.5c). The MCM loading factor Cdc6 was de novo synthesised during re-entry into the mitotic cell cycle and bound to chromatin by 9 hours. Although a small proportion of Cdt1 was already bound to chromatin at 0 hours, an increase in binding was detected between 6 and 9 hours with a continual accumulation until 18 hours and removal from chromatin at 21 hours. Binding of Cdc6 and Cdt1 was followed by recruitment of Mcm2, Mcm3, Mcm5 and Mcm7 from the soluble fractions to the chromatin-bound fraction between 9 and 21 hours after the release (Figure 3.5c). Like Cdc6, geminin was de novo synthesised during re-entry, however, notably, synthesis and association with chromatin did not occur until late in the G0-S transition post pre-RC assembly (18-21 hours after the release). Interestingly, the CDK inhibitor p27 accumulated in the nucleus and was detected in the chromatin-bound fraction during the permissive window of pre-RC assembly consistent with the recent finding that p27 interacts with Mcm7 and may act to prevent premature firing of origins in late G1 (Nallamshetty et
Figure 3.5. Regulation of replication licensing factors during re-entry into the cell cycle from quiescence. Re-entry of NIH/3T3 fibroblasts into the cell cycle from quiescence by density-dependent growth arrest was monitored by bi-variate flow cytometric analysis of (a) DNA content and (b) BrdU incorporation. Between 0 and 15 hours after release from quiescence cells have a DNA content equivalent to the DNA ploidy index 1.0, with transition into S phase occurring between 18 and 20 hours after release. The percentage of cells incorporating BrdU remains low for approximately 15 hours after the release, increasing to 11% at 15 hours, 30% at 18 hours, and 51% at 21 hours after release from G0. (c) Immunoblot analysis of Orc4, Cdc6, Cdt1, Mcm2, Mcm3, Mcm5, Mcm7, geminin (Gem), p27 and actin in sub-cellular fractions from NIH/3T3 cells following release from density-dependent growth arrest.
Evaluation of the DNA replication licensing machinery as an anti-proliferative target. Sarah Kingsbury

_al._, 2005). These data demonstrate that pre-RC assembly precedes reacquisition of proliferative capacity and that the origin licensing repressor geminin, which bans re-replication in mitotically active cells, is not required to suppress proliferative capacity in G0 (Figure 3.4c) (Kingsbury _et al._, 2005; see Appendix D).

3.3.3. _Conditional renewal tissues have an unlicensed replication licensing phenotype_

To determine whether the down-regulation of RLFs during withdrawal from cycle into the quiescent state _in vitro_ (Figure 3.4c) extends to conditional renewal tissue systems, regulation of Ki67, Mcm2 and geminin protein expression was analysed using immunohistochemistry on normal adult thyroid tissue obtained from surgical resection specimens (n=6). Representative immunostained tissue sections are illustrated in Figure 3.6 and mean labelling indices (percentage of positive cells) for each protein are shown in Figure 3.7. Expression of the standard proliferation marker Ki67 and Mcm2 was detected in a small proportion of thyrocytes (0.5% [95% CI=0-1.1%] and 1.3% [CI=0-3.7%] respectively), consistent with most follicular cells being in a replication-incompetent quiescent state. Importantly, geminin protein expression is restricted to a minute proportion of thyrocytes (0.1% [CI=0-0.3%]), indicating that loss of proliferative capacity in human stable tissues is independent of this origin licensing repressor, which appears to be redundant following down-regulation of the MCM replicative helicase subunits.

Graves Disease and thyroid adenoma are hyper-proliferative disorders of the thyroid.

1Technical assistance in the staining and analysis of histological sections was provided by Marco Loddo (Department of Pathology, UCL) and Ellen Obermann (visiting scholar, supported by Dr. Mildred Scheel Stiftung für Krebsforschung/Deutsche Krebshilfe). Guidance in the statistical analysis of data was provided by Thomas Fanshawe (University of Cambridge).
Figure 3.6. Expression of replication licensing factors is induced in stable tissues following re-entry into the mitotic cell division cycle. Indirect immunoperoxidase staining of normal thyroid, Graves Disease, and follicular adenoma with anti-Mcm2, anti-geminin and anti-Ki67 antibodies. Normal thyroid shows very low expression of Ki67 and Mcm2 with almost undetectable levels of geminin. In both hyper-proliferative conditions (Graves Disease and follicular adenoma) Ki67, Mcm2 and geminin protein expression levels are increased 10-30 fold (x125).
and represent in vivo correlates of re-entry into the mitotic cycle from G0, through a physiological and oncogenic stimulus respectively. In Graves Disease, stimulation of thyroid follicular cells by TSH receptor auto-antibodies results in thyroid growth through stimulation of the TSH-dependent mitogenic pathway (Chistiakov, 2003). Alternatively, oncogenic mutations in TSHR, ras or gsp can result in epithelial thyroid follicular tumourigenesis, follicular adenoma representing an early step in thyroid tumour progression (Moretti et al., 2000). To test whether acquisition of proliferative capacity following re-entry of quiescent thyrocytes into the mitotic cell cycle is linked to induction of RLFs, as shown in the in vitro fibroblast model (Figure 3.5c), regulation of Ki67, Mcm2 and geminin protein expression was immunohistochemically analysed in thyroid follicular adenoma (n=6) and Graves Disease (n=5). Increased expression of Ki67 and Mcm2 was detected in both hyper-proliferative conditions (Graves Disease: Ki67 [6.4%, CI=2.3-10.5%], Mcm2 [14.3%, CI=3.4-25.2%]; follicular adenoma: Ki67 [8.1%, CI=2.9-13.3%], Mcm2 [38.5%, CI=21.5-55.5%]), indicating re-entry of thyrocytes into the mitotic cell cycle, whilst increased geminin protein expression was also observed. Notably, geminin protein expression was detected in a subpopulation of thyrocytes (Graves Disease: 1.7% [CI=1.2-2.2%]; follicular adenoma: 2.2% [CI=0.7-3.7%]), a fraction less than Ki67 which is present throughout all phases of the mitotic cell cycle, in keeping with restriction of geminin protein expression to S, G2 and M phase in cultured human cells (Wharton et al., 2004). Taken together these data indicate that withdrawal of cells from cycle into the quiescent state in vitro and in vivo is coupled to down-regulation of the replication licensing machinery and the origin licensing inhibitor geminin. The repression of origin licensing may therefore act as a powerful downstream mechanism which contributes to loss of proliferative capacity in
Figure 3.7. Expression of replication licensing factors in normal thyroid, Graves Disease and follicular adenoma. Histogram summarising the percentage of nuclei expressing Ki67, Mcm2 and geminin in normal thyroid, Graves Disease and follicular adenoma (mean ± SD). Geminin identifies a growth fraction less than Ki67, consistent with an S-G2-M phase labelling index.
quiescence/conditional renewal tissues independently of the origin licensing inhibitor geminin.

3.3.5. *Prolonged cell cycle times of gut stem cells are due to exit of progenitor cells from cycle into an unlicensed quiescent state*

In order to define the replication licensing phenotype in self-renewing tissues, the data obtained for quiescence/conditional renewal tissues was extrapolated to the colon, a typical self-renewing system. As discussed above, self-renewing tissues are comprised of three cell populations: a terminally differentiated compartment containing functional cells, a transit amplifying compartment and a stem/progenitor cell compartment. To determine the replication licensing phenotype of these cell populations regulation of Ki67, Mcm2 and geminin protein expression was analysed using immunohistochemistry on normal distal colonic crypts obtained from surgical resection specimens (n=10).

Application of $^3$HTdR radioautography, vincristine mitotic accumulation (VCA) and per cent labelled mitosis (PLM) techniques has helped to define the cell cycle kinetics of stem cell hierarchies in gut epithelial cell populations (Figure 3.8, diagram) (Wright and Alison, 1984; Potten and Loeffler, 1987). These studies suggest that the stem cells of the gastrointestinal tract have unusually prolonged cell cycle times, about twice as long as committed progenitor cells of the transit amplifying populations (Potten, 1986). Two cell kinetic models are consistent with these observations, either stem cells are traversing through the mitotic cycle with a markedly prolonged G1 phase or, alternatively, have reversibly withdrawn from the proliferative cycle into G0. Since down-regulation of origin licensing factors and
Figure 3.8. Gut stem cells reside in an "unlicensed" quiescent state. Ki67, Mcm2 and geminin protein expression in colonic mucosa (x200 and x350). The schematic drawing of a colonic crypt shows the basal proliferative compartment (BC) containing the putative stem cell compartment (SCC) [4th position and below], and the transient amplified compartment (TAC). The luminal compartment contains differentiating mucus-secreting goblet cells that have lost proliferative potential (DC). A large fraction of cells in the BC express Ki67, Mcm2 and geminin. A subset of differentiating cells express Mcm2, with Ki67 and geminin rapidly down-regulated during the proliferation-differentiation switch. In contrast to the majority of cells in the BC, very low levels of Ki67, Mcm2 and geminin expression are observed in the putative SCC, indicating that the majority of these cells are unlicensed.
geminin defines the quiescent state *in vitro* and *in vivo*, it may be postulated that the dynamics of origin licensing can be exploited to distinguish between these two models.

The gastrointestinal tract is a well characterised model for studying stem cell populations with the stem cells thought to reside in the base of the crypt with unidirectional flux of differentiating cells to the surface (Figure 3.8, diagram). The precise position and number of stem cells in gut epithelium is based on circumstantial evidence, but the consensus opinion is that stem cells are located at the base of distal colonic crypts. The 'unitarian hypothesis' postulates that the epithelial cell lineages of the gastrointestinal tract are clonal populations derived from a single stem cell (Ponder *et al*., 1985; Griffiths *et al*., 1988), but opposing reports have suggested from 4-6 stem cells per crypt (Hendry *et al*., 1989; Potten & Loeffler, 1990) while others suggest up to 16 stem cells per crypt (Brittan and Wright, 2004). Stem cell number has also been shown to fluctuate throughout the crypt cycle (Loeffler *et al*., 1997) and throughout different regions of the gastrointestinal tract (Cai *et al*., 1997). An additional problem when considering stem cells is the definition of the term itself, since many different interpretations have been proposed in the literature. It has been put forward by Potten and Loeffler that 'stemness' is not necessarily a single property, but a number of properties which a cell has the capability of executing depending on the circumstances (Potten & Loeffler, 1990). From this definition of stemness it can be postulated that in addition to the presence of true (or actual) stem cells in the crypt base there may also exist a number of potential stem cells, most probably the first progeny of actual stem cells that are capable of stem cell function if necessary. Furthermore the concept of
complex stem cell niches has recently been invoked, niches that are characterised by their ability to support multiple stem cells, thus allowing the generation of multiple product cells (i.e. pleoclonal or polyclonal) (Ohlstein et al., 2004).

For this study, analysis of the distal colonic crypts was divided into three compartments (Figure 3.8, diagram). The luminal (differentiated) compartment (DC) lies in the upper two thirds of the crypt and contains the functionally-differentiated mucus-secreting goblet cells. The lower third of the crypt contains the basal proliferative compartment which is separated into the transit amplifying compartment (TAC) and the putative stem cell compartment (SCC). Based on published evidence (Hendry et al., 1989; Potten and Loeffler, 1990; Brittan and Wright, 2004), the stem cell compartment in the distal colonic crypts was defined as the 4th position and below. Representative immunostained tissue sections are illustrated in Figure 3.8 and mean labelling indices (percentage of positive cells) for each protein are shown in Figure 3.9. The labelling indices for Ki67, Mcm2 and geminin are highest for cells occupying the transient amplifying compartment (Ki67 [77.8%, CI=65.6-90.1%], Mcm2 [89.6%, CI=85.1-94.2%], geminin [28.2%, CI=20.4-36.0%]) with marked reductions in all three biomarkers of growth as cells lose their proliferative capacity, migrate into the differentiated compartment and adopt the terminally-differentiated phenotype (surface functional cells) (Ki67 [11.4%, CI=8.2-14.5%], Mcm2 [36.8%, CI=28.9-44.8%], geminin [3.0%, CI=1.6-4.5%]). Taken together these data confirm previous findings that engagement of the differentiation programme is coupled to down-regulation of RLFs and that geminin does not appear to function as an inducer or regulator of differentiation in somatic self-renewing systems (Stoeber et al., 2001; Eward et al., 2004).
Figure 3.9. Expression of replication licensing factors in distal colonic crypts. (a-b) Labelling indices (expressed as the percentage of Ki67, Mcm2 and geminin immunostained cells) in relation to stem cell (SCC), transient amplified (TAC) and differentiated (DC) anatomical compartments. Note that SCC and TAC together make up the basal proliferative compartment (BC). The proliferation-differentiation switch and loss of DNA replication competence as cells migrate from the BC to the DC are coupled to down-regulation of Ki67, Mcm2 and geminin. Terminally-differentiated cells are unlicensed. The SCC compartment contains unlicensed cells characterised by low level expression of Ki67, Mcm2 and geminin. Migration of cells from the SCC to the TAC is associated with a dramatic increase in expression of Ki67, Mcm2 and geminin, indicative of cell cycle re-entry. The quiescent (G0) and terminally-differentiated states (SCC v DC) show identical unlicensed replication phenotypes, indicative of withdrawal from cycle.
Intriguingly, the labelling indices for cells occupying the putative stem cell niche are indicative of a different origin licensing state compared to other cells of the basal proliferative compartment. In comparison with the transit amplifying compartment, there is down-regulation of Ki67 (26.8%, CI=16.9-36.7%), Mcm2 (52.1%, CI=41.5-62.8) and geminin (8.1%, CI=5.4-10.7%). In fact, the origin licensing state of the putative stem cell niche more closely resembles that of the differentiated compartment than that of the transit amplifying compartment (Wilcoxon signed-rank test: Ki67 $[P=0.005]$, Mcm2 $[P=0.005]$, geminin $[P=0.005]$). These data argue in favour of a cell kinetic model for gut stem cells in which a significant proportion of the progenitor cells in the stem cell niche are unlicensed and have therefore reversibly withdrawn from the mitotic cell cycle. Furthermore, the detection of a number of unlicensed cells in the putative stem cell zone supports models and experimental data pointing to the presence of multiple stem or stem-like cells in these zones (Ohlstein et al., 2004).

3.4. Discussion and Conclusions

The success of chemotherapeutics depends on their ability to kill tumour cell populations whilst causing minimal damage to normal cells. Therefore in the design of new therapeutic strategies it is critical to evaluate how agents may affect both normal and tumour cell populations. The DNA replication licensing machinery has recently emerged as a novel target for anti-cancer therapy, and the presence of an endogenous origin licensing repressor, geminin, raises the prospect that agents mimicking the activity of this protein may provide such therapeutic compounds. A comprehensive understanding of how these proteins are regulated during normal tissue maintenance and development is therefore crucial.
Down-regulation of the RLFs Cdc6 and Mcm2-7 has been linked to exit from the mitotic cell cycle into G0 (Stoeber et al., 1998, 2001; Yan et al., 1998; Berger et al., 1999; Petersen et al., 2000; Sun et al., 2000). The data presented here show that loss of proliferative capacity in G0 is a two step-process, and importantly is independent of the origin licensing repressor geminin (Figure 3.4c) (Kingsbury et al., 2005; see Appendix D). The immediate early response of fibroblasts to density-dependent growth arrest is a block to pre-RC assembly mediated through rapid down-regulation of the MCM loading factor Cdc6 and induction of the global CDK inhibitor p27 (Figure 3.4c). These data add further evidence to the emerging picture of Cdc6 as a "master regulator" of cell proliferation control in somatic cells (Kubota and Takisawa, 2003; Lea et al., 2003; Pelizon, 2003) and primary oocytes (Lemaitre et al., 2002; Whitmire et al., 2002). The late response involves complete down-regulation and removal from chromatin of the replicative Mcm2-7 helicase (Figure 3.4c). In recent years several levels of cellular proliferation control have been identified during exit from the mitotic cell cycle, illustrating the extensive measures to which metazoa go to prevent illegitimate DNA replication and cell division in organs with dormant growth potential such as liver and thyroid (Stoeber et al., 2001; Blow and Hodgson, 2002). It may therefore be expected that an additional mechanism to ensure loss of replicative capacity during exit into G0 may include the "inhibitory" presence of an antagonist of replication licensing coinciding with the observed down-regulation of key origin licensing factors. However consistent with the recent observations by Xouri et al. (Xouri et al., 2005), the data presented here show that geminin levels are not up-regulated during withdrawal of fibroblasts from cycle into G0 as might be expected for a putative tumour suppressor protein, but are down-regulated with similar kinetics to those observed for Cdc6 (Figure 3.4c). The
more rapid down-regulation of Cdc6 and geminin may reflect the fact that both molecules are targets for APC/C-mediated ubiquitin-dependent proteolysis (McGarry and Kirschner, 1998; Petersen et al., 2000) in contrast to MCM expression which is most likely regulated at transcriptional level (Schulte et al., 1996). Upon re-entry into the mitotic cell cycle and progression into S phase, Cdc6 and Mcm2-7 are re-synthesised and pre-RCs sequentially re-assembled onto chromatin (Figure 3.5c), in keeping with previous reports (Stoeber et al., 1998, 2001; Yan et al., 1998; Berger et al., 1999; Petersen et al., 2000; Sun et al., 2000). The CDK inhibitor p27 accumulates in the nucleus at the time of pre-RC assembly (Figure 3.5c), consistent with its main role in preventing activation of Cyclin A-associated kinase (Resnitzky et al., 1995), and is also associated with chromatin at this time, supporting recent findings that p27 can block premature origin firing through its interaction with Mcm7 (Nallamshetty et al., 2005). Geminin is de novo synthesised during re-entry but, importantly, synthesis and association with chromatin do not occur until late in the G0-S transition post pre-RC assembly (Figure 3.5c). Taken together, these data demonstrate that withdrawal of the “license” to replicate is sufficient for loss of replicative capacity during exit into G0 and that geminin is not required to suppress proliferative activity during this transition. Therefore, geminin does not appear to behave as a classic tumour suppressor protein. However the possibility does exist that geminin may play a role in the dissociation of pre-RCs during early stages of cell cycle exit. Acquisition of replication competence during re-entry is critically dependent on de novo expression of RLFs and re-assembly of pre-RCs onto chromatin. Importantly, geminin expression is delayed until late in the G0-S transition, suggesting that its absence during the permissive window of pre-RC assembly is a prerequisite for re-issuing of the replication license (Kingsbury et al.,
How geminin expression during re-entry into the mitotic cell cycle is temporally coordinated with pre-RC assembly is currently unclear. Interestingly geminin and Cdt1 constitute targets for various members of the E2F family of transcription factors and expression of geminin has been linked to the Rb/E2F pathway (Markey et al., 2004; Yoshida and Inoue, 2004). However, Cdt1 expression needs to precede geminin expression for pre-RC assembly to be completed before geminin protein levels reach an inhibitory threshold, suggesting additional levels of regulation for geminin expression.

It is not known whether the observed dynamics in origin licensing and the spatio-temporal regulation of geminin expression during G0 also apply to quiescent human cells in vivo. Expression profiling in thyroid shows that reversible loss of proliferative capacity is coupled to repression of origin licensing through down-regulation of Mcm2, and that the licensing repressor geminin is not required for G0 arrest in vivo (Figures 3.6 and 3.7). Graves Disease and thyroid adenoma are hyper-proliferative disorders of thyroid in response to inappropriate growth stimuli and represent in vivo correlates of cell cycle re-entry from G0. Upon escape from quiescence the standard proliferation marker Ki67, Mcm2 and geminin are re-synthesised in both conditions (Figure 3.7) with striking similarity to the fibroblast model system (Figure 3.5c). Taken together, these combined in vitro and in vivo data provide a unifying picture for the dynamics of origin licensing during reversible growth arrest in G0. Repression of origin licensing through down-regulation of key RLFs is a powerful downstream mechanism that contributes to loss of proliferative
capacity in G0 independent of the licensing antagonist geminin (Kingsbury et al., 2005; see Appendix D). Importantly, the down-regulation of RLFs and geminin during withdrawal of cells from cycle into G0, in conjunction with the absence of these factors in normal thyroid tissue suggests that conditional renewal tissues should be refractory to therapeutic origin licensing inhibitors.

Self-renewing tissue systems are comprised of three cell populations: a terminally-differentiated population at the luminal surface of the crypt, a transient amplifying population and a stem-cell population at the base of the crypt. Cells of the transient amplifying compartment display a replication phenotype predicted for cycling cells with expression of Ki67, Mcm2 and geminin. These data indicate that cells within this compartment would be sensitive to the action of a therapeutic origin licensing inhibitor, an issue which will be addressed in more detail in Chapter Six. Migration of cells into the terminally-differentiated compartment, where they obtain a functional phenotype and lose their proliferative capacity, is coupled to a marked reduction in expression of Ki67, Mcm2 and geminin. These data are consistent with previous reports that differentiation and DNA replication licensing are mutually exclusive processes in self-renewing and permanent tissues (Stoeber et al., 2001), in keeping with the concept of antagonism between the cellular circuits that control proliferation and differentiation (Olson and Spiegelman, 1999). In addition, the down-regulation of geminin during the differentiation process is consistent with an earlier report from our laboratory that geminin does not appear to function as an inducer or regulator of differentiation in somatic self-renewing systems (Eward et al., 2004). Importantly these results also suggest that differentiated cell populations in self-renewing tissues, and most likely in permanent tissues, should be refractory to
therapeutic origin licensing inhibitors.

Intriguingly Ki67, Mcm2 and geminin are also down-regulated in cells occupying the putative stem cell niche indicating that these primitive cells may reside in G0. Importantly as predicted from the \textit{in vitro} data (Figures 3.4 and 3.5), these cells also do not express the licensing repressor geminin. These data suggest that stem/progenitor cells may be distinguished from cycling committed progenitors of the proliferative compartment by absence of origin licensing factors and the antagonist geminin. This data is supported by recent findings that circulating CD34+ haemopoietic progenitor stem cells have an unlicensed phenotype (G.H. Williams and K. Stoeber, Department of Pathology, UCL, UK; personal communication). Recent studies have demonstrated that stem cells undergo asymmetrical divisions and that by discarding newly synthesised DNA which is more prone to replication induced mutation, the stem cell utilises an innate mechanism of genome protection (Cairns, 2002). The data presented here argue that genome protection may also be further ensured through withdrawal of stem cells into an “unlicensed” G0 state, thus restricting the potentially hazardous process of genome duplication and providing extended periods for DNA repair. Furthermore, these data suggest that stem/progenitor cell populations should be refractory to origin licensing inhibitors.

Since self-renewing tissue systems, including the gut, are prime targets for cytotoxic damage in response to classical chemotherapeutic regimes, the preservation of self-renewing stem/progenitor cell populations is extremely important when considering the development of novel therapeutic strategies.

In summary, the data presented in this chapter can be exploited to predict how
normal cell populations may respond to the presence of a therapeutic origin licensing inhibitor, such as a geminin mimetic. Functional cells, including quiescent, differentiated and stem/progenitor cell populations, have an unlicensed phenotype and should therefore be refractory to inhibition of DNA replication initiation. This contrasts with normal cycling cell populations which have a licensed phenotype and therefore present a target for therapeutic origin licensing inhibitors. However recent studies suggest that the putative origin licensing checkpoint may protect normal cycling cells from the effects of origin licensing inhibitors whilst transformed cells remain susceptible and are therefore killed (Shreeram et al., 2002). In order to validate the origin licensing checkpoint as a potential therapeutic target and to further investigate the differential response of normal and transformed cells to inhibition of DNA replication initiation, I have exploited the endogenous origin licensing repressor geminin (see Chapter 6). In addition to providing a molecular tool for early proof-of-principle studies, geminin may also provide a useful starting point for the development of mimetic compounds as anti-proliferative agents. In order for such compounds to be designed, a comprehensive molecular blueprint of geminin’s structure and the regions critical for mediating its activity must be established. In Chapter Four, I describe a dual approach to define the structure-function relationship of geminin and identify regions within the protein that are critical for mediating geminin’s activity as an origin licensing inhibitor.
CHAPTER FOUR

STRUCTURE-FUNCTION ANALYSIS OF GEMININ

4.1. Introduction

In the previous chapter I showed that withdrawal of cells from cycle into the quiescent and differentiated states is coupled to down-regulation of the replication licensing machinery. Moreover down-regulation of replication licensing factors in stem/progenitor cell populations indicates that these cells may reside for most of the time in an out-of-cycle, quiescent (G0) state. Taken together these findings raise the exciting prospect that therapeutic licensing inhibitors should not affect these cell populations in human tissues, thus ensuring that the majority of normal cells within the body are protected from potential cytotoxic effects of such agents. These data therefore further validate the replication licensing pathway as a potential anti-cancer target and raise the question as to how replication licensing could be inhibited therapeutically.

Theoretically, licensing inhibitors could act by interfering with protein-DNA interactions (e.g. ORC binding to DNA), protein-protein interactions (e.g. MCM helicase assembly), kinase activity (e.g. Cdc7-Dbf4), or phosphatase activity (e.g. protein phosphatase 2A), or by mimicking the activity of endogenous (e.g. geminin) or exogenous (e.g. HPV1 E4, see Chapter 5) replication licensing regulators. 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 and de Bono, 2005; see also Section 1.4.). Current knowledge of Cdc7-Dbf4 suggests that this
related kinase complex has a much narrower spectrum of action (Masai and Arai, 2002; see also Section 1.6.) and may therefore provide an attractive therapeutic target. As a result, development of Cdc7 kinase inhibitors is in progress in the commercial arena as well as in our laboratory. Whilst the screening for Cdc7 kinase inhibitors is ongoing, I have sought to exploit geminin for proof-of-principle studies evaluating the potential of the replication licensing machinery as an anti-proliferative target.

Human geminin is a highly conserved 212 amino acid, 23.5 kDa nuclear protein with known homologs in Xenopus (McGarry and Kirschner, 1998), Drosophila (Quinn et al., 2001), mouse (Yanagi et al., 2002) and Caenorhabditis elegans (Yanagi et al., 2005) (Figure 4.1a). Analysis of deletion mutants of geminin has identified three almost independent functional regions of the protein (Kroll et al., 1998; McGarry and Kirschner, 1998) (Figure 4.1b). The N-terminus of geminin contains a destruction sequence (DEAD box, RRTLKMIQP, amino acid residues 23-31) which mediates ubiquitin-dependent degradation via the APC/C-26S proteosome pathway during mitosis (McGarry and Kirschner, 1998). Deletion/mutation of this sequence generates a geminin molecule that is resistant to ubiquitin-dependent degradation (McGarry and Kirschner, 1998). Adjacent, and partially overlapping with the DEAD box, is a neuralisation domain (aa 28-79) that is necessary for geminin’s function in neuronal development (Kroll et al., 1998). Within the neuralisation domain are several clusters of basic amino acids (aa 50-62) that serve as a nuclear localisation signal (Benjamin et al., 2004). The central region of geminin consists of a highly conserved coiled-coil motif of 35 amino acid residues (aa 112-147) with 5 heptad repeats (Lupas, 1996; Burkhard et al., 2001), flanked by an N-terminal sequence rich
Figure 4.1. Amino acid sequence and structural domains of geminin. (a) Amino acid sequence alignment of mouse, rat, human, Xenopus, Zebrafish (Danio rerio) and Drosophila (D. mel) geminin. Highly conserved (*) and partially conserved (: and .) residues are indicated. The coiled-coil domain (shaded), and surrounding regions, show the highest inter-species homology. (b) Diagram illustrating the structural and functional domains of hsgeminin (NLS - Nuclear Localisation Sequence).
in basic amino acid residues and a C-terminus predicted to form a helix (Thépaut et al., 2004). Crystallisation of a synthetic peptide of this domain revealed that it forms a dimeric parallel two-stranded coiled-coil (Thépaut et al., 2002), whilst mutational analyses indicate that it is essential for inhibition of DNA replication (McGarry and Kirschner, 1998; Thépaut et al., 2002).

Geminin is expressed in S, G2 and M phase of the cell cycle (McGarry and Kirschner, 1998) and functions to prevent re-replication from fired origins by interacting with Cdt1 thus preventing re-assembly of pre-RCs (Wohlschlegel et al., 2000; Tada et al., 2001; Yanagi et al., 2002; Cook et al., 2004). Expression of geminin interferes with the interactions between Cdt1 and Mcm6, Cdt1 and Cdc6 (Cook et al., 2004) and Cdt1 and chromatin (Yanagi et al., 2002), whilst overexpression of Cdt1 reverses the block on licensing imposed by geminin and restores DNA replication (Wohlschlegel et al., 2000; Tada et al., 2001). It has recently been suggested that two pools of geminin-Cdt1 may exist in the cell. Geminin and Cdt1 bind chromatin at identical or closely adjacent sites (Kulartz and Knippers, 2004), with geminin remaining on chromatin after Cdt1 has dissociated (Maiorano et al., 2004). In addition, soluble geminin-Cdt1 complexes are present during late S and G2 phase, possibly as a means of protecting newly synthesised Cdt1 from degradation (Ballabeni et al., 2004). Ubiquitin-mediated degradation of geminin in mitosis would therefore release this new Cdt1 fraction and enable pre-RC assembly in early G1. This model is supported by knockdown experiments which demonstrate that whilst silencing of geminin expression in S phase results in re-replication (Melixetian et al., 2004), knockdown of geminin in M phase results in impaired pre-RC formation during the ensuing cell cycle (Ballabeni et al., 2004).
The importance of geminin in preventing re-replication during S phase is demonstrated by overexpression studies and the expression of non-degradable forms of geminin. Addition of geminin to in vitro replication reactions containing *Xenopus* egg extracts and sperm DNA prevents DNA replication (McGarry and Kirschner, 1998), whilst overexpression of *Drosophila* geminin results in DNA replication inhibition, induction of ectopic neuronal differentiation and apoptosis (Quinn *et al*., 2001). Adenoviral-induced overexpression of non-degradable human geminin in normal IMR90 diploid fibroblasts blocks origin licensing in G1 and thereby prevents transition of cells into S phase (Shreeram *et al*., 2002). In transformed cell lines the block to origin licensing does not impede transition into S phase, DNA replication is initiated from a few licensed origins and ultimately an apoptotic response is triggered either in S phase or G2/M phase depending on the genetic status of the cell (Shreeram *et al*., 2002; see also Section 1.5.4.).

Interestingly geminin has also been shown to play an essential role in allowing entry into mitosis, with geminin depletion resulting in centrosome overduplication, mitotic defects (Tachibana *et al*., 2005), and accumulation of Chk1 in its active phosphorylated form (McGarry, 2002; Melixetian *et al*., 2004). Geminin may also provide an intriguing link between the co-ordination of development and proliferative control. Geminin is essential for early neuronal specification in *Xenopus* (Kroll *et al*., 1998) and *Drosophila* (Quinn *et al*., 2001) with a loss of neural patterning in the presence of a putative dominant negative geminin lacking the neuralisation domain (Kroll *et al*., 1998) and *Dm geminin* mutants showing defective neural differentiation (Quinn *et al*., 2001). Recently geminin has been shown to directly interact with transcription factors of the *Hox* and *polycomb* protein families,
inhibiting their transcriptional activity (Del Bene et al., 2004; Luo et al., 2004), and to regulate neuronal differentiation by antagonising the activity of Brg1, a catalytic subunit of the SWI/SNF chromatin remodelling complex (Seo et al., 2005). It is possible therefore that competition for geminin binding results in the establishment of an equilibratory system which governs the decision to proliferate or differentiate (Luo and Kessel, 2004).

The role of geminin as a repressor of origin licensing and the fact that geminin is a relatively small molecule suggest that compounds mimicking geminin activity may provide therapeutic origin licensing inhibitors. Since the normal physiological activity of geminin does not induce any damage to the DNA, it can be postulated that an agent mimicking geminin activity should be non-genotoxic. In addition, the disruption of protein-protein interactions as a therapeutic strategy may be more robust than inhibiting kinase activity which can lack specificity and be susceptible to resistance. Furthermore no suppressor phenotype is known for geminin, whereas the requirement for Cdc7 activity is bypassed by the mcm5-bob1 mutant (Hardy et al., 1997). However, in order for therapeutic geminin mimetics to be developed, the mechanism by which geminin inhibits origin licensing, the protein-protein interaction(s) involved and the regions within the protein which are critical for mediating this activity must first be fully understood.

The geminin molecule also represents a powerful molecular tool that can be exploited to study the response of normal and cancer cells to inhibition of origin licensing. In this chapter I am going to discuss the development of a molecular blueprint of geminin for future design of geminin mimetics and the generation of a
small, non-degradable, biologically active fragment of geminin for use in proof-of-principle studies (see Chapter 6). To this end a dual approach was taken to study the structure-function relationship of geminin: working as part of a team to resolve the structure of geminin by transmission electron microscopy and complementing these structural studies with in vitro activity studies. For the latter a ‘loading assay’ was developed which, in conjunction with an established cell-free DNA replication assay, enabled the recruitment of replication licensing factors onto chromatin, and thus the ability of geminin and geminin derivatives to block pre-RC assembly, to be monitored.

4.2. Materials and Methods

For Transformation of E. coli (2.2.2.), Plasmid isolation (2.2.3.), Site-directed mutagenesis (2.2.8.), DNA sequencing (2.2.10), Antibodies (2.3.1.), Expression of recombinant hsgeminin and hsgeminin-FF in E. coli (2.3.3.), Purification of His6-hsgeminin and His6-hsgemininFF (2.3.6.), Peptide synthesis (2.3.11.), SDS-polyacrylamide gel electrophoresis (2.3.12), Coomassie Blue staining of SDS-PAGE gels (2.3.13.), Immunoblotting (2.4.1.), Immunofluorescence (2.4.2.), Preparation of total cell extracts (2.5.5.), Preparation of nuclei and cytosolic extracts (2.6.1.), Assaying for DNA synthesis in vitro (2.6.2.), Analysis of in vitro DNA synthesis products by confocal fluorescence microscopy (2.6.3.), Chromatin binding assay (2.6.4.) and Bioinformatic and database resources (2.7.1.) refer to Chapter Two (Materials and Methods). For Chemical crosslinking of recombinant geminin proteins (B.1.1.), Electron microscopy (B.1.2.) and Image processing and molecular modelling (B.1.3.) see Appendix B.
4.3. Results

4.3.1. Characterisation of an in vitro cell-free DNA replication assay

As discussed in Chapter Three, in cultured NIH/3T3 fibroblasts chromosomal replication origins are licensed for DNA synthesis during a defined “window of opportunity” between 16 and 18 hours after release from density-dependent growth arrest (Figure 3.5c). It has previously been established that nuclei prepared by dounce homogenisation during this window show tight synchrony and are capable of initiating a single round of semi-conservative DNA replication in a mammalian cell-free DNA replication system (Stoeber et al., 1998). In this assay, nuclei are incubated with S phase cytosol prepared from synchronised Hela cells along with ribonucleoside and deoxyribonucleoside triphosphates and an energy regeneration system to allow initiation of DNA replication in vitro (Krude et al., 1997; Stoeber et al., 1998) (Figure 4.2). Replication reactions contain biotin-16-dUTP as a marker to allow detection of DNA synthesised during the in vitro incubation by confocal fluorescence microscopy. The proportion of nuclei replicating is determined from printed images of nuclei stained for the presence of DNA by propidium iodide and for synthesis of DNA in vitro by fluorescein-linked streptavidin.

Since geminin’s biological role is to inhibit re-replication by blocking pre-RC assembly, I first developed an alternative readout to the cell-free system that allows the binding of replication licensing factors onto chromatin to be monitored (Figure 4.2). Chromatin-bound protein fractions are obtained by treatment of insoluble chromatin with DNase I and salt extraction following nuclear lysis and are resolved by SDS-PAGE. Following incubation in a physiological elongation buffer (Buffer A) G1 nuclei, prepared 16.25 hours after release from quiescence, have low levels of
Figure 4.2. A dual readout mammalian cell-free DNA replication system. Synchronised murine NIH/3T3 nuclei (N) prepared following release of cells from quiescence by subculturing into fresh serum are incubated with S phase HeLa cytosol (SC) or an elongation buffer (Buffer A/BA) which supports elongation, together with NTPs (0.1 mM each), dNTPs (0.1 mM each), an energy regeneration mix and biotin-16-dUTP for 3 hours. Recombinant proteins, antibodies, small molecules etc. can be titrated into reactions (x). To monitor DNA synthesis nuclei are washed, fixed in paraformaldehyde and stained with propidium iodide to reveal DNA (red) and with fluorescein-streptavidin (green) to detect biotin incorporation resulting from DNA synthesis. To monitor recruitment of replication licensing factors onto chromatin, chromatin-bound protein fractions are isolated by nuclear lysis followed by extraction with NaCl and DNasel. Proteins are resolved by SDS-PAGE gel electrophoresis and immunoblotted for replication licensing factors and Histone H1 as a loading control. Nuclei incubated in Buffer A have low levels of Mcm2 associated with chromatin, as a result of the small numbers of nuclei which have assembled pre-RCs prior to isolation in the G1 nuclear preparation. Incubation in S phase cytosol allows active recruitment of Mcm2 to chromatin in vitro.
chromatin-bound Mcm2, consistent with the small proportion of nuclei that have already assembled pre-RCs prior to preparation of the nuclear templates. These include both S phase contaminants that have already initiated DNA replication from licensed origins, and late G1 nuclei which have completed pre-RC assembly but have not yet initiated DNA replication. Chromatin-bound Mcm2 levels are significantly increased following incubation in S phase cytosol due to the completion of pre-RC assembly by early G1 nuclei. It was previously unclear whether pre-RCs are being actively assembled \textit{in vitro} or, alternatively, whether the initiation of DNA replication in this system is dependent on assembly of pre-RCs prior to nuclear preparation. These data demonstrate that initiation of DNA replication in the cell-free system is dependent to a large extent on dynamic \textit{in vitro} pre-RC assembly in addition to origin firing by CDKs and Cdc7-Dbf4. Since geminin inhibits DNA replication by blocking pre-RC assembly, active assembly of pre-RCs \textit{in vitro} is critical for analysis of geminin activity. An assay in which the initiation of DNA replication \textit{in vitro} is solely reliant on CDK/Cdc7-Dbf4 activity, as is true for alternative cell free systems (Krude, 2000), would be of no use for such studies. The \textit{in vitro} cell-free DNA replication system coupled to the chromatin-binding assay therefore provide a methodology for studying the activity of geminin, and derivatives thereof, \textit{in vitro}.

To assess the replication capacity of nuclei during the G1-S phase transition, nuclei were prepared at various timepoints after release from density-dependent growth arrest and incubated in HeLa S phase cytosol. Nuclei prepared from cells within 15 hours of release failed to initiate DNA synthesis in S phase cytosol (Figure 4.3). However, between 16 and 18 hours after release nuclei become competent to
Figure 4.3. NIH/3T3 nuclei become competent to replicate 15 hours after release from quiescence. Synchronised NIH/3T3 nuclei prepared at the indicated time after release from quiescence were incubated with HeLa S phase cytosol, which induces initiation in competent nuclei, or an elongation buffer (Buffer A), which only supports elongation DNA synthesis in nuclei that are already in S phase (Stoeber et al., 1998) along with NTPs, dNTPs, and an energy regeneration mix. After incubation for 3 hours with biotin-16-dUTP, nuclei were washed, fixed in paraformaldehyde and stained with propidium iodide (red) for the presence of DNA and with fluorescein-streptavidin (green) to detect biotin-16-dUTP incorporation resulting from DNA synthesis in vitro. Replicating nuclei appear yellow in merged images. Nuclei prepared within 15 hours after release fail to initiate DNA replication in S phase cytosol. Nuclei become competent to initiate replication at 15 hours and by 23 hours have entered S phase and show equivalent levels of replication in S phase cytosol or Buffer A.
replicate in S phase cytosol, consistent with the observation in Chapter Three that pre-RC assembly occurs at this time (Figure 3.5c). A small proportion of nuclei prepared between 16 and 18 hours are able to replicate DNA in Buffer A, which supports elongation DNA synthesis but does not allow initiation of DNA synthesis (Stoeber et al., 1998). These nuclei represent a small population of cells which have progressed back into cycle more rapidly following release from quiescence. At the point of nuclear preparation these S phase contaminants contain origins which have already fired, enabling DNA synthesis to continue at established replication forks. At 21 hours after release from quiescence the majority of cells have initiated DNA replication and equivalent levels of replication are observed in co-incubations with either S phase cytosol or Buffer A. These data demonstrate that competence of G1 nuclei to replicate in vitro corresponds to the binding of replication licensing factors onto chromatin, as has been previously suggested (Stoeber et al., 1998).

