Regulation of Mitotic Exit and Cytokinesis in

*Saccharomyces cerevisiae*

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Abstract

Cell proliferation depends upon the duplication of chromosomes and the segregation of duplicated sister chromatids to opposite poles of the cell prior to cell division (cytokinesis). Each of these processes must occur with high fidelity and in a strict order to ensure that the cell's genetic material is transferred to its progeny. Genetic instability resulting from aberrant cell division is implicated in tumour progression in multicellular organisms.

Much of our understanding of eukaryotic cell cycle control has been gained by studying model systems such as the budding yeast, *Saccharomyces cerevisiae*. Major cell cycle regulatory points are referred to as transitions and include START, which marks commitment to the entire cell cycle. Another occurs at M/G1, when cells undergo mitotic exit and cytokinesis. Mitotic exit requires destruction of mitotic cyclin-dependent kinase (CDK) activity (Cdc28-Clb in budding yeast) and is controlled by the mitotic exit network (MEN). Although many components of this protein network have been identified, including the G-protein Tem1, the kinases Cdc15, Dbf2 and Cdc5, the phosphatase Cdc14, and Mob1, until recently their regulation was poorly understood. I have investigated the order of function of the MEN and found that it forms a signal transduction cascade which functions in the order Tem1-Cdc15-Mob1-Dbf2-Cdc14. The polo-like kinase Cdc5 serves as both a negative and positive regulator of the MEN. MEN activation is necessary and sufficient for formation of the actomyosin contractile ring which is required for cytokinesis, and for CDK inactivation. Completion of cytokinesis requires an additional step, which may involve relocalisation of the MEN proteins to the bud neck.

Control of MEN activation operates through Tem1, and is mediated in part by Bub2 and Bfa1. Bub2 and Bfa1 were identified as components of a branch of the spindle assembly checkpoint (SAC) pathway, and are thought to form a 2-component GTPase activating protein (GAP) for Tem1. Consistent with this, Bub2 and Bfa1 both negatively regulate and form a complex with Tem1. To understand how they control the MEN, I have studied the regulation of Bub2 and Bfa1. Phosphorylation of Bfa1 by two cell cycle-regulated kinases, Cdc28 and Cdc5, is likely to activate Bub2/Bfa1 GAP activity. Rather than being a branch
of the SAC, Bub2/Bfa1 appear to function during a variety of mitotic arrests, forming a "spindle positioning checkpoint", which couples spindle elongation through the bud neck to initiation of mitotic exit and cytokinesis.
# Table of Contents

Abstract....................................................................................................................... 2
Table of contents......................................................................................................... 4
Table of figures........................................................................................................... 13
List of tables................................................................................................................ 18
List of abbreviations.................................................................................................. 19
Acknowledgements.................................................................................................... 22

Chapter 1.................................................................................................................... 23
Introduction.................................................................................................................. 23

1.1 Overview of the Eukaryotic Cell Cycle................................................................. 23
1.2 Checkpoints.......................................................................................................... 25
1.3 Cyclin-dependent kinases.................................................................................... 25
1.3.1 Cyclin-dependent kinase inhibitors (CDKIs).................................................. 27
1.4 Initiation of the cell cycle..................................................................................... 27
1.5 Licensing and initiation of DNA replication...................................................... 28
1.6 Mitosis................................................................................................................... 28
1.6.1 Sister chromatid cohesion.............................................................................. 28
1.6.2 Formation of the bipolar spindle................................................................. 29
1.6.2.1 The spindle pole body............................................................................. 29
1.6.2.2 SPB duplication..................................................................................... 29
1.6.2.3 Spindle polarity and positioning............................................................ 31
1.7 Regulation of mitotic progression....................................................................... 34
1.7.1 The anaphase promoting complex/cyclosome (APC/C)................................. 34
1.7.2 Regulation of the metaphase/anaphase transition by APC\textsuperscript{Cdc20}........ 35
1.7.2.1 Activation of APC<sub>Cdc20</sub> ................................................................. 35
1.7.2.2 Sister chromatid segregation ................................................................. 35
1.7.2.3 Checkpoints which target the APC/C<sub>Cdc20</sub>/Pds1 pathway .......... 36
1.7.2.3.1 The DNA checkpoint pathways ......................................................... 38
1.7.2.3.2 The Spindle assembly checkpoint (SAC) ........................................... 38
1.8 Mitotic exit ................................................................................................. 40
1.8.1 The mitotic exit network (MEN) ............................................................. 41
  1.8.1.1 Cdc14 .................................................................................................. 41
  1.8.1.2 Lte1/Tem1 ............................................................................................ 43
  1.8.1.3 Cdc15 .................................................................................................... 44
  1.8.1.4 Cdc5 ...................................................................................................... 45
  1.8.1.5 Dbf2 ...................................................................................................... 46
  1.8.1.6 Mob1 ..................................................................................................... 48
1.8.2 Coupling sister chromatid separation to mitotic exit ......................... 48
  1.8.2.1 The role of APC/CCdc20 .................................................................... 49
  1.8.2.2 Pds1 as an inhibitor of mitotic exit ....................................................... 50
  1.8.2.3 The Bub2/Bfa1 spindle position checkpoint ........................................ 50
1.8.3 The septation initiation network (SIN) .................................................. 56
1.8.4 Cytokinesis .............................................................................................. 59
1.9 Aims of the project ................................................................................. 61

Chapter 2 ...................................................................................................... 62

Materials and Methods ........................................................................... 62

2.1 Bacterial techniques ............................................................................... 62
  2.1.1 Bacterial strains .................................................................................... 62
  2.1.2 <i>E.coli</i> media and growth conditions ............................................... 62
  2.1.3 <i>E.coli</i> transformation ......................................................................... 63
2.2 Yeast techniques ....................................................................................... 63
2.2.1 Yeast strains .......................................................... 63
2.2.2 Yeast media and growth conditions ......................... 63
2.2.3 Determination of cell numbers ................................ 69
2.2.4 Growth synchronisation methods ............................ 69
2.2.5 Re-budding assay .................................................... 70
2.2.6 Yeast transformation ............................................... 70
2.2.7 Isolation of yeast genomic DNA ............................. 70

2.3 DNA manipulation ..................................................... 71
2.3.1 Restriction endnucleases and DNA modifying enzymes. 71
2.3.2 Polymerase chain reaction ........................................ 71
2.3.3 Agarose gel electrophoresis ..................................... 74
2.3.4 Recovery of DNA fragments from agarose gels .......... 74
2.3.5 DNA ligations ........................................................ 74
2.3.6 Preparation of plasmid DNA .................................... 74
2.3.7 Precipitation of PCR DNA ....................................... 75

2.4 Plasmid construction .................................................. 75
2.4.1 pGAL-6HisTEM1 .................................................. 75
2.4.2 pGAL-6HisCDC5 .................................................. 77
2.4.3 pGST.BFAI ....................................................... 77
2.4.4 pGST.BFAIANT270/300 .......................................... 77
2.4.5 pGST.BFAIΔCT270/300 ........................................... 77
2.4.6 YIPlac128-3HA.BFAI ........................................... 78
2.4.7 YIPlac128-3HA.BFAI ΔNT270/300 ......................... 78
2.4.8 YIPlac128-3HA.BFAI ΔCT270/300 ......................... 78
2.4.9 Bfa1 site-directed mutagenesis to construct YIPlac128-
            3HA.bfa1-6A ...................................................... 79

2.5 Strain construction .................................................. 79
2.5.1 Tetrad dissection ................................................... 79
2.5.2 Plasmid integration ................................................ 81
2.5.3 Deletion of BFA1 .................................................... 81
2.5.4 C-terminal tagging of BUB2 with a 13MYC epitope .... 82
Chapter 3

Order of function of the Mitotic Exit Network

3.1 Introduction

3.2 Results

3.2.1 Dbf2 kinase activity is dependent upon the MEN

3.2.2 Tem1 acts upstream of Cdc15 to control Dbf2

3.2.3 Cdc15 activates Dbf2 in a Mob1-dependent manner

3.2.4 Cdc5 kinase activity is not regulated by the MEN

3.2.5 Cdc5 over-expression prevents Dbf2 kinase activation

3.2.6 Inhibition of Dbf2 by Cdc5 is largely independent of Bub2

3.2.7 The timing of Cdc5 over-expression determines its effects on Dbf2 kinase activity

3.3 Discussion
Chapter 4

Regulation of Tem1 by Bub2 and Bfa1

4.1 Introduction
4.2 Results

Section 4.2.1 Inhibition of Dbf2 kinase following spindle damage requires Bfa1
Section 4.2.2 Bub2 and Bfa1 physically associate
Section 4.2.3 Bub2 and Bfa1 physically associate across the cell cycle
Section 4.2.4 Bub2 and Bfa1 associate with Tem1 during M phase and early G1
Section 4.2.5 Association with Tem1 requires both Bub2 and Bfa1
Section 4.2.6 Bub2 and Bfa1 oppose the function of Lte1
Section 4.2.7 Lte1 is not required for activation of Dbf2 kinase in the absence of Bub2

