MOLECULAR DISSECTION OF THE CENTROSOME
OVERDUPLICATION PATHWAY IN S-PHASE ARRESTED CELLS

Thesis submitted for the degree of
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by

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DECLARATION

The accompanying thesis submitted for the degree of Doctor of Philosophy, entitled “Molecular dissection of the centrosome overduplication pathway in S-phase arrested cells” is based on work conducted by the author in the Department of Biochemistry at the University of Leicester mainly during the period between October 2004 and May 2008. All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references. None of the work has been submitted for another degree in this or any other University.

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MOLECULAR DISSECTION OF THE CENTROSOME OVERDUPLICATION PATHWAY IN S-PHASE ARRESTED CELLS

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SUMMARY

The formation of a bipolar mitotic spindle is crucial for the even segregation of genetic material into two daughter cells during cell division. Each pole of the spindle is organised by a centrosome, therefore it is paramount that two, and only two, centrosomes are present in the cell at the time that it enters mitosis. The presence of too many centrosomes can lead to the formation of multipolar spindles and the uneven segregation of chromosomes. Indeed, cancer cells frequently display supernumerary centrosomes which may contribute to chromosome missegregation and aneuploidy. Normally, following the completion of mitosis, each cell contains a single centrosome. This then duplicates in a semi-conservative, templated manner that is tightly linked to the cell cycle. In some instances supernumerary centrosomes arise through uncoupling of this event from the cell cycle such that centrosomes overduplicate within a single cell cycle. Experimentally, centrosome overduplication can be induced in certain cell types by treatment with drugs, such as hydroxyurea, that inhibit DNA synthesis and thereby provoke an S-phase arrest. This assay has been exploited in CHO, U2OS and p53−/− MEF cells to decipher a molecular pathway for centrosome overduplication using a range of pharmacological inhibitors. Distinct granules containing the protein centrin were identified by fluorescence microscopy as early intermediates in this process and shown to form within the nucleus in a Cdk-dependent manner. These foci are then trafficked from the nucleus to the cytoplasm dependent upon the nuclear export machinery. Here, they recruit modified tubulin, PCM-1 and pericentrin, and resemble centriolar satellites. Microtubules and dynein are required to focus these satellites around the centrosome and the formation of centrioles as recognised by electron microscopy. Finally, Hsp90 is required for the recruitment of γ-tubulin to the newly-formed centrioles to construct functional microtubule organising centres. Significantly, intermediate steps in this pathway show similarities to events associated with the de novo centriole formation pathway and the centriolar and acentriolar pathways of ciliogenesis. Together, this work substantially increases our understanding of how supernumerary centrosomes are generated in cells and identifies key events that may be targeted to prevent centrosome overduplication in cancer cells.
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# CONTENTS

Declaration I  
Summary II  
Acknowledgements III  
Contents IV  
Abbreviations IX  
Figures XI

## CHAPTER 1  INTRODUCTION

1.1 Microtubule organising centres

1.1.1 The centrosome 

1.2 Centrosome function

1.2.1 Spindle assembly

1.2.2 Cilia and flagella

1.3 The centrosome cycle

1.3.1 Centriole duplication licences and blocks

1.3.2 Initiating centrosome duplication

1.4 Centriole formation

1.4.1 Templated semi-conservative centriole duplication

1.4.2 De novo centriole formation

1.4.3 Ciliogenesis

1.5 Centriole assembly

1.5.1 Structural assembly of centrioles

1.5.2 Proteins implicated in centriole duplication

1.5.3 A pathway for centriole formation

1.5.4 Conservation of the centriole formation pathway

1.6 Centrosomes and Cancer

1.7 Aims and objectives

## CHAPTER 2  MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents
2.1.2 Antibodies ........................................................................................................... 49
2.1.3 Drugs .................................................................................................................... 50
2.2 Cell culture ................................................................................................................. 51
  2.2.1 Cell maintenance ................................................................................................. 51
  2.2.2 Cell storage .......................................................................................................... 51
  2.2.3 Preparation of acid-etched sterile coverslips ......................................................... 52
  2.2.4 Centrosome overduplication assay ........................................................................ 52
  2.2.5 Transient transfections ......................................................................................... 52
    2.2.5.1 Lipofectamine 2000 ..................................................................................... 52
    2.2.5.2 FuGene 6 ..................................................................................................... 53
  2.2.6 Flow cytometry .................................................................................................... 53
2.3 Microscopy ................................................................................................................... 53
  2.3.1 Indirect immunofluorescence microscopy ........................................................... 53
  2.3.2 Centrosome and nuclear intensity measurements ................................................. 54
  2.3.3 Microinjection .................................................................................................... 54
  2.3.4 Live cell imaging ................................................................................................. 54
  2.3.5 Photobleaching experiments ............................................................................... 55
  2.3.6 Electron microscopy ........................................................................................... 55
2.4 Molecular biology techniques ..................................................................................... 56
  2.4.1 Plasmids ............................................................................................................. 56
  2.4.2 Bacterial transformations .................................................................................... 56
  2.4.3 Plasmid preparation ............................................................................................ 56
  2.4.4 Sequencing .......................................................................................................... 57
2.5 Analysis of proteins ................................................................................................... 57
  2.5.1 Cell extracts ....................................................................................................... 57
  2.5.2 BCA protein assay ............................................................................................. 57
  2.5.3 SDS-PAGE ........................................................................................................ 57
  2.5.4 Western blotting ................................................................................................. 58

CHAPTER 3  CENTROSOME OVERDUPICATION IN HYDROXYUREA-ARRESTED CHINESE HAMSTER OVARY CELLS
3.1 Introduction ............................................................................................................... 59
  3.1.1 Centrosome duplication ...................................................................................... 59
  3.1.2 Centrosome overduplication .............................................................................. 60
3.1.3 Supernumerary centrosomes and cancer cells................................. 60

3.2 Results................................................................................................. 62

3.2.1 Centrosomes overduplicate in CHO cells during S-phase arrest......... 62
3.2.2 Centrin localises to overduplicated centrosomes.......................... 64
3.2.3 Centrin1-GFP is incorporated into new centrioles......................... 67
3.2.4 Centrin1-GFP expression accelerates the rate of centrosome........... 67
            overduplication
3.2.5 Overduplicated centrosomes are positive for a range of ............... 72
            centrosomal markers
3.2.6 U2OS cells also undergo centrosome overduplication during......... 76
            prolonged S-phase arrest
3.2.7 Cells with supernumerary centrosomes organise abnormal............ 76
            mitotic spindles

3.3 Discussion.................................................................................................. 79

CHAPTER 4  THE EFFECT OF MICROTUBULE DEPOLYMERISATION ON CENTROSOLE
            OVERDUPlication

4.1 Introduction.......................................................................................... 83

4.1.1 Microtubules and centrosome assembly....................................... 83
4.1.2 Microtubules and centrosome duplication.................................... 83

4.2 Results....................................................................................................... 85

4.2.1 Microtubules are required for centrosome duplication.................... 85
4.2.2 Centrin foci accumulate in the absence of microtubules............... 85
4.2.3 Centrin foci do not nucleate microtubules.................................... 87
4.2.4 Centrin foci contain modified tubulins........................................ 92
4.2.5 Centrin foci are reminiscent of centriolar satellites....................... 96
4.2.6 Centrin localised in foci is more dynamic than that in centrioles..... 99
4.2.7 Centriolar satellites accumulate in cells with depolymerised......... 101
            microtubules during prolonged S-phase arrest
4.2.8 Multiple centrosomes rapidly accumulate following drug washout... 105

4.3 Discussion.................................................................................................. 108
CHAPTER 5  THE MICROTUBULE-BASED MOTOR PROTEIN DYNEIN IS REQUIRED FOR CENTROSOME OVERDUPLICATION

5.1 Introduction ............................................................................................................112
  5.1.1 Dynein .............................................................................................................112
  5.1.2 Dynein and the cell cycle ................................................................................114
  5.1.3 Dynein and centrosome assembly ..................................................................115

5.2 Results ....................................................................................................................118
  5.2.1 Dynamitin overexpression prevents centrosome overduplication ..............118
  5.2.2 Dynamitin overexpression causes the dispersal of centrin and PCM-1 ......119
  5.2.3 Dynamitin overexpression during prolonged S-phase arrest does not prevent the localisation of centrin and PCM-1 .........................................................................................123
  5.2.4 Dynein inhibition by vanadate perturbs centrosome overduplication ......123
  5.2.5 Dynein inhibition by EHNA prevents centrosome overduplication ...........126
  5.2.6 Vanadate and EHNA also prevent centrosome accumulation in U2OS cells .................................................................................................................................129
  5.2.7 Dynein is required for centriole accumulation .............................................133
  5.2.8 Monastrol does not prevent centrosome overduplication .........................133
  5.2.9 Injection of dynein-specific antibodies prevents centrosome overduplication during S-phase arrest ........................................................................................................137
  5.2.10 Multiple centrosomes rapidly accumulate following drug washout and release from dynein inhibition .........................................................................................141

5.3 Discussion ..............................................................................................................145

CHAPTER 6  HEAT SHOCK PROTEIN 90 IS REQUIRED FOR THE RECRUITMENT OF γ-TUBULIN TO OVERDUPLICATED CENTRIOLES

6.1 Introduction ............................................................................................................149
  6.1.1 Heat shock proteins .....................................................................................149
  6.1.2 Hsp90 ..........................................................................................................149
  6.1.3 Hsp70 ..........................................................................................................151

6.2 Results ....................................................................................................................153
  6.2.1 Hsp90 is required for centrosome overduplication .....................................153
  6.2.2 Centrioles form in the presence of Hsp90 inhibitors ...................................156
  6.2.3 Hsp90 inhibition prevents the recruitment of a range of centrosomal proteins to newly formed centrioles .................................................................159
6.2.4 γ-tubulin is lost from the centrosome upon Hsp90 inhibition ................. 159
6.2.5 Multiple centrosomes rapidly accumulate following drug washout ........ 163
and release from Hsp90 inhibition

6.3 Discussion ........................................................................................................ 167

CHAPTER 7 CDK ACTIVITY AND NUCLEAR EXPORT ARE REQUIRED FOR CENTROSOME OVERDUPlication

7.1 Introduction ...................................................................................................... 171
7.1.1 Centrosome duplication and cyclin-dependent kinases ......................... 171
7.1.2 Cdk regulators and centrosome duplication ......................................... 173
7.1.3 Cdk2 substrates and centrosome duplication ..................................... 174
7.1.4 Aims ............................................................................................................. 175

7.2 Results ............................................................................................................. 177
7.2.1 Cdk inhibition prevents centrosome overduplication ......................... 177
7.2.2 Cdk inhibition leads to the loss of centriolar satellites ....................... 179
7.2.3 Centrin accumulates in the nucleus upon Cdk inhibition ..................... 183
7.2.4 Nuclear export is required for centrosome overduplication ................. 186
7.2.5 Cdk inhibition prevents the formation of nuclear centrin foci ............. 191
7.2.6 Pericentrin is nucleolar upon inhibition of nuclear export ................. 191
7.2.7 Nuclear export is required for the formation of centriolar satellites .... 199
7.2.8 Cdk activity is required for an early step in centrosome reduplication ... 203

7.3 Discussion ........................................................................................................ 205

CHAPTER 8 DISCUSSION

8.1 Centrosome overduplication ....................................................................... 210
8.2 A pathway of centrosome overduplication ............................................. 211
8.3 A role for centriolar satellites in centrosome overduplication ............... 215
8.4 Centrin in centriole assembly ....................................................................... 216
8.5 Nuclear export and centrosome overduplication ...................................... 217
8.6 Cdk2 and centrosome overduplication ...................................................... 218
8.7 Perspectives .................................................................................................... 220

CHAPTER 9 BIBLIOGRAPHY ................................................................................. 222
APPENDIX ............................................................................................................ 245
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-AAG</td>
<td>17-(Allylamino)-17-demethoxygeldanamycin</td>
</tr>
<tr>
<td>APC/C</td>
<td>Anaphase-Promoting Complex/Cyclosome</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Calcium-dependent protein kinase II</td>
</tr>
<tr>
<td>Cdk</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Media</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>EHNA</td>
<td><em>erythro</em>-9-(2-hydroxy-3-nonly) adenine</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FLIP</td>
<td>Fluorescence loss in photobleaching</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>γ-TuRC</td>
<td>γ-tubulin ring complex</td>
</tr>
<tr>
<td>γ-TuSC</td>
<td>γ-tubulin small complex</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>HSPI-1</td>
<td>Heat shock protein inhibitor 1</td>
</tr>
<tr>
<td>HU</td>
<td>hydroxyurea</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>LMB</td>
<td>leptomycin B</td>
</tr>
<tr>
<td>LSCM</td>
<td>Laser scanning confocal microscope</td>
</tr>
<tr>
<td>MEFs</td>
<td>mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>mg</td>
<td>milli-gram</td>
</tr>
<tr>
<td>ml</td>
<td>milli-litre</td>
</tr>
<tr>
<td>mM</td>
<td>milli-molar</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MT</td>
<td>microtubule</td>
</tr>
<tr>
<td>MTOC</td>
<td>microtubule organising centre</td>
</tr>
<tr>
<td>NES</td>
<td>nuclear export signal</td>
</tr>
<tr>
<td>ng</td>
<td>nano-gram</td>
</tr>
<tr>
<td>nM</td>
<td>nano-molar</td>
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<tr>
<td>nm</td>
<td>nano-meter</td>
</tr>
<tr>
<td>Noc</td>
<td>nocodazole</td>
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<tr>
<td>NPM</td>
<td>nucleophosmin</td>
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<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>Optical Density (absorbance) at 600 nm</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCM</td>
<td>pericentriolar material</td>
</tr>
<tr>
<td>Plk</td>
<td>Polo-like kinase</td>
</tr>
<tr>
<td>RIPA</td>
<td>RadioImmuno Precipitation Assay Buffer</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
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<tr>
<td>ROCK 2</td>
<td>Rho-associated coiled-coil containing protein kinase 2</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SAS</td>
<td>spindle assembly</td>
</tr>
<tr>
<td>SCF</td>
<td>Skp1-Cullin-F-box</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl-sulphate</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SPD</td>
<td>spindle defective</td>
</tr>
<tr>
<td>SPB</td>
<td>spindle pole body</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
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<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
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<tr>
<td>µg</td>
<td>micro-gram</td>
</tr>
<tr>
<td>µl</td>
<td>micro-litre</td>
</tr>
<tr>
<td>µm</td>
<td>micro-meter</td>
</tr>
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<td>µM</td>
<td>micro-molar</td>
</tr>
<tr>
<td>v/v</td>
<td>volume / volume</td>
</tr>
<tr>
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<td>weight / volume</td>
</tr>
<tr>
<td>ZYG</td>
<td>zygote defective</td>
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FIGURES

1.1 The structure of the centrosome.......................................................... 3
1.2 Basal bodies template the formation of the cilia axoneme............... 9
1.3 The centrosome cycle........................................................................... 12
1.4 De novo centriole formation................................................................ 22
1.5 The centriolar and acentriolar pathways of ciliogenesis.................. 24
1.6 Centriole assembly pathway in C. elegans embryos......................... 35
1.7 A model for centriole assembly in human cells............................... 40
1.8 Deregulation of the centrosome cycle results in mitotic defects......... 43

3.1 Centrosome overduplication in HU-arrested CHO cells..................... 63
3.2 TEM analysis reveals the presence of extra centrioles in HU-arrested... 65
CHO cells
3.3 Centrin localises to both the centrioles and centriolar satellites......... 66
3.4 Overduplicated centrosomes in CHO cells stain for the centriolar...... 68
marker centrin
3.5 Centrin1-GFP is a centriole marker throughout the cell cycle and...... 69
is incorporated into new centrioles
3.6 Live cell imaging of CHO:centrin1-GFP during HU arrest reveals...... 70
the formation of multiple centrin dots
3.7 Centrin1-GFP is incorporated into overduplicated centrosomes......... 71
in CHO cells arrested with HU
3.8 Centrin1-GFP overexpression accelerates the rate of centrosome...... 73
overduplication in CHO cells
3.9 The rate of centrosome overduplication is comparable in three........ 74
individual CHO:centrin1-GFP cell lines
3.10 Pericentrin, rootletin, ninein and Nek2 all localise to overduplicated.... 75
centrosomes
3.11 U2OS cells also undergo centrosome overduplication during......... 77
prolonged HU arrest
3.12 CHO cells with overduplicated centrosomes form abnormal mitotic.... 78
spindles
4.1 Microtubules are required for centrosome overduplication………………..86
4.2 Centrin foci form in CHO cells treated with HU and nocodazole………..88
4.3 Centrin foci form in CHO:centrin1-GFP cells treated with HU and……….89
nocodazole
4.4 Live cell imaging of CHO:centrin1-GFP cells with depolymerised………90
microtubules during HU arrest reveals the formation of centrin aggregates
4.5 Centrin foci predominately localise around the nuclear periphery in……..91
an actin-dependent manner
4.6 Only γ-tubulin positive foci are able to nucleate microtubules……………93
4.7 Centrin aggregates cluster following microtubule regrowth………………94
4.8 Cytoplasmic centrin aggregates in HU-arrested CHO cells with……….95
depolymerised microtubules contain post-translationally modified tubulin
4.9 Cytoplasmic centrin aggregates in HU-arrested CHO cells with………..97
depolymerised microtubules stain for only a subset of centrosomal proteins
4.10 Cytoplasmic aggregates also form during HU-arrest in U2OS…………..98
   cells with depolymerised microtubules
4.11 FRAP analysis of centrin1-GFP in cytoplasmic aggregates and centrioles..100
4.12 FLIP analysis of centrin1-GFP in cytoplasmic aggregates and centrioles…102
4.13 FRAP analysis indicates that diffuse cytoplasmic centrin and……………103
   centrosomal Nek2 are both highly dynamic proteins
4.14 HU-arrested CHO cells with depolymerised microtubules contain……….104
   increased numbers of centriolar satellites than untreated cells
4.15 Drug washout reveals that cells in which microtubules had been………..107
   depolymerised during HU-arrest can rapidly accumulate multiple
   γ-tubulin dots
4.16 A model for the role of microtubules in centrosome overduplication……..110
   in S-phase arrested cells

5.1 Schematic illustration of dynactin subunits……………………………..113
5.2 Dynamitin overexpression perturbs centrosome overduplication in…….120
   CHO cells
5.3 Dynamitin overexpression perturbs centrosome overduplication in………121
   U2OS cells
5.4 Centrin and PCM-1 foci disperse in CHO cells overexpressing dynamitin..122
5.5 Centrin foci cluster together in CHO cells overexpressing dynamitin during prolonged S-phase arrest
5.6 PCM-1 foci cluster together in CHO cells overexpressing dynamitin during prolonged S-phase arrest
5.7 Inhibition of dynein by vanadate perturbs centrosome overduplication
5.8 Centrin and PCM-1 foci form around the centrosome of cells treated with vanadate during HU-arrest
5.9 Inhibition of dynein by EHNA perturbs centrosome overduplication
5.10 Centrin and PCM-1 foci form around the centrosome of cells treated with the dynein inhibitor EHNA during HU-arrest
5.11 Inhibition of dynein with EHNA and vanadate prevents centrosome overduplication in U2OS cells
5.12 Dynein is required for the overduplication of centrioles
5.13 Treatment of cells with the Eg5 inhibitor, monastrol, has no effect on centrosome overduplication in CHO cells
5.14 Treatment of cells with the Eg5 inhibitor, monastrol, has no effect on centrosome overduplication in U2OS cells
5.15 Microinjection of anti-dynein antibodies perturbs centrosome overduplication in CHO cells
5.16 Centrin foci cluster together in CHO cells injected with anti-dynein antibodies
5.17 Microinjection of anti-dynein antibodies perturbs centrosome overduplication in U2OS cells
5.18 Drug washout reveals that cells in which dynein has been inhibited during HU-arrest can accumulate multiple γ-tubulin dots
5.19 A model for the role of dynein in centrosome overduplication in S-phase arrested cells

6.1 Inhibition of Hsp90, but not Hsp70, blocks centrosome overduplication in S-phase arrested cells
6.2 Hsp90 inhibition prevents centrosome overduplication in U2OS cells arrested with HU
6.3 Centrin1-GFP dots accumulate around the centrosome during S-phase arrest in cells with inhibited Hsp90
6.4 Centrin dots form around the centrosome during S-phase arrest in cells with inhibited Hsp90

6.5 TEM analysis reveals the presence of extra centrioles formed in CHO cells during HU-arrest and Hsp90 inhibition

6.6 Centrioles generated in HU-arrested cells with inhibited Hsp90 do not recruit γ-tubulin, C-Nap1, pericentrin or ninein

6.7 PCM-1 signal is not lost in cells with inhibited Hsp90

6.8 γ-tubulin is stable in the presence of Hsp90 inhibitors, but is lost from the centrosome

6.9 Drug washout reveals that cells in which Hsp90 was inhibited can accumulate multiple γ-tubulin staining centrosomes

6.10 A model for the role of Hsp90 in centrosome overduplication in S-phase arrested cells

7.1 Cdk activity is required for centrosome overduplication

7.2 The Cdk inhibitor olomoucine also blocks centrosome overduplication

7.3 Cdk activity is required for centrosome overduplication in p53−/− MEFs

7.4 Cdk activity is required for the formation of centriolar satellites

7.5 Roscovitine prevents the accumulation of centrin aggregates in HU/nocodazole treated cells

7.6 Centrin accumulates in the nucleus of cells treated with the Cdk inhibitor roscovitine

7.7 Centrin aggregates accumulate in the nucleus when nuclear export is inhibited

7.8 Nuclear export is required for centrosome overduplication

7.9 Nuclear export and Cdk activity is required for centrosome overduplication in U2OS cells

7.10 Nuclear export is required for centrosome overduplication in p53−/− and p53−/−Cdk2−/− MEFs

7.11 Cdk activity is required for the formation of nuclear centrin granules in LMB treated cells arrested with HU

7.12 Formation of nuclear centrin granules occurs in LMB treated cells arrested with HU but not mimosine

7.13 Pericentrin accumulates in the nucleolus of cells treated with LMB
7.14 Centrin accumulates in the nucleolus of cells treated with LMB………………197
7.15 Nucleolar staining disappears when Cdks are inhibited.........................198
7.16 PCM-1 signal disappears when nuclear export is inhibited......................200
7.17 PCM-1 signal in U2OS cells disappears when nuclear export or………………201
Cdk activity is inhibited
7.18 OFD-1 signal disappears when nuclear export or Cdk activity is inhibited..202
7.19 Drug washout reveals that cells in which nuclear export was…………………..204
inhibited, but not in which Cdk activity was inhibited, can
rapidly accumulate multiple γ-tubulin dots
7.20 A pathway for the role of Cdk activity and nuclear export in………………208
centrosome overduplication in S-phase arrested cells

8.1 A pathway of centrosome overduplication in S-phase arrested cells…… 214
CHAPTER ONE

INTRODUCTION
1.1 Microtubule Organising Centres

Microtubules (MTs) are vital for a range of cellular processes in eukaryotes, including cell division, cell motility and cellular polarisation. MTs have a highly distinctive structure being formed from α-/β-tubulin heterodimers that are assembled to form a tubulin polymer composed of 13 tubulin protofilaments. MTs are nucleated from protein complexes that contain γ-tubulin (Moritz et al., 1995). In most cells, MTs form highly organised, polarised arrays, providing distinct tracks for the movement of vesicles, organelles and other cellular components, along with regulating cell shape and movement [reviewed in (Wade, 2007)]. In proliferating cells, these arrays undergo a dramatic reorganisation to form the bipolar spindle in mitosis. The organisation of MTs is primarily dictated by the distribution of the γ-tubulin containing complexes, along with the direction of elongation at nucleation. However, the function of MTs in cells is also governed by severing, bundling, transport and stabilisation following nucleation.

In eukaryotic cells, MT organising centres (MTOCs) function as the primary sites of MT nucleation. These specialised structures govern both the assembly and spatial distribution of MTs. Two morphologically diverse structures were identified by electron microscopy as MTOCs in yeast and animal cells. In yeast, the primary MTOC is the spindle pole body (SPB), a multilayered disc-like structure embedded in the nuclear envelope (Adams and Kilmartin, 2000; Jaspersen and Winey, 2004). Whilst in animal cells, the centrosome functions as the major MTOC. In contrast to SPBs, the centrosome consists of a pair of MT-based centrioles surrounded by an amorphous collection of proteins known as the pericentriolar material (PCM), which also contains the γ-tubulin complexes. Intriguingly, morphologically distinct MTOCs are absent in a proportion of higher plants. Instead, two modes of MT nucleation have been observed in plant cells. Most MTs are reported to form as branches on existing MTs, or “in regions where MTs once existed” [(Murata et al., 2005); reviewed by (Murata et al., 2007)]. It is also proposed that MTs in plants can be nucleated at the nuclear surface (Lambert, 1993), indeed isolated nuclei can nucleate MTs in vitro, demonstrating that the nuclear surface can act as a MTOC (Stoppin et al., 1996; Stoppin et al., 1994). MTs assembled at the nucleus are subsequently translocated and organised in the cell cortex. A similar mechanism of MT translocation to non-centrosomal sites has been described in animal cells (Mogensen et al., 1997). Whilst the structural organisation of MTOCs differs greatly between animal and plant cells, most components
involved in MT nucleation, including γ-tubulin, are conserved between metazoa and green plants. γ-tubulin is also a major element of SPBs (Oakley et al., 1990), suggesting that despite the differences in MTOCs, the components required for MT nucleation in eukaryotic cells are conserved.

1.1.1 THE CENTROSOME

The centrosome is a large non-membranous organelle approximately 1 μm in diameter, located in a central position in the cytoplasm of interphase cells, close to the nucleus. Centrosomes are structurally complex, consisting of two substructures: a pair of centrioles surrounded by PCM (Doxsey, 2001) (Figure 1.1). The PCM is a dynamic, but well organised matrix of fibres and protein aggregates that forms a tube-like structure around the centrioles (Dictenberg et al., 1998; Ou et al., 2003). This lattice provides a framework for anchoring proteins involved in MT nucleation. A number of electron dense centriolar satellites also associate with centrosomes. Whilst their relationship with centrosomes remains obscure, they are proposed to have roles in the trafficking of proteins to the centrosome and may coalesce with, and in part form, the PCM (Baron et al., 1994; Dammermann and Merdes, 2002; Kubo et al., 1999).

MT nucleation depends on the tubulin isoform γ-tubulin which is found in two highly conserved protein complexes: the γ-tubulin small complex (γ-TuSC) and the γ-tubulin ring complex (γ-TuRC) (Moritz et al., 1995; Oegema et al., 1999). γ-TuRCs are made of multiple copies of γ-TuSCs, along with a number of other proteins (Gunawardane et al., 2000; Gunawardane et al., 2003; Luders et al., 2006). Morphologically, γ-TuRCs are open ring structures that associate directly with MT minus ends (Moritz et al., 1995; Oegema et al., 1999). Salt-stripped purified centrosomes are no longer able to nucleate microtubules, although in this instance the PCM maintains a lattice-like structure composed of 12-15 nm filaments (Moritz et al., 1998; Schnackenberg et al., 1998; Schnackenberg and Palazzo, 2001). γ-TuRCs are recruited to this scaffold, restoring the MT nucleating capacity of salt-stripped centrosomes. The PCM is therefore thought to be the main functional component of the centrosome as it contains the sites of microtubule nucleation. However, nucleation and anchoring of MTs at the centrosome are two separate activities, performed by different proteins (Bornens, 2002).
Figure 1.1 The structure of the centrosome

A. The centrosome comprises of a pair of centrioles surrounded by an amorphous cloud of proteins, the pericentriolar material (PCM). The centrioles are linked at their proximal ends by a fibrous linker. Each centriole consists of nine triplet microtubules that form a barrel-like structure. Each microtubule is assigned a letter, A, B, or C, depending on its position. The two centrioles differ in age and structure: the older ‘mother’ centriole has distal and sub-distal appendages that are lacking in the younger ‘daughter’ centriole. B. These differences are apparent at the morphological level (i), scale bar 0.2 µm, with EM analysis revealing the intricate arrangement of microtubules that comprise the centriolar walls (ii). C. A number of electron dense centriolar satellites associate around the centrosome (arrowhead), and are involved in trafficking proteins to the centrosome and potentially coalesce with the PCM. A. and B(i). from Bettencourt-Dias and Glover (2007).
Centrioles are barrel-like structures, approximately 500 nm long and 200 nm in diameter, formed from a nine-fold symmetrical array of triplet microtubules (Marshall, 2001). The minus ends of the MTs within the centriolar wall are located at the proximal end of the centriole, with the plus ends located distally. Centriolar MTs have a number of post-translation modifications, including acetylation and polyglutamylation (Bobinnec et al., 1998b; Piperno and Fuller, 1985). This makes them extremely stable and resistant to MT depolymerising drugs. Injection of anti-polyglutamylated tubulin antibodies causes the loss of centriole structures, highlighting the importance of these modifications for centriole stability (Bobinnec et al., 1998a).

The two centrioles in a centrosome are structurally distinct. The older mature centriole is termed the ‘mother’ and can be distinguished by additional appendages at its distal end that are lacking in the younger immature ‘daughter’ centriole. These distal and sub-distal appendages are only acquired by a centriole in the cell cycle following their assembly (Vorobjev and Chentsov Yu, 1982). MT-anchoring activity also appears to be associated most tightly with the mother centriole (Piel et al., 2000). Indeed, a number of proteins involved in MT-anchoring, including ninein and dynactin, selectively localise to the mother centriole [reviewed by (Bornens, 2002; Doxsey, 2001)]. The two centrioles are held in close proximity to each other by a fibrous, intercentriolar linkage located at their proximal ends. C-Nap1, rootletin and Cep68 localise to this linkage and are involved in cell cycle regulated centrosome cohesion (Bahe et al., 2005; Fry et al., 1998a; Graser et al., 2007b; Mayor et al., 2000; Yang et al., 2006).

In vertebrate cells, centriolar walls are usually constructed from MT triplets. However, doublets and singlets are observed in other cell types. For example, Drosophila have centrioles with singlet, doublet and triplet MTs (Gonzalez et al., 1998). Singlet MTs are restricted to the early embryo, whilst doublets are seen later in development. Finally, centrioles with triplet MTs are observed in spermatocytes. C. elegans early embryos also contain singlet MTs (O'Toole et al., 2003b; Pelletier et al., 2006). The centrioles in both Drosophila and C. elegans embryos are also smaller than those in vertebrate cells being about 100 nm by 100-200 nm in size (O'Toole et al., 2003b; Pelletier et al., 2006). These differences may reflect the requirement for rapid centriole assembly in these embryos.
The role of the centrioles is believed to be in part to organise the PCM. Centrioles purified from *Xenopus* can organise electron-dense material containing PCM components such as \( \gamma \)-tubulin (Felix et al., 1994), whilst disruption of centrioles in cells is accompanied by dissolution of the PCM (Bobinnec et al., 1998a). The integrity of centrosome structure is therefore dependent upon the centriolar pair which organises centrosomal components into a single stable structure. Centrioles can also nucleate the formation of cilia. Centrioles are structurally related to basal bodies and only exist in species that have at least some ciliated cells. Almost all cells in the human body have a single non-motile primary cilium (Davis et al., 2006; Wheatley et al., 1996), that is nucleated from the elongation of the MTs at the distal end of the mother centriole. The appendages associated with the mother centriole appear to be important for cilia formation as depletion of a protein that localises to the distal appendages prevents primary cilium formation (Graser et al., 2007a). In multiciliated cells hundreds of basal bodies form, each nucleating a cilium.

One of the most fascinating aspects of the centrosome is its ability to faithfully duplicate its complex structure in a highly regulated manner once per cell cycle. Following the completion of cell division, each daughter cell contains only one centrosome, containing a pair of centrioles. During the course of the cell cycle, cells duplicate their centrosomes so that by the time they enter mitosis there are two centrosomes present, each containing a pair of centrioles. These centrosomes migrate to the two spindle poles helping to assemble a bipolar mitotic spindle.

### 1.2 Centrosome Function

#### 1.2.1 Spindle Assembly

As a MTOC, the centrosome has the ability to control MT events such as nucleation, anchoring and release. In interphase cells, the centrosome organises a radial MT array, whilst in mitosis centrosomes function in spindle assembly, forming the spindle poles. However, it is strongly suggested that centrosomes are dispensable for spindle assembly. Higher plants and many developmental systems lack centrosomes, yet are still able to organise spindles and undergo cell division. In many species, centrioles disappear during oogenesis, although PCM and centriolar components remain present in the cytoplasm of oocytes (Albertson and Thomson, 1993; Manandhar et al., 2005; Schatten, 1994). In this instance, female meiotic divisions occur in the absence of centrosomes, with centrioles
contributed at fertilisation by the male gamete. In mice there is no paternal or maternal contribution of centrioles. As a result, two acentriolar gametes fuse, and development of the early mouse embryo occurs without any centrioles (Abumuslimov et al., 1994; Calarco-Gillam et al., 1983). Additionally, spindle assembly was not affected when centrosomes were removed from human cells in culture. Functional bipolar spindles were still formed in somatic cells following laser ablation of centrosomes or centrosome removal by microsurgery (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). Furthermore, mutations in *Chlamydomonas* and *Drosophila* that result in the absence of centrioles does not affect cell viability (Basto et al., 2006; Matsuura et al., 2004). Indeed, mutant flies lacking centrioles can develop to adulthood with normal timing. Together, these observations clearly display that centrosome-independent methods of bipolar spindle assembly must exist within cells.

Bipolar spindles can assemble in vitro around DNA-coated beads incubated in *Xenopus* egg extracts (Heald et al., 1996). This system lacks centrosomes and kinetochores, suggesting that bipolarity is an intrinsic property of MTs assembling around chromatin in a mitotic cytoplasm. This chromatin-mediated MT nucleation pathway is regulated by the small GTPase Ran (Carazo-Salas et al., 1999). Following nuclear envelope breakdown, RCC1, a chromatin-bound nucleotide-exchange factor for Ran, generates a high local concentration of RanGTP around the chromosomes (Bastiaens et al., 2006; Carazo-Salas et al., 2001; Carazo-Salas et al., 1999; Kalab et al., 2006). This promotes the formation and organisation of MTs in the vicinity of mitotic chromosomes. Unsurprisingly, γ-tubulin is required to nucleate MTs in this pathway, although pre-formed MTs are also incorporated from the preceding interphase network (Gunawardane et al., 2000; Mahoney et al., 2006). The focusing of MTs into a functional bipolar spindle then requires a number of MT-based motor proteins, including cytoplasmic dynein, Eg5 (kinesin-5 protein), and Ncd (kinesin-14 protein), that aid the sliding of MTs across one another (Gaglio et al., 1997; Heald et al., 1996; Karsenti and Vernos, 2001; Vernos and Karsenti, 1996).

The chromatin-mediated pathway may not be sufficient for spindle assembly in all cells. Indeed, centrosomes may be required to promote the formation of well-organised spindles and the efficient segregation of chromosomes in embryos. *Drosophila cnn* mutants lack functional centrosomes and develop to the syncytial blastoderm stage. However, there are severe defects in spindle morphology and, as a consequence, the segregation of DNA
(Lucas and Raff, 2007; Megraw et al., 1999). Furthermore, *Drosophila* embryos that cannot support centrosome duplication arrest early in development (Stevens et al., 2007). Bipolar spindles also cannot form during the first mitotic division in *C. elegans* embryos that lack functional centrosomes (Hamill et al., 2002; Hannak et al., 2001; Kemp et al., 2004; Pelletier et al., 2004). The presence of centrosomes therefore appears to be vital for the formation of functional mitotic spindles in certain embryos. This suggests that the chromatin-mediated pathway may not occur fast enough for spindle assembly during the rapid divisions of the embryo. In support of this, the *Drosophila* cnn mutation leads to a lengthening of the duration of spindle assembly as compared to wild-type in somatic cells of the fly brain (Buffin et al., 2007). Mitotic spindle assembly is also reported to be slow in *Drosophila* mutants that completely lack centrosomes (Basto et al., 2006). The level of aneuploidy is also slightly raised in these cells, suggesting that acentrosomal spindles are marginally less efficient at segregating chromosomes than spindles formed by centrosomes.

The location of centrosomes at the spindle poles is essential for the formation of astral MTs, indeed acentrosomal spindles lack these structures. Astral MTs play important roles in cell division, including the correct positioning and orientation of the spindle and separation of the spindle poles. When present, centrosomes act dominantly to regulate spindle assembly (Heald et al., 1997), and are important, along with associated astral MTs, in spindle positioning (Schuyler and Pellman, 2001). The regulation of centrosomes in this process is essential as cells with structurally aberrant or supernumerary centrosomes organise dysfunctional mitotic spindles that missegregate chromosomes. Significantly, these types of defect have been observed in malignant cancer cells, suggesting that they contribute to genetic instability and tumour progression (Pihan et al., 1998; Pihan et al., 2003). Centrosome-mediated spindle assembly could provide a pathway to ensure high fidelity of chromosome segregation or provide a mechanism to ensure that centrosomes are inherited by each daughter cell (Doxsey, 2001).

The centrosome may also play a crucial role in cytokinesis. A number of centrosomal proteins have been reported to participate in cytokinesis (Kim et al., 2005; Tsang et al., 2006), whilst the removal of centrosomes from cells has been shown to cause a delay in cytokinesis completion (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). The mother centriole has been observed to move to the intercellular bridge that connects
dividing cells, at a time correlating with bridge narrowing and bridge MT depolymerisation (Piel et al., 2000). The centriole then moves away again before cell cleavage occurs. This suggests that centrosomes might be involved in activation of the final stages of cytokinesis or in the release of cells from a checkpoint that monitors mitosis completion (Doxsey, 2001). The centrosome could also function in the G1 to S transition as the presence of an intact centrosome is vital for cell cycle progression into S-phase [reviewed in (Doxsey et al., 2005)]. Direct evidence of a role for centrosomes in cell cycle progression from G1 into S-phase arises from experiments involving the removal of centrosomes by microsurgery (Hinchcliffe et al., 2001) or laser ablation (Khodjakov and Rieder, 2001). Ablation of one of the two centrosomes in prometaphase cells produces a centrosome-containing daughter cell that can continue to cycle and a centrosome-lacking daughter cell that is unable to enter S-phase. More recently, the loss of centrosome integrity induced by the depletion of individual centrosomal proteins has been shown to cause a G1-S arrest (Mikule et al., 2007). Together, these results support the requirement of an intact centrosome for the cell to progress into S-phase, and thus initiate both centrosome duplication and DNA replication. Whilst it is still unclear how these events are mediated, one consequence of centrosome-dependent checkpoints for cell cycle progression is to ensure that dividing cells receive the correct number of centrosomes following mitosis (Doxsey, 2001).

### 1.2.2 Cilia and Flagella

Cilia and flagella are comprised of a long microtubular axoneme surrounded by an external membrane, which is continuous with the plasma membrane of the cell. At its proximal end, the axoneme is nucleated from a basal body that anchors it in the cell (Beisson and Wright, 2003) (Figure 1.2). Primary cilia are found on virtually all mammalian cell types and are nucleated from the mother centriole of the centrosome (Davis et al., 2006; Wheatley et al., 1996). Ciliary resorption precedes entry into the cell cycle, with cilia reassembled following exit from mitosis. This process allows the centrioles to duplicate and switch from a basal body role to the assembly of the mitotic spindle poles [(Wheatley et al., 1996); reviewed by (Quarmby and Parker, 2005)]. In some exceptional cells, cilia are retained during division, with centrioles acting simultaneously as basal bodies and spindle pole organisers.
Basal bodies are structurally related to centrioles being comprised of a nine-fold symmetrical array of triplet microtubules. The axoneme that lies at the centre of a cilium is nucleated from and anchored by a basal body. Motile cilia have a nine plus two arrangement of doublet microtubules, as shown here. Immotile cilia lack the central pair of microtubules. Scale bars, 0.25 \( \mu \text{m} \). From Bettencourt-Dias and Glover (2007).
Multi-ciliated cells contain hundreds of basal bodies, formed during the process of ciliogenesis, with each giving rise to a cilium. Ciliogenesis consists of four stages: assembly of centrioles; migration of centrioles to the apical cell surface to become basal bodies; elongation of cilia containing the axoneme; and formation of accessory structures of basal bodies (Hagiwara et al., 2000). In most cells centrosomes duplicate only once per cell cycle, however in ciliated cells numerous replicating centrioles are observed simultaneously during ciliogenesis (Sorokin, 1968b). Whilst basal bodies and centrioles are structurally similar with nine triplet MTs in their walls, basal bodies lack the associated PCM and are instead attached to the axoneme and other structures, such as the rootlet, that anchor them to other parts of the cytoskeleton (Yang et al., 2005; Yang et al., 2002). Basal bodies also possess a ‘transition zone’ at their distal ends and undergo a process of maturation, acquiring appendages that allow them to dock at the cell surface (Dutcher, 2003a). Cilia assembly itself relies on a process of interflagellar transport, that allows the delivery of new axonemal subunits to their site of assembly at the cilium tip [reviewed by (Rosenbaum and Witman, 2002)].

Cilia have a number of roles, the most well-known being motility. This includes moving fluid over a surface, such as occurs in the respiratory epithelia, or cell propulsion. Cilia are also crucial to sensory signalling, including photoreceptors and the olfactory and auditory systems. Primary cilia play roles in sensory signalling, establishing polarity and, possibly, regulating the cell cycle [reviewed by (Quarmby and Parker, 2005)]. Due to the diverse roles of cilia, it is unsurprising that cilium dysfunction contributes to a wide range of debilitating human diseases (Badano et al., 2005; Dawe et al., 2007). Furthermore, a number of centrosomal proteins are being implicated in these diseases.

The arrangement of MTs in the ciliary axoneme differentiates cilia type (Dawe et al., 2007). Motile cilia possess axonemes consisting of nine outer doublet MTs and two central MTs. Radial spokes project inwards from the innermost tubule of the doublet, and are thought to sense motility-stimulating signals. Axonemes of motile cilia also have dynein molecules associated with the MT doublets. These allow sliding of the doublets relative to each other, providing the mechanical force for ciliary beating. Immotile cilia lack these additional features and also the central MT doublet. However, in some cases they are highly modified for specialised sensory functions, such as photoreception.
Basal bodies are indispensable for providing sites for the assembly of MTs in ciliary axonemes, if they are absent no cilia are formed. In contrast centrioles are not essential for the formation of cytoplasmic or mitotic MT arrays. It is strongly suggested that centrioles principally evolved to make cilia and that centrosomes provide a mechanism of centriole replication and inheritance (Rieder et al., 2001). Indeed, centrioles are lost in lineages that do not possess cilia, such as fungi and higher plants. The location of centrosomes at spindle poles therefore ensures that each daughter cell receives a pair of centrioles, maintaining the fidelity of centriole inheritance.

1.3 The centrosome cycle

The centrosome cycle describes the series of changes that the centrosome undergoes during the cell cycle. These steps coordinate centrosome duplication with the cell cycle such that by the time the cell enters mitosis it will have replicated both its centrosome and DNA. Additionally, the events contributing to centrosome duplication are strictly controlled so that centrosomes duplicate once and only once per cell cycle.

Four discrete steps constitute the centrosome cycle: centriole disengagement; centriole duplication; centrosome maturation; and centrosome disjunction [reviewed by (Nigg, 2007)] (Figure 1.3). In a metaphase cell, a centrosome, containing a pair of centrioles, resides at each of the two spindle poles. The centrioles at this point have a tight orthogonal arrangement. This close association between the two centrioles is lost upon exit from mitosis, or during early G1 (Kuriyama and Borisy, 1981; Tsou and Stearns, 2006b), with this centriole disengagement event proposed to licence the two centrioles for duplication (Tsou and Stearns, 2006a). However, the centriole pair remains tethered by the presence of an intracentriolar linkage that extends between their proximal ends (Bahe et al., 2005; Fry et al., 1998a; Mayor et al., 2000; Yang et al., 2006). Whilst remaining tethered the centrioles can move apart and acquire their own distinct PCM clouds. In G1-phase, the mother centriole maintains a relatively stationary position in the centre of the cell while the daughter centriole has been observed to move around the cytoplasm (Piel et al., 2000). However, towards the end of G1, the centrioles regain their close proximity to each other before centriole duplication commences.
Figure 1.3 The centrosome cycle
The centrosome cycle comprises of four distinct steps. Once cytokinesis is complete, each cell inherits a single centrosome comprising two disengaged centrioles. In S-phase, centriole duplication occurs with the appearance of procentrioles at the proximal ends of each existing centriole. These elongate throughout S and G2 phase. In late G2, the centrosome undergoes a process of maturation, involving the recruitment of additional PCM components. At the onset of mitosis the intracentriolar linkage is severed leading to centrosome disjunction and allowing the two centrosomes to move apart to form the poles of the bipolar mitotic spindle. The centriole pairs remain in an engaged configuration that prevents reduplication within the same cycle until late mitosis. The centriole pair becomes disengaged, licensing centriole duplication in the following cell cycle. Adapted from Nigg (2007).
Ultrastructural studies of the centrosome cycle revealed that centriole duplication begins in S-phase with the appearance of procentrioles that lie perpendicular to and in very close proximity with the proximal ends of the existing centrioles (Alvey, 1985; Kuriyama and Borisy, 1981; Robbins et al., 1968; Vorobjev and Chentsov Yu, 1982). This association establishes the orthogonal arrangement of the parental and progeny centrioles that is maintained until centriole disengagement. The two procentrioles then elongate in a proximal to distal direction, reaching full length in G2. Centrosome maturation occurs in late G2, characterised by an increase in centrosome size due to the recruitment of additional PCM (Blagden and Glover, 2003). This includes recruitment of γTuRCs in preparation for increased microtubule-nucleating activity at mitosis. The duplicated centrosome continues to act as a single MTOC until mitotic prophase. At this time, the tether between the parental centrioles is severed, leading to centrosome disjunction. The protein kinase Nek2 phosphorylates C-Nap1 and rootletin, components of the intercentriolar linker, inducing its disassembly (Bahe et al., 2005; Fry et al., 1998a; Fry et al., 1998b). Complete separation of the two centriole pairs, driven by motor proteins, yields the spindle poles (Sharp et al., 2000). Finally, completion of centriole biogenesis occurs over more than one cell cycle, with daughter centrioles not acquiring appendages until the cell cycle after they were formed.