4.3.2. Establishment of assay conditions for the evaluation of geminin activity in vitro

The in vitro cell-free DNA replication system and chromatin-binding assay (Figure 4.2) provide a ‘dual-readout’ for studying the structure-function relationship of geminin. Prior to analysing the activity of geminin with this assay, the approximate physiological concentration of endogenous geminin was determined. Total cell extracts were prepared from 2.5 x 10⁴ NIH/3T3 fibroblasts 12 hours (G1 phase) and 23 hours (S phase) after release from density-dependent growth arrest, resolved by SDS-PAGE and immunoblotted with a geminin antibody (Figure 4.4a). Comparisons with increasing amounts of recombinant geminin (see Chapter 2, Figure 2.2) indicate that 2.5 x 10⁴ S phase NIH/3T3 fibroblasts contain approximately 1 ng of
Figure 4.4. Recombinant His\textsubscript{6}-hsgeminin enters NIH/3T3 nuclei. (a) To determine the physiological concentration of endogenous geminin, total cell extracts (TCE) were prepared from NIH/3T3 fibroblasts 12 hours (G1 phase) and 23 hours (S phase after release from density-dependent growth arrest. Extracts from 2.5 x 10\textsuperscript{4} cells were resolved by SDS-PAGE against known quantities of recombinant geminin, and immunoblotted for geminin. 1 x 10\textsuperscript{5} S phase cells contain approximately 1 ng of endogenous geminin. (b & c) NIH/3T3 nuclei were incubated with recombinant His\textsubscript{6}-hsgeminin or a control buffer on ice for 30 minutes. Transport across the nuclear membrane was assessed by (b) immunofluorescence and (c) immunoblotting. For (b) nuclei were stained with an anti-His primary antibody and FITC-conjugated secondary antibody and counterstained with propidium iodide. For (c) nuclei were pelleted and the supernatant, containing untransported geminin, was removed. Nuclei were washed three times with PBS, and nuclear extracts were prepared and immunoblotted with an anti-His antibody.
endogenous geminin, which equates to approximately $10^6$ molecules per cell. This is approximately ten times higher than the number of MCM molecules per cell, which has been estimated at about $10^5$ (Kearsey and Labib, 1998). To achieve physiological concentrations of geminin in cell-free DNA replication reactions containing $1 \times 10^5$ nuclei, 4 ng of recombinant geminin would be required giving a final concentration of 4 nM.

I next sought to determine whether recombinant His$_6$-hsgeminin can enter NIH/3T3 nuclei prepared for in vitro replication reactions. Although geminin is a small protein of 23.5 kDa, published data suggest that it may oligomerise (Thépaut et al., 2002) which could impede its uptake into nuclei. To determine that recombinant geminin can enter nuclei in vitro, nuclei prepared by dounce homogenisation were incubated with 4 µM His$_6$-hsgeminin on ice for 30 minutes. Nuclei were spun onto coverslips, stained with a monoclonal anti-His primary antibody and a FITC-conjugated anti-mouse secondary antibody, counterstained with DAPI and analysed by confocal fluorescence microscopy (Figure 4.4b). Strong FITC staining was detectable in nuclei incubated with recombinant geminin, but not in nuclei which had been incubated with a control buffer (25 mM Tris pH 7.5, 250 mM NaCl, 10% glycerol). To independently confirm these results, nuclei were incubated with His$_6$-hsgeminin as above, washed three times with PBS, and nuclear extracts prepared by incubation in a whole cell extract lysis buffer. Extracts were immunoblotted for geminin and anti-His, revealing that approximately 50% of the recombinant His$_6$-hsgeminin had entered the nuclei (Figure 4.4c). Taken together these data indicate that recombinant geminin can enter NIH/3T3 nuclei in vitro.
To determine the optimal time at which to prepare nuclear templates for assessing geminin activity in the cell-free replication system, the effect of geminin on the replication capacity of nuclei prepared during release from G0 was examined (Figure 4.5a). Nuclei prepared between 15 and 23 hours after release from G0 were incubated with 4 μM His6-hsgeminin and S phase cytosol. A 64% reduction in replication was observed in nuclei prepared 16.25 hours after release from G0 in the presence of geminin (Figure 4.5a). This inhibition was reduced to 21% at 18.25 hours and 4% at 23 hours. The reduction in inhibition at later time-points can be explained by the increased proportion of nuclei with assembled pre-RCs in the nuclear preparations. Since geminin inhibits the assembly of pre-RCs, it is unable to block DNA replication from licensed origins. As the largest reduction in replication was observed in nuclei prepared 16.25 hours after release from G0, these nuclear templates were used in all subsequent experiments unless otherwise stated.

To determine the geminin concentration that causes maximum inhibition of DNA replication in vitro, and to provide evidence that replication inhibition is a direct result of geminin activity, His6-hsgeminin was titrated into replication reactions with 16.25 hour nuclei. His6-hsgeminin was purified to approximately 95% homogeneity (see Section 2.3.4, Figure 2.2b, fraction 27) and thus any titratable effect should be a result of geminin activity. A replication signal of 24% was observed upon incubation in S phase cytosol alone, with no reduction in replication in the presence of a control buffer (25 mM Tris pH 7.5, 250 mM NaCl, 10% glycerol). With increasing geminin concentrations there was a linear decrease in the percentage of nuclei replicating from 13.5% with 0.5 μM geminin to 6.4% with 4 μM geminin, suggesting that the inhibition is specific to geminin activity (Figure 4.5b). For all subsequent
Evaluation of recombinant geminin activity in the cell-free DNA replication system. (a) Synchronised NIH/3T3 nuclei prepared at the indicated time after release from quiescence were incubated with HeLa S phase cytosol or Buffer A and recombinant His$_6$-hsgeaminin. The addition of recombinant geminin caused a 64% reduction in replication at 16.25 hours. However, addition of geminin 23 hours after release caused only a 4% reduction in replication, indicating that geminin can not interfere with DNA replication in vitro once pre-RCs have been assembled. (b) His$_6$-hsgeaminin was titrated into replication reactions with 16.25 hour nuclei. Addition of control buffer had no effect on replication. As the concentration of recombinant geminin increased, the percentage of nuclei replicating linearly decreased.
experiments 4 μM recombinant geminin was used, which is approximately $10^3$ times the physiological concentration of endogenous geminin.

4.3.3. **Recombinant full-length geminin blocks initiation of DNA replication and assembly of pre-replication complexes in vitro**

Having established optimal conditions for analysing geminin activity in vitro, I next sought to determine that exogenous geminin retained its expected physiological function in the in vitro assay. Since geminin inhibits DNA replication by blocking pre-RC assembly, addition of geminin to nuclei in which pre-RCs have already been assembled or in which origins have fired prior to preparation of nuclear templates (i.e. entered S phase) should have no affect on DNA replication. In order to test this hypothesis, the ability of geminin to block DNA synthesis in G1 nuclei prepared 16.25 hours after release from quiescence (G0), a proportion of which should be competent to assemble replication complexes in vitro, and in S phase nuclei prepared 23 hours after release from G0 was assayed. Incubation of G1 nuclei in Buffer A resulted in 2.8% of nuclei synthesising DNA (Figure 4.6a). In comparison, 22% of the G1 nuclei replicated DNA upon incubation in S phase cytosol, the difference to the elongation control representing true initiation in vitro (Figure 4.6a). Addition of recombinant geminin to the reaction resulted in 7% of the nuclei replicating, indicating that 67% of replication-competent nuclei failed to synthesise DNA in vitro. Elongation of DNA replication was sufficiently supported upon incubation of S phase nuclei in either Buffer A (67% of nuclei replicating) or S phase cytosol (67%) (Figure 4.6b). Importantly, elongation was not affected by addition of recombinant geminin (64% of nuclei replicating). Taken together these data demonstrate that recombinant full-length geminin inhibits initiation of DNA replication in vitro, but is
Figure 4.6. Geminin blocks initiation but not elongation of DNA replication in vitro. NIH/3T3 fibroblasts were driven into density-dependent growth arrest to induce G0 and nuclei were isolated at (a) 16.25 hours (G1 phase) and (b) 23 hours (S phase) after release from contact-inhibition. A small percentage of S phase contaminants are detected in the G1 nuclear preparation (2.8%). Addition of recombinant geminin (final concentration: 4 μM) to co-incubations of G1 nuclei in S cytosol inhibits initiation of DNA replication in 67% of replication-competent nuclei, whilst S phase elongation DNA synthesis is refractory to the presence of recombinant geminin. Results are expressed as the percentage of nuclei replicating and summarised in the histograms (mean ± SD).
unable to inhibit elongation DNA synthesis, consistent with the physiological role of geminin in blocking re-assembly of pre-RCs (McGarry and Kirscher, 1998; Wohlschlegal et al., 2000; Tada et al., 2001).

To confirm that the loss of replicative capacity of G1 nuclei seen in the presence of recombinant geminin is due to inhibition of pre-RC assembly, identical replication reactions to those described above were performed, after which chromatin-bound protein fractions were resolved by gel electrophoresis and probed for Mcm2, recombinant geminin and Histone H1 as a loading control. Analysis of the chromatin-bound protein fraction of G1 nuclei incubated in Buffer A shows a small amount of Mcm2 bound to chromatin (Figure 4.7a), in keeping with a small proportion of nuclei which have assembled pre-RCs prior to preparation of nuclear templates. Incubation of G1 nuclei in S phase cytosol results in recruitment of Mcm2 protein to chromatin indicative of pre-RC assembly in vitro and corresponding to an 8-fold increase in the percentage of nuclei synthesising DNA in vitro (Figure 4.6a). Addition of recombinant geminin to co-incubations of G1 nuclei in S phase cytosol bans the replication license by blocking loading of Mcm2 to chromatin, corresponding to a 67% reduction in replication-competent nuclei. Notably, the block to pre-RC assembly in vitro coincides with binding of recombinant geminin to chromatin (Figure 4.7a) as has been reported by others for embryonic cell cycles (Li and Blow, 2004; Maiorano et al., 2004) and in somatic cells (Kulartz and Knippers, 2004). Consistent with the failure of geminin to inhibit elongation DNA synthesis, addition of recombinant geminin to co-incubations of S phase nuclei in S phase cytosol does not affect the level of Mcm2 protein bound to chromatin (Figure 4.7b). Taken together these data demonstrate that recombinant geminin inhibits initiation of
Figure 4.7. **Geminin inhibits initiation of DNA replication in vitro by blocking recruitment of replication licensing factors onto chromatin.** Immunoblots of Mcm2, recombinant His$_{6}$-geminin and Histone H1 in chromatin-bound protein fractions prepared from nuclei taken through in vitro replication reactions. Incubation of G1 nuclei in S cytosol results in recruitment of Mcm2 to chromatin, indicative of pre-RC assembly in vitro. Addition of recombinant geminin blocks loading of Mcm2 to chromatin, coinciding with binding of recombinant geminin to chromatin. Mcm2 is already bound to chromatin in S nuclei isolated post pre-RC assembly in vivo and its levels are unchanged in the presence of recombinant geminin.
DNA replication, but not elongation DNA synthesis, by actively blocking the assembly of pre-RCs, and that this inhibition is associated with the recruitment of recombinant geminin onto chromatin.

4.3.4. An N-terminal deletion mutant of geminin is fully functional at inhibiting origin licensing

To enable the future design of anti-proliferative agents mimicking geminin activity for testing in mouse tumour models, it is first necessary to understand the mechanism by which geminin inhibits the assembly of pre-RCs and to identify the regions within the protein that mediate this inhibitory activity. It has been reported for *Xenopus* geminin that the replication inhibition domain is comprised of residues 87 to 168, including the coiled-coil domain (McGarry and Kirschner, 1998). The data suggest that integrity of the coiled-coil motif is essential but probably not sufficient, and it has been postulated that adjacent N- and/or C-terminal sequences are required for inhibition of DNA replication. The minimal region of *hS* geminin required for inhibition of origin licensing is unclear. I therefore sought to identify a small fragment of geminin that retains biological activity. A number of strategies are available for producing protein fragments that can be assayed for activity. These include crude mechanisms such as shotgun cloning and more refined mechanisms such as limited proteolysis. In the work of this thesis, the latter option was used since it allowed the rapid generation of a small number of geminin fragments (Dr. A. Okorokov; personal communication). Full-length recombinant His₆-*hS* geminin was proteolytically cleaved with enterokinase, resulting in two geminin fragments. Enterokinase recognises the site DDDDK where D represents a negatively charged residue and generally contains one or more serine residues. Analysis by
**Figure 4.8. Production of two geminin fragments by enterokinase cleavage.** (a) Geminin fragments produced by enterokinase cleavage were resolved by SDS-PAGE gel electrophoresis and stained with Coomassie Blue, revealing a longer 15 kDa fragment and a shorter 9 kDa fragment with a 7 kDa degradation product. (b) Mass spectrometry analysis identified the 15 kDa fragment as amino acids 77-212 and the 9 kDa fragment as aa 77-160, with the 7 kDa fragment degradation product containing aa 94-160.
SDS-PAGE (Figure 4.8a) and mass spectrometry confirmed that the proteolysis products were a 15 kDa fragment containing amino acids 77 through to 212 and a 9 kDa fragment consisting of amino acids 77 to 160 that underwent degradation to produce a 7 kDa fragment (amino acids 94-160) (Figure 4.8b). The ability of these fragments to inhibit DNA replication initiation in vitro was assayed using the cell-free assay. In co-incubations of G1 nuclei with S phase cytosol 22% of nuclei were capable of DNA synthesis (Figure 4.9a), whilst incubation with full-length recombinant geminin resulted in a reduction in replicative capacity to 7%. A similar reduction in replication was observed with the 15 kDa proteolytic fragment (8% of nuclei replicating). However, no reduction in replication was observed following incubation with the 9,7 kDa fragments. Furthermore, as with full-length geminin (Figure 4.7a), a reduction in Mcm2 recruitment to chromatin was observed in the presence of the 15 kDa fragment (Figure 4.9b), whilst no reduction could be detected in the presence of the 9,7 kDa fragments. Taken together these data suggest that the coiled-coil motif is essential but not sufficient for inhibition of DNA replication, with N- and/or C-terminal sequences additionally being required.

4.3.5. Analysis of the molecular structure of geminin by transmission electron microscopy

In order to further understand the structure-function relationship of geminin and the active 15 kDa fragment, the structures of the two molecules were analysed using transmission electron microscopy (EM) combined with single particle analysis.

I wish to clearly state that the single particle EM, angular reconstitution image analysis and chemical cross-linking experiments described in 4.3.5. are entirely the
Figure 4.9. The N-terminal 80 amino acids of geminin are not required for inhibition of DNA replication. (a) Addition of the 15 kDa fragment to replication reactions containing G1 nuclei resulted in an inhibition of DNA replication almost identical to that achieved with full length geminin (7.65% and 6.8% respectively). Addition of the 9,7 kDa fragments had no effect on the replication signal indicating that the region between the coiled-coil and the C-terminus (aa 160-212) may play a critical role in the inhibition of DNA replication. Additionally aa 77-94 may be important since these are lost when the 9 kDa fragment degrades. (b) Addition of the 15 kDa geminin fragment to G1 nuclei prevents recruitment of Mcm2 onto chromatin, whilst the 9,7 kDa fragments have no effect on pre-RC assembly.
work of A. Okorokov (Department of Pathology, UCL, UK). These data are included here as part of the connected argument because they provide critical information to complement the functional studies described in 4.3.3., 4.3.4., 4.3.6., and 4.3.7. performed entirely by myself in the work of this thesis and enable an understanding of the structure-function relationship of geminin. In addition, they provide the rationale for the mutational analysis described in 4.3.7.

Since geminin has been reported to aggregate at acidic pH (Thépaut et al., 2002), alkaline conditions permissive for protein activity were used. EM raw images of full-length geminin appear as a homogenous dispersion of small elongated particles with a bulk at one end. A total of 3,300 molecular images were selected manually from negatives and subjected to image analysis using angular reconstitution to determine the orientations of characteristic views (van Heel et al., 1996). The structure of full-length geminin was refined by iterative procedures of alignment and classification to give a final three-dimensional (3D) map of geminin at a resolution of 17.5 Å at 0.5 threshold of Fourier shell correlation (Figure 4.10). The overall shape of the full-length geminin molecule resembles a ‘key’ of 175 Å in length with four visually defined parts: ‘tail’, central ‘body’, two ‘necks’ and a ‘head’ (Figure 4.11). The tail domain is ~45 Å long and ~22 Å in diameter at its widest cross-section. In the original images it appears longer and seems to be flexible, and is therefore not resolved in the reconstruction. The central body domain has a flattened conical shape which progresses from tail to head with a size of ~44 Å at the widest point, ~35 Å at the narrowest point and a length of ~70 Å. The overall volume of the body domain can accommodate ~28 kDa of protein mass assuming a unique density for the protein of 0.84 D/Å³. The top of the body domain splits into two sleeve-shaped ‘necks’
Figure 4.10. Three-dimensional reconstruction of geminin. Representative class averages together with the corresponding 3D surface representations at different 'tilt' angles $\beta$ and $\gamma$. (i) Class averages (representative views) of geminin particles obtained by multi-reference alignment and classification. (ii) 3D structure of geminin reprojected in the Euler angle directions found for the class averages in (i). The quality of the 3D reconstruction can be assessed by comparison between images in rows (i) and (ii). (iii) Surface representation of the 3D reconstruction viewed from directions identical to the Euler directions assigned to the corresponding class averages shown in (i). The bar represents 175 Å. (Figure adapted from Okorokov et al., 2004; see Appendix E).
Figure 4.11. Surface representation of the 3D reconstruction of geminin determined at a resolution of ~17.5 Å. The 'key'-shaped geminin molecule at different angle views. From left to right: main view, top view and side view with domain boundaries and their dimensions indicated accordingly. (Figure adapted from Okorokov et al., 2004; see Appendix E).
which enclose a large opening with ~26 Å between them and ~31 Å between the head and the central body. The neck domains support a large head which is also slightly flattened and measures 79 Å at its widest and 60 Å at its narrowest sides. The overall volume of the head is ~123 nm³ and can accommodate ~64 kDa of protein. The overall volume of the molecule is large enough to be fitted with 103 kDa of protein mass corresponding well to four geminin monomers of 23.5 kDa each.

To further confirm the tetrameric organisation suggested by the structural analysis, chemical cross-linking was performed with increasing concentrations of two different cross-linkers (BS³ and EGS). Analysis of the reaction products by SDS-PAGE shows that the cross-linked species of geminin predominantly migrate as dimeric and tetrameric forms of the protein (Figure 4.12). This supports previous data indicating that the coiled-coil domain of geminin has a dimeric organisation (Thepaut et al., 2002). Taken together with the structural information, these data suggest that the main structural organisation of geminin is a dimer that further oligomerises into tetramers or dimers of dimers, suggesting that full-length geminin has an additional level of spatial organisation.

It is unclear from the overall organisation of the full-length geminin molecule how to assign the amino- and carboxy-terminal domains. However, by resolving the structure of the 15 kDa fragment (gemininΔNt) it should be possible to distinguish between different structural domains of the protein. Moreover, structural analysis of the 15 kDa fragment should shed further light on the structure-function relationship of geminin as a repressor of origin licensing and may enable the design of smaller active peptides or mimetic molecules. GemininΔNt appeared as small thread-like
Figure 4.12. Chemical cross-linking of recombinant geminin. Full-length recombinant geminin was cross-linked with BS3 or EGS and analysed by SDS-PAGE. Geminin protein concentration was fixed at 10 mg/ml and concentrations of BS3 and EGS were increased from 0.5 to 5 mM as indicated by the ascending triangle. Cross-linked geminin species predominantly migrate as dimeric and tetrameric forms of the protein. (Figure adapted from Okorokov et al., 2004; see Appendix E).
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Elongated particles which were missing the head-like bulk observed for the full-length protein (Figure 4.13a). Approximately 300 molecular images were selected and subjected to multivariate statistical analysis (Serysheva et al., 1995; van Heel et al., 1996). GemininΔNt particles were uniformly shaped as short filaments of ~170 Å in length and ~25 Å in diameter. Since these particles were lacking the head domain observed for full-length geminin it can be concluded that this domain must contain the amino terminal part of geminin which is missing from gemininΔNt. The smaller diameter of gemininΔNt suggests that this fragment may have a dimeric rather than a tetrameric organisation. To confirm this hypothesis, gemininΔNt was cross-linked with increasing concentrations of BS³ and EGS. Analysis of the reaction products by SDS-PAGE shows that the cross-linked species of gemininΔNt predominantly migrates as a dimer (Figure 4.13b). Taken together with the structural data, this suggests that residues in the N-terminus of full-length geminin may mediate tetramer assembly.

In order to further understand the structural organisation of the geminin molecule, structural homology modelling was exploited to determine the location of the coiled-coil domain. Since the structure of the coiled-coil domain was unavailable, the potential dimeric coiled-coil domain was modelled using SwissModel structural homology modelling software using 1GK6 atomic structure as a template (Herrmann et al., 2000). This structure, originally used by Thépaut et al. for the molecular replacement method (Thépaut et al., 2002), was identified by the Threader 3 program (Jones et al., 1992) as a potential match for the geminin sequence. The resulting structural model is a leucine/isoleucine zipper (O’Shea et al., 1989; Struhl, 1989) that is coordinated into a parallel dimer of α-helices with the majority of hydrophobic
**Figure 4.13. Three dimensional reconstruction of gemininΔNt.** (a) Representative averages of gemininΔNt particles and their sum obtained by multi-reference alignment and classification. The bar represents 200 Å. (b) GemininΔNt forms a dimer in solution. Recombinant gemininΔNt was crosslinked with BS$_3^3$ or EGS and analysed by SDS-PAGE. Protein concentration was fixed at 10 μg/ml and concentrations of BS$_3^3$ and EGS were increased from 0.5 to 5 mM as indicated by the ascending triangle. (c) Space-filled representation of geminin's dimeric coiled-coil domain based on molecular modelling. Hydrophobic residues such as Leu, Ile and Val are shown in green. (Figure adapted from Okorokov et al., 2004; see Appendix E).
amino acid residues forming the interface between monomers (Figure 4.13c). The diameter of the dimer is ~22 Å, consistent with the diameter of gemininΔNt. To determine the location of this dimer within the full-length geminin tetramer, the atomic model was fitted interactively into the 3D map of geminin using O software (Jones and Kjeldgaard, 1997). This docking procedure indicated that the body domain of geminin could accommodate two dimeric coiled-coil domains, forming a 4-helix bundle domain that consists of two 2-strand parallel coiled-coils with their amino termini facing up towards the head of the molecule (Figure 4.14). The two dimeric coiled-coils form a ‘bouquet’-like shape, at the bottom of which the four helices form a rectangular configuration resembling a classic 4-strand coiled-coil (Walshaw and Woolfson, 2003). As the coiled-coils twist upwards, the two dimers move apart, with the formation resembling two conjoined parallel 3-strand coiled-coils. At this point one α-helix from each dimer appears to interact with the helix from the second dimer, potentially through the formation of salt bridges between glutamine and lysine residues of the coiled-coil domains. At the top of the central body domain the four helices lie almost flat in one plane, consistent with the observed flattened shape of the geminin tetramer. These data therefore allow the central body part of the 3D map of geminin to be assigned to the coiled-coil domain, with a tetrameric, or rather dimer of dimers formation of α-helices. At the top of the body the two dimers diverge to form two neck domains with a large opening between them that is potentially large enough to accommodate DNA. This opening is closed by the two co-joined head domains, containing the amino-terminal residues of geminin that are essential for geminin’s function in neuronal development (Kroll et al., 1998). The tail domain, which contains the carboxy-terminal domains of geminin, appears to be flexible and could not be sufficiently resolved for
Figure 4.14. Structural organisation of the human geminin tetramer. (a) Slice views of the 3D map of human geminin with fitted atomic coordinates of two coiled-coil dimers shown as cartoon α-helices. Left, the horizontal cross-section depicts the internal organisation within the 4-helix bundle domain. Middle, sliced side views on the right side of the panel show the positions of α-helices from each dimer within the molecule's shell. Right, position of the dimer within the molecular envelope. (b) Schematic representation of protein density divided into the independent domains with the protein sequence bar coloured accordingly to the right side. (Figure adapted from Okorokov et al., 2004; see Appendix E).
unambiguous separation of chains.

I wish to reiterate that the experiments described in the previous three pages were performed by A. Okorokov (Department of Pathology, UCL, UK) and that they are included to complement the functional studies described in 4.3.4., 4.3.4., 4.3.6., and 4.3.7., which are entirely the work of this thesis.

4.3.6. The coiled-coil domain may mediate oligomerisation of geminin monomers

Since the structural and cross-linking data indicate that full-length geminin has a tetrameric organisation whilst the 15 kDa fragment has a dimeric structure, it can be postulated that tetramer assembly is mediated by the N-terminus of geminin and that dimerisation may be mediated by residues in the C-terminus of the protein. This hypothesis is further supported by crystallisation studies of the coiled-coil domain which indicate that this domain alone is capable of dimerisation (Thépaut et al., 2002), suggesting that it is residues within the coiled-coil domain that are important for mediating dimerisation. In order to try to elucidate the region within the coiled-coil domain which may mediate this oligomerisation, peptide walking was used to design peptides spanning the coiled-coil domain and their ability to interfere with geminin activity was assayed using the cell-free DNA replication system. Four overlapping peptides were synthesised, encompassing the coiled-coil domain and short surrounding sequences (Figure 4.15). Incubation of G1 nuclei with each of the peptides had no significant effect on DNA replication (Figure 4.16a), consistent with previous findings that the coiled-coil domain is insufficient for inhibition of DNA replication (McGarry and Kirschner, 1998). Incubation of G1 nuclei with recombinant full-length geminin resulted in an approximate 75% reduction in
Figure 4.15. Synthesis of coiled-coil domain peptides. Design of four 20mer peptides containing overlapping regions of the coiled-coil domain.

□ = DEAD Box Domain
■ = Neuralisation Domain
■ = Five Coiled-Coil Domain
Figure 4.16. Oligomerisation of geminin may be mediated by the C-terminal region of the coiled-coil domain. (a) Addition of the coiled-coil domain peptides B1, C1, D1, and E1 to replication reactions containing G1 nuclei and S cytosol had no significant effect on the replication signal. However, in the presence of recombinant geminin (Gm), peptides D1 and E1 restored replication to 85% of that achieved with S cytosol and (b) restored recruitment of Mcm2 to chromatin to a similar level to that obtained with S cytosol. These data suggest that the C-terminal region of the coiled-coil domain may be involved in oligomerisation of geminin and peptides D1 and E1 may act in a dominant-negative manner to disrupt oligomerisation, thereby eliminating the inhibitory activity of geminin.
replication compared to S phase cytosol alone (Figure 4.16a). This decrease in replication was maintained when nuclei were incubated with either peptide B1 or C1 in conjunction with recombinant geminin. However, addition of either peptide D1 or E1 to replication reactions containing full-length geminin restored the replication signal to 85% of that obtained with S phase cytosol alone, indicating that the peptides are able to block the inhibitory action of recombinant geminin. Furthermore, addition of peptides D1 and E1 in conjunction with recombinant geminin resulted in a partial rescue of the recruitment of Mcm2 to chromatin, close to that observed with S cytosol alone (Figure 4.16b). Taken together these data suggest that the region encompassed by peptides D1 and E1, the C-terminal region of the coiled-coil domain, may be critical in mediating the interaction between geminin monomers. In addition these results suggest that dimerisation of geminin may be essential to the ability of geminin to inhibit pre-RC assembly.

4.3.7. The neck region of geminin is critical for mediating inhibition of origin licensing

To identify residues that may be important in mediating geminin’s activity as an origin licensing inhibitor, the sequence and structural data available for geminin were analysed. Based on our 3D structural model of geminin (Figure 4.14; Okorokov et al., 2004; see Appendix E), two potential binding interfaces for Cdt1 can be postulated, the narrow side of the central body domain and the neck region. The narrow side of the central body domain has multiple negative charges on its surface and is equivalent in size to the DNA double helix. It may therefore be able to mimic DNA and occupy the DNA binding pocket of Cdt1. This hypothesis is supported by the finding that the geminin-binding and DNA-binding domains of Cdt1 overlap
Figure 4.17. Identification of a putative Cdt1 binding site in the neck region of geminin. (a) Amino acid sequence alignment of the neck region of geminin in mouse, rat, human, Xenopus, Zebrafish (Danio rerio) and Drosophila (D mel). Sequence analysis identified two highly conserved residues, Y98 and W99 (blue and shaded), which may be involved in mediating the interaction of geminin with Cdt1. (b) Structure of a truncated mouse geminin-Cdt1 complex (Lee et al., 2004) highlighting amino acid residues Y98 and W99. Cdt1 (aa 179-365) is shown in blue, two geminin monomers (aa 79-157) are shown in yellow and green, residues 95 and 96 are highlighted in orange and blue. Note that for full-length geminin two additional geminin monomers would be present to form a tetrameric structure. The Y98 and W99 residues labelled in orange protrude into the opening between the neck regions, whilst the two residues labelled in blue protrude outwards from the loop region, potentially enabling interaction with Cdt1.
Figure 4.18. The neck region of geminin is critical for inhibition of origin licensing. (a) Addition of gemFF to co-incubations of NIH/3T3 G1 nuclei and HeLa S cytosol had no effect on the replication capacity of the nuclei, whereas in control reactions containing wildtype geminin a 50% reduction in replicative capacity was observed. (b) Consistent with its inability to block DNA replication gemFF had no significant effect on chromatin-bound Mcm2 levels compared to S phase cytosol whilst wildtype geminin was able to block Mcm2 binding to chromatin. Interestingly gemFF was still recruited to chromatin to the same extent as wildtype geminin.
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(Yanagi et al., 2002). The neck region of geminin is spaced out from the rest of the molecule and therefore well positioned to engage in protein-protein interactions. Analysis of the sequence homology data for this region of the protein revealed a highly conserved YW motif at residues 98 and 99 (Figure 4.17a). Analysis of the structure of this region of geminin, based on the predicted tetrameric organisation (Okorokov et al., 2004; see Appendix E), indicate that for two geminin monomers these residues protrude into the opening within the geminin molecule, whilst for the other two monomers they protrude outwards (Figure 4.17b), potentially enabling contact with another protein. The presence of aromatic hydrophobic residues on the surface of a protein often indicates an involvement in protein function, for example in protein-protein interactions, since such residues lacking any functional involvement are generally not conserved through evolution. In addition, the structural data indicate that these residues lie in a loop region and are unlikely to interact with each other, suggesting that they may be involved in mediating the interaction of geminin with Cdt1. To determine whether Y98W99 are critical for geminin's ability to repress origin licensing, site-directed mutagenesis was used to create a double phenylalanine mutant at these residues (gemininFF; see Chapter 2, Figure 2.4). The use of phenylalanine residues in the mutant ensures that the hydrophobic nature of the region is maintained and thus geminin should remain structurally intact. However, by removing the hydroxyl and amine groups of tyrosine and tryptophan respectively, there are no longer any functional groups for bonding available and therefore any interaction with Cdt1 that is mediated by this region of geminin may be impeded. To determine whether gemininFF is still functional at inhibiting origin licensing, its activity was analysed using the cell-free replication system. Incubation of G1 nuclei with gemininFF had no effect on the percentage of
nuclei replicating compared to S phase cytosol alone (Figure 4.18a). Furthermore, no reduction in Mcm2 recruitment to chromatin could be detected in the presence of gemininFF (Figure 4.18b). Interestingly however, a similar quantity of gemininFF could be detected on chromatin as wild-type geminin. Taken together these data indicate that the neck region of geminin (in particular residues Y98W99) plays a critical role in mediating the inhibition of origin licensing.

4.3. Discussion and Conclusions

The DNA replication licensing machinery represents an attractive, novel anti-cancer target. The existence of an endogenous inhibitor of origin licensing, geminin, provides a powerful molecular tool to carry out early proof-of-principle studies to validate this target in cell cultures for therapeutic intervention. Moreover, since geminin is a relatively small protein it may provide a useful starting point for the development of small mimetic compounds for use as therapeutic licensing inhibitors. The design of such compounds rests on a detailed biochemical understanding of the mechanism of geminin activity and the identification of the regions of the protein involved in mediating this activity. In this chapter I have analysed the structure-function relationship of geminin in order to further dissect the geminin molecule and identify regions critical for mediating inhibition of origin licensing. To analyse the activity of geminin in vitro, an assay was developed to monitor the recruitment of replication licensing factors onto chromatin. In conjunction with an established in vitro cell-free DNA replication system (Stoeber et al., 1998), the chromatin binding assay provides a highly sensitive ‘dual readout’ assay for analysing geminin activity in vitro. Moreover it has the potential for use as a secondary screen to identify compounds which mimic the activity of geminin as an inhibitor of origin licensing.
The cell-free nature of this assay allows the screening of both cell-permeable and cell-impermeable compounds with straightforward addition of reaction components and no background from cytotoxic compounds. In addition this assay is amenable to automation and has the potential to identify compounds which inhibit DNA replication initiation either by mimicking geminin activity (i.e. inhibition of pre-RC assembly) or by acting at different points in the pathway, for example Cdc7 kinase inhibitors (i.e. blocking origin firing).

Interestingly the ability of geminin to repress origin licensing in nuclei prepared during the G0-S transition provides further evidence to support the observation in Chapter Three that the absence of geminin in G0 may be critical for the re-acquisition of DNA replication competence during re-entry into the cell cycle. Geminin is absent in G0 and is expressed post pre-RC assembly approximately 18-21 hours after release from quiescence (Figures 3.4c and 3.5c). The ability of ectopic geminin to block Mcm2 loading onto chromatin in G1 nuclei prepared from fibroblasts traversing through the G0-S transition, and the resulting failure of these nuclei to initiate DNA synthesis in a cell-free replication assay, suggests that the delayed expression of geminin is critical for successful escape from quiescence. The presence of geminin in quiescence, or its expression early in the G0-S transition prior to pre-RC assembly, would inhibit pre-RC assembly and prevent the re-entry of cells from quiescence back into the cell cycle.

The structural and biochemical analyses of geminin indicate that the full-length protein assembles into a tetrameric ‘dimer-of-dimers’ resembling an elongated inflated key with a large opening between the conjoined amino-terminal domains
Evaluation of the DNA replication licensing machinery as an anti-proliferative target. Sarah Kingsbury

(Okorokov et al., 2004; see Appendix E). This structural organisation of geminin is in keeping with previous reports of its 'heavy molecular weight' behaviour on gel-filtration columns (Hodgson et al., 2002). The tetrameric organisation of full-length geminin is lost upon removal of the N-terminal region of the protein, with the 15 kDa N-terminal deletion mutant forming a dimeric structure (Okorokov et al., 2004; see Appendix E). This mutant provides a particularly useful tool for early proof-of-principle studies since it lacks the DEAD box domain that mediates ubiquitin-dependent proteolysis (McGarry and Kirschner, 1998), thereby rendering it resistant to such degradation. The retention of inhibitory activity by the dimeric fragment of geminin indicates that tetramer formation is not required for inhibition of DNA replication licensing, although it is possible that this additional level of organisation may be required for the protein to act as a neuralisation factor (Kroll et al., 1998). The minimum region of geminin required for activity as a neuralisation factor is amino acids 28-79 (Kroll et al., 1998). Determination of the oligomerisation status of this fragment by cross-linking analysis would therefore allow the minimum level of organisation required for geminin to act as a neuralisation factor to be established. In addition it is possible that the oligomerisation status of geminin may be cell cycle regulated with different complexes present at different stages of the cell cycle. To determine whether changes to the oligomerisation status of geminin occur during transition through the cell cycle, geminin complexes could be immunoprecipitated from synchronised cell populations, separated by gel filtration and analysed by immunoblotting.

A number of studies have implicated the coiled-coil domain (residues 112-147) in the inhibition of DNA replication licensing (McGarry and Kirschner, 1998; Thépaut
et al., 2002, 2004; Benjamin et al., 2004; Saxena et al., 2004), although it is apparent that the coiled-coil domain alone is insufficient for inhibitory activity. This is confirmed by retention of inhibitory activity by the 15 kDa fragment, containing residues 77-212, and the lack of inhibitory activity observed with the 9.7 kDa fragments which in the fully degraded 7 kDa form lacks amino acids 1-94 and 160-219 (Figures 4.8 and 4.9). Taken together with recent studies by other groups, which indicate that residues 70-152 are critical for geminin’s activity as a repressor of origin licensing (Saxena et al., 2004), it is apparent that the region N-terminal to the coiled-coil (residues 77-112), and possibly a short C-terminal sequence (residues 147-152) are critical for mediating inhibition of DNA replication, but that amino acids 152-212 may be redundant. The minimum region required to inhibit DNA replication licensing can therefore be defined as a 75 amino acid fragment containing residues 77-152. The importance of the coiled-coil domain is further supported by the loss of inhibitory activity observed in the presence of peptides representing the C-terminal region of the coiled-coil (Figure 4.16). The mechanism by which peptides D1 and E1 interfere with geminin activity can only be hypothesised from these studies. To elucidate the mechanism by which these peptides block geminin activity, further studies are necessary. To determine whether inhibition of geminin activity is a result of an alteration to the oligomerisation state of the protein, geminin could be synthesised by in vitro transcription-translation in the presence/absence of the peptides. Cross-linking of the reaction products followed by resolution by gel filtration and identification by immunoblotting should reveal changes in the oligomerisation status of geminin in the presence of the coiled-coil domain peptides. However the involvement of the coiled-coil domain in the dimerisation of geminin has been confirmed by other studies, with dimerisation of geminin abolished by
deletion of residues 117-160 (Benjamin et al., 2004) and by point mutation of residues 120, 124, 131 and 134 (Saxena et al., 2004). Taken together these data suggest that residues within this region are critical for mediating interaction between geminin monomers and that organisation of geminin monomers into a dimeric structure may be essential for inhibition of origin licensing.

Repression of origin licensing by geminin relies on its ability to interact with Cdt1 (Wohlschlegal et al., 2000; Tada et al., 2001; Benjamin et al., 2004; Saxena et al., 2004). The structural data presented here (Okorokov et al., 2004; see Appendix E) and mutational analyses carried out by other groups suggest that two regions may be required for interaction between the two proteins (Benjamin et al., 2004; Saxena et al., 2004). Residues 100-140, which lie within the coiled-coil domain, are essential for interaction with Cdt1 (Benjamin et al., 2004), although structural analysis of the interaction between mouse geminin and mouse Cdt1 indicates that residues 129-149 (residues 134-154 of human geminin) are required for inhibition of DNA replication but not interaction with Cdt1 (Lee et al., 2004). Interestingly a mutant containing residues 92-152 is capable of interacting with Cdt1 but not inhibiting replication (Saxena et al., 2004). This may be due to the requirement of an additional region of geminin that does not interact with Cdt1, which acts as a bulky mask to prevent access of the MCM complex to Cdt1 by steric hindrance (Lee et al., 2004). The surface of the coiled-coil domain is highly negatively charged suggesting that the interaction between the coiled-coil domain of geminin and Cdt1 may be mediated by strong electrostatic interaction. Elimination of the negatively charged residues on the surface of the coiled-coil domain inhibited the interaction of geminin with a fragment of Cdt1, but not with full length Cdt1 indicating that a second interaction also takes
place (Saxena et al., 2004). Interestingly, the interaction between geminin and Cdt1 was also blocked when dimerisation of geminin was inhibited (Saxena et al., 2004).