4.3 Discussion

Chapter 5

Bfa1 is phosphorylated in a cell cycle-dependent manner
5.2.3 Bub2 is required for Bfa1 phosphorylation ........................................ 137
5.2.4 The cell cycle-regulated phosphorylation of Bfa1 is prolonged in nocodazole .............................................................................................. 137
5.2.5 Bfa1 phosphorylation in an apc2-8-induced metaphase arrest resembles that in a nocodazole-induced metaphase arrest ................. 139
5.2.6 The enhanced phosphorylation of Bfa1 during a nocodazole-induced SAC arrest is dependent on the Mad branch of the spindle checkpoint ................................................................. 140
5.3 Discussion .............................................................................................. 142
5.3.1 Phosphorylation of Bfa1 may regulate Bub2/Bfa1 GAP activity ..... 142
5.3.2 Activation of the Bub2/Bfa1 pathway in metaphase-arrested cells... 146

Chapter 6 .......................................................................................... 147
Cdc5 is partially required for Bfa1 phosphorylation .............. 147

6.1 Introduction .................................................................................. 147
6.2 Results .......................................................................................... 147
6.2.1 Mps1 is not required for Bfa1 phosphorylation ....................... 147
6.2.2 Cdc5, but not Cdc15 or Dbf2, is partly required for Bfa1 phosphorylation ...................................................................................... 150
6.2.3 Cdc5 is required for the cell cycle-dependent phosphorylation of Bfa1 ................................................................................... 153
6.2.4 Cdc5 is required to maintain complete Bfa1 phosphorylation in nocodazole-arrested cells .............................................................. 155
6.2.5 cdc5 mutants do not display high Dbf2 kinase activity in metaphase-arrested cells ................................................................. 158
6.2.6 Cdc5 phosphorylates Bfa1 in vitro ........................................... 158
6.3 Discussion ..................................................................................... 161
Chapter 7..................................................................................... 166
Cdc28 is required for Bfa1 phosphorylation......................... 166

7.1 Introduction ......................................................................................... 166
7.2 Results ................................................................................................... 166
7.2.1 Cdc28 phosphorylates Bfa1 in vitro..................................................... 166
7.2.2 Bfa1 phosphorylation in vivo requires Cdc28................................. 166
7.2.3 Cdc28 is required to maintain Bfa1 phosphorylation in
nocodazole-arrested cells.................................................................... 167
7.2.4 Bfa1 phosphorylation is not affected following deletion of CLB1,
CLB2 or CLB5........................................................................................ 169
7.2.5 Deletion of CLB2 or CLB5 in cdc28-4 mutant cells alters the
phosphorylation pattern of Bfa1........................................................... 172
7.2.6 Mutating the putative Cdc28 phosphorylation sites within Bfa1
leads to loss of the protein................................................................. 174
7.2.7 Cdc14 is required for, but does not directly control, Bfa1
de-phosphorylation in late mitosis.................................................... 176
7.3 Discussion ............................................................................................. 179
7.3.1 Cdc28 controls Bfa1 phosphorylation............................................. 179
7.3.2 De-phosphorylation of Bfa1.............................................................. 182

Chapter 8..................................................................................... 185
Functional analysis of Bfa1 N-terminal and
C-terminal fragments........................................................................... 185

8.1 Introduction.......................................................................................... 185
Chapter 9..............................................................................................................197

Bub2 is essential to prevent mitotic exit in \textit{apc2-8} metaphase-arrested cells..............................................................197

9.1 Introduction..........................................................................................197
9.2 Results.....................................................................................................198
9.2.1 Bub2 restrains mitotic exit in metaphase-arrested cells..............198
9.2.2 Actin ring formation is controlled by the MEN and restrained by Bub2 in metaphase-arrested cells..........................200
9.2.3 Dbf2 does not re-localise to the bud neck in \textit{apc2-8 bub2A} cells......202
9.3 Discussion.............................................................................................204
9.3.1 Control of mitotic exit by the Bub2 checkpoint pathway........204
9.3.2 The role of the MEN in regulating cytokinesis.........................206

Chapter 10.................................................................................................209

Discussion......................................................................................................209

10.1 Order of function of the mitotic exit network.............................. 209
10.2 Control of Tem1 by Bub2/Bfa1.............................................................210
10.2.1 Bub2/Bfa1 appear to form a two-component GAP for Tem1........212
10.2.2 Bfa1 phosphorylation is likely to regulate GAP activity.............212
10.2.3 Bfa1 is phosphorylated by Cdc28 and Cdc5...............................213
10.3 Bub2 and Bfa1 enforce a dependency relationship between spindle elongation through the bud neck, and mitotic exit ................................................................. 214

10.4 Inactivation of Bub2/Bfa1 in late mitosis ............................................. 215
10.4.1 Lte1 overcomes the Bub2/Bfa1-mediated inhibition of Tem1 ......... 215
10.4.2 Bub2/Bfa1 are inactivated once the SPB_{daughter} interacts with the bud neck ................................................................. 216
10.4.3 Loss of Bfa1 phosphorylation may contribute to Bub2/Bfa1 inactivation ..................................................................................... 218

10.5 The requirement for the Bub2/Bfa1 pathway in metaphase-arrested cells ................................................................................. 219
10.5.1 The role of Bub2/Bfa1 in SAC-arrested cells .................................. 219
10.5.2 Bub2/Bfa1 and the DNA damage checkpoint pathway ............... 220
10.5.3 Metaphase arrests which may be independent of the Bub2 pathway 224

10.6 Regulation of cytokinesis by Bub2/Bfa1 and the MEN .................. 225

10.7 The role of the Bub2/Bfa1 pathway in higher eukaryotes .......... 225

References .................................................................................. 227
Publications .............................................................................. 242
## Table of figures

| Figure 1.1 | The spindle cycle in budding yeast | 30 |
| Figure 1.2 | Regulation of the Metaphase/Anaphase transition | 37 |
| Figure 1.3 | Control of mitotic exit in budding yeast | 42 |
| Figure 1.4 | Bub2 and Bfa1 may form a two-component GTPase-activating protein (GAP) for Tem1 | 52 |
| Figure 1.5 | Coupling mitotic exit to completion of anaphase | 53 |

| Figure 2.1 | Bfa1 site-directed mutagenesis protocol used to construct YIPlac128-3HA.bfa1-6A | 80 |
| Figure 2.2 | Bub2-13MYC is fully functional | 83 |
| Figure 2.3 | Combining 3HA-Bfa1 and Bub2-13MYC does not affect functionality | 84 |
| Figure 2.4 | 3HA-Bfa1 is fully functional | 85 |

| Figure 3.1 | Tem1, Cdc15, Mob1 and Cdc5, but not Cdc14 are required for Dbf2 kinase activity | 94 |
| Figure 3.2 | Tem1 activates Dbf2 kinase activity in a Cdc15-dependent manner | 96 |
| Figure 3.3 | Cdc15 activates Dbf2 kinase activity in a Mob1-dependent manner | 98 |
| Figure 3.4 | Cdc5 kinase activity is independent of the MEN | 100 |
| Figure 3.5 | Cdc5 over-expression inhibits Dbf2 kinase activation | 102 |
| Figure 3.6 | Inhibition of Dbf2 kinase by Cdc5 over-expression is largely independent of Bub2 | 104 |
| Figure 3.7 | Over-expressing Cdc5 in metaphase-arrested cells has no effect on Dbf2 kinase activity | 105 |
Figure 3.8 Model illustrating the order of function of the MEN.............108

Figure 4.1 Bfa1 negatively regulates Dbf2 kinase activation.............113
Figure 4.2 Bfa1 and Bub2 associate in mid-log phase cells and in
G1 or metaphase-arrested cells..............................................115
Figure 4.3 Bfa1 and Bub2 associate across the cell cycle...............117
Figure 4.4 Bfa1 and Bub2 physically associate with Tem1 in mid-log
phase cells and in G1 or metaphase-arrested cells.................118
Figure 4.5 Tem1 and Bfa1 associate in mid-log phase cells and in G1- and metaphase-arrested cells.................................120
Figure 4.6 Bfa1 and Bub2 do not physically associate with Tem1 if
either protein is absent..........................................................121
Figure 4.7 Deletion of BFA1 or BUB2 relieves the cold-sensitivity of
an lte1Δ strain.................................................................123
Figure 4.8 Deletion of Lte1 has no effect on Dbf2 kinase activation at
37°C..................................................................................124
Figure 4.9 Model illustrating possible regulation of Tem1 by Bub2,
Bfa1 and Lte1.................................................................126