1.3.1 CENTRIOLE DUPLICATION LICENCES AND BLOCKS

It is of great interest as to how centriole duplication is regulated so that it only occurs once per cell cycle. Cell fusion experiments led to the first suggestion that duplicated centrosomes have an intrinsic block to reduplication (Wong and Stearns, 2003). Cells at different cell cycle stages were fused to determine if centrioles could reduplicate when placed in a suitable cytoplasmic environment. Centrosomes from G1 cells were able to duplicate when placed in an S-phase cytoplasmic environment. However, G2 centrosomes could not reduplicate when placed in an S-phase environment, although the S-phase centrosome could undergo duplication. Furthermore, fusion of a G2 cell with a G1 cell also prevented reduplication of the G2 centrosome, but did not prevent duplication of the G1 centrosome. Together, this indicates that the block to reduplication is not imparted by the cytoplasm, but is intrinsic to the centrosome. More recently, this has been described as a physical block due to the tight orthogonal arrangement of parental and progeny centrioles in an ‘engaged’ configuration (Tsou and Stearns, 2006a). It is postulated that this engaged state blocks a site for new centriole nucleation, thereby preventing reduplication.
Centrioles maintain this engaged relationship throughout S, G2 and early M phases, only becoming disengaged in late mitosis or early G1 (Kuriyama and Borisy, 1981). Disengagement of centrioles would then license them for duplication in the subsequent S-phase (Tsou and Stearns, 2006a).

Regulated proteolysis may play a crucial role in the licensing step that leads to centriole disengagement. Inactivation of the Skp1-Cullin-F-box (SCF) ubiquitin ligase prevented centriole separation in Xenopus egg extracts (Freed et al., 1999). Similarly, proteasome inhibitors blocked both centriole separation in vitro and centrosome duplication in Xenopus embryos. Furthermore, active SCF is required to prevent centriole overduplication in Drosophila, with mutations affecting the Skp1 homologue SkpA or the F-box protein Slimb leading to centriole reduplication (Murphy, 2003; Wojcik et al., 2000). In both Xenopus and mice, a related SCF is also required to prevent centriole overduplication (Guardavaccaro et al., 2003; Margottin-Goguet et al., 2003). Together, these findings raise the possibility that SCF targets a protein that restricts centrosome duplication for degradation, although a candidate protein has yet to be identified.

Centriole disengagement in Drosophila embryos has been shown to depend on the metaphase to anaphase transition and the APC/C-activator Cdc20/Fizzy (Vidwans et al., 1999). Indeed, mutation of Cdc20/Fizzy delayed centriole disengagement in this system. This suggests that there is an APC/C target whose destruction leads to centriole disengagement in late mitosis. Recently, it was demonstrated, using purified centrosomes incubated in Xenopus egg extracts, that centriole disengagement required the APC/C and the protease separase (Tsou and Stearns, 2006b). Separase is required for sister chromatin separation and when cells are not dividing, separase is inhibited through its association with securin. However, at the metaphase to anaphase transition, ubiquitination and hydrolysis of securin by the APC/C releases active separase. Sister chromatids are held together by the cohesin (Nasmyth et al., 2000), with the protein Sgo1 protecting cohesin located at centromeres (McGuinness et al., 2005; Salic et al., 2004). A recent study raises the interesting possibility that an analogous separase-cohesin system operates in centriole cohesion, and that a protector analogous to Sgo1 prevents premature centriole disengagement (Wang et al., 2008). sSgo1, a smaller variant of full-length Sgo1, was identified as being required for maintaining the engaged state of paired centrioles in early mitosis. Indeed, loss of sSgo1 induced premature centriole splitting. Significantly, such
coordination of centriole disengagement and sister chromatid separation through similar mechanisms would prevent centriole disengagement before anaphase, which could otherwise lead to multipolar spindles (Hut et al., 2003).

1.3.2 INITIATING CENTROSOME DUPLICATION

The initiation of centrosome duplication at the G1/S transition intricately links centrosome duplication with DNA replication so that when a cell enters mitosis it will have duplicated both its DNA and centrosome (Hinchcliffe and Sluder, 2001; Sluder and Hinchcliffe, 1998). However, the two processes are not dependent upon one another. Indeed, in certain cell types, such as Chinese Hamster Ovary (CHO) and human U2OS cells, centrosomes can undergo reduplication during prolonged S-phase arrest in the absence of DNA replication (Balczon et al., 1995). Despite this, coordination of centrosome duplication and DNA replication would be achieved most effectively through regulation by common components that regulate cell cycle progression.

Cyclin-dependent kinases (Cdks), along with their regulatory cyclin partners are involved in the control of the cell cycle. The activities of Cdks together with their cyclin partners coordinate DNA replication with cell cycle progression by both activating DNA replication and preventing re-initiation of replication until completion of the cell cycle (Dahmann et al., 1995). Different Cdks are required sequentially throughout the cell cycle [reviewed in (Schwartz and Shah, 2005)]. For example, cyclin E-Cdk2 activity is required for the G1-S transition (Strausfeld et al., 1996). Control of Cdk activity is brought about by a number of inhibitors and activators, for example p21^{Waf1/Cip1} and p27^{Kip1} inhibit Cdk2, whilst Cdc25 phosphatases remove inhibitory phosphates. G1-Cdks together with G1/S-Cdks drive E2F-dependent expression of cyclins E and A through phosphorylation of Rb, leading to the formation of cyclin E-Cdk2 and cyclin A-Cdk2 complexes that together drive S-phase progression.

In support of a common regulator coupling the centrosome and DNA cycles together, a number of studies identified a requirement for Cdk2 in centrosome duplication (Hinchcliffe et al., 1999; Lacey et al., 1999; Matsumoto et al., 1999; Meraldi et al., 1999). Inhibition of cyclin E-Cdk2 in S-phase arrested Xenopus egg extracts prevented multiple rounds of centrosome duplication (Hinchcliffe et al., 1999). Similarly, injection of the Cdk2 inhibitors, p21 or p27, into one blastomere of a dividing Xenopus embryo prevented
centrosome duplication in that blastomere (Lacey et al., 1999). Inhibition of Cdk2 also prevented centrosome overduplication in mammalian somatic cells (Matsumoto et al., 1999; Meraldi et al., 1999). Overexpression of p21 or p27 prevented centriole reduplication (Lacey et al., 1999; Matsumoto et al., 1999; Meraldi et al., 1999), whilst cells lacking p21 or in which p21 had been inactivated were capable of centrosome amplification (Duensing et al., 2000; Tarapore et al., 2001). Additionally, murine cells in which p21 expression had been silenced accumulated excessive numbers of centrioles, whilst the inhibition of Cdk2 prevented this accumulation (Duensing et al., 2006a). Additionally, cyclin A appeared to be a more effective partner for Cdk2 activity than cyclin E in driving centrosome duplication in mammalian somatic cells (Meraldi et al., 1999).

Regulation of centrosome duplication by Cdk2 would limit initiation of duplication to the G1-S transition and S-phase of the cell cycle. Indeed, centrosomes in CHO cells do not undergo duplication when arrested in G1 with mimosine (Matsumoto et al., 1999). Overexpression of Cdk2 in these cells subsequently reversed this inhibition of duplication. However, recently it has been reported that centrosome duplication in CHO cells can proceed during mimosine-induced G1 arrest (Durcan et al., 2008). In this case, duplication was slower than that in cells that proceed into S-phase. Furthermore, whilst mimosine-arrested cells could assemble more than four centrioles, the extent of centrosome amplification seen was less than that in cells that enter S-phase. Perhaps more pertinently, during prolonged (40 hours) mimosine-arrest, Cdk2 activity was found to rise above that found in G0 cells, even if it did not reach the levels seen in S-phase arrested cells. This small rise in Cdk2 activity may well account for the centrosome duplication seen in these cells, with the slow activation of Cdk2 accounting for the delay in centrosome duplication and the slower rate of amplification.

It has been suggested that Cdk2 regulates centrosome duplication as it is required for centriole disengagement at the initiation of duplication. Separation of paired centrioles was observed in an in vitro assay system using Xenopus extracts, with depletion of Cdk2, cyclin E or cyclin A preventing this separation, indicating that it is dependent upon cyclin E/A-Cdk2 activity (Lacey et al., 1999). However, the relevance of this Cdk2 induced centriole separation in centrosome duplication is unclear. Indeed, centriole disengagement at anaphase has been shown to be independent of cyclin E-Cdk2, although new centriole
growth in interphase is dependent upon Cdk2 activity together with previously disengaged centrioles (Tsou and Stearns, 2006b).

One manner in which Cdk2 could initiate centriole duplication is through phosphorylation of proteins that promote or inhibit duplication. Nucleophosmin (NPM) was first implicated in centrosome duplication when it was demonstrated to localise specifically to unduplicated centrosomes (Okuda et al., 2000). NPM associates with the centrosome during mitosis due to phosphorylation by Cdk1 where it remains associated until the following G1/S transition. At this point, phosphorylation of NPM by Cdk2 causes it to dissociate from the centrosome; blocking this phosphorylation was found to prevent centrosome duplication (Okuda et al., 2000; Tarapore et al., 2002; Tokuyama et al., 2001). This led to the suggestion that localisation of NPM to the centrosome inhibits duplication. In fact, NPM is predominantly a nucleolar protein and trafficking of NPM to the centrosome is dependent upon the nuclear export complex, Ran-CRM1. In support of the above model, disruption of CRM1 caused loss of NPM from the centrosome, initiating premature centrosome duplication (Wang et al., 2005). Similar results were observed upon depletion of NPM by siRNA, and whilst this could be reversed upon expression of wild-type NPM, NPM containing a mutated NES (nuclear export signal) could not prevent premature centrosome duplication. Whilst these results suggest that NPM localised at the centrosome functions as a block to duplication, it is not a true block to reduplication as it is not present at the centrosome between S-phase and mitosis. However, NPM may also act as a positive regulator of duplication as, following phosphorylation by Cdk2, NPM has an increased affinity for ROCK 2 (Rho-associated coiled-coil containing protein kinase 2) and NPM binding strongly activates ROCK2 at centrosomes driving duplication (Ma et al., 2006b).

A further substrate of Cdk2 implicated in centriole duplication is the kinase, Mps1 (monopolar spindle 1). Mps1 was first identified as having a role in SPB duplication in budding yeast as Mps1 mutants arrested with unduplicated SPBs (Winey et al., 1991). Mps1 orthologues were subsequently found to have a spindle checkpoint role in a range of species, while their function in centrosome duplication is more open to question [reviewed by: (Fisk and Winey, 2004)]. Fisk and Winey (2001) first showed that the mouse ortholog of Mps1 (mMsp1) localised at the centrosome and that overexpression of mMps1 caused reduplication during S-phase arrest. Furthermore, inhibition of Cdk2 destabilised
centrosomal mMps1, preventing mMsp1 induced reduplication. However, the Nigg group reported that depletion of Mps1 by siRNA did not inhibit centrosome reduplication in HU-arrested U2OS cells, nor did overexpression of Mps1 accelerate duplication in human cells (Stucke et al., 2002). In direct contradiction of these results, the Fisk and Winey groups reported that overexpression of active Mps1 could accelerate reduplication in U2OS cells, whilst expression of dominant-negative Mps1 constructs prevented centrosome duplication (Fisk et al., 2003). More recently, this same group showed that prevention of Mps1 degradation at the centrosome was sufficient to drive centrosome reduplication (Kasbek et al., 2007). They hypothesise that phosphorylation of Mps1 by Cdk2 protects Mps1 from proteasome-mediated degradation thereby allowing Mps1 to stimulate centrosome duplication (Fisk and Winey, 2001). In line with this, depletion of Mps1 prevented centrosome reduplication in HeLa cells overexpressing cyclin A, supporting the idea that Mps1 is a downstream target of cyclin A-Cdk2 in centrosome reduplication. Moreover, the requirement for cyclin A in reduplication could be bypassed by preventing Mps1 degradation at the centrosome (Kasbek et al., 2007). Therefore, according to this group, control of Mps1 levels at the centrosome is crucial to regulating centrosome duplication. Interestingly, Mps1 has been shown to interact with and phosphorylate the Hsp70 family member mortalin (Kanai et al., 2007). In a positive-feedback loop, phosphorylated mortalin strongly activates Mps1 to drive centrosome duplication. Mortalin also promotes the dissociation of p53 from the centrosome to drive duplication (Ma et al., 2006a). Hence, one tentative pathway suggests that Cdk2 phosphorylation stabilises Mps1, which in turn phosphorylates mortalin, thereby promoting the dissociation of p53 from the centrosome. However, the role of centrosomal p53 in blocking centrosome duplication is far from clear.

Centrosome duplication may also be coupled to the cell cycle by changes in Ca\(^{2+}\) levels at the G1-to-S transition. Calmodulin is a major calcium signal-transducing factor in cells. The Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) was shown to be required for initiating centrosome duplication in *Xenopus* extracts (Matsumoto and Maller, 2002). Chelation of Ca\(^{2+}\) or inhibition of CaMKII in S-phase arrested egg extracts prevented centriole duplication. Furthermore, duplication could be restored by the addition of CaMKII and calmodulin. Ca\(^{2+}\)/calmodulin may also contribute to the mediation of cyclin E-Cdk2 activity at the G1-to-S transition. A calmodulin-binding motif has been identified in cyclin E, with calmodulin increasing Cdk2 activity via its binding to cyclin E [(Choi et al., 2006); reviewed by (Choi and Husain, 2006)].
1.4 CENTRIOLE FORMATION

1.4.1 TEMPLATED SEMI-CONSERVATIVE CENTRIOLE DUPLICATION

Morphologically, procentrioles are described as forming in close proximity to the proximal ends of existing centrioles (Alvey, 1985; Kuriyama and Borisy, 1981; Robbins et al., 1968). This observation resulted in the classical view that existing centrioles template the formation of daughter centrioles from a specific site on their walls. As the procentriole elongates, the centriole pair maintains an orthogonal configuration, providing a physical block to reduplication (Wong and Stearns, 2003). Subsequent disengagement of centrioles in mitosis licenses duplication in the next cell cycle by exposing the site from which procentrioles can form (Tsou and Stearns, 2006b). Labelled tubulin is only incorporated into newly forming centrioles such that following mitosis each daughter cell receives a newly formed centriole paired with an older centriole (Kochanski and Borisy, 1990). Therefore, centriole duplication, like DNA replication, is a templated semi-conservative process, with each existing centriole subtending the growth of a new centriole.

The assembly of a single new procentriole at a specific site on each of the existing centrioles provides an unambiguous counting mechanism for controlling centriole number during duplication. Attachment of a nascent procentriole to the wall of the existing centriole is proposed to prevent the formation of additional procentrioles by blocking the site at which they can form (Tsou and Stearns, 2006b; Wong and Stearns, 2003). However, the presence of a single site for procentriole formation on the wall of centrioles has been drawn into question following the demonstration that under certain experimental conditions multiple procentrioles can form concurrently around a single maternal template (Duensing et al., 2007; Kleylein-Sohn et al., 2007). In S-phase arrested cells, removal of the daughter centriole from the wall of the mother centriole by laser microsurgery induced centriole reduplication (Loncarek et al., 2008). Under these experimental conditions, the number of procentrioles formed did not necessarily match the number of ablated centrioles. Significantly, if the wall of the mother centriole was damaged during ablation of the daughter centriole the new centriole formed at a different site on the wall. Together, these results suggest that initiation of centriole formation is not restricted to a specific fixed spot on the mother centriole and it may not be the mother centriole that defines the number of centrioles that can form. In support of this, augmentation of the PCM following overexpression of the PCM component, pericentrin, was found to drive the formation of
multiple daughter centrioles that did not necessarily form in close association with existing centrioles (Loncarek et al., 2008). This led to the hypothesis that the formation of daughter centrioles is initiated within the PCM cloud, but not necessarily in association with the mother centriole. Hence, existing centrioles may promote centriole formation through their ability to organise PCM, instead of acting directly as a template. The mother centriole thereby provides a platform at which components required for procentriole formation can concentrate to critical levels for self-assembly (Rodrigues-Martins et al., 2007b; Salisbury, 2008). According to this argument, new centriole formation is restricted to the vicinity of existing centrioles solely due to their ability to recruit PCM (Dammermann et al., 2008; Dammermann et al., 2004; Loncarek et al., 2008).

However, it may be premature to discard the model of templated centriole duplication (Salisbury, 2008). It is unlikely that multiple procentrioles can form around a single template in cycling wild-type cells. The high concentration of components achieved in cells either overexpressing centrosomal components or regulators, or arrested in S-phase for prolonged periods, represents an artificial environment that would usually be circumvented by cell cycle progression. Formation of a single procentriole per existing centriole may indeed be templated, requiring the recruitment of relative low levels of components (Salisbury, 2008). Progression through S-phase would prevent these components accumulating further, thereby inhibiting the formation of additional procentrioles. In situations where these proteins accumulate rapidly, for example by overexpression, or when S-phase progression is blocked, many procentrioles can form. Cancer cells frequently exhibit overduplicated centrosomes, so it is likely that deregulation of templated duplication leading to the formation of many procentrioles could contribute to the accumulation of supernumerary centrosomes. Therefore, unravelling the mechanisms that lead to centriole overduplication is important for understanding how supernumerary centrosomes arise.

1.4.2 **DE NOVO CENTRIOLE FORMATION**

Centrioles can also arise de novo in the absence of existing centrioles. In many species, centrioles are lost during oogenesis so that at fertilisation centrioles are contributed by the paternal germline. By their very definition, parthenogenetic species lack the contribution of the male gamete and instead have to resort to de novo centriole formation in the zygote (Callaini et al., 1999). In mice there is neither a paternal nor maternal contribution of
centrioles so that early development of the mouse embryo occurs in the absence of centrioles. However, normal looking centrioles form de novo before the pre-implantation stage (Abumuslimov et al., 1994; Calarco-Gillam et al., 1983). During this process aggregates of the PCM component $\gamma$-tubulin form in which 25 nm ring structures that potentially serve as centriolar precursors are observed (Calarco, 2000). Artificially induced parthenogenesis can also activate de novo centriole formation in species that normally sexually reproduce and have paternally contributed centrioles (Kallenbach, 1983; Riparbelli and Callaini, 2003; Szollosi and Ozil, 1991). This suggests there are two pathways for centriole formation in cells: templated and de novo. These pathways are likely to be mutually exclusive as the presence of centrioles appears to suppress de novo formation.

De novo centriole formation is also observed in both transformed and untransformed mammalian somatic cells following experimental removal of the existing centrioles (Khodjakov et al., 2002; La Terra et al., 2005; Uetake et al., 2007). Significantly, de novo centriole formation only occurs during S-phase and takes much longer than templated duplication. Following laser ablation of centrioles, foci of PCM containing $\gamma$-tubulin assemble early in the de novo pathway (Khodjakov et al., 2002). Centrioles then form within this PCM cloud. A number of amorphous cytoplasmic ‘precentrioles’ are also observed which become morphologically recognisable as centrioles before mitosis (La Terra et al., 2005) (Figure 1.4). The number of newly formed centrioles ranges from 2 to 14 per cell, demonstrating that there is no strict control over the number of centrioles formed during the de novo pathway. Indeed, multipolar spindles and aberrant cell divisions are observed when cells with centrioles formed de novo during S-phase arrest are induced to enter mitosis (Khodjakov et al., 2002). In the next cell cycle de novo formed centrioles mature, becoming able to organise MTs and replicate. However, centrioles formed de novo in S-phase arrested cells do not undergo this maturation suggesting that it is not a time dependent process but requires progression into the next cell cycle. Significantly, if only one centriole of the pair is ablated, the de novo pathway is suppressed by the presence of the remaining centriole (La Terra et al., 2005).

Whilst the templated and de novo centriole assembly pathways are suggested to be mutually exclusive it has been hypothesised that the precentrioles observed during de novo
Figure 1.4 De novo centriole formation
Centrioles can form de novo following laser ablation of the existing centrosome. When one of the poles of a mitotic spindle was ablated, an acentriolar daughter cell is produced. As this cell enters S-phase multiple procenotrioles accumulate within the cytoplasm. These structures transform into morphologically recognisable centrioles before the onset of mitosis. In the subsequent cell cycle these de novo centrioles mature and are able to undergo normal templated duplication. From La Terra et al. (2005).
centriole assembly could also form during normal templated duplication (La Terra et al., 2005). In this instance, precentrioles represent centriolar precursors with a number of precentrioles forming during centriole duplication. However, only those precentrioles that ‘dock’ with an existing centriole will be stabilised and incorporated into new daughter centrioles, with any remaining, undocked precentrioles disappearing. Additionally, it is reported that de novo assembly occurs at a slower rate than templated duplication (Khodjakov et al., 2002; Marshall et al., 2001). Therefore, it may simply be progression through the cell cycle that prevents de novo centriole duplication in the presence of an existing centriole (Salisbury, 2007; Salisbury, 2008). Likewise, centriole formation may be dependent upon the localised concentration of precursor molecules. Assembly of new centrioles is favoured at existing centrioles where the local concentration of these proteins reaches the required levels. However, in the de novo pathway, high concentrations of centriole components are required before centrioles can form. The templated pathway would thus predominate as it occurs faster than the sites for de novo formation can assemble. In this manner, the de novo pathway would normally be prevented by cell cycle progression following templated centriole duplication (Salisbury, 2007).

1.4.3 CILIOGENESIS

The mother centriole acts as a basal body in cells that possess a single cilium. However, in multiciliated cells hundreds of basal bodies are required as each anchors a cilium. These cells therefore have the ability to assemble hundreds of centrioles as they undergo ciliogenesis. Two parallel pathways for centriole formation during ciliogenesis exist [reviewed by: (Hagiwara et al., 2004)] (Figure 1.5), both with similarities to the templated and de novo pathways in cycling cells. In the centriolar pathway, instead of a single procentriole, multiple centrioles are seen forming around the existing centrioles. Indeed, as many as 10 centrioles have been observed developing around a single centriole during ciliogenesis (Anderson and Brenner, 1971). In the acentriolar pathway, fibrous granules are the first recognisable structure (Anderson and Brenner, 1971). These structures are equivalent to centriolar satellites, containing many of the same components (Kubo et al., 1999). The fibrous granules aggregate and condense to form non-microtubule-based structures called deuterosomes (Anderson and Brenner, 1971; Sorokin, 1968a). Deuterosomes serve as organising centres for the formation of new centrioles with procentrioles assembling around them. Procentrioles are first observed as an amorphous structure formed by the fusion of fibrous granules; within this structure the cartwheel
Figure 1.5 The centriolar and acentriolar pathways of ciliogenesis
During differentiation of multiciliated cells hundreds of basal bodies are formed through two simultaneous pathways. **A.** In the centriolar pathway multiple centrioles can form around a single template (arrow). In the acentriolar pathway fibrous granules appear first that coalesce to form deutrosomes (arrowheads). These non-microtubular structures then nucleate the formation of multiple centrioles. From Hagiwara et al. (1992). **B.** Multiple centrioles (arrow) form around a single existing centriole (arrowhead) in the centriolar pathway. From Anderson and Brenner (1971). **C.** Up to nine centrioles (stars) form around a single deutroseome (arrow) in the acentriolar pathway. The size of deutrosomes differs between species and dictates the number of centrioles that can form around each one. From Dirksen (1971).
assembles and MTs are added to form centrioles (Anderson and Brenner, 1971). Most centrioles are formed via this acentriolar pathway, with the number of centrioles formed around one deuterosome depending upon its size (Dirksen, 1971; Hagiwara et al., 1992). Following assembly the centrioles move to the apical cell surface where they nucleate cilia.

There are four main differences between the assembly of centrioles during ciliogenesis and centrosome duplication in cycling cells. During ciliogenesis, more than two new centrioles are generated; an existing centriole can simultaneously nucleate more than one daughter centriole; non-centriolar structures nucleate centrioles; and centrioles are formed in non-dividing cells. However, both processes generate virtually identical structures, suggesting that similar mechanisms exist in both pathways. Indeed, during ciliogenesis, the expression of centrosomal proteins is up-regulated, whilst depletion of centriolar components prevents centriole formation (Laoukili et al., 2000; Vladar and Stearns, 2007). The first detectable sign of centriole assembly during ciliogenesis in mouse epithelial cells is the formation of foci of centrosomal proteins in the vicinity of the centrosome (Vladar and Stearns, 2007). Centrioles are then formed within these foci after they cluster together. The centrioles formed during ciliogenesis and centrosome duplication have similar protein constituents, demonstrating that their structure and components are seemingly identical. Basal bodies also go through a process of maturation similar to centrioles, although in multiciliated cells this does not require passage through mitosis into the next cell cycle (Vladar and Stearns, 2007). The process of assembly of centrioles in each instance is therefore likely to involve common proteins and intermediates, and occur through a similar pathway.

1.5 CENTRIOLE ASSEMBLY

1.5.1 STRUCTURAL ASSEMBLY OF CENTRIOLES

Centrioles and basal bodies display the same complex archetypal design: nine triplets of MTs arranged with nine-fold symmetry into a cylindrical structure. Each time new centrioles or basal bodies are formed this structure is maintained. Extensive studies in *Tetrahymena*, *Paramecium* and *Chlamydomonas* have provided insight into the assembly of basal bodies in these organisms and specifically the roles that members of the tubulin superfamily play in this process [reviewed by (Dutcher, 2003b)].
The MT triplets that form the walls of centrioles are comprised of α-/β-tubulin subunits that have undergone extensive post-translational modifications, including acetylation and polyglutamylation (Bobinnec et al., 1998b; Piperno and Fuller, 1985). Centriole MTs are turned over only very slowly and are extremely stable, being resistant to microtubule depolymerising drugs (Kochanski and Borisy, 1990). Injection of anti-glutamylated tubulin antibodies into cells causes centrioles to disappear, suggesting that post-translational modification of tubulin is critical for stability (Bobinnec et al., 1998a). Labelled tubulin is solely incorporated into newly formed centrioles during the duplication cycle, demonstrating that centriole formation is conservative (Kochanski and Borisy, 1990). Furthermore, polyglutamylation is an early marker of centriole assembly (Lechtreck and Geimer, 2000; Million et al., 1999). γ-tubulin has been demonstrated to be required for basal body duplication in Tetrahymena and Paramecium (Ruiz et al., 1999; Shang et al., 2002), whilst RNAi depletion of γ-tubulin in C. elegans embryos prevents centriole formation in the majority of cases (Dammermann et al., 2004). Where daughter centrioles do form in C. elegans embryos depleted of γ-tubulin, they are aberrant in structure and smaller than in wild-type embryos. In mammalian centrioles γ-tubulin localises within the centriole barrel, at the proximal ends, where the minus ends of the MTs are located (Fuller et al., 1995). In Chlamydomonas, γ-tubulin is found in the interior of basal bodies (Silflow et al., 1999). Consistent with its role in MT nucleation and location in centrioles, γ-tubulin is most likely required to nucleate centriolar MTs during centriole assembly.

Procentrioles form at sites at the proximal ends of the outer walls of existing centrioles. However, centriole assembly does not commence with MT nucleation but with the assembly of an amorphous disc-like structure adjacent to the existing centriole (Allen, 1969; Dippell, 1968). A cartwheel structure localises at the proximal end of nascent procentrioles and provides patterned assembly at the sites where centriole formation occurs. It is proposed that the cartwheel organises the efficient assembly of the outer MTs as it exhibits nine-fold symmetry (Anderson and Brenner, 1971). The Chlamydomonas protein Bld10p localises at the spoke tips of the cartwheel, being involved in MT assembly at these sites (Hiraki et al., 2007). Consistent with this role, bld10 null mutants lack centriolar structures (Matsuura et al., 2004), whilst C-terminal truncations of Bld10p cause cartwheels to form with shortened spokes and 8-fold, rather than 9-fold, symmetry (Hiraki et al., 2007). Additionally, Cep135, the human homolog of Bld10p, localises to the
proximal lumen of centrioles and appears to be necessary for centriole formation (Kleylein-Sohn et al., 2007). The cartwheel structures are also observed during de novo centriole formation, suggesting they are essential for templating centriole assembly in both duplication pathways (Mizukami and Gall, 1966; Renzaglia and Maden, 2000). MTs elongate from the cartwheel, first as nine singlets (A-tubules) to form a short centriole ‘bud’. The B-tubules are then added to form doublets, and finally, as they elongate, the C-tubules are added to form MT triplets (Dippell, 1968).

ε-tubulin localises to centrioles and is required for centriole duplication (Chang and Stearns, 2000; Dupuis-Williams et al., 2002; Dutcher et al., 2002). In Chlamydomonas mutants lacking ε-tubulin, centrioles are shorter than normal and composed of nine MT singlets rather than triplets (Dutcher et al., 2002; Goodenough and StClair, 1975). These aberrant centrioles are subsequently unable to nucleate formation of flagella. Paramecium also require ε-tubulin for basal body duplication (Dupuis-Williams et al., 2002), whilst in vertebrates, immunodepletion of ε-tubulin from Xenopus egg extracts prevents daughter centriole formation (Chang et al., 2003). ε-tubulin localises to the sub-distal appendages of the mother centriole in vertebrate cells (Chang et al., 2003) and to an analogous location in Chlamydomonas (Dutcher et al., 2002). However, ε-tubulin function in duplication may be restricted to centrioles that have triplet MTs. Neither Drosophila nor C. elegans, organisms whose centrioles have doublet and singlet MTs respectively, have ε-tubulin (Chang et al., 2003). However, triplet MTs are observed in Drosophila spermatocytes (Gonzalez et al., 1998), suggesting that ε-tubulin is not essential in these cells for forming triplet MTs. Furthermore, the need for ε-tubulin in Chlamydomonas bld2 null mutants could be circumvented by an extragenic suppressor mutation, rgn1 (Preble et al., 2001).

δ-tubulin is also required for the assembly of triplet microtubules. The C-tubule is aberrant in basal bodies in Chlamydomonas mutants lacking δ-tubulin (Dutcher and Trabuco, 1998; O'Toole et al., 2003a). Whilst it is missing from the length of the basal body, it is present at the distal end, suggesting that δ-tubulin is not essential for the assembly of the C-tubule, but may be required for its stability. Silencing of δ-tubulin in Paramecium causes similar defects (Garreau de Loubresse et al., 2001). δ-tubulin is also present in protozoa (Dutcher and Trabuco, 1998; Garreau de Loubresse et al., 2001) and mammalian cells (Chang and
Stearns, 2000; Smrzka et al., 2000), but not Drosophila, suggesting that triplet MTs in spermatocytes can form in the absence of δ-tubulin (Gonzalez et al., 1998).

η-tubulin distribution among organisms is much more limited that δ-tubulin and ε-tubulin, being identified in only four organisms to date (Dutcher, 2003b). η-tubulin is most closely related to δ-tubulin, suggesting that they could have overlapping functions. In Paramecium η-tubulin is required for basal body duplication and the localisation γ-tubulin (Ruiz et al., 2000). However, η-tubulin is unlikely to be a core centriole duplication protein as it has been identified in so few organisms.

1.5.2 PROTEINS IMPLICATED IN CENTRIOLE DUPLICATION

The SPB is the MTOC in yeast cells and like the centrosome must duplicate once per cell cycle. Whilst there is great diversity in structure between SPBs and centrosomes, many of the components are conserved. Yeast cdc31 mutants were found to arrest with single SPBs, suggesting that the gene product is essential for cell viability (Byers, 1981). Subsequently, the CDC31 protein was found to be crucial for SPB duplication in both S. cerevisiae and S. pombe (Paoletti et al., 2003; Spang et al., 1993). CDC31 localises to the half-bridge of the SPB, a structure that lies adjacent to the SPB and is required for its duplication. Centrins are homologues of CDC31 and are required for centriole and basal body duplication in a range of organisms (Koblenz et al., 2003; Salisbury et al., 2002; Stemm-Wolf et al., 2005). Centrins belong to the same calcium-binding EF-hand superfamily as calmodulin and localise to the distal lumen of centrioles (Laoukili et al., 2000; Paoletti et al., 1996; Salisbury, 1995). It has been suggested that centrin in this localisation stabilises the distal ends of centrioles (Paoletti et al., 1996). Centrins also localise to centriolar satellites and the PCM (Baron et al., 1994). Very early during centriole assembly, centrins are seen to localise to the sites of daughter centriole formation, in the procentriole bud (Paoletti et al., 1996). Additionally, centrins are markers of precentrioles that form during the de novo centriole assembly pathway and subsequently give rise to centrioles (La Terra et al., 2005; Uetake et al., 2007).

Four mammalian isoforms of centrin have been identified to date. Both centrin2 and centrin3 are ubiquitously expressed, whilst centrin1 and centrin4 are expressed in ciliated cells only (Gavet et al., 2003; Laoukili et al., 2000). Specifically, centrin1 has been shown
to only be expressed during ciliated cell differentiation as cells undergo ciliogenesis (Laoukili et al., 2000). Knockdown of centrin2 by RNAi in HeLa cells results in a failure of centriole duplication (Salisbury et al., 2002), whilst human centrin3 is a dominant-negative inhibitor of yeast SPB and *Xenopus* centrosome duplication (Middendorp et al., 2000). During ciliogenesis, centrin proteins are reported to localise to fibrous granules that are described as the first cytoplasmic structures involved in centriologenesis (Laoukili et al., 2000; Sorokin, 1968b). Furthermore, small centrin foci are formed early during de novo centriole formation following laser ablation of the existing centrosome (La Terra et al., 2005). Centrins are also implicated in basal body duplication in both *Chlamydomonas* and *Tetrahymena* (Koblenz et al., 2003; Marshall et al., 2001; Stemm-Wolf et al., 2005). Together these results suggest that centrins have a conserved role in centriole formation, specifically localising to sites of centriole assembly. However, RNAi depletion of centrin2 or centrin3 in *Paramecium* affects the site of probasal body formation and separation of newly formed basal bodies, but not the duplication process (Ruiz et al., 2005). Additionally, a genome-wide RNAi screen failed to implicate centrins in centriole duplication in *Drosophila* cells (Goshima et al., 2007), and depletion of neither centrin2 nor centrin3 blocked centriole overduplication in response to overexpression of Plk4 in mammalian cells (Kleylein-Sohn et al., 2007).

The role of centrins in centriole duplication is not mechanistically understood; however, a number of centrin-binding proteins have been identified. In yeast, Sfi1p was identified as a centrin-binding partner that localises to SPBs and is required for SPB duplication (Kilmartin, 2003; Li et al., 2006). Indeed, mutations in Sfi1p block SPB duplication. Sfi1p binds multiple centrin molecules through a series of 23 internal centrin-binding repeats (Kilmartin, 2003). The first event in SPB duplication is elongation of the half-bridge, from which initiation of daughter SPB formation occurs. Assembly of centrin/Sfi1 fibers allows lengthening of the half-bridge structure to provide the site for daughter SPB assembly (Li et al., 2006). Human Sfi1 localises to the centrosome and binds to centrin2 (Martinez-Sanz et al., 2006). Due to the conservation of these proteins and their interaction in yeast and mammals, it has been suggested that centrin/Sfi1 fibers may play analogous roles in both SPB and centriole duplication (Salisbury, 2007). Therefore, in the case of mammals, centrin/Sfi1 fibers could form part of the cartwheel structure, the earliest recognisable centriole precursor, to provide a site for centriole formation. The subsequent translocation of cartwheel structural elements, initially present at the proximal end of nascent centrioles,
via their association with the growing ends of the elongating centrioles, places centrin at the distal end (Geimer and Melkonian, 2005), where it may indeed provide stability to the centriole structure (Paoletti et al., 1996).

CP110 is another centrosomal protein that is required for centriole duplication and is reported to interact with both centrin and calmodulin (Chen et al., 2002; Kleylein-Sohn et al., 2007; Tsang et al., 2006). Additionally, centrin and CP110 were found to co-localise at the distal ends of centrioles. Depletion of CP110 in cells prevents both centriole duplication and experimentally induced centriole amplification (Chen et al., 2002; Kleylein-Sohn et al., 2007). Expression of CP110 is strongly induced at the G1/S transition and it can be phosphorylated by both Cdk2 and Cdk1 (Chen et al., 2002). In addition to a possible role in centrosome duplication, depletion of CP110, or expression of unphosphorylatable mutants, or those unable to bind calmodulin, results in cytokinesis defects (Chen et al., 2002; Tsang et al., 2006). CP110 may also have roles in centrosome separation as CP110 loss, or expression of mutants unable to be phosphorylated, results in unscheduled centrosome separation. More recently, a screen for proteins that associate with CP110 identified Cep97, a previously uncharacterised protein (Spektor et al., 2007). Depletion of Cep97, or expression of dominant-negative mutants, was found to cause CP110 to disappear from the centrosome. It was therefore postulated that Cep97 is required to recruit CP110 to the centrosome. The appearance of centrin and polyglutamylated tubulin, but not C-Nap1, at centrioles was also found to be greatly altered when either Cep97 or CP110 was depleted, suggesting that Cep97-CP110 complexes are required for the assembly of normal centrioles. Finally, there is some evidence that Cep97 and CP110 are involved in the regulation of cilia formation. Loss of CP110 or Cep97 led to primary cilia formation in growing cells. Conversely, expression of CP110 in quiescent cells prevented cilia formation. Therefore, CP110 appears to control several discrete functions in the centrosome cycle, although how it contributes to centriole duplication remains unclear. However, it has recently been proposed that CP110 forms a cap under which α-/β-tubulin dimers are inserted during procentriole elongation (Kleylein-Sohn et al., 2007). Indeed, it may be the removal of this cap in cells depleted of CP110 that leads to cilia formation.

A number of other kinases have also been associated with centriole duplication in mammalian cells. Depletion of Polo-like kinase 1 (Plk1) reduces centrosome
overduplication in S-phase arrested U2OS cells (Liu and Erikson, 2002). Plk2 is activated at the G1-to-S transition, with expression of a dominant-negative Plk2 construct perturbing centriole duplication in cycling U2OS cells (Warnke et al., 2004). Furthermore, depletion of Plk2 blocked centriole reduplication in S-phase arrested cells. Plk4 is also required for centriole duplication. Indeed, silencing of Plk4 causes a progressive reduction of centriole numbers in cycling cells (Habedanck et al., 2005), whilst overexpression of Plk4 causes the formation of more than one procentriole per maternal template [discussed in more detail in section 1.5.4 (Kleylein-Sohn et al., 2007)].

1.5.3 A PATHWAY FOR CENTRIOLE FORMATION

Whilst centrosome duplication has been extensively characterised at the morphological level and a number of factors required for duplication identified, the molecular pathway remains elusive. However, our understanding of the processes involved has been greatly enhanced by the use of the C. elegans embryo as a model system to decipher a molecular pathway for centriole formation. C. elegans centrioles are 150 nm in length and 100 nm in diameter, consisting of a central tube surrounded by nine singlet microtubules (O'Toole et al., 2003b). A number of proteins required for centriole duplication in C. elegans have been identified through genetic and genome-wide RNAi screens (Gonczy et al., 2000; O'Connell et al., 1998; Sonnichsen et al., 2005; Zipperlen et al., 2001). These include ZYG-1 (zygote defective 1), spindle assembly proteins SAS-4, SAS-5, SAS-6, and SPD-2 (spindle defective 2), all of which localise to centrioles and contribute to centriole formation (Dammermann et al., 2004; Delattre et al., 2004; Gonczy et al., 2000; Kemp et al., 2004; Kirkham et al., 2003; Leidel and Gonczy, 2003; O'Connell et al., 2001; O'Connell et al., 2000; Pelletier et al., 2004).

The C. elegans oocyte has no centrioles, but possesses a large store of centrosomal components. Upon fertilisation, the sperm donates two centrioles which must duplicate and recruit PCM from proteins in the maternal pool before the first mitotic division. RNAi-mediated silencing of the maternal expression of the coiled-coil proteins SAS-4, SAS-5, SAS-6, or the kinase ZYG-1, results in a characteristic phenotype (Dammermann et al., 2004; Delattre et al., 2004; Kirkham et al., 2003; O'Connell et al., 2001). Following fertilisation with wild-type sperm, the centrioles fail to duplicate. Nevertheless, they separate and recruit PCM to form a bipolar spindle so that mitosis completes normally. However, as cells enter mitosis at the two-cell stage only a single centriole, which is
unable to duplicate, is present so a monopolar spindle is formed and development ceases. Serial electron microscopy revealed that each spindle pole at the one-cell stage contained only a single centriole, confirming that centriole duplication had not occurred (Dammermann et al., 2004; Kirkham et al., 2003). In addition to a maternal requirement, if the paternal contributions of zyg-1, sas-4 or sas-5 are reduced, centrioles fail to duplicate during spermatogenesis and the sperm can only contribute a single centriole at fertilisation (Delattre et al., 2004; Leidel and Gonczy, 2003; O’Connell et al., 2001). In the case of ZYG-1, the centriole was shown to duplicate after fertilisation as the oocyte provides normal zyg-1 function. The centriole pair recruits PCM components, but as there is only a single centrosome present, a monopolar spindle forms at the first mitosis and cell division fails. At the next mitosis, a bipolar spindle forms as centrosome duplication has occurred normally. However, the embryos are tetraploid and eventually die (O’Connell et al., 2001). If both the maternal and paternal contributions of zyg-1 or sas-5 are reduced, the single centriole donated at fertilisation fails to duplicate and only monopolar spindles can ever be formed (O’Connell et al., 2001; Delattre et al., 2004).

In contrast to the other four centriole duplication proteins, SPD-2 depletion produces a different phenotype. Instead of arresting at the second mitosis, embryos lacking SPD-2 do not complete the first division event (Dammermann et al., 2004; Kemp et al., 2004; Pelletier et al., 2004). In addition to localising at centrioles throughout the cell cycle, SPD-2 is essential for centrosome maturation, being recruited to the PCM at mitosis (Kemp et al., 2004; Pelletier et al., 2004). In embryos depleted of SPD-2, sperm centrioles acquire a small amount of PCM following fertilisation. However, the accumulation of additional PCM during centrosome maturation fails to occur. Embryos lacking SPD-2 thus fail to nucleate sufficient MTs to form a mitotic spindle at the one-cell stage due to defects in centrosome maturation. An identical phenotype is observed when other proteins necessary for centrosome maturation, such as Aurora A (AIR-1) or SPD-5, are depleted (Dammermann et al., 2004; Hamill et al., 2002; Hannak et al., 2001). However, whilst embryos lacking SPD-5 arrest at the one-cell stage, they generate multiple centrosomes as they age. In contrast, embryos generate no additional centrioles in the absence of SPD-2 (Kemp et al., 2004). The use of marked mating experiments confirmed that centriole formation was perturbed in embryos depleted of SPD-2. SAS-4 is only incorporated into centrioles during assembly (Leidel and Gonczy, 2003), unlike SAS-5 that undergoes exchange between centriolar and cytoplasmic pools throughout the cell cycle (Delattre et
Expression of GFP-SAS-4 in oocytes subsequently fertilised with wild-type sperm with unlabelled centrioles enables newly formed, GFP-positive centrioles to be distinguished from the paternal centrioles. GFP-SAS-4 incorporation occurred normally in AIR-1-depleted embryos. However, no GFP-SAS-4 foci were detected in embryos lacking SPD-2. Together, these results provide evidence for SPD-2 functioning in both centriole formation and centrosome maturation, although maturation itself is not required for centriole assembly (Dammermann et al., 2004). Furthermore, mothers heterozygous for spd-2 and zyg-1 produce predominately dead embryos, indicating that SPD-2 and ZYG-1 function in the same pathway (Kemp et al., 2004).

Recent elegant studies have described a stepwise pathway in which the five centriole assembly proteins are recruited sequentially to the sites of newly forming centrioles (Delattre et al., 2006; Pelletier et al., 2006). Depletion of individual centriole proteins by RNAi allowed the order in which the proteins are recruited to be determined. Embryos lacking SPD-2 could no longer recruit any of the other centriole proteins. However, in embryos lacking ZYG-1, SPD-2 recruitment was unaffected, but SAS-4, SAS-5 and SAS-6 were absent from the site of centriole assembly. Furthermore, SAS-4 recruitment is blocked in embryos lacking either SAS-5 or SAS-6 (Dammermann et al., 2004; Delattre et al., 2004; Leidel et al., 2005), whilst SAS-4 depletion had no effect on the localisation of the other proteins. SPD-2 is therefore the most upstream protein in the pathway, being essential for the recruitment of the other four centriole proteins. SPD-2 and ZYG-1 are recruited to centrioles shortly after fertilisation, coincident with completion of meiosis of the maternal nucleus (Delattre et al., 2006; Pelletier et al., 2006). Recruitment of SPD-2 to the centrioles may be mediated by cyclin E-Cdk2, although Cdk2 activity is not required for centriole duplication in C. elegans as it is in other systems (Cowan and Hyman, 2006). SPD-2 and ZYG-1 are then required for the recruitment of SAS-5 and SAS-6, concurrent with pronuclear appearance. SAS-5 and SAS-6 physically interact, being co-dependent for their localisation to centrioles and may function as a structural heterodimer (Leidel et al., 2005). Both SAS-5 and SAS-6 are then essential for the recruitment of SAS-4, the final component in the pathway. SAS-4 remains in dynamic exchange between centriolar and cytoplasmic pools until it is stably incorporated in late prophase in a manner dependent upon γ-tubulin and MT assembly (Dammermann et al., 2008).
Combining electron tomography with the above approach to deciphering the pathway of centriole formation in the \textit{C. elegans} embryo, allowed the proteins to be assigned to specific intermediate structures that contribute to centriole assembly (Pelletier et al., 2006) (Figure 1.6). Initially, wild-type \textit{C. elegans} embryos were visualised by light microscopy until the desired cell cycle stage. At this point the embryos were rapidly frozen and processed for electron microscopy, allowing for precise correlation between the cell cycle and centriole structures seen at that time. This allowed for three distinct steps in centriole formation to be characterised: the formation of a central tube; elongation and widening of the central tube; followed by assembly of singlet MTs around the perimeter of the tube. Additionally, previously undescribed hook-like appendages were seen on the central tubes in the positions in which MTs attach. Coupling this approach with RNAi revealed that no daughter centrioles or intermediate structures were observed in embryos depleted of SPD-2, ZYG-1, SAS-5 or SAS-6. However, in SAS-4 depleted embryos a central tube was seen to form adjacent to the mother centriole. Whilst formation of the central tube represents a structural intermediate in centriole assembly, it was abnormal in the absence of SAS-4. The central tube was able to form and elongate, but it failed to widen and was defective in the assembly of the hook-like appendages and MTs. SAS-4 is therefore required for the assembly or maintenance of MTs onto the central tube, which itself may be required for widening of the tube. SAS-4 is incorporated strictly into newly forming daughter centrioles, and is stable at that location thereafter, much like tubulin dimers (Kirkham et al., 2003; Leidel and Gonczy, 2003). In fact, stable incorporation of SAS-4 into centrioles has recently been shown to be dependant upon MT assembly (Dammermann et al., 2008). As the central tube can assemble in embryos lacking SAS-4, a condition under which SAS-5 and SAS-6 recruitment is not inhibited, suggests that these proteins may contribute to the formation of the central tube (Pelletier et al., 2006). Indeed, SAS-5 and SAS-6 recruitment is coincident with central tube formation, leading to the proposal that they are structural components of the central tube, or act to maintain its structural integrity.