The structural data presented here suggested that the second region of geminin required for interaction with Cdt1 lies in the region N-terminal to the coiled-coil domain (Okorokov et al., 2004; see Appendix E). Consistent with this hypothesis, mutation of residues Y98 and W99 in the work of this thesis abolished the ability of geminin to inhibit DNA replication licensing in vitro, suggesting that an interaction between the two proteins may have been impeded (Figure 4.18). In this mutant both residues were substituted with phenylalanine residues thereby ensuring that the structural integrity of the protein is maintained, but that any hydrogen bonds between the side chains of these two amino acids are abolished. Structural analysis of this region suggests that it may form a bulky mass (Figure 4.17a) and could either act as a hook to interact with Cdt1 or block the interaction of Cdt1 with the MCM helicase or DNA by steric hindrance. Analysis of the ability of this mutant to interact with Cdt1 was beyond the scope of this thesis, however recent data suggest that mutation of this region does not abolish Cdt1 binding, but does eliminate inhibition of DNA replication licensing (Benjamin et al., 2004). It is unclear from this study, however, whether the binding affinity of this mutant with Cdt1 is altered with respect to the wild-type protein. The ability of this mutant to interact with Cdt1 to some extent is further supported by the finding that gemininFF is still recruited to chromatin (Figure 4.18), whilst additional studies suggest that although two interactions are required for inhibition of DNA replication, only one interaction is necessary for association of the two proteins (Saxena et al., 2004). Given that similar results are obtained when residues 98 and 99 are substituted for either phenylalanine (Figure 4.18) or alanine (Benjamin et al., 2004), and that phosphorylation of Y98 is not critical for activity
(Benjamin et al., 2004), it can be speculated that the functional hydroxyl and/or amine groups may be important for the interaction between geminin and Cdt1. Without the potential H-bond type interactions mediated by these residues the interaction between Cdt1 and geminin may be destabilised, thereby eliminating the inhibitory activity of the protein.

The thermodynamics of protein-protein interactions can be determined using high-sensitivity isothermal titration calorimetry (ITC). This technique allows the binding equilibrium between two molecules to be measured directly by determining the heat evolved on association of a ligand with its binding partner (Pierce et al., 1999). Protein-protein interactions can also be monitored by surface plasmon resonance (SPR), a technique in which one interaction partner is conjugated to the surface of a sensor chip and the other flows over the surface allowing interactions to be measured in real-time. Binding events result in changes in protein mass that, in turn, alter surface plasmon resonance, an electromagnetic phenomenon that dampens the intensity of light reflected off the surface of the sensor chip at a specific angle (Myszka, 1997). It would therefore be of interest in future work to use a combination of ITC and SPR to establish whether the Cdt1-geminin interaction is disturbed in the gemininFF mutant.

Changes to the stability of the geminin-Cdt1 complex may also affect the stability of the interaction of gemininFF with chromatin. This could be tested by altering the methodology for isolating chromatin-bound proteins from nuclei. For the experimental work presented in this chapter, chromatin-bound proteins were removed using treatment with DNase I and NaCl, a treatment which removes the
majority of chromatin-bound proteins including those which are strongly attached. However, by using a stepwise approach to remove proteins from chromatin, progressing from gentle to more stringent conditions, differences in the stability of attachment may be identified.

In summary, these studies have identified a 15 kDa fragment of geminin which retains full activity as an origin licensing inhibitor, but which lacks the DEAD box domain and should therefore be resistant to ubiquitin-mediated degradation. This fragment therefore provides a powerful molecular tool for carrying out further proof-of-principle studies evaluating the potential of the replication licensing machinery as a therapeutic target (see Chapter 6). However, the observation that a 15 kDa fragment of geminin is required for activity, that oligomerisation may also be essential for activity and that interaction between geminin and Cdt1 may involve multiple contacts suggests that the geminin blueprint may not be suited to the design of small molecule mimetics using medicinal chemistry approaches. Nonetheless the potential for the development of geminin mimetics cannot be completely discarded without further dissection of the geminin molecule and the complex that it forms with Cdt1. Future work may elucidate a mechanism by which the licensing of DNA can be therapeutically inhibited. Furthermore it is also interesting to note that although traditionally small molecules have provided the majority of anti-cancer agents, the emergence of targeted therapies has led to the expansion of molecules utilised for therapy including antibodies (e.g. Herceptin) and oligonucleotides (e.g. Oblimersen Sodium) (see also Section 1.4.). Therefore it can be envisioned that with the right delivery strategy the 15 kDa fragment of geminin (gemininΔNt) may provide a mechanism for therapeutically inhibiting origin licensing in itself, thereby
eliminating the need to develop small molecule or peptide mimetics. Further to providing a blueprint for the design of origin licensing inhibitors, geminin also represents a powerful molecular tool for early proof-of-principle studies evaluating the replication licensing machinery as a therapeutic target. In Chapter Six, geminin and the non-degradable 15 kDa fragment are exploited to study the response of normal and transformed cells to origin licensing repression.

In addition to endogenous regulators of cellular processes (for example geminin) exogenous and often pathogenic regulators such as viral proteins also exist. In order for viruses to propagate within their host environment, they utilise normal host cell activity to their own advantage. As a result, many viral proteins interact with cellular proteins and interfere with cellular processes, for example the human papilloma virus (HPV) E6 protein interacts with p53 whilst HPV E7 interacts with pRb, resulting in uncoupling of cellular growth and proliferation (Cheng et al., 1995). Recently a novel interaction between HPV1 E4 and two replication licensing factors, Mcm7 and Cdc6, was identified (S. Roberts, CR-UK Institute for Cancer Studies, University of Birmingham, UK; personal communication). These findings suggest that HPV1 E4 may impede cellular DNA replication in order to promote viral genome amplification. In Chapter Five I describe a functional analysis of the HPV1 E4 protein and the effect that its interaction with Mcm7 and Cdc6 has on DNA replication licensing. Since E4 is a small protein of only 17 kDa, it can be postulated that, like geminin, it may provide a starting point for the development of alternative mimetic compounds. It is also intriguing to note that since E4 and geminin target the same cellular process, together they represent a dual strategy for inhibition of origin licensing which may provide an opportunity for combination therapy.
CHAPTER FIVE

HPV1 E4 INHIBITS DNA REPLICATION LICENSING

5.1. Introduction

Initiation of DNA replication is dependent on sequential assembly of macromolecular pre-replicative complexes at origins of replication, resulting in chromatin being licensed for replication (see also Chapter 1, Section 1.5.). A key step in this process is recruitment of the MCM complex onto chromatin by the replication licensing factors Cdc6 and Cdt1 (Kelman et al., 1999; Chong et al., 2000; Labib et al., 2000; Lee and Hurwitz, 2000, 2001; Shechter et al., 2000; Labib and Diffley, 2001). Prevention of MCM recruitment to pre-RCs is a potential mechanism by which DNA replication initiation could be therapeutically inhibited (see also Section 4.1.). Two strategies by which Mcm2-7 could be prevented from assembling into pre-RCs include targeting the two parallel loading pathways, mediated by Cdc6 and Cdt1, which act in concert to recruit MCM onto chromatin. In Chapter Four, I discussed a biochemical analysis of the endogenous origin licensing inhibitor geminin, which blocks pre-RC assembly through its interaction with Cdt1. These studies have shed further light on the regions of geminin that are essential for interacting with Cdt1 and for blocking origin licensing, and suggest that an agent mimicking the activity of geminin may have potential as a therapeutic origin licensing inhibitor. Although no endogenous proteins are known to directly block the activity of Cdc6 in recruiting MCM onto chromatin, recent unpublished work has established an association between the human papillomavirus-1 (HPV1) E1^E4 protein (N.B. from hereon in the E1^E4 protein will be referred to as E4), Cdc6 and Mcm7 (Sally Roberts, CR-UK Institute for Cancer Studies, University of...
A key feature of viral infection is the disruption of normal host cell activity for the benefit of viral reproduction. The study of viral infection has thus greatly enhanced our understanding of normal cellular processes. For example, the tumour suppressor protein p53 was first identified as a complex with SV40 T-antigen in SV40-transformed cells (Lane and Crawford, 1979) whilst the existence of oncogenes was first realised following the identification of the Rous Sarcoma virus protein v-src (Rous, 1973). Moreover, in addition to furthering our understanding of biological processes, viruses have provided us with molecular tools for the manipulation of these processes. By understanding the mechanisms by which viruses disrupt host cell behaviour and the underlying protein-protein interactions, these activities may be exploited for drug discovery and development. For example, the ability of HIV Tat1 to transduce biological membranes has been harnessed for transportation of biomolecules into intact cells (see also Chapter 6 and Appendix B), whilst manipulation of viruses for gene transport has led to the emerging field of gene therapy as a novel therapeutic strategy (Young et al., 2006). ONYX-015 (dl1520), a replication competent adenovirus that lacks the ELB 55K gene and is therefore only able to replicate in cells with deficient p53 activity, is currently under development as a novel chemotherapeutic strategy (Heise et al., 1997; see also Section 1.4.). Although initial clinical trials with ONYX-015 have been disappointing (Reid et al., 2001; Vasey et al., 2002; Hamid et al., 2003; Hecht et al., 2003), these studies establish the principle for the manipulation and exploitation of viruses and viral proteins as a therapeutic strategy.

Two recent reports argue that viruses may engage with the cellular DNA replication
licensing machinery in order to support replication of their own genome. Infection by cytomegalovirus inhibits assembly of the pre-RC by promoting accumulation of geminin in infected cells (Biswas et al., 2003), as well as through the action of a viral protein, IE2 (Wiebusch et al., 2003). Although the underlying mechanism of repression of licensing by cytomegalovirus is unclear, these findings suggest that inhibition of cellular DNA replication could prove advantageous to viruses that depend on the host cell for the supply of essential host replication enzymes and nucleotides for viral DNA synthesis. Modulation of pre-RC activity has also been demonstrated for Epstein-Barr virus, although in this instance the virus has been shown to recruit cellular replication proteins, such as ORC and MCM, to its own origins in order to replicate its genome (Chaudhuri et al., 2001, Dhar et al., 2001). In the context of these studies, the finding that HPV1 E4 associates with Cdc6 and Mcm7 suggests that one of the roles of E4 in the viral life cycle may be the inhibition of cellular DNA replication licensing. In support of this hypothesis, addition of E4 to in vitro replication reactions resulted in loss of replicative capacity whilst microinjection of quiescent NIH/3T3 fibroblasts with an E4-expressing plasmid blocked transition into S phase (K. Stoeber, UCL, UK; personal communication).

Human papillomaviruses are small DNA tumour viruses that infect squamous epithelium and are associated with a broad range of clinical manifestations, including common hand and foot warts, genital condylomas and invasive cancers. The HPV replication cycle has an intimate relationship with epithelial cell differentiation (Fehrmann and Laimins, 2003). Thus, while HPV early gene expression occurs in proliferating and differentiating layers of the epithelium, vegetative viral genome replication, late gene expression, and assembly of new virions are restricted to differentiated cells. To support vegetative viral replication in non–dividing
differentiated cells, the early HPV proteins E6 and E7, through their interactions with p53 and pRb respectively, deregulate cell cycle progression and activate the host's DNA replication machinery (Cheng et al., 1995). Host cell replication fork assembly and activation would deplete the nucleotide pool and replication enzymes that are required by HPV for viral genome amplification, thereby interfering with the productive stages of the viral life cycle. The early E4 protein, produced from a spliced messenger RNA product E1^E4 that encodes five amino acids from the E1 open-reading frame (ORF) spliced to the protein encoded by the E4 ORF, accumulates to a high level at the onset of vegetative HPV genome amplification and persists in cells containing newly synthesised virions (Peh et al., 2002). As the viral life cycle progresses the 17 kDa spliced gene product is progressively proteolytically cleaved to produce 16 kDa, 11 kDa and 10 kDa species of E4 (Figure 5.1; Doorbar et al., 1988; Roberts et al., 1994a). In addition to modification by proteolysis, E4 is also modified by phosphorylation and self-multimerisation, with at least eight prominent species of E4 detected as protein doublets in HPV1 warts (Croissant et al., 1985; Doorbar et al., 1986; Grand et al., 1989). Multiple functions have been proposed for the E4 protein including inhibition of terminal differentiation to preserve cellular integrity and enhance virus synthesis (Rogel-Gaillard et al., 1993), involvement in vegetative viral DNA replication (Jareborg and Burnett, 1991), control of virus maturation (Doorbar et al., 1986; Palefsky et al., 1991) and mediation of virus release from the epithelial squames by disruption of the cytoskeleton (Doorbar et al., 1991). Cells containing papillomavirus genomes that are unable to express full-length E1^E4 proteins do not support amplification of the viral genome or S phase maintenance following cell differentiation (Peh et al., 2004; Wilson et al., 2005). The association of Mcm7 and Cdc6 with E4 and the ability of E4 to inhibit DNA replication in vitro raise the hypothesis that one of the roles of E4 in the viral life
Figure 5.1. Expression and processing of the HPV1 E4 protein throughout the viral life cycle. (a) HPV1 E4 is expressed as a 17 kDa spliced gene product containing the 5 N-terminal amino acids from the E1 ORF and is proteolytically cleaved during the viral life cycle to produce 16 kDa, 11 kDa and 10 kDa species of the protein. Full-length E4 contains 3 domains, an N-terminal leucine rich region which may be involved in interfilament interaction, an adjacent proline-rich region that is essential for mediating G2 arrest and a C-terminal homology domain. (b) HPV1 E4 is initially expressed in the parabasal cells coincident with the onset of vegetative viral genome amplification. The 16, 11 and 10 kDa species of the protein accumulate in a processive manner through the viral life cycle, with the more processed forms abundant in the superficial keratinocytes in which capsid protein expression (L1) and virion assembly occurs.
cycle may be to modulate cellular pre-RC function as a means of promoting viral DNA replication. These data suggest that E4-based mimetic compounds could potentially provide novel non-genotoxic anti-proliferative agents. In this chapter the preliminary studies by K. Stoeber have been extended to evaluate the potential of E4 as an inhibitor of DNA replication utilising the \textit{in vitro} cell-free DNA replication assay and, in particular, the chromatin-binding assay that was established in Chapter Four (see Section 4.3.1; Figure 4.3). In addition, the E4 molecule has been further dissected to identify regions of the protein critical for inhibition of DNA replication. Where necessary for the connected argument, the genetic, biochemical, and immunofluorescence investigations performed by S. Roberts are discussed.

5.2. Materials and methods

For Transformation of \textit{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.), Antibodies (2.3.1.), Expression of recombinant HPV1 E1\(^{\wedge}\)E4, E1\(^{\wedge}\)E4R45A and E1\(^{\wedge}\)E4\(\Delta\)44-48 in \textit{E. coli} (2.3.4.), Expression of recombinant \textit{XeCdc6} in Sf9 insect cells (2.3.5.), Purification of His\(_6\)-HPV1 E1\(^{\wedge}\)E4, His\(_6\)-E1\(^{\wedge}\)E4R45A and His\(_6\)-E1\(^{\wedge}\)E4\(\Delta\)44-48 (2.3.7.), Purification of baculovirus-expressed His\(_6\)-\textit{XeCdc6} (2.3.8.), SDS-polyacrylamide gel electrophoresis (2.3.12.), Coomassie Blue staining of SDS-PAGE gels (2.3.13), Immunoblotting (2.4.1.), Preparation of nuclei and cytosolic extracts (2.6.1.), Assaying for DNA synthesis \textit{in vitro} (2.6.2.), Analysis of \textit{in vitro} DNA synthesis products by confocal fluorescence microscopy (2.5.5.) refer to Chapter Two (Materials and Methods). For Expression and
purification of recombinant HPV1 E4 proteins (B.2.1.), *In vitro* binding assays (B.2.2.), Infection of keratinocytes and immunoprecipitations (B.2.3.), and Immunofluorescence (B.2.4.) see Appendix B.

5.3. Results

I wish to clearly state that the experiments described in 5.3.1. are entirely the work of S. Roberts (CR-UK Institute for Cancer Studies, University of Birmingham, UK). These unpublished data are included here as part of the connected argument because they provide the rationale for the functional studies described in 5.3.2., 5.3.3., 5.3.4. and 5.3.5. performed entirely by myself in the work of this thesis.

5.3.1. *HPV1 E4 associates with DNA replication licensing factors*

To identify cellular targets of HPV E4, a yeast two-hybrid analysis of an HaCaT keratinocyte cDNA library (Boukamp *et al*., 1988) was performed using a fusion protein between the DNA binding domain of GAL4 and the HPV1 E1\(^{+}\)E4 protein (amino acids 2 to 125), GAL4DB-E4, as the "bait". Eight out of 11 colonies carried partial cDNAs encoding a C-terminal fragment (128 amino acids) of the DNA replication licensing factor Mcm7.

To establish whether HPV1 E4 interacts with Mcm7 *in vitro*, baculovirus-expressed E4 protein was incubated with *in vitro*-translated Mcm7 (amino acids 117 to 719) and protein complexes isolated with a HPV1 E4 monoclonal antibody (MAb 4.37; Doorbar *et al*., 1988) bound to protein G-sepharose. Radiolabelled Mcm7 could be detected bound to the E4 matrix, while no Mcm7 binding could be detected bound to a control matrix without E4 protein (Figure 5.2a), confirming an interaction between
Figure 5.2. HPV1 E4 associates with Mcm7 and Cdc6 in vitro and in human keratinocytes. (a) In vitro-binding assays between $^{[35]}$S-Mcm7 and recombinant HPV1 and HPV16 E4 proteins. (b) Immunoblot of E4 immunoprecipitates prepared from nuclear fractions of SV40 immortalised keratinocytes infected with Ad-1E4 or Ad-βGal and blotted for E4, Cdc6 and Mcm7 proteins. (c) Immunofluorescence microscopy of Ad-1E4-infected SV40-immortalised keratinocytes, dual-stained for E4 (green) and Mcm7 (red) or Cdc6 (red). Nuclei were visualised using DAPI (4', 6'-diamidino-2-phenylindole; blue). Individual z-images were electronically merged to detect co-localisation (yellow). Mcm7 and Cdc6 co-localise with nucleolar E4 and co-localisation is maintained in cells pre-treated (1 min) with 0.1% Triton X-100 (lower panel). Co-localisation between nucleolar E4 and Mcm7 and Cdc6 following Triton X-100 pre-treatment was also detected using different Mcm7 and Cdc6 antibodies (inset panels). Specificity of anti-E4 is shown by absence of staining in cells not infected by Ad-HPV1E4 and by absence of staining in Ad-βGal-infected cells (data not shown).
HPV1 E4 and Mcm7 in vitro. To investigate whether this interaction was specific to HPV1 E4, binding studies were repeated with HPV16 E4, expressed as a glutathione-S-transferase (GST) fusion protein (GST-HPV16E4). GST-HPV16E4 bound radiolabelled Mcm7, but no Mcm7 could be detected bound to GST or an irrelevant GST-fusion protein (GST-FZA-B). Taken together these data demonstrate that the E4 proteins from HPV types 1 and 16 associate with Mcm7 protein in vitro.

The target cell type for infection by HPVs is keratinocytes. To determine whether E4 associates with endogenous Mcm7 in intact cells, immunoprecipitations were performed in extracts from SV40-immortalised skin keratinocytes (SVJD) (Roberts et al., 1993) infected with Ad-1E4 or a control virus expressing β-galactosidase (Ad-βGal). Mcm7 could only be detected in E4 immunoprecipitates of soluble nuclear fractions isolated from cells infected with the E4-expressing virus and not the control virus (Figure 5.2b). The interaction of E4 with Mcm7 raises the question as to whether E4 may also interact with other replication licensing factors. Further analysis of the immunoprecipitates revealed complexes containing the MCM loading factor Cdc6. E4-Cdc6 and E4-Mcm7 protein complexes were also immunoprecipitated from Ad-HPV1E4-infected human cervical intraepithelial neoplasia (CIN) 612-9E keratinocytes (Sally Roberts, personal communication; Meyers et al., 1992). Taken together these data show that HPV1 E4 associates with the cellular replication initiation factors Cdc6 and Mcm7 in human keratinocytes.

The low levels of Mcm7, and in particular Cdc6, in E4 complexes suggest that only subpopulations of E4, Cdc6 and Mcm7 may be associated. This hypothesis is supported by immunofluorometric analysis of E4, Cdc6 and Mcm7 protein distribution in SV40-immortalised human epidermal keratinocytes following
infection with a recombinant adenovirus expressing HPV1 E1^E4 (Ad-1E4). Consistent with previous studies (Roberts et al., 1993, 2003), the E4 protein was found in both the nucleus and the cytoplasm and co-localises with the keratin cytoskeleton. Although there was no evidence for re-distribution of Mcm7 to E4-keratin filaments, Mcm7 was found to be co-localised with E4 in the nucleolus (Figure 5.2c, upper panel) and co-localisation between nucleolar E4 and Mcm7 was also evident in a small percentage of cells in which E4 expression was restricted to the nucleus. Localisation of Mcm7 to the nucleolus in the absence of E4 indicates that this subnuclear distribution of Mcm7 is not E4-mediated. Cdc6 was also found to co-localise with nucleolar E4 in Ad1-E4-infected keratinocytes (but only in a small proportion (< 5%) of the E4-positive cells) and this association was maintained following extraction of cytoplasmic E4 with 0.1% Triton (Figure 5.2c, lower panel). Co-localisation of nucleolar E4 with these replication licensing factors was verified by using more than one Mcm7 and Cdc6 antibody (Figure 5.2c, inset in lower panel). Taken together these data suggest that subpopulations of E4, Cdc6 and Mcm7 associate in human keratinocytes.

I wish to reiterate that the experiments described in the previous three pages were performed by S. Roberts (CR-UK Institute for Cancer Studies, University of Birmingham, UK) and that they are included to introduce the following functional studies, which are entirely the work of this thesis.

5.3.2. HPV1 E4 inhibits the initiation but not elongation of DNA replication in a cell-free DNA replication system

The association of HPV1 E4 with the replication licensing factors Mcm7 and Cdc6 and the preliminary functional studies performed by K. Stoeber (Department of
Pathology, UCL, UK) indicate that an activity of E4 in the viral life cycle may be the modulation of cellular pre-RC function. To test this hypothesis, the effects of baculovirus- and bacterially-expressed E4 on DNA replication were assayed using the cell-free DNA replication assay. As described in Chapter Three, in cultured NIH/3T3 fibroblasts chromosomal replication origins are licensed for DNA synthesis during a defined "window of opportunity" between 16 and 18 hours after release from density-dependent growth arrest (Chapter 3, Figure 3.5c). Nuclei prepared during this period of pre-RC assembly have the capacity to complete origin licensing and to initiate DNA replication when incubated in HeLa S phase cytosol.

Incubation of G1 nuclei prepared 16.25 hours after release from quiescence (G0) in elongation buffer resulted in 2% of the nuclei synthesising DNA (Figure 5.3). In contrast 19% of nuclei were able to replicate DNA upon incubation in S phase cytosol, and addition of recombinant baculovirus-expressed E4 (final concentration 3 μM; a kind gift from S. Roberts, CR-UK Institute for Cancer Studies, University of Birmingham, UK) had no effect on the replication of G1 nuclei (20%). The co-immunoprecipitation and co-localisation data from Ad-HPV1E4-infected keratinocytes (Figure 5.2) show that only a subpopulation of Cdc6 molecules associates with E4, suggesting that Cdc6 may be required for a putative E4 function. As described in Chapter Three, during the G0 to S phase transition Cdc6 protein is synthesised de novo and is entirely chromatin-bound in G1 nuclei at the point of their preparation (Chapter 3, Figure 3.5c). However, in normal cycling cells Cdc6 can be found in both soluble and chromatin-bound protein fractions (Saha et al., 1998; Fujita et al., 1999; Jiang et al., 1999; Petersen et al., 1999). Hence to reconstitute

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1 Although the work described in 5.3.2. has previously been performed by K. Stoeber (Department of Pathology, UCL, UK), it was independently repeated in the work of this thesis since these experiments form the groundwork for the studies described in 5.3.3., 5.3.4., and 5.3.5.
Figure 5.3. HPV1 E4 inhibits initiation of DNA replication. Addition of baculovirus-expressed E4 to co-incubations of G1 nuclei and Hela S cytosol had no effect on DNA replication whilst addition of recombinant XeCdc6 had a slight stimulatory effect. Addition of both E4 and Cdc6 together resulted in a 50% drop in the replication potential of G1 nuclei. Results are expressed as the percentage of nuclei replicating and summarised in the histogram (mean ± SD).
more closely the microenvironment encountered by HPVs in proliferating cells, His6-XeCdc6 (see Chapter 2, Figure 2.6) was added to replication reactions in addition to E4. Consistent with previous findings that Cdc6 is a rate limiting factor in the initiation of DNA replication (Stoeber et al., 1998), addition of 0.65 μM of Cdc6 caused a 4% increase in DNA replication in G1 nuclei. Importantly in contrast to E4 alone, addition of E4 in conjunction with exogenous Cdc6 to co-incubations of G1 nuclei and S phase cytosol resulted in a 50% reduction in replication from 19% to 11% (Figure 5.3).

NIH/3T3 fibroblasts progress into S phase 21-23 hours after release from density-dependent growth arrest (Chapter 3, Figure 3.5). Once DNA replication has been initiated, agents which interfere with origin licensing can no longer prevent DNA replication, as demonstrated with the endogenous origin licensing repressor geminin in Chapter Four (Figure 4.6b), whilst agents that inhibit elongation DNA synthesis still remain effective. Hence by altering the time at which nuclear templates are prepared the cell-free DNA replication system can be used to determine whether inhibition of replication occurs at the initiation or elongation stage (see also Chapter 4, Section 4.3.3). To determine whether, as is the case for geminin, E4 is unable to prevent elongation DNA synthesis in vitro, replication reactions were repeated using NIH/3T3 S phase nuclei prepared 23 hours after release from G0 as the source of template. Elongation of DNA replication was sufficiently supported upon incubation of these nuclei in either elongation buffer (Buffer A; 55.6% of nuclei replicating) or S phase cytosol (57.2% of nuclei replicating) (Figure 5.4). Importantly, elongation was not effected by addition of recombinant E4 (final concentration 3 μM) either on its own (57.3% of nuclei replicating) or in conjunction with Cdc6 (55.8% of nuclei
Figure 5.4. HPV1 E4 fails to arrest ongoing DNA synthesis. Elongation of DNA replication in NIH/3T3 S phase nuclei prepared 23 hours after release from G0 was sufficiently supported in either elongation buffer (Buffer A) or S phase cytosol. Addition of E4 on its own or in conjunction with exogenous Cdc6 had no effect on the replication potential of the S phase nuclei. Results are expressed as the percentage of nuclei replicating and summarised in the histogram (mean ± SD).
replicating). Taken together these data demonstrate that E4 specifically inhibits the initiation of DNA replication but fails to arrest ongoing DNA synthesis.

5.3.3. **HPV1 E4 blocks the recruitment of replication licensing factors onto chromatin**

For the future design of E4-mimetic compounds as anti-proliferative agents, it is essential to elucidate the mechanism by which E4 inhibits the initiation of DNA replication. To determine whether the association of E4 with Cdc6 and Mcm7 blocks the recruitment of replication licensing factors onto chromatin, G1 and S phase nuclei were taken through *in vitro* replication reactions as described above, and chromatin-bound protein fractions resolved by gel electrophoresis and probed for Mcm2, Mcm7, Cdc6, E4 and Histone H1 as a loading control (Figures 5.5 and 5.6). Consistent with the results obtained for geminin in Chapter Four (Figure 4.7a), a small amount of Mcm2 and 7 are detectable bound to chromatin in G1 nuclei incubated in elongation buffer, whilst incubation in S phase cytosol results in recruitment of replication licensing factors to chromatin *in vitro* and an approximate two-fold increase in chromatin-bound Mcm2 and Mcm7 levels (Figure 5.5). In keeping with the findings from the *in vitro* replication reactions addition of recombinant E4 had no effect on the recruitment of replication licensing factors to chromatin, whilst addition of Cdc6 resulted in a further increase in recruitment of MCM and Cdc6 proteins to chromatin. Importantly addition of both Cdc6 and E4 to the reaction resulted in an approximate 50% reduction in recruitment of MCM proteins to chromatin, whilst levels of Cdc6 remained unchanged. E4 could not be detected associated with chromatin in any of the reactions, indicating that interactions between E4, Mcm7 and Cdc6 occur prior to recruitment of these
Figure 5.5. HPV1 E4 blocks recruitment of replication licensing factors to chromatin *in vitro*. Immunoblots of chromatin-bound protein fractions prepared from NIH/3T3 G1 phase nuclei taken through *in vitro* replication reactions. Addition of E4 and Cdc6 to co-incubations of G1 nuclei and HeLa S cytosol caused a 3.5-fold and 2-fold reduction in chromatin-bound Mcm2 and Mcm7 levels respectively, compared to addition of Cdc6 alone. Addition of E4 alone had no effect on chromatin-bound levels of Mcm2 and Mcm7. E4 could not be detected associated with chromatin and no changes in the level of exogenous chromatin-bound Cdc6 could be detected. Densities of protein bands were measured using ImageJ and are summarised in the histogram expressed relative to the band intensities for S cytosol.
Figure 5.6. HPV1 E4 is unable to alter levels of chromatin-bound replication licensing factors in S phase nuclei. Immunoblots of chromatin-bound protein fractions prepared from NIH/3T3 S phase nuclei taken through in vitro replication reactions. In S phase nuclei pre-RCs have fully assembled and initiation of DNA replication has already occurred prior to preparation of nuclear templates. Hence similar levels of chromatin-bound replication licensing factors are observed in co-incubations with Buffer A or HeLa S phase cytosol. Consistent with the inability of HPV1 E4 to inhibit elongation DNA synthesis, addition of E4 alone, or in conjunction with endogenous Cdc6, had no effect on the chromatin-binding status of Mcm2, Mcm7 or Cdc6. Densities of protein bands were measured using ImageJ and are summarised in the histogram expressed relative to the band intensities for S cytosol.
Evaluation of the DNA replication licensing machinery as an anti-proliferative target. Sarah Kingsbury

replication licensing factors to chromatin. An association between soluble Cdc6, Mcm7 and E4 is further supported by the requirement for exogenous soluble Cdc6 for E4’s inhibitory activity and the inability of E4 to affect the level of endogenous chromatin-bound Cdc6. Furthermore, protein complexes containing Cdc6 and Mcm7 were immunoprecipitated by an anti-E4 antibody (Doorbar et al., 1988) from soluble extracts of S phase HeLa cells containing exogenous recombinant HPV1 E4, confirming that the physical interaction between these proteins can occur in the absence of chromatin (S. Roberts; personal communication). Consistent with the inability of E4 to inhibit elongation DNA synthesis, E4 had no effect on chromatin-bound levels of Cdc6, Mcm2 or Mcm7 in NIH/3T3 S phase nuclei (Figure 5.6). Taken together these data suggest that E4 inhibits DNA replication licensing by blocking MCM loading onto chromatin.

To independently verify the in vitro replication data, identical reactions were carried out using bacterially-expressed E4 (see Chapter 2, Figure 2.5). Consistent with the results obtained for baculovirus-expressed E4, no reduction in replication was observed with E4 alone, whilst a similar reduction in replication was observed in the presence of exogenous Cdc6 (Figure 5.7a). Furthermore, addition of bacterially-expressed E4 to replication reactions in conjunction with exogenous Cdc6 blocked the recruitment of Mcm2 and Mcm7 onto chromatin (Figure 5.7b).

5.3.4. The N-terminal region of E4 is essential for the inhibition of DNA replication

As a prerequisite for future design of E4 mimetic compounds as anti-proliferative agents, it is essential to identify the regions within the E4 protein which are responsible for its activity in blocking origin licensing. During the HPV life cycle
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Figure 5.7. Inhibition of DNA replication and recruitment of replication licensing factors to chromatin by bacterially-expressed HPV1 E4. To demonstrate the specificity of replication inhibition, bacterially-expressed HPV1 E4 was added to co-incubations of G1 nuclei and S phase cytosol on its own, and with Cdc6. Consistent with the results obtained for baculovirus-expressed E4, no reduction in replication or in the recruitment of replication licensing factors to chromatin was observed with E4 alone, whilst a similar reduction in replication and Mcm2/7 recruitment to chromatin was observed in the presence of exogenous Cdc6 and E4.
E4 undergoes progressive N-terminal proteolysis producing smaller species of the protein, mediating different activities either as monomers or through the production of different hetero- and homodimers (Doorbar et al., 1988; Roberts et al., 1994a). The first such naturally occurring proteolytic cleavage removes the 15 N-terminal amino acids of the protein to produce a 16 kDa species of E4 (Figure 5.1). To determine whether these 15 amino acids are critical for the activity of E4 as an inhibitor of origin licensing, the activity of an N-terminal deletion mutant (ΔE4, a kind gift from S. Roberts, CR-UK Institute for Cancer Studies, University of Birmingham, UK), which is equivalent to the 16 kDa species of E4, was analysed using the cell-free DNA replication system. As with full-length E4, addition of ΔE4 alone to in vitro DNA replication reactions had no effect on the replication potential of G1 nuclei (Figure 5.8a). However when added to replication reactions together with Cdc6, ΔE4 failed to inhibit DNA replication initiation and did not prevent the assembly of both Mcm2 and Mcm7 onto chromatin (Figure 5.8b). Taken together these data indicate that the highly conserved N-terminal region of the E4 protein is critical in mediating its activity as an origin licensing inhibitor. Inhibition of DNA replication may therefore be specific to the full-length version of E4 which occurs earliest in HPV infection and may be turned off later in the viral life cycle by proteolytic cleavage of the protein, when capsid expression and virion assembly are initiated.

5.3.5. A proline rich sequence in E4 is necessary for inhibition of origin licensing

Expression of the 16 kDa form of HPV1 E4 in keratinocytes has recently been shown to arrest cells in G2 phase of the cell cycle with a small population of cells re-entering the cell cycle and continuing to replicate their DNA without intervening
Figure 5.8. The highly conserved N-terminal region of E4 is essential for inhibition of DNA replication. Addition of a baculovirus-expressed N-terminal deletion mutant (ΔE4) to co-incubations of NIH/3T3 G1 nuclei with HeLa S phase cytosol had no effect on either (a) replicative capacity of the G1 nuclei or (b) recruitment of replication licensing factors to chromatin either on its own or in conjunction with exogenous Cdc6.
cytokinesis (Knight \textit{et al.}, 2004). Interestingly, when full-length E4 was co-expressed with the 16 kDa species, the G2/M arrest was maintained but re-replication was repressed. Mutational analysis of full-length E4 suggested that amino acids 44-48, and in particular R45, may be important for mediating this repression of DNA synthesis (Gillian Knight and Sally Roberts, CR-UK Institute for Cancer Studies, University of Birmingham, UK; personal communication). To test whether these residues are involved in mediating the block to replication licensing that is observed with full-length E4 \textit{in vitro}, the activity of two E4 mutants, E4Δ44-48 and E4R45A (see Chapter 2, Figure 2.5) were analysed in the cell-free DNA replication assay. As with full-length wild type E4 (Figure 5.3), incubation of G1 nuclei with either E4Δ44-48 or E4R45A had no effect on the percentage of nuclei replicating compared to S cytosol alone (Figure 5.9a). Both mutants also failed to inhibit DNA replication when added to replication reactions together with exogenous Cdc6. Moreover, no loss of Mcm2 and Mcm7 recruitment to chromatin was observed following incubation of G1 nuclei with Cdc6 and either E4Δ44-48 or E4R45A. Taken together these data indicate that residues 44 to 48, and in particular R45, are critical in mediating the inhibition of DNA replication licensing by full-length E4.

5.4. Discussion and Conclusions

Molecular modelling and computer-aided design have emerged as important tools in drug discovery and pharmaceutical research (Zeng, 2000; Kitchen \textit{et al.}, 2004; Dahl and Sylte, 2005). These techniques utilise the information provided by structural, biochemical and biophysical analyses of target molecules to identify chemical compounds or peptides that will inhibit or stimulate the activity of the target, or mimic its function (Zeng, 2000). Structure-based design and screening strategies
Figure 5.9. A proline-rich sequence in E4 is necessary for inhibition of origin licensing. Addition of the bacterially-expressed point mutant E4R45A or the deletion mutant E4Δ44-48, either alone or in conjunction with exogenous Cdc6, to co-incubations of NIH/3T3 G1 nuclei and HeLa S phase cytosol had no effect on either (a) the replicative capacity of the G1 nuclei or (b) recruitment of replication licensing factors to chromatin. In contrast bacterially-expressed WTE4 caused a 50% reduction in replicative capacity and blocked the recruitment of Mcm2 and Mcm7 onto chromatin.
have influenced the development of a number of drugs, including HIV protease inhibitors (Rizzo et al., 2002; Kroeger-Smith et al., 2003; Udier-Blagovic et al., 2003a,b) and CDK inhibitors (Kontopidis et al., 2003; McInnes et al., 2003; Wu et al., 2003; Andrews et al., 2004). The experimental data presented in this chapter suggest that the HPV E4 protein may provide a molecular scaffold for the design of anti-proliferative therapeutic agents. Previous studies have identified an association between the HPV1 E4 protein and the replication licensing factors Mcm7 and Cdc6, suggesting that E4 may interfere with cellular DNA replication licensing and have a negative effect on DNA replication. Addition of E4, together with Cdc6, to in vitro DNA replication reactions containing G1 nuclei resulted in a reduction in DNA replication (Figure 5.3), coupled to a block to recruitment of replication licensing factors to chromatin (Figure 5.5). However, addition of E4 and Cdc6 to replication reactions containing S phase nuclei had no effect on DNA replication or recruitment of replication licensing factors to chromatin (Figures 5.4 and 5.6). These data indicate that E4 blocks the initiation of DNA replication by preventing the recruitment of replication licensing factors onto chromatin, but is unable to interfere with elongation DNA synthesis. Since E4 is a small protein, of only 125 amino acids, these studies suggest that E4-based mimetic compounds may provide a strategy for therapeutically inhibiting origin licensing. To enable the design of E4-based mimetic compounds it is critical to elucidate the mechanism by which E4 inhibits origin licensing, to identify the regions within the E4 protein that are critical for mediating this activity and to understand further the nature of the complex that is formed between E4, Cdc6 and Mcm7.

The precise mechanism by which E4 inhibits pre-RC assembly is still unclear, although a number of conclusions can be drawn. Since E4 could not be detected on
chromatin, is unable to affect binding of endogenous Cdc6 and requires soluble exogenous Cdc6 for its inhibitory activity, it is likely that the association of E4 with Cdc6 and Mcm7 occurs prior to their assembly into pre-RCs on chromatin. The ability of E4, Cdc6 and Mcm7 to interact in the absence of chromatin is further confirmed by the immunoprecipitation of protein complexes containing Cdc6 and Mcm7 from soluble extracts of HeLa cells containing exogenous HPV1 E4 (Sally Roberts; personal communication). Moreover, the low levels of Cdc6 and Mcm7 found in E4 complexes, in conjunction with the immunofluorescence data, suggests that only a subpopulation of E4, Cdc6 and Mcm7 are associated at any one time and that the interaction may therefore be transient. This raises the possibility that Cdc6 may act as a ‘molecular matchmaker’ allowing E4 to interact with Mcm7 and prevent its assembly into the MCM hexamer. Further understanding of the mechanism by which E4 inhibits DNA replication licensing may be revealed by a comprehensive analysis of the complex that is formed between E4, Cdc6 and Mcm7. Firstly it is important to determine whether the interaction between E4, Cdc6 and Mcm7 can occur in the absence of other factors. This can be achieved by carrying out immunoprecipitation and farwestern blotting analyses with recombinant proteins. In parallel with these studies, biophysical methodologies, including isothermal titration calorimetry (ITC, Pierce et al., 1999) and surface plasmon resonance (SPR, Myszka, 1997), can be exploited to provide information on the stoichiometry of the complex and the binding affinities of the protein-protein interactions. Coupled to structural analyses of E4, both alone and in complex with Mcm7 and Cdc6, these studies should provide a blueprint for the molecular modelling of E4-mimetic compounds and screening for lead compounds.