Figure 5.1 Bub2 is not regulated at the level of protein stability or
post-translational modification in mid-log phase cells or
in cells arrested in G1 or metaphase.................................131
Figure 5.2 Bub2 is not regulated at the level of protein stability or
post-translational modification during the cell cycle........132
Figure 5.3 Bfa1 is subject to cell cycle-dependent post-translational
modification.................................................................133
Figure 5.4 Bfa1 is a phospho-protein........................................135
Figure 5.5 Phosphorylation of Bfa1 is dependent upon Bub2.........136
Figure 5.6 Bfa1 undergoes cell cycle dependent phosphorylation that is protracted following nocodazole treatment ............... 138
Figure 5.7 Bfa1 shows the same phosphorylation pattern in cells arrested in metaphase following SAC activation or using an apc2-8 mutation ......................................................... 141
Figure 5.8 The enhanced phosphorylation of Bfa1 following SAC activation is dependent on Mad2 ......................... 143
Figure 5.9 Model illustrating how Bfa1 phosphorylation may regulate the putative Bub2/Bfa1 two-component GAP ......... 145

Figure 6.1 Mps1 is not required for Bfa1 phosphorylation ............... 149
Figure 6.2 Cdc15 and Dbf2 are not required for Bfa1 phosphorylation. 151
Figure 6.3 Cdc5 partly controls Bfa1 phosphorylation ...................... 152
Figure 6.4 Cdc5 controls Bfa1 phosphorylation during the cell cycle ... 154
Figure 6.5 Cdc5 is required to maintain Bfa1 phosphorylation in nocodazole-arrested cells ........................................ 156
Figure 6.6 Cdc5 is partly required for Bfa1 phosphorylation in an apc2-8-induced metaphase arrest ......................... 157
Figure 6.7 cdc5 mutants do not display high Dbf2 kinase activity in metaphase-arrested cells ................................. 159
Figure 6.8 GST-Bfa1 preparation for use as a substrate in in vitro kinase assays ......................................................... 160
Figure 6.9 Cdc5 and Cdc28 phosphorylate Bfa1 in vitro ................. 162
Figure 6.10 Purified Cdc5 phosphorylates Bfa1 in vitro ................. 163

Figure 7.1 Cdc28 is required for Bfa1 phosphorylation ....................... 168
Figure 7.2 Cdc28 is required to maintain Bfa1 phosphorylation in nocodazole ......................................................... 170
Figure 7.3 Deleting CLB1, CLB2 or CLB5 has no effect of Bfa1 phosphorylation ........................................ 171
Figure 7.4 Deletion of CLB2 or CLB5 when Cdc28 function is compromised, reduces or increases Bfa1 phosphorylation respectively ................................................................. 173
Figure 7.5 Putative Cdc28 phosphorylation sites within the Bfa1 C-terminus ...................................................... 175
Figure 7.6 Mutating the putative Cdc28 phosphorylation sites within Bfa1 leads to loss of Bfa1 protein .................. 177
Figure 7.7 Bfa1 phosphorylation is retained in the absence of functional Cdc14 ....................................................... 178
Figure 7.8 Purified Cdc14 does not dephosphorylate Bfa1 in vitro ..................................................................... 180
Figure 7.9 Regulation of Bfa1 by Cdc28 and Cdc5 .................................................. 184

Figure 8.1 Cartoon showing Bfa1 N-terminal and C-terminal deletion fragments ................................................. 187
Figure 8.2 Expression and SDS-PAGE mobility of the Bfa1 N-terminal and C-terminal deletion fragments .......... 188
Figure 8.3 Re-budding assay to assess functionality of the Bfa1 N-terminal and C-terminal deletion fragments ...... 190
Figure 8.4 Purification of GST-Bfa1 deletion fragments for use as in vitro kinase assay substrates ....................... 192
Figure 8.5 Cdc5 phosphorylates the N-terminus of Bfa1 in vitro................................................................. 193
Figure 8.6 Cdc28 and Cdc28-Clb2 phosphorylate the C-terminus of Bfa1 in vitro ................................................. 195

Figure 9.1 Bub2 restrains mitotic exit and initiation of the next cell cycle in apc2-8 metaphase-arrested cells .......... 199
Figure 9.2  Bub2 prevents premature actin ring formation in metaphase-arrested cells by restraining MEN function......201

Figure 9.3  Dbf2 does not re-localise to the bud neck in apc2-8 bub2Δ metaphase-arrested cells........................................203

Figure 9.4  Control of late mitotic events by the MEN, Sic1 and the APC/C.................................................................208

Figure 10.1  Model illustrating the control of mitotic exit in budding yeast.................................................................211

Figure 10.2  Speculative model illustrating how the activation of Tem1 in late mitosis may be regulated......................217

Figure 10.3  Model illustrating the role of the Bub2/Bfa1 checkpoint pathway following activation of the spindle assembly checkpoint........................................................................221

Figure 10.4  Model illustrating why both the Mad and Bub2 checkpoint pathways are required to induce a spindle assembly checkpoint arrest..............................................222

Figure 10.5  Model illustrating the possible differences between the mechanisms employed by the spindle assembly and DNA damage checkpoints to enforce a metaphase arrest.......223

17
List of tables

Table 1.1  Comparison of the mitotic exit network (MEN) and septation
           initiation network (SIN) components.............................................58

Table 2.1  Strains used in this study.........................................................64
Table 2.2  Oligonucleotides used in this study..........................................72
Table 2.3  Plasmids used in this study.......................................................76
Table 2.4  Antibodies used in this study.....................................................87
List of Abbreviations

\(\alpha F\)  \(\alpha\)-factor  alpha factor
\(\alpha\)-factor  alpha factor
APC/C  Anaphase-promoting complex/cyclosome ubiquitin ligase
APC/C\(^{\text{Cdc20}}\)  Cdc20-bound anaphase-promoting complex/cyclosome
APC/C\(^{\text{Cdh1}}\)  Cdh1-bound anaphase-promoting complex/cyclosome
APS  Ammonium persulphate
ATP  adenosine-5'-triphosphate
bp  base pairs
BSA  Bovine serum albumin
CAP  Calf alkaline phosphatase
CDK  Cyclin-dependent kinase
CDKI  Cyclin-dependent kinase inhibitor
dH\(_2\)O  distilled water
dNTPs  Deoxynucleotide triphosphates
DMSO  Dimethyl sulphoxide
DNA  Deoxyribonucleic acid
DTT  Dithiothreitol
\(E.\ coli\)  \textit{Escherichia coli}
EDTA  Ethylenediaminetetra-acetic acid
5-FOA  5-Fluoro-orotic acid
G1  Gap 1
G2  Gap 2
GAP  GTPase-activating protein
GDP  Guanosine-5’-diphosphate
GEF  Guanine nucleotide exchange factor
GFP  Green fluorescent protein
GTP  Guanosine-5’-triphosphate
HU  Hydroxyurea
HCl  Hydrogen chloride
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<tr>
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<tr>
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<td>Potassium sporulation medium</td>
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<td>Skp1-Cullin-F box ubiquitin ligase</td>
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<td>Sodium dodecyl sulphate</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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Chapter 1

Introduction

1.1 Overview of the Eukaryotic Cell Cycle

Cell proliferation depends upon the replication of chromosomes and segregation of the duplicated chromosomes into two daughter cells. DNA replication and chromosome segregation must occur with high fidelity and in an ordered sequence, if the cell's genetic information is to be accurately transmitted to its progeny. How the cell solves this complex problem is one of the most fundamental questions in cell biology.

Early in the cell cycle, the cell faces a decision point which marks commitment to the entire cell cycle. Passage through this decision point (START in *Saccharomyces cerevisiae*) is dependent upon the cell receiving the appropriate signals. In yeast, this decision is based primarily on nutrient availability and cell size, which are intimately linked (reviewed by Toone *et al.*, 1997). Passage through the restriction point in mammalian systems is controlled by multiple signalling pathways, and mutations in components of these pathways are associated with tumour development (reviewed by Nevins, 2001).

Following passage through START, there are a number of events that must take place if a cell is to complete a successful division cycle. The cell must accurately duplicate its DNA once per cell cycle, to ensure that both the mother and daughter cells receive a complete copy of the genetic information, whilst maintaining constant ploidy. The cell must then segregate the two sets of chromosomes between the mother and daughter, a process which requires the prior formation of a bipolar spindle. Finally, cell division or cytokinesis separates the mother and daughter cells and the cycle can begin again (reviewed by Nasmyth, 1996). The accuracy and order of these events is crucial for maintaining cell viability, and preventing tumourigenesis in multicellular organisms (Lengauer *et al.*, 1998).
Early microscopic and biochemical studies led to cell cycle progression being viewed in terms of four phases: G1 (Gap 1), S (DNA replication), G2 (Gap 2) and M (Mitosis) (reviewed by Nasmyth, 1996). Although it is now clear that the key cell cycle regulatory events do not always correspond to the boundaries between these phases (Nasmyth, 1996), the nomenclature still provides a useful framework when discussing the cell cycle. In addition, mitosis may be further subdivided into prometaphase, metaphase, anaphase A and B, and telophase. Chromosome condensation occurs during prometaphase, which is followed by metaphase, when the condensed chromosomes align on the metaphase plate. Since Saccharomyces cerevisiae chromosomes do not condense fully, these stages are difficult to identify in budding yeast. Sister chromatid separation takes place during anaphase. This is a two-stage process, involving loss of sister chromatid cohesion (anaphase A), and spindle elongation (anaphase B). Finally, telophase marks the period following DNA segregation, but preceding mitotic exit and cytokinesis. This state is transient in an unperturbed cell cycle, but cells defective in mitotic exit arrest the cell cycle in telophase (see 1.8.1) (Clarke and Gimenez-Abian, 2000).