1.5.4 Conservation of the Centriole Formation Pathway

Identification, and subsequent placement, of a number of proteins in a centriole duplication pathway in \textit{C. elegans} embryos has greatly increased our understanding of how centrioles are formed \textit{in vivo}. The identification of homologues for a number of these centriole duplication proteins in other species suggests that components of the assembly pathway are conserved in mammals and other eukaryotes. However, it must be kept in mind that
Figure 1.6 Centriole assembly pathway in *C. elegans* embryos

The sperm-donated centrioles recruit SPD-2 first, followed by the kinase ZYG-1, from the oocyte cytoplasm soon after fertilisation during meiosis. Both SPD-2 and ZYG-1 are required for the recruitment of the SAS proteins at a time coincident with daughter centriole tube formation (yellow). The presence of SAS-5 and SAS-6 is required for the recruitment of SAS-4. SAS-4 is necessary for central tube elongation and the assembly of singlet microtubules around the central tube (green). By the time of mitosis, two fully formed daughter centrioles are present. From Pelletier et al. (2006).
centrioles in *C. elegans* embryos differ from those in vertebrate cells in a number of ways. They are comprised of microtubule singlets, rather than triplets, lack prominent appendages and are somewhat shorter than centrioles in vertebrate cells (O’Toole et al., 2003b). Differences in structure may well reflect variations in the mechanisms controlling centriole assembly. Furthermore, a number of proteins, such as ε-tubulin and centrin, identified as being important in centriole formation in other species, are not present in the *C. elegans* proteome. Nevertheless, centriole formation in *C. elegans* may reflect a conserved pathway that contributes to centriole duplication in other species.

Homologues of SAS-6 were identified by sequence similarity in species that possess basal bodies or centrioles as members of this family each have a ~50 amino acid region of homology termed the PISA (present in SAS-6) motif (Leidel et al., 2005). Mass spectrometry initially identified HsSAS-6 as a probable centrosomal protein (Andersen et al., 2003), whilst fluorescently tagged HsSAS-6 was subsequently found to localise to centrosomes (Dammermann et al., 2004; Leidel et al., 2005). Furthermore, HsSAS-6 is essential for centriole duplication in somatic cells. Depletion of HsSAS-6 in cycling U2OS cells caused most cells to assemble monopolar spindles at mitosis, whilst loss of HsSAS-6 prevented centrosome overduplication in S-phase arrested U2OS cells (Leidel et al., 2005; Zhu et al., 2008). Additionally, depletion of SAS-6 prevented centriole assembly during ciliogenesis in mouse epithelial cells (Vladar and Stearns, 2007). Similarly, mutations in *Drosophila sas-6* result in severe reductions in centriole numbers (Peel et al., 2007; Rodrigues-Martins et al., 2007a), whilst mutations affecting the zebrafish orthologue of SAS-6 (cellular atoll) cause centrosome duplication defects in zebrafish embryos (Yabe et al., 2007).

Overexpression of SAS-6 orthologues can also drive centrosome overduplication (Leidel et al., 2005; Peel et al., 2007; Strnad et al., 2007). HsSAS-6 is required for procentriole formation, with increased protein levels promoting the formation of more than one procentriole per pre-existing centriole (Strnad et al., 2007). Centrin had previously been reported as the earliest marker of procentrioles (Salisbury et al., 2002); however, HsSAS-6 is recruited before and independently of centrin during procentriole formation (Strnad et al., 2007). Significantly, whilst depletion of centrin did not prevent HsSAS-6 recruitment, depletion of HsSAS-6 resulted in the absence of centrin. This suggests that HsSAS-6 is required for the formation of a site or structure to which centrin is then recruited.
Overexpression of DSAS-6 in unfertilised *Drosophila* eggs, which do not normally contain centrioles, resulted in the de novo formation of multiple MTOCs (Peel et al., 2007; Rodrigues-Martins et al., 2007a). Whilst Peel et al. (2007) described these as containing centriole-like structures, Rodrigues-Martins et al. (2007a) reported that the MTOCs formed upon high overexpression of DSAS-6 contained a ‘tube-like centriole scaffold’ at their centre. SAS-6 therefore appears to regulate the formation of a tubular centriole scaffold in both *C. elegans* and *Drosophila* (Pelletier et al., 2006; Rodrigues-Martins et al., 2007a). This structure appears to be central to centriole formation in these organisms by modulating centriole assembly and in particular the microtubules that form the centriolar walls. However, the generality of this tube structure in all species is not clear as in many organisms centriole assembly is initiated from a cartwheel-like structure (a hub with nine radiating spokes). *Chlamydomonas* SAS-6 localises to a central part of the cartwheel, with a null mutant of SAS-6, *bld12*, specifically lacking that part (Nakazawa et al., 2007). An identical localisation is seen for *Tetrahymena* SAS-6 (Kilburn et al., 2007). Whilst centrioles are formed in the *bld12* mutant, they are often abnormal, consisting of between 7 and 11 microtubule triplets rather than the normal nine (Nakazawa et al., 2007). It is likely that SAS-6 determines the radial shape of the cartwheel, thereby stabilising the 9-fold symmetry of the centriole. Therefore, in each organism studied to date, SAS-6 appears to be central to the formation of a structure that regulates the assembly of new centrioles, be it a tube or cartwheel.

HsSAS-6 is recruited to the centrosome during duplication, localising to the proximal part of the procentriole. HsSAS-6 levels are cell cycle regulated, with protein levels starting to accumulate at the end of G1 and the protein being degraded in mitosis (Strnad et al., 2007). Destruction of HsSAS-6 is mediated by the APC/C in association with its activator Cdh1. Cdh1 binds a KEN box in the C-terminus of HsSAS-6, targeting the protein for destruction by the 26S proteasome at telophase. Mutation of the KEN box prevents destruction of HsSAS-6, causing centriole overduplication. This effect was greater than that seen with HsSAS-6 overexpression alone suggesting that degradation of HsSAS-6 partially suppresses centriole overduplication. Regulation of HsSAS-6 proteins levels is therefore important in restricting procentriole formation to one per centriole. Consistent with this, blocking proteasome function with the inhibitor Z-L3VS lead to the formation of multiple centrioles around a single pre-existing centriole (Duensing et al., 2007).
SAS-6 interacts with SAS-5 (Leidel et al., 2005) and together they are required for both central tube formation and SAS-4 recruitment in *C. elegans* embryos (Delattre et al., 2006; Pelletier et al., 2006). However, to date, no orthologues of SAS-5 have been identified through sequence similarity. This may be somewhat surprising considering its association with SAS-6 in *C. elegans*, and the clear role that SAS-6 plays in centriole duplication in a range of organisms. On the other hand, SAS-4 has been shown to have a role in centriole formation in *Drosophila* and humans. Whilst *Drosophila* SAS-4 and *C. elegans* SAS-4 share only weak sequence similarity, DSAS-4 is required for centriole formation in embryos and somatic cells, with *DSas-4* mutants lacking centrioles and, as a consequence, cilia (Basto et al., 2006; Stevens et al., 2007). Additionally, overexpression of DSAS-4 in unfertilised eggs results in the de novo formation of centrioles (Peel et al., 2007). The human SAS-4 orthologue is known as CPAP or CENPJ, and both DSAS-4 and CPAP/CENPJ have been shown to localise to centrioles (Basto et al., 2006; Kleylein-Sohn et al., 2007). Mutations in the gene encoding CPAP/CENPJ have been identified in the disease, primary microcephaly, suggesting that this centriolar protein plays an important role during prenatal neurogenesis (Bond et al., 2005). More recently, RNAi depletion of CPAP/CENPJ has been shown to suppress Plk4-induced centriole overduplication (Kleylein-Sohn et al., 2007). Indeed, CPAP/CENPJ localises to the region between the parental centrioles and nascent procentrioles in cells overexpressing Plk4, suggesting that CPAP/CENPJ plays a direct role in centriole duplication.

ZYG-1 cannot be placed clearly into any of the known protein kinase families (O'Connell et al., 2001). However, Plk4 in humans, also known as Sak in *Drosophila*, has been suggested to be the functional homolog of ZYG-1. Plk4/Sak is necessary for centriole duplication in both *Drosophila* and human cells, acting early in the pathway, much in the same manner as ZYG-1 in *C. elegans* embryos (Bettencourt-Dias et al., 2005; Habedanck et al., 2005). Mutation or depletion of Sak in *Drosophila* cells leads to the loss of centrioles, whilst spermatids in Sak mutants lack centrioles and are therefore unable to make sperm axonemes (Bettencourt-Dias et al., 2005). Furthermore, overexpression of Sak in *Drosophila* embryos and unfertilised eggs causes centriole amplification and de novo centriole formation, respectively (Peel et al., 2007; Rodrigues-Martins et al., 2007b). In both these cases, centriole formation depended on the activity of DSAS-6 and DSAS-4, further implicating Sak in a similar pathway to ZYG-1. Loss of Plk4 in human cells also causes a step-wise reduction in centriole numbers (Bettencourt-Dias et al., 2005;
Habedanck et al., 2005). Additionally, active Plk4 is required for centriole reduplication in S-phase arrested U2OS cells. Plk4 is dependent on its ability to localise to the centrosome to phosphorylate substrates at the centrosome to promote centriole duplication (Habedanck et al., 2005). Overexpression of Plk4 causes centriole overduplication by promoting the formation of multiple procentrioles around a single existing centriole during S-phase (Habedanck et al., 2005; Kleylein-Sohn et al., 2007). Interestingly, Plk-4 induced centriole overduplication is dependent upon Cdk2 activity, whilst Cdk2 is unable to cause centrosome overduplication in the absence of Plk4 (Duensing et al., 2007; Habedanck et al., 2005).

Plk4-induced centriole biogenesis has been coupled with RNAi to screen centriolar proteins for a role in centriole formation in human cells (Kleylein-Sohn et al., 2007). Plk4 recruitment was confirmed as an early step in centriole formation, not dependent upon any of the other proteins tested. Depletion of HsSAS-6 suppressed Plk4-induced procentriole assembly and prevented the recruitment of other centriolar proteins (Habedanck et al., 2005; Kleylein-Sohn et al., 2007). Centriole biogenesis was also suppressed upon depletion of CPAP, Cep135 or γ-tubulin. Depletion of CP110 also prevented procentriole formation (Habedanck et al., 2005; Kleylein-Sohn et al., 2007), but not the recruitment of HsSAS-6. HsSAS-6, CPAP, Cep135 and γ-tubulin are therefore recruited early after Plk4 induction to form procentrioles. These proteins are mutually dependent on each other and are required for the development of procentrioles, whilst CP110 functions at a later stage. Depletion of centrin2 and 3 did not prevent Plk4-induced procentriole formation or the recruitment of CPAP, Cep135 nor CP110 (Kleylein-Sohn et al., 2007). Centrin is also not required for the localisation of HsSAS-6, whilst depletion of HsSAS-6 causes the loss of centrin (Strnad et al., 2007). Therefore, Plk4, HsSAS-6, CPAP, Cep135, γ-tubulin and CP110 are required at different stages of procentriole formation, with immunoelectron microscopy revealing that they associate with different centriolar structures (Kleylein-Sohn et al., 2007) (Figure 1.7). HsSAS-6 associates only transiently with nascent procentrioles, whilst CPAP and Cep135 form a core structure located within the proximal lumen of both parental centrioles and nascent procentrioles. Cep135 is a homolog of *Chlamydomonas* Bld10p (Ohta et al., 2002), a protein that localises to the cartwheel structure and stabilises the 9-fold symmetry of the centriole (Hiraki et al., 2007; Matsuura et al., 2004). In fact, *bld10* null mutants lack discrete centriolar structures, whilst mutants lacking Bld12p (SAS-6) form abnormal
Figure 1.7 A model for centriole assembly in human cells
Plk4 (red) induces the formation of procentrioles on the walls of the existing centrioles. hSAS-6 (green) is recruited early to the procentriole bud and remains at the proximal end of the growing centriole. γ-tubulin, CPAP and Cep135 (brown) are recruited after hSAS-6 and reside in the proximal lumen of the procentriole. The procentriole grows through the addition of microtubules under a CP110 cap (yellow), such that CP110 always appears at the distal end of the procentriole regardless of its length. Polyglutamylation of tubulin in the centriole walls stabilises the newly incorporated microtubules. From Kleylein-Sohn et al. (2007)
centrioles with between 7 and 11 microtubule triplets (Nakazawa et al., 2007). CP110 associates with procentrioles and then localises to the distal tips of all centrioles, regardless of their elongation state (Kleylein-Sohn et al., 2007). This suggests that α-/β-tubulin dimers are inserted underneath a CP110 cap, such that CP110 always remains at the distal end during centriole elongation. The localisation and function of Plk4, HsSAS-6 and CPAP in centriole formation in human cells therefore appears to be very similar to that of ZYG-1, SAS-6 and SAS-4 in C. elegans embryos, whilst other known centriolar proteins have been shown to contribute directly to this pathway. This implies that the fundamental aspects of centriole formation have been conserved during evolution. Orthologues of SPD-2 have been proposed for a number of organisms on the basis of sequence similarity (Kemp et al., 2004; Pelletier et al., 2004). Whilst the role of SPD-2 in centrosome maturation appears to be conserved between species, its requirement for centriole duplication remains unclear. Drosophila SPD-2 is essential for PCM recruitment and hence microtubule nucleation (Dix and Raff, 2007; Giansanti et al., 2008). However, quantification of centriole numbers in fixed brain preparations from DSpd-2 mutants revealed that there was no decrease in centriole numbers as compared to wild-type (Dix and Raff, 2007). Furthermore, spermatocyte analysis in DSpd-2 mutants suggests that SPD-2 is required for maintenance of centriole cohesion, but not centriole duplication (Giansanti et al., 2008). Mammalian SPD-2, known as Cep192, also regulates PCM recruitment and centrosome maturation (Gomez-Ferreria et al., 2007; Zhu et al., 2008). During centrosome maturation, Cep192 and pericentrin are mutually dependent for their localisation to centrosomes and are required for the subsequent assembly of γ-TuRCs (Zhu et al., 2008). However, Gomez-Ferreria et al. (2007) reported that centriole duplication in HeLa cells was not perturbed upon depletion of Cep192, but that loss of Cep192 induces premature centriole disengagement. Additionally, Cep192 was not required for Plk4-induced centriole overduplication (Kleylein-Sohn et al., 2007). To the contrary, Zhu et al. (2008) reported that RNAi-mediated depletion of Cep192 in HeLa cells prevented centriole duplication in over 80% of treated cells. Furthermore, Cep192 depletion prevented centriole overduplication in S-phase arrested U2OS cells. Whilst these results appear to be contradictory, the discrepancies in centriole duplication phenotype upon depletion of Cep192 may reflect differences in silencing efficiency achieved in these studies. If this is the case, it suggests that the phenotypes are dosage-specific to the amount of Cep192 remaining in the cells.
Clearly, a number of the proteins identified in *C. elegans* as being essential for centriole duplication are conserved in other species. However, a range of additional proteins, as discussed earlier, have also been implicated in centriole duplication in these species. Additionally, for example, centrobin is a daughter centriole-associated protein implicated in centriole duplication. Depletion of centrobin prevented both centriole duplication in HeLa cells and centrosome reduplication in S-phase arrested U2OS cells (Zou et al., 2005). Together, this suggests that centriole formation is a more complex process in higher organisms, possibly due to differences in centriole structure between *C. elegans* and vertebrates.

### 1.6 Centrosomes and Cancer

The fidelity of genetic material segregation into the two daughter cells during mitosis greatly depends on the establishment of a bipolar mitotic spindle. As each of the two spindle poles are organised by centrosomes, the presence of two centrosomes at mitosis is crucial to spindle bipolarity. Therefore, like DNA, the centrosome must duplicate once and only once in each cell cycle, so that there are two centrosomes present by the time the cell enters mitosis. Synchronisation of the centrosome duplication and DNA replication cycles thus produces a strict correlation between the number of centrosomes and the ploidy of the cell. Deregulation of the centrosome cycle would result in centrosome abnormalities or amplification, increasing the chances of mitotic defects and uneven chromosome segregation [reviewed in (Fukasawa, 2007)] (Figure 1.8A). Failure of duplicated centrosomes to separate would result in the formation of monopolar spindles, whilst centrosomes that do not undergo an appropriate maturation process would be unable to form a functional mitotic spindle. Additionally, failure to maintain structural integrity or centriole pairing would result in centrosome fragmentation. Such centriole pair splitting is seen when p16 is lost from cells (McDermott et al., 2006). If the fragments retained the ability to nucleate MTs, they would be able to form multipolar spindles. Similarly, centrosome amplification can also lead to the formation of aberrant mitotic spindles organised by more than two spindle poles, although in some instances supernumerary centrosomes can coalesce into two spindle poles [reviewed in (Nigg, 2002)].

Cancer cells frequently display supernumerary centrosomes which is in turn correlated with chromosome instability (D'Assoro et al., 2002; Fukasawa, 2005). Supernumerary
Figure 1.8 Deregulation of the centrosome cycle results in mitotic defects

A. The presence of two centrosomes (yellow) at mitosis is crucial to the formation of a bipolar spindle. If the duplicated centrosomes fail to separate at the onset of mitosis a monopolar spindle is formed. If more than two centrosomes are present, a multipolar spindle can form. DNA shown in blue, α-tubulin shown in red. B. There are four prominent models for the appearance of supernumerary centrosomes in cells. In model I centrosomes undergo more than one round of duplication in a single cell cycle. In models II and III, aborted division and cell fusion results in tetraploidy and doubling of centrosome numbers. In the fourth model, centrosome amplification can be caused by inappropriate activation of the de novo centriole formation pathway (not shown). From Nigg (2002). C. Centrosome overduplication may occur through deregulation of templated duplication. This could involve more than one round of duplication per cell cycle or the formation of more than one progeny centriole per maternal template. From Nigg (2007).
centrosomes can arise through, at least, four distinct mechanisms [reviewed in (Nigg, 2002)] (Figure 1.8B). Firstly, deregulation of centrosome duplication may lead to several rounds of duplication within the same cell cycle. This includes loss of cell cycle control, resulting in centrosome reduplication and thus more than one round of duplication per cell cycle, and loss of copy number control, leading to the generation of multiple centrioles around a single template (Nigg, 2007) (Figure 1.8C). Secondly, aborted cell division would result in a tetraploid cell containing two centrosomes. Thirdly, cell fusion would also result in tetraploidization and doubling of centrosome number. Fourthly, inappropriate activation of the de novo centriole assembly pathway would contribute excessive centriole numbers. Significantly, mammalian cells appear to lack checkpoints for tetraploidization, supernumerary centrosomes, and cytokinesis failure (Uetake and Sluder, 2004; Wong and Stearns, 2005), demonstrating how easily each of these mechanisms could contribute to cancer progression. Although these mechanisms are not mutually exclusive, it is unclear whether one predominates over the others as there is a lack of data with regard to how frequently each mechanism operates in vivo.

Deregulation of centrosome duplication may result in either the sequential or simultaneous assembly of new centrioles [reviewed in (Nigg, 2006)]. Centrioles could form sequentially through repeated rounds of templated reduplication within a single cell cycle. This would require prolongation of interphase, which may be achieved through a checkpoint-induced cell cycle arrest. Such an arrest may occur, for example, in response to DNA damage, providing opportunity for centrosomes to reduplicate during a delay in cell cycle progression. The simultaneous assembly of centrioles could occur through activation of the de novo centriole formation pathway or the creation of multiple procentrioles around a single maternal template, as is seen when Plk4 is overexpressed (Kleylein-Sohn et al., 2007). Failure of division does not cause such assembly of extra centrioles, but does lead to the presence of too many centrosomes. Aurora A is a mitotic kinase that is frequently overexpressed in tumor cells, causing division defects. This leads to both centrosome amplification and increased ploidy of the cells. Such conditions would favour the survival of genetically unstable and, therefore, potentially harmful cells [reviewed in (Nigg, 2006)].

Aberrant centrosome numbers are found in many cancer types and are thought to be a key event in tumour progression in breast, prostate and colon cancers (Saavedra et al., 2003). Furthermore, amplified centrosome numbers are often more pronounced in advanced stage
cancers and recurrent tumours [reviewed in (D'Assoro et al., 2002)]. Supernumerary centrosomes have been observed following the deregulation of several gene products, such as BRCA1 and BRCA2, which have been implicated in human cancer. This observation is often interpreted to reflect a role for these proteins in controlling centrosome duplication. However, they may act indirectly by disrupting the cell cycle, and thereby allowing centrosome reduplication in a single cell cycle. For example, loss or inactivation of proteins involved in homologous recombination, as part of the DNA damage repair mechanism, also results in centrosome amplification [reviewed in (Fukasawa, 2007)].

BRCA2, a familial breast cancer susceptibility gene, functions in homologous recombination. Loss of BRCA2 leads to centrosome amplification, implying a link between defective DNA repair and centrosome amplification (Tutt et al., 1999). BRCA1 is also involved in DNA repair, as well as other cellular functions. Mice lacking full-length BRCA1 display a high frequency of centrosome amplification. Whilst it remains unclear whether BRCA1 localises at centrosomes, it may be involved in maintaining centrosome integrity through ubiquitylation of γ-tubulin (Starita et al., 2004). In support of this, expression of non-ubiquitylatable γ-tubulin results in centrosome amplification. Homologous recombination is also mediated by the Rad51 protein family. Loss of members of this family induces a prolonged G2-arrest and leads to centrosome amplification (Bertrand et al., 2003; Dodson et al., 2004). G2-arrest allows centrosomes to re-acquire duplication competency and reduplicate, however this may only occur when the p53-dependent G2/M checkpoint is compromised.

The most commonly mutated gene in cancer is the tumour suppressor p53. p53 is a transcription factor that induces cell cycle arrest, at G1/S or G2/M, or apoptosis in response to DNA damage. Loss of p53 results in supernumerary centrosomes in both tissue culture cells and tumours (Carroll et al., 1999; Fukasawa et al., 1996). Furthermore, deletion of p21, a transcriptional target of p53 and an inhibitor of cyclin-Cdk2 complexes, also leads to centrosome amplification (Duensing et al., 2000; Tarapore et al., 2001). p53 is therefore involved in the control of centrosome duplication through its transcriptional regulation of p21. Indeed, the human papillomavirus (HPV) type 16 E7 oncoprotein stimulates centrosome reduplication by inactivation of p21. In vitro, cells that lack functional p53 can be induced to undergo centrosome amplification during prolonged S-phase arrest caused by inhibiting DNA replication with drugs such as hydroxyurea (HU) or aphidicolin (Balczon et al., 1995; Bennett et al., 2004; Tarapore and Fukasawa, 2002). However, such
centrosome overduplication requires several hours and would need a substantial delay in cell cycle progression in culture cells.

Understanding the pathway leading to centrosome overduplication in S-phase arrested cells is of clinical importance. Many chemotherapies target DNA synthesis, with transient exposure to subtoxic concentrations of such drugs shown to induce centrosome amplification in cells lacking p53 (Bennett et al., 2004). When released from arrest these cells suffer extensive chromosome destabilisation. It is hypothesised that this effect may explain why recurrent tumours, occurring after chemotherapy treatment, often exhibit more malignant characteristics that the original tumours. During chemotherapy, tumour cells exposed to subtoxic levels of DNA synthesis inhibiting drugs will arrest in S-phase and undergo centrosome overduplication in the absence of p53. When the chemotherapy is stopped, these cells will be released from arrest and re-enter the cell cycle. The presence of supernumerary centrosomes will lead to multipolar divisions causing mitotic catastrophe and chromosome instability, driving tumour progression. If centrosome overduplication could be prevented in these cells it would limit tumour re-emergence following seemingly successful chemotherapy. However, the pathway leading to centrosome amplification has not been explored in much detail.

1.7 AIMS AND OBJECTIVES

The presence of two centrosomes at mitosis is vital for the formation of a functional bipolar spindle and therefore the equal segregation of genetic material into the daughter cells. Supernumerary centrosomes are frequently observed in cancer cells, leading to the formation of multipolar spindles. This contributes to chromosome missegregation and aneuploidy. Still, the pathway that leads to centrosome overduplication is largely unknown.

As measured by C-Nap1 and/or γ-tubulin staining, Cdk2 activity and microtubules have previously been shown to be required for centrosome overduplication in HU-arrested CHO cells (Balczon et al., 1999; Matsumoto et al., 1999; Meraldi et al., 1999). Dynein on the other hand has been reported not to be required for normal templated duplication, as observed by centrin staining (Quintyne and Schroer, 2002). Studies investigating centrosome duplication generally focus solely on single centrosome markers. However, many recent studies have demonstrated the sequential recruitment of proteins to newly
forming centrioles, suggesting that some proteins will be early markers of centriole assembly, whilst other will be late markers. Therefore, we propose to use a range of centrosomal markers to investigate the possibility of observing intermediates formed in the centrosome overduplication pathway, rather than exclusively focusing on either early or late markers.

Experimentally, centrosome overduplication can be induced by exposing p53-deficient cells to drugs such as HU that arrest cells in S-phase through inhibiting DNA replication. Using this approach in CHO, U2OS and p53\textsuperscript{-/-} MEF cells, combined with a range of pharmacological inhibitors, this study aims to investigate the molecular pathway that leads to centrosome overduplication. Using specific inhibitors, the roles of MTs, dynein, heat shock proteins, Cdk5 and nuclear export in centrosome overduplication in CHO and U2OS cells will be investigated. This will be coupled with the employment of a range of centrosomal markers to decipher intermediates formed during this process.
CHAPTER TWO

MATERIALS AND METHODS
### 2.1 MATERIALS

#### 2.1.1 REAGENTS

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precision Plus all blue protein standards</td>
<td>Bio-Rad (Hemel Hempstead, UK)</td>
</tr>
<tr>
<td>Hoechst 33258</td>
<td>Calbiochem (Darmstadt, Germany)</td>
</tr>
<tr>
<td>EDTA; EGTA; NaCl; KCl; Na$_2$HPO$_4$; KH$_2$PO$_4$</td>
<td>Fisher Scientific (Loughborough, UK)</td>
</tr>
<tr>
<td>Ethanol</td>
<td></td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
</tr>
<tr>
<td>BSA (Fraction V)</td>
<td>Fluka (Gillingham, UK)</td>
</tr>
<tr>
<td>D-MEM with GlutaMAX$^{TM}$-I</td>
<td>Invitrogen (Paisley, UK)</td>
</tr>
<tr>
<td>F-12 Nutrient Mixture (Hams) with GlutaMAX$^{TM}$-I</td>
<td></td>
</tr>
<tr>
<td>Opti-MEM$^{®}$ with GlutaMAX$^{TM}$-I</td>
<td></td>
</tr>
<tr>
<td>Heat-inactivated Foetal Bovine Serum</td>
<td></td>
</tr>
<tr>
<td>Penicillin-Streptomycin Solution</td>
<td></td>
</tr>
<tr>
<td>0.05% Trypsin-EDTA</td>
<td></td>
</tr>
<tr>
<td>Lipofectamine$^{TM}$ 2000</td>
<td></td>
</tr>
<tr>
<td>Glass bottom culture dishes 33 mm with coverslip 0.175 mm thick</td>
<td>Iwaki (Tokyo, Japan)</td>
</tr>
<tr>
<td>12 or 27 mm in diameter</td>
<td></td>
</tr>
<tr>
<td>ProtoFLOWgel (30% w/v acrylamide)</td>
<td>Flowgen Bioscience (Nottingham, UK)</td>
</tr>
<tr>
<td>Bacto-agar</td>
<td>Oxoid (Basingstoke, UK)</td>
</tr>
<tr>
<td>Bacto-tryptone</td>
<td></td>
</tr>
<tr>
<td>Yeast Extract</td>
<td></td>
</tr>
<tr>
<td>BCA Protein Assay</td>
<td>Pierce (Rockford, USA)</td>
</tr>
<tr>
<td>Skimmed milk powder (Marvel)</td>
<td>Premiere Beverages (Stafford, UK)</td>
</tr>
<tr>
<td>Qiafilter Plasmid Maxipreps</td>
<td>Qiagen (Hilden, Germany)</td>
</tr>
<tr>
<td>FuGene 6 Transfection Reagent</td>
<td>Roche (Lewes, UK)</td>
</tr>
<tr>
<td>ProTran Nitrocellulose membrane</td>
<td>Schleicher &amp; Schuell (Dassel, Germany)</td>
</tr>
<tr>
<td>Coverslips 22 mm diameter, No 1.5</td>
<td>VWR International (Lutterworth, UK)</td>
</tr>
<tr>
<td>Glass slides</td>
<td></td>
</tr>
<tr>
<td>Whatman® 3MM Chromatography Paper</td>
<td>Whatman (Maidstone, UK)</td>
</tr>
</tbody>
</table>

All chemicals were of analytical grade or higher. Unless indicated in the table above, all chemicals were purchased from Sigma (Poole, UK).
2.1.2 Antibodies

The following antibodies were used for indirect immunofluorescence microscopy, probing Western blots or microinjection into mammalian cells. The working dilution is given for each antibody with the corresponding final concentration where known.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution ([antibody])</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-α-tubulin (mouse)</td>
<td>1:2000 (0.3 µg/ml)</td>
<td>Sigma</td>
</tr>
<tr>
<td>anti-γ-tubulin (clone GTU-88)</td>
<td>1:1000</td>
<td>Sigma</td>
</tr>
<tr>
<td>anti-γ-tubulin (rabbit)</td>
<td>1:500</td>
<td>Sigma</td>
</tr>
<tr>
<td>anti-acetylated tubulin (mouse)</td>
<td>1:5000 (0.2 µg/ml)</td>
<td>Sigma</td>
</tr>
<tr>
<td>anti-centrin (rabbit)</td>
<td>1:500 (1.4 µg/ml)</td>
<td>Abcam</td>
</tr>
<tr>
<td>anti-centrin2 (N17; rabbit)</td>
<td>1:100 (2 µg/ml)</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>anti-C-Nap1 (rabbit)</td>
<td>1:750 (1 µg/ml)</td>
<td>A.M. Fry (Fry et al., 1998a)</td>
</tr>
<tr>
<td>anti-dynein intermediate chain (clone 70.1)</td>
<td>2 mg/ml; in 0.75x PBS</td>
<td>Sigma</td>
</tr>
<tr>
<td>anti-GFP (clone GFP-20)</td>
<td>1:2000 (1 µg/ml)</td>
<td>Sigma</td>
</tr>
<tr>
<td>anti-GFP (rabbit)</td>
<td>1:1000 (0.5 µg/ml)</td>
<td>Abcam</td>
</tr>
<tr>
<td>anti-golgin-97 (clone CDF4)</td>
<td>1:200 (1 µg/ml)</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>anti-myc (clone 9B11)</td>
<td>1:1000 (0.5 µg/ml)</td>
<td>Cell Signalling</td>
</tr>
<tr>
<td>anti-Nek2 (monoclonal)</td>
<td>1:250 (1 µg/ml)</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>anti-ninein (rabbit)</td>
<td>1:1000</td>
<td>M.M. Mogensen (Moss et al., 2007)</td>
</tr>
<tr>
<td>anti-nucleophosmin (mouse)</td>
<td>1:500</td>
<td>(Shu et al., 2005)</td>
</tr>
<tr>
<td>anti-OFD-1 (rabbit)</td>
<td>1:100</td>
<td>(Romio et al., 2003)</td>
</tr>
<tr>
<td>anti-PCM-1 (rabbit)</td>
<td>1:500</td>
<td>A. Merdes (Dammermann and Merdes, 2002)</td>
</tr>
<tr>
<td>anti-pericentrin (rabbit)</td>
<td>1:500 (1 µg/ml)</td>
<td>Abcam</td>
</tr>
<tr>
<td>anti-polyglutamylated tubulin (mouse)</td>
<td>1:5,000 (0.2 µg/ml)</td>
<td>Abcam</td>
</tr>
<tr>
<td>anti-rootletin (rabbit)</td>
<td>1:500 (0.52 µg/ml)</td>
<td>H.L. Tsai and A.M. Fry</td>
</tr>
<tr>
<td>goat anti-mouse IgG Alexa 488</td>
<td>1:200 (10 µg/ml)</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>goat anti-mouse IgG Alexa 594</td>
<td>1:200 (10 µg/ml)</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>goat anti-mouse IgG AP conjugate</td>
<td>1:7500</td>
<td>Promega</td>
</tr>
<tr>
<td>goat anti-rabbit IgG Alexa 488</td>
<td>1:200 (10 µg/ml)</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>goat anti-rabbit IgG Alexa 594</td>
<td>1:200 (10 µg/ml)</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>goat anti-rabbit IgG AP conjugate</td>
<td>1:7500</td>
<td>Promega</td>
</tr>
<tr>
<td>mouse IgGs</td>
<td>2 mg/ml; in 0.75x PBS</td>
<td>Sigma</td>
</tr>
</tbody>
</table>
2.1.3 Drugs

The following drugs were used to treat cells at the indicated final concentration. The appropriate drug was added to fresh pre-warmed culture media and mixed well before being added to cells. More details about each compound can be found in the appendix.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Full name</th>
<th>[stock]</th>
<th>[final]</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-AAG</td>
<td>17-(Allylamino)-17-demethoxygeldanamycin</td>
<td>1 mM in DMSO</td>
<td>1 µM</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Cytochalasin D</td>
<td></td>
<td>10 mg/ml in DMSO</td>
<td>10 µg/ml</td>
<td>Sigma</td>
</tr>
<tr>
<td>EHNA</td>
<td>erythro-9-(2-hydroxy-3-nonly)adenine</td>
<td>300 mM in DMSO</td>
<td>300 µM</td>
<td>Sigma</td>
</tr>
<tr>
<td>Geldanamycin</td>
<td></td>
<td>1 mM in DMSO</td>
<td>1 µM</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>HSPI-1</td>
<td>Heat Shock Protein Inhibitor 1 (3,4-Methyleneoxy-benzylidine-γ-butyrolactam)</td>
<td>50 mM in DMSO</td>
<td>100 µM</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>HU</td>
<td>Hydroxyurea</td>
<td>100 mM in ddH₂O</td>
<td>2 or 16 mM (Balczon et al., 1995)</td>
<td>Sigma</td>
</tr>
<tr>
<td>LMB</td>
<td>Leptomycin B</td>
<td>37 µM in ethanol</td>
<td>20 nM</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Mimosine</td>
<td>(S)-α-Amino-β-[1-(3-hydroxy-4-oxopyridine)]propionic acid</td>
<td>10 mM in DMSO</td>
<td>0.5 mM</td>
<td>Sigma</td>
</tr>
<tr>
<td>Monastrol</td>
<td></td>
<td>100 mM in ethanol</td>
<td>100 µM (Kapoor et al., 2000)</td>
<td>Tocris</td>
</tr>
<tr>
<td>Nocodazole</td>
<td>Methyl-(5-[2-thienylcarbonyl]-1H-benzimidazol-2-yl) carbamate</td>
<td>5 mM in DMSO</td>
<td>5 µM</td>
<td>Sigma</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Olomoucine</td>
<td>2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine</td>
<td>50 mM in DMSO</td>
<td>360 µM</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Roscovitine</td>
<td>2-(R)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine</td>
<td>50 mM in DMSO</td>
<td>180 µM</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Vanadate</td>
<td>Sodium orthovanadate</td>
<td>100 mM in ddH₂O</td>
<td>1-20 µM</td>
<td>Sigma</td>
</tr>
</tbody>
</table>
2.2 CELL CULTURE

2.2.1 CELL MAINTENANCE

Chinese Hamster Ovary (CHO) cells and CHO cells stably transformed with constructs expressing centrin1-GFP (CHO:centrin1-GFP) were cultured in F-12 Nutrient Mixture (Hams) with GlutaMAX™-I supplemented with 10% v/v heat-inactivated foetal bovine serum (FBS) and penicillin/streptomycin at 100 units/ml and 100 µg/ml, respectively. Generation of the CHO:centrin1-GFP cell line has previously been described (Crookes, 2004). p53−/− mouse embryonic fibroblasts (MEFs) (gift from S. Macip), p53−/− Cdk2−/− MEFs (gift from P. Kaldis) and U2OS cells were cultured in Dulbecco’s Modified Eagle’s Media (DMEM) with GlutaMAX™-I supplemented with 10% v/v FBS and penicillin/streptomycin at 100 units/ml and 100 µg/ml, respectively.

Cells were maintained at 37°C in a humidified 5% CO₂ atmosphere and passaged before reaching confluence. Growth media was aspirated and cells washed with phosphate buffered saline (PBS) (137 mM NaCl, 8.1 mM Na₂HPO₄, 2.7 mM KCl, 1.4 mM KH₂PO₄). Cells were detached using either PBS containing 0.5 mM EDTA or 0.05% Trypsin-EDTA and seeded into pre-warmed growth media at an appropriate density.

2.2.2 CELL STORAGE

Cells were washed in PBS, detached using PBS-EDTA and resuspended in pre-warmed culture media. Cells were then pelleted by centrifugation at 1100 rpm for 5 minutes at room temperature. The supernatant was discarded and the cell pellet resuspended in 10% v/v DMSO in FBS (CHO and U2OS cells) or 10% v/v DMSO in DMEM (MEFs) and transferred to cryotubes (Nunc). The cryotubes were then placed in an isopropanol filled Nalgene Cryo 1°C Freezing Container and placed at -80°C overnight. The next day the cryotubes were transferred to liquid nitrogen for long-term storage.

To thaw cells, a cryotube was removed from liquid nitrogen storage and rapidly thawed in a 37°C waterbath. Cells were then washed with pre-warmed media, pelleted at 1100 rpm for 5 minutes at room temperature, resuspended in media and transferred to a culture dish.
2.2.3 Preparation of acid-etched sterile coverslips
Coverslips were first rinsed in sterile water, before being placed in 1M HCl for 30 minutes. The acid was then discarded and the coverslips placed in 100% ethanol for at least 30 minutes. The coverslips were then individually air-dried, placed together in a glass dish and baked at 240°C for 4 hours.

2.2.4 Centrosome overduplication assay
To reproduce the centrosome overduplication assay as first described by Balczon et al. (1995), cells were seeded onto acid-etched sterile coverslips at a density of 1 x 10^5 cells/ml in 6-well dishes and cultured overnight. The following day, cells were washed once with PBS and placed in fresh growth media containing 2 mM HU for CHO cells, p53^-/- MEFs and p53^-/- Cdk2^-/- MEFs; or 16 mM HU for U2OS cells. Additional inhibitors were either added concurrently with the HU or after 18 hours depending on whether the inhibitor would cause a G1 arrest. Following the desired assay duration, cells were then fixed and processed for immunofluorescence microscopy.

2.2.5 Transient transfections
For transient transfection studies, cells were seeded onto acid-etched sterile coverslips at a density of 1 x 10^5 cells/ml in 6-well dishes and cultured overnight. The next day they were transfected with plasmid DNA using either Lipofectamine 2000 or FuGENE 6 transfection reagents.

2.2.5.1 Lipofectamine 2000
On the day of transfection, a Lipofectamine : DNA mix was prepared at a ratio of 3 µl : 1 µg in OptiMEM (serum and penicillin-streptomycin free) according to the manufacturer’s instructions. The standard growth media on the cells was replaced with serum and penicillin-streptomycin free media and the Lipofectamine : DNA mix added dropwise to the cells. The dishes were then returned to the incubator and following 5 hours the media was replaced with standard growth media (with serum and penicillin-streptomycin). The cells were then cultured for a minimum of 16 hours to allow for expression before being used in subsequent assays.
2.2.5.2 FuGENE 6

On the day of transfection, a FuGENE : DNA mix was prepared at a ratio of 6 µl : 1 µg in serum and penicillin-streptomycin free growth media according to the manufacturer’s instructions. The growth media on the cells was replaced with fresh complete growth media and the FuGENE : DNA mix added dropwise to the cells. The cells were then cultured for a minimum of 16 hours to allow for expression before being used in subsequent assays.

2.2.6 Flow cytometry

Adherent cells were washed with PBS, detached with PBS-EDTA and collected by centrifugation at 1100 rpm for 5 minutes at room temperature. Cells were then washed with ice-cold PBS and slowly resuspended in 70% ice-cold ethanol pre-cooled at -20°C whilst vortexing to disperse cell clumps. Fixed cells were then stored at -20°C for at least 16 hours. Immediately prior to flow cytometry analysis, cells were washed again with ice-cold PBS and resuspended in staining solution (0.02 mg/ml propidium iodide, 0.2 mg/ml RNaseA in PBS). After incubation at room temperature for 30 minutes the DNA content of the samples was measured using a FACSCAN II (Becton Dickinson) instrument and analysed with CellQuest software.

2.3 Microscopy

2.3.1 Indirect immunofluorescence microscopy

Adherent cells were rinsed once with 1x PBS and fixed with ice-cold 100% methanol pre-cooled at -20°C and incubated for a minimum of 10 minutes at -20°C. Alternatively, cells were fixed with 4% w/v paraformaldehyde-PBS for 5 minutes at room temperature, followed by three 5 minute washes with PBS and permeabilisation with 0.25% v/v Triton in PBS for 5 minutes. Cells were then rehydrated/washed four times for 5 minutes each with PBS. Coverslips were then blocked by the addition of 1% w/v BSA-PBS for 10 minutes at room temperature. Blocking solution was then replaced with primary antibody diluted in 3% w/v BSA-PBS and incubated for 50 minutes in a humidity chamber at room temperature. Coverslips were then washed three times with PBS and incubated with secondary antibody in 3% w/v BSA, 0.3 µg/ml Hoechst 33258 in PBS for 50 minutes at room temperature in a humidity chamber protected from light. Again coverslips were washed three times with PBS, then dipped in H2O and inverted onto a drop of anti-fade
mounting solution (80% v/v glycerol, 3% w/v n-propyl gallate in PBS) on glass slides and sealed with clear nail varnish.

Fluorescence microscopy was performed using a Nikon TE300 inverted microscope using a Plan Apo 100x DIC oil immersion objective (NA 1.4). Fluorescence images were captured using an Orca ER CCD camera (Hamamatsu, Japan) using Openlab 5.0 software (Improvision, UK) and processed using Adobe Photoshop 7. Alternatively, microscopy was performed on a Leica TCS SP5 laser scanning confocal microscope (LSCM) equipped with a Leica DMI 6000B inverted microscope using a Plan Apo 63x oil objective (NA 1.4). Images were captured and processed using Leica LAS AF software.

2.3.2 CENTROSOME AND NUCLEAR INTENSITY MEASUREMENTS
Cells were processed for indirect immunofluorescence microscopy and images captured on the same day using constant exposure times and gain settings determined to be within the linear range of the camera. A 3 µm x 3 µm region of interest (ROI) was positioned either in the nucleus or to encompass the centrosome. The mean fluorescence intensity of this ROI, minus background, was then measured using Openlab 5.0 software.

2.3.3 MICROINJECTION
Microinjection was performed on a Nikon TE300 inverted microscope using a Plan Fluor 40x Ph2 objective (NA 0.6). Cells were cultured on Iwaki glass bottom culture dishes (No. 1.5 coverglass) and maintained at 37°C on the stage. Antibodies at 2 mg/ml in 0.75x PBS were injected into cells using an Eppendorf Micromanipulator System equipped with Eppendorf Femtotips. Cells were returned to the incubator and HU added to the media 4 hours post-injection.

2.3.4 LIVE CELL IMAGING
Time lapse imaging of cells was performed on a Leica TCS SP5 LSCM equipped with a Leica DMI 6000B inverted microscope and SuperZ Galvo stage. Cells were cultured on glass-bottom culture dishes (No. 1.5 coverglass, Iwaki) and maintained on the stage at 5% CO₂ and 37°C using a microscope temperature control system (The Cube and The Box, Life Imaging Services). Cells were located under brightfield and GFP fluorescence monitored with 10% of a 488 nm argon laser using an HCX Plan Apo 63x oil objective (NA 1.4). An area of cells was selected for imaging with a scan zoom of 2. Typically, z-
section parameters of 30-40 steps of 0.3-1 µm were set. A z-stack was collected every 10 minutes for the duration of the experiment, often up to 60 hours. Z-stacks were collapsed into a single maximum intensity projection per timepoint using Leica LAS AF software.

2.3.5 Photobleaching experiments
Photobleaching experiments were performed on a Leica TCS SP5 LSCM equipped with a Leica DMI 6000B inverted microscope using an HCX Plan Apo 63x oil objective (NA 1.4) and a scan zoom of 3. Cells were cultured in glass-bottom culture dishes and maintained on the stage at 5% CO₂ and 37°C using a microscope temperature control system. For FRAP, ROIs 3 µm x 3 µm were bleached with 5 iterations at 100% laser power (488 nm argon laser). Two or three images were captured prior to bleaching and 30 images after bleaching at intervals of 10 seconds, or 15 images at intervals of 2 seconds using 10% laser power. For FLIP, an image was acquired every 5 seconds for 20 seconds prior to bleaching and between each bleach cycle with 10% laser power. A bleach area encompassing the whole cell but excluding areas of interest was bleached with 10 iterations of 100% laser power. Ten bleach cycles were performed per cell. For both FRAP and FLIP, the fluorescence intensity of the ROIs (P) was determined using Leica LAS AF software for each timepoint. The fluorescence intensity of the background (B) was determined and the corrected fluorescence intensity (P-B) calculated; correction for photobleaching due to imaging was not required. The amount of fluorescence recovery was calculated as the corrected fluorescence intensity value of a given frame divided by the correct fluorescence intensity value of the first frame before photobleaching and was expressed as a percentage.