Further information regarding the regions of E4, Cdc6 and Mcm7 involved in
mediating complex formation and origin licensing inhibition can be elucidated by the use of the techniques described above in conjunction with mutational analyses and in vitro activity studies. The experimental data presented in this chapter have identified two regions of E4 which are essential for inhibiting origin licensing and which may therefore be involved in protein-protein interactions. The activity of E4 as an origin licensing inhibitor is dependent on the N-terminal 15 amino acids of the protein (Figure 5.8) and on residues 44-48 (Figure 5.9), or at the very least R45. Intriguingly, Mcm7- and Cdc6-containing complexes could be immunoprecipitated from soluble HeLa cell extracts containing ΔE4 (S. Roberts; personal communication), indicating that the N-terminal region of E4 is not essential for mediating complex formation. However, since this interaction is insufficient to block DNA replication licensing (Figure 5.8) it can be postulated that complexes formed between ΔE4, Cdc6 and Mcm7 may be unstable. It would be interesting therefore to analyse the binding affinities of the ΔE4/Cdc6/Mcm7 complex using ITC and SPR and similarly to assess the role of amino acids 44-48 in mediating complex formation. In addition peptide walking and shotgun proteolysis could be exploited to generate small fragments of the E4 protein that can be assayed for inhibitory activity, using the in vitro DNA replication system and chromatin-binding assay, and for their ability to interact with Cdc6 and/or Mcm7, using immunoprecipitation and biophysical techniques. It would also be of interest to use shotgun proteolysis to fragment Cdc6 and Mcm7 in order to determine the regions within these two proteins which interact with E4. Alternatively interacting regions may be determined by cross-linking the complex formed by recombinant Cdc6, Mcm7 and E4, removing non-interacting regions by proteolysis and identifying the remaining peptides, and hence the interacting regions, by mass spectrometry. Such studies will allow the critical
residues required for mediating complex formation and inhibition of DNA replication licensing to be identified. Moreover, by identifying the binding interfaces between the three proteins this information can be utilised for the molecular modelling of E4 mimetics for use as anti-proliferative agents. The inhibition of protein-protein interactions, as opposed to interfering with enzyme function (e.g. CDKIs), is an attractive strategy for therapeutic agents since such agents generally show higher target specificity and less susceptibility to the evolution of drug resistance (see also Chapter 1, Section 1.4).

It is also of interest to note that the functional effect of E4 mimics the endogenous origin licensing repressor geminin, which inhibits the initiation of DNA replication by blocking incorporation of MCM proteins into pre-RCs. However whilst geminin acts through the MCM loading factor Cdt1, E4 targets the MCM loading factor Cdc6. Therefore together E4 and geminin represent a dual strategy for inhibition of origin licensing, targeting the two parallel pathways required for recruitment of the MCM complex onto chromatin. Consequently, development of E4 and geminin-based mimetic compounds provides an opportunity for a non-genotoxic combination therapy. The use of two therapeutic origin licensing inhibitors in combination should increase tumour cell kill leading to improved overall response of the tumour and reduce the opportunity for the development of drug resistance.

In addition to providing support for the development of E4-based mimetic compounds as novel anti-proliferative agents, the work discussed in this chapter sheds further light on the role of E4 in the viral life cycle. An integral part of the HPV viral life cycle is the reactivation of cellular genes required to support DNA replication. Early in HPV infections the E6 and E7 proteins are expressed leading to
uncoupling of cellular growth and proliferation through their interactions with p53 and pRb respectively. Inhibition of p53 and pRb results in upregulation of S phase gene activity (Cheng et al., 1995), creating an environment in the cell that supports active DNA replication. Use of these replication factors for replication of the cellular genome would deplete the host cell nucleotide pool and replication enzymes that are required by HPV for viral genome amplification, thereby interfering with the productive stages of the viral life cycle. The genetic, biochemical and functional in vitro replication data suggest that E4, through its interaction with Cdc6 and Mcm7 and subsequent inhibition of host cell DNA synthesis, may play a role in ensuring that the virus gets maximum benefit from this replication-supporting environment. In support of this hypothesis, the induction of S phase gene activity and the switch to vegetative viral replication is associated with induction of high levels of E4 protein and suppression of DNA synthesis (Peh et al., 2002; Nakahara et al., 2005). Moreover, loss of E4 expression is associated with a defect in vegetative genome replication in systems that recapitulate the papillomavirus productive replication life cycle (Peh et al., 2004; Wilson et al., 2005; Nakahara et al., 2005). It can therefore be postulated that in differentiating keratinocytes E4 allows efficient replication of the virus genome by inhibiting the recruitment of host cell replication factors to cellular origins of replication. The lack of inhibitory activity in the N-terminal deletion mutant of E4 (ΔE4) (Figure 5.8), representative of the 16 kDa form of E4, suggests that inhibition of DNA replication may be confined to the full length version of E4 which occurs earliest in HPV infection. Posttranslational modification may therefore allow E4 to have a pleiotropic role in productive HPV infections.

One constraint of this study is that the data were generated using a number of different model systems. Hence these data constitute a circular rather than a direct
argument for the biological role of E4 as an inhibitor of cellular replication licensing. To confirm that the association of E4, Cdc6 and Mcm7 that was detected by immunoprecipitation and immunofluorescent studies in keratinocytes is responsible for inhibition of DNA replication licensing, the functional studies could be extended into a human keratinocyte cell line, the target cell type for HPV infection. One mechanism by which this could be achieved is through use of novel small molecule carriers (SMoC; see also Chapter 6), to transport recombinant E4 into human keratinocytes. If the association of E4 with Cdc6 and Mcm7 is responsible for blocking DNA replication licensing then recruitment of replication licensing factors to chromatin and the replicative capacity of the keratinocytes should be impeded in the presence of exogenous E4.

In summary, the data presented in this chapter suggest that one of the roles of E4 in the viral life cycle may be the inhibition of cellular DNA replication licensing, thereby supporting efficient amplification of the viral genome. The ability of E4 to block origin licensing suggests that E4-based mimetic compounds may provide a molecular scaffold for the design of novel non-genotoxic anti-proliferative agents. However, the apparent requirement for interaction with both Mcm7 and Cdc6 for inhibition of DNA synthesis indicates that multiple regions of E4 may be required for mediating this inhibition. Therefore, as with geminin, mimicking the activity of E4 with a small molecule or peptide may be complex. Further understanding of E4's mechanism of action is therefore critical to elucidate whether mimicking the function of E4 with a small molecule or peptide is viable. However, as with geminin, it can be envisaged that with the correct delivery strategy E4 itself may provide a mechanism for therapeutically inhibiting origin licensing. Together with the analysis of the endogenous origin licensing repressor geminin in Chapter Four, these data further
validate the prevention of MCM recruitment to chromatin as a potential mechanism for the therapeutic inhibition of origin licensing. In the following chapter, these studies have been extended to investigate the effects of blocking origin licensing in normal and transformed cell lines. To achieve this the endogenous origin licensing repressor geminin was exploited as a tool for triggering a block to origin licensing, delivering the protein into cells using a novel small molecular carrier (SMoC) which can transport biomolecules across cellular membranes.
CHAPTER SIX

NORMAL AND TRANSFORMED CELLS RESPOND DIFFERENTLY TO INHIBITION OF ORIGIN LICENSING

6.1. Introduction

In Chapter Three, I showed that the replication licensing machinery is down-regulated as cells withdraw from cycle into the out-of-cycle states of quiescence and differentiation (Figures 3.4, 3.6, 3.7, 3.8, and 3.9). Furthermore, the majority of cells in the stem/progenitor compartment of the colonic crypt displayed an unlicensed phenotype (Figures 3.8 and 3.9). Taken together these data suggest that therapeutic origin licensing inhibitors should have no effect on these cell populations. On the contrary, the rapidly proliferating cells in the transit amplifying compartment of distal colonic crypts contain high levels of replication licensing proteins. Consequently these cells would be expected to be affected by origin licensing inhibitors, indicating that such inhibitors could have severe cytotoxic effects, particularly in the rapidly proliferating gastrointestinal and haemopoietic systems. However, preliminary data presented in a recent report (Shreeram et al., 2002) argue against this conclusion and suggest that inhibition of origin licensing may provoke a differential response in normal and transformed cells (see also Section 1.8.; Shreeram et al., 2002). The authors report that adenoviral-induced overexpression of non-degradable geminin in untransformed IMR-90 fibroblasts prevents transition into S phase, causing cells to arrest with characteristic traits of a G1 cell population (Shreeram et al., 2002). In contrast, osteosarcoma-derived Saos2 and U20S cells are able to progress into S/G2 phase, where they subsequently apoptose. These findings point towards the existence of a novel checkpoint in G1 phase that monitors the
assembly of pre-replication complexes at a critical number of origins in normal cells, but that may be defective in transformed cells. Up to 30,000 replication origins are spread along the chromosomes (Todorovic et al., 1999), and it can be postulated that the number of licensed origins must reach a threshold before cells can proceed into S phase and initiate DNA replication. An origin licensing inhibitor could reduce the number of origins that are licensed, thus triggering activation of the putative ‘origin licensing’ checkpoint and stalling normal cells in G1. If this checkpoint is defective, DNA replication initiation may commence with only a subset of replication origins licensed, ultimately leading to impaired DNA synthesis and DNA damage. It may be postulated that dependent on the genetic background of the cancer cell, DNA damage may trigger intra-S phase and G2/M checkpoint pathways, ultimately resulting in an apoptotic response. The induction of apoptosis in S phase, as observed for U20S (p53+/pRb+) cells (Shreeram et al., 2002), is likely to be triggered by stalled replication forks. Although replication fork stalling is a common occurrence during S phase, under normal circumstances replication can be continued from an adjacent replication origin. However if an insufficient number of origins are licensed, then the space between origins may impede the recovery of stalled replication forks, resulting in the generation of an intra-S phase checkpoint signal. Defects in the intra-S phase checkpoint will allow the continuation of DNA synthesis in the presence of stalled replication forks, as observed for Saos2 (p53-/pRb-) cells (Shreeram et al., 2002), and progression into mitosis with large unreplicated stretches of DNA. The attempt to segregate incompletely replicated chromosomes during mitosis will result in the generation of DNA double strand breaks, triggering the activation of G2/M checkpoints and the induction of apoptosis (Shreeram et al., 2002).
The published report by Shreeram and co-workers had two constraints, both linked to the adenoviral delivery system used in the study. Firstly, some of the observed responses may have been due to secondary effects of the viral infection. For example, p53 activation, which was attributed to ATM/ATR checkpoint activity in this study (Abraham, 2001), is commonly observed in adenoviral infection (Debbas and White, 1993; Lowe and Ruley, 1993; Grand et al., 1994; Lomonosova et al., 2005). More importantly, the difficulty in removing the block to origin licensing due to the irreversible nature of adenoviral infections prevented Shreeram et al. from determining whether normal cells can recover from the G1 arrest that results from a block to origin licensing. This is critical in the context of therapeutic origin licensing inhibitors since if normal cells were able to recover following treatment, cytotoxicity would be dramatically reduced. These remaining doubts highlight the need for independent experimental verification of the differential response to origin licensing inhibition and an investigation of whether origin licensing inhibition is reversible in normal cells. To address these points and to characterise the putative origin licensing checkpoint as a therapeutic target, a novel delivery system was exploited to reversibly deliver geminin at near physiological levels into normal and transformed cells.

6.2. Materials and Methods

For Transformation of E. coli (2.2.2.), Antibodies (2.3.1), Expression of recombinant hsgeminin, hsgeminin-ΔNt and hsgemininFF in E. coli (2.3.3.), Purification of His6-hsgeminin, His6-hsgeminin-ΔNt and His6-hsgemininFF (2.3.6.), Coupling of proteins to SMoC (2.3.9.), Assaying for efficiency of SMoC-protein coupling (2.3.10.), SDS-polyacrylamide gel electrophoresis (2.3.11.), Coomassie Blue staining of SDS-PAGE
gels (2.3.12), Immunoblotting (2.4.1.), Immunofluorescence (2.4.2.), Cell culture and synchronisation (2.5.1.), Assaying for cell proliferation (2.5.3), Assaying for apoptosis (2.5.4.), Preparation of total cell extracts (2.5.5.) and Biochemical subcellular fractionation (2.5.6.) refer to Chapter Two (Materials and Methods). For Design of small molecule carriers, Synthesis of small molecule carriers and Molecular modelling please see Appendix B.

6.3. Results

6.3.1. 4G-SMoC rapidly transports FITC into intact cells

In order to deliver geminin into cells, a novel delivery system in the form of small molecule carriers (SMoCs), which are biphenyl, alpha helical mimetics of peptide protein transduction domains (PTDs), was exploited (Figure 6.1; see also Appendices B and F [Okuyama et al., manuscript submitted] for additional information on SMoCs). To study the uptake of SMoCs into cells, the accumulation of a 4G-SMoC-FITC conjugate in NIH/3T3 fibroblasts was monitored. Cells were incubated with 10 μM 4G-SMoC-FITC for 5, 10, 15, 30 and 60 minutes, fixed, counter-stained with DAPI and visualised by confocal fluorescence microscopy. Cells incubated with 4G-SMoC-FITC showed strong intracellular FITC staining whereas no intracellular staining was detectable in cells incubated with unconjugated FITC (Figure 6.2). Diffuse cytoplasmic staining and some nuclear staining could be detected after five minutes whilst after ten to fifteen minutes the staining could be resolved as being predominantly nuclear. These data demonstrate that delivery of FITC into cells by 4G-SMoC is both rapid and efficient. No morphological features of apoptosis were noted, indicating that SMoCs are non-toxic.
Figure 6.1. Chemical structure of small molecular carriers (SMoC). (a) Molecular model of a typical alpha helix showing the similar spatial orientation of the i and i + 3 positions with the 2 and 2' positions on the biphenyl ring (purple). (b) Chemical structure of 2G-SMoC-FITC and (c) 4G-SMoC-FITC. (d) A late stage 4G-SMoC-amine intermediate allows a flexible range of attachment chemistries to be simply instituted depending on the application.
Figure 6.2. 4G-SMoC-FITC rapidly enters intact cells. NIH/3T3 fibroblasts were incubated with 10 μM 4G-SMoC-FITC for the indicated time periods, fixed, counterstained with DAPI and visualised by confocal fluorescence microscopy. Cells incubated with 4G-SMoC-FITC showed strong intracellular staining whereas no staining could be detected in cells incubated with unconjugated FITC.
The mechanism by which SMoCs transport biomolecules into cells is unclear, and is beyond the scope of this thesis. Preliminary studies indicate that uptake of SMoC-FITC is not affected by temperature or by the presence of cytochalasin D or nocodazole, and hence appears to be an energy- and endocytosis-independent process (Laman H and Selwood D, Wolfson Institute for Biomedical Research, UCL; personal communication). In contrast uptake of SMoC-geminin appears to be temperature dependent, suggesting that SMoC may utilise a number of different mechanisms to cross cellular membranes depending on the biomolecule to which it is conjugated, as is the case for PTDs (Joliot and Prochiantz, 2004). For a more detailed discussion of the SMoC delivery mechanism see Appendices B and F (Okuyama et al., manuscript submitted).

6.3.2. 4G-SMoC transports proteins into intact cells

The linker chemistry of SMoCs can be altered to allow conjugation to different biomolecules (Figure 6.1d). Conjugation to proteins can be achieved by synthesising SMoC with a thiopyridal derivation (SMoC-SSPyr) which allows coupling to proteins via the formation of disulphide bonds at cysteine residues in the target protein (Figure 6.3a). Geminin has two cysteine residues in its C-terminus (residues 193 and 208) which should enable coupling to SMoC. Coupling of geminin to 4G-SMoC was achieved by reducing the two reactive thiol groups at residues 193 and 208 using β-mercaptoethanolamine.HCl and mixing with 4G-SMoC-SSPyr reagent resulting in coupling via disulphide bond formation (Figure 6.3a). To determine the efficiency of this coupling, free sulfhydryl groups were quantitated after reduction by β-mercaptoethanolamine.HCl and conjugation to 4G-SMoC using Ellman’s reagent (Figure 6.3b). Following reduction by β-mercaptoethanolamine.HCl geminin showed
Figure 6.3. 4G-SMoC can be efficiently conjugated to proteins via formation of disulphide bonds. (a) Schematic showing protocol for coupling of 4G-SMoC to geminin. Following reduction of the reactive thiol groups at cysteine residues 193 and 208, geminin is coupled to 4G-SMoC-SSPyr via disulphide bond formation. (b) Quantification of sulphydryl groups with Ellman's reagent before and after coupling indicates that coupling of geminin to 4G-SMoC approaches 100% efficiency.
an increased absorbance at 412 nm. After incubation with 4G-SMoC the absorbance was reduced to zero, indicating that all free sulfhydryl groups that were present following β-mercaptoethanolamine.HCl treatment were no longer free after conjugation with 4G-SMoC and hence that coupling efficiency was close to 100% (Figure 6.3b).

To assess whether 4G-SMoC-geminin could enter into live cells, NIH/3T3 fibroblasts were incubated with 1.5 µM 4G-SMoC-geminin or unconjugated geminin eight hours after release from quiescence (G0), prior to expression of endogenous geminin (see Chapter 3, Figure 3.5c). Following incubation for one hour, cells were fixed and immunostained with a rabbit anti-geminin polyclonal antibody (Wharton et al., 2004) and a FITC-conjugated anti-rabbit secondary antibody. Diffuse cytoplasmic and nuclear FITC staining could be detected in cells incubated with 4G-SMoC-geminin but not in cells treated with unconjugated geminin (Figure 6.4a). To further confirm these results, total cell lysates were prepared from asynchronously proliferating NIH/3T3 fibroblasts following incubation with 1.5 µM 4G-SMoC-geminin or unconjugated geminin for one hour. Proteins were resolved by gel electrophoresis and immunoblotted with an anti-His antibody to detect 4G-SMoC-geminin (Figure 6.4b). Exogenous recombinant geminin could be detected in cells incubated with 4G-SMoC-geminin but not in cells incubated with unconjugated geminin. Taken together, these data confirm that 4G-SMoC can be efficiently coupled to geminin and that conjugated geminin rapidly enters intact mammalian cells in culture (Okuyama et al., manuscript submitted; see Appendix F).
Figure 6.4. 4G-SMoC-geminin rapidly enters intact cells. (a) NIH/3T3 fibroblasts were incubated with 4G-SMoC-geminin for one hour, eight hours after release from quiescence, when no endogenous geminin is present, fixed, stained with an anti-geminin polyclonal antibody, a FITC-conjugated anti-rabbit secondary antibody and DAPI, and visualised by confocal fluorescence microscopy. Diffuse cytoplasmic and nuclear FITC staining could be detected in cells incubated with 4G-SMoC-geminin but not in cells treated with unconjugated geminin. (b) Detection of 4G-SMoC-geminin with an anti-His antibody in total cell extracts prepared from NIH/3T3 fibroblasts incubated with 4G-SMoC-geminin or unconjugated geminin for one hour.
6.3.3. 4G-SMoC-geminin blocks G0-S progression in mouse fibroblasts in a concentration-dependent manner

To first determine that geminin retains its biological activity following conjugation to SMoCs, the ability of SMoC-geminin to block origin licensing was tested in our characterised in vitro system for the re-entry of quiescent fibroblasts into the mitotic cell division cycle (see Chapter 3). In addition, this experiment allows the question of whether the putative origin licensing checkpoint is active during re-entry into the cell cycle from G0 to be addressed. In cultured NIH/3T3 fibroblasts chromosomal replication origins are licensed for DNA synthesis during a defined “window of opportunity” between 16 and 18 hours after release from density-dependent growth arrest (see Chapter 3, Figure 3.5c; Stoeber et al., 1998; Kingsbury et al., 2005) and progression into S phase can be easily monitored by pulse-labelling with bromodeoxyuridine (BrdU) 21 hours after release from contact-inhibition (Figure 6.5a). To assess the ability of SMoC-geminin to inhibit origin licensing and activate the putative origin licensing checkpoint during re-entry into cycle, 4G-SMoC-geminin was added at concentrations ranging from 0.5 μM to 10 μM to NIH/3T3 cells eight hours after release from G0. Cells were pulsed for one hour with BrdU 21 hours after release, analysed by confocal fluorescence microscopy and the percentage of cells synthesising DNA determined (Figure 6.5b). With increasing concentrations of 4G-SMoC-geminin the percentage of cells incorporating BrdU gradually decreased from 65% in the control population to 36% at 10 μM 4G-SMoC-geminin. Substitution of 4G-SMoC-geminin with uncoupled geminin or an equivalent volume of control buffer did not affect BrdU incorporation.

Full-length wild type geminin undergoes ubiquitin-dependent proteolysis in M phase,
Evaluation of the DNA replication licensing machinery as an anti-proliferative target. Sarah Kingsbury

**Figure 6.5. 4G-SMoC-geminin blocks G0-S progression in mouse NIH/3T3 fibroblasts.** (a) To determine whether 4G-SMoC-geminin can block re-entry into cycle from G0 4G-SMoC-geminin was added to NIH/3T3 fibroblasts 8 hours after release from quiescence, prior to pre-RC assembly at 16-18 hours, and entry into S phase monitored by pulsing with BrdU at 21 hours. Inhibition of G0-S progression increases with (b) 4G-SMoC-geminin and (c) 4G-SMoC-ΔNt concentration. Results are expressed as the reduction in the percentage of cells incorporating BrdU relative to the control population. (d) A 50% reduction in G0-S progression was observed following treatment with 10 μM 4G-SMoC-geminin and 4G-SMoC-ΔNt, but not with the loss-of-function mutant 4G-SMoC-gemininFF.
mediated by the destruction box in the N-terminus of the protein (see Chapter 4, Figure 4.1b). The 15 kDa N-terminal deletion mutant of geminin (gemininΔNt) discussed in Chapter Four (Figures 4.8 and 4.9; see also Chapter 2, Figure 2.3) lacks this motif and therefore should be resistant to ubiquitin-dependent proteolysis. Treatment of NIH/3T3 fibroblasts with increasing concentrations of 4G-SMoC-ΔNt resulted in a similar reduction in BrdU incorporation to that seen with full-length geminin (Figure 6.5c). Maximal inhibition for both SMoC-geminin and SMoC-ΔNt was observed with 10 μM, and therefore this concentration was used for all subsequent experiments.

To determine that the reduction in BrdU incorporation observed with 4G-SMoC-geminin and 4G-SMoC-ΔNt was dependent on geminin activity and not a non-specific effect of SMoC, the ability of a loss-of-function geminin mutant (gemininFF, see Chapter 4, Figure 4.18) to inhibit BrdU incorporation was assessed. No reduction in BrdU incorporation was observed with 4G-SMoC-gemininFF (Figure 6.5d) whilst a 50% reduction was observed with both 4G-SMoC-geminin and 4G-SMoC-ΔNt. Taken together these results demonstrate that delivery of geminin into cells by 4G-SMoC does not affect its activity as an origin licensing inhibitor (Okuyama et al., manuscript submitted; Appendix F). Furthermore, these data suggest that by blocking pre-RC assembly with exogenous geminin during re-entry into the cell cycle, an as yet unknown checkpoint activity may be triggered preventing cells from initiating DNA replication from the few origins which may be licensed.
6.3.4. 4G-SMoC-ΔNt blocks G1-S progression by repressing origin licensing in normal asynchronously cycling cells

The ability of 4G-SMoC-geminin and 4G-SMoC-ΔNt to inhibit re-entry of NIH/3T3 fibroblasts into the cell cycle from G0, potentially by activating a putative origin licensing checkpoint, raises the question as to whether cell cycle progression could also be prevented in normal cycling cells. WI-38 human diploid fibroblasts were treated with 10 μM 4G-SMoC-ΔNt for 24 hours (approximately one doubling time) to assess the ability of geminin to block cell cycle progression. During the final hour of treatment cells were pulsed with BrdU and the percentage of cells incorporating BrdU calculated. A 50% reduction in BrdU incorporation was observed in treated populations compared to control populations (Figure 6.6a). Since cells will only be sensitive to geminin activity during G1 phase, treatment of cells for one population doubling may not be enough to catch all cells, which may explain why cell cycle progression was only blocked in 50% of cells. In addition it is possible that 4G-SMoC-ΔNt may be susceptible to degradation independently of the ubiquitin-26S proteasome pathway, and therefore after 24 hours the intracellular concentration of 4G-SMoC-ΔNt in some cells may have been insufficient to block cell cycle progression. Longer time-courses, with replenishment of 4G-SMoC-ΔNt every 12 or 24 hours, are therefore necessary to determine whether cell cycle progression can be blocked in a higher proportion of cells. However these results still demonstrate that exogenous geminin can block cell cycle progression, and activate the putative origin licensing checkpoint, in normal cycling cells.

To determine the biochemical mechanism by which DNA replication is inhibited upon treatment with 4G-SMoC-ΔNt, changes in chromatin-bound Mcm2 levels were
Evaluation of the DNA replication licensing machinery as an anti-proliferative target. 

Figure 6.6. 4G-SMoC-ΔNt blocks G1-S progression by repressing origin licensing in normal asynchronously cycling WI-38 human diploid fibroblasts. (a) Following treatment with 4G-SMoC-ΔNt for 24 hours a 50% reduction in BrdU incorporation was observed in WI-38 human diploid fibroblasts. This reduction in proliferation was coupled to a block to Mcm2 chromatin-binding as detected by (b) immunofluorescence and (c) immunoblotting. For detection by immunofluorescence cells were treated with Triton X-100 prior to fixation to remove soluble proteins and stained with an anti-Mcm2 antibody, FITC-conjugated secondary antibody and propidium iodide. For detection by immunoblotting, cytosolic (CF), nucleosolic (NF) and chromatin-bound (CBF) proteins were prepared, resolved by gel-electrophoresis and immunoblotted for Mcm2 and anti-His to detect 4G-SMoC-ΔNt. The reduction in Mcm2 association with chromatin is coupled to recruitment of 4G-SMoC-ΔNt to chromatin.
analysed using immunofluorescence and alternatively, immunoblotting of subcellular protein fractions. Following treatment with 10 μM 4G-SMoC-geminin for 24 hours, WI-38 fibroblasts were either treated to remove soluble proteins and immunostained for Mcm2 or biochemically fractionated and analysed by immunoblotting. Treatment with 4G-SMoC-ΔNt resulted in a 75% reduction in the relative intensity of Mcm2 staining (Figure 6.6b) indicating that 4G-SMoC-ΔNt inhibits G1-S progression by blocking MCM loading onto chromatin. A reduction in chromatin-bound Mcm2 levels was also observed in the immunoblot data following treatment with SMoC-ΔNt and interestingly, was coupled to increased levels of the protein in the cytosolic fraction (Figure 6.6c). Consistent with previous findings that repression of origin licensing is associated with the presence of geminin on chromatin (see Chapter 4, Figure 4.7), exogenous geminin was clearly detected in the cytosolic, nucleosolic and chromatin-bound fractions following treatment with 4G-SMoC-ΔNt. Taken together these data confirm the ability of 4G-SMoC to efficiently enter cell lines of different origin and to retain the biological activity of its protein cargo (Okuyama et al., manuscript submitted; Appendix F). Furthermore these data suggest that inhibition of origin licensing in normal cells may invoke an as yet unknown checkpoint activity which prevents cells from initiating DNA synthesis with an insufficient number of licensed origins.

6.3.5. 4G-SMoC-ΔNt triggers a differential response in normal and transformed cell lines

To determine whether normal and transformed cells respond differently to origin licensing inhibition, as has been previously suggested (Shreeram et al., 2002), the response of normal and transformed cells to 4G-SMoC-ΔNt was analysed. WI-38
human diploid fibroblasts were chosen as a normal cell line and osteosarcoma-derived U20S (p53+/pRb+) cells were chosen as an example for a transformed cell line. Since induction of apoptosis may not be detectable after treatment for 24 hours, cells were treated with 10 μM 4G-SMoC-ΔNt for 72 hours, replenishing with fresh medium and 4G-SMoC-ΔNt every 12 hours to ensure that the 4G-SMoC-ΔNt concentration remained constant. After treatment, cells were assayed for cell proliferation by monitoring BrdU incorporation and for apoptosis by TUNEL staining. BrdU incorporation in both cell lines dropped to less that 10% of the control populations following treatment (Figure 6.7). This indicates that the 50% reduction in BrdU incorporation observed for WI-38s in the initial experiments (Figure 6.6a) was a result of the short period of treatment. Interestingly, when the BrdU images are observed more closely differences in the intensities of staining can be noted between the different populations. In the control populations of both cell lines and in the treated WI-38s, the majority of positive cells show strong FITC staining. In contrast, the majority of positive cells in the treated U20S populations, and indeed a proportion of cells which would be considered negative, show faint punctuated FITC staining (Figure 6.7, white arrow), suggesting that although cells have initiated DNA replication they fail to successfully progress through S phase. These findings are in keeping with the report by Shreeram et al which suggests that U20S cells arrest in S phase prior to committing apoptosis. WI-38 fibroblasts showed none of the morphological changes associated with induction of apoptosis, whilst U20S cells showed typical changes characteristic of apoptotic cell death, including cell shrinkage, cytoplasmic blebbing and chromatin condensation (Figure 6.8). Apoptosis is associated with inter-nucleosomal degradation of genomic DNA which can be monitored by TUNEL assay (Gavrieli et al., 1992). 72 hrs after treatment, U20S cells
Figure 6.7. 4G-SMoC-ΔNt blocks proliferation in normal WI-38 human diploid fibroblasts and transformed U20S cells. Treatment of (a) WI-38 human diploid fibroblasts and (b) U20S osteosarcoma-derived cells for 72 hours with 4G-SMoC-ΔNt resulted in a 90% reduction in cells stained positive for BrdU incorporation in both cell lines compared to control populations. However, 4G-SMoC-ΔNt-treated U20S cells that are positive for BrdU incorporation show much fainter, punctuated staining, compared to treated WI-38 fibroblasts, indicative of an inability to complete S phase (white arrow). Moreover isolated BrdU foci can be detected in some of the treated U20S cells which were recorded as negative, suggesting that many U20S cells have attempted to initiate DNA synthesis from the few origins which were licensed in the presence of 4G-SMoC-ΔNt.
Figure 6.8. 4G-SMoC-ΔNt induces apoptosis in transformed U20S cells but not WI-38 human diploid fibroblasts. Following treatment of WI-38 human diploid fibroblasts and U20S cells with 4G-SMoC-ΔNt for 72 hours, apoptosis was assessed by monitoring morphological features and by TUNEL assay. WI-38 fibroblasts retained normal morphological features, although the cell density was significantly reduced compared to the control population, and showed no TUNEL staining. U20S cells on the contrary showed characteristic features of apoptosis including cell shrinkage, cytoplasmic blebbing and abundant TUNEL staining.
showed abundant TUNEL staining compared to the control population (Figure 6.9). In contrast, no TUNEL staining could be detected in treated WI-38 fibroblasts. These findings argue that 4G-SMoC-geminin invokes a cell-cycle arrest in normal cells which prevents them from initiating DNA synthesis but does not invoke an apoptotic response. On the contrary, transformed cells are able to overcome the inhibition to origin licensing and progress into S phase where they ultimately undergo apoptosis.

6.3.6. Normal cells continue proliferating upon removal of the block to origin licensing

The finding that exogenous geminin induces cell cycle arrest in normal WI-38 human diploid fibroblasts whilst transformed U20S cells appear to attempt to enter S phase, is indicative of checkpoint activity. A critical question, particularly in the context of origin licensing inhibition as a therapeutic strategy, is whether the G1 arrest observed in normal cells as a result of this checkpoint activity is reversible. To address this question, WI-38 fibroblasts were treated with 10 μM 4G-SMoC-ΔNt for 72 hours, following which cells were washed and incubated in medium which did not contain 4G-SMoC-ΔNt for 24 hours and DNA replication assessed by monitoring BrdU incorporation. Treatment of cells with 4G-SMoC-ΔNt caused a 70% reduction in the number of cells incorporating BrdU compared to control populations. After removal of 4G-SMoC-ΔNt for 24 hours, the percentage of cells incorporating BrdU was approaching the levels found in the control population (Figure 6.9). These data suggest that upon removal of the block to origin licensing normal cells have the capacity to continue pre-RC assembly and proceed through the cell cycle, whilst the fate of transformed cell lines is already determined in the irreversible activation of an apoptotic response.
Figure 6.9. Normal WI-38 human diploid fibroblasts continue proliferating upon removal of the block to origin licensing. Following treatment of WI-38 human diploid fibroblasts with 4G-SMoC-ΔNt for 72 hours, cells were washed and incubated in medium which did not contain 4G-SMoC-ΔNt. 24 hours after removal of 4G-SMoC-ΔNt the percentage of cells incorporating BrdU was approaching the levels found in the control population.
6.4. Discussion and Conclusions

The response of normal proliferating cell populations to chemotherapeutic agents is critical in determining dose-limiting toxicities. The DNA replication licensing machinery, which has been proposed as a potential new therapeutic anti-cancer target, is dysregulated early in carcinogenesis suggesting that inhibitors of origin licensing may have high efficacy in tumour cells (Shreeram and Blow, 2003; Gonzalez et al., 2005). Replication licensing factors are also expressed in normal cycling cells, suggesting that such agents may have severe cytotoxic effects in tissues containing rapidly proliferating cell populations. However work by Shreeram et al. argues that inhibition of origin licensing may induce a differential response in normal and transformed cell lines, with normal cells arresting in G1 and transformed cells undergoing apoptosis (Shreeram et al., 2002). Due to the nature of the experiments conducted these preliminary findings required independent verification and the important question of whether the G1 arrest observed following inhibition of origin licensing in normal cells is reversible needed to be addressed. To achieve this, a novel delivery strategy (SMoCs) was exploited to transport a non-degradable, biologically active fragment of geminin (see Chapter 4, Figure 4.9) into normal and transformed cell lines to investigate their response to inhibition of origin licensing and to determine whether normal cells can recover from this inhibition.

Delivery of recombinant gemininANt into cells by conjugation to 4G-SMoC resulted in a block to G0-S transition in NIH/3T3 fibroblasts (Figure 6.5) and to G1-S transition in asynchronously cycling WI-38 human diploid fibroblasts (Figure 6.6). Moreover, treatment of transformed U20S cells with 4G-SMoC-ANt resulted in induction of a striking apoptotic response, supporting the preliminary data by
Shreeram et al., with no apoptosis detectable in treated WI-38 fibroblasts (Figure 6.8). Critically removal of the block to origin licensing in normal cells resulted in progression into S phase. These results suggest that the G1 arrest observed following inhibition of origin licensing in normal cells may be the result of a checkpoint response. Upon removal of the block to origin licensing, stimulation of the putative checkpoint response is alleviated and cells can therefore continue proliferating normally.

It can be postulated that this checkpoint response acts to monitor the assembly of a critical number of replication complexes onto chromatin during G1, before allowing cells to progress into S phase. Two mechanisms by which this could be achieved can be envisioned. Firstly, the soluble levels of a replication factor may need to be reduced to a certain level, by its assembly into replication complexes, before replication can be initiated. Alternatively, assembly of pre-RCs at a critical subset of origins (sensor origins) may be required. This latter model is particularly attractive since it may provide a direct connection between the inappropriate licensing of origins and the inhibition of transition into S phase. Gene transcription and pre-RC assembly are intricately linked, with the presence of replication complexes at origins of replication having both a negative and positive effect on transcription (Muller et al., 2000; Aladjem and Fanning, 2004; Danis et al., 2004; Niedusynski et al., 2005). For example, replication licensing factors may compete with transcription factors for DNA binding, with the assembly of pre-RCs therefore having a negative effect on transcription (Nieduszynski et al., 2005). In addition transcription of some genes, potentially including proteins involved in maintaining a checkpoint response, may be activated by the absence of pre-RCs. Alternatively licensing of origins may have an
enhancer effect on promoter regions, leading to increased gene transcription. The location of ‘sensor’ origins within the promoters of genes that are essential for S phase entry, for example E2F-regulated genes, would therefore allow a direct mechanism by which S phase entry could be inhibited in the absence of sufficient origin licensing. Interestingly interaction of the retinoblastoma protein (pRb), a regulator of E2F transcription complex activity, with Mcm7 has been shown to negatively regulate transition into S phase (Sterner et al., 1998). Under normal conditions mitogenic stimulation triggers phosphorylation of pRb by Cyclin D/CDK4/6 (Gladden and Diehl, 2003) leading to release of E2F and Mcm7, transcription of E2F-regulated genes and entry into S phase. However in the absence of sufficient origin licensing transcription of E2F-regulated genes will be impeded, leading to reduced phosphorylation of pRb and an inadequate supply of S-phase promoting factors. Moreover the reduction in pRb phosphorylation may prevent the release of Mcm7 for further pre-RC assembly (Gladden and Diehl, 2003), thereby reinforcing the block to S phase entry. Further to changes at the transcriptional level, activation of the origin licensing checkpoint may also invoke post-translational modifications, including phosphorylation, ubiquitination and sumoylation, leading to activation/inactivation of proteins and protein degradation.

Whilst activation of the origin licensing checkpoint causes cells to stall in G1 until origins are correctly licensed, a defect in this checkpoint would allow the initiation of replication to occur from the few origins which were to able to assemble replication complexes in the presence of an origin licensing inhibitor. The genome contains approximately 30,000 origins of replication (Todorovic et al., 1999), ensuring that failure of a few origins to initiate does not impede accurate replication of DNA.
However if, for example, 60% of origins remained unlicensed upon entry into S phase, then the space between licensed origins would likely be such that replication complexes would breakdown or stall prior to reaching the next origin, leaving large unreplicated stretches of DNA. In addition it has been suggested that if an insufficient number of origins are active, DNA replication may occur in the opposite direction than under normal circumstances leading to collisions with RNA polymerases (Deshpande and Newlon, 1996) and impeding progress through ‘slow zones’ (Cha and Kleckner, 2002). In some instances, as observed for U20S cells, this stalling of replication forks may invoke a checkpoint response in S phase which triggers apoptosis (Osborn et al., 2002; Tercero et al., 2003; Bartek et al., 2004; Gottifredi and Prives, 2005). This checkpoint response most likely involves the ATM/ATR checkpoint pathway (Hekmat-Nejad et al., 2000; Osborn et al., 2002; Lavin, 2005) and appears to be dependent on pRb (Shreeram et al., 2002). However, if defects exist in the intra-S phase checkpoint then cells will not respond to the presence of stalled replication forks and will complete S phase with large stretches of unreplicated DNA. Studies in both S. cerevisiae Mec1 mutants (Lopes et al., 2001) and in human ATR mutants (Cha and Kleckner, 2002) suggest that the inability to respond to stalled replication forks during S phase results in the generation of DSB in G2/M phase, consistent with the finding that Saos2 cells commit apoptosis in G2/M (Shreeram et al., 2002). An alternative hypothesis suggests that entry into S phase with an insufficient number of licensed origins may induce apoptosis independent of intra-S phase checkpoint pathways (Burhans et al., 2002; Weinberger et al., 2005). Studies with Orc2-l mutants in yeast indicate that apoptosis may be triggered by the irreversible collapse of replication forks that occurs when the number of active replication origins is below the threshold required to activate checkpoints and/or
rescue stalled replication forks (Labib et al., 2001; Shimada et al., 2002; Burhans et al., 2003; Tercero et al., 2003). In the absence of checkpoint restraint, replication proteins are inappropriately phosphorylated leading to their dissociation from replication origins (Dimitrova and Gilbert, 2000) and inducing irreversible collapse of replication forks (Burhans et al., 2003). This hypothesis supports previous findings that proteolytic destruction of Cdc6 has been shown to contribute to apoptosis in mammalian cells (Blanchard et al., 2002; Pelizon et al., 2002; Yim et al., 2003). In order to distinguish between these two hypotheses, experiments could be performed in the presence of inhibitors of key intra-S phase checkpoint proteins, including ATM and Chk1. If checkpoint activity is required for the induction of apoptosis in U20S cells following inhibition of origin licensing, then these cells would be expected to show a similar phenotype to Saos2 cells (Shreeram et al., 2002), complete S phase and apoptose with a G2/M DNA profile. However, if checkpoint activity is redundant for the induction of apoptosis then no change in the response to inhibition of origin licensing should be observed. In addition, the levels and phosphorylation status of replication licensing proteins can be monitored to determine whether they are involved in triggering the apoptotic programme.