Detailed genetic studies in yeast have identified many of the proteins responsible for regulating the eukaryotic cell cycle (Hartwell et al., 1974; Johnston and Thomas, 1982; Nurse and Thuriaux, 1980). Cell cycle progression is primarily controlled by a family of protein kinases known as cyclin-dependent kinases (CDKs) (1.3). CDK activity is high during S, G2 and M phases but low during G1 phase, and this alternation between low (G1) and high (S, G2, M) CDK activity is thought to drive the cell cycle (reviewed by Nasmyth, 1996). In addition, there is evidence, at least in budding yeast, that a CDK-independent cell cycle oscillator also exists. This oscillator, which has not been identified, will continue to activate G1 events such as bud formation with normal cell cycle timing, in the absence of CDK-Clb function and may contribute to the timing of CDK activation in an unperturbed cell cycle (Haase and Reed, 1999).

Other aspects of cell cycle control include regulated gene transcription, and targeted ubiquitin-mediated proteolysis of key regulatory proteins by the Skp1-cullin F-box (SCF)
and anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligases, with the latter complex being a key component of mitotic control (see 1.7.1) (reviewed by Tyers and Jorgensen, 2000).

1.2 Checkpoints

To ensure that cell cycle progression occurs in an ordered fashion, the initiation of late events must be dependent upon the successful completion of earlier events. This dependency may be an intrinsic feature of the events themselves, if one is biochemically dependent upon the other. However, if the two processes are biochemically unrelated, a control mechanism is necessary to enforce the dependency. Such controls are referred to as checkpoints (Hartwell and Weinert, 1989). Mutations in checkpoint genes abolish the dependency relationship, and may seriously compromise cell cycle fidelity.

Most budding yeast checkpoint genes are non-essential, since the intrinsic timing of cell cycle events is sufficient to maintain their ordered sequence in an unperturbed cell cycle (Clarke and Gimenez-Abian, 2000; Hartwell and Weinert, 1989). In the absence of checkpoint controls, cells do exhibit a moderate decrease in cell cycle fidelity, such as loss or gain of chromosomes. However, if the intrinsic timing of cell cycle events is perturbed, by mutation or by drugs, checkpoints become essential for viability, emphasising the importance of dependency relationships during cell division (Hartwell and Weinert, 1989). Specific checkpoint pathways are discussed in sections 1.3, 1.7.2.3 and 1.8.2.3.

1.3 Cyclin-dependent kinases

Cyclin-dependent kinases (CDKs) are the central co-ordinators of the cell cycle, and are conserved from yeast through to mammals (reviewed by Nasmyth, 1996). As the name implies, CDK activation requires the binding of a cyclin subunit, and different cyclins are thought to confer substrate specificity to their CDK partners (reviewed by Mendenhall and Hodge, 1998). Cyclins are unstable proteins, which are subject to transcriptional control and targeted proteolysis, generating periodic waves of cyclin protein which correspond to
peaks of CDK activity (Arellano and Moreno, 1997; Evans et al., 1983). Specific cyclin-CDK complexes promote different cell cycle events, although they also display functional redundancy. Thus regulated peaks of CDK-cyclin activity are crucial for controlling the timing of cell cycle events. CDK complexes are also subject to stimulatory or inhibitory phosphorylation, although the importance of these modifications varies between systems (Mendenhall and Hodge, 1998).

In *Saccharomyces cerevisiae*, the major CDK is Cdc28, which is homologous to *Schizosaccharomyces pombe* Cdc2, and human CDK1 (Mendenhall and Hodge, 1998). Cdc28 has nine different cyclins, the Cln1-3 G1 cyclins and the Clb1-6 B-type cyclins (Mendenhall and Hodge, 1998). Unusually, although Cln3 is subject to transcriptional regulation, its protein levels are not strongly cell cycle-regulated (Cross and Blake, 1993; Tyers et al., 1993). The remaining cyclins are transcriptionally activated in the order Cln1/2, Clb5/6, Clb3/4 and Clb1/2 (Epstein and Cross, 1992; Fitch et al., 1992; Richardson et al., 1992; Wittenberg et al., 1990). Cln1-3 are involved in regulating G1 transcription and the passage through START (Dirick et al., 1995), whilst Clb5/6 promote DNA replication (Schwob et al., 1994), Clb3/4 activate spindle formation (Fitch et al., 1992; Richardson et al., 1992) and Clb1/2 trigger nuclear division (Nasmyth, 1996; Surana et al., 1991). However, these roles are not fixed, as shown, for example, by the genetic redundancy amongst the *CLB1-6* genes (Mendenhall and Hodge, 1998).

At least one checkpoint in budding yeast acts by targeting Cdc28. The morphogenesis checkpoint prevents nuclear division when bud formation is delayed due to transient depolarisation of the actin cytoskeleton, by activating the Swe1 kinase, which in turn phosphorylates and inhibits Cdc28, giving a G2 arrest. This arrest allows actin to repolarise and complete bud formation, thus preventing the generation of binucleate cells (reviewed by Lew, 2000).
1.3.1 Cyclin-dependent kinase inhibitors (CDKIs)

CDK-cyclin complexes are also subject to negative regulation by cyclin-dependent kinase inhibitors (CDKIs) (reviewed by Mendenhall and Hodge, 1998). The most important budding yeast CDKI is Sic1, which inhibits Cdc28-Clb kinase complexes, and is involved in down-regulating Cdc28-Clb kinase in late mitosis, and maintaining low Cdc28-Clb kinase activity during G1 (Donovan et al., 1994; Mendenhall, 1993; Schwob et al., 1994). Sic1 is unstable from late G1 until late mitosis, when Cdc28 kinase is active, since Cdc28 phosphorylates Sic1, and phospho-Sic1 is recognised by the SCF ubiquitin ligase which targets it for ubiquitin-mediated proteolysis (Tyers and Jorgensen, 2000). Activation of Sic1 in late mitosis is a two-step process. Firstly, Sic1 transcription is up-regulated following the de-phosphorylation of its transcription factor, Swi5, which allows Swi5 to enter the nucleus (Moll et al., 1991; Toyn et al., 1997; Visintin et al., 1998). Secondly, Sic1 is itself dephosphorylated, rendering it resistant to the SCF, and allowing accumulation of the protein (Visintin et al., 1998).

1.4 Initiation of the cell cycle

It remains unclear precisely how the cell assesses whether conditions are right for passage through START. However, the signals appear to converge at the Cdc28-Cln3 kinase. During G1, Cdc28-Cln3 activity reaches a level sufficient to activate two transcription factors, SCB-binding factor (SBF) and MCB-binding factor (MBF), possibly by phosphorylating Swi6, a component of both transcription factors (reviewed by Mendenhall and Hodge, 1998). SBF and MBF activate transcription of a number of genes, including the G1 cyclins Cln1 and 2 (Nasmyth and Dirick, 1991; Ogas et al., 1991). Cdc28-Cln1/2 trigger duplication of the yeast microtubule organising centres (the spindle pole bodies or SPBs) (Segal and Bloom, 2001; Winey and O'Toole, 2001), and the actin polarisation necessary for bud formation (Lew and Reed, 1993). They also phosphorylate the Sic1 CDKI, targeting it for destruction by SCF-dependent ubiquitin-mediated proteolysis and alleviating its inhibitory effect on Cdc28-Clb5/6 kinase, which is required to trigger DNA replication (Mendenhall and Hodge, 1998).
1.5 Licensing and initiation of DNA replication

It is vitally important that DNA replication only occurs once per cell cycle. Re-replication in the absence of chromosome segregation results in aneuploidy, which is characteristic of certain tumours (Lengauer et al., 1998). Initiation of DNA replication requires the formation of the pre-replicative complex (pre-RC) at origins of replication. Pre-RC formation can only occur when CDK activity is low, during a window in the cell cycle after cell division but before START. Since Cdc28-Clb5/6 kinase activity is required to trigger origin firing, initiation of DNA replication occurs after the switch from low to high CDK activity in late G1. Once this switch occurs, the formation of pre-RC complexes is inhibited until the cell has exited mitosis and re-established a state of low kinase activity. Thus pre-RC formation is a "licensing event", ensuring that the cell cannot re-replicate its DNA until after the duplicated chromosomes have divided, and that ploidy is maintained (reviewed by Toone et al., 1997).