2.3.6 Electron microscopy
Transmission electron microscopy (TEM) analysis was performed by The Electron Microscope Laboratory at the University of Leicester. Briefly, adherent cells were rinsed with PBS, lifted with PBS-EDTA and collected by centrifugation at 1100 rpm for 5 minutes at room temperature. The cells were then washed twice with PBS, fixed with 3% v/v glutaraldehyde in PBS and post-fixed in 1% w/v OsO₄. Following washing with PBS, samples were stained en-bloc with 2% w/v uranyl acetate in 30% v/v ethanol and completely dehydrated through an ethanol series. Samples were infiltrated in propylene oxide/Spurr resin before final embedding and polymerisation in Spurr resin. Sections approximately 80 nm thick were cut using a Reichert Ultracut E ultramicrotome, collected on copper mesh grids and counter stained with Reynolds’ lead citrate. Sections were
observed using a JEOL 1220 electron microscope using an accelerating voltage of 80 kV. Images were recorded using a SIS Megaview III digital camera.

2.4 MOLECULAR BIOLOGY TECHNIQUES

2.4.1 PLASMIDS

<table>
<thead>
<tr>
<th>Protein and tag</th>
<th>Vector</th>
<th>From</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrin1-GFP</td>
<td>pEGFP-N1</td>
<td>M. Bornens</td>
</tr>
<tr>
<td>GFP-Nek2A-WT</td>
<td>pEGFP-C1</td>
<td>A.M. Fry (Hames and Fry, 2002)</td>
</tr>
<tr>
<td>Myc-laminA</td>
<td>pCIneo</td>
<td>S. Shackleton (Lloyd et al., 2002)</td>
</tr>
<tr>
<td>Myc-dynamitin</td>
<td>pCMV-myc</td>
<td>R. Vallee (Helfand et al., 2002)</td>
</tr>
</tbody>
</table>

Large scale DNA preparations were made of each plasmid and confirmed by DNA sequencing.

2.4.2 BACTERIAL TRANSFORMATIONS

Chemically-competent *E. coli* (DH5α) were thawed on ice and 100 µl added to 100 ng ice-cold DNA and mixed by gentle tapping before incubation on ice for 30 minutes. The mixture was then heat-shocked at 42°C for 45 seconds and immediately returned to ice for 2 minutes before adding 400 µl Luria Broth (LB) (1% w/v tryptone, 1% w/v NaCl, 0.5% w/v yeast extract). The cells were then incubated at 37°C, 225 rpm for 1 hour. Following incubation, the cells were plated out onto LB-agar (LB plus 2% w/v agar) containing the appropriate antibiotic for selection (ampicillin at 100 µg/ml, or kanamycin at 50 µg/ml). Plates were then incubated at 37°C for 16 hours.

2.4.3 PLASMID PREPARATION

Single colonies were used to inoculate starter cultures of 200 µl LB-antibiotic and were incubated at 37°C, 225 rpm for 8 hours. This was then diluted to 100 ml with LB-antibiotic and incubated at 37°C, 225 rpm for 16 hours. The culture was then centrifuged at 6000 g for 20 minutes at 4°C and the supernatant discarded. Plasmid DNA was then purified from the pellet according to the QiaFilter Maxiprep protocol and eluted with ddH₂O. Yield was quantified by measuring OD₆₀₀ and the DNA preparation diluted to a concentration of 1 µg/µl.
2.4.4 Sequencing
Sequencing was performed by Lark Technologies (Takely, UK) and data analysed by Gene Jockey software.

2.5 Analysis of Proteins

2.5.1 Cell extracts
Whole cell lysates were prepared in RIPA buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% w/v SDS, 1% v/v Nonidet P-40, 0.5% w/v deoxycholate, 2 mM EDTA, 1 mM PMSF, 1 mM DTT, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1% v/v aprotonin). Briefly, cells were washed with PBS and lysed directly in RIPA buffer for 30 minutes on ice. Insoluble material was then pelleted by centrifugation at 14,000 rpm, for 10 minutes at 4°C and the total protein concentration of the supernatant determined by BCA assay.

2.5.2 BCA Protein Assay
Protein content of cell lysates was determined using the BCA protein assay. BCA working reagent was prepared according to the manufacturer’s instructions and 1 ml added to 5 µl of cell lysate. The assay mixture was then incubated at 37°C for 30 minutes, allowed to cool to room temperature and the absorbance at 562 nm measured. A serial dilution of BSA standards was prepared and assayed alongside the protein samples to allow construction of a standard curve from which the protein concentration of the samples could be determined.

2.5.3 SDS-PAGE
Protein samples were resolved on 12 or 15% polyacrylamide gels by electrophoresis. Gels were cast and resolved using the Mini Protean II polyacrylamide gel system from Bio-Rad. Resolving gel (40 or 50% ProtoFLOWgel (30% w/v acrylamide), 375 mM Tris-HCl (pH 8.8), 0.1% w/v SDS, 0.12% w/v APS, 0.1% v/v TEMED) was overlaid with stacking gel (40 or 50% ProtoFLOWgel (30% w/v acrylamide), 125 mM Tris-HCl (pH 6.8), 0.1% w/v SDS, 0.12% APS, 0.1% TEMED). Samples were mixed with an appropriate volume of 2 x protein solubilising buffer (0.05 M Tris-HCl (pH 6.8), 2% w/v SDS, 20% v/v glycerol, 0.05 mg/ml bromophenol blue, 10 mM DTT) and denatured at 95°C for 5 minutes. Precision plus all blue protein standards were loaded alongside samples. Electrophoresis
was performed in Tris-Glycine running buffer (25 mM Tris-base, 192 mM glycine, 0.1% w/v SDS) at 180 V for 1 hour.

2.5.4 WESTERN BLOTTING
Proteins were transferred to nitroceullulose membrane for immunodetection using semi-dry blotting. Transfer was performed by soaking the gel in transfer buffer (25 mM Tris, 192 mM glycine, 10% v/v methanol) for 5 minutes, along with 6 pieces of Whatman 3MM filter paper and a piece of nitrocellulose (0.2 or 0.45 µm pore size). These were then sandwiched together in a semi-dry blotter (Hoefer Science), as directed by the manufacturer. Transfer was carried out at 70 mA for 1 hour. The blots were then removed and stained with Ponceau Red solution (0.1% w/v Ponceau S, 5% v/v acetic acid) and the markers and lane positions annotated in pencil. Blots were then blocked in 5% w/v non-fat milk powder in 0.1% v/v Tween-20 in PBS for 1 hour and incubated with primary antibody in 5% w/v non-fat milk powder in 0.1% v/v Tween-20 - PBS for 1 hour on a rocking platform. The membrane was then washed for 30 minutes with six changes of washing buffer (0.1% v/v Tween-20 in PBS) and incubated in alkaline phosphate conjugated secondary antibody in 5% w/v non-fat milk powder in 0.1% v/v Tween-20 - PBS for 1 hour on a rocking platform. The membrane was then washed as before, with a final wash in AP buffer (100 mM Tris-Hcl (pH 9.5), 100 mM NaCl, 5 mM MgCl₂). The blots were then developed in AP buffer containing NBT (0.33 mg/ml) and BCIP (0.16 mg/ml). At the appropriate moment, the staining reaction was stopped by washing the membrane in PBS.
CHAPTER THREE

CENTROSONE OVER DUPPLICATION IN HYDROXYUREA-ARRESTED
CHINESE HAMSTER OVARY CELLS
3.1 INTRODUCTION

3.1.1 CENTROSOme DUPLICATION

The establishment of a bipolar spindle is essential for the equal segregation of genetic material into the two daughter cells during mitosis. Spindle bipolarity is dependent upon the presence of two centrosomes that organise one spindle pole each. At the completion of mitosis each daughter cell possesses one centrosome, which must then duplicate before the next mitotic event. Whilst centrosome duplication has been well defined at the morphological level in mammalian cells (Alvey, 1985; Kuriyama and Borisy, 1981), the molecular events involved remain enigmatic.

Classically, centrosome duplication has been described as occurring in a semi-conservative templated manner, with each existing centriole subtending the growth of a new centriole. As one new centriole forms in association with each pre-existing centriole the semi-conservative templated duplication process provides a counting mechanism for controlling centriole number. In G1-phase of the cell cycle, a centrosome consists of two centrioles surrounded by PCM. As cells progress into S-phase, procentrioles form at the proximal ends of the existing centrioles. The position of the procentriole extending from the wall of the existing centriole defines an orthogonal arrangement for the pair that exists from duplication through to mitosis. Initiation of procentriole formation at the G1/S transition intricately links centrosome duplication with DNA replication so that when a cell enters mitosis it will have duplicated both its DNA and centrosome (Hinchcliffe and Sluder, 2001; Sluder and Hinchcliffe, 1998).

Throughout S-phase procentriole extension occurs in a proximal to distal direction with centrosome maturation, characterised by the recruitment of additional PCM components, occurring in late G2 (Blagden and Glover, 2003). The duplicated centrosome continues to act as a single microtubule organising centre until prophase, when centrosome disjunction occurs and the duplicated centrosomes separate to form the two poles of the spindle (Bahe et al., 2005; Fry et al., 1998a; Fry et al., 1998b). In a licensing event for centrosome duplication in the next cell cycle, the two centrioles within each spindle pole lose their perpendicular orientation, exposing the site from which a new pro-centriole can form; this event is known as centriole disengagement (Tsou and Stearns, 2006a; Tsou and Stearns, 2006b).
3.1.2 CENTROSOME OVERDUPICATION

The physical separation of the licensing and duplication events into different cell cycle stages prevents overduplication within a single cell cycle. However, centrosome duplication can be uncoupled from the cell cycle, resulting in centrosome amplification. Balczon et al. first showed in 1995 that prolonged arrest in S-phase with HU led to CHO cells accumulating multiple centrosomes (Balczon et al., 1995). Subsequent studies have revealed that it is the loss of p53 that enables cells to undergo centrosome overduplication during prolonged S-phase arrest (Bennett et al., 2004; Tarapore and Fukasawa, 2002). Specifically, the loss of p53 leads to reduced levels of the Cdk inhibitor, p21, and subsequent activation of Cdk2. The role of Cdk2 in centrosome duplication will be discussed in detail in Chapter 7. Hence, in simple terms, centrosome duplication can be uncoupled from the DNA replication cycle in cells with compromised p53 function.

This overduplication assay has since been extensively utilised in both CHO and U2OS cells to investigate the role of a range of factors in centrosome duplication including, for example, microtubules, Plk1, Plk2, Plk4, Cdk2, Cep192 and centrobin (Balczon et al., 1999; Kleylein-Sohn et al., 2007; Liu and Erikson, 2002; Matsumoto et al., 1999; Meraldi et al., 1999; Warnke et al., 2004; Zhu et al., 2008). However, the mechanisms involved in centrosome overduplication remain poorly understood.

3.1.3 SUPERNUMERARY CENTROSOMES AND CANCER CELLS

Understanding how centrosome amplification occurs in cells is of clinical importance as the vast majority of cancer cells are characterised by extra, or supernumerary, centrosome numbers (Brinkley, 2001; D'Assoro et al., 2002; Pihan et al., 2003). Supernumerary centrosomes may arise through a number of mechanisms. The failure of cells to complete cytokinesis would lead to tetraploidization and doubling of centrosome number. Cell fusion would also lead to tetraploidization and centrosome doubling, although how often this occurs is not known. Alternatively, uncoupling of the centrosome and DNA cycles would lead to multiple rounds of centrosome duplication within a single cell cycle (Nigg, 2006). The human papillomavirus type 16 E7 oncoprotein has been shown to induce centrosome overduplication and MEFs lacking the p53 tumour suppressor protein accumulate multiple functional centrosomes during a single cell cycle (Duensing et al., 2000; Fukasawa et al., 1996).
Supernumerary centrosomes can coalesce to organise bipolar spindles in a manner dependent on the microtubule-based motor protein dynein (Brinkley, 2001; Quintyne et al., 2005). However, the presence of more than two centrosomes can result in the formation of multipolar spindles and thus abnormal chromosome segregation leading to aneuploidy, the most common feature of cancer cells. Aneuploidy and chromosome instability drive cancer progression, leading to heterogenous populations of cells that can contain cells with more malignant or drug resistant properties (Lengauer et al., 1998).

Many commonly used anti-cancer agents, including actinomycin D, arabinoside C and 5-fluorouracil, function by inhibiting DNA replication. As the most commonly mutated gene in cancer is the tumour suppressor p53 it can be envisaged that application of these drugs could inadvertently promote centrosome amplification during S-phase arrest (Bennett et al., 2004). Any cells that subsequently survive the treatment will therefore have the potential to develop a high level of chromosome instability and form a highly malignant population.

The aim of this study was to examine the molecular events required for centrosome overduplication by utilising the HU assay in CHO cells. The initial starting point was therefore to reproduce this assay in our laboratory and test a range of centrosomal markers that will be used to characterise the centrosome overduplication pathway.
3.2 RESULTS

3.2.1 CENTROSOMES OVERDUPPLICATE IN CHO CELLS DURING S-PHASE ARREST

The first step was to reproduce the centrosome duplication assay first described by Balczon et al. using suitable centrosomal markers (Balczonek et al., 1995). It was essential that the centrosomal markers used would localise to duplicated centrosomes and as such would be able to provide reliable information on the number of centrosomes in a cell. γ-tubulin localises to the PCM of centrosomes and is responsible for the nucleation of microtubules (Moritz et al., 1995). Cells enter G1 with a single centrosome containing a pair of centrioles. However, the two centrioles can move slightly apart and acquire their own distinct region of PCM. As cells proceed into mitosis the amount of γ-tubulin at each centrosome increases considerably in preparation for increased nucleating activities required to form the mitotic spindle (Blagden and Glover, 2003) (Figure 3.1A). Therefore, γ-tubulin was selected as a suitable marker for the centrosome and used to score the number of centrosomes within a cell.

Centrosome duplication can be dissociated from the cell cycle by arresting cells at the G1/S transition or in S-phase with HU treatment (Balczonek et al., 1995). HU inactivates the enzyme ribonucleoside reductase which is responsible for the synthesis of deoxynucleotides (Timson, 1975). As the nucleotide pool is depleted DNA synthesis is inhibited and cell cycle arrest in S-phase results. However, in cells with compromised p53 function centrosome duplication continues leading to supernumerary centrosomes (Figure 3.1B).

To study centrosome overduplication, CHO cells were incubated with HU at a final concentration of 2 mM. At 0, 24 and 48 hours following treatment the cells were fixed and processed for immunofluorescence microscopy. γ-tubulin along with C-Nap1 (centrosomal Nek2 associated protein 1) antibodies were used to detect centrosomes. C-Nap1 is a 280 kDa protein which forms part of the linkage between the mother and daughter centrioles. It is therefore most concentrated at the proximal ends of centrioles and is an excellent marker for the centrosome (Fry et al., 1998a). Using these centrosomal markers it can clearly be seen that cells had undergone centrosome overduplication during prolonged S-phase arrest (Figure 3.1C). Flow cytometry was used to confirm the cell cycle arrest (data not shown). Quantification of the number of γ-tubulin staining centrosomes in each cell revealed that
Figure 3.1 Centrosome overduplication in HU-arrested CHO cells

A. CHO cells were fixed and stained with γ-tubulin (green) and α-tubulin (red) antibodies. Merge panels include DNA stained with Hoechst (blue). Cells in interphase or mitosis are seen. Scale bars, 10 µm. B. Schematic representation of HU arrest that leads to the formation of multiple centrosomes in CHO cells. C. CHO cells were treated with HU and fixed at 0, 24 and 48 hours. Coverslips were processed for immunofluorescence microscopy and cells stained with γ-tubulin (red) and C-Nap1 (green) antibodies. Merge panels include DNA stained with Hoechst (blue). Scale bar, 10 µm. D. Histogram indicates the number of centrosomes as detected by γ-tubulin staining in CHO cells treated for 0, 24 and 48 hours with HU. At least 200 cells were counted per experiment and the results are shown as the mean of three experiments. Standard deviations are indicated. E. Histogram indicates the mean number of centrosomes per cell in CHO cells treated with HU for 0, 24 and 48 hours with HU. Data are shown as the average of three experiments and standard deviations are indicated. Approximately 200 cells were counted per experiment.
centrosomes increased in number over the treatment period as expected (Figure 3.1D). At 0 hours only 14% of cells contained three or more centrosomes. However, following 24 and 48 hours treatment with HU this increased to 68% and 90%, respectively. In fact at 48 hours the mean number of centrosomes per cell was approximately 4.2, compared with less than two at 0 hours (Figure 3.1E).

Centrosomes consist of a pair of centrioles surrounded by pericentriolar material. TEM analysis of centrioles in untreated and HU-arrested CHO cells revealed that in cells undergoing centrosome overduplication multiple centrioles do form (Figure 3.2). In addition, a number of electron dense centriolar satellites are seen in association with the centrioles (Figure 3.2). These results are consistent with those published by Balczon et al. (1995) and Kubo et al. (1999).

### 3.2.2 Centrin localises to overduplicated centrosomes

Centrin is widely used as a centriole marker due to its localisation in the distal lumen of centrioles (Paoletti et al., 1996). It is also reported to be one of the first proteins to localise at sites of newly forming centrioles in both the templated and de novo assembly pathways [reviewed in: (Salisbury, 2007)]. However, in addition to its localisation in centrioles, centrin is also found in the PCM and centriolar satellites (Baron and Salisbury, 1988; Kuriyama et al., 2007; Paoletti et al., 1996; White et al., 2000), although localisation to these additional structures may well be cell type-dependent. Staining of asynchronous cells with a commercial antibody from Santa Cruz raised against a peptide mapping to the N-terminus of human centrin2 showed co-localisation of two centrin dots with each γ-tubulin staining centrosome in mitotic cells. In interphase cells, additional centrin dots were observed clustered around the centrosome (Figure 3.3). This suggests that this cenrin2 antibody stains both centrioles and centriolar satellites.

To investigate the staining pattern of centrin in cells with overduplicated centrosomes CHO cells were exposed to HU and fixed and stained at 0, 24 and 48 hours. Staining with the centrin2 antibody again revealed co-localisation of a subset of centrin dots with γ-tubulin along with extra dots clustered around the centrosomes. Each γ-tubulin dot co-localised with at least one centrin dot, confirming that the γ-tubulin dots associated with at least one centriole. Staining with an alternative commercial centrin antibody from Abcam,
Figure 3.2 TEM analysis reveals the presence of extra centrioles in HU-arrested CHO cells
Untreated (A) or HU-arrested (B) CHO cells were cultured for 48 hours at 37°C. Cells were then collected, fixed and processed for transmission electron microscopy. Centrosomes consist of two centrioles, HU-arrested cells contain multiple centrioles as centrosomes become overduplicated during prolonged S-phase arrest. Each centriole has a number of associated electron dense centriolar satellites (arrowheads in selected images). C. and D. Serial sections are shown of the indicated region of two individual cells treated with HU for 48 hours. Scale bars, 500 nm.
Figure 3.3 Centrin localises to both the centrioles and centriolar satellites
CHO cells, representing different stages of the cell cycle as indicated, were fixed and stained with centrin2 (red) and γ-tubulin (green) antibodies. Merge panels include DNA stained with Hoechst (blue). Magnified images of centrosomes are shown. Note that in interphase, centrin2 antibodies stain centriolar satellites (small red dots) as well as centrioles.
raised against amino acids 157-172 of human centrin1, a sequence that is found also in
centrin2, but not centrin3, produced a different staining pattern. There was strong co-
localisation between the centrin and γ-tubulin signals suggesting that this centrin antibody
stains both centrioles and PCM (Figure 3.4).

3.2.3 Centrin1-GFP is Incorporated Into New Centrioles

A CHO cell line stably expressing centrin1-GFP had previously been generated in our
laboratory (Crookes, 2004). Many studies have utilised GFP-tagged centrin1 to monitor
centriole dynamics in live cells [for example: (Loncarek et al., 2008; Piel et al., 2000)] and
to follow the formation of centrioles along the de novo pathway (La Terra et al., 2005;
Uetake et al., 2007). We found that centrin1-GFP is a centriole marker throughout the cell
cycle and is incorporated into new centrioles during centrosome duplication in
asynchronous cells (Figure 3.5). In addition, live cell imaging of CHO:centrin1-GFP cells
treated with HU revealed the formation of additional centrin1-GFP dots during the time
course of the experiment (Figure 3.6). To confirm that these centrin1-GFP dots localised to
overduplicated centrosomes, fixed CHO:centrin1-GFP cells were analysed by
immunofluorescence microscopy with γ-tubulin antibodies (Figure 3.7A). There was
strong co-localisation between the GFP signal and γ-tubulin pattern, with at least one
centrin1-GFP dot per γ-tubulin dot. Therefore, centrin1-GFP is incorporated into
overduplicated centrosomes. In addition, quantification of the number of γ-tubulin staining
centrosomes in each cell revealed that centrosomes increased in number over the treatment
period as expected (Figure 3.7B).

3.2.4 Centrin1-GFP Expression Accelerates the Rate of Centrosome
Overduplication

Comparison of the centrosome overduplication profiles at each time point for the CHO:WT
and CHO:centrin1-GFP cell lines revealed that centrosome overduplication was
accelerated in cells expressing centrin1-GFP (Figure 3.8A). Prior to treatment with HU the
mean number of γ-tubulin staining centrosomes per cell was comparable for each cell line.
Therefore, expression of centrin1-GFP in asynchronous cells did not lead to cells with
abnormal numbers of centrosomes. However, following HU treatment, more centrosomes
were observed in cells expressing centrin1-GFP compared to the wild-type cell line.
Following 48 hours treatment, the mean number of centrosomes in CHO and
Figure 3.4 Overduplicated centrosomes in CHO cells stain for the centriolar marker centrin

A. CHO cells were treated with HU and fixed at 0, 24 and 48 hours. Coverslips were processed for immunofluorescence microscopy and cells stained with γ-tubulin (red) and centrin (Abcam; green) antibodies. Scale bar, 10 µm. B. CHO cells were treated with HU and fixed at 0, 24 and 48 hours. Coverslips were processed for immunofluorescence microscopy and cells stained with γ-tubulin (red) and centrin2 (Santa Cruz; green) antibodies. Scale bar, 10 µm.
Figure 3.5 Centrin1-GFP is a centriole marker throughout the cell cycle and is incorporated into new centrioles

A. Asynchronous CHO:centrin1-GFP cells were plated on glass bottom dishes and imaged for a total duration of 50 hours using a Leica TCS SP5 LSCM. Cells were located under brightfield (upper images) and the corresponding GFP fluorescence monitored with 10% power of a 488 nm argon laser (lower images). Images are shown as the maximum intensity projections of the z-stack collapsed into a single image per timepoint. Time shown as hours:minutes. Scale bar, 10 µm. B. Images from a time-lapse movie of centrosome duplication in asynchronous CHO:centrin1-GFP cells.
Figure 3.6 Live cell imaging of CHO:centrin1-GFP during HU arrest reveals the formation of multiple centrin dots

CHO:centrin1-GFP cells grown on glass bottom culture dishes were treated with HU and imaged for a total of 38 hours. A stack of z-sections consisting of 25 steps each 1 μm in size was acquired every ten minutes for the duration of the experiment. Images are shown as the maximum intensity projection of the z-stack. Time shown as hours:minutes. Scale bar, 10 μm.
Figure 3.7 Centrin1-GFP is incorporated into overduplicated centrosomes in CHO cells arrested with HU
A. CHO:centrin1-GFP cells were treated with HU and fixed at 0, 24 and 48 hours. Coverslips were processed for immunofluorescence microscopy and cells stained with γ-tubulin (red) antibodies and the GFP fluorescence (green) monitored. Merge panels include and DNA stained with Hoechst (blue). Scale bars, 10 µm. B. Histogram indicates the number of centrosomes as detected by γ-tubulin staining in CHO:centrin1-GFP cells treated for 0, 24 and 48 hours with HU. At least 200 cells were counted per experiment and the results are shown as the mean of three experiments. Standard deviations are indicated.
CHO:centrin1-GFP cells was 4.2 and 5.9, respectively (Figure 3.8B). Additionally, the maximum number of centrosomes observed per cell was 9 in wild-type cells, compared to 12 in the CHO:centrin1-GFP cells line (Figure 3.8A). Western blot analysis with centrin antibodies revealed that centrin1-GFP was expressed at a level approximately 2.5 times above endogenous centrin in the stable cell line (Figure 3.8C and D).

To ensure that the rate of centrosome overduplication in CHO:centrin1-GFP cells was not an artefact of a particular cell line, centrosome overduplication was assayed in three individual CHO:centrin1-GFP cell lines (A1, A5 and C1) previously generated in the laboratory (Crookes, 2004). Fluorescence microscopy revealed the formation of multiple centrin1-GFP dots in each of the cell lines (Figure 3.9A) and comparison of the mean number of γ-tubulin staining centrosomes revealed that the rate of centrosome overduplication was equivalent in each (Figure 3.9B).

3.2.5 OVERDUPlicated CENTROSOMES ARE POSITIVE FOR A RANGE OF CENTROSOMAL MARKERS

To increase the choice of centrosomal markers that can be used in this study into the mechanisms of centrosome overduplication, CHO cells treated with HU for 48 hours were stained with a range of antibodies against centrosomal proteins. Pericentrin is a 220 kDa protein that forms complexes with γ-tubulin and localises to the PCM (Doxsey et al., 1994). In CHO cells pericentrin co-localised with γ-tubulin at the centrosome and also stained a number of cytoplasmic dots, some of which clustered around the centrosome. Following 48 hours HU treatment pericentrin was also found to localise to overduplicated centrosomes (Figure 3.10A). In addition, overduplicated centrosomes were found to stain for rootletin, ninein and Nek2 (Figure 3.10B).

To investigate whether protein levels of the key centrosomal markers that would be used in this study changed upon prolonged S-phase arrest Western blots of cell extracts of CHO cells treated with HU for 0 or 48 hours were probed with antibodies against centrin, γ-tubulin and α-tubulin (Figure 3.10C). The abundance of each protein was comparable in untreated and treated cells showing that these proteins were not altered in abundance due to the S-phase arrest.
Figure 3.8 Centrin1-GFP overexpression accelerates the rate of centrosome overduplication in CHO cells

A. Histograms compare the number of γ-tubulin staining centrosomes in CHO and CHO:centrin1-GFP cells following 0, 24 and 48 hours treatment with HU. Approximately 200 cells were counted per time-point, per experiment and data are shown as the mean +/- standard deviation of three independent experiments. B. Histogram compares the mean number of γ-tubulin positive centrosomes per cell in CHO and CHO:centrin1-GFP cells treated with HU for 0, 24 and 48 hours. Data are shown as the average of three experiments and standard deviations are indicated. Approximately 200 cells were counted per experiment. C. Asynchronous CHO:centrin1-GFP cell extracts were prepared and analysed by Western blot probed with centrin antibodies. D. Quantification of centrin and centrin1-GFP abundance in the CHO:centrin1-GFP cell line based on densitometry of the Western blot shown in C. Results are shown relative to endogenous centrin set on 1.
Figure 3.9 The rate of centrosome overduplication is comparable in three individual CHO:Centrin1-GFP cell lines

A. CHO:Centrin1-GFP cell lines A1, A5 and C1 were treated with HU for 48 hours. Following treatment cells were fixed and GFP fluorescence monitored. Merge panels include DNA stained with Hoechst 33258 (blue). B. Quantification of the mean number of γ-tubulin staining centrosomes at 48 hours post-HU treatment. Approximately 200 cells were counted per experiment and data are shown as the mean +/- standard deviation of three independent experiments.
Figure 3.10 Pericentrin, rootletin, ninein and Nek2 all localise to overduplicated centrosomes

A. CHO cells were treated with HU and fixed at 0 and 48 hours. Cells were processed for immunofluorescence microscopy and stained with pericentrin and γ-tubulin antibodies. B. CHO cells were treated with HU for 48 hours and processed for immunofluorescence microscopy. Cells were stained with antibodies against γ-tubulin (red) and rootletin, ninein or Nek2 (green). Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 µm. C. Cell extracts were prepared from CHO cells treated with HU for 0 and 48 hours. Western blots were probed with antibodies against centrin, γ-tubulin and α-tubulin.
3.2.6 U2OS CELLS ALSO UNDERGO CENTROSOME OVERDUPICATION DURING PROLONGED S-PHASE ARREST

U2OS cells also have compromised p53 function and as such are able to undergo centrosome overduplication during S-phase arrest (Stucke et al., 2002). However, to achieve an efficient arrest, U2OS cells had to be treated with a higher dose of HU and for longer time periods compared to CHO cells. Typically, U2OS were treated with 16 mM HU for 68 hours before being fixed and processed for immunofluorescence microscopy. Using the centrosomal markers γ-tubulin and C-Nap1 multiple centrosomes were seen to accumulate during the arrest period (Figure 3.11A). Additionally, centrin2 staining revealed the presence of supernumerary centrioles co-localising with γ-tubulin staining centrosomes (Figure 3.11B). Quantification of the number of cells with greater than 2 centrosomes before and after HU revealed that over 60% of cells undergo centrosome overduplication (Figure 3.11C). Therefore, U2OS cells provide an alternative cell type in which to investigate centrosome overduplication.

3.2.7 CELL WITH SUPERNUMERARY CENTROSOMES ORGANISE ABNORMAL MITOTIC SPINDLES

The effects of HU are reversible, so cells placed into drug-free media following the centrosome overduplication assay will continue into mitosis. To visualise the mitotic spindles formed by cells with too many centrosomes, CHO cell were treated with HU for 48 hours and then placed in drug-free media for 10 hours before being fixed. Staining of cells with α-tubulin antibodies revealed that cells organise abnormal mitotic spindles when they enter mitosis (Figure 3.12). These included many examples of multipolar spindles, as well as monopolar and bipolar spindles that each contained multiple centrosomes per spindle pole. Clearly, supernumerary centrosomes can contribute to aneuploidy as the genetic material is highly unlikely be faithfully segregated into the two daughter cells under these circumstances.
Figure 3.11 U2OS cells also undergo centrosome overduplication during prolonged HU arrest

U2OS cells were treated with HU for 68 hours, fixed and processed for immunofluorescence microscopy. Cells were stained with antibodies against γ-tubulin (red) and C-Nap1 (green) (A) or and γ-tubulin (green) and centrin2 (red) (B). Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 µm. C. Quantification of the percentage of U2OS cells with more than two centrosomes per cell as scored by γ-tubulin staining following 68 hours HU treatment. Approximately 200 cells were counted per experiment and data are shown as the mean +/- standard deviation of three independent experiments.
Figure 3.12 CHO cells with overduplicated centrosomes form abnormal mitotic spindles
CHO cells were treated with HU for 48 hours. The drug was then washed out for 10 hours and the cells fixed and processed for immunofluorescence microscopy. Cells were stained with antibodies against C-Nap1 (red) and α-tubulin (green). Images include DNA stained with Hoechst (blue). A series of mitotic cells are shown. Scale bar, 5 μm.
3.3 DISCUSSION

Centrosome duplication is a carefully controlled process tightly coupled with the DNA replication cycle. However, the two events can be dissociated experimentally in cells with compromised p53 function by inducing a prolonged S-phase arrest (Balczon et al., 1995). These cells continue to undergo centrosome duplication in the absence of DNA replication, accumulating supernumerary centrosomes. The presence of abnormal numbers of centrosomes is a common feature of cancer cells and drives aneuploidy through the construction of aberrant mitotic spindles (Brinkley, 2001; D'Assoro et al., 2002; Pihan et al., 2003). Supernumerary centrosomes are likely to have arisen at least in some cells by the overduplication of centrosomes within a single cell cycle (Nigg, 2006). However, whilst the mechanisms by which centrosome overduplication occur are poorly understood, the centrosome overduplication assay can be used to investigate the molecular events involved.

Overduplicated centrosomes form in both CHO and U2OS cells during prolonged S-phase arrest as both these cell types lack functional p53 pathways. These centrosomes stain with markers for both centrioles (centrin, C-Nap1, Nek2 and ninein) and the PCM (γ-tubulin and pericentrin), suggesting that these are bona fide functional centrosomes that have formed. Indeed, they all have the capacity for microtubule nucleation. Additionally, the presence of multiple centrioles in these cells was confirmed by TEM. Centriolar components are therefore formed into centrioles and PCM proteins recruited to them.

Centrin localises to the distal lumen of centrioles and is reported to be one of the first proteins to localise in newly forming centrioles being found in the pro-centriole bud (Paoletti et al., 1996; Salisbury, 2007). However, the role of centrin in centriole formation remains obscure, although it has been suggested that centrin/Sfi1 fibers form part of the cartwheel structure that is the earliest recognisable centriole precursor (Salisbury, 2007).

The role of centrin in the duplication of the yeast SPB is better understood than its function in centriole formation in mammalian cells. Human centrins were identified as homologues of Cdc31p from yeast. As a component of the half-bridge structure, Cdc31p is essential for the initial stages of SPB duplication. Indeed, conditional mutations in the centrin gene of yeast, CDC31, result in failure of the SPB to duplicate (Paoletti et al., 2003). The discovery
of a centrin-binding partner in yeast, Sfi1p, which contains a series of 23 internal centrin-binding repeats, brings new insight into the role of centrin in SPB/centriole formation (Kilmartin, 2003; Li et al., 2006; Salisbury, 2004). The assembly of centrin/Sfi1 fibers allows elongation of the half bridge structure, from which initiation of daughter SPB formation occurs (Li et al., 2006). Due to the high degree of conservation of centrin and Sfi1 proteins in yeast and mammals, it is suggested that centrin/Sfi1 fibers play similar roles in both SPB and centriole formation (Salisbury, 2007).

Roles for both centrin2 and centrin3 in centrosome duplication have previously been proposed. Knockdown of centrin2 by RNAi results in the failure of centriole duplication in HeLa cells and subsequent defects in cell cycle progression (Salisbury et al., 2002). Meanwhile, expression of human centrin3 has a dominant negative effect on centrosome duplication in yeast and *Xenopus* eggs, suggesting that human centrin3 can bind factors required for SPB/centrosome duplication in these species (Middendorp et al., 2000). Additionally, this study revealed that overexpression of centrin3 can promote centrosome duplication in mammalian cells. However, centrins have recently been demonstrated to be dispensable for the formation of multiple procentrioles around a single template as induced by Plk4 overexpression (Kleylein-Sohn et al., 2007). This suggests that centrin is not required for the formation of procentrioles, at least when Plk4 is overexpressed, but does not preclude a role for centrin in the stabilisation of the growing procentrioles.

In our experiment, overexpression of centrin1-GFP accelerated the rate of centrosome overduplication in CHO cells. Whilst centrin1 is predominately expressed in neuronal, ciliated and male germ cells (Laoukili et al., 2000), it is extensively used as a marker for normal and overduplicated centrioles in cultured cells (La Terra et al., 2005; Piel et al., 2000). In support of this we find that centrin1-GFP is a marker for centrioles throughout the cell cycle and is incorporated into newly formed centrioles. The centrin1 and 2 proteins are 84% identical, whilst centrin3 is 54% identical to both centrin1 and 2 (Middendorp et al., 1997). Additionally, according to amino acid comparison between centrin proteins, centrin1 and 2 belong in one subgroup, whilst centrin3 and yeast centrins belong to another (Gavet et al., 2003). It is possible that centrin1 adopts a centrin2 like function when ectopically expressed in cells and that acceleration of the rate of centrosome overduplication is a result of the increased abundance of a centriole precursor protein.
It is unclear whether overduplication is the result of unregulated templated duplication or whether de novo formation of centrioles also occurs, and in fact it may not be as simple as one or other pathway. During the licensing event, disengagement of paired centrioles requires the activation of the APC/C and separase (Tsou and Stearns, 2006b). It is unlikely that these conditions are met in cells in S-phase arrest. This suggests that disengagement occurs by a different mechanism in these cells or that de novo formation of centrioles occurs. Indeed, the rapid induction of aberrant centriole numbers detected under these experimental conditions is not readily explained solely by templated centriole formation. A recent study of centrosome amplification in HU-arrested CHO cells expressing GFP-tagged centrin2 has suggested that duplication is initially templated but that prolonged treatment with HU overrides the suppression of excess centriole replication in cells with existing centrioles leading to de novo centriole formation (Kuriyama et al., 2007).

The presence of a pre-existing centriole was reported to inhibit the de novo pathway (Khodjakov et al., 2002; La Terra et al., 2005), thus the pathway should be prohibited in these S-phase arrested cells. However, in the de novo pathway centrioles arise at sites of high concentration of centriole components. Indeed, de novo centriole formation in unfertilised *Drosophila* eggs can be induced by the overexpression of centriolar proteins, including Sas-6 and Sas-4 (Peel et al., 2007). It is therefore plausible to envisage that these sites can form during the extended periods of this assay, overriding the licensing step and templated duplication. This would also suggest that duplication along the templated semi-conservative pathway predominates in cycling cells as it occurs faster than the sites for de novo formation can assemble. Centriole formation via the de novo pathway would therefore normally be prevented by cell cycle progression and/or the inability of proteins to reach a critical abundance (Salisbury, 2007).

There may be several similarities between the different pathways, and it has been suggested, although not shown, that they may not be mutually exclusive (La Terra et al., 2005). Indeed during ciliogenesis, multiple basal bodies form through both centriolar and acentriolar pathways (Anderson and Brenner, 1971), reminiscent of both templated and de novo centriole formation. Additionally, under some experimental conditions, individual centrioles have been reported to act as templates for the initiation of multiple centrioles (Duensing et al., 2007; Kleylein-Sohn et al., 2007; Loncarek et al., 2008). The human papillomavirus type 16 E7 oncoprotein induces centrosome overduplication within a single
cell cycle (Fukasawa et al., 1996). This occurs, at least in part, through the formation of more than one pro-centriole per maternal template (Duensing et al., 2007). If single maternal centrioles can concurrently generate multiple daughter centrioles this overrides the requirement for the licensing step. Disengagement of the daughter centriole from the site of pro-centriole formation is no longer required as many sites must exist on each centriole. The generation of multiple daughter centrioles around a single maternal template has been shown to be dependent upon cyclin E-Cdk2 and Plk4 (Duensing et al., 2007; Kleylein-Sohn et al., 2007). Additionally, increased Sas-6 levels have been shown to promote the formation of more than one procentriole per centriole (Strnad et al., 2007). Therefore, Sas-6 levels also play a significant role in regulating the number of pro-centrioles formed around a maternal template.

Each pathway quite possibly contributes to centrosome overduplication in both cancer cells and the centrosome overduplication assay. Regardless of whichever of the pathways predominates in centrosome overduplication, the molecular pathway involved is not understood, so whilst this study will not speculate on whether centrosome overduplication is templated or de novo it will endeavour to elucidate the molecular events involved.
CHAPTER FOUR

THE EFFECT OF MICROTUBULE DEPOLYMERISATION ON CENTROSOME OVERDUPPLICATION
4.1 INTRODUCTION

4.1.1 MICROTUBULES AND CENTROSONE ASSEMBLY

As the microtubule-organising centre of the cell, the centrosome sits at the heart of a microtubule array. Microtubules may aid the trafficking of proteins to the centrosome through microtubule-associated proteins, particularly motor proteins. Dyneins are a family of minus-end directed motor proteins that generally move along microtubules towards the centrosome. Indeed, centriolar satellites move towards the centrosome in a dynein-dependent manner (Dammermann and Merdes, 2002; Kim et al., 2004; Kubo et al., 1999). However, not all centrosomal proteins rely on microtubules for their delivery to the centrosome. The dynamic exchange of γ-tubulin throughout the cell cycle does not require microtubules (Khodjakov and Rieder, 1999). Additionally, microtubules are only implicated in the long-range transport of Nek2 (Hames et al., 2005).

Depolymerisation of microtubules results in the dispersal of a number of centrosomal proteins, including centrin, pericentrin, ninein and PCM-1 (Dammermann and Merdes, 2002). PCM-1 is a 230 kDa specific component of centriolar satellites that localises around centrosomes (Kubo et al., 1999). PCM-1 containing centriolar satellites have been observed to move along microtubules towards centrosomes in an ATP-dependent manner (Kubo et al., 1999). Additionally, small granules of GFP-tagged PCM-1 have been directly followed by video microscopy, shuttling along microtubules between the cytoplasm and the centrosome (Dammermann and Merdes, 2002). Inhibition or depletion of PCM-1 leads to reduced targeting of centrin, pericentrin and ninein to the centrosome. Comparable effects are seen after inhibition of dynein following overexpression of the dynactin subunit, dynamitin (Dammermann and Merdes, 2002). Therefore, PCM-1 mediates the transport of these centrosomal components along microtubules in a dynein-dynactin dependent manner and microtubules play an important role in their recruitment to the centrosome.

4.1.2 MICROTUBULES AND CENTROSONE DUPLICATION

The centrosome must be replicated once, and only once, during each cell cycle. To achieve this, cells must have the capacity to coordinate the synthesis of centrosomal components with other cell cycle progression events, transport the subunits to the centrosome, and then assemble the subunits into a functional organelle.
Studies with unfertilised oocytes and fertilised eggs have demonstrated that these cells contain large pools of centrosomal subunits that can be recruited to assemble functional centrosomes (Gard et al., 1990; Palazzo et al., 1992; Sluder et al., 1990). Whilst fertilised eggs contain large cytoplasmic stockpiles of centrosomal proteins, somatic cells must synthesise centrosome proteins anew each cell cycle. Indeed both an intact nucleus and protein synthesis are required for centrosome duplication to occur in somatic cells (Kuriyama and Borisy, 1981; Kuriyama et al., 2007; Phillips and Rattner, 1976). Some centriolar proteins, such as Sas-6, are degraded in mitosis and start to accumulate again at the end of G1 in the following cell cycle (Strnad et al., 2007). Once synthesised, centrosome proteins must then be targeted to the parental centrosome and assembled into a functional centrosome complex. One advantage of a mediated transport system over diffusion-based mechanisms is that it has the potential to increase the local concentration of transported molecules.

Using the centrosome overduplication assay, Balczon et al. (1999) demonstrated that centrosome duplication is perturbed when microtubules are depolymerised, although it was shown that the synthesis of centrosomal proteins in these cells was not affected (Balczon et al., 1999). This suggests that cytoplasmic microtubules are either required for the transport of components to the centrosome for duplication or contribute to the association of proteins with the centrosome and centrosome organisation. However, whilst the microtubules of existing centrioles are insensitive to microtubule depolymerising drugs, the lack of centrosome duplication may simply reflect that centriolar microtubules cannot form in the presence of microtubule depolymerising agents. In fact, it has been demonstrated that daughter centriole nucleation and elongation is diminished in the presence of colcemid, a microtubule depolymerising drug (Kuriyama, 1982). To support the role of microtubules in centriole formation, microtubule depolymerising drugs also prevent basal body formation during ciliogenesis in oviducts (Lemullois et al., 1988).

Taken together, these results strongly suggest that centriolar microtubules cannot form in the presence of microtubule depolymerising drugs. However, the role of microtubules in recruiting centrosomal components essential for centrosome duplication is more obscure. Therefore, using the centrosome overduplication assay and a range of centrosomal markers, we decided to carefully investigate the role of microtubules in this process.
4.2 Results

4.2.1 Microtubules are required for centrosome overduplication

To commence our investigation into the role of microtubules in centrosome overduplication, we first aimed to reproduce the findings of Balczon et al. (1999). Using CHO cells arrested in S-phase with HU they used the microtubule depolymerising drug nocodazole to determine whether microtubules were necessary for the accumulation of centrosomes in these cells. Staining for the centrosomal marker γ-tubulin revealed that centrosome duplication was perturbed, therefore Balczon et al. (1999) concluded that microtubules are required for centrosome duplication.

Similarly, we used CHO cells treated concurrently with HU and nocodazole at final concentrations of 2 mM and 5 µm, respectively. Following 48 hours treatment, cells were fixed and stained for α-tubulin to demonstrate microtubule depolymerisation and γ-tubulin to detect the centrosome. Immunofluorescence microscopy revealed the presence of only two γ-tubulin staining centrosomes per cell following 48 hours treatment with HU and nocodazole (Figure 4.1A). Therefore, cells had failed to undergo centrosome overduplication. Quantification of the number of γ-tubulin staining centrosomes in cells confirmed that approximately 80% of cells had failed to accumulate more than two centrosomes (Figure 4.1B). Additionally, the mean number of centrosomes in cells treated with HU and nocodazole was 2.1, compared to 4.2 in cells treated with HU alone (Figure 4.1C). Our finding thus confirm those of Balczon et al. (1999) and we can conclude that microtubules are required for the overduplication of centrosomes as measured by γ-tubulin staining.

4.2.2 Centrin foci accumulate in the absence of microtubules

To further investigate the role of microtubules in centrosome overduplication, we utilised a range of centrosomal markers available in the laboratory. Centrin is reported to be one of the first proteins to localise to newly forming centrioles being found in the pro-centriole bud (Paoletti et al., 1996; Salisbury, 2007), it is therefore an early maker for centriole formation. CHO cells treated with HU and nocodazole for 48 hours were stained with the two commercial centrin antibodies; centrin and centrin2 (see section 3.2.2).
Figure 4.1 Microtubules are required for centrosome overduplication

A. CHO cells were treated with HU and nocodazole for 48 hours. Cells were processed for immunofluorescence microscopy and stained with γ-tubulin (green) and α-tubulin (red) antibodies. Merge panels include DNA stained with Hoechst (blue). Scale bar, 10 μm.

B. Histogram represents the number of γ-tubulin staining centrosomes per cell following 48 hours treatment with HU and nocodazole. Data are shown as the average of three independent experiments +/- standard deviation. At least 200 cells were counted per experiment.