The proteins involved in monitoring, activating and maintaining the putative origin licensing checkpoint are currently unclear, although increased levels of p21 and p53 and reduction of pRb phosphorylation have been observed in response to inhibition of origin licensing (Shreeram et al., 2002; Montagnoli et al., 2004). Intriguingly, the reduction in chromatin-bound Mcm2 levels observed in response to inhibition of origin licensing in WI-38s (Figure 6.6) was coupled to a dramatic increase in cytosolic levels of the protein, whilst in the control population no Mcm2 was
detected in the cytosolic fraction. It can be postulated that activation of the origin licensing checkpoint may trigger an increase in cytosolic Mcm2 by two mechanisms. Firstly the block to origin licensing may lead to exclusion of Mcm2 from the nucleus, thereby removing the pool of Mcm2 available for licensing until conditions are permissible for licensing to continue. Alternatively, the cell may respond to insufficient origin licensing by de novo Mcm2 synthesis in order to increase the cytosolic pool of Mcm2 and ultimately provide a supply of Mcm2 for licensing to continue. Other candidates for involvement in the origin licensing checkpoint include the checkpoint proteins ATM, ATR, Chk1, Chk2, RPA, γ-H2AX and Cdc25 (Longhese et al., 1996; Abraham, 2001; Feijoo et al., 2001; Shiloh, 2001; Ward and Chen, 2001; Gottifredi and Prives, 2005). In addition to their potential involvement in the origin licensing checkpoint, many of these proteins may also be involved in mediating the induction of apoptosis in transformed cells. The involvement of these proteins in mediating the response of cells to inhibition of origin licensing can be determined by monitoring changes to protein levels and phosphorylation status following treatment of normal and transformed cells with 4G-SMoC-ΔNt. These studies should shed some light on the mechanism by which the putative origin licensing checkpoint is mediated, and on the differential response observed in transformed cells. In addition, further factors involved in monitoring or co-ordinating the response to inhibition of origin licensing can be identified using comparative proteomics and transcriptomics. Activation of the origin licensing checkpoint may invoke changes in both the transcriptome and proteome, with the latter potentially involving changes in protein levels, or post-translational modifications including phosphorylation, ubiquitination, sumoylation and neddylation status. Generic changes in the transcriptome can be identified by affymatrix arrays whilst changes to
the proteome can be determined by utilising different comparative proteomic techniques. For example to identify alterations in ubiquitination status, cells can be transfected with a tagged-ubiquitin construct, treated with 4G-SMoC-ΔNt in the presence of a proteasome inhibitor and ubiquitinated proteins purified and identified. In addition to ascertaining changes which occur in normal cells upon activation of the origin licensing checkpoint, these techniques can also be utilised to observe the differences between normal and transformed cell populations to determine why transformed cells fail to arrest in G1 upon inhibition of origin licensing. Furthermore, a range of tumour cell lines with varying genetic backgrounds can be screened to determine whether the response to inhibition of origin licensing is influenced by genotype.

The data presented in this chapter are particularly exciting when considering the therapeutic potential of origin licensing inhibitors. Rapidly proliferating cells, for example in the gastrointestinal tract and haemopoietic systems, are a prime site for cytotoxic damage in response to classical chemotherapeutic agents. However, these results suggest that upon administration of a therapeutic origin licensing inhibitor these cell populations would arrest in G1 and, following metabolism of the drug and removal of the block to origin licensing, would be able to progress into S phase and continue their proliferative cycle. Taken together with the evidence presented in Chapter Three that functional cell populations, including stem/progenitor cells, have an unlicensed phenotype and should hence be refractory to origin licensing inhibitors, these results suggest that origin licensing inhibitors should have little, or no, cytotoxic effects in normal cell populations and should not cause secondary cancers or infertility. Consequently licensing inhibitors should have a high
therapeutic index, allowing patients to tolerate high doses of the drug which will have a greater insult on the tumour cell population and will reduce the risk of the evolution of drug-resistant tumour clones. Moreover, these results also reinforce previous findings that transformed cells respond to origin licensing inhibition by committing apoptosis (Shreeram et al., 2002). It remains to be seen whether this response is shared by all transformed cell populations or whether it is dependent on genetic background. Collectively, these results further reinforce the concept of the DNA replication licensing machinery as an attractive and promising target for the development of novel non-genotoxic therapies to combat cancer.
CHAPTER SEVEN

CONCLUSIONS AND IMPLICATIONS

In the developed world one in three people will develop cancer over their life time and approximately one in four will die from the disease. Despite significant advances in chemotherapeutic regimes, a dramatic improvement in mortality rates has not been realised (Landis et al., 1998). There is therefore a profound need for identification of novel targets, and for continual enhancement of chemotherapeutic regimes.

Cell cycle specific agents targeting DNA synthetic pathways are important therapeutic tools; however inhibiting elongation DNA synthesis can invoke genomic damage, infertility and neoplastic transformation. To circumvent these problems, the DNA replication licensing machinery has been proposed as a novel therapeutic target (Shreeram and Blow, 2003). Origin licensing lies at the convergence point of all oncogenic signalling and transduction pathways, and can be regarded as a ‘molecular switch’ coupling these upstream pathways to chromosomal replication. Unlike these branched, redundant and parallel pathways, there appears to be only a single mechanism of DNA replication initiation which is highly conserved in eukaryotes. Notably dysregulation of the DNA replication licensing machinery is an early event in tumourigenesis that has already been exploited in diagnostic and prognostic clinical applications (reviewed in Tachibana et al., 2005). Licensing inhibitors should therefore be of clinical benefit in a wide range of solid and haematopoietic cancers, independent of genetic background and histological type, in contrast to agents targeting upstream events where great heterogeneity exists between subgroups of even single tumour types.
From a therapeutic perspective targeting the replication licensing machinery may at first seem counterproductive. Although early dysregulation of replication licensing suggests that licensing inhibitors should have high efficacy in cancer cells, there is no obvious rationale for a differential response between normal proliferating cells and tumour cells. It is also unclear how germ cells, stem/progenitor cells and quiescent or differentiated cell populations would respond to licensing inhibitors, and what the long term effects on the patient may be. Moreover the sequential, multi-step assembly of functional replication complexes at chromosomal origins provides a conundrum as to which step may represent the most promising target. The work of this thesis answers some of these questions and has generated data which argue strongly for therapeutic targeting of the replication licensing machinery.

The finding that functional cells in self-regenerating, stable and permanent tissues, including quiescent, differentiated and stem/progenitor cell populations, have an unlicensed phenotype indicates that these cells should be refractory to origin licensing inhibitors (see Chapter 3). Hence the vast majority of normal cells within the human body do not present a target, minimising potential cytotoxic effects of licensing inhibitors. However rapidly proliferating cells, for example cells in the transit amplifying compartment of colonic crypts, are a target to such agents.

Tantalising clues have been reported which suggest that normal and transformed cells may respond differently to licensing inhibitors (Shreeram et al., 2002). These preliminary data however, which have been generated by overexpressing a mutant form of geminin, are far from conclusive since protein overexpression often has pleiotropic effects, whilst some of the observed responses in normal and transformed
cells may be attributed to the adenoviral delivery system. Moreover this study did not address the critical question of whether normal cells can recover from G1 arrest and re-enter the cell cycle upon removal of the block to origin licensing. The data shown in this thesis take the concept further and prove that the replication licensing machinery is a promising therapeutic target. Cancer cell specific killing was observed following treatment with a licensing inhibitor, whereas normal cells arrested in G1 retaining the capacity to continue cell cycle progression upon removal of the inhibitor (see Chapter 6). The observed differential response to origin licensing repression appears to be due to a checkpoint mechanism that monitors assembly of replication licensing complexes at a critical number of origins in normal cells, and which appears to be defective or lost in cancer cells. Therefore paradoxically, the resistance to growth arrest that is one of the hallmarks of malignant cells (Hanahan and Weinberg, 2002) can be exploited to achieve selective cancer cell death. Future studies on a large set of selected cancer cell lines derived from solid and haematopoietic cancers will reveal whether the findings shown in this work can be generalised for cancers of different genetic background and histological type.

The arrest of human diploid fibroblasts in G1 phase following origin licensing inhibition raises questions about the molecular nature of the putative origin licensing checkpoint. A working model favoured by some researchers in the field features so-called ‘sensor’ origins located in the vicinity of promoter regions for S-phase inducing genes. In an unperturbed cell cycle licensing of these sensor origins could have an enhancer-like function for the expression of S-phase inducing genes and thus drive the cell into S phase. Blocking origin licensing would result in loss of enhanced function and reduced gene expression leading to G1 arrest. To date no experimental
data have been reported in support of this model and the elucidation of the checkpoint pathway will undoubtedly have to await data from transcriptomic and proteomic studies.

The sequential assembly of pre-RCs and their dynamic transition to pre-IC structure offers a number of different strategies for blocking origin licensing and/or firing. Small molecular inhibitors or macrodrugs may act by blocking protein-DNA interactions (e.g. ORC binding to DNA), protein-protein interactions (e.g. interactions between Mcm subunits) and kinase (e.g. Cdc7-Dbf4) or phosphatase (protein phosphatase 2A) activity. Detailed structural and molecular investigations are required to determine the feasibility of these approaches; however the biology of some pre-RC/pre-IC constituents already provides important clues for shortlisting potential therapeutic targets. Blocking ORC binding to DNA, for example, may not prove to be an optimal therapeutic strategy since ORC has additional roles in transcription, chromatin remodelling, chromosome segregation and cytokinesis (Fox et al., 1995; Huang et al., 1998; Iizuka and Stillman, 1999; Pak et al., 1997; Prasanth et al., 2002; Chesnokov et al., 2003; Prasanth et al., 2004) which may determine side effect profiles. Dbf4-dependent Cdc7 kinase has a narrow spectrum of action, its only target being the Mcm2-7 complex (Masai and Arai, 2002), and may therefore represent an alternative to the recently developed CDKIs for which phase I and II clinical trials have shown poor results (Blagden and Bono, 2005). With Cdc7 being at the centre stage of current basic and drug discovery research, ongoing and future studies will search for as yet unknown targets of this kinase and possible suppressor mutations such as the known mcm5-bobl mutant (Hardy et al., 1997) which bypasses Cdc7 requirement for replication initiation. Interestingly siRNA knockout of Cdc7
function in human cancer cells revealed a similar response to that observed for
geminin, suggesting that origin firing, which is temporally regulated during S phase,
may also be governed by checkpoint activity (Montagnoli et al., 2004). Interfering
with the assembly and/or function of the Mcm2-7 replicative helicase, for example
through blocking interaction with other pre-RC constituents or between MCM
subunits, may yet prove to be the best strategy. Although MCM proteins have been
linked to damage response, transcription and chromatin modelling (Forsburg, 2004)
these functions do not depend on the complete MCM complex (Ishimi et al., 2001;
Fitch et al., 2003). Furthermore the tight down-regulation of Mcm2-7 in cell
populations residing in out-of-cycle states (Madine et al., 2000; Stoeber et al., 2001;
see Chapter 3) suggests that side effect profiles should be minimal, in contrast to
ORC, for example, which is not down-regulated due to its additional roles.
Moreover, inhibiting protein-protein interactions is less susceptible to the evolution
of drug resistance than, for example, blocking the ATP binding pocket of a kinase
such as Cdc7, and is more target specific.

In the work of this thesis the endogenous origin licensing repressor geminin (see
Chapter 4) and a viral pathogen HPV1 E4 (see Chapter 5) were exploited in proof-of-
principle studies aimed at evaluating the potential of inhibiting origin licensing by
blocking MCM loading. Although both molecules are capable of blocking MCM
loading in vitro resulting in the inhibition of DNA replication initiation, questions
remain about their suitability for drug development programmes. Our quaternary
structure of the geminin molecule, combined with crystal data for geminin fragments,
indicates that the geminin-Cdt1 interaction involves at least two contact patches.
Thus mimicking geminin function with a small molecule may prove difficult. This
also seems to be the case for E4, which associates with both Cdc6 and Mcm7 suggesting that multiple regions of the protein may be required for mediating origin licensing inhibition. However, computer-aided design and molecular modelling may live up to this challenge and identify chemical compounds or peptides with the capacity to mimic the activity of these biological licensing blockers. Alternatively with the optimal delivery strategy geminin or E4 polypeptides may themselves find use as ‘biologics’ in treatment regimes. If SMoC-mediated uptake could be adapted into humans with the efficacy observed here in vitro, and that has been observed for PTDs in in vivo models (Selivanova et al., 1997; Gius et al., 1999; Hollinger et al., 1999), then SMoC-geminin or SMoC-E4 derivatives may provide potent anti-proliferative agents for a wide range of applications. To evaluate the potential of SMoC-protein conjugates as therapeutic agents the ability of SMoCs to transport a variety of cargo across cell layers, into a range of cell types and across the blood-brain barrier must be investigated, whilst the stability of SMoC derivatives and chemical protection to prolong the half-life of the molecule must also be determined.

Origin licensing inhibitors may find use for a range of applications and could potentially be administered systemically or through other administrative routes. For example, intraperitoneal infusion would allow treatment of ovarian tumours in which morbidity and mortality is linked to transcoelomic spread, whereas cerebrospinal fluid (CSF) administration would be used in the treatment of malignant glial tumours. Autologous bone marrow transplantation which is compromised by failure of engraftment or can be complicated by relapse as a consequence of contaminating tumour cells is another possible application. Origin licensing inhibitors could be exploited to purge grafts of contaminating tumour cells. Cycling stem cells are
resistant to engraftment, therefore arresting stem cells with a non-genotoxic agent, especially after genetic manipulation which stimulates cell cycle re-entry, could help improve engraftment rates. Importantly by targeting components of the assembly process whose roles are unique to this process, the possibility of a side-effect profile linked to actions in other cellular processes is removed. This has not been the case with the majority of other approaches to the inhibition of the cell cycle. This approach also circumvents problems associated with cytotoxicity, which is linked to hair loss, infertility and suppression of self-renewing tissues such as the haematopoietic and gastrointestinal systems. Low blood counts and gut-related symptoms, for example gastrointestinal haemorrhage, are rate-limiting side effects for conventional cytotoxic agents. In contrast cyclical treatment with origin licensing inhibitors should have minimal effects on normal tissues and therefore be associated with a much greater tolerance. Levels and activities of replication licensing factors and their regulators, as determined by immunohistochemical staining of biopsy sections, may have predictive value for such therapeutic interventions. Origin licensing inhibitors may also provide powerful tools to enhance the tumour kill of classical chemotherapeutic drugs. Both cell cycle-specific and cell cycle non-specific agents have been shown to have more potent effects on cycling cells. In contrast to cancer cell cycles, licensing inhibition in normal cells results in stalling of the cell cycle in G1 phase and therefore should provide protection against cytotoxic injury.

In conclusion the work presented in this thesis provides a strong rationale for therapeutic targeting of the DNA replication licensing machinery in hyperproliferative diseases and cancer, independent of genetic background and tissue
type. The findings discussed above pave the ground for future experimental work towards two major goals. Firstly, detailed transcriptomic and proteomic studies of untransformed human cells in the presence and absence of origin licensing inhibitors will help to characterise the molecular nature of the origin licensing checkpoint and shed some light on its mechanism of action. Such studies will further advance our understanding of how origin activation is linked to the cellular circuits that control proliferation, DNA damage response and apoptosis. With mechanistic knowledge of the molecular machinery that triggers the origin licensing checkpoint, comparative studies of matched pairs of normal and transformed human cell lines will help to understand loss of this checkpoint function in cancer cells. Secondly the identification of a promising new therapeutic target will start the process of drug development, which involves further additional target validation particularly in human tissues and the optimisation of potential lead compounds. Whether SMoC-geminin derivatives themselves constitute potent macrodrugs or ‘biologic’ agents for the treatment of cancer, will largely depend on their success in \textit{in vivo} Xenograft model systems and their properties in pharmacokinetic and ADMET (absorption, distribution, metabolism, excretion, toxicity) screens. Perhaps more straightforward than the development of macrodrugs targeting the licensing machinery is the screening for small molecule inhibitors of the Dbf4^{ASK}-dependent Cdc7 kinase, a joint venture that our group is currently pursuing in collaboration with the Cancer Research UK Drug Development group.


CHAPTER EIGHT

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Sarah Kingsbury


Yan, Z., DeGregori, J., Shohet, R., Leone, G., Stillman, B., Nevins, J. R. & Williams,


<table>
<thead>
<tr>
<th>Class of compound</th>
<th>Mode of action</th>
<th>Compounds in clinical use</th>
<th>Principle tumour types treated</th>
<th>Principle toxicities</th>
<th>Mechanisms of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen mustards</td>
<td>DNA alkylation at N7 and O6 positions of guanine leading to DNA interstrand cross-links (Thomas et al., 1978; Erikson et al., 1980; Garcia et al., 1988)</td>
<td>Mechlorethamine, Chlorambucil, Melphalan, Ifosphamide</td>
<td>Hodgkin's lymphoma, chronic lymphocytic leukaemia, multiple myeloma, ovarian and breast cancer (DeVita et al., 1972; George et al., 1972; Lerner, 1978)</td>
<td>Nausea, vomiting, alopecia, bone marrow depression, development of secondary malignancies (Van Putten and Lilieveld, 1971; Harris, 1976; Penn, 1976; Tucker et al., 1988)</td>
<td>Drug inactivation due to reaction with cellular thiols (Meister, 1988), decreased drug uptake, (Goldenberg and Begleiter, 1984) increased excision repair (Ewig and Kohn, 1977)</td>
</tr>
<tr>
<td>Nitrosoureas</td>
<td>Alkylation and carbamoylation at guanine O6 producing interstrand cross-links (Reed et al., 1975)</td>
<td>Carmustine, Lomustine, Semustine, Streptozotocin, Chlorozotocin</td>
<td>Central nervous system, brain, lung, and colorectal cancers, myeloma, Hodgkin's lymphoma (Wasserman et al., 1975)</td>
<td>Delayed and cumulative thrombocytopenia and leucopenia, renal damage, nausea, vomiting (Ramirez et al., 1972; Smith, 1989)</td>
<td>Increased alkytransferase activity (Breit et al., 1985; Aida and Bodell, 1987; Fox et al., 1989; D'Incalci et al., 1988)</td>
</tr>
<tr>
<td>Aziridines</td>
<td>As nitrogen mustards (Iyer and Szybalski, 1963; Dubois et al., 1990)</td>
<td>Thiotepa, Altretamine, Mitomycin</td>
<td>Lymphoma, ovarian adenocarcinoma, metastatic breast cancer, (Crocket and Bradner, 1976)</td>
<td>Anorexia, nausea, vomiting, bone marrow depression, neurotoxicity (Legha et al., 1976)</td>
<td>As nitrogen mustards (Dorr et al., 1987; Duhanty et al., 1989; Hoban et al., 1990)</td>
</tr>
<tr>
<td>Alkane sulphonates</td>
<td>Bifunctional alkylating agents that react with guanine N7 to form a diguanyl derivative and DNA interstrand cross-links (Tong and Ludlam, 1980; Farmer, 1987)</td>
<td>Busulfan</td>
<td>Chronic granulocytic leukaemia (Galton, 1969)</td>
<td>Nausea, vomiting, bone marrow depression, fibrosis, sterility, pulmonary infiltration, impotence, amenorrhrea (Sostman et al., 1977; Wilson, 1978)</td>
<td>Increased repair of DNA cross-links (Bedford and Fox, 1982)</td>
</tr>
</tbody>
</table>
Table A.2. Anti-metabolites. Table showing the classes of anti-metabolites that have been developed, their mode of action, examples of compounds that are in clinical use and the principle tumour types they are used to treat, the principle toxicities associated with treatment and the mechanisms by which resistance can develop.

<table>
<thead>
<tr>
<th>Class of compound</th>
<th>Mode of action</th>
<th>Compounds in clinical use</th>
<th>Principle toxicities</th>
<th>Mechanisms of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anti-folates</strong></td>
<td>Inhibition of DHFR (Schweitzer et al., 1990) and thymidylate synthase (Chu et al., 1990)</td>
<td>Methotrexate, Trimetrexate</td>
<td>Neurotoxicity, bone-marrow depression, renal failure (Condit et al., 1969; Dahl et al., 1971; Jacobs et al., 1976; Bleyer et al., 1981)</td>
<td>Overexpression/mutation of DHFR, altered drug transport (Gosick et al., 1997)</td>
</tr>
<tr>
<td><strong>Anti-pyrimidines</strong></td>
<td>Inhibition of thymidylate synthase (Chu et al., 1990)</td>
<td>Raltretrexad</td>
<td>Colorectal, breast, gastric and pancreatic cancers (Graa, 1990)</td>
<td>Neurotoxicity, bone-marrow depression, renal failure (Condit et al., 1969; Dahl et al., 1971; Jacobs et al., 1976; Bleyer et al., 1981)</td>
</tr>
<tr>
<td><strong>Anti-purines</strong></td>
<td>Inhibition of ribonucleotide reductase (Seckes et al., 1997)</td>
<td>Hydroxyurea</td>
<td>Colorectal, breast, gastric and pancreatic cancers (Graa, 1990)</td>
<td>Nausea, vomiting, ulceration of the oral cavity and bowel, alopecia, bone-marrow depression (Condit et al., 1969; Dahl et al., 1971; Jacobs et al., 1976; Bleyer et al., 1981)</td>
</tr>
<tr>
<td><strong>Anti- pyrimidines</strong></td>
<td>Inhibition of thymidylate synthase, incorporation into DNA/RNA (Weiss and Pitot, 1994; Uribe-Luna et al., 1997)</td>
<td>5-FU, 6-thioguanine, Pentostatin</td>
<td>Severe side effects, hemaaturnia (Shorey et al., 1972; Jackson et al., 1975)</td>
<td>Overexpression/mutation of DHFR, altered drug transport (Gosick et al., 1997)</td>
</tr>
</tbody>
</table>

Table A.2. Anti-metabolites. Table showing the classes of anti-metabolites that have been developed, their mode of action, examples of compounds that are in clinical use and the principle tumour types they are used to treat, the principle toxicities associated with treatment and the mechanisms by which resistance can develop.
### Table A3. Anti-tumour antibiotics. Table showing the classes of anti-tumour antibiotics that have been developed, their mode of action, examples of compounds that are in clinical use and the principle tumour types they are used to treat, the principle toxicities associated with treatment and the mechanisms by which resistance can develop.

<table>
<thead>
<tr>
<th>Class of compound</th>
<th>Mode of action</th>
<th>Compounds in clinical use</th>
<th>Tumour types treated</th>
<th>Principle toxicities</th>
<th>Mechanisms of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycin</td>
<td>Inhibition of DNA synthesis and DNA-directed RNA synthesis by blocking chain elongation (Reich et al., 1962)</td>
<td>Dactinomycin</td>
<td>Paediatric solid tumours (e.g. Wilms’ tumour, Ewing’s sarcoma), lymphoma, testicular cancer (Frei III, 1974)</td>
<td>Nausea, vomiting, myelosuppression, alopecia, acneiform skin lesions, secondary neoplasms (Svoboda et al., 1970; Frei III, 1974)</td>
<td>Increased drug efflux due to P-glycoprotein amplification (Diddens et al., 1987)</td>
</tr>
<tr>
<td>Anthracyclins</td>
<td>Interacts with DNA preventing strand-reunion reaction of topol and causing generation of double strand breaks (Tewey et al., 1984; Glisson et al., 1986; Wang et al., 1987)</td>
<td>Doxorubicin, Daunorubicin</td>
<td>Doxorubicin is used for a wide range of tumours, daunorubicin is used solely for acute myelogenous leukaemia (Carter, 1975)</td>
<td>Cardiotoxicity, myelosuppression, immunosuppression, nausea, vomiting, secondary neoplasms (Marquardt et al., 1976; Vecchi et al. 1976; O'Bryan et al., 1977)</td>
<td>Decreased topol activity (Pommier et al., 1986), increased glutathione peroxidase activity (Sinha et al., 1989)</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>DNA fragmentation and generation of free radicals (Twentyman, 1984)</td>
<td>Bleomycin</td>
<td>Squamous cell carcinoma, lymphoma (Blum, 1973)</td>
<td>Pulmonary toxicity, nausea, vomiting, stomatitis, alopecia (Blum, 1973)</td>
<td>Increased DNA repair (Miyaki et al., 1973), bleomycin inactivation (Sebi et al., 1991), altered glutathione S-transferase activity (Giacia et al., 1991)</td>
</tr>
<tr>
<td>Anthracene-dione</td>
<td>Intercalation and electrostatic interactions with DNA (Lown et al., 1984; Bowden et al., 1985)</td>
<td>Mitoxantrone</td>
<td>Leukaemias, lymphomas (Silver et al., 1991)</td>
<td>Nausea, vomiting, myelosuppression, cardiotoxicity (Silver et al., 1991)</td>
<td>Increased DNA repair, decreased topol activity (Faulds et al., 1991)</td>
</tr>
</tbody>
</table>
### Table A.4: Endocrine therapy

<table>
<thead>
<tr>
<th>Class of compound</th>
<th>Mode of action</th>
<th>Compounds in clinical use</th>
<th>Tumour types treated</th>
<th>Principle toxicities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestrogens</td>
<td>Inhibition of leutethinising hormone production leading to reduced testosterone levels (Bishop et al., 1985; Mashchak et al., 1982)</td>
<td>Diethylstilbestrol, Ethinyl estradiol</td>
<td>Prostate cancer, (postmenopausal breast cancer)</td>
<td>Tumour flare, impotence, gynecomastia, secondary neoplasms (Cartes et al., 1977; Claysse, 1985; Bergvis et al., 1989)</td>
</tr>
<tr>
<td>Anti-oestrogens</td>
<td>Competition for binding of oestrogen to oestrogen receptors (Furr and Jordan, 1984; MacGregor and Jordan, 1998)</td>
<td>Tamoxifen</td>
<td>Advanced breast cancer</td>
<td>Tumour flare, thrombocytopenia, leukopenia, nausea</td>
</tr>
<tr>
<td>Progestins</td>
<td>Promote maturation of secretory epithelium of the endometrium, efficacy correlates with presence of progesterone receptors (Kneale, 1986)</td>
<td>Medroxyprogesterone acetate, Megestrol acetate</td>
<td>Advanced endometrial carcinoma</td>
<td>Hypercalcaemia, weight gain, tumour flare, thrombophlebitis, embolism</td>
</tr>
<tr>
<td>Androgens</td>
<td>Suppression of leutethinising hormone production leading to reduction in oestrogen synthesis (Swinnen et al., 2004)</td>
<td>Fluoxymestrone</td>
<td>Metastatic breast cancer</td>
<td>Masculinisation</td>
</tr>
<tr>
<td>Anti-androgens</td>
<td>Competition for binding of dihydrotestosterone to androgen receptor (Neumann and Topert, 1986)</td>
<td>Cyproterone acetate, Flutamide</td>
<td>Prostate cancer</td>
<td>Gynecomastia</td>
</tr>
<tr>
<td>Aromatase inhibitors</td>
<td>Inhibition of aromatisation, the final step in oestrogen synthesis (Lonning and Kvinsland, 1988; Nemoto et al., 1989; Smith and Dowsett, 2003)</td>
<td>Aminoglutethimide, Testolactone</td>
<td>Metastatic breast cancer</td>
<td>CNS depression, masculinisation</td>
</tr>
<tr>
<td>Glucocorticoids</td>
<td>Induction of endonuclease activity resulting in programmed cell death (Melby, 1977)</td>
<td>Cortisol, Prednisome, Prednisolone</td>
<td>Leukaemias, lymphomas</td>
<td>Osteoporosis, congestive heart failure, impotence, neurotoxicity</td>
</tr>
</tbody>
</table>
### Table A.5 Immunotherapy

Table showing the classes of immunotherapies that have been developed, their mode of action, examples of compounds that are in clinical use, and their principle toxicities associated with their use.

<table>
<thead>
<tr>
<th>Class of compound</th>
<th>Mode of action</th>
<th>Compounds in clinical use</th>
<th>Tumour types treated</th>
<th>Principle toxicities</th>
<th>Principle drawbacks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micro-organisms</td>
<td>Activation of macrophages, T/B lymphocytes and type II immunological responses, inhibition of tumour cell motility</td>
<td>Bacillus Calmette-Guérin (BCG), C. parvum</td>
<td>Metastatic disease, cutaneous melanoma</td>
<td>Sepsis, leukopenia, respiratory failure, hypertension</td>
<td>Low activity</td>
</tr>
<tr>
<td>Interferons</td>
<td>Enhance cytotoxicity of immunocompetent cells, suppression of neovascularisation, enhanced expression of MHC I and II</td>
<td>IFN-α, IFN-β, IFN-γ</td>
<td>Hairy-cell leukaemia, chronic myeloid leukaemia</td>
<td>Asthenia, neutropenia, nausea, vomiting, thrombocytopenia</td>
<td>Responses are usually only partial</td>
</tr>
<tr>
<td>Interleukins</td>
<td>Enhanced growth of activated T cells, proliferation of lymphoid cells, lysis of tumour cells, regression of distant metastases, stimulation of release of other cytokines</td>
<td>IL-1, IL-2, IL-3, IL-4</td>
<td>Renal cell carcinoma, metastatic melanoma, acute myeloid lymphoma</td>
<td>Toxic fatalities in up to 10% of patients, angina, multi-organ malfunction, thrombocytopenia</td>
<td>Severe toxicity</td>
</tr>
<tr>
<td>Tumour necrosis factor</td>
<td>Induction of apoptosis, promotion of intravascular thrombosis causing tumour necrosis, activation of immunocompetent cells</td>
<td>TNF-α</td>
<td>Advanced melanoma, sarcoma</td>
<td>CNS toxicity, impairment of renal function, thrombocytopenia, hepatotoxicity,</td>
<td>Very low (&lt;5%) response rate, severe toxicity</td>
</tr>
<tr>
<td>Monoclonal antibodies</td>
<td>Antibody-dependent cellular cytotoxicity (ADCC)</td>
<td>Unconjugated/native mAbs, mAbs-enzyme conjugates</td>
<td>Haematological malignancies</td>
<td>HAMA (human anti-mouse antiglobulin response)</td>
<td>Low antibody uptake, antigenic heterogeneity, poor stability</td>
</tr>
</tbody>
</table>
Appendix B: Methods performed in collaboration

All work performed in collaboration and included because it forms part of the connected argument has been clearly identified. This appendix describes the methods used in that collaborative work.

B.1. CHAPTER FOUR

B.1.1. Chemical crosslinking of recombinant geminin proteins

Crosslinking of geminin or gemininΔNt proteins at 10 μg/ml was performed in 1 ml of buffer (20 mM HEPES pH 7.5, 200 mM KCl, 10 mM DTT, 1 mM EDTA) containing either bis-[sulfosuccinimidyl]suberate (BS₃) (Sigma) or ethyleneglycolbis-succinimidylsuccinate (EGS) (Pierce). BS₃ and EGS concentrations ranged from 0.5 to 5 mM. Reactions were quenched after 45 min at RT by adding 0.05 ml 1 M Tris-HCl pH 8.0. Cross-linked protein was precipitated by adding 0.115 ml 100% trichloroacetic acid. Protein was collected by centrifugation at 10,000 g for 5 min, washed once with 1 ml of acetone, air dried, resuspended in SDS-PAGE loading dye and analysed by 4-20% SDS-PAGE.

B.1.2. Electron microscopy

For structural analysis, 5 μl of purified full-length geminin or gemininΔNt (0.05 mg/ml in buffer [25 mM Tris-HCl pH 8.0, 100 mM NaCl]) were applied to carbon-coated copper grids (400 mesh, freshly glow-discharged in air). After 2 min excess buffer was removed by blotting and specimens were stained twice with 5 μl 2% w/v methylamine vanadate, pH 8.0, or 5 μl 2% w/v methylamine tungstate, pH 6.8 (NanoVan and Nano-W, Nanoprobes Inc., NY, USA) for 1 min. Micrographs were
recorded on Kodak SO163 film using a FEI Tecnai T10 transmission electron microscope in low-dose mode, operated at an accelerating voltage of 100 kV and with a magnification of 44,000×. Negatives were developed in Kodak full strength developer D-19 for 12 min and their quality was assessed by optical diffraction.

**B.1.3. Image processing and computer molecular modelling**

Micrographs were digitized on a Zeiss SCAI microdensitometer (Z/I Imaging) with a step size of 7 μm and images were coarsened by averaging 2×2 pixels corresponding to a pixel size of 3.18 Å at specimen level. Micrographs were scanned and particle images were selected manually. Image analysis was performed using IMAGIC (van Heel et al., 1996). The resulting set of images was normalised to the same sigma and filtered: low-resolution cut-off was ~100 Å to remove uneven background on particle images; high-resolution cut-off was ~7 Å. Images were centered using rotationally averaged total sum of images. The following multivariate statistical analysis allowed us to find references for multi-reference alignment. The alignment procedure was followed by the next round of statistical analysis of images (Serysheva et al., 1995).

The first approximate model of the molecule was obtained using angular reconstitution from the best 10-15 classes. The model was centered and its reprojections were used to refine alignment and classification of images. The final reconstruction was calculated out of ~200 classes containing 8-15 images each. 3D maps were calculated using the exact-filter back projection algorithm (Harauz and van Heel, 1986; Radermacher, 1988). Resolution of the map was assessed using the 0.5 threshold of Fourier Shell Correlation (Saxton & Baumeister, 1986) which corresponds to ~17.5 Å. Multiple sequence alignments between geminin and known structures were made using Threader3 (Jones et al., 1992)
Evaluation of the DNA replication licensing machinery as an anti-proliferative target. Sarah Kingsbury

(http://bioinf.cs.ucl.ac.uk/threader/). After alignment, the 1GK6 structure previously used in X-ray analysis for molecular replacement (Thépaut et al., 2002) was chosen as a template for modelling geminin's coiled-coil domain structure with the SwissModel software package (http://swissmodel.expasy.org/) (Schwede et al., 2003). Domain fitting by small translational and rotational movements into the 3D map of geminin was performed interactively using O (Jones and Kjeldgaard, 1997). The secondary structure prediction was performed by using PredictProtein server (EMBL, Heidelberg, http://www.embl-heidelberg.de/predictprotein/predictprotein.html). Illustrations were generated using Deep View Swiss-PDB viewer (Guex and Peitsch, 1997) (http://ca.expasy.org/spdbv/), PyMOL (http://pymol.sourceforge.net/) and IRIS Explorer (http://www.nag.co.uk/visual/IE/iecb/products/Product.html). Surface representations are displayed at a threshold level of 1σ corresponding to ~100% of the expected mass. This threshold was determined assuming the mass of the complex is ~105 kDa and the specific density is 0.833 kDa Å⁻³.

B.2. CHAPTER FIVE

B.2.1. Expression and purification of recombinant HPV1 E4 proteins

HPV1 E4 proteins were expressed in Sf9 insect cells using recombinant baculoviruses and purified using non-denaturing conditions as previously described (Roberts et al., 1994b).

B.2.2. In vitro binding assays

[³⁵S]-methionine-labelled Mcm7 protein was synthesised using a TNT T7/T3 coupled rabbit reticulocyte system (Promega). Recombinant HPV1 E4 (5 µg) was incubated with [³⁵S]-Mcm7 for 2 h at 4°C in ICB: 10 mM Tris-HCl pH 7.4, 150 mM
NaCl, 0.1% NP-40, 100 μg/ml PMSF, 0.5 μg/ml leupeptin and pepstatin A. E4 protein was collected by incubating with anti-E4 MAb 4.37 (Doorbar et al., 1988) bound to protein-G-Sepharose and analysed by SDS-PAGE and fluorography. HPV16 E1^E4 cDNA (Roberts et al., 1993) was cloned into pGEX-2T (Amersham) and GST-16E4 fusion protein expressed in E. coli. GST fusion proteins bound to glutathione agarose beads were mixed with [35S]-Mcm7 for 1 h at 4°C in PBS/0.1% NP-40, and complexes analysed as described above.

**B.2.3. Infection of keratinocytes and immunoprecipitations**

A recombinant adenovirus expressing HPV1 E4 was generated by homologous recombination in 293 cells as previously described (Graham and van der Eb, 1973; Wilkinson and Akrigg, 1992). Human keratinocyte cell lines were infected with recombinant adenoviruses at a multiplicity of infection (m.o.i.) of 50 for 2 h and harvested after 24 h. SVJD keratinocytes were infected with a recombinant SV40 virus (SV40-ΔE4) as previously described. Total cell lysates were prepared in ICB or extraction buffer. Nuclear pellet fractions were prepared and lysed as described (Kimura et al., 1994). Soluble extracts from S phase HeLa cells containing baculovirus-expressed HPV1 E4 (3 μM) and XeCdc6 (0.65 μM) were incubated at 37°C for 2 h and diluted in extraction buffer. All lysates and extracts were precleared with IgG-protein-G-Sepharose, incubated with antibodies for 1 h and precipitated with protein-G-Sepharose. Isolated immune complexes were resolved by SDS-PAGE, immunoblotted, and visualised by ECL (Amersham Pharmacia). HPV1 E4/ΔE4 proteins were detected by anti-E4 MAb 4.37 and rabbit polyclonal antibodies were used to detect Cdc6 and Mcm7 (Fujita et al., 1996).
B.2.4. Immunofluorescence

Human keratinocytes grown on glass slides were infected with recombinant adenoviruses at a m.o.i. of 25 to 50. After 24 h, cells were fixed as described (Roberts et al., 1993). HPV1 E4 was detected using anti-E4 MAb 4.37 and Cdc6 and Mcm7 using rabbit polyclonal antibodies to Cdc6 and Mcm7 (Fujita et al., 1996). Immune complexes were detected using Alexa 488-conjugated anti-mouse IgG (Molecular Probes, Invitrogen) and Texas Red-conjugated anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL, USA). Nuclei were counter-stained with DAPI. Z-images (0.5 μm) were acquired and digitally deconvolved using Openlab (Improvision, Lexington, MA, USA).

B.3. CHAPTER SIX

B.3.1. Design of small molecule carriers

Small molecule carriers (SMoCs) were designed to mimic the structure and activity of protein transduction domains (PTDs), which have recently been highlighted as a novel mechanism for the transport of biomolecules into cells and a potential new drug delivery strategy (Schwarze et al., 2000). Over recent years studies have shown that a variety of peptides, many of which are present in viral proteins, have the ability to cross biological membranes in various different cell types. Examples include the basic region (47-57) of HIV-1-tat (Frankel and Pabo, 1988; Green and Loewenstein, 1988), the third helix of the Drosophila antennapedia homeodomain (penetratin) (Joliot et al., 1991; Derossi et al., 1994) and designed variants such as Pep1. These peptides, known as ‘protein transduction domains’ (PTDs), can be linked to a variety of molecules with limited bioavailability (e.g. peptides, proteins, DNA), thereby enabling them to traverse biological membranes. Unlike traditional based techniques,
including fusion to receptor ligands (Ng et al., 2002) and packaging into caged liposomal carriers (Abu-Amer et al., 2001), protein transduction does not appear to be affected by cell type and can efficiently transduce almost 100% of cells both in vitro and in vivo with no apparent toxicity (Nagahara et al., 1998). Furthermore PTDs can deliver equal amounts of protein to 100% of treated cells, in contrast to viral delivery strategies which achieve only 30-50% transduction efficiency with highly variable levels of expression within these cells (Barka et al., 2000). In addition it has been claimed that some PTDs, for example Tat, have no limit in the size of molecule that they can transport into cells (Joliot and Prochiantz, 2004). A major hindrance in drug delivery is accessibility of the central nervous system since most hydrophilic substances are unable to cross the blood-brain barrier. However studies have shown that PTD fusion molecules introduced into mice exhibit delivery to all tissues, including traversal of the blood-brain barrier (Schwarze and Dowdy, 2000; Denicourt and Dowdy, 2003). Taken together, these studies suggest that tethering potential drugs to PTDs may circumvent some of the problems associated with current drug delivery strategies (Schwarze et al., 2000). However, there are a number of drawbacks to the use of PTDs including reduced expression levels of PTD-fusion proteins (Cashman et al., 2003), altered biological activity compared to the native protein and susceptibility to rapid degradation by cellular enzymes.