1.6 Mitosis

1.6.1 Sister chromatid cohesion

Cohesion between sister chromatids is essential for their correct segregation during mitosis. Firstly, it ensures that sister chromatids are segregated from each other, rather than homologous chromatids. Secondly, it is important for formation of the bipolar spindle, since it provides the opposing force to that exerted by the spindle microtubules (Michaelis et al., 1997). Cohesion is mediated by cohesin, a conserved protein complex composed of four subunits: Scc1/Mcd1, Scc3, Smc1 and Smc3 (Michaelis et al., 1997; Toth et al., 1999). Another protein complex, containing the Scc2 and Scc4 proteins, is required to load cohesin onto chromatin (Ciosk et al., 2000).

Sister chromatid cohesion is established during DNA replication (Uhlmann and Nasmyth, 1998), and persists until the metaphase/anaphase transition, when loss of cohesion allows
sister chromatids to segregate to opposite poles of the cell (Nasmyth et al., 2000). Loss of cohesion at sister kinetochores may in fact occur as early as S phase, when biorientation is established, and the forces exerted by the spindle microtubules cause the paired kinetochores to split apart (Goshima and Yanagida, 2000). However, cohesion is maintained at the chromosome arms, preventing premature sister chromatid separation (Goshima and Yanagida, 2000).

1.6.2 Formation of the bipolar spindle

Chromosome segregation in anaphase is dependent on the prior formation of the bipolar mitotic spindle. This is organised by two spindle pole bodies (SPBs), situated at opposite ends of the nuclear envelope which nucleate the intra-nuclear microtubules responsible for chromosome segregation, and the cytoplasmic microtubules responsible for nuclear positioning (reviewed by Winey and O'Toole, 2001).

1.6.2.1 The Spindle Pole Body

In G1, yeast cells contain one microtubule organising centre, the SPB mentioned above, which is responsible for nucleating all the microtubules within the cell. It is embedded within the nuclear envelope, but has faces in both the nucleus (the inner plaque) and cytoplasm (the outer plaque), which nucleate the nuclear and cytoplasmic (astral) microtubules respectively. Before the mitotic spindle can form, the SPB must be duplicated (reviewed by Adams and Kilmartin, 2000).

1.6.2.2 SPB duplication

SPB duplication is initiated during early G1, but complete duplication is dependent upon the activation of Cdc28-Cln1/2 kinase as the cells pass START (Figure 1.1, upper panel) (Byers and Goetsch, 1976). SPB duplication is not yet well understood, although a number of gene products are known to be required, including the Mps1 protein kinase (Winey et al., 1991). Like DNA replication, SPB duplication must only occur once every cell cycle to
Figure 1.1  The spindle cycle in budding yeast (upper panel). During G1 phase the cell contains one SPB which is embedded in the nucleus. SPB duplication is completed following activation of Cdc28-Cln1/2 kinase as cells pass Start. During S phase cytoplasmic microtubules emanating from the SPB$_{daughter}$ attach to the bud cortex, whilst the SPB$_{mother}$ establishes microtubule interactions with the mother cortex. Following SPB separation a short spindle forms. Spindle alignment and nuclear movement to the bud neck are mediated by the cytoplasmic microtubules. During anaphase the spindle elongates and the chromosomes are partitioned between the mother and daughter cells. Anaphase is followed by mitotic exit and cytokinesis. [Adapted from Segal and Bloom (2001)].

Deleting components of the Kip3 or Dynein pathways leads to a spindle positioning defect (middle panel). The spindle cycle initially proceeds as in the wild-type, but cells fail to align the spindle correctly. Notably, cytoplasmic microtubules fail to attach to the bud cortex. Nuclear segregation occurs within the mother cell and the cells arrest in anaphase due to the spindle position checkpoint pathway which prevents mitotic exit or cytokinesis until the SPB$_{daughter}$ penetrates the bud neck. Loss of checkpoint function results in completion of mitosis, generating one anucleate and one binucleate cell.

Cdc28-Clb5 kinase is involved in establishment of spindle polarity (lower panel). In $cde28-4$ clb5A diploid cells (see text) microtubules from both the SPB$_{mother}$ and SPB$_{daughter}$ attach to the bud cortex. The undivided nucleus is pulled through the bud neck, and the cells arrest in metaphase, presumably due to activation of a checkpoint. Loss of this checkpoint is predicted to lead to completion of mitosis, producing one anucleate and one binucleate cell, whilst correction of the fault should allow mitosis to proceed as normal.
**Wild-type**

Cytoplasmic microtubule

SPB

Nucleus

Start

| G1 | S | G2 | M |

Cdc28-Cln1/2

Spindle

Cdc28-Clb3/4

**dyn1 Δ/ kar9Δ/ bni1Δ**

Checkpoint

Correction

**cdc28-4 clb5Δ**

Checkpoint

Correction
prevent formation of multiple spindles and aberrant chromosome segregation. It has therefore been suggested that a licensing event in late mitosis or early G1 is necessary before SPB duplication can take place (Luca and Winey, 1998), which may be loss of Cdc28-Clb kinase activity as cells exit mitosis (Haase et al., 2001).

Following duplication, the two SPBs are initially held together by a bridge structure. They then separate, to form a short bipolar spindle, a process which requires Cdc28-Clb3/4 kinase activity (Fitch et al., 1992; Haase et al., 2001) and either the Kip1 or Cin8 kinesin-like motor proteins (Roof et al., 1992). The bipolar spindle consists of two types of nuclear microtubules: kinetochore microtubules, which attach to the chromosome kinetochores, and core microtubules, which form the central spindle (Winey et al., 1995). It is not clear exactly when the kinetochore microtubules attach to the chromosomes, although this may occur as early as S phase (Goshima and Yanagida, 2000; Winey and O'Toole, 2001).

1.6.2.3 Spindle polarity and positioning

Concomitant with SPB separation, spindle polarity and orientation are established. Spindle polarity dictates which SPB will separate to the mother or daughter cells respectively during anaphase, whilst spindle orientation refers to the alignment of the spindle along the cell polarity axis (reviewed by Segal and Bloom, 2001). In Saccharomyces cerevisiae, the cell polarity axis is predetermined by the site of bud emergence: the spindle must align along the mother-bud axis to ensure that nuclear segregation occurs between the mother and daughter cells (Figure 1.1) (Chant et al., 1995). Failure to do so could result in nuclear segregation occurring entirely within the mother cell, resulting in one anucleate and one binucleate cell (Figure 1.1, middle panel).

The establishment of spindle orientation and positioning (and hence nuclear positioning) is dependent upon the attachment of cytoplasmic microtubules to cell cortical determinants, and the subsequent action of microtubule-based motor proteins, which position the spindle, perhaps by controlling microtubule dynamicity (reviewed by Schuyler and Pellman, 2001;
Segal and Bloom, 2001). Two redundant pathways are involved: the Kip3 pathway, and the dynein/dynactin pathway (Cottingham and Hoyt, 1997; Heil-Chapdelaine et al., 1999; Lee et al., 1999; Yeh et al., 2000). Strains lacking components of either pathway show a spindle positioning defect, and loss of functionality in both pathways is lethal (Yeh et al., 2000). Although these pathways are redundant, the Kip3 pathway is thought to function primarily to align and position the nucleus at the neck, whilst the dynein pathway provides the driving force required to insert the daughter-bound SPB and chromosomes through the neck during anaphase B (Yeh et al., 2000).

The spindle positioning defect observed when either the Kip3 or dynein/dynactin pathways are compromised can lead to chromosome segregation occurring within the mother cell (Figure 1.1, middle panel). However, a checkpoint pathway exists which prevents these cells completing mitosis until the correct spindle alignment has been attained, and the chromosomes have been partitioned between both mother and daughter cells (Figure 1.1, middle panel). This spindle position checkpoint pathway will be discussed in detail in section 1.8.2.3.