C. Histogram indicates the mean number of γ-tubulin staining centrosomes per cell in CHO cells treated with HU alone or HU plus nocodazole. Data are shown as the average of three independent experiments +/- standard deviation. At least 200 cells were counted per experiment.
Immunofluorescence microscopy revealed that many small centrin foci as stained with either of these antibodies were present around the periphery of the nucleus. A number of these centrin foci were also seen to cluster into larger aggregates made up of individual foci. However, γ-tubulin only co-localised with one or two of these aggregates (Figure 4.2), suggesting that an early step in the overduplication pathway may proceed in the absence of microtubules.

To extend these studies further, we turned to the CHO:centrin1-GFP cell line. Correspondingly, these cells were treated with HU and nocodazole for 48 hours and processed for immunofluorescence microscopy. Centrin1-GFP foci were seen to form around the periphery of the nucleus, reminiscent of the centrin staining in wild-type cells treated with HU and nocodazole (Figure 4.3A and B) and again only two γ-tubulin staining centrosomes were seen per cell (Figure 4.3B and D). Examination of the three CHO:centrin1-GFP cell lines available demonstrated that these foci form and display a similar pattern in each cell line (Figure 4.3C).

Time-lapse imaging of CHO:centrin1-GFP cells commencing at the addition of HU and nocodazole revealed that individual foci appeared in the cytoplasm within two hours of treatment. These foci then became more apparent and formed aggregates over the next 6 hours (Figure 4.4). The foci were distributed in a perinuclear manner and were generally static, suggesting an association with the nuclear envelope (Figures 4.2, 4.3, 4.4 and 4.5). Treatment of cells with cytochalasin D to disrupt the actin network led to the loss of this perinuclear distribution (Figure 4.5C), suggesting a role for actin in this association.

**4.2.3 CENTRIN FOCI DO NOT NUCLEATE MICROTUBULES**

The ability of the centrin foci to nucleate microtubules was investigated by transferring cells briefly back to nocodazole-free media to allow microtubule regrowth. Specifically, CHO cells were treated with HU alone for 48 hours and then the microtubules briefly depolymerised and allowed to regrow for one minute. Multiple microtubule foci as visualised with α-tubulin antibodies were seen co-localising with the multiplied γ-tubulin staining centrosomes (Figure 4.6A). This confirms that overduplicated centrosomes are functional in so much as they can nucleate microtubules. When microtubule aster formation was similarly examined in CHO cells following 48 hours treatment with HU and
Figure 4.2 Centrin foci form in CHO cells treated with HU and nocodazole
CHO cells were treated with HU and nocodazole for 48 hours. Cells were processed for immunofluorescence microscopy and stained with centrin (A) or centrin2 (B) (green) antibodies and co-stained for either α-tubulin or γ-tubulin (red). Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 µm.
Figure 4.3 Centrin foci form in CHO:centrin1-GFP cells treated with HU and nocodazole

CHO:centrin1-GFP cells were treated with HU and nocodazole for 48 hours. Cells were processed for immunofluorescence microscopy and stained with α-tubulin (A) or γ-tubulin (B) (red) antibodies. Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 µm.

C. Three individual CHO:centrin1-GFP cell lines (A1, A5 and C1) were treated with HU and nocodazole for 48 hours and the GFP fluorescence monitored. Scale bar, 10 µm.

D. Histogram indicates the mean number of γ-tubulin staining centrosomes per cell in CHO:centrin1-GFP cells treated with HU alone or HU plus nocodazole. Data are shown as the average of three independent experiments +/- standard deviation. At least 200 cells were counted per experiment.
Figure 4.4 Live cell imaging of CHO:centrin1-GFP cells with depolymerised microtubules during HU arrest reveals the formation of centrin aggregates
CHO:centrin1-GFP cells grown on glass bottom culture dishes were treated with HU and nocodazole and imaged for a total of 29 hours. A z-stack consisting of 25 steps each 1 µm in size was acquired every ten minutes for the duration of the experiment. Images are shown as the maximum intensity projection of the z-stack. Time shown as hours:minutes. Scale bar, 10 µm.
Figure 4.5 Centrin foci predominately localise around the nuclear periphery in an actin-dependent manner

**A.** CHO:centrin1-GFP cells were treated for 48 hours with HU and nocodazole and imaged for brightfield and GFP fluorescence. Magnified images of GFP fluorescence is shown. Arrowheads in merge indicate location of aggregates. **B.** Two further examples of GFP fluorescence from cells treated as in A. **C.** Following 48 hours treatment as in A, cytochalasin D was added to the culture media for 2 hours. Cells were processed for immunofluorescence microscopy and stained for α-tubulin (red). Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 μm.
nocodazole, only one or two microtubule organising centres were observed (Figure 4.6B and D). These co-localised with the \( \gamma \)-tubulin staining centrosomes as expected since \( \gamma \)-tubulin is responsible for the nucleation of microtubules (Moritz et al., 1995). In the CHO:centrin1-GFP cell line only one or two of the centrin aggregates co-localised with the microtubule aster, confirming that the centrin foci lacking \( \gamma \)-tubulin were incapable of nucleating microtubules (Figure 4.6C).

Time-lapse imaging commencing when CHO:centrin1-GFP cells treated with HU and nocodazole for 48 hours were then placed in nocodazole-free media revealed that the centrin foci were able to coalesce when the microtubules reform (Figure 4.7A). The foci moved away from the nuclear periphery and were relatively dynamic whilst they came together. Immunofluorescence microscopy of cells fixed following 5 hours nocodazole washout showed that these collections of foci consisted of many centrin dots and that additional \( \gamma \)-tubulin had been to be recruited to their vicinity (Figure 4.7B).

### 4.2.4 Centrin Foci Contain Modified Tubulins

Depolymerisation of microtubules in CHO cells has been reported to result in the dispersal of the centrosomal proteins centrin, pericentrin, ninein and PCM-1 (Dammermann and Merdes, 2002). However, in this study, large cytoplasmic aggregates of these proteins were observed, suggesting that they may be different to the small centrin foci we see. To examine the properties of the centrin foci in more detail, CHO:centrin1-GFP cells treated with HU and nocodazole were stained for a range of centrosome associated proteins. It had already been noted that the centrin foci appeared to co-localise with \( \alpha \)-tubulin (Figures 4.2, 4.3 and 4.8). This suggested that the centrin foci contain nocodazole-resistant tubulin.

Many stabilised tubulins have post-translational modifications including those found in centrioles. Centrioles contain glutamylated tubulin, and loading of HeLa cells with anti-glutamylated tubulin antibodies provokes the transient disappearance of centrioles (Bobinnec et al., 1998a; Bobinnec et al., 1998b). Polyglutamylation of tubulin has been shown to be an early event in the centriole assembly process. During ciliogenesis polyglutamylated tubulin is detected in fibrous granules and newly forming basal bodies (Million et al., 1999). In contrast, glycylation is only detected along axonemal tubulin. To
Figure 4.6 Only γ-tubulin positive foci are able to nucleate microtubules

A. CHO cells were treated with HU for 48 hours before the microtubules were briefly depolymerised with nocodazole and allowed to regrow for 1 minute. Cells were fixed and processed for immunofluorescence microscopy. Cells were stained for γ-tubulin (red) and α-tubulin (green).

B. CHO cells were treated with HU and nocodazole for 48 hours, followed by a 2 minute washout to allow microtubule regrowth. Cells were processed for immunofluorescence microscopy and stained with γ-tubulin (red) and α-tubulin (green) antibodies.

C. CHO:centrin1-GFP cells were treated with HU and nocodazole for 48 hours, followed by a 2 minute washout. Cells were processed for immunofluorescence microscopy and stained for α-tubulin (red). Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 µm.

D. Histogram represents the number of microtubule asters per CHO cell treated with HU and nocodazole for 48 hours followed by a 2 minute drug washout. At least 200 cells were counted per experiment and data are shown as the average of three experiments. Standard deviations are indicated.
Figure 4.7 Centrin aggregates cluster following microtubule regrowth
A. CHO:centrin1-GFP cells grown on glass bottom culture dishes were treated with HU and nocodazole for 48 hours. The drugs were then washed out and the cells imaged for a total of 13 hours. A z-stack consisting of 25 steps each 1 μm in size was acquired every ten minutes for the duration of the experiment. Images are shown as the maximum intensity projection of the z-stack. Time shown as hours:minutes. B. CHO:centrin1-GFP cells were treated for 48 hours with HU and nocodazole. The drugs were then washed out for 5 hours and the cells processed for immunofluorescence microscopy. Cells were stained for γ-tubulin (red) and GFP fluorescence monitored. Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 μm.
Figure 4.8 Cytoplasmic centrin aggregates in HU-arrested CHO cells with depolymerised microtubules contain post-translationally modified tubulin

CHO:centrin1-GFP cells were treated with HU and nocodazole for 48 hours. Cells were processed for immunofluorescence microscopy and stained for α-tubulin, acetylated tubulin or polyglutamylated tubulin (red). Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 μm.
investigate whether the centrin foci contained modified tubulin, CHO:centrin1-GFP cells treated with HU and nocodazole were stained with antibodies specific for acetylated and glutamylated tubulin. Both antibodies gave a strong co-localisation with the GFP signal confirming that the centrin foci contain modified tubulins (Figure 4.8).

4.2.5 CENTRIN FOCI ARE REMINISCENT OF CENTRIOLAR SATELLITES

PCM-1 transport to the centrosome is dependent upon microtubules (Balczó et al., 1999; Dammermann and Merdes, 2002). PCM-1 is component of centriolar satellites and is detected in fibrous granules during ciliogenesis (Kubo et al., 1999). Centrin is also reported to be a component of centriolar satellites and can bind to PCM-1 (Baron et al., 1994; Dammermann and Merdes, 2002). Depolymerisation of microtubules leads to the mislocalisation of centriolar satellites, with the dispersal of PCM-1 and centrin along with pericentrin and ninein, but not γ-tubulin (Dammermann and Merdes, 2002). To determine if the centrin foci we observe contain other proteins reminiscent of centriolar satellites, CHO:centrin1-GFP cells treated with HU and nocodazole were stained with a range of antibodies. Staining for PCM-1 and pericentrin revealed a strong co-localisation between each of these proteins and the GFP fluorescence. In contrast, antibodies against ninein, C-Nap1, rootletin or Nek2 only localised to one or two foci which we propose to be the original centrosomes (Figure 4.9).

Microtubule depolymerisation also prevented centrosome overduplication in U2OS cells during prolonged S-phase arrest. Less than 5% of cells had greater than two γ-tubulin staining centrosomes following 68 hours treatment with HU and nocodazole, as compared to over 65% of cells treated with HU alone (Figure 4.10A and C). Centrin and pericentrin foci were also found to have formed in these cells confirming that this observation is not specific to a single cell type (Figure 4.10A and B).

In conclusion, the centrin foci contain a subset of proteins typically found in centriolar satellites and fibrous granules. These foci are different to the large cytoplasmic aggregates reported to form upon microtubule depolymerisation in CHO cells (Dammermann and Merdes, 2002) and live cell imaging showed that no such large aggregates formed during our assay (Figure 4.4). The centrin foci are smaller and more discrete than those observed by Dammermann and Merdes (2002). This suggests that whilst they might just represent
Figure 4.9 Cytoplasmic centrin aggregates in HU-arrested CHO cells with depolymerised microtubules stain for only a subset of centrosomal proteins. CHO:centrin1-GFP cells were treated with HU and nocodazole for 48 hours. Cells were processed for immunofluorescence microscopy and stained for Rootletin, C-Nap1, Pericentrin, PCM-1, Nek2 or ninein (red). Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 μm.
Figure 4.10 Cytoplasmic aggregates also form during HU-arrest in U2OS cells with depolymerised microtubules

U2OS cells were treated for 68 hours with HU and nocodazole. Cells were processed for immunofluorescence microscopy and stained for γ-tubulin (red) and centrin (A) or pericentrin (B) (green). Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 µm. C. Histogram represent the percentage of U2OS cells with overduplicated centrosomes as determined by γ-tubulin staining following the indicated treatments. At least 200 cells were counted per experiment and the data are shown as the average of three experiments. Standard deviations are indicated.
the mislocalisation of centriolar satellites, they could be early precursors to centriole formation as they form during prolonged S-phase arrest that ordinarily leads to overduplicated centrosomes in these cells. Microtubules would then be required for their trafficking to the centrosome where they would contribute to centriole formation.

4.2.6 Centrin Localised in Foci is More Dynamic Than That in Centrioles

A range of proteins associate with the centrosome either stably or transiently at specific points in the cell cycle (Lange, 2002). The manner in which proteins associate with the centrosome can be determined using photobleaching techniques. As proteins are turned over there must be a dynamic exchange between cytoplasmic and centrosomal protein pools. Proteins which stably associate with the centrosome show slow dynamics and are therefore more likely to have a structural role. Conversely, those proteins that undergo rapid turnover are more dynamic and are thus more likely to be regulatory, such as Nek2 (Hames et al., 2005).

The dynamics of fluorescently tagged proteins can be measured in live cells using fluorescence recovery after photobleaching (FRAP) or fluorescence loss in photobleaching (FLIP). To study the dynamics of centrin association with the centrioles, FRAP was first performed on untreated CHO:centrin1-GFP cells. A region of interest (ROI) encompassing the centrioles was selected for bleaching. Two further ROIs were placed in the cytoplasm. Three images were acquired before bleaching and 30 images at 10 second intervals were acquired post-bleach. The ROIs were bleached with 5 iterations of 100% laser power. The GFP intensity within the ROI encompassing the centrioles was calculated for each timepoint and the relative intensity expressed as a percentage (Figure 4.11C). In the five minutes that immediately followed bleaching approximately 29% of centrin was exchanged. These results indicate that the centrin within centrioles of untreated cells is highly stable. Photobleaching of multiple centrioles in CHO:centrin1-GFP cells treated with HU for 48 hours showed an almost identical pattern of recovery (Figure 4.11C).

FRAP in CHO:centrin1-GFP cells treated with HU and nocodazole for 48 hours was performed with three ROIs placed over three separate centrin foci. This was to ensure that at least some foci other than the original centrosome were being analyzed. Results showed that centrin in these foci is more dynamic than that in centrioles as a greater level of recovery was observed (46%; Figure 4.11C). On the assumption that some of the ROIs in
Figure 4.11 FRAP analysis of centrin1-GFP in cytoplasmic aggregates and centrioles
FRAP was performed using a Leica TCS SP5 confocal microscope on untreated CHO:centrin1-GFP cells and cells treated with either HU alone or HU and nocodazole for 48 hours. Three ROIs were bleached per cell. For untreated and HU-treated cells, one ROI included the centrosome, whilst the other two were cytoplasmic. For cells treated with HU and nocodazole, three distinct centrin foci were bleached per cell to ensure that at least one foci other than the original centrosomes was bleached. Images show pre- and post-bleach along with the final image for A. untreated and B. HU plus nocodazole treated cells. C. FRAP data were analysed using Leica LAS AF software, and the relative GFP intensity within the bleached area was recorded as a measure of time. Each data point represents the average intensity +/- standard deviation from at least 12 cells.
this experiment represented the original centrosome, this is probably an underestimate of the real dynamics of centrin in these foci.

FLIP was also performed with untreated CHO:centrin1-GFP cells and those treated with HU alone or HU plus nocodazole for 48 hours. In this instance, an area was selected for bleaching that encompassed the entire cell except for the centrosome/s in untreated and HU treated cells or three centrin foci in cells treated with HU and nocodazole. Ten bleach cycles were performed with 100% laser power. Prior to bleaching and between each bleach cycle 5 images were captured of the whole cell at 5 second intervals. The GFP intensity of the centrosome/s or foci was measured and the relative intensity displayed as a percentage for each bleach cycle. Untreated and HU treated cells showed a fluorescence loss of approximately 30% each. Cells treated with HU and nocodazole showed a fluorescence loss of approximately 45% (Figure 4.12). These figures are comparable for the level of protein exchange seen with the FRAP experiments.

Centrin in unduplicated and overduplicated centrioles is therefore highly stable suggesting a structural role and supporting its position in the lumen of centrolioles where exchange would be slower. Centrin foci in cells treated with HU and nocodazole exhibited a higher level of mobility, possibly suggesting that these are structurally less complex. However, the centrin1-GFP in these foci was still much less dynamic than the highly dynamic centrosomal protein Nek2 or free cytoplasmic centrin1-GFP [Figure 4.13 and (Hames et al., 2005)]. This suggests that centrin1-GFP is still somewhat restricted in these structures.

4.2.7 Centriolar satellites accumulate in cells with depolymerised microtubules during prolonged S-phase arrest

Centriole nucleation and elongation is perturbed in the presence microtubule depolymerising drugs (Kuriyama, 1982). To confirm that centriole formation was not occurring in our cells with depolymerised microtubules TEM analysis was performed. In cells treated with HU alone, multiple centrioles were detected. However, in untreated cells or cells treated with HU and nocodazole, no more than two centrioles per section were observed (Figure 4.14A).

Centriolar satellites are morphologically characterised by electron microscopy as densely-staining granules in the vicinity of centrioles (Kubo et al., 1999). Indeed, under each
Figure 4.12 FLIP analysis of centrin1-GFP in cytoplasmic aggregates and centrioles

FLIP was performed on untreated CHO:centrin1-GFP cells and cells treated for 48 hours with HU alone or HU plus nocodazole. An area was selected for bleaching that encompassed the entire cell except for the centrosomes in untreated (A) and HU treated cells or three centrin foci in cells treated with HU and nocodazole. Ten bleach cycles were performed on this area with 100% laser power for 10 iterations (upper panels). Prior to bleaching and between each bleach cycle, 5 images of the whole cell were captured 5 seconds apart using standard imaging conditions (lower panels). Insets show enlargements of centrosomes. B. FLIP analysis of untreated CHO:centrin1-GFP cells and cells treated for 48 hours with HU alone or HU plus nocodazole. Each data point represents the average intensity +/- standard deviation from at least 12 cells.
Figure 4.13 FRAP analysis indicates that diffuse cytoplasmic centrin and centrosomal Nek2 are both highly dynamic proteins
FRAP was performed using a Leica TCS SP5 confocal microscope on untreated CHO:centrin1-GFP cells and CHO cells transiently transfected with GFP-Nek2A-WT. For CHO:centrin1-GFP cells a ROI in the cytoplasm, not including any aggregates, was bleached. For CHO cells expressing GFP-Nek2A-WT a ROI encompassing the centrosome was bleached. Following bleaching one image was captured every 2 seconds for the duration of the experiment. The relative fluorescent intensity of the ROI was calculated and plotted against time. Data are shown as the average of at least 10 cells +/- the standard deviation.
Figure 4.14 HU-arrested CHO cells with depolymerised microtubules contain increased number of centriolar satellites than untreated cells

A. TEM analysis of centrioles in CHO:centrin1-GFP cells that were either untreated or treated for 48 hours with HU alone or HU plus nocodazole. Centrioles (arrows) and centriolar satellites (arrowheads) are indicated. Scale bars, 500 nm.

B. Histogram shows the mean number of centriolar satellites within the EM sections that also contained centrioles from CHO:centrin1-GFP cells that were either untreated or treated for 48 hours with HU alone or HU plus nocodazole. At least 30 sections were examined for each condition and the standard deviation is indicated.
condition a number of electron dense granules were observed in association with the centrioles (Figure 4.14A). Strikingly, the number of these granules was greatly increased in cells treated with HU and nocodazole (Figure 4.14B). Centriolar satellites are indistinguishable from the fibrous granules involved in ciliogenesis. As centriolar satellites/fibrous granules contain centrin, polyglutamylated tubulin and PCM-1 (Baron et al., 1994; Kubo et al., 1999; Laoukili et al., 2000; Million et al., 1999), it is likely that the centrin foci observed by fluorescence microscopy are indeed the same structures. As fibrous granules have been localised around the nuclear envelope and contribute to basal body formation during ciliogenesis (Anderson and Brenner, 1971), it is possible that the centrin foci we see are precursors to centriole formation during centrosome duplication.

4.2.8 MULTIPLE CENTROSONES RAPIDLY ACCUMULATE FOLLOWING DRUG WASHOUT
To determine the potential of cells treated with HU and nocodazole to form multiple centrosomes a drug washout was performed and the number of γ-tubulin positive centrosomes present in each cell was quantified. It was proposed that if the centrin foci observed in HU/nocodazole treated cells were precursors to centrosome duplication that the cells would be able to rapidly convert these into centrosomes and recruit γ-tubulin following microtubule regrowth. CHO cells treated with either mimosine, HU or HU and nocodazole for 48 hours were placed in drug-free media for 10 hours. Staining with γ-tubulin antibodies allowed the number of centrosome per cell to be counted. As expected, cells treated with mimosine had only two centrosomes before and after washout as cells do not undergo centrosome overduplication during G1 arrest. There was no significant increase in centrosome number in HU treated cells following washout. However, in cells treated with HU and nocodazole the number of centrosomes increased from around two before washout to over five following drug removal (Figure 4.15). This suggests that rapid centriole formation followed by γ-tubulin recruitment could occur under conditions where centrin foci had already formed.

In conclusion, whilst microtubules are required for centrosome overduplication, they are not necessary for the formation of centrin foci that are highly reminiscent of centriolar satellites/fibrous granules. However, these are not recruited to the centrosome in the absence of microtubules, thus microtubules play a role in the trafficking of centriolar satellites to the centrosome. Finally, the accumulation of these centriolar satellites during
prolonged S-phase arrest suggests that they may contribute to centrosome overduplication by providing precursors of new centrosomes.
Figure 4.15 Drug washout reveals that cells in which microtubules had been depolymerised during HU-arrest can rapidly accumulate multiple γ-tubulin dots

A. CHO cells were treated with either mimosine, HU alone or HU plus nocodazole for 48 hours. The drugs were then washed out for 10 hours. Cells were processed for immunofluorescence microscopy and stained for γ-tubulin. Scale bar, 10 μm.

B. Histogram shows the number of γ-tubulin dots per cell following 48 hours drug treatment (grey bars) and 48 hours treatment followed by 10 hours washout (black bars). At least 200 cells were counted per experiment and data are shown as the average of three experiments. Standard deviation is indicated.
4.3 DISCUSSION

Microtubules are essential for centrosome duplication, as centrosomes cannot duplicate in cells in which the microtubules have been depolymerised (Balczon et al., 1999). Our findings support this conclusion. At its most basic, this may reflect a fundamental role for microtubules to form the walls of new centrioles. Daughter centriole nucleation and elongation, along with basal body formation during ciliogenesis, is perturbed in the presence of microtubule depolymerising agents (Kuriyama, 1982; Lemullois et al., 1988). Additionally, centrioles cannot be formed along the de novo pathway when microtubules have been disassembled by the drug nocodazole (Khodjakov et al., 2002). However, microtubules may also serve a role in the trafficking of centrosomal components to the centrosome during replication.

Centrosome duplication is dependent upon the localised concentration of proteins at the centrosome, with centriole precursors accumulating in the region of the centrosome through a microtubule-based mechanism (Salisbury, 2008). Centriolar satellites are trafficked to the centrosome along microtubules in a dynein-dynactin dependent manner (Dammermann and Merdes, 2002; Kubo et al., 1999). The relationship between centriolar satellites and centrosomes remains obscure, although the evidence suggests that the satellites are at least in part responsible for the delivery of proteins to the centrosome. It has also been suggested that the satellites may coalesce with, and in part form, the PCM (Baron et al., 1994). Depolymerisation of microtubules results in the dispersal of a number of centrosomal proteins associated with the centriolar satellites (Dammermann and Merdes, 2002). Additionally, inhibition or depletion of the centriolar satellite component PCM-1 leads to reduced targeting of centrin, pericentrin and ninein to the centrosome.

It is likely that the accumulation of centrin foci we observed in S-phase arrested cells with depolymerised microtubules reflects the accumulation of centriolar satellites. As the centrin foci contain stabilised tubulin, pericentrin and PCM-1 they are highly reminiscent of centriolar satellites and indeed we observed an increase in satellite number in these cells as detected by electron microscopy. Centriolar satellites are equivalent to fibrous granules that act as precursors to basal body formation during ciliogenesis (Anderson and Brenner, 1971). Fibrous granules also contain centrin, PCM-1 and stabilised tubulin and in studies of oviduct ciliogenesis have been associated with the nuclear envelope (Anderson and
Brenner, 1971; Kubo et al., 1999; Laoukili et al., 2000; Million et al., 1999). Therefore, there are many similarities between the centrin foci we observe and centriolar satellites/fibrous granules.

As centriolar satellites accumulate in S-phase arrested cells with depolymerised microtubules, it is likely that satellite formation is an early step in the overduplication pathway. Therefore, a model can be proposed where satellites can form in the absence of microtubules, but an intact microtubule network is required for their delivery to the centrosome (Figure 4.16). Once at the centrosome, centriolar satellites in the very least contribute to increase the localised concentration of proteins.

The formation of centrin aggregates has been reported to be the first step in precentriole formation along the de novo pathway in HeLa cells (La Terra et al., 2005). Centriole assembly begins with the formation of small cytoplasmic centrin aggregates that appear during S-phase following the removal of resident centrioles. However, it is unlikely that the centrin foci we observe are the same, as the presence of a pre-existing centrosome is thought to suppress the de novo pathway. The exact nature of the precentrioles described in the de novo pathway has not yet been determined. The accumulation of a number of minuscule centrin aggregates in control cells during their de novo study led to the postulation that the de novo and templated centriole formation pathways are not mutually exclusive. These centrin aggregates disappeared within a few hours of their formation, leading the researchers to suggest that there are in fact precentrioles forming in cells with existing centrioles. It is thus proposed that precentrioles form during templated centrosome duplication and it is only those precentrioles that ‘dock’ with the existing centrioles that will form new centrioles (La Terra et al., 2005). Whether centriolar satellites contribute directly to the de novo pathway remains to be seen. However, centrioles formed along the de novo pathway are reported to form within a well defined PCM lattice and centriolar satellites are thought to contribute to the PCM (Baron et al., 1994; Khodjakov et al., 2002).

Intriguingly, microtubules have been implicated in the de novo centriole formation pathway. A cloud of PCM is reported to form as part of the de novo pathway in S-phase arrested CHO cells expressing γ-tubulin-GFP in which the centrosome has been laser ablated (Khodjakov et al., 2002). The appearance of this PCM cloud, often located in an invagination of the nuclear envelope, occurs within 4 to 5 hours of centrosome removal.
Figure 4.16 A model for the role of microtubules in centrosome overduplication in S-phase arrested cells
In this model for the contributions of microtubules to centosome overduplication, centrin foci, containing PCM-1, pericentrin and modified tubulins form around the periphery of the nucleus in the absence of microtubules. In the presence of microtubules these can be trafficked to the centrosome where they promote centrosome overduplication.
The size of the PCM focus then rapidly increases in size over the next 4 hours. Whilst the formation of the PCM cloud was not perturbed by the depolymerisation of microtubules, several individual PCM foci were observed instead of a relatively large single PCM focus (Khodjakov et al., 2002). This suggests that smaller amounts of PCM are initially assembled before being transported to a single site in a microtubule-dependent manner. This also adds weight to the requirement for a high local concentration of centriolar/centrosomal proteins for centriole formation to occur.

Whatever the link between precentrioles and centriolar satellites/fibrous granules, and the de novo and templated centriole formation pathways, our results have highlighted the importance of using a range of centrosomal markers when looking at the contribution of microtubules to centrosome duplication. Looking at \( \gamma \)-tubulin, C-Nap1, rootletin, ninein or Nek2 alone would confirm that microtubules are required for centrosome duplication. However, using an early centriole marker such as centrin revealed the presence of a number of centrin foci, which also contained modified tubulin, pericentrin and PCM-1 and in fact are most likely centriolar satellites. Without using a range of markers this observation would have been missed. Therefore, when looking at the mechanisms of centrosome duplication it is important to use markers for centriolar satellites, centrioles and PCM.
CHAPTER FIVE

THE MICROTUBULE-BASED MOTOR PROTEIN
DYNEIN IS REQUIRED FOR CENTROSONE OVERDUPPLICATION
5.1 INTRODUCTION

5.1.1 DYNEIN

Cytoplasmic dynein is a minus-end directed microtubule based ATPase motor protein involved in numerous activities including intracellular transport and cell division. A megadalton-sized multi-subunit protein complex, dynein consists of two heavy chains, along with various intermediate, light intermediate and light chains (reviewed in: (King, 2000)). Each of the two heavy chains fold to form the enzymatically active heads of the motor that converts energy from ATP hydrolysis into force. However, dynein requires another multi-subunit protein complex, called dynactin, to efficiently carry out its role in cells.

Initially isolated as a factor required to activate dynein-dependent transport in vitro (Gill et al., 1991; Schroer and Sheetz, 1991), dynactin is best described as an activator of dynein and is required for all cellular functions in which cytoplasmic dynein is involved (for review see: (Schroer, 2004)). Dynactin facilitates dynein-based movement by acting as both a processivity factor and a binding platform which mediates the association of dynein with its cargo (King and Schroer, 2000). Whilst dynein can bind some cargoes independently of dynactin, dynactin is still required in these instances to establish a fully functional dynein-cargo link.

Dynactin contains at least eleven different subunits, including p150\textsuperscript{Glued}, dynamitin (p50), p24 and Arp1 (Figure 5.1). These subunits allow dynactin to have distinct cargo-, dynein-, and microtubule binding domains (for an extensive review see (Schroer, 2004)). Arp1 forms the basis of an Arp1 rod, the components of which are more highly conserved than the other dynactin subunits. This Arp1 rod forms a large cargo binding domain by which dynactin can associate with a range of proteins. The projecting arm of dynactin consists of p150\textsuperscript{Glued} and p24, and allows binding to both dynein and microtubules. Dynamitin plays a profound role in dynamitin structure by linking the Arp1 rod to the projecting arm. Overexpression of dynamitin in cells disrupts dynactin, leading to dynein redistribution, Golgi dispersal and a prometaphase arrest (Burkhardt et al., 1997; Echeverri et al., 1996; Melkonian et al., 2007).
Figure 5.1 Schematic illustration of dynactin subunits
Dynactin consists of 11 different polypeptides, some present in more than one copy per complex. The main subunits are illustrated. Arp1 (red), an actin-related protein, forms a filament structure that is the basis of the so called Arp1 rod. This forms a large cargo binding domain via which dynactin can associate with a range of proteins. Together, p150GLUED (green) and p24 (yellow) form a side-arm that enables dynactin to bind to both dynein and microtubules. Dynamitin (blue) forms the link between the Arp1 rod and side-arm. Overexpression of dynamitin in cells disrupts this linkage, releasing free side-arms that are still able to bind to dynein and microtubules. Adapted from Schroer (2004).
5.1.2 Dynein and the Cell Cycle

Phosphorylation appears to play a role in the regulation of dynein function and localisation in cells. The interaction between dynein and dynactin seems to be tightly regulated as dynactin and dynein do not always colocalise (Holleran et al., 1998; Karki and Holzbaur, 1999; Quintyne et al., 1999; Quintyne and Schroer, 2002). Phosphorylation of a serine residue (S84) in the dynein intermediate chain has been proposed to regulate the dynein-dynactin interaction (Vaughan et al., 2001). Additionally, mitotic phosphorylation of the dynein light intermediate chain is mediated by the kinase Cdk1 (Dell et al., 2000). This phosphorylation drives dynein from an organelle positioning role to its localisation at spindle poles, kinetochores and mitotic spindles in mitosis.

Dynein binds the centrosome in a cell-cycle dependent manner, whereas dynactin is present at the centrosome throughout the cell cycle. Dynein recruitment to the centrosome slightly precedes centriole duplication and is lost again following mitosis (Quintyne and Schroer, 2002). Dynactin acts independently of dynein at the centrosome to anchor microtubules (Quintyne et al., 1999). Disruption of dynein-based motility, by overexpression of the dynactin subunits dynamitin, p24 or p150\textsuperscript{Glued}, prevented the recruitment of dynactin, dynein and other pericentriolar proteins to the centrosome (Dammermann and Merdes, 2002; King et al., 2003; Quintyne et al., 1999; Quintyne and Schroer, 2002). This led to destabilisation of both centrosomes and the microtubule array, interfering with cell cycle progression during mitosis.

Dynein is recruited to the centrosome during S-phase, suggesting that the motor protein may play a role in centrosome duplication. Surprisingly, injection of cells with the cDNAs of inhibitory dynactin subunits and subsequent release of cells synchronised at the G1/S boundary had no affect on centriole duplication as measured by centrin staining (Quintyne and Schroer, 2002). However, the recruitment of a range of proteins that constitute the pericentriolar material is dependent on dynein-dynactin mediated transport [for review see: (Zimmerman and Doxsey, 2000)]. Conversely, overexpression a truncated p150\textsuperscript{Glued} construct (p150\textsuperscript{926-1049}) or p24, dynactin inhibitors that have no measurable effect on dynein-based motility (Quintyne et al., 1999), but which disrupt the centrosomal dynactin pool, somehow delay S-phase entry (Quintyne and Schroer, 2002). The authors suggest this may be due to a requirement for the correct balance of centrosomal dynactin subunits to satisfy the control mechanism for G1 to S transition and may involve centriole coupling.
Dynein inhibition on the other hand has the most dramatic effects during mitosis when formation of the mitotic spindle is disrupted. Spindle assembly is spatially and temporally regulated, with microtubule organisation and chromosome positioning achieved via a range of forces. Both microtubule dynamics and the actions of opposing microtubule-based motor proteins contribute to these forces (Barton and Goldstein, 1996; Fuller and Wilson, 1992; Gaglio et al., 1996; Inoue and Salmon, 1995). As a minus-end directed microtubule-based motor protein, dynein has been demonstrated to contribute to the organisation of the mitotic spindle. Inhibition of dynein and/or dynactin in both cell-free systems for mitotic aster assembly and in cultured cells prevents focusing of microtubule minus-ends and disruption of spindle formation (Echeverri et al., 1996; Gaglio et al., 1997; Gaglio et al., 1996). Therefore, dynein and dynactin are required for the formation and maintenance of the organisation of microtubule minus-ends at the spindle poles. Inhibition of their function leads to splaying of the minus end of the microtubules, disrupting the connection between the poles and spindle. Indeed, inhibitors of the dynein-dynactin interaction, predicted to interfere with the centrosome cycle, have little effect until mitosis (Quintyne and Schroer, 2002).

5.1.3 Dynein and Centrosome Assembly

Centrosome overduplication is prevented when microtubules are depolymerised ((Balczon et al., 1999); Chapter 4). Whilst it is likely that centriole formation is perturbed in the presence of microtubule depolymerising drugs (Kuriyama, 1982; Lemullois et al., 1988), an intact microtubule network may also be required for the trafficking of proteins to the centrosome during duplication. A functional dynein-dynactin interaction is required for proper microtubule organisation and for the transport and localisation of a number of centrosomal components (King et al., 2003; Zimmerman and Doxsey, 2000). One advantage of a dynein-mediated transport system over diffusion-based mechanisms is that it has the potential to increase the local concentration of transported molecules. It may also facilitate protein assembly onto centrosomes and spindle poles, providing proper orientation of components and aid anchoring or activation of the transported molecules (Zimmerman and Doxsey, 2000). Additionally, it may ensure that those centrosomal components whose localisation is cell cycle regulated arrive at the centrosome at the appropriated time in the cell cycle.
Centriolar satellites have been demonstrated to move towards the centrosome in a microtubule and ATP-dependent manner (Dammermann and Merdes, 2002; Kubo et al., 1999). Additionally, inhibition of dynein-dynactin based transport by dynamitin overexpression leads to reduced targeting of centrin, pericentrin, ninein and PCM-1 to the centrosome (Dammermann and Merdes, 2002). BBS4 localises to centriolar satellites and acts as an adaptor of p150Glued to recruit PCM-1 and its associated cargoes (Kim et al., 2004). BBS4 interacts with the C-terminus of PCM-1 and silencing of BBS4 leads to PCM-1 mislocation. Additionally, dynamitin overexpression causes the loss of BBS4 from satellites. Dynein therefore serves an important role in the localisation of centriolar satellites along with their associated proteins to the centrosome.

Other centrosomal proteins also require dynein for their localisation. Ninein and ninein-like protein (Nlp) are both involved in microtubule organisation at the centrosome (Mogensen et al., 2000; Rapley et al., 2005). Both proteins interact with the dynein-dynactin complex, with overexpression of the dynactin-binding domain of either ninein or Nlp causing Golgi and lysosome dispersal (Casenghi et al., 2005). Similarly, pericentrin has been shown to bind directly to the dynein light intermediate chain, with overexpression of pericentrin perturbing proper dynein function (Purohit et al., 1999). Centrosomal levels of pericentrin increase progressively from G1 to metaphase (Dictenberg et al., 1998) with live cell imaging of GFP-tagged pericentrin revealing that it moves along microtubules at speeds compatible with dynein-mediated transport (Young et al., 2000).

Whilst there is strong evidence that a range of proteins utilise dynein for their trafficking, some proteins may arrive at the centrosome independently of both dynein and microtubules. It is reported that the dynamic exchange of γ-tubulin between the cytoplasm and centrosome occurs independently of microtubules (Khodjakov and Rieder, 1999). However, dynein inhibition by antibody injection, along with dynamitin overexpression has been reported to inhibit the centrosomal recruitment of γ-tubulin (Young et al., 2000). This leads to the suggestion that the two mechanisms are redundant, possibly with one predominating over the other at different stages of the cell cycle (Young et al., 2000; Zimmerman and Doxsey, 2000). So, dynein might function to speed the trafficking of proteins that would otherwise localise at the centrosome through slower diffusion.
It is surprising that whilst dynein is implicated in the trafficking of a number of centrosomal proteins, it is dispensable for normal centrosome duplication as measured by centrin dot formation (Quintyne and Schroer, 2002). The requirement for dynein in centrosome overduplication has not yet been investigated. It is plausible to suggest that there are enough centriolar components present at the centrosome to allow one round of templated duplication to be completed in the absence of functional dynein. However, centrosome overduplication may require a continual supply of centrosomal components to sustain centriole formation. Therefore, utilising the centrosome overduplication assay we tested the effect of a range of dynein inhibitors on this process.
5.2 RESULTS

5.2.1 DYNAMITIN OVEREXPRESSION PREVENTS CENTROsome OVER Duplication

A functional dynein-dynactin complex is essential for the centrosomal accumulation of PCM-1, centrin, pericentrin and ninein, but not γ-tubulin (Dammermann and Merdes, 2002). Based on the finding by Quintyne and Schroer (2002) that dynein is recruited to the centrosome just before centriole replication, but is not required for normal centrosome duplication, we decided to look at whether dynein is required for centrosome overduplication.

Dynactin is an activator of dynein, facilitating dynein-based movement by acting both as a processivity factor and an adaptor that mediates dynein binding to its cargoes. Additionally, dynactin is also required for microtubule anchoring at centrosomes (Quintyne et al., 1999). The p150Glued subunit of dynactin is responsible for interactions with both dynein and microtubules. Dynamitin overexpression disrupts the endogenous pool of cellular dynactin yielding a free pool of p150Glued that can still bind to dynein (Melkonian et al., 2007). This prevents proper targeting of dynein to its cargo, thus perturbing dynein-based transport of cargoes to the centrosome. Under these conditions, accumulation of dynactin and dynein at the centrosome is prohibited and the radial microtubule array becomes defocused due to redistribution of centrosomal dynactin (Quintyne and Schroer, 2002). Dynamitin overexpression has been extensively used to investigate the role of dynein in the recruitment of centrosomal proteins. We therefore first used dynamitin overexpression to investigate the role of dynein-dynactin in centrosome overduplication.

CHO cells were transiently transfected with a myc-dynamitin construct for 24 hours prior to exposure to HU. Control cells were transfected with a myc-lamin A construct. Following 48 hours treatment with HU, cells were fixed and processed for immunofluorescence microscopy. Staining with anti-myc antibodies revealed transfected cells, whilst anti-γ-tubulin antibodies were used to identify centrosomes (Figure 5.2A). When HU-arrested cells were examined, it was found that centrosome overduplication was perturbed in cells overexpressing dynamitin, but not in those overexpressing lamin A. Quantification of the number of centrosomes per cell in CHO cells overexpressing dynamitin at 0, 24 and 48 hours following the addition of HU revealed no increase in
centrosome number with time (Figure 5.2B). The mean number of centrosomes in cells overexpressing dynamitin following 48 hours treatment with HU was less than two. This compared to mean numbers of over four centrosomes in untransfected cells and those overexpressing lamin (Figure 5.2C).

U2OS cells also accumulate multiple centrosomes during prolonged HU-treatment. However, overexpression of dynamitin, but not lamin A, was found to similarly prevent centrosome accumulation in these cells as visualised with γ-tubulin staining (Figure 5.3A). Less than 10% of cells had more than two centrosomes, compared to over 60% of control cells (Figure 5.3B). Overexpression of dynamitin therefore perturbs centrosome overduplication during prolonged S-phase arrest as measured by γ-tubulin staining.

5.2.2 Dynamitin overexpression causes the dispersal of Centrin and PCM-1

Dynamitin overexpression has been reported to cause the dispersal of PCM-1 containing centriolar satellites along with centrin, pericentrin, ninein and BBS4, but not γ-tubulin (Dammermann and Merdes, 2002; Kim et al., 2004). This subsequently leads to the reduced targeting of these proteins to the centrosome. To investigate the role of dynein-dynactin in the targeting of centrin and PCM-1 to the centrosome, CHO cells were transfected with a myc-dynamitin construct and stained for centrin or PCM-1.

Initially, CHO cells were transiently transfected with a myc-dynamitin construct and fixed following 24 hours incubation. Cells were stained with anti-myc antibodies to reveal transfected cells and either anti-centrin2 or anti-PCM-1 antibodies. Centrin2 staining revealed a discrete localisation at the centrioles, but dispersal of the centrin signal corresponding to centriolar satellites (Figure 5.4A). Similarly, the PCM-1 signal was also found to be dispersed throughout the cell (Figure 5.4B). Therefore, inhibition of the dynein-dynactin complex by dynamitin overexpression prevents the localisation of PCM-1 and centrin containing satellites in the vicinity of the centrosome, however centrin is not lost from the centrioles.
Figure 5.2 Dynamitin overexpression perturbs centrosome overduplication in CHO cells

A. CHO cells were transfected with myc-dynamitin or myc-lamin A for 24 hours before HU was added to the culture media. Following 48 hours treatment with HU, cells were processed for immunofluorescence microscopy and stained with anti-myc (green) and anti-γ-tubulin (red) antibodies. Merge panels include DNA stained with Hoechst (blue). Scale bar, 10 µm. B. Histogram represents the number of γ-tubulin staining centrosomes per cell in CHO cells overexpressing dynamitin and treated with HU for 0, 24 and 48 hours. Data are shown as the mean +/- standard deviation of three experiments. Approximately 200 cells were counted per experiment. C. Histogram shows the mean number of γ-tubulin staining centrosomes in CHO cells either untransfected or overexpressing dynamitin or lamin A and treated with HU for 48 hours. Data are shown as the mean +/- standard deviation of three experiments. Approximately 200 cells were counted per experiment.
Figure 5.3 Dynamitin overexpression perturbs centrosome overduplication in U2OS cells

A. U2OS cells were transfected with myc-dynamitin or myc-lamin A for 24 hours before HU was added to the culture media. Following 68 hours treatment with HU, cells were processed for immunofluorescence microscopy and stained with anti-myc (green) and anti-γ-tubulin (red) antibodies. Merge panels include DNA stained with Hoechst (blue). Scale bar, 10 μm.

B. Histogram represents the percentage of cells with more than two γ-tubulin staining centrosomes per cell in U2OS cells either untransfected or overexpressing dynamitin or lamin A and treated with HU for 68 hours. Data are shown as the mean ± standard deviation of three experiments. Approximately 200 cells were counted per experiment.
Figure 5.4 Centrin and PCM-1 foci disperse in CHO cells overexpressing dynamitin
CHO cells were either untransfected or transfected with myc-dynamitin for 24 hours as indicated and processed for immunofluorescence microscopy. Cells were stained with anti-myc (green) and anti-centrin2 (A) or anti-PCM-1 (B) (red) antibodies. Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 μm.
5.2.3 Dynamitin overexpression during prolonged S-phase arrest does not prevent the localisation of centrin and PCM-1

To investigate the effect of dynamitin overexpression on centrin localisation during centrosome overduplication, CHO and CHO:centrin1-GFP cells were transfected with myc-dynamitin for 24 hours as above and then treated with HU for 48 hours. Staining for centrin2 or visualisation of GFP fluorescence revealed that centrin foci cluster tightly together in these cells (Figure 5.5). This is dramatically different to the dispersal of centrin seen in cells transfected with myc-dynamitin for 24 hours, but similar to the centrin2 staining pattern seen in untransfected cells (Figure 5.4A) or cells overexpressing lamin A and treated with HU for 48 hours (Figure 5.5).

Similarly, when PCM-1 staining was examined in cells overexpressing dynamitin during prolonged S-phase arrest a tight focus of PCM-1 dots was seen. This staining pattern is comparable to that seen in untransfected cells (Figure 5.4B) or cells overexpressing lamin A during S-phase arrest (Figure 5.6), but significantly different to that in cells overexpressing dynamitin for 24 hours (Figure 5.4B). Together, these results suggest that whilst dynamitin overexpression causes the dispersal of centrin and PCM-1, that during prolonged periods of S-phase arrest these proteins can be recruited to the centrosome via an alternative, but presumably slower mechanism.

5.2.4 Dynein inhibition by vanadate perturbs centrosome overduplication

It is reported that dynamitin can bind several proteins besides dynactin, therefore the inhibitory effects seen upon dynamitin overexpression could occur through pathways independent of dynein-based motility. The use of additional dynein inhibitors was thus recommended to determine whether processes that no longer occur in the presence of excess dynamitin truly require dynein (Schroer, 2004).

Vanadate is a known inhibitor of dynein-ATPase activity (Gibbons et al., 1978; Penningroth, 1986). As a phosphate analogue, vanadate and phosphate compete for the same binding site on the enzyme. Inhibition is thus brought about by the formation of a dynein-ADP-vanadate complex instead of the dynein-ADP-phosphate intermediate (Shimizu and Johnson, 1983).
Figure 5.5 Centrin foci cluster together in CHO cells overexpressing dynamitin during prolonged S-phase arrest

**A.** CHO cells were transfected with myc-dynamitin or myc-lamin A for 24 hours before HU was added to the culture media. Following 48 hours treatment with HU, cells were processed for immunofluorescence microscopy and stained with anti-myc (green) and anti-centrin2 (red) antibodies. Merge panels include DNA stained with Hoechst (blue).