Small molecule carriers (SMoCs) were designed to circumvent the problem associated with PTDs, and provide a novel strategy for in vitro and in vivo delivery of various cargo moieties into cells (D. Selwood, Wolfson Institute for Biomedical Research, UCL; personal communication). The design of SMoCs was based on the observation that although small peptides (<20 amino acids) generally lack any
structure in aqueous media, peptides such as the third helix of the antennapedia sub-domain adopt a 3,10 helix in trifluoroethanol solution (which is close to the hydrophobic nature of a lipid bilayer) whilst HIV-1-tat 47-57 analogues are more efficient transporters when their predicted helicity is increased (Ho et al., 2001). Furthermore, the first step in translocation of lysine/phenylalanine co-polymers across biological membranes involves a conformation transition from a random coil form to a helix-rich form (Shibata et al., 2003). It has been postulated from these studies that the helical structure may serve to present the amino acid side chains such that the overall molecule is amphipathic in nature. Several reports have highlighted the ability of polyphenyl systems (Orner et al., 2001) to mimic the display of amino acid side chains in a peptide helix. In particular the 2, 2’ positions of a biphenyl system are able to closely mimic the spatial orientations of the i, i+3 or i+4 positions of an alpha helix (Jacoby, 2002) (Figure 6.1a). SMoCs are comprised of a simple biphenyl system displaying guanidine groups at the 2 and 2’ positions (2G-SMoC) to mimic display of arginine residues on one face of the helix (Figure 6.1b). A key feature of transportation of PTDs across cellular membranes is the formation of bidentate hydrogen bonds between the guanidinium head groups of the PTD and anionic structures on the cell surface (Rothbard et al., 2005). For this reason unsubstituted guanidine groups were used in the design of SMoCs. A further analogue displaying 4-guanidine groups (4G-SMoC) was also designed; in this case the precise mimicry of the helix is lost though the amphipathicity of the molecule is maintained and the molecule displays more guanidine groups per phenyl unit (4 in a 6 Å distance compared to 3 for an alpha helix) (Figure 6.1c). An important feature of PTDs is their ability to transport a variety of different cargo. In order to mimic this feature in SMoCs the synthesis was designed such that a late stage intermediate
would contain a free amine group, allowing a very straightforward variation of the linking chemistry (Figure 6.1d).

**B.3.2. Synthesis of small molecule carriers**

A scheme of the synthesis of 2G-SMoC is shown in Figure A.1. Initial reduction and diazotization-halogenation of the 2-nitro group in 1 provides the 2-bromo-5-methylanisole 2. The key step in the synthesis is the palladium [0] catalysed Suzuki coupling of the 3-methoxyaryl boronic acid to give the biphenyl system 2 in 63% yield. Radical bromination of the aryl methyl group using N-bromosuccinimide, followed by reaction with potassium phthalimide yields the protected amine intermediate 3. Removal of the methyl ethers and alkylation using the Mitsunobu reaction provides 4. Removal of the tertbutyloxycarbonyl (Boc) protecting groups with trifluoroacetic acid and guanidinylation using the bis-Boc triflate provides the Boc protected biphenyl mimic 5. Finally, removal of the phthalimide group may be effected using hydrazine and the amine reacted with fluorescein isothiocyanate (FITC) which, after Boc removal, gave the required compound 2G-SMoC-FITC. 4G-SMoC-FITC and 4G-SMoC-SS-py were synthesised by similar routes.

**B.3.3. Molecular modelling**

Molecular modelling was carried out using Sybyl 6.5. Peptides were modelled starting from the penetratin structure (pdb code 1kz0) using the Biopolymer tools. The peptides were energy minimised to a gradient of less than 0.05 kcal/Å using the Kollman all atom forcefield and charges and Powell minimisation algorithm. Non-bonded cutoffs were applied at 12 Å and a distance dependent dielectric of 1.0r was used. Small molecules were modelled using Chem3DPro and minimised with MM2.
Figure A.1. Chemical synthesis of 2G-SMoC-FITC. The key biphenyl formation is established early in synthesis by means of a Suzuki Pd(0) coupling of 1 and 3-methoxy boronic acid. Guanidinylation of the side chains (4 to 5) is achieved through use of bis (Boc) triflate.
## Appendix C: Summary of data from *in vitro* DNA replication reactions

### Table A.6. Summary of data obtained from the cell-free *in vitro* DNA replication system. Data is expressed as the number of nuclei positive/negative for biotin-16-dUTP incorporation. Three individual experiments were performed for each data set and the mean percentage of nuclei replicating calculated.

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Appendix D

Supplementary Data

Functional characterisation of recombinant human Geminin

Prior to structural analysis by EM we characterised recombinant Geminin for its DNA replication inhibition activity in the cell-free DNA replication assay\(^1\). To test the activity of recombinant Geminin, in vitro DNA replication reactions were performed in the presence of recombinant his-tagged human Geminin (H\(_6\)-haGeminin). The purified recombinant Geminin was homogeneous and migrated as a single peak corresponding to a molecular weight of \(~240\) kDa, indicating either an unusual shape of the protein and/or assembly into oligomers. G1 nuclei incubated in a physiological buffer supporting DNA elongation resulted in 2.2 % of nuclei synthesising DNA, in keeping with a small amount of S phase contaminants\(^1\) (Supplementary Fig. 1a,i online). In contrast, 22% of G1 nuclei synthesised DNA upon incubation in S phase cytosol, representing true initiation in vitro (Supplementary Fig. 1a,ii online). Addition of Geminin to the reaction resulted in 6.7% of the nuclei replicating, indicating that 66% of replication-competent nuclei failed to synthesise DNA in vitro (Supplementary Fig. 1a,iii). In control reactions, DNA elongation was not affected by addition of recombinant Geminin to S phase nuclei with 64% of them continuing to replicate (data not shown). Taken together, these data demonstrate that recombinant Geminin is a potent inhibitor of the initiation of DNA replication in vitro, but does not arrest DNA elongation from origins that have fired in vivo prior to preparation of the nuclear templates from intact cells.

We next analysed chromatin-bound protein fractions from the reactions identical to those described above. There was no Mcm2 protein bound to chromatin in fractions prepared from G1 nuclei incubated in elongation buffer (Supplementary Fig. 1b online). Incubation of G1 nuclei in S phase cytosol resulted in recruitment of Mcm2 to chromatin, indicative of pre-RC assembly in vitro (Supplementary Fig. 1b online). Addition of Geminin to co-incubations of G1 nuclei in
S phase cytosol banned the replication license by blocking loading of Mcm2 onto chromatin (Supplementary Fig. 1b online). Importantly, this block to pre-RC assembly coincides with binding of recombinant Geminin to chromatin (Supplementary Fig. 1b online). This suggests that the replication inhibitory activity of Geminin may also be coupled to its potential to bind DNA. These data demonstrate that bacterially expressed recombinant Geminin subjected to structural analysis is fully functional in repressing origin licensing and blocking initiation of DNA replication.

Properties of an amino-terminal truncated form of human Geminin

To distinguish between different structural domains in our model, we analysed a truncated form of Geminin with deletion of the amino terminal part of the protein, which is predicted to form an independent structural and functional domain (Supplementary Fig. 3a online). In *Xenopus* it has been demonstrated that the first 80 amino acid residues of the frog ortholog are not required for Geminin’s replication inhibition activity, suggesting that the truncated protein folds into the active form. We therefore engineered a Geminin-ΔNt truncated derivative of human Geminin lacking the first 80 aa residues. Prior to analysis by EM, the Geminin-ΔNt recombinant protein was tested for its biochemical activity in the cell-free replication assay and showed activity (inhibition of 65.5% of competent nuclei) almost identical to that of the full-length protein (Supplementary Fig. 1a,iii online), indicating that overall folding of either the truncated protein or domains involved in replication control have not been affected by the deletion.

Next we analysed Geminin-ΔNt by EM at conditions identical to those employed for full-length Geminin. Raw images of Geminin-ΔNt appeared as small, thread-like elongated particles. In contrast to particles of the full-length protein, the head-like bulk at the end was absent. Approximately 300 molecular images were selected and subjected to multivariate statistical
Five typical raw images of Geminin-ΔNt and one of the class averages are shown in Supplementary Fig. 4d online. All particles of Geminin-ΔNt are uniformly shaped as a short filament of ~25Å in diameter and ~170Å in length, thinner and slightly longer than we expected. Since the molecular images did not show the head-like domain at one end of the filaments, we conclude that the head-like part in the structure represents the amino terminal part of Geminin. The smaller diameter of Geminin-ΔNt particles in comparison to particles of the full-length protein is indicative of dimeric rather than tetrameric organisation of the molecule. In keeping with this observation, crosslinking experiments show that the major crosslinked species of Geminin-ΔNt is a protein band migrating as a dimer (Supplementary Fig. 4b online). This is in keeping with crystallographic data on a peptide representing the coiled coil domain of Geminin.

Domain structure modelling of human Geminin

Since the structure of the coiled coil domain is unavailable at present, we modelled the potential Geminin dimer with SwissModel structural homology modelling software using the 1GK6 atomic structure as a template. This structure, originally used by Thepaut and co-authors for the molecular replacement method, was identified as a potential match for the Geminin sequence by the Threader 3 program. The resulting structural model is a leucine/isoleucine zipper that is coordinated into a parallel dimer of α-helices, with almost all hydrophobic aa residues forming the interface between monomers (Supplementary Fig. 4c online). The diameter of the dimer is ~22Å, consistent with our findings for Geminin-ΔNt particles. The length of the 37 aa dimer was measured as ~60Å. Considering that Geminin-ΔNt is 132 residues long, it is plausible to suggest that the overall length of the putative long helical structural organisation could be close to ~170Å as determined for Geminin-ΔNt. The extended length could be explained by the absence of
dimer-dimer interactions within the Geminin molecule and thus straightening of the Geminin-ΔNt dimer.

References


Supplementary Methods

Protein expression and purification

Geminin and Geminin-ΔNt were expressed using modified pET-15b vector with thrombin site substituted for the recognition sequence of TEV protease. Rosetta pLysS strain (Novagen) was used for protein expression. Target proteins were purified by NiNTA chromatography followed by removal of the tag using His-tagged TEV protease (Invitrogen) and a subsequent second NiNTA purification step. The proteins were further purified by gel filtration chromatography on a Superose 6 (Pharmacia) column using 250mM NaCl, 25 mM Tris-HCl pH 8.5 buffer.

Chemical crosslinking of recombinant Geminin proteins

Crosslinking of Geminin or Geminin-ΔNt proteins at 10 μg ml⁻¹ was performed in 1 ml of buffer (20 mM HEPES pH 7.5, 200 mM KCl, 10 mM DTT, 1 mM EDTA) containing either bis-[sulfo succinimidyl] suberate (BS²⁺) (Sigma) or ethyleneglycol-bis-succinimidylsuccinate (EGS) (Pierce). BS²⁺ and EGS concentrations ranged from 0.5 to 5 mM. Reactions were quenched after 45 min at RT by adding 0.05 ml 1 M TrisHCl pH 8.0. Crosslinked protein was precipitated by adding 0.115 ml 100% trichloroacetic acid. Protein was collected by centrifugation at 10,000 g for 5 min, washed once with 1 ml of acetone, air dried, resuspended in SDS-PAGE loading dye and analyzed by 4-20% SDS-PAGE (Invitrogen).
Cell culture, *in vitro* DNA replication and chromatin binding assay

NIH3T3 and HeLa S3 tissue culture cells were cultured and synchronised as described\(^1,2,3\). Nuclei and cytosolic extracts were prepared from synchronised cells as described\(^1,3,4\). DNA replication in vitro reactions were performed as previously described\(^1,3\). H6-hsGeminin protein (or Geminin-ΔNt ) in 50 mM Na-Phosphate was added to a final concentration of 4 μM. An equal volume of buffer was added to control replication reactions. Confocal fluorescence microscopy of random fields of nuclei was performed on a Leica TCS DMRE confocal microscope and images were processed and analysed as described\(^1\). For chromatin binding assay nuclei were lysed as described\(^5\). Insoluble chromatin was washed and resuspended in buffer (10 mM KCl, 1.5 mM MgCl\(_2\), 10 mM HEPES pH 7.9, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 0.1 mM PMSF) plus 1000 U ml\(^{-1}\) DNase I, incubated for 30 min at RT followed by 30 min incubation at 4°C with 1 volume of 0.5 M NaCl. Solubilised chromatin bound proteins were clarified by centrifugation, separated by 10% SDS-PAGE and immunoblotted with antibodies against Mcm2 (BD Biosciences, #610701), His-tag (BD Biosciences, #631212) and Histone H1 (Santa Cruz, #10806).

**Electron Microscopy**

For structural analysis, 5 μl of purified full-length Geminin or Geminin-ΔNt (0.05mg ml\(^{-1}\) in buffer [25 mM Tris-HCl pH 8.0, 100 mM NaCl]) were applied to carbon-coated copper grids (400 mesh, freshly glow-discharged in air). After 2 min excess buffer was removed by blotting and specimens were stained twice with 5 μl 2% w/v methylamine vanadate, pH 8.0, or 5 μl 2% w/v methylamine tungstate, pH 6.8 (NanoVan and Nano-W,
Nanoprobes Inc.) for 1 min. Micrographs were recorded on Kodak SO163 film using a FEI Tecnai T10 transmission electron microscope in low-dose mode, operated at an accelerating voltage of 100 kV and with a magnification of 44,000×. Negatives were developed in Kodak full strength developer D-19 for 12 min and their quality was assessed by optical diffraction.

**Image processing and computer molecular modelling**

Micrographs were digitized on a Zeiss SCAI microdensitometer (Z/I Imaging) with a step size of 7 μm and images were coarsened by averaging 2×2 pixels corresponding to a pixel size of 3.18Å at specimen level. Micrographs were scanned and particle images were selected manually. Image analysis was performed using IMAGIC. The resulting set of images was normalized to the same sigma and filtered: low-resolution cut-off was ~100Å to remove uneven background on particle images; high-resolution cut-off was ~7Å. Images were centred using rotationally averaged total sum of images. Subsequent multivariate statistical analysis allowed us to find references for multi-reference alignment. The alignment procedure was followed by the next round of statistical analysis of images. The first approximate model of the molecule was obtained using angular reconstitution from the best 10-15 classes. The model was centred and its reprojections were used to refine alignment and classification of images. The final reconstruction was calculated out of ~200 classes containing 8-15 images each. 3D maps were calculated using the exact-filter back projection algorithm. Resolution of the map was assessed using the 0.5 threshold of Fourier Shell Correlation, which corresponds to ~17.5Å. Multiple sequence alignments between Geminin and known structures were
made using Threader3\textsuperscript{12} (http://bioinf.cs.ucl.ac.uk/threader/). After alignment, the 1GK6 structure previously used in X-ray analysis for molecular replacement\textsuperscript{13} was chosen as a template for modelling Geminin’s coiled coil domain structure with the SwissModel software package (http://swissmodel.expasy.org/)\textsuperscript{14}. Domain fitting by small translational and rotational movements into the 3D map of Geminin was performed interactively using O\textsuperscript{11}. The secondary structure prediction was performed by using PredictProtein (EMBL, Heidelberg, http://www.embl-heidelberg.de/predictprotein/predictprotein.html). Illustrations were generated using Deep View Swiss-PDB viewer\textsuperscript{15} (http://ca.expasy.org/spdbv/), PyMOL (http://pymol.sourceforge.net/) and IRIS Explorer (http://www.nag.co.uk/visual/IE/iecbb/Product.html). Surface representations are displayed at a threshold level of 1\sigma corresponding to \~100\% of the expected mass. This threshold was determined assuming the mass of the complex is \~105 kDa and the specific density is 0.833 kDa Å\textsuperscript{3}.

References


Appendix F

Small molecule mimics of an α-helix for rapid and efficient transport of proteins into cells

Authors
Masahiro Okuyama, Heike Laman, Sarah R Kingsbury, Cristina Visintin, Elisabetta Leo, Kathryn Leigh Eward, Kai Stoeber, Chris Boshoff, Gareth H Williams, David L Selwood.

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1Biological and Medicinal Chemistry Group,
2Viral Oncology Group and 3Cancer Research UK Chromosomal Replication Group, Wolfson Institute for Biomedical Research, University College London, Gower Street, London, WC1E 6BT, UK.
4Department of Pathology, Royal Free and University College Medical School, University College London, London, WC1E 6JJ, UK.
5These authors contributed equally to this work.
6Current address: Division of Virology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QP, UK.
7Current address: Department of Life Sciences, Faculty of science and Technology, Anglia Ruskin University, East Road, Cambridge, CB1 1PT, UK.
8Correspondence should be addressed to D.L.S. (d.selwood@ucl.ac.uk).
Abstract

We have designed and synthesized small molecule, biphenyl, alpha helical mimics of a peptide protein transduction domain (PTD). These small molecule carriers, which we have termed SMoCs, efficiently deliver dye molecules and proteins into a variety of cell types and can be easily coupled to recombinant proteins. The SMoCs were designed using molecular modelling techniques. As an example of a protein cargo, we have applied this new technology to the internalization of the DNA replication licensing repressor geminin, \textit{in vitro}, providing evidence that a SMoC-geminin, delivered extra-cellularly, can have an anti-proliferative effect on U2OS human osteosarcoma cells. The mechanism of uptake of SMoC-geminin was shown to be via clathrin mediated endocytosis.
Introduction

A convenient method to deliver exogenous proteins into cells would have wide ranging applications in cell biology. Expression of the protein following gene delivery is a widely used technique. However, the amount of protein synthesized is largely dependent on the ability of the cell of interest to express the protein. The currently available methods to deliver native proteins include micro-injection, which is time and labour intensive, or complexing the protein with a lipid-membrane permeablizing agent, which is inefficient and often toxic to cells.1,2

One solution to the delivery problem is to utilize a protein transduction domain (PTD) to internalize the protein. A number of PTDs have been identified, mostly small, less than 30 amino acid peptides. Well known examples include the basic region (47-57) of HIV-1-tat, the third helix of antennapedia homeodomain (penetratin)3,4 and designed variants such as Pep15, which is not covalently linked to its cargo. PTDs generally contain multiple basic residues and in many cases adopt an amphipathic helical structure in a membrane-like environment. The field was galvanised by a report by Schwarze and Dowdy6 on the ability of HIV-1-tat fusion proteins to deliver β-galactosidase to multiple sites, including the brain, in mice. More recently reports on delivery of functionally active proteins in vivo have started to appear.7,8 Despite these notable successes, the use of PTDs to deliver proteins has yet to become commonplace in cell biology, as their use necessitates further experiments. PTD-fusion proteins must be engineered, expressed and purified from bacteria, and PTDs have been reported to reduce expression levels in some cases.9 Reagents based on synthetic PTDs share the disadvantages common to all peptides, including high cost of manufacture and the potential for biodegradation.
We sought to design a small molecule mimic of an amphipathic helix cell penetrating peptide. Ideally a carrier would be of minimal size and molecular weight and easily attached to proteins and other cargo for their transport into cells. As the carrier is a small molecule, it would not be subject to the same biodegradation processes as peptides. Such a carrier would deliver the protein cargo into cells in its native form, so there would be no disruption of protein function. Attachment to a carrier after a protein's cloning, expression and purification would allow researchers to easily utilise their existing recombinant proteins. In this article we describe the design (using molecular modelling techniques), synthesis and biological application of biphenyl mimics of peptide PTDs, which we have named small molecule carriers (SMoCs). First we demonstrate the effectiveness of these molecules in transporting SMoC-dyes into cells; and second, as an example for protein cargos, we show that SMoCs deliver the DNA replication licensing repressor protein geminin into cells in a functionally active native form, suppressing origin licensing and progression into S phase.
Materials and Methods

For chemistry synthesis, see supplementary information.

Molecular modelling. Molecular modelling was carried out using Sybyl 6.5 (Tripos Inc, St Louis, MO, USA). Peptides were modelled starting from the penetratin structure (pdb code 1kz0) using the Biopolymer tools. The peptides were energy minimised to a gradient of less than 0.05 kcal/Å using the Kollman all atom forcefield and charges and Powell minimisation algorithm. Non-bonded cutoffs were applied at 12 Å and a distance dependent dielectric of 1.0r was used. Small molecules were modelled using Chem3DPro (Cambridgesoft Corporation, Cambridge, MA, USA) and minimised with MM2. Figures were generated using Accelrys ViewerPro (Accelrys Software Inc., San Diego, CA, USA).

Cell Culture. Human U2OS, WI-38 human diploid fibroblasts (HDF), HeLa S3, and murine NIH/3T3 fibroblasts were cultured in DMEM supplemented with 10% FCS, 100 U/mL penicillin and 0.1 mg/mL streptomycin. NIH/3T3 fibroblasts were subjected to density-dependent growth arrest as previously described.11 Human peripheral blood mononuclear cells were isolated from whole blood, and CD3+ cells were isolated by MACS separation (Miltenyi Biotech, Belgium).

Cell viability assays. Cell viability assays were performed using CellTiter-Glo™ (Promega, Southampton, UK). U2OS cells were seeded in 96 well dishes and treated with 10 μM of each compound, as indicated, and incubated for 24 or 48 h. Samples were processed as per manufacturer’s protocol. Four independent experiments were performed and replicated twice.
Protein expression and coupling to 4G-SMoC and AlexaFluor488. Geminin, ΔNt-geminin, a truncated derivative of human geminin lacking the first 80 amino acid residues, and Tev protein were expressed and purified as described.\textsuperscript{12,13} For coupling of proteins to 4G-SMoC 2 mg protein (1mg/mL in 0.1 M sodium phosphate buffer, 5 mM EDTA, pH 6.00), was treated with 14 mg 2-mercaptoethylamine·HCl, and incubated at 37°C for 90 min. Excess 2-mercaptoethylamine. HCl was removed by buffer exchange using an Amersham PD-10 (exclusion limit (Mr) $5 \times 10^3$ globular protein) desalting column pre-equilibrated with PBS-EDTA buffer. The eluted, reduced protein was concentrated to approximately 1mg/mL using an ultrafiltration centrifugation cartridge (Sartorius AG, Goettigen, Germany) and coupled with 4G-SMoC-SS-Py. Coupling was performed for 90 min at rt using 0.1 mg SMoC reagent per 2 mg protein. Following coupling 4G-SMoC-geminin was purified by buffer exchange using the desalting column as above.

Conjugation of geminin with AlexaFluor488 was performed using the Molecular Probe Protein Labeling Kit, according to the manufacturer’s protocol (Invitrogen, CA, USA). Geminin-AlexaFluor488 and Tev-AlexaFluor488 were then coupled with 4G-SMoC following the procedure described above, which generated 4G-SMoC-geminin-AlexaFluor488 and 4G-SMoC-Tev-AlexaFluor488. The efficiency of conjugation reactions, protein concentration and absence of unincorporated label in the solution were measured and confirmed with spectroscopic and electrophoretic methods.
**Assay for efficiency of coupling.** Efficiency of 4G-SMoC coupling to geminin was assessed by quantification of sulfhydryl groups using Ellman's Reagent (Pierce, Rockford, IL, USA) according to Pierce protocol 22582.

**Biochemical fractionation and immunoblotting** For analysis of subcellular protein fractions, WI-38 HDFs were biochemically fractionated as previously described.\(^\text{14}\) Protein fractions were resolved by SDS-PAGE and immunoblotted with antibodies against Mcm2 (BD Transduction Laboratories™, Lexington, KY, USA, #610701), geminin\(^\text{15}\) and β-Actin (Sigma Aldrich, Gillingham, UK, #A5441).

**Subcellular localization studies.** For visualization of SMoC-FITC uptake in live cells, U2OS cells were cultured on glass coverslips and pulsed with 10 μM FITC, TAT-FITC or 2G- or 4G-SMoC-FITC for 15 min. For analysis of uptake kinetics in live cells, cells were incubated with 10 μM 2G- or 4G-SMoC-FITC for 10-80 min prior to immediate visualization. The efficiency and sub-cellular localization of SMoC delivery in live cells was assessed by confocal fluorescence microscopy on a Leica TCS DMRE confocal microscope or quantified by FACS analysis (FACS Calibur, BD Biosciences, NJ, USA), where cells were washed in PBS and treated with 50 μg/mL propidium iodide, to enable discrimination of dead cells. 10000 Cells were collected for each sample and analyzed using CellQuest™ software (BD Biosciences, NJ, USA).

For detection of 4G-SMoC-geminin-AlexaFluor488 and 4G-SMoC-Tev-AlexaFluor488 uptake into live cells, exponentially growing cells (WI-38 HDF, NIH/3T3, U2OS and Saos2) were cultured on glass coverslips. Cells were washed
once in PBS, and incubated with fresh medium containing 10 μM 4G-SMoC-Geminin-AlexaFluor488 or 10 μM 4G-SMoC-Tev-AlexaFluor488. After appropriate time incubation, coverslips were washed extensively in PBS, placed in a plate containing medium without Red Phenol (Gibco) and observed by live confocal fluorescence microscopy (MP-UV, Leica Microsystems, GmbH, Wetzlar, Germany) using a 40x and a 63x water immersion objective.

For detection of Mcm2 associated with chromatin and/or the nuclear matrix by immunofluorescence, WI-38 HDFs were treated with 4G-SMoC-ΔNt-geminin for 24 h. Soluble proteins were detergent extracted by treatment with 0.1% Triton-X 100 for 30 s, cells were fixed with 4% paraformaldehyde for 5 min, washed in 2x PBS for 10 min followed by 1x PBS for 5 min and permeabilized in 0.1% Triton-X100 for 5 min. Non-specific interactions were blocked with 1% BSA for 15 min and cells stained with a monoclonal anti-Mcm2 antibody, a FITC-conjugated anti-mouse secondary antibody and counterstained with propidium iodide/RNase A (both at 50 ng/mL, Sigma Aldrich, Dorset, UK). Fluorescence intensity was quantified using ImageJ (http://rsb.info.nih.gov/ij).

**Mechanism of cellular uptake studies.** Studies to determine whether uptake of 2G-SMoC-FITC or 4G-SMoC-geminin-AlexaFluor488 by WI-38 or U2OS cells could be affected by inhibitors of trafficking utilized the following compounds: 14 μM chlorpromazine, 25 μM nystatin, 25 μM (eip)amiloride, 4 μM cytochalasin D, and 40 nM nocodazole. Cells were pre-incubated for 1 h prior to the addition of either 2G-SMoC-FITC or 4G-SMoC-geminin-AlexaFluor488. To determine whether uptake could be affected by decreased temperature, cells were placed either at 37°C or in an
ice bath prior to the addition of either 2G-SMoC-FITC or 4G-SMoC-geminin-AlexaFluor488. Incubation continued for an additional hour. Cells were then washed extensively with PBS, and either visualised on a fluorescence microscope or trypsinized and stained with 33 μg/mL propidium iodide prior to being collected by FACS analysis.

**Cell proliferation assays.** To assess the ability of 4G-SMoC-geminin and 4G-SMoC-ΔNt-geminin to block re-entry into cycle from G0, NIH/3T3 fibroblasts were driven into G0 by density-dependent growth arrest and after 5 days were released back into the cell cycle by sub-culturing into fresh growth medium. Eight hours after release from G0, 10 μM of 4G-SMoC-geminin/4G-SMoC-ΔNt-geminin or the equivalent volume of PBS was added to the cells. At 21 h following release, cells were pulse-labelled for 1 h with 50 μM BrdU. Subsequently, cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and DNA was denatured by treatment with 2 M HCl to allow detection of incorporated BrdU. Cells were stained with FITC-conjugated anti-BrdU (1:20 dilution; Alexis Biochemicals, Nottingham, UK) and propidium iodide/RNase A (both at 50 ng/mL). Confocal fluorescence microscopy of random fields was performed on a Leica TCS DMRE confocal microscope.

To assess the ability of 4G-SMoC-ΔNt-geminin to block cell proliferation in cycling cells WI-38 HDFs were seeded onto glass slides at a density of 1 x 10^4/mL. After 12 h 10 μM of 4G-SMoC-ΔNt-geminin was added to the cells and replenished after a further 12 h. Twenty-three hours after the initial treatment, cells were pulse-labelled for 1 h with 50 μM BrdU. BrdU incorporation was monitored as described above.
Results

Design of the amphipathic helix mimics. Although small peptides (<20 amino acids) generally lack any structure in aqueous media, peptides such as the third helix of the antennapedia sub-domain can adopt a helical conformation in phospholipid vesicles as can the HIV-1 Rev-(34-50) peptide in methanol solution. An analogous conformational switch into a helical form has been reported for lysine/phenylalanine co-polymers. HIV-1-tat 47-57 analogues became more efficient transporters when their predicted helicity was increased. Thus the helical structure may serve to present the amino acid side chains such that the overall molecule is amphipathic in nature (Figure 1b). Several reports, notably by Hamilton, have highlighted the ability of polyphenyl systems to mimic the display of amino acid side chains in a peptide helix. In a detailed molecular modelling study the 2, 2' positions of a biphenyl system are able to closely mimic the spatial orientations of the i, i+3 or i+4 positions of an alpha helix (Figure 1a). We designed a simple biphenyl system displaying guanidine groups at the 2 and 2' positions (2G-SMoC) to mimic display of arginine residues on one face of the helix (Figure 1c). Unsubstituted guanidine groups were used, since bivalent coordination of guanidine by phosphate, which forms a tight ion pair, is thought to be essential for the function of cell penetrating peptides. The amphipathic nature of the antennapedia third helix compared to the designed biphenyl compound is shown in Figure 1b. Another analogue displaying 4-guanidine groups (4G-SMoC) was also designed (Figure 1d); in this case the precise mimicry of the alpha helix is lost, however, the amphipathicity of the molecule is maintained and the molecule displays more guanidine groups per phenyl unit (4 in a 6 Å distance compared to 3 for an alpha helix). We designed the synthesis such that a late stage
intermediate would contain a free amine group (Figure 1e). This allows a straightforward variation of the chemistry to link the SMoC to the protein cargo. Here we utilize the DNA replication licensing repressor, geminin\textsuperscript{10} as a protein cargo to couple to an SMoC. Geminin is a 23.5 kDa protein which blocks DNA replication licensing by competitively binding Cdt1 to prevent loading of the hexameric MCM complex (Mcm2-7) onto chromatin. We employed a thiopyridyl derivative of 4G-SMoC to enable its coupling to geminin through a bio-reducible disulfide bond to two cysteine residues at amino acids 194 and 208 in the C-terminus of geminin.

*Synthesis of SMoCs.* A scheme of the synthesis of 2G-SMoC is shown in Figure 2. Initial reduction and diazotization-halogenation of the 2-nitro group in 1 provides the 2-bromo-5-methylanisole 2. The key step in the synthesis is the palladium [0] catalysed Suzuki coupling of the 3-methoxyaryl boronic acid to give the biphenyl system 2 in 63\% yield. Radical bromination of the aryl methyl group using N-bromosuccinimide, followed by reaction with potassium phthalimide yields the protected amine intermediate 3. Removal of the methyl ethers and alkylation using the Mitsunobu reaction provides 4. Removal of the tertbutyloxycarbonyl (Boc) protecting groups with trifluoroacetic acid and guanidinylation using the bis-Boc triflate provides the Boc protected biphenyl mimic 5. Finally, removal of the pthalimide group may be effected using hydrazine and the amine reacted with fluorescein isothiocyanate (FITC) which, after Boc removal, gave the required compound 2G-SMoC-FITC. 4G-SMoC-FITC and 4G-SMoC-SS-py were synthesized by similar routes (see supplementary information).
**SMoC delivery and sub-cellular localization.** In order to test the ability of the di-guanidine (2G-SMoC) and tetra-guanidine (4G-SMoC) compounds to deliver small molecules, they were coupled to fluorescein as described above. Human U2OS osteosarcoma cells were incubated with 10 μM of each compound, and live cells were visualized by confocal fluorescence microscopy. Delivery was compared to a TAT-FITC conjugate and to FITC alone. At lower magnification (10×), cells treated with either of the SMoC-FITC compounds or with TAT-FITC appeared strongly fluorescent (Figure 3a inset panels), while cells treated with FITC alone were not visible. Assessment of sub-cellular localization of the FITC was possible under higher magnification (40×). Cells treated with 2G-SMoC-FITC or 4G-SMoC-FITC had both nuclear and cytoplasmic fluorescence, however, 4G-SMoC-FITC showed more intense nuclear fluorescence (Figure 3a). These results demonstrate the delivery of FITC to both the nucleus and cytoplasm of cells by 2G-SMoC and 4G-SMoC. Next a time course of 2G-SMoC-FITC uptake in live U2OS cells was performed. Cells were incubated with 10 μM 2G-SMoC-FITC for the indicated times and visualized with confocal microscopy which revealed that 2G-SMoC-FITC was rapidly transported into cells (Figure 3b). Identical results were seen when cells were treated with 4G-SMoC-FITC and fixed for visualization (data not shown). After ten minutes, 2G-SMoC-FITC showed diffuse cytoplasmic staining that steadily increased in intensity up to 80 minutes after addition. Thus delivery of FITC by the SMoCs was rapid, and upon higher magnification (60x), the localization appeared to be in vesicles located in the cytoplasm of the cell, possibly the Golgi apparatus or the endoplasmic reticulum (Figure 3c).
Next the efficiency of SMoC mediated fluorescein delivery was determined by FACS analysis. U2OS cells were treated with 10 μM of SMoCs, then analysed for the percentage of live fluorescent cells. As can be seen in Figure 3d, >99% of live cells treated with either 2G-SMoC-FITC or 4G-SMoC-FITC were fluorescent. These data were comparable to TAT-FITC, for which 97% of cells were fluorescent after treatment. Similar results were obtained with 293T cells and for primary human cells, CD3+ cells, isolated from freshly harvested peripheral blood mononuclear cells (data not shown). Thus both primary and cultured human cells were susceptible to the delivery of FITC by SMoCs.

SMoCs do not affect the viability of cultured cells. The experiments described above demonstrate the rapid and efficient delivery of SMoC-FITC to cells, which occurred within 10 minutes of application. To test the effects of longer exposure on viability, cells were incubated for 24 and 48 hours with 2G-SMoC-FITC and 4G-SMoC-FITC, ranging in concentration from 0.1 μM to 10 μM (Figure 3e and data not shown). No decrease in viability was seen with SMoC treatment compared to DMSO alone. Viability was compared to cells treated with cycloheximide (CHX), a protein synthesis inhibitor, where prolonged exposure is toxic to cells. These results indicated that 48 hour incubation of cells with SMoCs did not affect their viability.

4G-SMoCs coupled proteins are transported into live cells.

To determine whether SMoCs would deliver a protein cargo to cells, 4G-SMoC-SSPy was coupled to the nuclear protein, geminin, a repressor of origin licensing. We selected 4G-SMoC due to its enhanced ability to deliver FITC to the nucleus (Figure 3a). Endogenous geminin protein levels are down-regulated during G1 phase, which
allows the licensing of replication origins for DNA synthesis. Geminin levels increase during S-G2-M, when the protein binds competitively to the MCM loading factor Cdt1, to block the re-licensing of origins.\textsuperscript{24} The reducing agent 2-mercaptoethanolamine was used to ensure that the two reactive thiols (i.e., cysteines at amino acid 194 and 208) in geminin were in the free thiol form, and the protein was mixed with 4G-SMoC-SSPy reagent (Figure 1e). The efficiency of coupling full-length human geminin or a truncated protein to 4G-SMoC approached 100\%, as determined by quantification of free sulphydryl groups before and after the coupling reaction with Ellman’s reagent (Figure 4a).

The uptake and the distribution of 4G-SMoC-geminin-AlexaFluor488 in live cells was followed in WI-38 HDFs, NIH/3T3 mouse fibroblasts, U2OS and Saos2 osteosarcoma cells by live confocal fluorescence microscopy (Figure 4b, data not shown for Saos2). No fluorescence was observed in controls (AlexaFluor488 or geminin only) nor in samples treated with geminin-AlexaFluor488, suggesting that coupling to SMoC is essential for cellular uptake of geminin. In contrast cells incubated with 10 \( \mu \)M 4G-SMoC-geminin-AlexaFluor488 appeared fluorescent with preferential perinuclear and nuclear staining. In order to obtain similar fluorescence intensities, WI-38, NIH/3T3 and U2OS cells required incubation with the protein conjugate for 1, 2 and 5 hours respectively.

To confirm that SMoC can deliver proteins other than geminin into cells, we sought a protein with free, solvent exposed cysteine and lysine residues (required for SMoC and AlexaFluor488 conjugation). We chose Tev-protease, which has 4 cysteines and 12 lysines on its surface, and no reported toxicity for human cells.\textsuperscript{13} WI-38 HDFs
were incubated with 10 μM 4G-SMoC-Tev-AlexaFluor488 as described above for 4G-SMoC-geminin-AlexaFluor488. Live fluorescence microscopy revealed that this conjugated protein was able to enter the cells whereas, Tev-AlexaFluor488 lacking SMoC was not. The intensity of fluorescence inside the cells was similar to that obtained with 4G-SMoC-geminin-AlexaFluor488.

To independently assess the ability of SMoCs to transport geminin into cultured cells, WI-38 HDFs were biochemically fractionated into cytosolic, nucleosolic and chromatin-bound protein fractions following incubation with 4G-SMoC-geminin for 24 hours. Proteins were resolved by gel electrophoresis and immunoblotted for geminin. In the treated cell population exogenous geminin can be clearly detected in the cytosolic, nucleosolic and chromatin bound fractions, consistent with previous findings that repression of origin of licensing is associated with the presence of geminin on chromatin.25

**Cellular uptake mechanisms for SMoC coupled molecules.** In order to determine the mechanisms by which SMoCs are taken up by cells, chemical inhibitors were employed to block specific endocytic pathways,. These included chlorpromazine, which induces the misassembly of clathrin-coated pits at the plasma membrane;26 nystatin which sequesters cholesterol and thus inhibits lipid-raft mediated endocytic pathways; (epi)amiloride, an inhibitor of the Na⁺/H⁺ exchange required for macropinocytosis;27 cytochalasin D,28 an F-actin elongation inhibitor; and nocodozole, which inhibits microtubule polymerization. Cells were treated with these inhibitors prior to the addition of 2G-SMoC-FITC and then visualized by fluorescence microscopy. Both untreated and treated cells appeared equally fluorescent (Figure 5a).
This finding was verified by flow cytometric analysis of the mean fluorescence intensity of live cells (Figure 5b), which confirmed that no significant differences were observed upon treatment with these inhibitors. These data suggest that either 2G-SMoC-FITC can be taken up by cells by several distinct entry routes or that macropinocytosis, and clathrin-mediated and lipid-raft mediated endocytic pathways are not required for 2G-SMoC-FITC.

To determine whether the cellular uptake of 2G-SMoC was energy dependent, the effect of reduced temperature was assessed. A large reduction in the mean intensity of fluorescence was observed when incubated with 2G-SMoC-FITC at a lower temperature (Figure 5c), suggesting that it did utilize energy dependent pathways for uptake into cells.