The first step in spindle orientation, is the capture by bud cortical determinants of cytoplasmic microtubules which emanate from the daughter-bound SPB (SPB_{daughter}) (Segal et al., 1998; Shaw et al., 1997; Yeh et al., 2000). The microtubule-bud cortex interactions require a number of proteins including the microtubule binding protein Bim1/Yeb1, Kar9, Bni1, Bud6 and the kinesin motor protein Kip3 (Cottingham and Hoyt, 1997; Heil-Chapdelaine et al., 1999; Lee et al., 1999; Yeh et al., 2000). Bim1 localises to the microtubule plus ends (Lee et al., 2000), and binds to Kar9 (Korinek et al., 2000; Lee et al., 2000), and this linkage presumably provides the cortical attachment for the cytoplasmic microtubules, since Kar9 is localised to the bud tip in a Bni1/Bud6-dependent manner (Miller et al., 1999). Kar9 is transported to the bud tip along actin cables, which may explain the requirement for actin in spindle orientation (Beach et al., 2000; Yin et al., 2000).
Whilst capture of SPB$_{\text{daughter}}$-specific microtubules is crucial for spindle orientation, it is equally important that microtubules emanating from the SPB$_{\text{mother}}$ do not become associated with the bud cortex. To facilitate this, organisation of cytoplasmic microtubules by the SPB$_{\text{mother}}$ is delayed until after the SPB$_{\text{daughter}}$-bud cortex interactions have been established, when the SPBs are 1μm apart (Segal et al., 1998; Shaw et al., 1998). Failure to enforce this lag results in both poles becoming daughter-bound. The delay is dependent upon Cdc28-Clb5 kinase activity, since in cells containing a mutant form of $CDC28$ and a $clb5\Delta$ deletion ($cdc28\Delta-4\; clb5\Delta$), both SPBs begin to organise microtubules early, and become "daughter-bound". As a result, the pre-anaphase spindle and undivided nucleus translocate into the bud, giving a cell-cycle arrest (Figure 1.1, lower panel) (Segal et al., 1998). Although it is not clear how Cdc28-Clb5 functions to specify spindle polarity, these studies emphasise its importance.

Once SPB$_{\text{daughter}}$-bud cortex interaction have been established, as the SPBs separate, the relatively rigid intra-nuclear spindle forces the second SPB away from the neck, and the microtubules emanating from the SPB$_{\text{mother}}$ form cortical interactions progressively further away from the bud (Figure 1.1, upper panel) (Segal and Bloom, 2001; Yeh et al., 2000). Cytoplasmic microtubules are unstable, and the dynamic instability is also believed to play a role in orienting the spindle. This is known as the "search and capture" model whereby microtubules from the two SPBs search for and attach to cortical sites progressively further apart (Schuyler and Pellman, 2001; Segal and Bloom, 2001).

Once the spindle has assembled correctly and aligned along the mother-bud axis, chromosome segregation can occur. This stage in the cell cycle is equivalent to metaphase, although yeast cells do not condense their chromosomes or align them on the metaphase plate, as is seen in many other cell types (Winey and O'Toole, 2001).
1.7 Regulation of mitotic progression

1.7.1 The anaphase-promoting complex/cyclosome (APC/C)

Progression through mitosis is largely mediated by the anaphase-promoting complex or cyclosome (APC/C), which triggers the ubiquitin-mediated proteolysis of key regulatory proteins (reviewed by Morgan, 1999). The APC/C is thought to function as an E3 ubiquitin ligase, which co-operates with an E1 ubiquitin-activating enzyme and an E2 ubiquitin-conjugating enzyme to covalently attach the small protein ubiquitin to a lysine residue of the target protein. This process is repeated to produce a poly-ubiquitin chain, which functions as a degradation signal for the 26S proteasome. E3 enzymes such as the APC/C are though to impart specificity to the ubiquitination reaction (Jorgensen and Tyers, 1999).

The budding yeast APC/C is a large complex, composed of at least 12 subunits (Hwang and Murray, 1997; Irniger et al., 1995; Kramer et al., 1998; Zachariae et al., 1998b; Zachariae et al., 1996). Strains harbouring temperature-sensitive mutations in APC/C components arrest in metaphase at the restrictive temperature, indicating that the APC/C is crucial for entry into anaphase (Kramer et al., 1998).

The APC/C activates two crucial transitions during mitosis, the metaphase/anaphase transition and the exit from mitosis (reviewed by Morgan, 1999; Zachariae and Nasmyth, 1999). Progression through these two transitions requires the targeted proteolysis of distinct proteins, and is mediated by two different forms of the APC/C, defined by two different cofactors, Cdc20 and Cdh1/Hct1 (Schwab et al., 1997; Visintin et al., 1997). These related WD40-containing proteins are required to activate the APC/C and are believed to contribute to its substrate specificity. APC/C\(^{C\text{dc20}}\) acts at the metaphase/anaphase transition, whilst APC/C\(^{C\text{dh1}}\) controls mitotic exit (Schwab et al., 1997; Visintin et al., 1997).
1.7.2 Regulation of the metaphase/anaphase transition by APC/C^{Cdc20}

1.7.2.1 Activation of APC/C^{Cdc20}

Since the transitions mediated by the APC/C are irreversible, it is crucial that they are tightly regulated. Control of the APC/C is predominately exerted at the level of Cdc20 and Cdh1, but its activity is also regulated by phosphorylation (reviewed by Zachariae and Nasmyth, 1999).

The regulation of APC/C^{Cdc20} remains something of an enigma. One contributory factor is the cell cycle control of Cdc20 protein levels. During G1, Cdc20 is targeted for degradation by APC/C^{Cdh1}, but as cells enter S phase, APC/C^{Cdh1} is inactivated and Cdc20 accumulates. It is unlikely that this gradual accumulation explains the abrupt activation of APC/C^{Cdc20} that occurs in metaphase (Zachariae and Nasmyth, 1999).

APC/C^{Cdc20} function also requires phosphorylation by Cdc28-Clb kinase (Rudner et al., 2000; Rudner and Murray, 2000). However, Cdc28-Clb kinase is also activated prior to metaphase, indicating that there must be a further level of control operating. One other kinase which could regulate the APC/C is the Cdc5 polo-like kinase, since Cdc5 overexpression can activate the ubiquitin ligase activity of the APC/C (Charles et al., 1998). Moreover, cdc5 mutants affect APC/C phosphorylation in vivo, and Cdc5 phosphorylates APC/C subunits in vitro (Rudner and Murray, 2000). In mammalian cells, polo-like kinase has been shown to regulate the APC/C (Kotani et al., 1999), but it is unclear whether Cdc5 controls APC/C^{Cdc20} activity directly in budding yeast (Rudner and Murray, 2000).

1.7.2.2 Sister chromatid segregation

Segregation of sister chromatids during anaphase requires the loss of sister chromatid cohesion. This is thought to be triggered when Scc1 is cleaved by Esp1, a caspase-related protease (Uhlmann et al., 1999). Cleavage of Scc1 by Esp1 generates a 33kDa C-terminal fragment which must be degraded by the N-end rule pathway to completely abolish
cohesion (Rao et al., 2001). Esp1 is maintained in an inactive form by the anaphase inhibitor Pds1 until the metaphase/anaphase transition, when Pds1 is destroyed by APC/C<sup>Cdc20</sup> (Figure 1.2) (Ciosk et al., 1998). In the absence of Cdc20, cells are unable to degrade Pds1 and arrest in metaphase, with a similar phenotype to <i>apc</i> mutants (Shirayama et al., 1999; Visintin et al., 1997).

Interestingly, deletion of <i>PDS1</i> does not result in premature loss of cohesion, indicating that there is an additional level of regulation (Alexandru et al., 1999). A recent study showed that phosphorylation of Scc1 by the Polo-like kinase Cdc5, is necessary for efficient cleavage of Scc1 by Esp1 (Figure 1.2) (Alexandru et al., 2001). Since Cdc5 kinase activity is cell cycle-regulated, peaking during mitosis (Cheng et al., 1998; Fesquet et al., 1999), this explains why premature activation of Esp1 in the absence of Pds1 protein does not lead to loss of cohesion.

Following the loss of sister chromatid cohesion, Esp1 also functions in anaphase to regulate spindle elongation (Jensen et al., 2001). This activity requires Pds1, which assists in localising Esp1 to the spindle, indicating that a pool of Pds1 escapes proteolysis during metaphase (Jensen et al., 2001). Esp1 is therefore likely to be involved in coupling sister chromatid separation to spindle elongation during anaphase B (Jensen et al., 2001).