**B.** CHO:centrin1-GFP cells were transfected with myc-dynamitin for 24 hours before HU was added to the culture media. Following 48 hours treatment with HU, cells were processed for immunofluorescence microscopy and stained with anti-myc (red) antibodies. Merge panels include GFP fluorescence (green) and DNA stained with Hoechst (blue). Scale bars, 10 µm.
Figure 5.6 PCM-1 foci cluster together in CHO cells overexpressing dynamitin during prolonged S-phase arrest
CHO cells were transfected with myc-dynamitin or myc-lamin A for 24 hours as indicated before HU was added to the culture media. Following 48 hours treatment with HU, cells were processed for immunofluorescence microscopy and stained with anti-myc (green) and anti-PCM-1 (red) antibodies. Merge panels include DNA stained with Hoechst (blue). Scale bar, 10 \( \mu \)m.
CHO cells were treated with HU and vanadate concurrently for 48 hours. Cells were then fixed and stained with antibodies against both γ-tubulin and C-Nap1. Analysis of the staining pattern with these antibodies revealed the presence of only two centrosomes per cell (Figure 5.7A). Quantification of the number of centrosomes per cell in CHO cells treated with HU and vanadate for 0, 24 and 48 hours revealed an identical profile at each timepoint (Figure 5.7B). When the mean number of centrosomes in cells was calculated, a dose response to vanadate was seen. At the lowest concentration of vanadate (1 µM) little inhibition of centrosome overduplication was seen, however at the highest dose (20 µM) centrosome accumulation was prevented (Figure 5.7C). Therefore, inhibition of dynein by vanadate similarly prevents centrosome overduplication during prolonged S-phase arrest, supporting the result seen with dynamitin overexpression.

Vanadate has previously been shown to inhibit the movement of PCM-1 at concentrations that did not affect the motor protein kinesin (Kubo et al., 1999). Additionally, AMP-PNP inhibited PCM-1 movement at concentrations that affect both dynein and kinesin, but not at concentrations that affect kinesin alone. When the localisation of centrin1-GFP in the CHO:centrin1-GFP cell line, and centrin2 and PCM-1 in the CHO:WT cells was examined following 48 hours treatment with HU and vanadate, tight clusters of foci of each protein were seen around the γ-staining centrosomes (Figure 5.8). These staining patterns were comparable to those seen in cells overexpressing dynamitin during prolonged S-phase arrest (Figures 5.5 and 5.6). This supports the hypothesis that both centrin and PCM-1 still accumulate around the centrosome in the absence of functional dynein by an alternative mechanism.

5.2.5 Dynein inhibition by EHNA prevents centrosome overduplication

Whilst dynein is the motor protein most sensitively inhibited by vanadate, it is known that vanadate can inhibit kinesin at higher concentrations. Therefore, to investigate the specificity of the inhibition of centrosome overduplication seen with vanadate treatment, we repeated the assay using another chemical inhibitor of dynein. EHNA (erythro-9-[3-(2-hydroxynonyl)]adenine) is a phosphodiesterase inhibitor with a very high affinity for the dynein ATPase (Bouchard et al., 1981; Penningroth, 1986).
Figure 5.7 Inhibition of dynein by vanadate perturbs centrosome overduplication

A. CHO cells were treated with HU and vanadate for 48 hours and processed for immunofluorescence microscopy. Cells were stained with anti-\( \gamma \)-tubulin (red) and anti-C-Nap1 (green) antibodies. Merge panels include DNA stained with Hoechst (blue). Scale bar, 10 \( \mu \)m.

B. Histogram represents the number of \( \gamma \)-tubulin staining centrosomes in CHO cells treated with HU and vanadate for 0, 24 and 48 hours. Data are shown as the average of three experiments with approximately 200 cells counted per experiment. Standard deviations are indicated.

C. Histogram represents the mean number of \( \gamma \)-tubulin staining centrosomes per cell in CHO cells treated with HU alone or HU plus vanadate for 48 hours. Data are shown as the mean +/- standard deviation of three experiments. Approximately 200 cells were counted per experiment.
Figure 5.8 Centrin and PCM-1 foci form around the centrosome of cells treated with vanadate during HU-arrest
A. CHO:centrin1-GFP cells were treated with HU and vanadate for 48 hours and processed for immunofluorescence microscopy. Cells were stained with anti-γ-tubulin (red) and anti-GFP (green) antibodies. Merge panels include DNA stained with Hoechst (blue). CHO cells were treated with HU and vanadate for 48 hours and processed for immunofluorescence microscopy. Cells were stained with anti-γ-tubulin (red) and anti-centrin2 (B) or anti-PCM-1 (C)(green) antibodies. Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 μm.
CHO cells were treated simultaneously with HU and EHNA for 0, 24 and 48 hours. Cells were fixed and stained with antibodies against γ-tubulin and C-Nap1 to identify the centrosome. Examination of the cells revealed the presence of only two centrosomes in each cell (Figure 5.9A). Quantification of the number of centrosomes in each cell revealed an identical profile at each timepoint, confirming the absence of centrosome overduplication (Figure 5.9B). Indeed the mean number of centrosomes in cells treated with HU and EHNA was only 2.2, as compared to 4.4 in cells treated with HU alone (Figure 5.9C). The use of EHNA therefore confirms the results seen with both vanadate and dynamitin overexpression, and supports a role for dynein in centrosome overduplication.

When the localisation of centrin1-GFP in the CHO:centrin1-GFP cell line, and centrin2 and PCM-1 in the CHO:WT cells was examined following 48 hours treatment with HU and EHNA, tight clusters of foci of each protein were seen around the γ-staining centrosomes (Figure 5.10). These staining patterns are comparable to those seen in cells overexpressing dynamitin or treated with vanadate during prolonged S-phase arrest (Figures 5.5, 5.6 and 5.8). This confirms that centrin and PCM-1 can still cluster around the centrosome in the absence of a functional dynein-dynactin complex, although this localisation is clearly not enough to support centrosome overduplication as measured by γ-tubulin staining.

5.2.6 Vanadate and EHNA also prevent centrosome accumulation in U2OS cells

To confirm the results seen with vanadate and EHNA in CHO cells, the effects of these chemical inhibitors on centrosome overduplication was also examined in U2OS cells. U2OS cells were treated with HU and either vanadate or EHNA for 68 hours, prior to being fixed and stained with antibodies against γ-tubulin and C-Nap1. Only two centrosomes were seen in cells treated with either vanadate or EHNA during prolonged S-phase arrest (Figure 5.11A). Indeed, less than 10% of cells treated with HU and vanadate or EHNA have more than two centrosomes, compared to over 65% of cells treated with HU alone (Figure 5.11B).
Figure 5.9 Inhibition of dynein by EHNA perturbs centrosome overduplication

A. CHO cells were treated with HU and EHNA for 0, 24 and 48 hours before being fixed and processed for immunofluorescence microscopy. Cells were stained with anti-γ-tubulin (red) and anti-C-Nap1 (green) antibodies. Merge panels include DNA stained with Hoechst (blue). Scale bar, 10 µm.

B. Histogram represents the number of γ-tubulin staining centrosomes in CHO cells treated with HU and EHNA for 0, 24 and 48 hours. Data are shown as the average of three experiments with approximately 200 cells counted per experiment. Standard deviations are indicated.

C. Histogram represents the mean number of γ-tubulin staining centrosomes per cell in CHO cells treated with HU alone or HU plus EHNA for 48 hours. Data are shown as the mean +/- standard deviation of three experiments. Approximately 200 cells were counted per experiment.
Figure 5.10 Centrin and PCM-1 foci form around the centrosome of cells treated with the dynein inhibitor EHNA during HU-arrest

A. CHO:centrin1-GFP cells were treated with HU and EHNA for 48 hours and processed for immunofluorescence microscopy. Cells were stained with anti-γ-tubulin (red) and anti-GFP (green) antibodies. Merge panels include DNA stained with Hoechst (blue). CHO cells were treated with HU and EHNA for 48 hours and processed for immunofluorescence microscopy. Cells were stained with anti-γ-tubulin (red) and anti-centrin2 (B) or anti-PCM-1 (C) (green) antibodies. Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 µm.
Figure 5.11 Inhibition of dynein with EHNA and vanadate prevents centrosome overduplication in U2OS cells

A. U2OS cells were treated with HU and either EHNA or vanadate for 68 hours and processed for immunofluorescence microscopy. Cells were stained with anti-γ-tubulin (red) and anti-C-Nap1 (green) antibodies. Merge panels include DNA stained with Hoechst (blue).

B. Histogram represents the percentage of U2OS cells with more than two γ-tubulin staining centrosomes following treatment with HU alone or HU plus EHNA or vanadate. Data are shown as the mean of three experiments +/- standard deviation. Approximately 200 cells were counted per experiment.

C. To demonstrate dynein inhibition, untreated U2OS cells or those treated with HU plus nocodazole, EHNA or vanadate for 68 hours were processed for immunofluorescence microscopy and stained for the Golgi marker, golgin (red), and γ-tubulin (green). Dynein inhibition leads to dispersal of the Golgi apparatus. DNA was stained with Hoechst (blue). Scale bars; 10 µm.
To confirm inhibition of dynein with EHNA and vanadate, the localisation of the Golgi complex was examined. Golgi localisation depends upon an intact microtubule network and its accumulation in the vicinity of the centrosome is entirely dependent upon cytoplasmic dynein (Corthesy-Theulaz et al., 1992). Perturbation of dynein function would thus result in Golgi mislocalisation (Hirokawa, 1998). Untreated U2OS cells and those treated with HU plus nocodazole, EHNA or vanadate for 68 hours were fixed and stained with anti-golgin antibodies to identify the Golgi network and anti-γ-tubulin antibodies to identify the centrosome (Figure 5.11C). In untreated cells, the Golgi complex was seen to localise in the vicinity of the centrosome. Disruption of the microtubule network with nocodazole led to scattering of Golgi throughout the cell. Similarly, dynein inhibition with either EHNA or vanadate led to Golgi dispersal. This confirms that dynein has been inhibited and is unable to traffic proteins and complexes towards the centrosome.

5.2.7 DYNEIN IS REQUIRED FOR CENTRIOLE ACCUMULATION
Quintyne and Schroer (2002) reported that dynein is not required for centrosome duplication as measured by centriole formation visualised by centrin dot doubling. This suggests that dynein is dispensable for normal templated duplication. However, our results demonstrate that centrosome overduplication is prevented upon dynein inhibition. To investigate whether centriole formation continues in cells with inhibited dynein during prolonged S-phase arrest, TEM analysis of CHO cells treated with HU and EHNA for 48 hours was performed. TEM revealed that extra centrioles had not accumulated in these cells (Figure 5.12A), however the number of centriolar satellites per section was greatly increased. In fact, cells treated with HU and EHNA had an average of over 17 satellites per section, compared to approximately 9 in cells treated with HU alone and 4 in untreated cells (Figure 5.12). This confirms that centriolar satellites can still accumulate around the centrosome in cells with inhibited dynein during prolonged S-phase arrest, but that extra centrioles do not form.

5.2.8 MONASTROL DOES NOT PREVENT CENTROSOme OVERDUPICATION
To confirm that perturbation of centrosome overduplication was not solely due to a global effect of the chemical inhibitors used, the effect of a specific kinesin inhibitor on centrosome duplication was investigated. Monastrol, so called because it arrests cells in mitosis with monopolar spindles, is a specific inhibitor of kinesin Eg5 motility (Mayer et al., 1999). Eg5, a member of the mitotic kinesin subset of the kinesin superfamily, is a
Figure 5.12 Dynein is required for the overduplication of centrioles
A. TEM analysis of centrioles in CHO cells treated with HU and EHNA for 48 hours. Scale bar, 500 nm. B. Histogram shows the mean number of centriolar satellites within the EM sections that also contained centrioles from untreated CHO cells or those treated with HU alone or HU + EHNA. Error bars show standard deviation.
plus-end directed motor protein required for spindle bipolarity. In cells treated with monastrol the bipolar mitotic spindle is replaced by a mono-astral microtubule array surrounded by a ring of chromosomes. Monopolar spindles may be generated in three different ways: failure of centrosome duplication (Sluder et al., 1989; Winey et al., 1991); inhibition of centrosome separation (Heck et al., 1993; Hoyt et al., 1992; Sawin et al., 1992); or spindle collapse following normal centrosome duplication and subsequent separation (Sharp et al., 1999).

Monastrol has been shown to inhibit centrosome separation, but not duplication (Kapoor et al., 2000). Electron micrographs of monastrol-treated cells reveal four centrioles, corresponding to two centrosomes, at the centre of the mono-aster. Therefore, centrosome number is unaffected in monastrol treated cells, but centrosome separation is perturbed. Additionally, monastrol leads to disassembly of assembled bipolar spindles, although the spindle poles remain organised (Kapoor et al., 2000). NuMA is a structural protein that localises to the spindle poles and interacts with dynein (Merdes et al., 1996). Monastrol induced mono-asters have NuMA localised at their centres, indicating that the microtubules are focused at the centre of these structures. Monastrol treated bipolar spindles have NuMA staining and microtubule organisation that show the poles to be focused even at late stages of spindle disassembly (Kapoor et al., 2000). Therefore, monastrol does not inhibit activities, such as dynein motility, required to maintain the localisation of structural proteins at the poles and the focusing of the minus ends of microtubules. Additionally, no effect on the localisation and organisation of the Golgi apparatus or lysosomes is observed upon monastrol treatment thus supporting the notion that monastrol is not a general inhibitor of motor proteins (Mayer et al., 1999).

CHO cells were treated with HU and monastrol for 48 hours. Staining with antibodies against γ-tubulin and C-Nap1 revealed the accumulation of multiple centrosomes in these cells (Figure 5.13A). CHO:centrin1-GFP cells were treated similarly and stained for γ-tubulin and the GFP fluorescence visualised. Similarly, centrosome accumulation was observed to have occurred in these cells (Figure 5.13B). Comparison of the mean number of centrosomes in CHO and CHO:centrin1-GFP cells treated with either HU alone or HU plus monastrol showed similar levels of centrosome accumulation under each drug condition (Figure 5.13C). Therefore, monastrol has no effect on centrosome overduplication.
Figure 5.13 Treatment of cells with the Eg5 inhibitor, monastrol, has no affect on centrosome overduplication in CHO cells

A. CHO cells were treated with HU and monastrol for 48 hours and processed for immunofluorescence microscopy. Cells were stained with anti-γ-tubulin (red) and anti-C-Nap1 (green) antibodies. Merge panels include DNA stained with Hoechst (blue). B. CHO:centrin1-GFP cells were treated with HU and monastrol for 48 hours and processed for immunofluorescence microscopy. Cells were stained with anti-γ-tubulin (red) and anti-GFP (green) antibodies. Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 µm. C. Histogram represents the mean number of γ-tubulin staining centrosomes per cell in CHO and CHO:centrin1-GFP cells treated with HU alone or HU plus monastrol for 48 hours. Data are shown as the mean +/- standard deviation of three experiments. Approximately 200 cells were counted per experiment.
These results were confirmed in U2OS cells treated with HU and monastrol for 68 hours. Staining for γ-tubulin and C-Nap1 in these cells revealed the presence of supernumerary centrosomes (Figure 5.14A). The percentage of cells with greater than two centrosomes was comparable between cells treated with HU alone or HU plus monastrol (Figure 5.14B). This confirms that monastrol does not inhibit centrosome overduplication.

5.2.9 **Injection of Dynein-Specific Antibodies Prevents Centrosome Overduplication During S-Phase Arrest**

In a final approach to inhibit dynein, microinjection of neutralizing anti-dynein antibodies was employed. This method has been extensively used in a range of studies to investigate the role of dynein in a number of processes [including: (Gaglio et al., 1997; Kubo et al., 1999)].

CHO cells were injected in the cytoplasm with either control antibody (mouse IgGs) or a dynein-specific antibody (dynein intermediate chain mAb 70.1). At 4 hours post-injection, HU was added to the culture media and cells incubated for 48 hours. Cells were fixed and stained with anti-γ-tubulin antibodies to identify centrosomes and anti-mouse secondary antibodies to locate injected cells. In control cells multiple centrosomes had formed. However, in cells injected with dynein antibodies only two centrosomes were observed (Figure 5.15A). Comparison of the number of γ-tubulin staining centrosomes in control cells with cells injected with dynein antibodies revealed that centrosome overduplication occurred in the former, but was prohibited in cells in which dynein was inhibited (Figure 5.15B).

Investigation of centrosome overduplication using centrin staining in CHO cells injected with control or dynein antibodies revealed distinct staining patterns. In control cells, discrete centrin dots were seen corresponding to the formation of multiple centrioles (Figure 5.16A). On the other hand, in dynein injected cells tight clusters of individual foci were seen with both centrin and centrin2 antibodies (Figure 5.16B). This suggests that whilst centriole formation is prevented upon dynein inhibition, centriolar satellites containing centrin can still cluster together in the absence of a functional dynein-dynactin complex.
Figure 5.14 Treatment of cells with the Eg5 inhibitor, monastrol, has no affect on centrosome overduplication in U2OS cells

A. U2OS cells were treated with HU and monastrol for 68 hours and processed for immunofluorescence microscopy. Cells were stained with anti-γ-tubulin (red) and anti-C-Nap1 (green) antibodies. Merge panels include DNA stained with Hoechst (blue). Scale bar, 10 µm. B. Histogram represents the percentage of cells with more than two γ-tubulin staining centrosomes per cell in U2OS cells treated with HU alone or HU plus monastrol for 68 hours. Data are shown as the mean +/- standard deviation of three experiments. Approximately 200 cells were counted per experiment.
Figure 5.15 Microinjection of anti-dynein antibodies perturbs centrosome overduplication in CHO cells
A. Anti-dynein intermediate chain antibodies or control mouse IgGs were microinjected into CHO cells. After 4 hours cells were treated with HU for 48 hours and processed for immunofluorescence microscopy. Cells were stained with anti-γ-tubulin (red) antibodies. Cells were identified by anti-mouse secondary antibodies (green). Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 μm. B. Histogram represents the number of γ-tubulin staining centrosomes per cell in CHO cells microinjected with dynein antibodies or mouse IgGs and treated with HU for 48 hours. Data are shown as the mean +/- standard deviation of three experiments. Approximately 200 cells were counted per experiment.
Figure 5.16 Centrin foci cluster together in CHO cells injected with anti-dynein antibodies

CHO cells were injected with control mouse IgGs (A) or anti-dynein intermediate chain antibodies (B). After 4 hours, cells were treated with HU for 48 hours and processed for immunofluorescence microscopy. Cells were stained with anti-centrin or anti-centrin2 (red) antibodies. Injected cells were identified by anti-mouse secondary antibodies (green). Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 μm.
Microinjection of U2OS cells with dynein antibodies also prevented centrosome overduplication during prolonged S-phase arrest. Staining with γ-tubulin antibodies revealed the presence of only two centrosomes per cell. In control cells, multiple centrosomes form (Figure 5.17A). Less than 10% of cells injected with dynein antibodies contained more than two centrosomes, compared to over 50% of control cells (Figure 5.17B). These results confirm that dynein is required for centrosome overduplication during prolonged S-phase arrest.

**5.2.10 Multiple centrosomes rapidly accumulate following drug washout and release from dynein inhibition**

To determine the potential of cells treated concurrently with HU plus EHNA or vanadate to form multiple centrosomes, a drug washout was performed and the number of γ-tubulin positive centrosomes present in each cell quantified. If precursors to centrosome formation had accumulated in cells in which dynein had been inhibited during S-phase arrest, it was proposed that these would rapidly convert to centrosomes and recruit γ-tubulin upon drug washout. CHO cells treated with HU alone, or HU plus EHNA or vanadate for 48 hours were placed in drug-free media for 10 hours. Staining for γ-tubulin allowed the number of centrosomes in each cell to be quantified. As expected there was no further increase in centrosome number in HU treated cells following washout. However, in cells treated with HU and either of the dynein inhibitors, EHNA or vanadate, the number of centrosomes increased from two before washout to 4 and 3.6, respectively, following washout (Figure 5.18). This suggests that centriole formation, followed by γ-tubulin recruitment, had occurred in these cells following washout.

In conclusion, inhibition of dynein by a variety of methods prevents centrosome overduplication during prolonged S-phase arrest. Centriolar satellites as demonstrated by TEM analysis, along with centrin and PCM-1 staining, can still accumulate around the centrosome under these conditions. Dynein has been demonstrated to be necessary for the trafficking of centriolar satellites and indeed we show that dynamitin overexpression for 24 hours in cells leads to the dispersal of centrin and PCM-1 staining foci. This suggests that under these prolonged treatment periods centriolar satellites are recruited to the centrosome via an alternative mechanism which is slower than dynein-mediated transport.
Additionally, centrioles fail to form when dynein is inhibited even though centrin and centriolar satellites still accumulate around the centrosome.
Figure 5.17 Microinjection of anti-dynein antibodies perturbs centrosome overduplication in U2OS cells

A. Anti-dynein intermediate chain antibodies or control mouse IgGs were microinjected into U2OS cells. After 4 hours, cells were treated with HU for 68 hours and processed for immunofluorescence microscopy. Cells were stained with anti-\( \gamma \)-tubulin (red) antibodies. Injected cells were identified by anti-mouse secondary antibodies (green). Merge panels include DNA stained with Hoechst (blue). Scale bar, 10 \( \mu \)m.

B. Histogram represents the percentage of cells with greater than two \( \gamma \)-tubulin staining centrosomes per cell in U2OS cells microinjected with anti-dynein antibodies or mouse IgGs and treated with HU for 68 hours. Data are shown as the mean +/- standard deviation of three experiments. Approximately 200 cells were counted per experiment.
Figure 5.18 Drug washout reveals that cells in which dynein has been inhibited during HU-arrest can accumulate multiple γ-tubulin dots

A. CHO cells were treated with either HU alone or HU plus vanadate or EHNA for 48 hours. The drugs were then washed out for 10 hours. Cells were processed for immunofluorescence microscopy and stained for γ-tubulin. Scale bar, 10 μm. B. Histogram shows the number of γ-tubulin staining centrosomes per cell following 48 hours drug treatment (grey bars) and 48 hours treatment followed by 10 hours washout (black bars). Data are shown as the mean +/- standard deviation of three experiments. Approximately 200 cells were counted per experiment.
5.3 Discussion

A functional dynein-dynactin complex has been implicated in the trafficking of a range of proteins to the centrosome. Inhibition of dynein-mediated transport leads to the dispersal of centrin, pericentrin, ninein, PCM-1 and BBS4 (Dammermann and Merdes, 2002; Kim et al., 2004). Ninein and Nlp also require dynein for their localisation (Casenghi et al., 2005). However, a previous study has reported that dynein is not required for centrosome duplication during normal cell cycle progression (Quintyne and Schroer, 2002). To investigate whether dynein is required for centrosome overduplication we employed a range of methods to inhibit the dynein-dynactin complex. Centrosome overduplication, as measured by γ-tubulin and C-Nap1 staining, was found to be consistently perturbed in the presence of any one of the dynein inhibitors used.

Whilst these results support a role for a functional dynein-dynactin complex in centrosome overduplication, it was surprising to observe that the accumulation of centrin and PCM-1 foci in the vicinity of the centrosome was not prevented by dynein inhibition. These foci are proposed to be centriolar satellites as TEM analysis revealed a large increase in the number of satellites in cells treated with HU and EHNA as compared to cells treated with HU alone. An intact microtubule network and functional dynein-dynactin complex has been reported to be necessary for the movement and localisation of PCM-1 containing centriolar satellites around the centrosome (Dammermann and Merdes, 2002; Kubo et al., 1999). Indeed, in the absence of microtubules during prolonged S-phase arrest we see these foci localised around the periphery of the nucleus (Chapter 4). However, unlike the perinuclear distribution seen in the absence of microtubules, the centriolar satellites are seen to cluster around the pre-existing centrosome when dynein is inhibited during these prolonged assay periods. This suggests that an intact microtubule array but not dynein is essential for the localisation of centriolar satellites.

Dynamitin overexpression can be used to inhibit the dynein-dynactin complex and has been shown to disrupt the trafficking of centriolar satellites (Dammermann and Merdes, 2002; Kim et al., 2004). Similarly, we have shown that 24 hours overexpression of dynamitin in cells leads to the dispersal of PCM-1 and centrin. However, PCM-1 and centrin foci were found to still cluster around the centrosome during prolonged S-phase arrest in cells with inhibited dynein. This suggests that whilst dynein inhibition over short
periods in a normal cell cycle leads to the dispersal of centriolar satellites, that during prolonged periods of S-phase arrest these foci can be recruited to the centrosome via an alternative, although slower mechanism.

The abundance of a number of centrosomal proteins through their association with centriolar satellites is greatly increased in the vicinity of the centrosome during prolonged S-phase arrest in cells with inhibited dynein. However, no extra centrioles are seen to form. The recruitment of $\gamma$-tubulin to the centrosome has been reported to occur via both microtubule-independent (Khodjakov and Rieder, 1999) and dynein-dependent (Young et al., 2000) mechanisms. It is mostly like that we do not see the accumulation of $\gamma$-tubulin positive centrosomes though as extra centrioles have not formed due to dynein inhibition. This suggests that an early step in centrosome overduplication has been blocked and prevents centriole formation followed by subsequent PCM recruitment.

Together, these observations allow us to propose a model for the contribution of the dynein-dynactin complex to centrosome overduplication (Figure 5.19). Centriolar satellites can cluster around the centrosome in a dynein-independent manner. However, centriole formation is prevented in the absence of a functional dynein-dynactin complex and consequently the accumulation of functional $\gamma$-tubulin containing centrosomes is prevented.

There are a number of ways in which dynein may play a role in centrosome overduplication. It is plausible that dynein may be required for the trafficking of centriolar precursors that initiate centriole formation. However, these precursors must be unable to locate to the centrosome in the absence of functional dynein. It is more likely that dynein or indeed dynactin is required for an anchoring or stabilisation role during centriole formation. It is known that dynactin has protein anchoring roles at the centrosome independent of dynein (Echeverri et al., 1996; Quintyne et al., 1999; Quintyne and Schroer, 2002). Additionally, inhibition of the dynein-dynactin complex leads to the loss of centrosomal dynactin (Quintyne and Schroer, 2002). Therefore, the loss or destabilisation of centrosomal dynactin may well contribute to the inhibition of centrosome overduplication. In fact, overexpression of dynactin subunits that have no effect on dynein motility (Quintyne et al., 1999), but that destabilise the centrosomal pool of dynactin,
Figure 5.19 A model for the role of dynein in centrosome overduplication in S-phase arrested cells
In this model for the contribution of dynein to centrosome overduplication, centriolar satellites, containing centrin and PCM-1, cluster around the centrosome when dynein is inhibited; however, multiple centrioles do not accumulate. Dynein is required for the recruitment of additional components or anchoring of centriolar proteins that leads to centrosome overduplication.
delayed entry into S-phase during a normal cell cycle (Quintyne and Schroer, 2002). It was observed that the centrioles in these cells were no longer tightly coupled in G1, but become coupled again during S-phase. This implies that centrosomal dynactin is important for this coupling and thus cell cycle progression, and may suggest that it serves a role in centrosome duplication.

Whether it is dynactin or dynein alone, or a dynein-dynactin complex that is responsible for aiding centriole formation at the centrosome, there are two possible mechanisms by which they may act. They may function by anchoring centriole precursors at the site for centriole formation on the parental centrioles. Alternatively, they may function to stabilise the centriole architecture of nine microtubule triplets. These mechanisms are not mutually exclusive and it has previously been suggested that dynein-dynactin may provide the proper orientation of components and aid anchoring of molecules during centrosome assembly (Zimmerman and Doxsey, 2000).

Whilst dynein may be dispensable for normal templated duplication (Quintyne and Schroer, 2002), it is possible that in this instance there are sufficient levels of dynein and dynactin at the centrosome to facilitate one round of centriole formation; or that this requirement at the G1/S transition has been fulfilled before dynein has been inhibited. However, during our prolonged treatment periods, these centrosomal pools would be completely depleted leading to an inhibition of centrosome overduplication.
CHAPTER SIX

HEAT SHOCK PROTEIN 90 IS REQUIRED FOR THE RECRUITMENT OF γ-TUBULIN TO OVERDuplicated CENTRIOLES
6.1 INTRODUCTION

6.1.1 HEAT SHOCK PROTEINS

Heat shock proteins (HSPs) were first identified as a group of proteins that undergo a rapid increase in synthesis following exposure of Drosophila larvae to temperatures exceeding those optimal for growth (Tissieres et al., 1974). It is now known that a range of physiological stresses can initiate this response, with HSP synthesis bestowing on cells tolerance to stress (Parsell and Lindquist, 1993). However, HSPs are also present in unstressed cells and are essential for normal growth (Hendrick and Hartl, 1993; Hendrick and Hartl, 1995).

HSPs are some of the most conserved proteins known, existing in all organisms from bacteria to humans (Schlesinger, 1990). They function as molecular chaperones, stabilising other proteins by binding to them and influencing higher order protein structure. By controlled binding and subsequent release of proteins, molecular chaperones can facilitate the correct fate of these proteins in the cell (Ellis and van der Vies, 1991). This may be via protein folding, complex assembly, aiding transport or control of switching between active and inactive conformations. HSPs thus mediate many diverse cellular processes.

HSPs are grouped into five major classes and, due to significant overlaps in their function, are classified according to their molecular weights. Both Hsp70 (70 kDa) and Hsp90 (90 kDa) are reported to bind to microtubules [reviewed in: (Liang and MacRae, 1997)] and have been identified at the centrosome. Here they may modulate the assembly of centrosomal proteins or act to protect the centrosome from damage. Indeed, whilst heat shock leads to the dispersal of pericentriolar material, thermotolerant cells have an enhanced capacity to repair centrosomal damage (Vidair et al., 1993). This suggests that molecular chaperones may play a role in this process.

6.1.2 HSP90

Hsp90 comprises at least 1% of total cellular protein making it one of the most abundant proteins in eukaryotic cells (Welch and Feramisco, 1982). Along with a number of co-chaperones, Hsp90 functions in the folding of at least 200 different proteins (Buchner, 1999; Csermely et al., 1998; Pratt and Toft, 2003). Hsp90 is predominately cytoplasmic and is reported to localise to the centrosome under basal conditions, suggesting that the
chaperone may perform a centrosome-specific role (Wigley et al., 1999). This study also placed the proteasomal machinery along with HSPs at the centrosome, indicating that centrosomal Hsp90 may participate in protein degradation.

Hsp90 may also function in centrosome assembly or ensure proper centrosome function. In yeast, heat shock transcription factor (Hsf1) mutants are defective in the synthesis of members of the Hsp90 family of chaperones (Zarzov et al., 1997). Examination of the SPB, the yeast equivalent of the centrosome, reveals that duplication is perturbed in cells without Hsp90. Mutant cells possess only one SPB with an unusually long half-bridge structure. Hsp90 is a core centrosomal component structure in vertebrate cells, but is not required for centriole replication (Lange et al., 2000). Inactivation of Hsp90 in nonsynchronized cell cultures was found to cause the formation of aberrant mitotic spindles. This correlated with the presence of severely disrupted and/or fragmented centrosomes as visualised with antibodies against γ-tubulin and pericentrin. Centriole replication had been completed normally in these cells, but the PCM had become dispersed. One function of Hsp90 is therefore related to maintaining centrosome integrity (Lange et al., 2000). In fact, a role for Hsp90 in the recruitment and/or stabilisation of PCM during centrosome maturation is suggested from the observation that mitotic centrosomes are much more sensitive to the loss of Hsp90 function than interphase centrosomes (Lane and Nigg, 1996).

An interaction between Hsp90 and Polo/Polo-like kinases has been demonstrated in both Drosophila and human cells (de Carcer et al., 2001; Simizu and Osada, 2000). In Drosophila, Hsp90 is required to maintain levels of Polo, with inhibition of Hsp90 leading to inactivation of Polo kinase activity (de Carcer et al., 2001). As a consequence, cells with inhibited Hsp90 arrest in G2 or at the metaphase to anaphase transition, with Hsp90 function seemingly reflected in Plk stability (de Carcer, 2004). Centrosomes in HeLa cells injected with antibodies against Plk1 fail to recruit γ-tubulin (Lane and Nigg, 1996). This kinase is thus stabilised and regulated at the centrosome by its association with Hsp90 and contributes to centrosome maturation by its role in recruiting the γ-tubulin ring complex [reviewed in: (Glover, 2005)]. Additionally, HSPs have been implicated in the folding of γ-tubulin (Melki et al., 1993).
6.1.3 Hsp70

Many members of the Hsp70 family were originally identified as microtubule-associated proteins before their role as molecular chaperones was identified. It has since been suggested that Hsp70 may function either directly or indirectly to regulate microtubule assembly (reviewed by: (Liang and MacRae, 1997)). Like Hsp90, Hsp70 also localises to the centrosome and spindle poles under normal cell growth conditions (Rattner, 1991; Wigley et al., 1999). Indeed, Hsp70 associates with centrosomes in a range of species, from humans to unicellular eukaryotes (Perret et al., 1995). One member of the Hsp70 family (Hsp73) is reported to have a role in centrosome repair, with inhibition blocking centrosome assembly following heat treatment of cells (Brown et al., 1996). Additionally, the abundance of Hsp70 at the centrosome is found to be specifically increased under conditions of high levels of misfolded proteins (Wigley et al., 1999).

The localisation of Hsp70 at the centrosome suggests that it may participate in modulating centrosome assembly and/or behaviour during the cell cycle. An interaction between the centrosomal protein, centrin, and Hsp70 and Hsp90 was identified in CSF-arrested Xenopus eggs (Uzawa et al., 1995). A substantial pool of centrin is present in eggs and is required for centrosome assembly during early development. Hsp70 and Hsp90 form a complex with centrin to prevent it from associating with other centrosomal precursors. Upon activation, centrin is released from this complex by a rise in Ca\(^{2+}\) levels making it available for centriole formation.

More recently, comparative proteomic analyses of unduplicated and duplicated centrosomes from HeLa cells has identified a role for mortalin, a member of the Hsp70 family, in controlling centrosome duplication (Ma et al., 2006a). Mortalin localises to the centrosome in late G1, before centrosome duplication commences. Dissociation of p53 from the centrosome is promoted by mortalin and drives centrosome duplication. Mutant p53 that is unable to bind mortalin remains at the centrosome, thereby suppressing duplication. Additionally, mortalin overexpression circumvents p53-dependent suppression of centrosome duplication. This consequently leads to centrosome overduplication during HU-arrest in HeLa cells that do not normally accumulate centrosomes during prolonged S-phase arrest.
The localisation of molecular chaperones at the centrosome suggests that they may serve roles in the accumulation of correctly folded centrosomal components. HSPs could also function in centrosome assembly and duplication. Whilst Hsp90 is implicated in spindle pole body duplication in yeast (Zarzov et al., 1997), it appears to be dispensable for centriole formation in mammalian cells (Lange et al., 2000). However, the formation or maintenance of the PCM does appear to require Hsp90. We therefore examined the role of Hsp90 in centrosome overduplication using Hsp90 specific inhibitors and a range of centrosomal markers.
6.2 RESULTS

6.2.1 Hsp90 is required for centrosome overduplication

A range of Hsp90 inhibitors have been developed as potential chemotherapy agents. Indeed, the first Hsp90 inhibitor, geldanamycin, had clear antitumour effects (Supko et al., 1995; Whitesell et al., 1994). The Hsp90 ATP-binding site is highly conserved between species and is vital for regulating Hsp90 function (Panaretou et al., 1998). Geldanamycin is a specific competitive inhibitor of Hsp90, docking with its ATP-binding site (Prodromou et al., 1997; Stebbins et al., 1997). Hsp90 bound by geldanamycin is unable to form complexes, leading to the destabilisation of proteins that rely on Hsp90. 17-AAG (17-allylamino-17-demethoxy-geldanamycin) is a synthetic analog of geldanamycin (Schulte and Neckers, 1998). It inhibits Hsp90 in the same manner as geldanamycin, but displays much lower toxicity.

CHO cells were treated with HU along with either geldanamycin or 17-AAG to inhibit Hsp90. Following 48 hours treatment, cells were fixed and stained with antibodies against γ-tubulin and C-Nap1 to identify the centrosome. Examination of the cells revealed the presence of no more than two centrosomes per cell following treatment with either Hsp90 inhibitor (Figure 6.1A). Conversely, cells treated with HU and Heat Shock Protein Inhibitor 1 (HSPI-1), an inhibitor of Hsp70 (Yokota et al., 2000), were able to accumulate multiple centrosomes (Figure 6.1A). Comparison of the mean number of centrosomes in cells treated with HU plus geldanamycin, 17-AAG or HSPI-1 confirmed that centrosome overduplication as measured by γ-tubulin staining was perturbed upon Hsp90 inhibition (Figure 6.1B). Cells in which Hsp70 had been inhibited had a mean number of approximately five centrosomes per cell. On the other hand, cells in which Hsp90 had been inhibited with either geldanamycin or 17-AAG had a mean of only two centrosomes per cell. This suggests that Hsp90 plays a role in centrosome overduplication, with inhibition preventing centrosome accumulation during S-phase arrest.

Similarly, treatment of U2OS cells with Hsp90 inhibitors during prolonged S-phase also prevented centrosome overduplication. U2OS cells were treated with HU and either geldanamycin, 17-AAG or HSPI-1 for 68 hours. Staining of cells with antibodies against γ-tubulin and C-Nap1 revealed the presence of only two centrosomes in those cells with inhibited Hsp90. On the other hand, multiple centrosomes were found to have accumulated
Figure 6.1 Inhibition of Hsp90, but not Hsp70, blocks centrosome overduplication in S-phase arrested cells

A. CHO cells were treated with HU plus either Hsp90 inhibitors, geldanamycin or 17-AAG, or the Hsp70 inhibitor, HSPI-1, for 48 hours. The cells were processed for immunofluorescence microscopy and stained with antibodies against γ-tubulin (red) and C-Nap1 (green). Merge panels include DNA stained with Hoechst (blue). Scale bar, 10 µm.

B. Histogram represents the mean number of γ-tubulin staining centrosomes in cells treated with HU plus either geldanamycin, 17-AAG or HSPI-1. Data are shown as the average of three experiments +/- standard deviation. Approximately 200 cells were counted per experiment.
Figure 6.2 Hsp90 inhibition prevents centrosome overduplication in U2OS cells arrested with HU

A. U2OS cells were treated with HU plus either geldanamycin, 17-AAG or HSPI-1 for 68 hours. The cells were processed for immunofluorescence microscopy and stained with antibodies against γ-tubulin (red) and C-Nap1 (green). Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 μm. B. Histogram represents the percentage of cells with greater than two γ-tubulin staining centrosomes in cells treated with HU plus either geldanamycin, 17-AAG or HSPI-1. Data are shown as the average of three experiments +/- standard deviation. Approximately 200 cells were counted per experiment.
in cells treated with HSPI-1 (Figure 6.2A). Indeed, over 55% of cells treated with HU alone or HU plus HSPI-1 had greater than two γ-tubulin staining centrosomes, compared to approximately 15% of cells in which Hsp90 had been inhibited during HU-arrest (Figure 6.2). This therefore confirms that Hsp90 is required for the accumulation of γ-tubulin staining centrosomes during prolonged S-phase arrest.

### 6.2.2 Centrioles Form in the Presence of Hsp90 Inhibitors

Treatment of cells with geldanamycin has previously been reported not to prevent centriole formation (Lange et al., 2000). Using centrin1-GFP as a centriole marker we then looked at the role of HSPs in the formation of centrin foci during HU-arrest. CHO:centrin1-GFP cells were treated with HU plus geldanamycin, 17-AAG or HSPI-1 for 48 hours. Cells were then fixed and stained with antibodies against γ-tubulin and the GFP fluorescence visualised. Examination of cells treated with either of the Hsp90 inhibitors again revealed the presence of only two γ-tubulin staining centrosomes. However, multiple centrin foci, reminiscent of centrioles, were found to have formed around the existing centrosome. At least two of these dots co-localised with the γ-tubulin signal, whilst the remaining foci were not associated with γ-tubulin (Figure 6.3A). Treatment of cells with HSPI-1 on the other hand did not prevent centrosome overduplication, with the γ-tubulin and GFP signals co-localising at each centrosome (Figure 6.3B). Quantification of the number of centrin1-GFP dots in cells treated with HU plus geldanamycin, 17-AAG or HSPI-1 revealed that under each treatment condition cells contained approximately the same number of centrin foci (Figure 6.3C). This led to the postulation that multiple centrioles could form in the presence of either Hsp90 or Hsp70 inhibitors, but that Hsp90 is required for the recruitment of γ-tubulin to these structures.

To investigate this further, staining of endogenous centrin in cells treated with Hsp90 inhibitors was performed. CHO cells were treated with HU and either geldanamycin or 17-AAG for 48 hours. Staining with antibodies against γ-tubulin confirmed the presence of only two centrosomes in each cell. However, staining with anti-centrin2 or anti-centrin antibodies revealed the presence of multiple centrin foci, similar to centrioles, in the vicinity of the pre-existing centrosomes (Figure 6.4). This supports the observations from the CHO:centrin1-GFP cell line.
Figure 6.3 Centrin1-GFP dots accumulate around the centrosome during S-phase arrest in cells with inhibited Hsp90

CHO:centrin1-GFP cells were treated with HU plus either geldanamycin or 17-AAG (A); or HSPI-1 for 48 hours (B). The cells were processed for immunofluorescence microscopy and stained with antibodies against γ-tubulin (red) and GFP fluorescence monitored. Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 µm. C. Histogram represents the mean number of γ-tubulin and centrin1-GFP dots in cells treated with HU plus either geldanamycin, 17-AAG or HSPI-1. Data are shown as the average of three experiments +/- standard deviation. Approximately 200 cells were counted per experiment.
**Figure 6.4 Centrin dots form around the centrosome during S-phase arrest in cells with inhibited Hsp90**

CHO cells were treated with HU plus either geldanamycin or 17-AAG for 48 hours. The cells were processed for immunofluorescence microscopy and stained with antibodies against γ-tubulin (red) and centrin2 (A) or centrin (B) (green). Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 μm.
To determine whether centrioles could indeed form in cells with inhibited Hsp90 during prolonged S-phase arrest, TEM analysis was performed on CHO cells treated with HU and geldanamycin. Examination of sections revealed the presence of multiple centrioles within cells (Figure 6.5). This confirms that Hsp90 is dispensable for centriole formation, but is likely to be required for at least the recruitment of γ-tubulin to these newly formed centrioles.

6.2.3 Hsp90 inhibition prevents the recruitment of a range of centrosomal proteins to newly formed centrioles

Inhibition of Hsp90 by geldanamycin has been reported to cause the dispersal of the PCM as visualised with antibodies against γ-tubulin and pericentrin (Lange et al., 2000). Indeed, centrosomes are most sensitive to the loss of Hsp90 function during maturation when additional PCM components are recruited to the centrosome (Lane and Nigg, 1996). This suggests an important role for Hsp90 in the recruitment or stabilisation of PCM components. In support of these observations we have found that newly formed centrioles are unable to recruit γ-tubulin when Hsp90 has been inhibited (Figures 6.3 and 6.4). The lack of additional C-Nap1 staining in CHO and U2OS cells treated with HU and geldanamycin or 17-AAG (Figures 6.1 and 6.2) also suggests that it may not be just the recruitment of γ-tubulin that is affected upon Hsp90 inhibition.

CHO cells were treated with HU and geldanamycin for 48 hours. Cells were then fixed and stained for γ-tubulin plus C-Nap1, pericentrin or ninein. Visualisation of cells revealed that C-Nap1, pericentrin and ninein only co-localised with the γ-tubulin staining centrosomes (Figure 6.6). This suggests that these proteins are not recruited to newly formed centrioles in HU-arrested cells with inhibited Hsp90. Staining for PCM-1 on the other hand revealed that centriolar satellites still cluster in the vicinity of the centrioles formed in CHO:centrin1-GFP cells treated with HU and geldanamycin (Figure 6.7). Hsp90 therefore appears to be vital to the recruitment of a number of proteins to the centrosome, but is not required to localise centriolar satellites.

6.2.4 γ-Tubulin is lost from the centrosome upon Hsp90 inhibition

Polo kinase was demonstrated to undergo rapid degradation upon inhibition of Hsp90 in Drosophila SL2 cells (de Carcer et al., 2001). In contrast, the cellular abundance of a
Figure 6.5 TEM analysis reveals the presence of extra centrioles formed in CHO cells during HU-arrest and Hsp90 inhibition
CHO cells were treated with HU plus geldanamycin for 48 hours. The cells were then collected, fixed and processed for electron microscopy. Sections containing centrioles are shown from three individual cells. A. Multiple centrioles are shown in one section of a cell. B. and C. Serial sections are shown from two different cells revealing the presence of multiple centrioles in each cell. Scale bars, 500 nm.
Figure 6.6 Centrioles generated in HU-arrested cells with inhibited Hsp90 do not recruit γ-tubulin, C-Nap1, pericentrin or ninein

CHO cells were treated with HU plus geldanamycin for 48 hours. Cells were processed for immunofluorescence microscopy and stained with antibodies against γ-tubulin (red) and C-Nap1 (A), pericentrin (B), or ninein (C) (green). Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 µm.
Figure 6.7 PCM-1 signal is not lost in cells with inhibited Hsp90

CHO:centrin1-GFP cells were treated with HU and geldanamycin for 48 hours. Cells were processed for immunofluorescence microscopy and stained with antibodies against PCM-1 (red) and GFP fluorescence monitored. Merge panels include DNA stained with Hoechst (blue). Scale bar, 10 µm.
number of proteins, including γ-tubulin and α-tubulin, were unaffected. To confirm that our observations were not the result of a change in γ-tubulin levels, Western blot analysis of equalised cell extracts from untreated cells and those treated with HU alone or HU plus geldanamycin, 17-AAG or HSPI-1 was performed. The total abundance of γ-tubulin, α-tubulin and centrin were found to be comparable between each treatment suggesting these proteins remain stable in the presence of both Hsp70 and Hsp90 inhibitors (Figure 6.8A).