To test whether endocytic processes are involved in the uptake of a protein cargo, we next tested the effect of reduced temperature on the delivery of 4G-SMoC-geminin-Alexafluor488. Cells incubated with 4G-SMoC-geminin-Alexafluor488 at a lower temperature had a significant reduction in the intensity of fluorescence (Figure 5d), suggesting that it also used endocytic pathways for uptake. We next tested these chemical inhibitors (chlorpromazine, nystatin, epi-amiloride, and cytochalasin D) for their ability to inhibit 4G-SMoC-geminin-Alexafluor488 uptake. As shown in Figure 5e, cells treated with chlorpromazine showed a 70% reduction in fluorescence intensity compared to the control cells. Treatment with other inhibitors, did not significantly alter the fluorescence intensity of the cells (data not shown). This indicates that uptake of 4G-SMoC-geminin-Alexafluor488 is dependent upon clathrin-mediated endocytosis. Moreover, these data together suggest that the SMoCs
are capable of efficiently delivering different cargo, which retain functionality, and which use multiple pathways, dependent on the cargo, for uptake into cells.

**SMoCs can deliver functionally active protein cargo into cells of different tissue origin.** In cultured NIH/3T3 fibroblasts, chromosomal replication origins are licensed for DNA synthesis during a defined “window of opportunity” between 16 and 18 hours after release from density-dependent growth arrest (Figure 6a). In this *in vitro* model system, DNA synthesis can be monitored by pulse-labelling with bromodeoxyuridine (BrdU). To determine whether, SMoC-mediated cell delivery affects protein functionality, 4G-SMoC-geminin was added at concentrations ranging from 0.5 μM to 10 μM to NIH/3T3 cells at eight hours after release from G0. We then examined whether geminin was functional in repressing origin licensing and thus the initiation of DNA synthesis by determining the percentage of cells incorporating BrdU at 21 hours, which is the time they would normally enter S phase (Figure 6b). Both 4G-SMoC-geminin and a truncation mutant of geminin (ΔNt-geminin) delivered by 4G-SMoC inhibited G0-S progression in a concentration dependent manner with a maximal inhibition of ~50% occurring with 10 μM 4G-SMoC-geminin/ΔNt-geminin (Figure 6b and Supplementary Figure 1) in keeping with geminin's biological function as a repressor of origin licensing. However when 4G-SMoC was coupled to a geminin derivative with a double point mutation (Y98F, W99F) at a predicted Cdt1 binding site (4G-SMoC-gemininFF), no decrease in the percentage of cells incorporating BrdU was observed, indicating that inhibition of DNA replication in these experiments was dependent on wild-type geminin and not linked to a non-specific effect of the SMoC (Figure 6c). Substitution of 4G-SMoC-geminin with equal molar concentrations of uncoupled geminin protein or an
equivalent volume of control buffer did not affect BrdU incorporation. Trypan blue exclusion showed no loss of cell viability compared with controls (data not shown). These data demonstrate that geminin delivered by 4G-SMoC retained its biological activity to block entry into S phase in cells re-entering the mitotic cell division cycle from G0.14

To determine whether human cells of epithelial, mesenchymal and haematopoietic origin were susceptible to geminin delivered by 4G-SMoC, WI-38 HDFs, HeLa S3 epithelial adenocarcinoma cells and MOLT-4 leukaemic lymphoblasts were treated with 10 μM 4G-SMoC-ΔNt-geminin for 24 hours. Consistent with the data from NIH/3T3 fibroblasts, the percentage of cells synthesizing DNA was reduced by approximately 50% compared to control populations in the three different cell lines tested, WI-38: 50% [control] vs 26% [treated], Figure 6c; HeLa S3: 40% vs 17%; MOLT-4: 42% vs 22% (data not shown).

To confirm that the reduction in DNA synthesis that occurs following treatment with 4G-SMoC-ΔNt-geminin is due to geminin's biological function as an origin licensing repressor, changes in chromatin-bound Mcm2 levels were analysed using immunofluorescence and subcellular fractionation. Following treatment with 10 μM 4G-SMoC-ΔNT-geminin for 24 hours WI-38 HDFs were either treated to remove soluble proteins and immunostained for Mcm2 or biochemically fractionated and analysed by immunoblotting. Treatment with 4G-SMoC-ΔNt-geminin resulted in a 75% reduction in the relative intensity of Mcm2 staining indicating that 4G-SMoC-ΔNt-geminin inhibits G1-S progression by blocking MCM loading onto chromatin (Figure 6d). These results are confirmed by the immunoblot data, which show a
reduction in chromatin bound mcm2 levels following treatment with 4G-SMoC-ΔNt-geminin (Figure 6e), indicating that 4G-SMoC-Nt-geminin is specifically interfering with the recruitment of replication licensing factors onto chromatin in treated cells. Taken together these data confirm the ability of 4G-SMoC to efficiently transduce cell lines of different origin and to retain the biological activity of its protein cargo.

Discussion

It is significant that 4G-SMoC is capable of delivering its protein cargo in such a way that it retains its endogenous cellular function. Specifically, 4G-SMoC conjugated to a truncated derivative of human geminin was capable of both inhibiting quiescent cells from re-entering the proliferative cycle and of preventing cycling cells from progressing through the G1-S transition. Origin licensing was repressed, by prevention of loading of MCM subunits onto chromatin. In addition, the decision to utilize 4G-SMoC as the geminin transporter on the basis of the nuclear localization of 4G-SMoC-FITC was vindicated as the sub-cellular localization of the 4G-SMoC-geminin was predominantly nuclear. Currently the reasons for this localization are unclear but one intriguing possibility is that 4G-SMoC is mimicking a short highly basic nuclear localisation signal such as the SV40 sequence PKKKRKV or the vJun sequence GKKRSKV. This ability to target SMoC cargo to different sub-cellular locations is likely to be a useful property. An alternative scenario is that once native geminin is generated in the cell (by cleavage of the SMoC-SS-geminin bond) it is localised to the nucleus by virtue of its own structure and sequence characteristics. 4G-SMoC was able to efficiently transduce a number of geminin variants into several cell lines of epithelial, mesenchymal and haematopoetic origin. Thus our novel small molecule PTD mimics are able to transport proteins into a wide variety of cell types.
The mechanisms of entry of PTDs into cells are a matter of some debate, with both energy dependent and non-dependent processes being advanced. In particular the mechanism of HIV-1-tat PTD i.e. the short tat peptide, entry has been proposed to be via the clathrin mediated endocytotic pathway, while a recent report indicated that the process for tat fusion-proteins was in fact macropinocytosis. Our experiments using fluorescently labelled geminin protein in live cells show that the uptake process is inhibited at 4°C and by the presence of chlorpromazine, which induces the misassembly of clathrin-coated pits at the plasma membrane and therefore inhibits this endocytotic pathway. In contrast nystatin, which sequesters cholesterol and blocks lipid raft mediated endocytosis and (epi)-amiloride, which inhibits macropinocytosis by blocking the Na+/H+ pump, were ineffective. Similarly cytochalasin D (actin elongation) and nocodazole (microtubule polymerisation) were without effect. There appears to be a difference in the mechanism of uptake for the SMoC-dye molecules, as none of the inhibitors had any significant effect, though 2G-SMoC-FITC was still inhibited at 4°C. Our experiments indicate that uptake of the SMoC-geminin molecules is via classical clathrin mediated endocytosis. Calveolae mediated endocytosis or macropinocytosis, do not appear to be involved. However we should caution that at least for PTD’s the uptake pathways are both cargo and cell type dependent.

In summary, we have designed and synthesized a small molecule, biphenyl, alpha helical mimic of a peptide PTD. These small molecule PTDs, which we have termed SMoCs, efficiently deliver dye molecules and proteins into a variety of cell types and can be easily coupled to recombinant proteins. As an example for a protein cargo, we have applied this new technology to the internalization of geminin *in vitro*, providing
evidence that 4G-SMoC-geminin, delivered extra-cellularly, can have an anti-proliferative effect on tumour cells. Future investigations will establish the utility of the SMoCs for other types of cargo such as peptides, antibodies and oligonucleotides.

Supporting information available

Full experimental details of the chemical syntheses are provided.

Acknowledgements

We thank Mitsubishi Pharma Corporation for a visiting fellowship for MO and funding for CV. HL was funded by the Association for International Cancer Research. We are grateful to Andrei Okorokov for advice on the generation of geminin loss-of-function mutants and the ΔNt-geminin construct, and to Dr Snezana Djordjevic for the gift of pRET 3a-TEV plasmid. We thank Amanda Stuart for advice on inhibitors of pathways of endocytosis and to Hanna Engel for technical assistance. We thank Annahita Farshchi and Sharon Paige for technical assistance with site-directed mutagenesis and protein expression. We are grateful to Lauren Rakes and Lorena Suarez-Delgado for assistance with cell proliferation assays. The geminin studies were funded by Cancer Research UK Programme Grant C428/A2281. S.R.K. is supported by an MRC Research Studentship.
Figure Legends

Figure 1. (a) Molecular model of a typical alpha helix showing the similar spatial orientation of the i and i + 3 positions with the 2 and 2' positions on the biphenyl ring (purple). (b) The amphipathic nature of the helix mimic 2G-SMoC is shown compared to the alpha helix of antennapedia 44-57 (penetratin). Electrostatic potential is shown as a wire mesh with blue (positive), red (negative) and grey (neutral). Both molecules are drawn to the same scale. (c) Chemical structures of 2G-SMoC-FITC and (d) 4G-SMoCo-FITC. (e) A late stage 4G-SMoC-amine intermediate allows a flexible range of attachment chemistries to be simply instituted depending on the application.

Figure 2. Chemical synthesis of 2G-SMoC-FITC; the key biphenyl formation is established early in the synthesis by means of a Suzuki Pd[0] coupling of 1 and 3-methoxy boronic acid. Guanidinylation of the side chains (4 to 5) is achieved through use of the bis(Boc) triflate

Figure 3. SMoCs are rapid and efficient transporters of FITC in primary and cultured cells. (a) Visualization of delivery of 2G-SMoC and 4G-SMoC coupled to FITC compared to TAT-FITC and FITC alone in live cells. There is widespread distribution of FITC in nuclear and cytoplasmic compartments delivered by SMoCs and TAT, compared to minimal uptake of FITC alone. (b) Time course of delivery of 2G-SMoC-FITC. Cells were treated for the times indicated prior to visualization. Fluorescence is detectable within five minutes of application of 2G-SMoC-FITC, with increasing intensity being detected after 80 minutes. (c) Visualization of 2G-SMoC uptake at higher magnification in live cells. There is widespread distribution of FITC
in perinuclear, vesicular structures in the cytoplasmic compartment. (d) Efficiency of delivery of 2G-SMoC and 4G-SMoC coupled to FITC, TAT-FITC and FITC alone in live cultured cells as determined by FACS analysis. Cells were treated for 10 minutes prior to washing with PBS and staining with propidium iodide to exclude dead cells. (e) Cell viability of cells is not affected by SMoC-FITC. Cells were incubated for 48 hours with 10 µM of the indicated compounds prior to assaying for viability. Cycloheximide (CHX) was included as a positive control for inducing loss of cell viability.

**Figure 4.** Coupling of geminin to SMoC allows rapid transportation of active protein into cells. (a) Quantification of sulfhydryl groups with Ellman's reagent before and after coupling indicates that coupling of geminin to 4G-SMoC approaches 100% efficiency. (b) Geminin and Tev proteins were coupled with AlexaFluor 488 (AF488) and 4G-SMoC. WI-38 HDFs (i), osteosarcoma-derived U20S cells (ii) and mouse NIH/3T3 fibroblasts (iii) were treated with 10 µM 4G-SMoC-geminin-AF488 for 1, 5 and 2 hours respectively and analysed by live confocal fluorescence microscopy. The presence of fluorescence inside the cells (particularly localised in perinuclear and nuclear areas) proves the internalization of the protein complex. (iv) WI-38 human diploid fibroblasts were incubated with 10 µM 4G-SMoC-Tev-AlexaFluor488 and cells were analysed after 1 hour. (c) For detection of geminin internalization by immunoblotting, cytosolic (CF), nucleosolic (NF) and chromatin-bound (CBF) protein fractions were prepared from WI-38 HDFs following treatment with 4G-SMoC-geminin. Protein fractions were resolved by gel-electrophoresis and immunoblotted for geminin. In the treated population exogenous geminin can be clearly detected in the cytosolic, nucleosolic and chromatin-bound protein fractions.
Figure 5. Effect of chemical inhibitors and temperature on uptake of SMoCs with different cargo. (a) Images of U2OS cells treated with the compounds indicated and then incubated with 2G-SMoC-FITC. (b) Histograms of FACS profiles of live cells treated with various compounds as indicated prior to addition of 2G-SMoC-FITC. (c) Histogram of FACS profile of live cells treated with 2G-SMoC-FITC at 37°C or 4°C. (d) Histogram of FACS profile of live cells treated with 4G-SMoC-geminin-Alexafluor488 at 37°C or 4°C. (e) Histograms of FACS profiles of live cells treated with chlorpromazine prior to addition of 4G-SMoC-geminin-Alexafluor488.

Figure 6. The biological activity of protein cargo is not affected by SMoC-mediated delivery. (a) In cultured NIH/3T3 fibroblasts, chromosomal replication origins are licensed for DNA synthesis during a defined “window of opportunity” between 16 and 18 hours after release from density-dependent growth arrest. Progression into S phase can be easily monitored by pulse-labelling with BrdU 21 hours after release. To determine whether SMoC-mediated cell delivery affects protein functionality 4G-SMoC-geminin was added to NIH/3T3s eight hours after release from G0 and re-entry into S phase monitored at 21 hours. (b) Treatment of NIH/3T3 fibroblasts with 10 μM 4G-SMoC-geminin or 4G-SMoC-ΔNt-geminin eight hours after release from G0 resulted in a 50% reduction in BrdU incorporation compared to control populations. In contrast, no reduction in BrdU incorporation was observed in a geminin derivative with a double point mutation (Y98F, W99F) at a possible Cdt1 binding site (4G-SMoC-FFgeminin). (c) Asynchronous WI-38 HDFs were treated for 24 hours with 10 μM 4G-SMoC-ΔNt-geminin and pulse-labelled with BrdU for one hour. G1/S progression was inhibited by approximately 50% in treated cells compared to a control population. (d and e) The reduction in DNA synthesis was coupled to a block
to Mcm2 chromatin-binding as detected by (d) immunofluorescence and (e) immunoblotting. For detection by immunofluorescence, WI-38 HDFs were treated with detergent prior to fixation to remove soluble proteins and stained with a mouse anti-Mcm2 MAb, a FITC conjugated secondary anti-mouse antibody and propidium iodide. Treatment with 4G-SMoC-ΔNt-geminin reduced the intensity of fluorescently-labelled bound Mcm2 by 75%. For detection by chromatin-bound (CBF) protein fractions were prepared from WI-38 HDFs following treatment with 4G-SMoC-ΔNt-geminin. Protein fractions were resolved by gel electrophoresis and immunoblotted for Mcm2 and actin as a loading control. Treatment with 4G-SMoC-ΔNt-geminin caused a reduction in chromatin-bound Mcm2 levels, indicating that 4G-SMoC-ΔNt-geminin blocks DNA replication by specifically interfering with recruitment of replication licensing factors onto chromatin.
Reference List


2. Roberts, J. P. Buyer's guide to protein transduction reagents - A range of chemicals and peptides are available to help get proteins into cells. Scientist 18, 42-43 (2004).


**Figure 1**

(a) Diagram showing molecular structure with labeled parts 1, 2, 2', and 1+3.

(b) Colorful molecular model, possibly depicting the same structure as in (a).

(c) Chemical structures:
- **2G-SMoC-FITC**
- **4G-SMoC-FITC**

(d) Another set of chemical structures:
- **4G-SMoC-H**

(e) Diagram illustrating reactions:
- Maleimide for reaction with protein SH
- Acid group for further conjugation
- Dye tagged SMoCs
- Forms disulfides with proteins
Figure 2

1) SnCl₂, MeOH
2) 'BuONO
3) CuBr₂

1) TFA
2) SO₂CF₃
BocHN'NHBoc
iPr₂EtN, DMF

1) H₂NNH₂
2) FITC, iPr₂EtN, DMF
3) TFA
FITC = HO₂C-O-OH

90%

35%

63%

63%

56%
Figure 3

a) Images of different cell fluorescence markers over time:

- **FITC**: Green fluorescence markers.
- **2G-SMoC-FITC**: Green fluorescence markers in a specific phase.
- **4G-SMoC-FITC**: Green fluorescence markers in a different phase.
- **TAT-FITC**: Green fluorescence markers with TAT peptide.

b) Time-lapse images showing cell growth and fluorescence over 80 minutes:

- 0 min, 10 min, 20 min, 40 min, 80 min.


c) Fluorescence microscopy images of cell morphology:

- **Phase**: phase-contrast imaging.
- **FITC**: fluorescence imaging with FITC.
- **2G-SMoC-FITC**: fluorescence imaging with 2G-SMoC-FITC.


d) Flow cytometry plots showing fluorescence intensity:

- Control, 2G-SMoC-FITC, 4G-SMoC-FITC, TAT-FITC.


e) Graph showing luminescence values:

- DMSO, 4G-SMoC-FITC, 2G-SMoC-FITC, TAT, CHX, FITC.
Figure 4

(a) Bar graph showing the change in A412 before and after coupling.

(b) Images illustrating the effect of coupling on cell morphology.

(c) Table comparing Geminin expression in different conditions:

<table>
<thead>
<tr>
<th></th>
<th>ΔNt</th>
<th>4G-SMoC-ΔNt</th>
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<td>CF</td>
<td>NF</td>
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Geminin
Figure 6

(a) Schematic diagram of the experimental design. Cells were synchronized in G0 and then pulsed with BrdU for 16-22 hours. Quantitation of number of replicating cells was performed using fluorescence confocal microscopy.

(b) Representative images of cells stained for BrdU incorporation.

(c) Quantification of BrdU incorporation in different groups. Control: 50%, 4G-SMoC-geminin: 25%.

(d) Western blot analysis showing Mcm2 levels in control and 4G-SMoC-geminin treated cells.

(e) Western blot analysis showing Mcm2 and Actin levels in ΔNt-geminin and 4G-SMoC-geminin treated cells.
**Supporting information**

Small molecule mimics of an α-helix for rapid and efficient transport of proteins into cells

Authors
Masahiro Okuyama, Heike Laman, Sarah Kingsbury, Cristina Visintin, Elizabetta Leo, Kathryn Leigh Eward, Kai Stoeber, Chris Boshoff, Gareth Williams, David Selwood*.

Chemistry Experimental methods

All starting materials were either commercially available synthesised by methods reported in the literature. \(^1\)H and \(^{13}\)C spectra were recorded on a Bruker AMX-300 spectrometer. Chemical shifts are reported as ppm relative to TMS as internal standard. Mass spectra were recorded on either a VG ZAB SE spectrometer (EI, FAB) or a Gilson- Finningen AQA LC-mass spectrometer using C-18 column (Hypersil BDS 100 x 4.6 mm, 5 μm). Microanalysis was carried out by the Analytical Services Section, Department of Chemistry, University College London. Purification was by reverse-phase HPLC (Gilson) using preparative C-18 columns (Hypersil PEP 100 x 21 mm, 5μm). Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected.

**Synthesis of 2G-SMoC-FITC**

See the chemical scheme in the manuscript

2-Methoxy-4-methyl-phenylamine

\[
\begin{align*}
\text{NH}_2 \\
\text{O}
\end{align*}
\]

A solution of 2-methoxymethyl-1-nitrobenzene (3.00 g, 17.9 mmol) and SnCl\(_2\)·2H\(_2\)O (20.2 g, 89.5 mmol) in MeOH (120 mL) was refluxed for 3 h. The solvent was removed under vacuum and the residue was dissolved in Ethyl acetate (200 mL). The solution was washed with saturated aqueous NaHCO\(_3\) (3 x 200 mL), and dried over Na\(_2\)SO\(_4\) to afford the product (2.37 g, 95% yield).

\(^1\)H-NMR (CDCl\(_3\)): \(\delta\) 2.27 (s, 3H), 3.84 (s, 3H), 6.56-6.66 (m, 3H).

\(^{13}\)C-NMR (CDCl\(_3\)): \(\delta\) 21.0, 55.4, 111.5, 115.1, 121.2, 128.1, 133.5, 147.4.

Theoretical Mass: (M + H) 409.30120. Measured Mass: (M + H) 409.30140

Bromo-2-methoxy-4-methyl-benzene (1)
To a suspension of cupric bromide (4.88 g, 21.8 mmol) in CH$_3$CN (20 mL) at rt was added tert-butyl nitrite (2.13 mL, 17.9 mmol), the mixture was then warmed to 65°C. A solution of 2-methoxy-4-methyl-phenylamine (2.24 g, 16.3 mmol) in CH$_3$CN (20 mL) was then added slowly and stirred for an additional 15 min. The solvent was removed under vacuum and the residue was dissolved in cyclohexane (150 mL), washed with aqueous ammonia (3 x 150 mL), water (150 mL), and dried over Na$_2$SO$_4$. The solvent was removed under vacuum and the product purified by silica gel flash chromatography using CHCl$_3$ as eluent to afford (1) (1.92 g, 59% yield).

$^1$H-NMR (CDCl$_3$): δ 2.32 (s, 3H), 3.86 (s, 3H), 6.64 (dd, $J$ = 8.0, 1.4 Hz, 1H), 6.72 (d, $J$ = 1.4 Hz, 1H), 7.39 (d, $J$ = 8.0 Hz, 1H).


Theoretical Mass: (M + H) 199.98313. Measured Mass: (M + H) 199.98387

2, 3'-Dimethoxy-4-methyl-biphenyl (2)

To a solution of bromo-2-methoxy-4-methyl-benzene (610 mg, 3.03 mmol) in 1,2-dimethoxyethane (12 mL) and EtOH (3 mL) was added 3-methoxy-phenylboronic acid (553 mg, 3.64 mmol), followed by 2 M Na$_2$CO$_3$ solution (6 mL) and Pd(PPh$_3$)$_4$ (176 mg, 0.152 mmol). The suspension was then refluxed for 17 h under nitrogen. The reaction mixture was cooled to rt, diluted with hexane/Ethyl acetate (1/1, 20 mL), washed with water (3 x 20mL), brine (20 mL), and dried over Na$_2$SO$_4$. The solvent was removed under vacuum and the product was purified by silica gel flash chromatography using hexane/dichloromethane (2/1) as eluent to afford (2), (529 mg, 76% yield).

$^1$H-NMR (CDCl$_3$): δ 2.43 (s, 3H), 3.82 (s, 3H), 3.86 (s, 3H), 6.83-6.91 (m, 3H), 7.11-7.15 (m, 2H), 7.25 (d, $J$ = 7.6 Hz, 1H), 7.34 (t, $J$ = 7.6 Hz, 1H).
13C-NMR (CDCl3): δ 21.6, 55.3, 55.6, 112.3, 115.4, 121.5, 122.1, 127.8, 128.9, 130.6, 138.8, 140.0, 156.4, 159.3.
Theoretical Mass: (M + H) 229.1223. Measured Mass: (M + H) 229.14446.

2-(2, 3'-Dimethoxy-biphenyl-4-ylmethyl)-isoindole-1,3-dione (3)

To a solution of 2,3'-dimethoxy-4-methyl-biphenyl (529 mg, 2.32 mmol) in CCl4 (48 mL) was added N-bromosuccinimide (392 mg, 2.20 mmol) followed by AIBN (34 mg) and the reaction refluxed for 2.5 h. The mixture was then cooled to 0°C; and filtered. The solvent was removed under vacuum to afford the crude 4-(bromomethyl)-2,5'-dimethoxybiphenyl, which was used without further purification. The crude material was dissolved in DMF (7.5 mL) and potassium phthalimide (430 mg, 2.32 mmol) was added. The mixture was then stirred at 80°C for 1.5 h. The suspension was then cooled to rt and diluted with hexane/ethyl acetate (1/1, 20 mL) and washed with water (3 × 20 mL), brine (20 mL), and dried over Na2SO4. The solvent was removed under vacuum and the product purified by silica gel flash chromatography using hexane/ethyl acetate (3/1) as eluent to afford (3) as a cream solid. mp 110-112°C.

IR ν max KBR/cm⁻¹ 2940(CH₃), 1718 (C=ON)

1H-NMR (CDCl3): δ 3.81 (s, 6H), 4.87 (s, 2H), 6.86 (dd, J = 8.0, 2.4 Hz, 1H), 7.02-7.10 (m, 4H), 7.24-7.29 (m, 2H), 7.71 (dd, J = 5.4, 3.0 Hz, 2H), 7.86 (dd, J = 5.4, 3.0 Hz, 2H).

13C-NMR (CDCl3): δ 41.6, 55.2, 55.7, 111.8, 112.6, 115.2, 121.0, 122.0, 123.4, 128.9, 130.2, 131.0, 132.2, 134.0, 137.0, 139.5, 156.6, 159.2, 168.1.
Theoretical Mass: (M + H) 347.13923. Measured Mass: (M + H) 347.13891.
Calculated C_{23}H_{19}NO_{4}: C 73.98%, H 5.13%, N 3.75%, found C 73.36%, H 5.08%, N 3.86%

2-[2, 3'-Bis(2-tert-butyloxycarbonylamino-ethyloxy)-biphenyl-4-ylmethyl]-isoindole-1,3-dione (4)

2-(2, 3'-Dimethoxy-biphenyl-4-ylmethyl)-isoindole-1,3-dione (72 mg, 1.9 mmol) was dissolved in dichloromethane (5 mL) and treated with a 1.0 M solution of BBr₃ (1.0 mL) in dichloromethane at 0°C, allowed to warm to room temperature and stirred overnight. The reaction mixture was then cooled to 0°C and MeOH (3 mL) was added, and the solvent was removed under vacuum. The residue was dissolved in ethyl acetate (20mL) and washed with water (3 x 20 mL), brine (20 mL), and dried over Na₂SO₄. The solvent was removed under vacuum and the product obtained was purified by short silica gel flash chromatography using hexane/ethyl acetate (1/1) as eluent to give 2-[(2, 5'-dihydroxybiphenyl-4-yl)methyl]isoindoline-1,3-dione (61 mg).

To a mixture of the crude bis-phenol (56 mg, 0.16 mmol), 2-tert-butyloxycarbonylamino-ethanol (65 mg, 0.41 mmol) and triphenylphosphine (106 mg, 0.41 mmol) in THF (2 mL) was added diethylazido dicarboxylate (71 mg, 0.41 mmol) and the mixture stirred at r.t. for 24 h. The reaction mixture was diluted with ethyl acetate (20 mL), washed with 1M Na₂CO₃ (2 x 20 mL), and dried over Na₂SO₄. The solvent was removed under vacuum and the product purified by silica gel flash chromatography eluting with hexane/ethyl acetate (2/1) to afford (4) as a oil (36 mg, 35% yield).

^1H-NMR (CDCl₃): δ 1.41 (s, 9H), 1.43 (s, 9H), 3.41 (m, 2H), 3.52 (m, 2H), 3.99-4.05 (m, 4H), 4.73 (br s, 1H), 4.85 (s, 2H), 5.02 (br s, 1H), 6.85 (dd, J = 8.0, 2.4 Hz, 1H), 7.00-7.12 (m, 4H), 7.24-7.32 (m, 2H), 7.72 (dd, J = 5.4, 3.0 Hz, 2H), 7.86 (dd, J = 5.4, 3.0 Hz, 2H).
$^{13}$C-NMR (CDCl$_3$): $\delta$ 28.40, 40.0, 40.1, 41.5, 67.2, 68.2, 113.0, 113.6, 115.7, 121.7, 122.3, 123.5, 129.0, 130.5, 131.0, 132.1, 134.1, 137.2, 139.5, 155.5, 155.8, 155.9, 168.1.

Theoretical Mass: (M + H) 632.29718. Measured Mass: (M + H) 632.29602.

2-{$\text{2', 3'-Bis[2-(N,N'-bis-boc-guanidino)-ethyloxy]-biphenyl-4-ylmethyl}$}-isoindole-1,3-dione (5)

IR $\nu_{\text{max}}$ KBR/$\text{cm}^{-1}$ 1723 (C=O), 1717 (C=ON)

$^1$H-NMR (CDCl$_3$): $\delta$ 1.47 (s, 18H), 1.49 (s, 18H), 3.74-3.86 (m, 4H), 4.04-4.10 (m, 4H), 4.85 (s, 2H), 6.87 (dd, $J = 8.0, 2.0$ Hz, 1H), 6.97 (br s, 1H), 7.04 (s, 1H), 7.09 (d, $J = 7.7$ Hz, 1H), 7.21 (d, $J = 7.7$ Hz, 1H), 7.26-7.31 (m, 2H), 7.71 (dd, $J = 5.4$, 3.0 Hz, 2H), 7.86 (dd, $J = 5.4$, 3.0 Hz, 2H), 8.62 (br t, $J = 5.6$ Hz, 1H), 8.74 (br t, $J = 5.1$ Hz, 1H).
\[^{13}\text{C}-\text{NMR } (\text{CDCl}_3): \delta 28.0, 28.3, 40.1, 40.3, 41.5, 66.3, 66.9, 112.8, 113.3, 115.8, 121.5, 123.0, 123.5, 128.9, 130.3, 131.2, 132.1, 134.1, 137.0, 139.3, 152.9, 153.0, 155.4, 156.38, 156.43, 158.4, 163.30, 163.33, 168.1. \]

Theoretical Mass: \((M + H) 632.29718\). Measured Mass: \((M + H) 632.29602\).

2-[2, 3'-Bis(2-guanidino-ethyloxy)-biphenyl-4-ylmethyl]-thioureido-fluorescein (6)

\[
\text{NH} \\
\text{NH} \\
\text{NH} \\
\text{NH} \\
\text{OH}
\]

To a solution of 2-[2, 3'-bis[2-(N, N'-bis-boc-guanidino)-ethyloxy]-biphenyl-4-ylmethyl]-isoindole-1,3-dione (22 mg, 0.024 mmol) in EtOH (0.6 mL) was added hydrazine monohydrate (12 \(\mu\)L, 0.24 mmol) and the mixture stirred at rt for 18 h. The solvent was then evaporated under vacuum, and the residue was dissolved in dichloromethane (15 mL) and the precipitate removed by filtration. The filtrate was washed with brine (10 mL), and the water layer was extracted with dichloromethane (3 \(\times\) 10 mL). The combined organic layer was dried over Na\(_2\)SO\(_4\). The solvent was removed under vacuum to afford crude amine which was used without further purification.

Crude amine was dissolved in DMF (1.0 mL) and fluorescein isothiocyanate isomer I (19 mg, 0.048 mmol) and \(i\)-Pr\(_2\)EtN (17 \(\mu\)L, 0.096 mmol) were added and the reaction mixture was stirred in the dark at rt for 20 h. The reaction mixture was diluted with
ethyl acetate (10 mL) and washed with 1N HCl (3 × 10 mL), water (10 mL), brine (10 mL), and dried over Na₂SO₄. The solvent was removed under vacuum. The residue was dissolved in THF (1.5 mL) and treated with PS-trisamine (Argonaut Technologies Inc., 4.17 mmol/g, 20 mg) to remove the excess of fluorescein isothiocyanate. After shaking the mixture at room temperature for 15 min, the resin was filtered off and the solvent was removed. The residue was dissolved in TFA (1.5 mL) and stirred at rt for 2 h. TFA was removed under vacuum, and the crude material was purified by HPLC using a gradient of 3-97% acetonitrile in 0.1 aq. TFA over 30 min at a flow rate of 10 mL/min. Retention time 8.0 min., to afford (6) (8 mg, 30 % yield).

¹H-NMR (MeOH-d₄): δ 3.54 (m, 2H), 3.61 (m, 2H), 4.15 (m, 4H), 4.91 (s, 2H), 6.64 (d, J = 9.0 Hz, 2H), 6.78-6.83 (m, 4H), 6.93 (d, J = 7.9 Hz, 1H), 7.05-7.35 (m, 9H), 7.81 (d, J = 8.2 Hz, 1H), 8.22 (s, 1H).

MS (MALDI) m/z 774 (calcd), 775 (M + 1, found).

Theoretical Mass: (M + Na) 797.24817. Measured Mass: (M + Na) 797.24955.
**Synthesis of 4G-SMoC amine intermediate**

1. $\text{OMe}$
   - n BuLi
   - BrCCl$_2$CCl$_2$Br
   - 36%

2. $\text{OMe}$
   - $\text{B(OH)}_2$
   - Pd(PP$_3$)$_4$
   - Na$_2$CO$_3$aq
   - DME/ EtOH
   - 92%

3. $\text{OMe}$
   - 1) NBS/AIBN
   - 2) $\text{K_2CO_3}$
   - 3) $\text{Ac}_2\text{O}$

4. $\text{BocNH}$
   - $\text{TeO}^+$
   - $\text{NH}$
   - $\text{NHBoc}$
   - $\text{Cs}_2\text{CO}_3$, DMF
   - 2 steps 66%

5. 1) TFA
   - 2) $\text{N}_3\text{SO}_2\text{CF}_3$
   - iPr$_2$EtN
   - DMF

6. 2 steps 74%

7. 2 steps 74%

8. $\text{S8}$

9. $\text{S9}$

10. $\text{S10}$

11. $\text{S11}$

12. $\text{S12}$
**Synthesis of 4G-SMoC-FITC and 4G-SMoC-SS-py.**

1. **1-Bromo-2, 3-dimethoxy-4-methyl-benzene (S7)**

   \[
   \text{n-BuLi} \ 1.6 \text{ M in hexane (18.5 mL, 29.6 mmol) was added dropwise at 0°C to a solution of 2, 3-dimethoxytoluene (3.00 g, 19.7 mmol) and TMEDA (2.97 mL, 19.7 mmol) in anhydrous ether (50 mL) under nitrogen and stirred at room temperature for 2 h. The reaction mixture was cooled to -78°C and (CBrCl)\textsubscript{2} (9.64 g, 29.6 mmol)}
   \]
was added. After stirring for a further 10 min, the cooling bath was removed and the reaction vessel allowed to warm to rt. The reaction mixture was then diluted with ether (50 mL) and washed with water (950 mL), 1N HCl (2 × 50 mL), brine (50 mL), and dried over Na₂SO₄. The solvent was removed under vacuum and purified by silica gel flash chromatography using hexane/dichloromethane (5/1) as eluent to afford (S7) as a colourless oil (1.62 g, 36% yield).

¹H-NMR (CDCl₃): δ 2.22 (s, 3H), 3.85 (s, 3H), 3.88 (s, 3H), 6.79 (d, J = 8.3 Hz, 1H), 7.16 (d, J = 8.3 Hz, 1H).

¹³C-NMR (CDCl₃): δ 15.7, 60.4, 60.6, 114.6, 126.7, 127.4, 132.1, 150.4, 152.5.

Theoretical Mass: (M + Na) 252.98346. Measured Mass: (M + Na) 252.98370.

2, 3, 2', 3'-Tetramethoxy-4-methyl-biphenyl (S8)

To a solution of 1-bromo-2, 3-dimethoxy-4-methyl-benzene (S1) (500 mg, 2.16 mmol) in 1,2-dimethoxyethane (8.7 mL) and EtOH (2.2 mL), was added 2, 3-dimethoxy-phenylboronic acid (472 mg, 2.59 mmol) and 2M Na₂CO₃ solution (4.3 mL), followed by Pd(PPh₃)₄ (125 mg, 0.108 mmol). The mixture was refluxed for 18 h under nitrogen. The reaction mixture was cooled to rt and diluted with hexane/ethyl acetate (1/1, 10 mL) and washed with water (3 × 20 mL), brine (20 mL), and dried over Na₂SO₄. The solvent was removed under vacuum and the product was purified by silica gel flash chromatography using dichloromethane as eluent to afford (S8) as a solid (573 mg, 92% yield). Mp : 30-31 °C

¹H-NMR (CDCl₃): δ 2.31 (s, 3H), 3.63 (s, 3H), 3.67 (s, 3H), 3.87 (s, 3H), 3.90 (s, 3H), 6.85 (dd, J = 7.6, 1.3 Hz, 1H), 6.91-6.94 (m, 3H), 7.07 (t, J = 7.9 Hz, 1H).

¹³C-NMR (CDCl₃): δ 15.9, 55.8, 60.1, 60.4, 60.6, 111.6, 123.3, 123.4, 125.0, 125.7, 130.8, 131.7, 133.0, 146.9, 150.8, 151.3, 152.8.

2-(2, 3, 2', 3'-Tetramethoxy-biphenyl-4-ylmethyl)-isoindole-1, 3-dione (S9)

To a solution of 2, 3, 2', 3'-tetramethoxy-4-methyl-biphenyl (573 mg, 1.99 mmol) in CCl₄ (42 mL), was added N-bromosuccinimide (336 mg, 1.89 mmol), and AIBN (29 mg). After 2 h reflux, the mixture was cooled to 0°C; and filtered. The solvent was removed under vacuum to afford crude 4-(bromomethyl)-2, 3, 5', 6'-tetramethoxybiphenyl, which was used without further purification.

A solution of the crude bromomethyl compound and potassium phthalimide (369 mg, 1.99 mmol) in DMF (6.5 mL) was stirred at 80 °C for 1.5 h. The mixture was then cooled to rt and diluted with hexane/ethyl acetate (20 mL, 1/1) and washed with water (5 x 20 mL), brine (20 mL), and dried over Na₂SO₄. The solvent was removed under vacuum and the product was purified by silica gel flash chromatography using hexane/ethyl acetate (3/1) as eluent to give (S9) (552 mg, 64% yield). Mp 135-136 °C

IR ν_max, KBR/cm⁻¹ 2936(CH₃), 1712 (C=ON)

¹H-NMR (CDCl₃): δ 3.62 (s, 3H), 3.65 (s, 3H), 3.89 (s, 3H), 3.99 (s, 3H), 4.98 (s, 2H), 6.81 (dd, J = 7.6, 1.5 Hz, 1H), 6.90-6.93 (m, 2H), 6.98 (d, J = 8.0 Hz, 1H), 7.06 (t, J = 7.9 Hz, 1H), 7.72 (dd, J = 5.4, 3.0 Hz, 2H), 7.86 (dd, J = 5.4, 3.0 Hz, 2H).

¹³C-NMR (CDCl₃): δ 36.4, 55.8, 60.3, 60.59, 60.63, 111.8, 122.9, 123.1, 123.36, 123.45, 125.9, 129.5, 132.2, 132.5, 132.7, 134.0, 146.9, 151.2, 151.4, 152.8, 168.2.


2-[2, 3, 2', 3'-Tetra(2-tert-butyloxycarbonylamino-ethyloxy)-biphenyl-4-ylmethyl]-isoindole-1,3-dione (S10)
2-(2, 3, 2', 3'-Tetramethoxy-biphenyl-4-ylmethyl)-isoindole-1,3-dione (250 mg, 0.577 mmol) was dissolved in dichloromethane (15 mL) and treated with a solution of BBr₃ in dichloromethane 1.0 M (6.0 mL) at 0 °C. The mixture was then allowed to warm to room temperature and stirred overnight. The reaction mixture was then cooled to 0 °C and MeOH (3 mL) was added. The solvent was removed under vacuum, and the residue was dissolved in ethyl acetate (20 mL), washed with 1N HCl (2 x 20 mL), water (20 mL), brine (20 mL), and dried over Na₂SO₄. The solvent was removed under vacuum to give the tetramine (248 mg), which was used without further purification.