### 1.7.2.3 Checkpoints which target the APC/C<sup>Cdc20</sup>/Pds1 pathway

It is essential that the cell does not segregate its chromosomes until after DNA replication and spindle assembly have been completed. The DNA damage, DNA replication and spindle assembly checkpoint pathways prevent passage through the metaphase/anaphase transition when DNA is damaged or when DNA replication or spindle assembly respectively are incomplete.
Figure 1.2 Regulation of the Metaphase/Anaphase transition. Sister chromatid segregation requires destruction of the anaphase inhibitor Pds1 which binds to and inhibits the Esp1 protease. Pds1 is destroyed by APC/C<sup>Cdc20</sup>-dependent ubiquitin-mediated proteolysis, liberating Esp1. Esp1-dependent cleavage of Scc1 leads to loss of sister chromatid cohesion and triggers sister chromatid separation. Phosphorylation of Scc1 by Cdc5 promotes its cleavage, and Cdc5 may also activate the metaphase/anaphase transition by phosphorylating and activating APC/C<sup>Cdc20</sup>. The DNA damage, DNA replication, and spindle assembly checkpoint pathways arrest cells in metaphase. Activation of the DNA damage checkpoint leads to inhibition of Cdc5, and phosphorylation of Pds1, which renders it resistant to APC/C<sup>Cdc20</sup>. In contrast, the spindle assembly checkpoint is though to stabilise Pds1 indirectly, by sequestering Cdc20 and preventing activation of APC/C<sup>Cdc20</sup>. 
DNA damage checkpoint

Spindle assembly checkpoint

Cdc5 → ? → APC/C

Pds1

Esp1

Scc1

Metaphase

Sister chromatid separation

Anaphase
The DNA checkpoint pathways

The DNA damage and replication checkpoint pathways are extremely complex. DNA damage generated by ultraviolet or ionising radiation, or stalled DNA replication (for example by addition of hydroxyurea) are sensed by different proteins but the signals appear to converge at the Mec1 kinase (reviewed by Lowndes and Murguia, 2000). In response to DNA damage, Mec1 activates a number of signalling cascades, including a Rad53-dependent pathway which inhibits Cdc5 (Sanchez et al., 1999) and consequently exerts a negative influence on the metaphase/anaphase transition. Mec1 also activates the Chkl kinase, which phosphorylates Pds1, rendering it resistant to APC/C-mediated proteolysis (Lowndes and Murguia, 2000; Sanchez et al., 1999). Activation of the DNA replication (S-phase) checkpoint pathway also leads to stabilisation of Pds1, but the mechanism is less well defined (Clarke and Gimenez-Abian, 2000). Thus DNA damage or incomplete DNA replication both result in a checkpoint arrest in metaphase, giving the cell time to correct the fault (Figure 1.2). It is worth noting that cells do not arrest indefinitely following checkpoint activation, since if the cell is unable to correct the problem it is better to attempt mitosis than to die. This phenomenon is termed adaptation, and interestingly, in budding yeast, Cdc5 is involved in adaptation to the DNA damage response, perhaps by overcoming its Rad53-dependent inhibition (Toczyski et al., 1997).

The spindle assembly checkpoint (SAC)

The spindle assembly checkpoint (SAC) arrests the cell cycle in metaphase in response to defects in the mitotic spindle (reviewed by Skibbens and Hieter, 1998). These include defects in spindle pole body duplication, kinetochore function, microtubule polymerisation and microtubule motor proteins (Hardwick et al., 1999). Experimentally, the checkpoint is usually activated by microtubule depolymerising drugs such as nocodazole or benomyl (Skibbens and Hieter, 1998).

The components of the budding yeast SAC were identified by screening for mutants which fail to arrest the cell cycle in the presence of microtubule depolymerising drugs (Hoyt et al.,
1991; Li and Murray, 1991). These screens identified six genes necessary for SAC function, MAD1-3 and BUB1-3, although it has since been shown that BUB2 forms a separate branch of the SAC (see 1.8.2.3). The MPS1 protein kinase, which functions in SPB duplication (1.6.2.2) is also required for spindle checkpoint function, since unlike other SPB duplication mutants, mps1-1 cells do not display a SAC arrest (Weiss and Winey, 1996). The MAD and BUB genes are not essential for viability in budding yeast, although cells exhibit elevated levels of chromosome loss if the checkpoint genes are deleted, suggesting that the checkpoint is important for high fidelity chromosome segregation in an unperturbed cell cycle. In higher eukaryotes, the checkpoint components are essential genes, suggesting that the checkpoint has evolved into a fundamental element of cell cycle control (reviewed by Skibbens and Hieter, 1998).

It is not clear how the SAC components sense spindle abnormalities. Experiments in vertebrate cells suggest that the checkpoint responds to a failure to establish either kinetochore-microtubule attachment, or microtubule tension (Skibbens and Hieter, 1998). These two possibilities are not exclusive, and the cell may sense both attachment and tension (Skoufias et al., 2001). Consistent with both models, components of the SAC localise to kinetochores (Skibbens and Hieter, 1998). Moreover, the Xenopus Mad2 homologue localises predominantly to kinetochores with fewer microtubules attached (Chen et al., 1996), suggesting that Mad2 senses attachment of spindle microtubules to kinetochore binding sites.

Although the order of function of the SAC has not been established, the Mps1 and Bub1 kinases are thought to function together upstream of the other SAC components, since over-expression of Mps1, or a dominant mutant form of Bub1, induces a metaphase arrest, which is dependent upon the remaining SAC components (including Bub1 and Mps1 respectively) (Farr and Hoyt, 1998; Hardwick et al., 1996). Mps1 phosphorylates Mad1 in vitro and in vivo (Hardwick et al., 1996), whilst Bub1 phosphorylates Bub3 in vitro, suggesting that Bub3 may be a Bub1 substrate (Murray, 1994).
Several SAC proteins are known to associate and these complexes seem to be important for function. Bub3 binds to Bub1 through the cell cycle, and this interaction is though to recruit Bub1 to kinetochores (Roberts et al., 1994). The association of Mad1 with Mad2 is necessary for checkpoint function and Mad1 phosphorylation (Chen et al., 1999). Recent data indicate that following checkpoint activation, Bub1 and Bub3 bind to Mad1 and that formation of this complex is crucial for checkpoint function (Brady and Hardwick, 2000). However, the roles of these complexes remain to be elucidated.

Like the DNA damage checkpoint, the spindle assembly checkpoint arrests cells in metaphase by preventing the function of APC/C\(^{Cdc20}\). However, the SAC arrest is thought to be due to sequestration of Cdc20 (Figure 1.2). In human cells, Mad2 binds to APC/C\(^{Cdc20}\) and directly inhibits its activity (Fang et al., 1998), whilst in budding yeast, Cdc20 and Mad2 associate, and cells over-expressing Cdc20 fail to establish a SAC arrest (Hwang et al., 1998). Thus the DNA damage and spindle assembly checkpoints both regulate the metaphase/anaphase transition, by subtly different methods.

1.8 Mitotic exit

For the purposes of this study, mitotic exit refers to the destruction of Cdc28-Clb kinase activity. This effectively marks the end of the cell cycle and the beginning of the subsequent one, since it permits licensing of bud formation, SPB duplication and DNA replication (Haase et al., 2001; Lew and Reed, 1993; Noton and Diffley, 2000). CDK inactivation is generally achieved by a combination of Sic1-mediated inhibition of Cdc28-Clb complexes, and APC/C\(^{Clb}\)-dependent destruction of cyclin subunits. However, two further events are necessary to complete the cell cycle, namely disassembly of the mitotic spindle and cytokinesis, which both occur shortly after CDK inactivation. Spindle disassembly is dependent upon APC\(^{Clb}\)-mediated destruction of Ase1, whilst cytokinesis is thought to be triggered by CDK inactivation (reviewed by Zachariae and Nasmyth, 1999).
1.8.1 The mitotic exit network (MEN)

A number of genes control exit from mitosis in budding yeast, and these are known collectively as the mitotic exit network or MEN (Jaspersen et al., 1998). Cells containing temperature-sensitive mutations in MEN components fail to complete mitotic exit and cytokinesis, and arrest in telophase, with an elongated spindle and divided nuclei (Jaspersen et al., 1998). MEN genes include the GTP-binding protein TEM1, its putative exchange factor LTE1, the kinases CDC5, CDC15 and DBF2, the DBF2 homologue DBF20, the phosphatase CDC14, and the CDK inhibitor SIC1 (Donovan et al., 1994; Johnston et al., 1990; Kitada et al., 1993; Mendenhall et al., 1995; Shirayama et al., 1994; Toyn and Johnston, 1994). Together, these proteins are thought to form a signal transduction cascade which activates mitotic exit (Jaspersen et al., 1998).

Mitotic exit is dependent upon two events: activation of APC/C\(^{Cdh1}\), and up-regulation of Sic1. Together, APC/C\(^{Cdh1}\) and Sic1 co-operate to remove mitotic CDK activity, and trigger mitotic exit (reviewed by Zachariae and Nasmyth, 1999). However, APC/C\(^{Cdh1}\) and Sic1 are themselves negatively regulated by mitotic CDK activity. Cdc28-Clb kinase maintains Cdh1 in a phosphorylated state from S phase until anaphase, and this phosphorylation prevents its association with the APC/C (Zachariae et al., 1998a). Phosphorylation of the Sic1 transcription factor, Swi5, excludes it from the nucleus, and prevents activation of Sic1 transcription, whilst phosphorylation of Sic1 itself targets it for SCF-mediated proteolysis (Feldman et al., 1997; Moll et al., 1991; Toyn et al., 1997). Thus exit from mitosis requires the de-phosphorylation of Cdh1, Swi5 and Sic1. These de-phosphorylation events are mediated by a single phosphatase, Cdc14 (Figure 1.3). Activation of Cdc14 at the end of mitosis is therefore a key event in mitotic exit, and is tightly regulated (Visintin et al., 1998).