As γ-tubulin abundance remains constant in the presence of geldanamycin and 17-AAG it is likely that Hsp90 inhibition instead causes a defect in the recruitment of γ-tubulin to the centrosome. Quantification of γ-tubulin intensity at the centrosome revealed a drop in centrosomal γ-tubulin levels upon Hsp90 inhibition. U2OS cells were treated with HU alone or HU plus either geldanamycin, 17-AAG or HSPI-1 for 24 hours and stained for γ-tubulin. Images of cells were then obtained using constant imaging conditions so that the intensity of γ-tubulin at the centrosome could be compared between cells (Figure 6.8B). The relative intensity of γ-tubulin was found to be approximately 3-fold higher in cells treated with HU alone or HU plus HSPI-1 or EHNA, as compared to cells treated with either Hsp90 inhibitor. Hsp90 inhibition therefore causes the loss of γ-tubulin from centrosomes in S-phase arrested cells.

6.2.5 Multiple centrosomes rapidly accumulate following drug washout and release from Hsp90 inhibition

To determine the potential of the extra centrioles formed in cells with inhibited Hsp90 during prolonged S-phase arrest to recruit γ-tubulin, a drug washout was performed. CHO cells were treated with HU alone or HU plus geldanamycin, 17-AAG or HSPI-1 for 48 hours. Cells were then transferred to fresh growth media for 10 hours before being fixed and stained with anti-γ-tubulin antibodies. Examination of the cells revealed the presence of greater than two centrosomes following drug washout (Figure 6.9A). Quantification of the number of centrosomes per cell revealed that there were between four and five centrosomes in cells treated with HU alone, or HU plus HSPI-1 before and after washout (Figure 6.9B). Therefore, these cells do not accumulate extra centrosomes following drug washout. In contrast, the number of centrosomes in cells treated with Hsp90 inhibitors increased from two prior to washout to nearly four following washout. This suggests that upon drug washout, the extra centrioles formed in these cells are able to recruit γ-tubulin.
In conclusion, centrioles can form in cells with impaired Hsp90 function, however the recruitment of PCM components, including γ-tubulin and pericentrin, is perturbed. Hsp70 does not appear to be required for either of these processes.
Figure 6.8 γ-tubulin is stable in the presence of Hsp90 inhibitors, but is lost from the centrosome

A. Cell extracts were prepared from untreated CHO cells or CHO cells treated with HU alone or HU plus geldanamycin, 17-AAG, or HSPI-1. Western blots were probed with anti-α-tubulin, anti-γ-tubulin and anti-centrin antibodies. B. U2OS cells were treated with HU alone or HU plus either geldanamycin, 17-AAG, or HSPI-1 for 24 hours. The cells were processed for immunofluorescence microscopy and stained with antibodies against γ-tubulin. Three examples are shown of each. Scale bar, 10 µm. C. The mean intensity of γ-tubulin at the centrosome is shown for cells treated with HU alone, or HU plus geldanamycin, 17-AAG, HSPI-1 or EHNA. Data are shown as the mean intensity for 50 cells +/- standard deviation.
Figure 6.9 Drug washout reveals that cells in which Hsp90 was inhibited can accumulate multiple $\gamma$-tubulin staining centrosomes

A. CHO cells were treated with either HU alone or HU plus HSPI-1, geldanamycin, or 17-AAG for 48 hours, followed by 10 hours washout. Cells were processed for immunofluorescence microscopy and stained with antibodies against $\gamma$-tubulin. Scale bar, 10 $\mu$m. B. Histogram represents the number of $\gamma$-tubulin dots per cell following 48 hours drug treatment (grey bars) and 48 hours drug treatment followed by 10 hours washout (black bars). At least 200 cells were counted per experiment and data are shown as the average of three experiments. Standard deviation is indicated.
6.3 DISCUSSION

Molecular chaperones mediate many diverse cellular processes through an influence on higher order protein structure. Indeed, a close relationship exists between chaperones and the cytoskeleton, including roles in microtubule nucleation [reviewed by: (Liang and MacRae, 1997)]. Hsp90 is a core centrosomal component required at different stages of the centrosome cycle to ensure proper centrosome function (Lange et al, 2000). Treatment of mammalian cells with Hsp90 inhibitors leads to abnormal centrosome separation and maturation. Additionally, Hsp90 is also essential for the stability of key centrosome regulators, including Plk1 (de Carcer, 2004). In yeast, Hsp90 is required for SPB duplication (Zarzov et al., 1997), but not for centriole formation in mammalian cells (Lange et al., 2000).

Using the centrosome overduplication assay we have shown that the inhibition of Hsp90 prevents the accumulation of functional centrosomes. However, centrin staining, supported by TEM analysis, revealed the presence of multiple centrioles in cells with inhibited Hsp90. Thereby, we propose a model for centrosome overduplication in which Hsp90 is essential for the recruitment of PCM components, including \( \gamma \)-tubulin and pericentrin, to newly formed centrioles (Figure 6.10). But, Hsp90 is dispensable for centriole formation itself. Conversely, we found no effect on centrosome overduplication when Hsp70 was inhibited.

It may be somewhat surprising that centrioles can form when PCM recruitment is perturbed. Numerous studies have proposed a role for PCM in centriole formation, indeed \( \gamma \)-tubulin is likely to be required for the nucleation and assembly of centriolar microtubules (Dutcher, 2003b; Haren et al., 2006; Kleylein-Sohn et al., 2007). Further evidence that supports a role for \( \gamma \)-tubulin in centriole formation comes from a centriole assembly assay in *C. elegans* embryos. SPD-5 is an essential component of the PCM, whose depletion causes the loss of \( \gamma \)-tubulin around the centrioles (Dammermann et al., 2004; Hamill et al., 2002). Depletion of \( \gamma \)-tubulin, or SPD-5, in this assay system causes the failure of daughter centriole initiation approximately 50% of the time (Dammermann et al., 2004). Centrioles that do form contain reduced amounts of SAS-4 and fail to reach their full length. Further to this, these authors have recently demonstrated that \( \gamma \)-tubulin is required to stabilise
Figure 6.10 A model for the role of Hsp90 in centrosome overduplication in S-phase arrested cells

In this model for the contribution of Hsp90 to centrosome overduplication, centrioles are able to form in the absence of Hsp90 activity, however they are unable to recruit a number of centrosomal proteins, including γ-tubulin, pericentrin, ninein and C-Nap1.
SAS-4 in new centrioles, mostly likely by γ-tubulin-mediated addition of centriolar microtubules (Dammermann et al., 2008). The requirement of γ-tubulin for centriole formation in this system lead to the suggestion that new centrioles only form adjacent to pre-existing centrioles due to their ability to recruit PCM, including γ-tubulin (Dammermann et al., 2008; Dammermann et al., 2004).

PCM also appears to be important in the de novo centriole formation pathway. PCM foci that stain for γ-tubulin assemble following laser ablation of the existing centrosome (Khodjakov et al., 2002). New centrioles subsequently form within these PCM clouds. More recently, PCM has been proposed to control the number of daughter centrioles that can form around an existing centriole (Loncarek et al., 2008). Removal of the daughter centriole from the mother by laser microsurgery induces centriole reduplication in S-phase arrested cells. Under these experimental conditions, multiple daughter centrioles can form around a single mother. However, the number of daughter centrioles formed did not necessarily match the number of ablated centrioles. This suggested that it may not be the mother centriole that specifically defines the number of centrioles that can form. Loncarek et al. (2008) subsequently found that augmentation of the PCM by overexpression of pericentrin could drive the formation of multiple daughter centrioles in S-phase arrested cells. However, pericentrin itself is reported to be dispensable for centriole formation (Kleylein-Sohn et al., 2007), suggesting that this observation is due to the general increase in PCM. Indeed, Loncarek et al. (2008) suggest that the formation of daughter centrioles is initiated within the PCM cloud, but not necessarily in association with the mother centriole. A model is thereby proposed where the mother centriole provides a localised environment for centriole formation by its ability to organise a foci of PCM, rather than acting directly as a template. New centriole formation would therefore be restricted to the vicinity of exiting centrioles that can recruit PCM (Dammermann et al., 2008; Dammermann et al., 2004; Loncarek et al., 2008).

All this evidence supports an indispensable role for PCM in centriole formation. However, we observe continued centriole formation even when the recruitment of the PCM components, γ-tubulin and pericentrin, to the centrosome had been perturbed by Hsp90 inhibition. Dammermann et al. (2004) postulated that γ-tubulin may accelerate the kinetics of centriole assembly. Therefore, centrioles may fail to elongate upon γ-tubulin depletion.
in their assay system due to the time constraints imposed by the rapid cell cycles of the early \textit{C. elegans} embryo. If indeed \(\gamma\)-tubulin functions in centriole formation in this manner it is plausible that during our prolonged assay periods there is enough time for complete centrioles to assemble or that there is still sufficient \(\gamma\)-tubulin present at the centrosome to nucleate centriole formation. In the study by Dammermann et al. (2004), \(\gamma\)-tubulin was depleted to approximately 2\% of wild-type levels, suggesting that little, if any, remained at the centrioles. However, in our cells \(\gamma\)-tubulin levels are unaffected and staining was still observed at the pre-existing centrosomes, albeit at a reduced intensity when compared to cells with functional Hsp90. Thus, there is still a percentage of functional PCM material localised at the centrosome. It is possible that the \(\gamma\)-tubulin localised here is sufficient to support centriole formation without additional PCM recruitment. Indeed, the amount of \(\gamma\)-tubulin remaining at the centrosome may provide a limit to the number of new centrioles that can form.
CHAPTER SEVEN

CDK Activity and Nuclear Export are Required for Centrosome Overduplication
7.1 INTRODUCTION

7.1.1 CENTROsome DUPLICATION AND CYCLIN-DEPENDENT KINASES

The centrosome cycle is tightly coupled to the DNA cycle to ensure that by the time a cell enters mitosis it will have replicated both its centrosome and DNA. This synchronisation is achieved through regulation by a common component, Cdk2, which initiates both centrosome and DNA duplication. Studies in *Xenopus* embryos and egg extracts first identified a need for cyclin E-Cdk2 activity in centrosome duplication (Hinchcliffe et al., 1999; Lacey et al., 1999). Inhibition of Cdk2 was also found to prevent centrosome overduplication in S-phase arrested CHO cells (Matsumoto et al., 1999; Meraldi et al., 1999). Similarly, loss of the Cdk2 inhibitor p21 has been shown to trigger centriole overduplication (Duensing et al., 2006a; Duensing et al., 2000; Tarapore et al., 2001). A requirement for Cdk2 activity in centrosome duplication limits the initiation of replication to the G1-S transition and S-phase of the cell cycle. Indeed, CHO cells arrested in G1 do not undergo centrosome overduplication to the extent seen in S-phase arrested cells due to limited Cdk2 activity (Durcan et al., 2008; Matsumoto et al., 1999).

Somewhat unexpectedly, though, more recent investigations into the requirement of Cdk2 for centrosome duplication reveal that it may be dispensable for normal templated duplication and in fact may only drive centrosome reduplication. The first suggestion for this came from the observation that Cdk2<sup>−/−</sup> MEFs complete normal centrosome duplication, maturation and bipolar spindle formation (Duensing et al., 2006b). In contrast, Cdk2 deficiency or inhibition prevents centrosome amplification as caused by the E7 oncoprotein of human papillomavirus type 16 (HPV-16) (Duensing et al., 2004) and the recently reported formation of multiple daughter centrioles at single maternal templates is dependent upon Cdk2 activity (Duensing et al., 2007). Additionally, *Xenopus* extracts, depleted for cyclin E-Cdk2 could duplicate, but not reduplicate their centrosomes (Hinchcliffe et al., 1999). However, whilst these results strongly support a role for Cdk2 in centrosome reduplication, the apparent ability for centrosomes to undergo normal duplication in the absence of Cdk2 may reflect a redundancy between the actions of the different Cdk s.

As Cdk2<sup>−/−</sup> mice are viable, it is suggested that in these cells Cdk1 can substitute for Cdk2 in its absence (reviewed by: Berthet and Kaldis, 2007). Indeed, Cdk1 has been
demonstrated to be sufficient to drive the mammalian cell cycle in mice lacking all the interphase Cdk s (Cdk2, Cdk3, Cdk4 and Cdk6) (Santamaria et al., 2007). Therefore, Cdk1 appears to be the only essential cell cycle kinase. Recently, it has been reported that Cdk1 activity can compensate for the absence of Cdk2 to regulate centrosome duplication (Hochegger et al., 2007). However, the presence of a single Cdk2 allele drives S-phase progression independent of Cdk1, suggesting that Cdk2 is the dominant kinase. This provides further support that Cdk1 can take over the role of Cdk2 in its absence, and suggests that centrosome duplication can precede in the absence of Cdk2 due to Cdk1.

Centrosome overduplication during S-phase arrest occurs preferentially in the absence of p53 (Bennett et al., 2004; Fukasawa et al., 1996; Tarapore and Fukasawa, 2002). If functional p53 is present, reduplication is prevented by p21-mediated repression of Cdk2 activity. However, there is some evidence to suggest that the loss of functional p53 is not sufficient to drive centrosome reduplication. Kawamura et al. (2004) found that both cyclin E overexpression and loss of p53 were required to efficiently induce supernumerary centrosomes in human bladder cancer cells. Neither overexpression of cyclin E or loss of p53 alone could induce centrosome amplification. This suggests that Cdk2 activation is under some order of control in these cells, even in the absence of p53, and that higher levels of cyclin E are required to bring about sufficient Cdk2 activity to induce centrosome reduplication. Therefore, defects in both p53 function and increased Cdk2 activity are required to drive centrosome reduplication.

It remains unclear as to which cyclin partner for Cdk2 is vital to drive centrosome amplification. Indeed, centrosome amplification in cancer cells correlates with increased expression of both cyclin A and cyclin E (Kronenwett et al., 2003). Although HU-arrested HeLa cells have elevated cyclin E levels, they are incapable of centrosome overduplication (Balczon, 2001). This is presumably due to HeLa cells possessing functional p53 and suggests that increased cyclin E alone is not sufficient to drive centrosome reduplication. However, HeLa cells harbor HPV and, as such, express the E6 oncoprotein that promotes the degradation of p53, resulting in HeLa cells having low levels of functional p53. In a clonal derivative of HeLa cells, the HeLa1 cell line, E6 has been transcriptional silenced, resulting in these cells containing functional intact p53 at levels similar to normal cells. However, centrosome overduplication during S-phase arrest can be induced in HeLa1 cells by RNAi silencing of p53 expression (Ma et al., 2006a), suggesting that the low levels of
p53 in the parental cell line are sufficient to prevent centrosome overduplication. Investigating the differences between cells that can and cannot undergo centrosome overduplication during prolonged S-phase arrest revealed differences in cyclin A levels (Balczont, 2001). The comparison of HeLa and CHO cells during HU-arrest revealed that the former has depressed cyclin A levels compared to the latter, and thus reduced cyclin A-Cdk2 activity. Subsequently, overexpression of cyclin A, but not cyclin E, in HeLa cells was found to induce centrosome overduplication during S-phase arrest (Balczon, 2001). Furthermore, cyclin A appeared to be a more effective partner for Cdk2 activity than cyclin E in driving centrosome duplication in S-phase arrested CHO cells (Meraldi et al., 1999).

7.1.2 Cdk regulators and centrosome duplication

As cyclin-Cdk complexes function in the initiation of centrosome duplication, many proteins that regulate cyclin-Cdk activity are also implicated in the regulation of duplication. Indeed, overexpression of the Cdk inhibitors, p21 and p27, has been used extensively to investigate the role of Cdk2 in centrosome replication (Lacey et al., 1999; Matsumoto et al., 1999; Meraldi et al., 1999). Not surprisingly, p21 deficiency leads to the accumulation of multiple centrioles (Duensing et al., 2006a; Duensing et al., 2000; Tarapore et al., 2001). The Cdc25 phosphatases are required to activate Cdk by the removal of inhibitory phosphate groups. Three isoforms of Cdc25 exist in mammalian cells, although like Cdk there may be some overlap in their function. It was initially thought that each Cdc25 isoform had a specific role at defined stage of the cell cycle; however, it has recently been demonstrated that all isoforms can participate in the control of early and late cell cycle transitions, targeting all cyclin-Cdk complexes (Boutros et al., 2006). Furthermore, Cdc25B and Cdc25C knockout mice are viable (Ferguson et al., 2005), whilst Cdc25A seems to be the only essential Cdc25 phosphatase (Ray and Kiyokawa, 2007; Ray et al., 2007). Cdc25s, particularly isoforms A and B, are reported to be overexpressed in a number of cancers, representing poor clinical prognosis (Boutros et al., 2007a; Kristjansdottir and Rudolph, 2004). Whilst Cdc25A can shuttle between the nucleus and cytoplasm (Kallstrom et al., 2005), Cdc25B is the only isoform that has been reported to localise at the centrosome from S-phase until mitosis (Lindqvist et al., 2005). Overexpression of Cdc25B under an inducible promoter in S-phase synchronised U2OS cells results in rapid centrosome overduplication (Boutros et al., 2007b), whilst depletion of Cdc25B by siRNA causes the accumulation of cells in G2 with two separated centrosomes, each containing only a single centriole (Boutros and Ducommun, 2008).
Thus, Cdc25 presumably regulates centrosome duplication via its control of Cdk2 activation.

7.1.3 Cdk2 substrates and centrosome duplication

As described above, there is strong evidence supporting a role for Cdk2 in the regulation of centrosome duplication. However, the exact mechanisms for this remain elusive. One possibility is that Cdk2 is required for centriole disengagement at the initiation of duplication. Indeed, separation of paired centrioles in *Xenopus* extracts was dependent on cyclin-Cdk2 activity (Lacey et al., 1999). However, the relevance of this Cdk2-induced centriole disengagement in centrosome duplication is unclear.

Initiation of centrosome duplication at the G1-S transition coincides with Cdk2-dependent phosphorylation of a number of proteins that localise at the centrosome. These targets include nucleophosmin (NPM) (Okuda et al., 2000), Mps1 (mono-polar spindle 1) (Fisk and Winey, 2001) and CP110 (Chen et al., 2002), each of which has been implicated in centrosome duplication. NPM localises at the centrosome from mitosis until the G1/S transition in the following cell cycle (Okuda et al., 2000). Phosphorylation of NPM by Cdk2 displaces it from the centrosome and, significantly, blocking this phosphorylation prevents centrosome duplication (Okuda et al., 2000; Tarapore et al., 2002; Tokuyama et al., 2001). Localisation of NPM to the centrosome depends upon the nuclear export complex Ran-CRM1. Inhibition of this trafficking of NPM led to its displacement from the centrosome and initiation of premature centrosome duplication (Wang et al., 2005). This suggests that nuclear and centrosomal pools of NPM are in constant exchange and, if this exchange is prevented, centrosomal NPM is lost triggering duplication. Phosphorylation of NPM can therefore be envisaged to prevent its localisation rather than directly driving its displacement from the centrosome. Whilst the presence of NPM at the centrosome inhibits the initiation of centrosome duplication, it is not a true block to reduplication as it is not present at the centrosome between S-phase and mitosis.

The requirement for Mps1 in centrosome duplication remains controversial. In yeast, Mps1 is essential for SPB duplication (Winey et al., 1991). Mouse Mps1 was subsequently found to be able to induce centrosome reduplication during S-phase arrest in a Cdk2-dependent manner (Fisk and Winey, 2001). However, Mps1 was initially found to be dispensable for reduplication in human cells (Stucke et al., 2002). Further studies now suggest, in direct
contradiction to these results, that overexpression of Mps1 can accelerate centrosome reduplication in U2OS cells (Fisk et al., 2003), whilst the prevention of Mps1 degradation was sufficient to drive reduplication (Kasbek et al., 2007). This led to the hypothesis that phosphorylation of Mps1 by Cdk2 protects Mps1 from degradation, allowing Mps1 to promote centrosome duplication. One target of Mps1 in driving centrosome duplication may be the Hsp70 member mortalin (Kanai et al., 2007). In a tentative model, Cdk2 phosphorylation stabilises Mps1, which in turn phosphorylates mortalin. Mortalin then promotes the dissociation of p53 from the centrosome which drives duplication, although it remains far from clear as to how centrosomal p53 regulates centrosome duplication.

CP110 was first isolated in a screen for Cdk substrates and was subsequently found to localise at the centrosome (Chen et al., 2002). CP110 expression is strongly induced at the G1/S transition and it can be phosphorylated by both Cdk2 and Cdk1. Depletion of CP110 by siRNA was shown to prevent centrosome reduplication in HU-arrested U2OS cells, implicating CP110 in centrosome duplication. More recently, CP110 was demonstrated to be necessary for the formation of multiple centrioles around a single template as induced by Plk4 overexpression (Kleylein-Sohn et al., 2007). Although CP110 is reported to bind to centrin (Tsang et al., 2006), its role in centrosome duplication has not been fully elucidated. However, due to the presence of CP110 at the ends of growing procentrioles regardless of their length, it has been postulated that CP110 forms a cap under which MTs are added during procentriole extension (Kleylein-Sohn et al., 2007). Recently, CP110 was shown to interact with Cep97, a previously uncharacterised protein (Spektor et al., 2007). Disruption of Cep97 led to the loss of CP110 from centrioles and the apparent elongation of the centriole walls as visualised by polyglutamylated tubulin staining. Indeed, loss of either CP110 or Cep97 led to the formation of primary cilia in growing cells. Together, this suggests that CP110 is required to restrict the size of centrioles by modulating the addition of MTs to their walls. However, it remains unknown as to how Cdk2 regulates CP110 function in centriole duplication.

### 7.1.4 AIMS

A role for Cdk2 in centrosome reduplication has been demonstrated by many different groups. However, the mechanism by which Cdk2 promotes centrosome duplication, and the possible downstream roles of NPM, Mps1 and CP110, remain to be defined. As γ-tubulin has prominently been employed as a centrosomal marker in these studies, we wished to
investigate whether Cdk2 was required for the formation of the centrin foci we see early in the overduplication pathway. As Cdk2 is predominantly a nuclear protein, it remains unclear as to whether it phosphorylates nuclear or centrosomal targets to promote centrosome duplication therefore we also wished to investigate whether nuclear export is necessary for centrosome overduplication.
7.2 RESULTS

7.2.1 CDK INHIBITION PREVENTS CENTROSOME OVERTDPLICATION

Cdk activity in cells can be perturbed by the use of selective chemical inhibitors (Vesely et al., 1994). Roscovitine and olomoucine are selective inhibitors of Cdk2, Cdk1 and Cdk5, with very little effect on Cdk4 or Cdk6 (Meijer, 1996; Meijer et al., 1997). Additionally, a screen of over 150 kinases revealed that most other kinases are not affected by roscovitine (Bach et al., 2005). A requirement for Cdk2 in centrosome reduplication has previously been demonstrated by combining the use of roscovitine with prolonged HU-arrest in CHO cells (Matsumoto et al., 1999). We proposed to use roscovitine and olomoucine to investigate the formation of centrin foci as an early step in centrosome overduplication. Both roscovitine and olomoucine have previously been shown to induce a G1-arrest in human cells by inhibiting Cdk2 activity (Alessi et al., 1998). To ensure that cells were not prevented from entering a state permissive for centrosome overduplication, cells were first treated with HU for 18 hours prior to the addition of either roscovitine or olomoucine. Treating cells for a period of time corresponding approximately to one cell cycle would allow cells to become arrested at the G1/S transition or during S-phase. It would be expected that these cells would complete one round of normal templated duplication; however, reduplication in this time period would be limited as there is reported to be at least a 20 hour delay before reduplication occurs during HU-arrest (Balczon et al., 1995).

CHO:centrin1-GFP cells were first treated with HU for 18 hours followed by 30 hours treatment with HU and roscovitine. Immunofluorescence microscopy revealed the presence of only two γ-tubulin positive centrosomes per cell. These corresponded to four centrin1-GFP dots confirming that these cells had completed one round of duplication, but had not overduplicated their centrosomes. Strikingly, there were no additional centrin foci located anywhere within the cells (Figure 7.1A). Quantification of the number of γ-tubulin positive centrosomes in cells treated with either HU alone for 18 or 48 hours, or HU for 18 hours followed by HU plus roscovitine for 30 hours confirmed the block to centrosome overduplication. Cells treated with the Cdk inhibitor had an identical profile to cells treated with HU for 18 hours, verifying that no further duplication had occurred upon addition of roscovitine (Figure 7.1B). To confirm these results, the experiment was repeated in CHO:WT and CHO:centrin1-GFP cells using olomoucine in place of roscovitine. Again immunofluorescence microscopy revealed the absence of centrosome overduplication as
Figure 7.1 Cdk activity is required for centrosome overduplication

A. CHO:centrin1-GFP cells were treated with HU for 18 hours followed by 30 hours with HU and the Cdk inhibitor, roscovitine. Cells were processed for immunofluorescence microscopy and stained with γ-tubulin (red) and GFP (green) antibodies. Merge panels includes DNA stained with Hoechst (blue). Scale bar, 10 µm. Insets show magnified centrosome regions. B. Histogram indicates the number of centrosomes as detected by γ-tubulin staining in CHO:centrin1-GFP cells treated for 18 hours with HU (grey bars), 18 hours with HU followed by 30 hours with HU and roscovitine (white bars) or for 48 hours with HU (black bars). At least 200 cells were counted per experiment and the results are shown as the mean of three experiments. Standard deviations are indicated.
visualised by γ-tubulin staining. Furthermore, no additional centrin foci were observed in either cell line (Figure 7.2A and B). Comparison of the mean number of γ-tubulin positive centrosomes revealed an average of two centrosomes per cell in those treated with HU for 18 hours and those treated with HU first followed by olomoucine. In contrast, cells treated with HU alone for 48 hours had a mean number of approximately 6 centrosomes per cell (Figure 7.2B).

p53−/− MEFs are also capable of centrosome amplification during prolonged S-phase arrest [Figure 7.3A; (Tarapore et al., 2001)]. However, we found that inhibition of Cdk activity could also perturb centrosome amplification in these cells. p53−/− MEFs were treated with HU for 18 hours followed by HU plus roscovitine for 30 hours and stained for γ-tubulin and centrin. It was noted that the p53−/− MEFs display strong nuclear envelope staining with centrin2 antibodies (Figure 7.3); in fact, centrin2 has recently been reported to localise to nuclear pores in mammalian cells (Resendes et al., 2008). Inhibition of Cdk activity prevented centrosome overduplication in these cells, with only two γ-tubulin positive centrosomes in each cell (Figure 7.3B). Significantly, p53−/−Cdk2−/− MEFs were also unable to undergo centrosome overduplication during prolonged S-phase arrest (Figure 7.3C), suggesting that it is Cdk2, rather than Cdk1, that is required for centrosome overduplication. Therefore, taken together, these results support a requirement for Cdk2 in centrosome overduplication, and in the formation of centrin foci.

7.2.2 Cdk inhibition leads to the loss of centriolar satellites

To investigate whether additional centrioles could form in the absence of Cdk activity, TEM analysis was performed on CHO cells treated for 18 hours with HU followed by 30 hours with HU and roscovitine. As expected, no accumulation of centrioles was observed in any of the examined sections. Remarkably, it was noticed that there was also a complete absence of centriolar satellites (Figure 7.4A). Immunofluorescence staining for the centriolar satellite component, PCM-1, revealed a drastic change its localisation and intensity. In CHO:centrin1-GFP cells treated with HU alone, PCM-1 staining satellites clustered tightly around the overduplicated centrioles. However, in cells treated for 18 hours with HU, followed by HU and roscovitine for 30 hours, the PCM-1 satellites were much reduced in number and intensity, with those that remained being scattered throughout the cytoplasm (Figure 7.4B). Furthermore, whilst PCM-1 satellites clustered
Figure 7.2 The Cdk inhibitor olomoucine also blocks centrosome overduplication

A. CHO or B. CHO:centrin1-GFP cells were treated with HU for 18 hours followed by 30 hours with HU and olomoucine. Cells were processed for immunofluorescence microscopy and stained with γ-tubulin (red) and centrin or GFP (green) antibodies, respectively. Merge panels include DNA stained with Hoechst (blue). Scale bar, 10 μm. Insets show magnified centrosome regions.

C. Histogram indicates the mean number of centrosomes as detected by γ-tubulin staining in CHO cells treated for 18 hours with HU, 18 hours with HU followed by 30 hours with HU and olomoucine or for 48 hours with HU. At least 200 cells were counted per experiment and the results are shown as the mean of three experiments. Standard deviations are indicated.
Figure 7.3 Cdk activity is required for centrosome overduplication in p53<sup>−/−</sup> MEFs

p53<sup>−/−</sup> MEFs were treated with HU alone for 48 hours (A); or HU for 18 hours followed by
30 hours with HU and roscovitine (B); whilst p53<sup>−/−</sup>Cdk2<sup>−/−</sup> MEFs were treated with HU
alone for 48 hours (C). Cells were processed for immunofluorescence microscopy and
stained with γ-tubulin (red) and centrin2 (green) antibodies. Merge panels in (C) include
DNA stained with Hoechst (blue). Scale bars, 10 µm.
Figure 7.4 Cdk activity is required for the formation of centriolar satellites

A. CHO cells were treated with HU for 18 hours followed by 30 hours with HU and roscovitine. Cells were then processed for TEM analysis. Scale bar, 500 nm. B. CHO:centrin1-GFP cells were treated with HU for 48 hours or HU for 18 hours followed by 30 hours with HU and roscovitine. Cells were then processed for immunofluorescence microscopy and stained for PCM-1 (red) and GFP (green). Merge panels include DNA stained with Hoechst (blue). C. p53−/− and p53−/−Cdk2−/− MEFs were treated with HU or HU plus roscovitine as indicated. Cells were stained for PCM-1 (green), γ-tubulin (red) and DNA (blue). Scale bars in B and C, 10 µm.
around overduplicated centrosomes in p53−/− MEFs, they were absent in p53−/−Cdk2−/− MEFs and p53−/− MEFs treated with roscovitine (Figure 7.4C). These observations would suggest that Cdk2 activity is required for the formation and centrosomal accumulation of centriolar satellites.

We have shown that numerous perinuclear centrin foci form upon microtubule depolymerisation during prolonged S-phase arrest (Chapter 4 and Figure 7.5A). As these share many features of centriolar satellites, we argued that they are likely to be the same structure and potential early precursors to centrosome overduplication. Cdk inhibition prevented the accumulation of centrin foci during overduplication and in addition caused the loss of centriolar satellites. To directly determine if Cdk activity contributes to the formation of perinuclear centrin foci in cells treated with HU and nocodazole, roscovitine was added to these cells. Immunofluorescence microscopy of CHO:centrin1-GFP cells treated with HU for 18 hours, followed by HU plus nocodazole and roscovitine for 30 hours revealed the absence of centrin foci in these cells (Figure 7.5B). This suggests that Cdk activity is indeed required for the formation of these foci. As the prominent Cdk active during HU-arrest should be Cdk2 it was proposed that these foci would not form in cells arrested in G1 before Cdk2 becomes active. CHO:centrin1-GFP cells were therefore treated with mimosine alone or mimosine plus nocodazole for 48 hours. Immunofluorescence staining revealed the absence of both centrosome overduplication and centrin foci (Figure 7.5C and D). This indicates that centrin foci can only be formed in cells arrested in S-phase with active Cdk2.

7.2.3 CENTRIN ACCUMULATES IN THE NUCLEUS UPON CDK INHIBITION

Less than 10% of centrin is localised at the centrosome, with the remaining centrin distributed evenly between the cytoplasm and nucleus (Paoletti et al., 1996). In our experiments using roscovitine and olomoucine it was noted that centrin appeared to accumulate within the nucleus upon Cdk inhibition (Figures 7.1, 7.2 and 7.6A and B). To quantify this observation, CHO cells were treated with either HU alone for 48 hours or HU for 18 hours, followed by 30 hours with HU and roscovitine and stained with anti-centrin2 and anti-γ-tubulin antibodies. The intensity of centrin and γ-tubulin in the nucleus was then quantified for each of the treatments by measuring mean nuclear pixel intensities. The amount of γ-tubulin within the nucleus varied little between the two treatments. On the
Figure 7.5 Roscovitine prevents the accumulation of centrin aggregates in HU/nocodazole treated cells

CHO:centrin1-GFP cells were treated with HU and nocodazole for 48 hours (A); HU for 18 hours followed by 30 hours with HU, nocodazole and roscovitine (B); mimosine for 48 hours (C); or mimosine and nocodazole for 48 hours (D). Cells were processed for immunofluorescence microscopy and stained with γ-tubulin (red) and GFP (green) antibodies. Merge panels include DNA stained with Hoechst. Scale bars, 10 µm.
Figure 7.6 Centrin accumulates in the nucleus of cells treated with the Cdk inhibitor roscovitine

CHO cells were treated with HU for 18 hours followed by 30 hours with HU and the Cdk inhibitor roscovitine. Cells were processed for immunofluorescence microscopy and stained with γ-tubulin (red) and centrin (A) or centrin2 (B) (green) antibodies. Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 μm. Insets show magnified centrosome regions. C. The intensity of centrin2 and γ-tubulin in the nucleus (n>40) was quantified, and displayed as a fold-change over cells treated with HU alone.
other hand, there was nearly 4-fold more nuclear centrin in cells subjected to the combined HU-roscovitine treatment compared to those treated with HU alone (Figure 7.6C).

Centrin has previously been demonstrated to accumulate within the nucleus upon inhibition of nuclear export (Keryer et al., 2003). The CRM1/exportin1 export factor can be specifically inhibited using leptomycin B (LMB) (Kudo et al., 1999). In addition to the nuclear accumulation of centrin upon treating cells with LMB for 3 hours, Keryer et al. (2003) also found pericentrin to be blocked within the nucleus. On the contrary, the localisation of γ-tubulin and ninein was not affected. LMB can also cause cells to arrest in G1 and G2-phases of the cell cycle, therefore we continued to use a HU-treatment for 18 hours before the addition of HU and LMB for 30 hours. CHO cells were treated in this manner and stained with anti-γ-tubulin and anti-centrin or anti-centrin2 antibodies. γ-tubulin did not accumulate within the nucleus and the γ-tubulin centrosomal staining was not depleted. Staining with either centrin antibody revealed the accumulation of centrin within the nucleus, although some did remain at the centrosome (Figure 7.7A and B). Quantification of the intensity of centrin and γ-tubulin in the nucleus was performed for cells treated with HU-alone for 48 hours or the combined HU-LMB treatment. The amount of γ-tubulin within the nucleus varied little between the two conditions. On the other hand, there was nearly 3-fold more nuclear centrin in cells subjected to the combined HU-LMB treatment compared to those treated with HU alone (Figure 7.7C). It was also noted that cells in which nuclear export had been inhibited during prolonged HU-arrest contained only two γ-tubulin positive centrosomes. In addition, many nuclear centrin foci were observed upon LMB treatment in S-phase arrested cells, in sharp contrast to the diffuse nuclear centrin staining pattern seen in those cells treated with Cdk inhibitors. Therefore, we went on to investigate the requirement of nuclear export for centrosome overduplication.

7.2.4 Nuclear export is required for centrosome overduplication

It has been demonstrated that nuclear events are dispensable for centrosome duplication in embryonic cells, with duplication completely under cytoplasmic control (Sluder and Lewis, 1987; Sluder et al., 1990; Sluder et al., 1986). However, centrosome assembly does not occur in enucleated somatic cells, suggesting that the nucleus contributes something to
Figure 7.7 Centrin aggregates accumulate in the nucleus when nuclear export is inhibited
CHO cells were treated with HU for 18 hours followed by 30 hours with HU and the nuclear export inhibitor, LMB. Cells were processed for immunofluorescence microscopy and stained with γ-tubulin (red) and centrin (A) or centrin2 (B) (green) antibodies. Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 µm. C. The intensity of centrin2 and γ-tubulin in the nucleus of cells treated with HU and LMB was quantified and displayed as a fold-change over cells treated with HU alone (n>50).
centrosome duplication (Kuriyama and Borisy, 1981; Kuriyama et al., 2007; Maniotis and Schliwa, 1991). Cdk2 is a nuclear enzyme, although it could be postulated that nucleocytoplasmic shuttling of Cdk2 with its cyclin partners would allow for phosphorylation of centrosomal Cdk2 targets within the cytoplasm (Jackman et al., 2002). Yet, we were intrigued by the perinuclear association of centrin foci formed in cells treated with HU and nocodazole, the accumulation of centrin within the nucleus of cells treated with HU and roscovitine or olomoucine, and the apparent formation of nuclear centrin foci in cells treated with HU plus LMB. To confirm the requirement of nuclear export for centrosome overduplication, CHO:centrin1-GFP cells were treated with HU and LMB as described above. Immunofluorescence microscopy revealed the presence of only two γ-tubulin staining centrosomes present in each cell (Figure 7.8A), confirming the observation seen with the wild-type CHO cells. Additionally, centrin1-GFP accumulated within the nucleus, with a number of distinct nuclear centrin1-GFP foci observed. Quantification of the mean number of γ-tubulin staining centrosomes in cells treated with either HU alone or the combined HU plus LMB treatment clearly demonstrated the inhibition of centrosome overduplication caused by perturbing nuclear export. Only two centrosomes were seen in cells treated with LMB, compared with 4.5 in cells treated with HU alone (Figure 7.8B). These results strongly support a role for nuclear export in centrosome overduplication, with nuclear centrin foci formation potentially being a very early step in this pathway.

To investigate the relevance of these nuclear centrin foci, we set out to determine whether they form in other cell types during centrosome overduplication. Firstly, U2OS cells were treated with either HU alone for 68 hours, or HU for 18 hours followed by HU plus LMB or roscovitine for 50 hours. Immunofluorescence microscopy revealed that U2OS cells treated with HU alone accumulate multiple centrin staining centrioles. Additionally, centrin staining is seen throughout the nucleus and cytoplasm (Figure 7.9A). In cells in which either nuclear export or Cdk activity had been inhibited no more than four centrin dots corresponding to centrioles were seen. Under both these treatments centrin was also seen to accumulate within the nucleus. Following Cdk inhibition this nuclear staining was relatively diffuse, whilst in cells treated with LMB a number of small, intense, nuclear centrin foci were observed (Figure 7.9B and C). Less than 10% of cells accumulated more than two γ-tubulin staining centrosomes when either nuclear export or Cdk activity were inhibited, compared to over 60% of cells treated with HU alone (Figure 7.9D).
Figure 7.8 Nuclear export is required for centrosome overduplication

A. CHO:centrin1-GFP cells were treated with HU for 18 hours followed by 30 hours with HU and the nuclear export inhibitor, LMB. Cells were processed for immunofluorescence microscopy and stained for γ-tubulin (red) and GFP (green). Merge panels include DNA stained with Hoechst (blue). Scale bar, 10 μm. B. Histogram represents the number of centrosomes per cell as observed by γ-tubulin staining in cells treated with HU alone or HU plus LMB. At least 200 cells were counted per experiment and data are shown as the mean of three experiments. Standard deviation is indicated.
Figure 7.9 Nuclear export and Cdk activity is required for centrosome overduplication in U2OS cells
U2OS cells were treated with HU alone for 68 hours (A); or HU for 18 hours followed by 50 hours with HU and LMB (B) or HU and roscovitine (C). Cells were processed for immunofluorescence microscopy and stained with centrin2 antibodies. Two examples are shown of each treatment. Scale bar, 10 µm. Insets indicate over duplicated centrosomes. Arrowheads indicate centriole pairs. D. Histogram indicates the percentage of U2OS cells with greater than two centrosomes in untreated cells or cells treated with HU alone, HU and roscovitine or HU and LMB. At least 200 cells were counted per experiment and the data are shown as the average of three experiments. Standard deviations are indicated.
Inhibition of nuclear export similarly prevented centrosome overduplication in both p53\(^{-/-}\) and p53\(^{-/-}\) Cdk2\(^{-/-}\) MEFs (Figure 7.10). As these MEFs display very strong nuclear envelope centrin2 staining, it was difficult to determine any change in the level of nuclear centrin following treatment with LMB. However, discrete centrin foci were observed within the nucleus of these cells when nuclear export was inhibited (Figure 7.10A). Intriguingly, p53\(^{-/-}\) Cdk2\(^{-/-}\) MEFs did not accumulate any similar centrin foci upon treatment with LMB (Figure 7.10B). This confirmed that the formation of these foci is dependent upon Cdk2 activity.

### 7.2.5 Cdk inhibition prevents the formation of nuclear centrin foci

It was noted that whilst the inhibition of both Cdk activity and nuclear export resulted in the nuclear accumulation of centrin, there was still a significant difference between the two treatments. Nuclear centrin in cells with inhibited Cdk activity was relatively uniform across the whole nucleus. On the other hand, individual discrete centrin foci were detected in the nuclei of cells in which nuclear export had been inhibited. Together, with the observation that nuclear centrin foci did not form in LMB treated p53\(^{-/-}\) Cdk2\(^{-/-}\) MEFs, this suggested that the formation of these centrin foci was dependent upon Cdk activity. To confirm this suspicion, cells were subjected to a combined treatment of HU plus LMB and Cdk inhibitor. CHO, CHO:centrin1-GFP cells or p53\(^{-/-}\) MEFs were treated with HU for 18 hours, followed by HU plus LMB and roscovitine for 30 hours. Visualisation of centrin, centrin2 or GFP confirmed the nuclear accumulation of centrin and the absence of nuclear centrin foci (Figure 7.11 A, B and C). These results were confirmed with centrin2 staining in CHO cells treated with olomoucine instead of roscovitine (Figure 7.11D). Therefore, Cdk activity is required for the formation of nuclear centrin foci, with centrin accumulating in the nucleus in a diffuse form in the absence of Cdk activity. Consistent with this requirement, we found that nuclear centrin foci were unable to form in LMB-treated cells arrested in G1 with mimosine (Figure 7.12).

### 7.2.6 Pericentrin is nucleolar upon inhibition of nuclear export

To determine whether the nuclear centrin foci observed upon inhibition of nuclear export contained other known centrosomal proteins, CHO cells treated with HU and LMB were stained with a range of antibodies. The foci did not stain for modified tubulins (data not shown), pericentrin (Figure 7.13A), or PCM-1 (Figure 7.16A) suggesting that they are distinct from the cytoplasmic foci seen in cells treated with HU and nocodazole. Indeed,
Figure 7.10 Nuclear export is required for centrosome overduplication in p53^-/- and p53^-/-Cdk2^-/- MEFs
p53^-/- (A) and p53^-/-Cdk2^-/- (B) MEFs were treated with HU for 18 hours followed by 30 hours with HU and LMB. Cells were processed for immunofluorescence microscopy and stained with γ-tubulin (red) and centrin2 (green) antibodies. Scale bar, 10 μm.
Figure 7.11 Cdk activity is required for the formation of nuclear centrin granules in LMB treated cells arrested with HU

A. CHO:centrin1-GFP, B. CHO cells or C. p53-/- MEFs were treated with HU for 18 hours followed by HU, LMB and roscovitine for 30 hours. Cells were processed for immunofluorescence microscopy and stained for \(\gamma\)-tubulin (red) and GFP, centrin or centrin2 (green) as indicated. Merge panels in (A) and (B) include DNA stained with Hoechst (blue).

D. CHO cells were treated with HU for 18 hours followed by HU, LMB and olomoucine for 30 hours. Cells were processed for immunofluorescence microscopy and stained with centrin2 (green) antibodies. Merge panels include DNA stained with Hoechst (blue). Each experiment was repeated three times and at least 200 cells examined on each occasion. Scale bars, 10 \(\mu\)m.
Figure 7.12 Formation of nuclear centrin granules occurs in LMB treated cells arrested with HU but not mimosine

CHO:centrin1-GFP cells were treated with mimosine for 18 hours (G1 arrest) followed by mimosine and LMB for 30 hours (A); or HU for 18 hours (S-phase arrest) followed by HU and LMB for 30 hours (B). GFP fluorescence was then monitored. Images are shown as the maximum intensity projection of z-sections collapsed into a single image. Scale bars, 10 µm.
Figure 7.13 Pericentrin accumulates in the nucleolus of cells treated with LMB

A. CHO:centrin1-GFP cells were treated with HU for 18 hours followed by HU and LMB for 30 hours. Cells were processed for immunofluorescence microscopy and stained for GFP (green) and pericentrin (red).

B. CHO cells were treated with HU alone for 48 hours or 18 hours followed by HU and LMB for 30 hours. Cells were processed for immunofluorescence microscopy and stained for pericentrin (green) and NPM (red). Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 µm.

C. The intensity of pericentrin and NPM in the nucleolus was quantified in cells treated with HU alone (grey bars) and those treated HU plus LMB (black bars) and displayed as mean pixel intensity (n>30).
they may act as precursors to these foci with centrin foci first forming within the nucleus and then these proteins being added to the centrin foci upon nuclear export. However, pericentrin has been reported to accumulate within the nucleus following 3 hours treatment of cells with LMB (Keryer et al., 2003). During our prolonged treatment with LMB during S-phase arrest we found pericentrin to have a very specific and distinct localisation within the nucleus (Figure 7.13A). This staining was reminiscent of nucleoli, and indeed staining cells with antibodies against the nucleolar protein, NPM, revealed a strong co-localisation between the NPM and pericentrin signals (Figure 7.13B). Quantification of pericentrin and NPM within nucleoli in cells treated with HU alone or HU plus LMB revealed that the intensity of each protein approximately doubled upon inhibition of nuclear export (Figure 7.13C). Using NPM staining to identify nucleoli revealed that a fraction of centrin also localised to nucleoli (Figure 7.14A). Similar to pericentrin, nucleolar centrin also increased upon inhibition of nuclear export with a nearly 3-fold increase in intensity (Figure 7.14B and C).