To a solution of the tetramine (105 mg) in DMF (3.0 mL) was added Cs₂CO₃ (654 mg, 2.00 mmol) and tert-butyl N-(2-tosyloxyethyl)-carbamate (526 mg, 1.67 mmol) and the mixture heated to 80 °C and stirred under nitrogen for 4 h. After this time additional Cs₂CO₃ (654 mg, 2.00 mmol) and tert-butyl N-(2-tosyloxyethyl)-carbamate (526 mg, 1.67 mmol) were added and the mixture stirred for a further 15 h. The reaction mixture was diluted with ethyl acetate (15 mL). The organic layer was washed with 1N HCl (2 x 10 mL) water (15 mL), brine (15 mL), and dried over Na₂SO₄. The solvent was removed under vacuum and the product purified by preparative TLC (CHCl₃/MeOH = 10/1). The product obtained, was dissolved in DMF (2 mL) and acetic anhydride (0.5 mL) was added and the mixture stirred at 80 °C for 1 h. The mixture was diluted with ethyl acetate (10 mL) and washed with water (3 x 10 mL), Na₂CO₃ saturated aqueous solution (15 mL), brine (15 mL), and dried over Na₂SO₄. The solvent was removed under vacuum and purified by preparative TLC (ethyl acetate/hexane = 1/1) to afford (S10) (38 mg, 16 % yield).

¹H-NMR (CDCl₃): δ 1.38 (s, 9H), 1.42 (s, 9H), 1.43 (s, 9H), 1.45 (s, 9H), 3.10 (m, 2H), 3.19 (m, 2H), 3.58 (m, 4H), 3.78 (m, 4H), 4.11 (m, 2H), 4.23 (m, 2H), 4.64 (br s,
1H), 4.95 (s, 2H), 5.20 (br s, 1H), 5.44 (br s, 1H), 5.78 (br s, 1H), 6.85 (br d, J = 6.7 Hz, 1H), 6.93 (br d, J = 7.4 Hz, 1H), 6.98 (d, J = 8.0 Hz, 1H), 7.08 (t, J = 7.9 Hz, 1H), 7.14 (d, J = 8.0 Hz, 1H), 7.73 (dd, J = 5.4, 3.0 Hz, 2H), 7.87 (dd, J = 5.4, 3.0 Hz, 2H).

Theoretical Mass: (M + H) 950.47625. Measured Mass: (M + H) 950.47705.

2-{2, 3', 2'-Tetra[2-N, N'-bis(tert-butoxycarbonyl)guanidino-ethyloxy]-biphenyl-4-ylmethyl}-isoindole-1,3-dione (SI 1)

![Chemical Structure of SI 1]

2-[2, 3', 2'-Tetra[2-tert-butyloxycarbamylamino-ethyloxy]-biphenyl-4-ylmethyl]-isoindole-1,3-dione (38 mg, 0.040 mmol) was dissolved in TFA (2 mL) and stirred at rt for 2 h. The solvent was removed under vacuum to give the unprotected tetramine, which was used without further purification. To a solution of the crude tetramine in DMF (1.0 mL), was added N, N-di-boc-N'-trifluoromethanesulfonyl-guanidine (94 mg, 0.24 mmol), and i-Pr$_2$EtN (84 μL, 0.48 mmol) and the mixture stirred at rt for 15 h. The reaction mixture was diluted with ethyl acetate (5 mL) and washed with 1N HCl (3 × 5 mL), followed by water (5 mL), brine (5 mL), and dried over Na$_2$SO$_4$. The solvent was removed under vacuum and purified by preparative TLC (hexane/ethyl acetate 3/2) to give (S11) (45 mg, 74% yield) as a colourless oil.

$^1$H-NMR (CDCl$_3$): δ 1.36 (s, 9H), 1.41 (s, 9H), 1.44 (s, 9H), 1.45 (s, 18H), 1.48 (s, 27H), 3.45 (m, 4H), 3.88 (m, 8H), 4.16 (m, 2H), 4.39 (m, 2H), 4.98 (s, 2H), 6.83-7.03 (m, 5H), 7.71 (dd, J = 5.4, 3.0 Hz, 2H), 7.86 (dd, J = 5.4, 3.0 Hz, 2H), 8.46 (br s, 1H), 8.57 (br s, 1H), 8.79 (br s, 1H), 8.89 (br s, 1H).

Theoretical Mass: (M + H) 1518.7731. Measured Mass: (M + H) 1518.78005.

[2, 3, 2', 3'-Tetra(2-guanidino-ethyloxy)-biphenyl-4-ylmethyl]-thioureido-fluorescein (S13)
To a solution of 2-{2, 3, 2', 3'-tetra[2-N, N'-bis(tert-butoxycarbonyl)guanidino-ethyloxy]-biphenyl-4-ylmethyl}-isoindole-1,3-dione (45 mg, 0.030 mmol) in EtOH (0.7 mL) was added hydrazine monohydrate (15 µL, 0.30 mmol) and the mixture stirred at rt for 16 h. The solvent was then evaporated under vacuum, the residue was dissolved into dichloromethane (5 mL), and the precipitate filtered off. The filtrate was washed with brine (5 mL) and the aqueous layer was extracted with dichloromethane (3 × 5 mL). The combined organic layer was then dried over Na₂SO₄. The solvent was removed under vacuum to afford crude amine (S12), which was used without further purification. The crude material from the previous reaction dissolved in DMF (1.0 mL) and fluorescein isothiocyanate isomer I (18 mg, 0.045 mmol) and i-Pr₂EtN (16 µ, 0.090 mmol) were added and the mixture was stirred in the dark for 15 h. To the reaction mixture was added PS-trisamine (Argonaut Technologies Inc., 4.17 mmol/g, 25 mg) to remove the excess FITC. After shaking the mixture at rt 30 min, the resin was filtered off and the filtrate was diluted with ethyl acetate (10 mL), washed with 1N HCl (3 × 5 mL) followed by water (5 mL), brine (5 mL), and dried over Na₂SO₄. The solvent was removed under vacuum to give the fully protected product, which was dissolved in TFA (2.0 mL) and stirred at rt for 2 h. TFA was removed under vacuum and the residue was purified by reverse-phase HPLC using a preparative C-18 column (0.1 % TFA H₂O / acetonitrile) to afford (S13) (9 mg, 20 % yield).
$^1$H-NMR (MeOH-d$_4$): $\delta$ 3.34 (m, 4H), 3.69 (m, 4H), 3.94-3.97 (m, 4H), 4.22-4.30 (m, 4H), 4.96 (s, 2H), 6.61 (d, $J = 8.7$ Hz, 2H), 6.76-6.78 (m, 4H), 6.90 (d, $J = 7.3$ Hz, 1H), 7.04-7.24 (m, 5H), 7.79 (d, $J = 8.1$ Hz, 1H), 8.19 (s, 1H).

MS (MALDI) $m/z$ 976 (calcd), 977, 978, 979 (found).

N-{2, 3, 2', 3'-Tetra[2-NN'-bis(tert-butoxycarbonyl)guanidino]-ethyloxy}-biphenyl-4-ylmethyl)-3-[2-pyridyl]dithio]propionamide

To a solution of 2-{2, 3, 2', 3'-tetra[2-NN'-bis(tert-butoxycarbonyl)guanidino]-ethyloxy}-biphenyl-4-ylmethyl]-isoindole-1,3-dione (8 mg, 0.005 mmol) in EtOH (0.5 mL) was added hydrazine monohydrate (3 $\mu$L, 0.06 mmol) and the mixture was stirred at rt for 17 h then the solvent was evaporated under vacuum. The residue was dissolved into DCM (5 mL) and the precipitate was filtered off. The filtrate was washed with brine (5 mL), and the aqueous layer was extracted with dichloromethane (3 x 5 mL). The combined organic layer was dried over Na$_2$SO$_4$, and the solvent was removed under vacuum to afford crude amine (S12), which was used without further purification.

To a solution of the crude amine and i-Pr$_2$EtN (2 $\mu$L, 0.01 mmol) in dichloromethane (0.3 mL) was added N-succinimidyl-3-(2-pyridyldithio)propionate (3 mg, 0.01 mmol) and the mixture was stirred in the dark for 24 h. The solvent was removed under vacuum and purified by preparative TLC (ethyl acetate/hexane 1/1) to afford the product (4 mg, 50 % yield).

IR $\nu_{max}$ KBR/cm$^{-1}$ 3336 (NH), 1722 (C=O), 1641 (C=ON)

$^1$H-NMR (CDCl$_3$): $\delta$ 1.43-1.51 (m, 72H), 2.70 (t, $J = 7.2$ Hz, 2H), 3.11 (t, $J = 7.2$ Hz, 2H), 3.45-3.50 (m, 4H), 3.84-3.93 (m, 8H), 4.15-4.21 (m, 4H), 4.46 (d, $J = 5.6$ Hz,
2H), 6.89-6.92 (m, 2H), 6.99-7.08 (m, 4H), 7.57-7.68 (m, 2H), 8.36 (m, 1H), 8.46 (br t, 1H), 8.55 (br t, 1H), 8.80 (br t, 2H).

Theoretical Mass: (M + H) 1585.76459. Measured Mass: (M + H) 1585.05043.

N-[2, 3, 2', 3'-Tetra(2-guanidino-ethyloxy)-biphenyl-4-ylmethyl]-3-[2-pyridyl]dithio]propionamide (14)

N'-Bis(tert-butoxycarbonyl)guanidino]-ethyloxy]-biphenyl-4-ylmethyl]-3-[2-pyridyl]dithio]propionamide (4 mg, 0.0024mmol) was dissolved in TFA (0.8 mL) and stirred at rt for 3 h. TFA was removed under vacuum to give (S14), as a colourless oil (4 mg).

IR v Max KBR/cm': 3376 (br NH, NH2), 1381 (C=ON)

1H-NMR (MeOH-d4):  δ 2.73 (t, J = 6.6 Hz, 2H), 3.10 (t, J = 6.6Hz, 2H), 3.23-3.30 (m, 4H), 3.62-3.67 (m, 4H), 3.88 (m, 2H), 3.96 (m, 2H), 4.22 (m, 4H), 4.47 (s, 2H), 6.87 (dd, J = 7.4, 1.7 Hz, 1H), 7.02 (d, J = 7.9 Hz, 1H), 7.08-7.24 (m, 4H), 7.76-7.80 (m, 2H), 8.38 (d, J = 4.7 Hz, 1H), 8.70 (br t, 1H).

ESMS; m/z calcd; 784, found; 899[(M + 1) + TFA], 785 (M + 1), 507 [(M + 2) + 2TFA], 450 [(M + 2) + TFA], 393 (M + 2), 338[(M + 3) + 2TFA].
Inhibition of G0-S progression increases with (a) 4G-SMoC-geminin and (b) 4G-SMoC-ΔNt-geminin concentration, with a maximum inhibition of 50% occurring with 10 μM of 4G-SMoC-geminin/4G-SMoC-ΔNt-geminin. Treatment with an equal molar concentration of uncoupled 4G-SMoC-geminin/4G-SMoC-ΔNt-geminin or an equivalent volume of PBS-EDTA had no effect on G0-S progression. Results are
expressed as the reduction in the percentage of cells incorporating BrdU relative to the control population.
Appendix G

Loss of Proliferative Capacity and Induction of Senescence in Oxidatively Stressed Human Fibroblasts

Jian-Hua Chen§, Kai Stoeber¶, Sarah Kingsbury¶**, Susan E. Ozanne†, Gareth H. Williams¶, and C. Nicholas Hales‡

From the Department of Clinical Biochemistry, University of Cambridge, Cambridge CB2 2QK, United Kingdom, the Wolfson Institute for Biomedical Research, University College London, London WC1E 6BT, United Kingdom, and the Department of Histopathology, University College London, London WC1E 6JJ, United Kingdom

Cellular senescence can result from short, dysfunctional telomeres, oxidative stress, or oncogene expression, and may contribute to aging. To investigate the role of cellular senescence in aging it is necessary to define the time-dependent molecular events by which it is characterized. Here we investigated changes in levels of key proteins involved in cell cycle regulation, DNA replication, and stress resistance in senescing human fibroblasts following oxidative stress. An immediate response in stressed cells was dephosphorylation of retinoblastoma (Rb) and cessation of DNA synthesis. This was followed by sequential induction of p53, p21, and p16. Increase in hypophosphorylated Rb and induction of p53 and p21 by a single stress treatment was transient, whereas sustained induction or dephosphorylation were achieved by a second stress. Down-regulation of the critical DNA replication initiation factor Cde6 occurred early after stress concurring with p53 induction, and was followed by a decrease in Mcm2 levels. A late event in the stress-induced molecular sequence was the induction of SOD1, catalase, and HSP27 coinciding with development of the fully senescent phenotype. Our data suggest that loss of proliferative capacity in oxidatively stressed cells is a multistep process regulated by time-dependent molecular events that may play differential roles in induction and maintenance of cellular senescence.

Normal human fibroblasts only have a limited proliferative capacity in vitro and will become senescent eventually (1). Although cellular senescence is mainly observed with cells in culture, several lines of evidence suggest that a similar process occurs in vivo and contributes to aging (Ref. 2 and references therein). Telomere shortening because of the end replication problem is believed to be the prime intrinsic cause that limits the cell proliferation capacity because critically shortened telomeres serve to activate the senescence process (3). However, various stresses such as DNA damaging agents, oxidative stress, and certain oncogene overexpression can also induce cellular senescence (4-7). The cell culture condition of 20% O2 might represent an oxidative stress when compared with the much lower in vivo physiological pressure of O2 (8-10). Thus telomere shortening may be driven to a large extent by stress, and telomere shortening by the end replication problem may set an upper limit to the replicative lifespan, which might only seldom be reached (11).

The free radical theory of aging proposes that reactive oxygen species produced in mitochondria cause damage to cellular macromolecules, thus resulting in a decline of cellular function and organism aging (12). This theory has gained strong support from studies that linked oxidative stress to longevity using model organisms including yeasts, nematodes, flies, and rodents (13, 14). Mutations that increase longevity are often associated with increased antioxidant activity and improved resistance to oxidative stress (15-17).

For the past decade we have studied the long term consequences of poor early growth using rodent models (18). One of our most striking observations is that the rate of growth in early life has profound effects on longevity. Fetal growth restriction followed by postnatal catch-up growth in male rats and mice results in reduced longevity (19, 20). Male rats most frequently die of renal failure (19). We have shown that the reduced longevity of fetally growth restricted, postnatally growth caught-up male rats is associated with accelerated shortening of telomeres in the kidney (21). We have suggested that this shortening is associated with accelerated senescence of renal cells with earlier renal failure and death. It seems unlikely that replicative senescence alone could lead to the degree of shortening observed and therefore telomere shortening consequent upon oxidative damage may be involved (21, 22). We have further speculated that telomeres not only function as a measure of the number of cell divisions undergone by telomerase-negative cells but also, by virtue of their GGG content, monitor oxidative damage by the accelerated telomere shortening that results (23).

The present study was undertaken to study a selection of key proteins involved in DNA damage response, cell cycle regulation, DNA replication licensing, and stress resistance in oxidatively senescing cells by exploiting a model cell line in vivo. Knowledge of the molecular markers of oxidative stress-induced cellular senescence will allow us to study the progress of cell senescence in vivo. It has been shown that early passage human fibroblasts can be induced into senescence prematurely by oxidative stress (4, 24, 25). Because the cells develop features of senescence within 7 days and the time course for developing senescent features is well defined, oxidatively stressed human fibroblasts provide a useful experimental model to study the underlying mechanism and markers of senescence-associated aging. Here we show that oxidative stress causes loss of proliferative capacity and induction of
The abbreviations used are: PD, population doubling; BrdUrd, 2-bromo-5-deoxyuridine; SA-β-gal, senescence-associated β-galactosidase; PBS, phosphate-buffered saline; Rb, retinoblastoma; SOD, superoxide dismutase; HSP, heat shock protein; PCNA, proliferating cell nuclear antigen; PML, promyelocytic leukemia protein.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and H$_2$O$_2$ Treatment**—IMR-90 cells at population doubling (PD)$^1$ 24.5 were obtained from the American Type Culture Collection (ATCC). The cells were grown in ATCC modified Eagle’s minimal essential medium supplemented with 10% fetal bovine serum. Cells for H$_2$O$_2$ treatment were prepared from exponential phase around PD30. H$_2$O$_2$ treatment was carried out 24 h after seeding by incubating 2 × 10$^5$ cells in 100-mm dishes in 4 ml of the culture medium containing 600 μM H$_2$O$_2$ for 2 h. For a second H$_2$O$_2$ treatment the cells were split 1:3 after being cultured for 4 days following the first treatment and treated again for 2 h with 600 μM H$_2$O$_2$ 24 h after seeding. IMR-90 cells reached replicative senescence at PD59.

**BrdUrd Labeling and SA-β-Gal Assay**—Cells were seeded and treated as described above in dishes containing autoclaved coverslips. For BrdUrd labeling, coverslips were transferred into a 6-well plate and incubated in 2 ml of the culture medium containing 10 μM BrdUrd for 48 h. Cells on coverslips were then washed with PBS, fixed in 4% paraformaldehyde in PBS, washed twice in PBS, permeabilized in 0.2% Triton X-100, and washed in PBS. DNA was denatured by incubation in 2 N HCl for 1 h followed by three washes with PBS. Coverslips were incubated for 1 h with fluorescein-conjugated mouse anti-BrdUrd monoclonal antibody (1:20 dilution; Alexis Biochemicals) and with DNA stain TOTO-3'-iodide (0.5 μM; Molecular Probes), washed three times with PBS, and mounted in 90% glycerol, 10% PBS containing p-phenylenediamine (1 mg/ml; Sigma). For senescence-associated β-galactosidase (SA-β-gal) assay, cells on coverslips were washed twice in PBS, fixed for 5 min in 4% paraformaldehyde in PBS, washed three times in PBS, and incubated at 37 °C overnight in fresh SA-β-gal staining solution (1 mg of 5-bromo-4-chloro-3-indolyl β-D-galactosidase (stock = 100 mg/ml of dimethylformamide)/ml of 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl in 40 mM citric acid/sodium phosphate, pH 6.0) (26). At least 500 cells from each time point were scored under microscope for BrdUrd- or SA-β-gal assay.

**Western Blotting**—Whole cell lysate was prepared by scraping cells in Laemmli buffer (0.12 M Tris, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol with protease inhibitors (mixture from Sigma)). Protein concentration was determined by the bicinchoninic acid (BCA) method (Sigma) with bovine serum albumin as a standard. 25 μg of proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. Blots were probed with the following antibodies: anti-p53 antibody (ab7757, abcam), anti-p21 antibody (H-164, Santa Cruz), anti-p16 antibody (H-156, Santa Cruz), anti-Rb antibody (G3-245, BD Pharmingen), anti-Cdc6 antibody (185.2, Santa Cruz), anti-Mcm3 antibody (BM28, Transduction Laboratories), anti-Mcm5 antibody (27), anti-PCNA antibody (ab29, abcam), anti-Catalase antibody (ab1877, abcam), anti-SOD1 antibody (FL-154, Santa Cruz), anti-HSP70 antibody (W27, Santa Cruz), and anti-HSP27 (H-77, Santa Cruz). The

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**Fig. 1.** Inhibition of DNA synthesis and induction of SA-β-gal activity in H$_2$O$_2$-treated human fibroblasts. Logarithmically growing IMR-90 cells were seeded at a density of 2 × 10$^5$ cells in 100-mm dishes and treated with H$_2$O$_2$ for 2 h 24 h after seeding. Cells were split 1:3 4 days after the first H$_2$O$_2$ treatment and treated for 2 h with H$_2$O$_2$ again 24 h later. A, BrdUrd was added to the cells at the indicated time points after a 48-h incubation and its incorporation was detected with fluorescein-conjugated antibody (green). Nuclei are stained with TOTO-3 to reveal DNA (red). The inset is a representative BrdUrd incorporation assay of control cells. Data represent the mean ± S.E. of three independent experiments.

**B** SA-β-gal activity was detected by incubating the fixed cells in SA-β-gal solution at 37 °C overnight. The inset is a representative SA-β-gal assay of H$_2$O$_2$-induced senescent cells. The mean ± S.E. of three independent experiments are shown.

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$^1$ The abbreviations used are: PD, population doubling; BrdUrd, 2-bromo-5-deoxyuridine; SA-β-gal, senescence-associated β-galactosidase; PBS, phosphate-buffered saline; Rb, retinoblastoma; SOD, superoxide dismutase; HSP, heat shock protein; PCNA, proliferating cell nuclear antigen; PML, promyelocytic leukemia protein.
bound primary antibodies were detected by horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences), followed by enhanced chemiluminescence (Amersham Biosciences). The densities of the bands were quantified using an Alpha Imager (Alpha Innotech Corporation, San Leandro, CA).

Preparation of Nuclei and Cytosolic Extracts—Nuclear and cytosolic extracts were prepared by hyptonically swelling cells in ice-cold LS buffer (20 mM potassium-HEPES, pH 7.8, 5 mM potassium acetate, 0.5 mM MgCl₂, 0.5 mM dithiothreitol), a buffered mixture of NTPs, spermidine trihydrochloride, and Dounce homogenization as described previously (28) with the following minor modifications. Cytoplasmic supernatant was taken from the first spin (2,000 x g in a Whatman Micro-Centrifuge for 2 min) and respun at 2,000 x g for 10 min. Supernatant fractions were then aliquoted, processed, and stored in liquid nitrogen. Nuclear pellets from the first spin were resuspended in an equal volume of ice-cold SuNaSp/bovine serum albumin solution (250 mM sucrose, 75 mM NaCl, 0.5 mM spermine tetrahydrochloride, 0.15 mM spermidine trithydrochloride, 3% bovine serum albumin) and respun at 20,000 x g for 2 min. The supernatant was discarded and the packed nuclear pellet was resuspended in an equal volume of SuNaSp/bovine serum albumin, frozen, and stored at -80 °C.

In Vitro DNA Synthesis Assay—DNA replication reactions containing 30 μl of elongation buffer (60 mM KCl, 15 mM Tris-HCl, pH 7.4, 15 mM NaCl, 1 mM β-mercaptoethanol, 0.5 mM spermine tetrathydrochloride, 0.5 mM spermidine trihydrochloride), a buffered mixture of NTPs, dNTPs, and an energy regeneration system (yielding a final concentration of 40 mM K-HEPES, pH 7.8, 7 mM MgCl₂, 3 mM ATP, 0.1 mM each of GTP, CTP, UTP, 0.1 mM each of dATP, dGTP, and dCTP, 0.25 μM biotin-16-dUTP, 0.5 mM dithiobiotreitol, 40 mM creatine phosphate, and 5 μg of phosphocreatine kinase) and 1 x 10⁶ nuclei were performed as previously described (28, 29). For detection by immunofluorescence, the nucleotide mixture was supplemented with a final concentration of 0.25 μM biotin-16-dUTP (Roche Diagnostics). In vitro DNA replication reactions were incubated at 37 °C for 3 h. Nuclei in the DNA replication assays were resuspended in PBS (0.5 ml) and fixed for 5 min by adding an equal volume of 8% paraformaldehyde. After fixation, nuclei were spun through 30% sucrose/PBS onto polysilane-coated coverslips. All subsequent washing and staining steps were carried out in PBS, 0.2% Triton X-100, 0.04% SDS. Coverslips were washed, stained for incorporated biotin-16-dUTP with fluorescein-linked streptavidin (1:100 dilution, Amersham Biosciences) and for DNA with propidium iodide/ RNase A (both at 50 μg/ml, Sigma), washed again, and mounted in vectashield (Vector Laboratories, Peterborough, UK). Confocal fluorescence microscopy of random fields of nuclei was performed on a Leica TCS DMRE confocal microscope. Images were collected, and merged pictures of the propidium iodide channel (red) and fluorescein channel (green) were obtained using Adobe Photoshop with standardized brightness- and contrast-enhanced operations of all samples. Images were printed, and the number of nuclei incorporating biotin-16-dUTP in vitro (yellow) and non-replicating nuclei (red) were counted. Routinely, 800–1000 nuclei were scored for each replication reaction and quantitated as percentages of the total number of nuclei that synthesized DNA in vitro.

RESULTS

Cessation of DNA Replication and Induction of Senescence in the H₂O₂-treated Cells—To understand molecular mechanisms underlying aging, we investigated key cell cycle regulatory proteins and stress-resistant factors in normal human diploid fibroblasts that were subjected to oxidative stress. We adopted H₂O₂ treatment to induce premature cellular senescence. Dose-response experiments showed that the cells could tolerate up to...
600 µM H₂O₂ for 2 h with very few lethal effects under the conditions used in this study (data not shown). A small fraction of the cells that received a single H₂O₂ treatment were able to recover and re-enter the cell cycle. This was manifested by the gradual increase of cell number after the treatment. Indeed, about 20% of the treated cells incorporated BrdUrd 5 days after a first H₂O₂ treatment (Fig. 1A). We therefore reseeded the cells at a ratio of 1:3 4 days after the first treatment and performed a second treatment 24 h later. The second H₂O₂ treatment caused a permanent cell cycle arrest for over 95% cells. As shown in Fig. 1A BrdUrd positive cells decreased to less than 4% after the second H₂O₂ treatment and eventually became undetectable 4 days later. Cells stressed with H₂O₂ twice eventually developed senescence phenotypic features including enlarged and flattened morphology and SA-β-gal activity. The fully developed phenotypic features appeared 4 days after the second H₂O₂ treatment when over 95% cells displayed SA-β-gal activity (Fig. 1B). This result shows that a second oxidative stress is necessary to induce a high incidence of premature senescence.

Induction of Cell Cycle Checkpoint Proteins in the H₂O₂-treated Cells—Among the cell cycle checkpoint proteins investigated, p53 was rapidly increased by oxidative stress (Fig. 2A). This was followed by the induction of p21, which is a target of p53 (Fig. 2A). Both proteins tended to return to basal level if the induction was caused by a single H₂O₂ treatment. Sustained induction of p53 and p21 was seen in the cells that received two H₂O₂ treatments (Fig. 2A). An elevated p16 level was only observed in the cells that received two H₂O₂ treatments and developed senescent phenotypic features (Fig. 2A).

Dephosphorylation of Rb was also induced by H₂O₂ treatment (Fig. 2B). The ratio of hyperphosphorylated Rb (pRb) to dephosphorylated Rb decreased from 53% in control cells to 43% in cells a half-hour after a 2-h H₂O₂ treatment (Fig. 2B). The ratio returned to the control level 5 days after the treatment. However, the second H₂O₂ treatment caused a sustained decrease in hyperphosphorylated Rb, which eventually became undetectable 1 day after the second treatment and remained dephosphorylated thereafter (Fig. 2B).

Decrease of DNA Replication Proteins in the H₂O₂-treated Cells—Cdc6, Mcm2, Mcm5, and PCNA are proteins involved in DNA replication (30). Apart from Mcm5 that remained largely unaffected, they generally decreased in the H₂O₂-treated cells (Fig. 3A). Cdc6 became undetectable in the cells that received two H₂O₂ treatments and had the senescent phenotype fully developed (Fig. 3A). However, a residual amount of Mcm2 and a substantial amount of PCNA remained detectable in the twice H₂O₂-treated cells (Fig. 3A).

Effects of Oxidative Stress on Antioxidant Enzymes and Stress-response Proteins—Levels of antioxidant enzymes SOD1 and catalase were not immediately affected by H₂O₂ treatment, but an increase in the levels of these two enzyme proteins was clearly detected in the stressed cells when senescent phenotypic features were fully developed (Fig. 3B). Levels of the stress response protein HSP70 did not change noticeably in the H₂O₂-treated cells, whereas HSP27 showed a slight increase.
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Molecular Events in Replicatively Senescing Cells—To confirm that molecular events induced by oxidative stress are similar to those in replicatively senescing cells, we examined changes in p53, p21, p16, and Mcm2 in cells when they were in early population doubling (PD30), in a near senescent state (PD57), and in the fully established replicative senescent state (4 weeks into replicative senescence). As shown in Fig. 4, p53 and p21 increased substantially in the near replicative senescent cells but declined in the fully senescent cells. A significant increase in p16 was only observed in the fully senescent cells (Fig. 4). On the other hand, Mcm2 decreased substantially in the near replicative senescent cells and was undetectable in the fully senescent cells. These data suggest that time-dependent changes in molecular events observed in the oxidatively senescent cells occur similarly in the replicatively senescent cells but over a much longer time frame.

Molecular Events in Early Time Points after H2O2 Treatment—Oxidative stress is a potential source of DNA damage. To investigate the immediate consequences of oxidative stress in the cells we examined molecular events in earlier time points after H2O2 treatment. Induction of p53 can be detected at the end of the 2-h H2O2 treatment (Fig. 5). The highest induction was observed 2 h later. Thereafter it declined gradually and returned to almost the basal level 5 days later. This rapid induction of p53 was repeated after a second H2O2 treatment (Fig. 5). Induction of p21 was detected 2 h later than that of p53 with the highest induction being seen 6 h after the 2-h H2O2 treatment (Fig. 5). Expression of p16 was not affected by H2O2 treatment during this early time course (Fig. 5). Mcm2 treatment caused a decrease in the ratio of hyperphosphorylated Rb to dephosphorylated Rb in the cells. This change was detected in the cells that received treatment for only 1 h (Fig. 5). Densitometry analysis showed that the ratio decreased from 60% in the control cells to 46% in the 1-h H2O2-treated cells. Further decreases were seen in subsequent time points and the hyperphosphorylated Rb eventually became undetectable 10 h after the 2-h H2O2 treatment (Fig. 5). The hyperphosphorylated Rb reappeared a day after the treatment and returned to the control level 5 days later (Fig. 5). The second H2O2 treatment caused a similar change in the ratio in the cells at the end of the treatment (Fig. 5). In contrast, the DNA replication protein Mcm2 remained unchanged during the initial 24 h after the first H2O2 treatment with a slight decrease only being seen 5 days later and 2 h after a second H2O2 treatment (Fig. 5). Changes in levels of Cdc6 showed a similar pattern to that of Mcm2 (data not shown).

Cessation of DNA Replication Is a Rapid Responsive Event in the H2O2-treated Cells and Is Because of the Inhibition of DNA Elongation—H2O2 treatment caused a rapid cessation of DNA replication as demonstrated by a rapid decrease of BrdUrd positive cells in the oxidatively stressed cells (Fig. 6A). A 1-h H2O2 treatment caused an almost 10-fold decrease in DNA synthesis as compared with the untreated cells (83% BrdUrd positive cells). At the end of the 2-h H2O2 treatment BrdUrd positive cells decreased to 2.7%. A further decrease was observed 2 h later. A slight recovery was observed 4 h after the 2-h H2O2 treatment (Fig. 6A). Thus cessation of DNA replication was a rapid responsive event that preceded any detectable changes in protein levels of p53, p21, p16, Mcm2, and Cdc6 (see Fig. 5).

Rapid cessation of DNA replication in the H2O2-treated cells suggested that any ongoing DNA synthesis during S-phase must have been halted by the stress. To test this hypothesis we employed a cell-free in vitro DNA replication assay and compared DNA synthesis in elongation buffer using nuclei prepared from control and 2-h H2O2-treated cells. As shown in Fig. 6B, only 1.8% of nuclei prepared from H2O2-stressed cells were capable of DNA synthesis in contrast to the control in which about 10% of nuclei were synthesizing DNA (Fig. 6B). Note that the relatively low percentage of replicating nuclei compared with that detected by a 48-h BrdUrd labeling (Fig. 6A) is because of the short incubation period of the in vitro replication.
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Fig. 6. Acute inhibition of DNA synthesis is because of inhibition of DNA elongation in \( \text{H}_2\text{O}_2 \)-treated cells. A, control and \( \text{H}_2\text{O}_2 \)-treated IMR-90 cells were incubated with BrdUrd for 48 h and its incorporation was detected with fluorescein-conjugated antibody. The data represent the mean of two independent experiments. Insets are merged images from a representative BrdUrd incorporation assay for control and 1-h \( \text{H}_2\text{O}_2 \)-treated cells. B, nuclei were prepared from control and 2-h \( \text{H}_2\text{O}_2 \)-treated cells and incubated for 3 h in elongation buffer substituted with ribonucleotide and deoxyribonucleotide triphosphates and an energy regeneration system. Nuclei are stained with propidium iodide to reveal DNA (red) and with fluorescein-streptavidin (green) to detect biotin-dUTP incorporation resulting from in vitro DNA synthesis. Merged images are shown. Results are expressed as the percentage of nuclei replicating and summarized in the histogram. The data represent the mean ± S.E. of three independent experiments.

reaction (3 h). This suggests that elongation during S-phase was inhibited by \( \text{H}_2\text{O}_2 \) treatment.

**DISCUSSION**

We have established a unique rodent model in which longevity can be significantly increased or decreased by a minor manipulation of maternal diet (19, 20). A causative link between longevity and cellular senescence *in vivo* remains to be established. As a first step toward elucidating the molecular mechanisms underlying cellular senescence and such a profound effect of growth rate during fetal and postnatal development on aging, we used an *in vitro* human fibroblast model to study key proteins involved in cell cycle control, DNA replication, and stress resistance in oxidatively stressed cells. We demonstrated that around 20% of cells exposed to a single \( \text{H}_2\text{O}_2 \) treatment were able to recover and re-enter the cell cycle despite the initial abrupt cessation of DNA synthesis. This is consistent with an earlier report that showed that over 30% of the BrdUrd positive cells were detected in single \( \text{H}_2\text{O}_2 \)-treated IMR-90 cells (24). Presumably this is because these cells were able to repair the DNA damage caused by a single \( \text{H}_2\text{O}_2 \) treatment. It is not clear as to why some cells are able to recover and re-enter cell cycle while the majority become permanently arrested and eventually senesce. One possibility is the heterogeneity in DNA damage caused by \( \text{H}_2\text{O}_2 \) treatment. A high incidence of cell senescence was induced only by a second subcytotoxic \( \text{H}_2\text{O}_2 \) treatment. Indeed, cellular senescence *in vivo* is more likely to be induced by repeated oxidative insults.

Mitochondria are major producers of reactive oxygen species and, at the same time, particularly susceptible to oxidative damage. In particular, damage to mtDNA results in faulty mitochondrial proteins, with a consequent decrease of electron transfer, leading to further production of reactive oxygen species and thus exposing cells to a vicious circle of oxidative stress (31).

In our time course study of oxidative senescence, we observed sequential inductions of cell cycle checkpoint proteins including p53, p21, and p16 with the induction of p16 coinciding with an increase in the activity of SA-β-gal. Increased p16 expression was also observed in replicative senescent cells. This is in keeping with cells undergoing replicative senescence in that p53 senses stresses, which then activates expression of p21 resulting in cell cycle arrest, whereas p16 is responsible for the maintenance of the senescent state (32, 33). The induced p53 and p21 by a single \( \text{H}_2\text{O}_2 \) treatment tended to return to basal levels. The transient induction of p53 may be because of the regulation of the p53-Mdm2 feedback loop. In the p53-Mdm2 feedback loop, elevated p53 induces Mdm2 transcription, which in turn binds p53 and promotes p53 degradation via the ubiquitin-proteasome pathway (34). Sustained elevations of p53 and p21 were observed only after the cells received a second treatment. This suggests that the p53-Mdm2 feedback loop may be disrupted by repeated oxidative stress.

Louria-Hayon *et al.* (35) recently showed that PML (promyelocytic leukemia protein) is critical for the accumulation of p53 in response to DNA damage. PML protects p53 from Mdm2-mediated ubiquitination and degradation by prolonging the stress-induced phosphorylation of p53 on serine 20 (35). PML is essential for the formation of a subnuclear structure called PML nuclear bodies, which serve as an important meeting point for p53 regulation, including phosphorylation, acetyla-
PCNA were not significantly affected immediately after H2O2 was not because of the proteolytic degradation of DNA replication proteins, as the protein levels of Cdc6, Mcm2, Mcm5, and PCNA in the H2O2-treated cells were decreased. The presence of Cdc6 and decreased levels of Mcm2 and PCNA in the H2O2-induced senescent cells are probably a consequence of cell cycle arrest in the G1 phase (24). The sequential down-regulation of Cdc6 and Mcm2 in the oxidatively stressed cells suggests that repression of DNA origin licensing is responsible for the loss of proliferative capacity upon oxidative stress. The presence of residual Mcm2 in cells that received two H2O2 treatments but not in control cells indicates that PML is involved in the induction of premature cell senescence (36).

The present study also showed that H2O2-induced cell senescence is accompanied by the decline of several DNA replication proteins including Cdc6, Mcm2, and PCNA. Cdc6 and Mcm proteins are components of the DNA replication licensing complex, which is assembled during the G1 phase, whereas PCNA is a DNA sliding clamp for replicative DNA polymerases, which is an essential component of the eukaryotic chromosomal DNA replisome during S-phase (30). These proteins undergo cell cycle-regulated synthesis, chromatin association, and proteolysis, thereby ensuring that DNA is replicated once and only once during a single cell division cycle (30, 45, 46). The absence of Cdc6 and decreased levels of Mcm2 and PCNA in the H2O2-induced senescent cells are probably a consequence of cell cycle arrest in the G1 phase (24). The sequential down-regulation of Cdc6 and Mcm2 in the oxidatively stressed cells suggests that repression of DNA origin licensing is responsible for the loss of proliferative capacity upon oxidative stress. The presence of residual Mcm2 in cells that received two H2O2 treatments but not in control cells indicates that PML is involved in the induction of premature cell senescence (36).

Indeed, we observed that a small fraction of cells retained DNA replication potential even after receiving two H2O2 treatments (data not shown).

DNA damage rapidly activates DNA checkpoint machinery that stop a proliferating cell in the G1, S, or G2 phases of the cell cycle, contributing to the maintenance of genome integrity (47). Our cell-free in vitro assay indicated that the abrupt cessation of DNA replication caused by H2O2 treatment was because of inhibition of ongoing DNA elongation. This rapid inhibition was not because of the proteolytic degradation of DNA replication proteins, as the protein levels of Cdc6, Mcm2, Mcm5, and PCNA were not significantly affected immediately after H2O2 treatment. Although H2O2 treatment stabilized p53 and caused subsequent induction of p21, these events only occurred after cessation of DNA replication had already occurred. It is therefore unlikely that p21 is directly involved in the inhibition of DNA replication under these conditions. Cicchillitti et al. (48) showed that a rapid inhibition of DNA synthesis in human umbilical vein endothelial cells (HUVEC) by H2O2 treatment was correlated with a rapid hypophosphorylation of the retinoblastoma family proteins Rb, p107, and p130 (48). This event did not require p53 or p21 and was not associated with cyclin/CDK down-modulation, but was rather dependent on the activity of protein phosphatase 2A (48). It was speculated that, upon cell treatment with H2O2, Rb family members might bind in their dephosphorylated form directly to the DNA replication origins thereby inhibiting their firing (48). Indeed, upon DNA damage by γ-irradiation, Rb binds to selected DNA replication origins, inhibiting DNA replication in S-phase (49). In this study we observed a decrease in the ratio of hyperphosphorylated Rb to hypophosphorylated Rb in the cells at the earliest time point (Fig. 5). It is conceivable, therefore, that dephosphorylation of Rb may play an important role in the immediate inhibition of DNA replication in the H2O2-treated cells. It also remains possible that Rb re-location from certain chromatin structures to chromatin locations that contain origins of DNA replication contributes to the rapid inhibition of DNA synthesis upon oxidative damage (49).

In conclusion, our study demonstrated that oxidative stress induced an array of molecular events. This involved increases and decreases in the levels of various proteins as well as changes in phosphorylation status. These events were time dependent and resulted in loss of proliferative capacity and ultimately the development of a senescent state that was indistinguishable from replicatively senescent cells. One of the initial events in this multistep process is the immediate inhibition of ongoing DNA elongation that is likely to be controlled by dephosphorylated Rb. Down-regulation of Cdc6 may contribute to the early response of cells to oxidative stress by inhibiting the pre-replicative complex from licensing during the G1 phase. In contrast down-regulation of Mcm proteins appeared to constitute a late response in the process. Sustained loss of proliferative capacity and high induction of cellular senescence are achieved by a second oxidative stress that is presumably necessary to cause irreparable DNA damage in human fibroblasts.

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