NPM has been identified at unduplicated centrosomes with phosphorylation by Cdk2 leading to its dissociation. If this phosphorylation is prohibited, NPM remains at the centrosome and duplication is prevented (Okuda et al., 2000; Tarapore et al., 2002; Tokuyama et al., 2001). NPM is also involved in the nucleocytoplasmic transport of proteins, interacting with CRM1 through a NES (Borer et al., 1989; Wang et al., 2005). Inhibition of CRM1 by LMB prevents NPM targeting to the centrosome, leading to its dissociation and premature duplication. Due to our initial treatment of cells with HU for 18 hours before adding LMB, most cells complete one round of normal templated duplication before nuclear export is inhibited. It could thus be envisaged that to complete this duplication event Cdk2 has phosphorylated NPM, causing its loss from the centrosome. Inhibition of nuclear export then prevents centrosome reduplication. Intriguingly, inhibition of Cdk activity during S-phase arrest led to the apparent loss of nucleoli, with the dispersal of NPM, centrin and pericentrin nucleolar signals (Figure 7.15). It was also noted that that whilst the treatment of cells with Cdk inhibitors led to the nuclear accumulation of centrin, pericentrin was completely absent from the nucleus when Cdk activity was inhibited (Figure 7.15).
Figure 7.14 Centrin accumulates in the nucleolus of cells treated with LMB
CHO cells were treated with HU alone for 48 hours (A); or HU for 18 hours followed by HU and LMB for 30 hours (B). Cells were processed for immunofluorescence microscopy and stained for centrin2 (green) and NPM (red). Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 μm. C. The intensity of centrin in the nucleolus in cells treated with HU alone or HU plus LMB was quantified and displayed as mean pixel intensity (n>35).
Figure 7.15 Nucleolar staining disappears when Cdk5 are inhibited
CHO cells were treated with HU for 18 hours followed by HU and roscovitine for 30 hours. Cells were processed for immunofluorescence microscopy and stained for NPM (red) and centrin2 (A) or pericentrin (B) (green). Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 µm.
7.2.7 Nuclear export is required for the formation of centriolar satellites

Inhibition of Cdk activity during prolonged S-phase arrest led to the loss of centriolar satellites. To investigate whether inhibition of nuclear export has a similar effect, CHO:centrin1-GFP cells treated with HU and LMB as described above were fixed and stained with antibodies against the centriolar satellite component, PCM-1. In cells treated with HU alone, a strong PCM-1 signal, corresponding to many satellites, is seen clustered tightly around overduplicated centrosomes (Figure 7.16A). However, upon LMB treatment the PCM-1 signal is greatly reduced in intensity and is dispersed throughout the cytoplasm (Figure 7.16A). This observation is highly similar to the PCM-1 signal seen in cells treated with roscovitine (Figure 7.4C). Additionally, the PCM-1 signal in U2OS cells also disappears upon nuclear export or Cdk inhibition (Figure 7.17). It has previously been reported that PCM-1 mRNA must be synthesized anew each cell cycle (Balczonz et al., 1995), and that roscovitine inhibits RNA synthesis (Ljungman and Paulsen, 2001). It is therefore possible that LMB blocks mRNA export of critical centriolar satellite mRNAs, including that of PCM-1. Nevertheless, analysis of total cellular abundance of PCM-1, along with γ-tubulin, α-tubulin and centrin, by Western blot, showed that neither roscovitine nor LMB altered the levels of these proteins within cells as compared to cells treated with HU alone (Figure 7.16B). Therefore, despite these treatments, these centrosomal proteins remain available within the cell and it can be concluded that both Cdk activity and nuclear export are required for the formation of PCM-1 containing centriolar satellites.

OFD-1 is a centrosome-associated protein encoded by OFD1, the gene mutated in oral-facial-digital type 1 syndrome (Ferrante et al., 2001; Romio et al., 2003). OFD-1 localises to centrosomes and the basal body of primary cilia and has recently been identified in the nucleus (Giorgio et al., 2007; Romio et al., 2004). Moreover, data from our laboratory indicates that OFD-1 also co-localises with PCM-1 in centriolar satellites (C. Lopes, unpublished data). In CHO cells treated with HU for 48 hours, anti-OFD-1 antibodies display a strong localisation at overduplicated centrosomes (Figure 7.18A). However, whilst there is no nuclear accumulation when nuclear export or Cdk activity is inhibited, OFD-1 signal is completely lost from the centrosome when cells were treated with LMB or roscovitine (Figure 7.18B and C). This provides further support that a range of centrosomal proteins rely on both Cdk activity and nuclear export for their localisation.
Figure 7.16 PCM-1 signal disappears when nuclear export is inhibited

A. CHO:centrin1-GFP cells were treated with HU alone for 48 hours, or HU for 18 hours followed by HU and LMB for 30 hours. Cells were processed for immunofluorescence microscopy and stained for GFP (green) and PCM-1 (red). Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 µm.

B. Cell extracts were prepared from CHO cells treated with either HU alone or HU plus roscovitine or LMB. Western blots were probed with anti-PCM-1, anti-γ-tubulin, anti-α-tubulin and anti-centrin2 antibodies.
Figure 7.17 PCM-1 signal in U2OS cells disappears when nuclear export or Cdk activity is inhibited

U2OS cells were treated with HU alone for 68 hours (A); HU for 18 hours followed by HU and LMB for 50 hours (B); or HU for 18 hours followed by HU and roscovitine for 50 hours (C). Cells were processed for immunofluorescence microscopy and stained for PCM-1. DNA was stained with Hoechst. Inset shows normal PCM-1 signal. Scale bars, 10 µm.
**Figure 7.18** OFD-1 signal disappears when nuclear export or Cdk activity is inhibited

CHO cells were treated with HU alone for 48 hours (A); HU for 18 hours followed by HU and LMB for 30 hours (B); or HU for 18 hours followed by HU and roscovitine for 30 hours (C). Cells were processed for immunofluorescence microscopy and stained for γ-tubulin (green) and OFD-1 (red). Merge panels include DNA stained with Hoechst (blue). Scale bars, 10 µm.
7.2.8 CDK activity is required for an early step in centrosome reduplication

To determine how rapidly cells in which nuclear export or Cdk activity had been inhibited during S-phase arrest could accumulate functional centrosomes containing γ-tubulin, a 10 hour washout was performed following treatment of cells as described in the above experiments. Upon washout, cells treated with HU alone do not accumulate any extra centrosomes. Similarly, those cells treated with Cdk inhibitors could not accumulate centrosomes upon drug washout, with only an average of two γ-tubulin positive centrosomes present before and after washout (Figure 7.19). To the contrary, cells treated with HU plus LMB were able to accumulate extra γ-tubulin positive centrosomes upon washout, with an increase from two to nearly four centrosomes per cell. Cells treated with HU, LMB and roscovitine had an average of only two centrosomes following washout (Figure 7.19). Together, these results suggest that intermediate centrosome precursors form in the nucleus of cells in which nuclear export has been inhibited. Upon drug washout, these structures leave the nucleus and rapidly contribute to the formation of extra centrosomes. Inhibition of Cdk activity prevents the accumulation of these precursors, so that extra centrosomes are unable to rapidly form upon washout due to the absence of intermediate structures that can facilitate their formation. Therefore, Cdk activity is an early and, potentially rate limiting, step in the centrosome overduplication pathway.
Figure 7.19 Drug washout reveals that cells in which nuclear export was inhibited, but not in which Cdk activity was inhibited, can rapidly accumulate multiple \( \gamma \)-tubulin dots

**A.** CHO cells were treated with either HU alone or HU plus roscovitine; olomoucine; LMB; or LMB plus roscovitine for 48 hours. The drugs were then washed out for 10 hours. Cells were processed for immunofluorescence microscopy and stained for \( \gamma \)-tubulin. Two examples are shown of each. Scale bar, 10 \( \mu \)m.

**B.** Histogram shows the number of \( \gamma \)-tubulin dots per cell following 48 hours drug treatment (grey bars) and 48 hours treatment followed by 10 hours washout (black bars). At least 200 cells were counted per experiment and data are shown as the average of three experiments. Standard deviation is indicated.
7.3 DISCUSSION

Morphologically, centrosome duplication commences in late G1 or early S-phase, with the appearance of a procentriole next to the proximal ends of each of the existing centrioles (Kuriyama and Borisy, 1981). Whilst they are not dependent upon one another (Balczon et al., 1995; Kuriyama et al., 1986; Rattner and Phillips, 1973), co-ordination of the centrosome duplication and DNA replication cycles would ensure that by the time a cell enters mitosis it will have duplicated both its DNA and centrosome. This could be achieved by common components regulating both events such that they are initiated at the same time. Cdk2 is one such cell cycle regulator that could accomplish this. Indeed, a number of studies have implicated Cdk2 in centrosome duplication (Hinchcliffe et al., 1999; Lacey et al., 1999; Matsumoto et al., 1999; Meraldi et al., 1999). However, recent work has suggested that Cdk2 is dispensable for normal centrosome duplication (Duensing et al., 2006b), but required for centrosome amplification. Inhibition of Cdk2 activity prevents centriole overduplication as triggered by a range of methods (Duensing et al., 2007; Duensing et al., 2004). However, completion of normal centrosome duplication in Cdk2−/− MEFs may simply reflect the ability of Cdk1 to compensate for the absence of Cdk2 (Hochegger et al., 2007).

A requirement for Cdk2 activity in the overduplication of γ-tubulin-stained centrosomes in HU-arrested CHO cells has previously been demonstrated (Matsumoto et al., 1999; Meraldi et al., 1999). Using specific Cdk inhibitors, we confirmed a requirement for Cdk activity in centrosome overduplication during prolonged S-phase arrest in CHO, U2OS and p53−/− MEFs. Furthermore, p53−/−Cdk2−/− MEFs were unable to undergo centrosome overduplication during HU-arrest, suggesting that it is Cdk2 rather than Cdk1 that is required for centrosome amplification. It is interesting to note that Cdk1 does not appear to be able to compensate for Cdk2 in this circumstance. Strikingly, Cdk inhibition also caused the disappearance of centriolar satellites as observed by electron microscopy and immunofluorescence staining of PCM-1. Additionally, centrin was observed to accumulate in the nucleus in the absence of Cdk activity. Centrin has previously been reported to undergo nucleocytoplasmic shuttling and concentrates within the nucleus upon inhibition of nuclear export with LMB (Keryer et al., 2003). We found that treating cells with LMB during prolonged S-phase arrest prevented centrosome overduplication, but gave rise to centrin foci within the nucleus.
Immunofluorescence studies revealed cyclin E and cyclin A to be predominantly nuclear proteins (Girard et al., 1991; Ohtsubo et al., 1995; Pines and Hunter, 1991). Cdk2 is also a nuclear protein, phosphorylating chromatin-associated replication factors (Blow and Dutta, 2005). A number of centrosomal proteins have been demonstrated to localise to the nucleus (Keryer et al., 2003). Therefore, the nucleocytoplasmic shuttling of these centrosomal proteins could control their function in centrosome duplication. However, cyclins E and A have also been localised to the centrosome (Matsumoto and Maller, 2004) and live cell imaging has revealed that cyclin E/A-Cdk2 complexes do indeed shuttle between the nucleus and cytoplasm (Jackman et al., 2002). So whilst the substrates that Cdk2 phosphorylates to regulate centrosome duplication could be nuclear, it is likely that the kinase can act directly on substrates localised at the centrosome to initiate duplication. Indeed, cyclin A-Cdk2 has recently been demonstrated to co-ordinate centrosomal and nuclear events in late G2 by activating cyclin B-Cdk1 both at the centrosome and in the nucleus (De Boer et al., 2008).

Whilst blocking nuclear export with LMB is reported to not inhibit shuttling of cyclin E/A-Cdk2 (Jackman et al., 2002), we found that LMB treatment completely prevented centrosome overduplication. Nuclear export may be required to move centrosomal mRNAs or proteins from the nucleus to the cytoplasm, where Cdk2 can then phosphorylate the encoded protein. However, Cdk2 activity may be required within the nucleus to initiate the assembly of centrosomal components that are then transported to the cytoplasm in a CRM1-dependent manner.

When nuclear export was inhibited during HU-arrest, a number of centrin foci were observed within the nucleus. Importantly, the appearance of these foci was shown to be dependent upon Cdk2 activity. Co-treatment of cells with LMB and roscovitine prevented the formation of nuclear centrin foci, and whilst Cdk inhibition alone caused nuclear accumulation of centrin, no such foci were observed within cells treated with roscovitine or olomoucine. Therefore, in the absence of Cdk activity centrin accumulates in the nucleus in a diffuse form. Furthermore, inhibition of Cdk activity prevented the formation of perinuclear centrin foci observed in cells treated with HU and nocodazole. The inability of centrin foci to form in cells arrested in G1 provides further support for the requirement of Cdk2 activity in their assembly. It is likely that the nuclear centrin foci are precursors to
the perinuclear foci seen upon microtubule depolymerisation. We have proposed that the latter are in fact centriolar satellites. It is therefore significant that both inhibition of Cdk activity and nuclear export leads to the loss of centriolar satellites. Taken together, we propose that these results support a model in which Cdk2 activity is required for the generation of nuclear centrin-containing foci and that nuclear export is then necessary for the trafficking of these foci to the cytoplasm (Figure 7.20). Once exported, additional proteins, such as PCM-1, would be recruited and the granules/centriolar satellites are then targeted to the centrosome.

The substrate that Cdk2 phosphorylates to initiate the formation of nuclear centrin foci remains unknown. However, centrin is known to interact indirectly with CP110, a protein that when phosphorylated by Cdk2 promotes centrosome duplication (Chen et al., 2002; Tsang et al., 2006). It is plausible that Cdk2 phosphorylation of proteins, such as CP110, would promote the recruitment of centrin and thus the assembly of early centrosomal precursors. However, extensive screening for other centrosome proteins in the nucleus following LMB treatment revealed only the presence of pericentrin. Whilst the accumulation of pericentrin in the nucleus upon inhibition of nuclear export has previously been reported (Keryer et al., 2003), during our prolonged treatment periods pericentrin was specifically found to tightly localise to the nucleolus. The significance of this localisation to centrosome duplication is unclear, particularly as pericentrin is reported to be dispensable for centriole formation (Kleylein-Sohn et al., 2007). However, as pericentrin is found in the centrin foci seen in cells treated with HU and nocodazole, it follows that pericentrin must at some point be recruited to the centrin foci. This may occur within the nucleus or following export of both pericentrin and the centrin-containing foci.

Intriguingly, pericentrin was precluded from the nucleus, and thus the nucleolus, upon inhibition of Cdk activity. Perhaps more pertinently, Cdk inhibition also caused the loss of nucleoli as observed by NPM staining. NPM may be involved in the trafficking or folding of centrosomal proteins as, generally, NPM functions as a chaperone protein and is involved in the nucleocytoplasmic transport of proteins (Borer et al., 1989; Szebeni and Olson, 1999). However, NPM also localises at the centrosome from mitosis to the G1/S transition (Okuda et al., 2000), at which point phosphorylation of NPM by Cdk2 causes its dissociation from the centrosome, driving duplication. Inhibition of this phosphorylation
In this model for the contribution of Cdk activity and nuclear export to centrosome overduplication, the earliest precursor structures, that include the protein centrin, assemble in the nucleus dependent upon Cdk activity. Nuclear export is required for the translocation of these precursors to the cytoplasm where they can recruit additional components to form centriolar satellites and centrioles, which then recruit γ-tubulin.
prevents duplication, whilst its loss leads to premature initiation of centriole formation (Okuda et al., 2000; Tarapore et al., 2002; Tokuyama et al., 2001; Wang et al., 2005). In our study it is likely that NPM had dissociated from the centrosome in response to Cdk2 phosphorylation during the 18 hour pre-treatment with HU before the addition of Cdk or nuclear export inhibitors. Furthermore, as NPM does not re-associate with the centrosome until mitosis, it is not a true block to reduplication and as such should be dispensable for centrosome overduplication. LMB treatment thus prevents centrosome reduplication due to blocking centrin-containing granules within the nucleus and increases the nucleolar abundance of NPM as it can no longer be exported.

As a NES has not yet been identified in centrin, it is possible that centrin interacts with CRM1 through another protein, possibly even NPM. Indeed, NPM interacts with CRM1 through a NES, with LMB treatment causing NPM to be lost from the centrosome (Wang et al., 2005). Recently, centrin2 has been shown to localise to nuclear pores, interacting with major nuclear pore subunits (Resendes et al., 2008). Yeast centrin had previously been shown to localise at nuclear pores and function in mRNA export (Fischer et al., 2004; Rout et al., 2000). Centrin2 is also required for mRNA export in mammalian cells. Additionally, overexpression of the N- or C-terminus of centrin2 affects CRM1-mediated protein export but had no affect whatsoever on protein import (Resendes et al., 2008).

It remains to be shown how the nuclear centrin foci formed upon inhibition of nuclear export contribute to centrosome assembly. However, support for the hypothesis that they are centrosome precursors comes from washout experiments. After a 10 hour washout, cells in which nuclear export had been inhibited accumulated extra centrosomes, suggesting that intermediates had formed within these cells that could rapidly be converted into functional centrosomes. In sharp contrast, cells in which Cdk activity had been inhibited, either alone or in conjunction with inhibition of nuclear export, did not accumulate any extra centrosomes. Hence, we speculate that Cdk inhibition prevents the formation of intermediate structures and, as such, Cdk-dependent activation is an early step in the centrosome overduplication pathway. Inhibiting Cdk2 may therefore be the best strategy to prevent centrosome overduplication during the use of chemotherapeutic strategies that arrest p53-defective cancer cells in S-phase.
CHAPTER EIGHT

DISCUSSION
8.1 CENTROSOME OVERDUPPLICATION

The presence of supernumerary centrosomes in cells can lead to the formation of multipolar spindles, thereby contributing to chromosome missegregation and aneuploidy. Apart from cell division failure and cell fusion, abnormal centrosome numbers can be generated through deregulation of the centrosome cycle (Nigg, 2002). Centrosome duplication may become uncoupled from the cell cycle, such that more than one round of duplication occurs in a single cell cycle; or more than one procentriole may form around each template, as is seen when Plk4 is overexpressed or when existing procentrioles have been laser-ablated (Kleylein-Sohn et al., 2007; Loncarek et al., 2008). Inappropriate activation of the de novo centriole formation pathway would also lead to the generation of supernumerary centrosomes. In cells with defective p53 pathways, centrosome overduplication can be induced by provoking a prolonged S-phase arrest (Balczon et al., 1995; Bennett et al., 2004; Tarapore and Fukasawa, 2002). Treating such cells with drugs that prevent DNA synthesis uncouples the centrosome and DNA duplication cycles, leading these cells to accumulate multiple centrosomes in the absence of DNA replication. However, it is unclear as to which mechanism of centrosome overduplication contributes to this phenomenon.

Licensing of centriole duplication requires separase, which becomes active at the metaphase to anaphase transition (Tsou and Stearns, 2006b). This suggests that for centrosome overduplication in HU-arrested cells to occur via a templated mechanism, separase would need to be active in these cells, or centriole disengagement to occur by a different mechanism. Indeed, there is a substantial delay before duplicated centrioles undergo reduplication, suggesting that they need to regain duplication competency before reinitiating duplication (Balczon et al., 1995). However, under certain experimental conditions multiple procentrioles are observed forming around a single template (Duensing et al., 2007; Kleylein-Sohn et al., 2007). Indeed, more than one procentriole per mother centriole has been reported in S-phase arrested CHO cells (Loncarek et al., 2008). This demonstrates that the disengagement of centrioles is not necessarily required for the formation of multiple daughters from a single maternal template. Furthermore, the de novo pathway may well become activated within these cells, contributing to the formation of extra centrioles. Indeed, a recent study reports that centrosome duplication in HU-arrested CHO cells is initially templated, but following prolonged periods of arrest centrioles are
formed along the de novo pathway (Kuriyama et al., 2007). Whilst the presence of a pre-existing centriole was reported to inhibit the de novo pathway (Khodjakov et al., 2002; La Terra et al., 2005), the exact mechanism of this control is not understood and may well be lost in these cells. Moreover, multiple centrioles are formed through simultaneous centriolar and acentriolar pathways during ciliogenesis (Anderson and Brenner, 1971). These pathways have striking similarities to both templated and de novo centriole formation pathways, suggesting that they can each operate within cells at the same time. Whilst it remains unknown as to which, if any, pathway predominates in centrosome overduplication, it is likely that many of the molecular events involved in each are the same as they all result in the formation of excess centrioles. Therefore, in this study the centrosome overduplication assay was used to investigate this process and in particular elucidate a pathway for the molecular events that lead to the accumulation of supernumerary centrosomes.

### 8.2 A PATHWAY FOR CENTROSOME OVERDUPPLICATION

The mechanisms by which centrosomes overduplicate are far from fully understood. However, by combining the centrosome overduplication assay with a range of pharmacological inhibitors we have identified a series of novel molecular events that lead to the formation of functional supernumerary centrosomes. The key to this study was the observation that particular treatments could block centrosome overduplication as measured by some centrosomal markers, but not others. Indeed, using γ-tubulin staining, MTs have previously been shown to be necessary for centrosome overduplication in CHO cells (Balczon et al., 1999). We similarly found that MT depolymerisation prevented the accumulation of γ-tubulin positive centrosomes during HU-arrest. However, many centrin foci were found to accumulate around the periphery of the nucleus within these cells, suggesting that they may in fact be intermediates formed in the centrosome overduplication pathway. Furthermore, these centrin foci contained modified tubulins, PCM-1 and pericentrin, all of which are found in centriolar satellites. Indeed, TEM analysis revealed that the number of centriolar satellites was greatly increased in cells treated with HU and nocodazole as compared to controls. Together, this suggests that these centrin foci are in fact centriolar satellites.
Inhibition of both Cdk activity and nuclear export was found to prevent centrosome overduplication and, intriguingly, also caused the loss of centriolar satellites. Centrin foci were observed to form within the nucleus upon inhibition of nuclear export, suggesting that these form as an early intermediate in the centrosome overduplication pathway. Significantly, the assembly of these nuclear centrin foci, and the cytoplasmic centrin foci seen in cells treated with HU and nococadazole, was found to be dependent upon Cdk activity. However, the nuclear centrin foci did not associate with any other proteins, suggesting that PCM-1, pericentrin and modified tubulin are added to these structures in the cytoplasm.

Whilst the centrin foci accumulate around the periphery of the nucleus in the absence of MTs, they cluster around the pre-existing centrosome in the presence of a MT array. Centriolar satellites are reported to be trafficked to the centrosome in a dynein-dependent manner (Dammermann and Merdes, 2002). However, inhibition of dynein by a range of methods did not prevent their accumulation around the centrosome. Whilst this indicates that dynein is not required for their transport, it is plausible to suggest that in the presence of an intact MT network the centriolar satellites are able to be recruited to the centrosome via an alternative, but presumably slower, mechanism during these prolonged assay periods. Indeed, short periods of dynamitin overexpression caused the dispersal of centriolar satellites, although when overexpression was combined with prolonged HU-arrest the satellites still clustered around the centrosome.

Dynein has previously been reported to be dispensable for normal templated centrosome duplication (Quintyne and Schroer, 2002). However, we found that inhibition of dynein prevented the accumulation of γ-tubulin positive centrosomes in S-phase arrested cells. Additionally, centrioles did not accumulate within these cells. This suggests that during our prolonged treatment periods, the centrosomal pools of proteins that rely on dynein for their trafficking to the centrosome become depleted and are therefore no longer able to contribute to newly forming centrosomes. These proteins may include dynein itself or dynactin, which could both have anchoring or stabilisation roles that allow the assembly of new centrioles.

Finally, in the case of Hsp90 inhibition, the accumulation of γ-tubulin positive centrosomes was prevented, although TEM analysis revealed the presence of multiple centrioles. This
suggests that this chaperone is required for the recruitment of proteins such as γ-tubulin and C-Nap1 to the newly formed centrioles. Although γ-tubulin is proposed to be necessary for the nucleation of the MTs in centriolar walls (Dammermann et al., 2004; Haren et al., 2006; Kleylein-Sohn et al., 2007), it is likely that this role is fulfilled by the γ-tubulin that resides at the pre-existing centrosome. Hsp90 is therefore required for a later step in the pathway that leads to the formation of supernumerary centrosomes. Indeed, it was found that functional centrosomes could rapidly accumulate upon drug washout in cells in which nuclear export, MTs, dynein or Hsp90 had been inhibited. This supports the theory that intermediates had formed within these cells and that these can be rapidly converted into functional centrosomes. However, this was not the case following inhibition of Cdk2, confirming that Cdk2 acts early in the pathway and that no intermediates had formed within these cells.

Together, these results allow us to propose a novel model for the contribution of Cdk2, nuclear export, MTs, dynein and Hsp90 to centrosome overduplication. Nuclear centrin foci are formed early in this pathway in a Cdk2 dependent manner. Nuclear export is then required for their movement to the cytoplasm where they accumulate modified tubulins, PCM-1 and pericentrin. At this point they resemble centriolar satellites and move to the centrosome in a MT-dependent manner. Functional dynein and MT assembly are then required for the formation of new centrioles, to which γ-tubulin is recruited, dependent on the chaperone protein, Hsp90 (Figure 8.1).

At the present time it is unclear as to how this pathway fits with the emerging model for centriole assembly. In *C. elegans* embryos, SPD-2 and ZYG-1 are recruited early to the sites of newly forming centrioles and are essential for the subsequent recruitment of SAS-5 and SAS-6 (Delattre et al., 2006; Pelletier et al., 2006). These proteins contribute to the formation of the daughter centriolar central tube and recruit SAS-4. The recruitment of SAS-4 is vital for elongation of the central tube and the assembly of MTs that form the centriole wall (Pelletier et al., 2006). In mammalian cells, HsSAS-6 localises to an analogous position in the procentriole bud, whilst overexpression of Plk4, the putative functional homologue of ZYG-1, leads to the formation of multiple procentrioles around a single template (Kleylein-Sohn et al., 2007). Furthermore, centrin relies on HsSAS-6 for its recruitment to procentrioles as depletion of HsSAS-6 cause the loss of centrin from newly
Figure 8.1 A pathway for centrosome overduplication in S-phase arrested cells

In this model of centrosome overduplication, the earliest precursors, that include centrin, assemble in the nucleus dependent upon Cdk2 activity (green dots). Nuclear export is required for translocation of these precursors to the cytoplasm where they recruit modified tubulins, PCM-1 and pericentrin, and now resemble centriolar satellites (grey dots). MTs and dynein contribute to the concentration of centriolar satellites around the existing centrosome and the formation of new centrioles. Finally, Hsp90 is required for the recruitment of γ-tubulin to the new centrioles to form functional centrosomes capable of microtubule nucleation.
forming procentrioles (Strnad et al., 2007). It would therefore be very interesting to use antibodies against mammalian homologues of these proteins to determine which structures they stain in the centrosome overduplication pathway.

8.3 A ROLE FOR CENTRIOLAR SATELLITES IN CENTROSOME OVERDUPlication

In our study, centriolar satellites have been identified as intermediates in the centrosome overduplication pathway. Significantly, we found that their assembly was dependent upon both Cdk activity and nuclear export, whilst inhibition of MTs or dynein, that blocked centrosome overduplication, led to their accumulation within cells. However, the relationship between centriolar satellites and the centrosome remains obscure. At the very least they contribute to the trafficking of proteins to the centrosome (Dammermann and Merdes, 2002), and may coalesce and interconvert with the PCM (Baron et al., 1994). Centriolar satellites could therefore be very important for increasing the localised concentration of proteins during centriole formation. Indeed, they may be vital for the delivery of centrin that appears within the distal lumen of centrioles and as such is an early marker of centriole formation (Paoletti et al., 1996). Interestingly, we have found that centriolar satellites form in a Cdk2-dependent manner, and that this is an early event in the centrosome overduplication pathway. However, it remains unclear whether the centriolar satellites are directly incorporated into newly forming centrioles, or more simply act as a source of proteins that are assembled into centrioles.

Centriolar satellites are almost certainly equivalent to the fibrous granules that are reported to form early in the acentriolar pathway of centriole formation in ciliogenesis (Anderson and Brenner, 1971). These fibrous granules coalesce to form deuterosomes, around which multiple centrioles form. Fibrous granules may also contribute to the centriolar pathway as a number are seen associating with existing centrioles and the nascent centrioles that form around them (Anderson and Brenner, 1971). Remarkably, fibrous granules have been reported to form in the pockets of the nuclear envelope (Anderson and Brenner, 1971; Nayak et al., 1976), an observation that has striking similarities to the concentration of centrin foci around the nuclear envelope in the absence of MTs. Ciliogenesis is accompanied by an increase in expression levels of centrin and other centrosomal proteins (Laoukili et al., 2000; Vladar and Stearns, 2007). Furthermore, centrin is observed in
precursor cytoplasmic structures proposed to be required for the assembly of centrioles as epithelial cells commence differentiation. Immuno-EM analysis subsequently confirmed electron-dense fibrous granules contain centrin (Laoukili et al., 2000). Together, this suggests that the centrin foci that we see forming during centrosome overduplication show a significant similarity to the structures assembled during ciliogenesis.

Centrin foci are also observed early in the de novo centriole assembly pathway in HeLa cells (La Terra et al., 2005). Whilst the presence of a pre-existing centriole is believed to suppress the de novo pathway, it has been reported that de novo centriole assembly contributes to centrosome overduplication in S-phase arrested CHO cells (Kuriyama et al., 2007). The exact nature of these centrin foci, termed precentrioles, has not yet been determined and it is suggested that they form ordinarily during templated duplication (La Terra et al., 2005). However, only those precentrioles that ‘dock’ with the existing centrioles are able to form new centrioles, with excess precentrioles apparently disappearing. It has not been described whether these centrin foci represent centriolar satellites, and it is unknown if, or how, centriolar satellites contribute to the de novo pathway. Although, it could be postulated that they contribute to the PCM cloud that forms during the de novo pathway and in which new centrioles are formed (Baron et al., 1994; Khodjakov et al., 2002).

8.4 CENTRIN IN CENTRIOLE ASSEMBLY

Centrin has long been associated with centriole duplication. The yeast homologue of centrin, CDC31, is required for SPB duplication (Byers, 1981; Paoletti et al., 2003; Spang et al., 1993), whilst centrin is required for centriole and basal body duplication in a range of organisms (Koblenz et al., 2003; Salisbury et al., 2002; Stemm-Wolf et al., 2005). Centrin localises to the procentriole early in daughter centriole formation and resides in the distal lumen of centrioles (Paoletti et al., 1996). This localisation of centrin is proposed to stabilise the distal ends of centrioles and newly formed procentrioles. Indeed, centrin2 binds to Sfi1, forming calcium sensitive fibers (Martinez-Sanz et al., 2006), and it is suggested that these form part of the cartwheel structure to provide a site for centriole formation (Salisbury, 2007). Centrin would then presumably translocate with the growing centriole walls to establish its position at the distal end of the centriole (Geimer and Melkonian, 2005). However, it has recently been shown that depletion of centrin2 and 3
does not prevent excess procentriole formation induced by Plk4 overexpression (Kleylein-Sohn et al., 2007). Furthermore, depletion of HsSAS-6 prevents the formation of procentrioles and, probably as a consequence, centrin localisation (Kleylein-Sohn et al., 2007; Strnad et al., 2007). This suggests that HsSAS-6 is required before centrin for the assembly of a structure to which centrin is then recruited. However, HsSAS-6 only associates transiently with newly formed centrioles, in contrast to centrin which is stably incorporated (Kleylein-Sohn et al., 2007). Whilst it would appear that centrin is not required for the formation of procentrioles, at least when Plk4 is overexpressed, this does not mean that centrin is not required to aid stability of the centriole structure. Indeed, the structures formed in cells overexpressing Plk4 but depleted of centrin may be in some way aberrant, but this may only become apparent upon cell cycle progression. Additionally, centrin is known to interact with the Cdk2 substrate CP110, co-localising at the distal ends of centrioles (Chen et al., 2002; Tsang et al., 2006). CP110 was initially implicated in centriole duplication as depletion prevented centrosome overduplication in HU-arrested U2OS cells. More recently, CP110 has been proposed to act as a cap under which microtubules are added during procentriole extension (Kleylein-Sohn et al., 2007). It is plausible that centrin may help to stabilise this cap and thereby aid the stability of the growing MTs in the centriole wall.

While some debate remains over whether centrins are absolutely essential for centriole duplication (Kleylein-Sohn et al., 2007; Middendorp et al., 2000; Salisbury et al., 2002), this study does not speculate on whether centrin is an essential component for centriole assembly. Instead, we find that, as well as being a centriolar marker, centrin is a marker for centrosome intermediates that form when the overduplication of functional centrosomes is prevented by the inhibition of nuclear export or dynein, or the depolymerisation of MTs. Significantly, Cdk2 activity is required for the formation of these intermediates within the nucleus, whilst a functional CRM1/exporting 1 complex is required for their export to the cytoplasm.

**8.5 Nuclear Export and Centrosome Overduplication**

In embryonic cells, nuclear events are dispensable for centrosome duplication (Sluder and Lewis, 1987; Sluder et al., 1990; Sluder et al., 1986); however, enucleated somatic cells are unable to undergo centrosome duplication (Kuriyama and Borisy, 1981; Maniotis and
Schliwa, 1991). This suggests that, at least in somatic cells, the nucleus contributes something to centrosome duplication. In our study, the perinuclear localisation of the centrin foci in cells treated with HU and nocodazole was very interesting. Furthermore, centrin has previously been reported to accumulate within the nucleus when nuclear export is inhibited and more recently has been localised to the nuclear pores (Keryer et al., 2003; Resendes et al., 2008). We were therefore interested to see if blocking nuclear export would affect centrosome overduplication and, more specifically, the formation of centrin foci. Treating S-phase arrested cells with the nuclear export inhibitor, leptomycin B, was found to completely prevent the accumulation of γ-tubulin positive centrosomes, demonstrating that nuclear export is required for centrosome overduplication. As expected, centrin accumulated within the nucleus in these cells. Strikingly, whilst most centrin was diffuse within the nucleus, a number of distinct nuclear centrin foci were also observed. Furthermore, the assembly of these foci was found to be dependent upon Cdk activity. Despite extensive screening of centrosomal markers, no other centrosomal proteins were found to localise to these nuclear centrin foci. However, it was observed that inhibition of nuclear export led to the loss of centriolar satellites, suggesting that their formation also requires nuclear export. From our observations, we postulate that the nuclear centrin foci recruit known components of centriolar satellites upon export from the nucleus. Centriolar satellites are therefore formed following nuclear export of simpler centrin granules that then accumulate modified tubulins, PCM-1 and pericentrin in the cytoplasm.

8.6 Cdk2 and Centrosome Overduplication

A requirement for Cdk2 activity for centrosome overduplication in HU-arrested CHO cells has previously been demonstrated (Matsumoto et al., 1999; Meraldi et al., 1999). Whilst Cdk2 is predominantly a nuclear protein (Blow and Dutta, 2005), it can, along with its cyclin partners, undergo nucleocytoplasmic shuttling that is not blocked by nuclear export inhibitors (Jackman et al., 2002). However, it remains unclear as to whether Cdk2 phosphorylates nuclear or centrosomal targets, or indeed both, to initiate centrosome overduplication. Using both pharmacological inhibitors and genetically-modified cells we similarly found that Cdk2 activity is required for the overduplication of functional centrosomes. Significantly, loss of Cdk2 activity also prevented the formation of nuclear centrin foci and caused the loss of centriolar satellites. This would suggest that Cdk2 has at least one nuclear target involved in centrosome duplication and that its phosphorylation is
necessary for the formation of nuclear centrin foci. These observations also support the suggestion that the nuclear centrin foci are precursors to centriolar satellites and that Cdk2 activity is required for their formation.

Co-ordination of centrosome duplication and DNA replication is most likely achieved through the late G1 specific activation of cyclin E-Cdk2. A number of inhibitory studies implicated Cdk2 in centrosome duplication (Hinchcliffe et al., 1999; Lacey et al., 1999; Matsumoto et al., 1999; Meraldi et al., 1999). Indeed, unduplicated centrosomes rapidly respond to active cyclin E-Cdk2 and initiate duplication (Mussman et al., 2000). However, more recent studies have demonstrated that Cdk2 is dispensable for normal templated duplication (Duensing et al., 2006b), and it is postulated that Cdk1 can substitute for Cdk2 in its absence [(Hochegger et al., 2007); reviewed by (Berthet and Kaldis, 2007)]. Despite this, Cdk2 is required for centrosome overduplication, as shown in this and other studies (Duensing et al., 2007; Duensing et al., 2004; Matsumoto et al., 1999; Meraldi et al., 1999). Moreover, whilst Cdk2 is unable to promote centrosome overduplication in the absence of Plk4; Plk4-induced centriole overduplication is dependent upon Cdk2 activity (Duensing et al., 2007; Habedanck et al., 2005). However, the exact mechanisms by which Cdk2 and Plk4 cooperate to initiate centrosome duplication remain unclear.

To date, three Cdk2 substrates have been implicated in centrosome duplication: NPM, Mps1 and CP110. However, as Cdk2 is predominantly a nuclear kinase, it is intriguing to know whether Cdk2 phosphorylates targets in the nucleus or at the centrosome to initiate duplication. Indeed, recently it has been demonstrated that Cdk2 coordinates centrosomal and nuclear events in late G2 by synchronising cyclin B-Cdk1 activation at the centrosome and in the nucleus (De Boer et al., 2008). We found that Cdk2 activity is required for the formation of centrin foci within the nucleus, suggesting that their formation requires the phosphorylation of a nuclear target. However, the identity of such a target remains unknown. Centrin is known to bind to CP110, a protein whose phosphorylation by Cdk2 promotes centrosome duplication (Chen et al., 2002; Tsang et al., 2006), although the mechanism involved in this is unclear. NPM is also a Cdk2 substrate (Okuda et al., 2000). Phosphorylation of NPM promotes its dissociation from centrosomes, which in turn drives centrosome duplication. NPM is a nucleolar protein that undergoes nucleocytoplasmic shuttling (Borer et al., 1989; Szepesi and Olson, 1999; Yung et al., 1985). However, it is
not clear if Cdk2 phosphorylates NPM in nucleoli or at the centrosome. Interestingly, when nuclear export was inhibited in this study, centrin and pericentrin were found to accumulate within the nucleoli. Centrin foci are assembled in the nucleus in response to Cdk2 activity, which are then exported to the cytoplasm in a CRM1/exporting 1-dependent manner. We therefore speculate that Cdk2 phosphorylation of nuclear proteins, such NPM, may promote the formation of these centrin foci in an early event in the centrosome overduplication pathway.

8.7 PERSPECTIVES

The work presented within this thesis identifies a pathway for the molecular events that contribute to centrosome overduplication in cells. These events include the generation of centrin foci within the nucleus, their export to the cytoplasm, the recruitment of additional centrosome components to these foci, their MT-dependent trafficking to the centrosome, assembly of new centrioles and the recruitment of γ-tubulin. Whilst intermediates in the centrosome overduplication pathway were observed upon inhibition of nuclear export, MTs, dynein or Hsp90, no such structures were observed upon Cdk2 inhibition. This suggests that Cdk2 is potentially the earliest component in this pathway that could be targeted to prevent centrosome overduplication in cells.

Many cancer cells display supernumerary centrosomes, although it remains unclear whether this is a cause or consequence of cancer progression (Nigg, 2002). Significantly, the HPV-16 E7 oncoprotein is able to induce centrosome amplification in a single cell cycle, which correlates with cell division defects (Duensing et al., 2008; Duensing et al., 2001; Duensing and Munger, 2003). Loss or inhibition of Cdk2 perturbed E7-induced centrosome overduplication (Duensing et al., 2004), demonstrating that Cdk2 is a suitable target for preventing centrosome amplification in cells. Our results would suggest this is due to preventing the formation of early centrosomal precursors through the inhibition of Cdk2.

It has been postulated that the use of chemotherapeutic regimes that arrest cancer cells in S-phase may lead to the generation of supernumerary centrosomes in those cells that receive a subtoxic dose (Bennett et al., 2004). When the chemotherapy is removed, these cells are able to re-enter the cell cycle and potentially organise multipolar spindles due to
the presence of supernumerary centrosomes. This would lead to the re-emergence of
tumours following seemingly successful chemotherapy. If combination therapies were used
that prevented centrosome overduplication at the same time as DNA synthesis was
inhibited, then this may well help to reduce the re-emergence of tumours.


The following table provides details of each of the drugs used in the course of this study. This information has been taken from the product sheets relating to each compound.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Full name</th>
<th>Details of inhibition</th>
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<tbody>
<tr>
<td><strong>Cell cycle arrest</strong></td>
<td></td>
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<tr>
<td>HU</td>
<td>Hydroxyurea</td>
<td>Specific inhibitor of DNA synthesis, does not affect RNA and protein synthesis. Inhibition of the enzyme ribonucleotide reductase, and thus, the synthesis of deoxyribonucleotides from ribonucleotides. Inactivates ribonucleoside reductase by forming a free radical nitroxide that binds a tyrosyl free radical in the active site of the enzyme. This blocks the synthesis of deoxynucleotides, which inhibits DNA synthesis and induces synchronization or cell death in S-phase.</td>
</tr>
<tr>
<td>Mimosine</td>
<td>(S)-α-Amino-β-[1-(3-hydroxy-4-oxopyridine)]propionic acid</td>
<td>Plant amino acid and potential inhibitor of the cell cycle giving rise to growth arrest in G1-phase. Can induce a cell cycle arrest of human somatic cells in late G1 phase, before establishment of active DNA replication forks.</td>
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<td><strong>Inhibitors of microtubules or actin</strong></td>
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<tr>
<td>Nocodazole</td>
<td>Methyl-(5-[2-thienylcarbonyl]-1H-benzimidazol-2-yl)carbamate</td>
<td>Interferes with the structure and function of microtubules in interphase and mitotic cells. Thought to bind directly to tubulin causing conformational changes resulting in increased exposure of some sulfhydryl and possibly tyrosine residues.</td>
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<tr>
<td>Cytochalasin D</td>
<td></td>
<td>Potent inhibitor of actin polymerization; disrupts actin microfilaments; activates the p53-dependent pathways; inhibits smooth muscle contraction; inhibits insulin-stimulated glucose transport.</td>
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<td><strong>Inhibitors of motor proteins</strong></td>
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<tr>
<td>EHNA</td>
<td>erythro-(2-hydroxy-3-nonly)adenine</td>
<td>Adenosine deaminase inhibitor; dynein inhibitor; does not inhibit proteasome function.</td>
</tr>
<tr>
<td>Vanadate</td>
<td>Sodium orthovanadate</td>
<td>Phosphate analogue. Inhibits ATPase, alkaline phosphatase and tyrosine phosphatase.</td>
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<tr>
<td>Monastrol</td>
<td>1,2,3,4-Tetrahydro-4-(3-hydroxyphenyl)-6-methyl-2-thiox o-5-pyrimidinecarboxylic acid, ethyl ester</td>
<td>Selective inhibitor of mitotic kinesin Eg5. Potent, cell-permeable, small molecule mitosis inhibitor that does not interact with tubulin. Arrests cells in mitosis and specifically inhibits the motility of the mitotic kinesin Eg5, a motor protein required for mitotic spindle formation and maintenance.</td>
</tr>
<tr>
<td>Drug</td>
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<td>Details of inhibition</td>
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<tr>
<td><strong>Heat shock protein inhibitors</strong></td>
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<tr>
<td>Geldanamycin</td>
<td>(4E,6Z,8S,9S,10E,12S,13R,14S,16R)-13-hydroxy-8,14,19-trimethoxy-4,10,12,16-tetramethyl-3,20,22-trioxo-2-azabicyclo[16.3.1]docosa-1(21),4,6,10,18-pentaen-9-yl carbamate</td>
<td>A potent antitumor antibiotic active at nanomolar concentration against 60 cell lines. Binds specifically to the heat shock protein Hsp90 and to its endoplasmic reticulum homologue GP96, and thus interferes with conformational maturation of proteins and the cellular stress response.</td>
</tr>
<tr>
<td>17-AAG</td>
<td>17-(Allylamino)-17-demethoxygeldanamycin</td>
<td>A less toxic, potent, synthetic derivative of geldanamycin that binds to Hsp90 and regulates its function. It induces apoptosis and displays antitumor effects.</td>
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<tr>
<td>HSPI-1</td>
<td>Heat shock protein inhibitor 1 (3,4-Methylenedioxy-benzylidine-γ-butyrolactam)</td>
<td>A benzylidene lactam compound that inhibits the induction of heat shock proteins, including Hsp70, Hsp72 and Hsp105. Blocks the development of thermostolerance in a dose-dependent manner. Does not affect the thermosensitivity of nontolerant cells. Does not have an affect on the activities of PKA, PKC, or PTK.</td>
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<td><strong>Cdk inhibitors</strong></td>
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<tr>
<td>Olomoucine</td>
<td>2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine</td>
<td>Potent and selective inhibitor of Cdk2/cyclinA/E, Cdk5/p35 and MAPK. Acts by competing for the ATP binding domain of the kinase. Exhibits reduced sensitivity towards related kinases (Cdk4, Cdk6). Does not significantly affect the activity of other protein kinases. Known to inhibit DNA synthesis and triggers G1 arrest.</td>
</tr>
<tr>
<td>Roscovitine</td>
<td>2-(R)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine</td>
<td>Potent and selective inhibitor of cyclin-dependent kinases. Exhibits greater inhibitory potency compared to olomoucine. Inhibits Cdk2 and Cdk5 by competing for the ATP binding domain of these kinases. Exhibits very low sensitivity towards related kinases and does not significantly affect the activity of other protein kinases. Compared to olomoucine, roscovitine displays increased anti-mitotic activity at the G1/S and G2/M phases of the cell cycle.</td>
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<tr>
<td><strong>Nuclear export inhibitor</strong></td>
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<tr>
<td>LMB</td>
<td>Leptomycin B (2E,5S,6R,7S,9R,10E,12E,15R,16Z,18E)-17-ethyl-6-hydroxy-3,5,7,9,11,15-hexamethyl-19-[(2S,3S)-3-methyl-6-oxo-2,3-dihydropyran-2-yl]-8-oxononadeca-2,10,12,16,18-pentaenoic acid</td>
<td>Inhibitor of nuclear export because of its ability to interact with and impair the function of the nuclear export factor CRM1. Blocks the Rev-dependent export of mRNA into cytoplasm. LMB causes a G1 and G2 cell cycle arrest in mammalian cells.</td>
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