1.8.1.1 Cdc14

Cdc14 is kept inactive from G1 until late mitosis, by Cfi1/Net1, a component of the RENT complex (regulator of nucleolar silencing and telophase), which sequesters Cdc14 in the
Figure 1.3  Control of mitotic exit in budding yeast. Mitotic exit requires inactivation of mitotic CDK (predominantly Cdc28-Clb2). This is controlled by APC/C^{Cdh1}, which targets Clb2 for ubiquitin-mediated proteolysis, and Sic1, which directly inhibits the Cdc28-Clb2 complex. APC/C^{Cdh1} and Sic1 are activated in late mitosis by the Cdc14 phosphatase. Cdc14 dephosphorylates Cdh1, allowing it to associate with the APC/C. Cdc14 also dephosphorylates Sic1 and its transcription factor Swi5, allowing the latter to enter the nucleus and activate Sic1 transcription and preventing SCF-mediated proteolysis of the former. Cdc14 is sequestered in the nucleolus for much of the cell cycle, and its release in late anaphase ("activation") is dependent upon a number of MEN proteins, including Tem1, Cdc15, Cdc5, Dbf2 and Mob1. Activation of the MEN is though to be partly due to the interaction of Tem1 with its GEF, Lte1, which occurs when the SPB_{daughter} enters the bud neck during anaphase.
Spindle
Nucleus
Lte1 localises to the bud
Tem1 localises to the SPB

Tem1 interacts with Lte1

Lte1

Tem1

Active

Cdc15
Cdc5 Dbf2 Mob1

Cdc14

Swi5 Sic1

Sic1

Cdc14

Cdc28

Cdc28

Anaphase
Mitotic exit
Cytokinesis
nucleolus and may also inhibit its phosphatase activity (Shou et al., 1999; Visintin et al., 1999). Release of Cdc14 occurs in late anaphase, and is dependent upon Tem1, Cdc15, Cdc5, Dbf2 and Dbf20 (Shou et al., 1999; Visintin et al., 1999). Thus the sole function of these MEN genes in mitotic exit seems to be the release of Cdc14. Consistent with this, over-expression of Cdc14, or deletion of CFII/NET1 rescues the temperature-sensitive lethality of cdc15-2, dbf2-2 and tem1-3 mutants (Jaspersen et al., 1998; Visintin et al., 1999). An understanding of how these MEN components regulate Cdc14 release is therefore critical.

Since the components of the MEN required for Cdc14 release include a Ras-like GTP-binding protein (1.8.1.2), its putative exchange factor, and several kinases, it is likely that they form a signal transduction cascade which culminates in release of Cdc14 from the nucleolus. If this hypothesis is correct, it is likely that the Tem1 GTP-binding protein would act near the top of this signalling network, and that activation of Tem1 would be a key regulatory event.

1.8.1.2 Lte1/Tem1

Tem1 is a member of the ras family of small GTP-binding proteins, which undergo structural changes in response to binding of GDP or GTP (Shirayama et al., 1994). The GDP-bound (inactive) form and the GTP-bound (active) form interact with different proteins, and consequently act as molecular switches in the cell. The association of the GTP-bound form with its effector proteins leads to activation of downstream signalling pathways (Cherfils and Chardin, 1999). A key stage in the regulation of GTP-binding proteins is consequently the switch from the GDP-bound state to the GTP-bound state (activation), facilitated by guanine nucleotide exchange factors (GEFs). The subsequent hydrolysis of GTP to GDP (inactivation) requires the activity of GTPase activating proteins (GAPs) which enhance the intrinsic GTPase activity of the GTP-binding protein (Cherfils and Chardin, 1999).
Lte1 is thought to act as the exchange factor (GEF) for Tem1 (Shirayama et al., 1994). Although it is a non-essential gene, strains lacking LTE1 are cold-sensitive, and arrest in telophase at low temperatures. Over-expression of TEM1 suppresses this cold-sensitivity phenotype, supporting the idea that Lte1 is required to activate Tem1 at low temperatures (Shirayama et al., 1994). It is not clear why Lte1 is not required at higher temperatures, although it is possible that Tem1 has a high intrinsic exchange activity which is compromised in the cold (Bardin et al., 2000).

A recent analysis of Tem1 and Lte1 localisation has provided an elegant model which explains how activation of Tem1 and mitotic exit may be coupled to sister chromatid segregation (Bardin et al., 2000). The authors found that Tem1 localised to the outer plaque of the daughter-bound SPB during late S phase, G2 and mitosis. This localisation was independent of the MEN components Cdc15, Cdc5 and Dbf2 (Bardin et al., 2000). In contrast, Lte1 localises to the emerging bud, and this localisation pattern persists until after spindle disassembly when Lte1 is present in both the mother and daughter cells (Bardin et al., 2000). Thus, Tem1 and Lte1 are restricted to different parts of the cell until late anaphase, when the daughter-bound SPB enters the bud as the chromosomes segregate. Tem1 and Lte1 can then interact, triggering activation of Tem1 and subsequent mitotic exit (Figure 1.3) (Bardin et al., 2000).

It is not clear how the remaining members of the MEN interact with each other to facilitate Cdc14 release from the nucleolus. The following sections describe what is known about these MEN components.

1.8.1.3 Cdc15

Cdc15 is a protein kinase which is thought to function early in the MEN pathway (Jaspersen et al., 1998). It is phosphorylated in a cell-cycle dependent manner by Cdc28-Clb kinase until anaphase/telophase, when it is dephosphorylated by Cdc14 (Jaspersen and Morgan, 2000; Xu et al., 2000). This phosphorylation has no effect on its kinase activity (Jaspersen and Morgan, 2000), but appears to negatively regulate Cdc15, since a non-
phosphorylatable mutant (Cdc15-7A) is a more potent stimulator of mitotic exit than the wild-type protein, and suppresses several MEN temperature-sensitive mutant strains (Jaspersen and Morgan, 2000). The de-phosphorylation (and activation) of Cdc15 by Cdc14 indicates that at least one positive feedback loop exists within the MEN.

Cdc15 associates with Tem1 during metaphase and telophase when Tem1 is localised to the SPBs, suggesting that Tem1 could regulate Cdc15 localisation (Bardin et al., 2000). Consistent with this, localisation studies (Cenamor et al., 1999; Menssen et al., 2001; Xu et al., 2000) have shown that Cdc15 is situated at the SPBs, although there are disagreements regarding the timing and polarity of this association. One report found Cdc15 localised at the SPB from anaphase until telophase, when a portion of the protein relocated to the bud neck (Xu et al., 2000), whilst a second, similar, study suggested that Cdc15 localised to the outer plaque of the SPB from anaphase until cytokinesis, but with a preference for the daughter-bound SPB (Cenamor et al., 1999). In contrast, Menssen et al., (2001) suggested that Cdc15 localises to the mother-bound SPB until after activation of the MEN, when it also associates with the SPB<sub>daughter</sub>. A cdc15 mutant which failed to localise to the SPB completed mitotic exit, but was defective in cytokinesis (see 1.8.4), emphasising the importance of localisation in MEN function (Menssen et al., 2001).

1.8.1.4 Cdc5

CDC5 belongs to the family of polo-like kinases which share homology to the Drosophila polo gene (reviewed by Glover et al., 1998). Polo family members share a conserved amino-terminal catalytic domain, and three conserved sequences in their C-termini, the polo boxes. CDC5 is an essential gene, and the only polo-like kinase (plk) in Saccharomyces cerevisiae, although higher eukaryotes have multiple plk genes (Glover et al., 1998). Human Plk1 can complement a cdc5-1 mutation, suggesting that Plk family members are highly conserved (Lee and Erikson, 1997).

Consistent with its role in mitosis, Cdc5 protein levels are cell-cycle regulated. Cdc5 accumulates during G2/M, but its levels abruptly decline as cell exit mitosis (Cheng et al.,
1998), following the activation of APC/C\textsuperscript{Cdc1}, which targets Cdc5 for ubiquitin-mediated proteolysis during G1 (Shirayama et al., 1998). Cdc5 kinase activity is also regulated across the cell cycle, peaking in late mitosis (Cheng et al., 1998). The delay in Cdc5 kinase activation relative to its protein levels indicates that it is post-translationally regulated, perhaps by phosphorylation, since phosphatase treatment reduces Cdc5 kinase activity (Cheng et al., 1998).

Although most of the cdc5 mutant alleles identified to date are defective in mitotic exit, this is not the only transition it regulates. Cdc5 may regulate DNA replication through its interaction with Dbf4 (Hardy and Pautz, 1996; Kitada et al., 1993), although its degradation during G1 precludes a major role early in the cell cycle (Cheng et al., 1998). Cdc5 also acts at the metaphase/anaphase transition, phosphorylating Scc1, and possibly also APC/C\textsuperscript{Cdc20} (1.7.2.1; Figure 1.2) (Alexandru et al., 2001; Rudner and Murray, 2000). Finally, recent evidence suggests that Cdc5 is involved in the regulation of cytokinesis (see 1.8.4) (Song et al., 2000; Song and Lee, 2001).

It is clear that Cdc5 is involved in activating a number of cell cycle events, presumably by phosphorylating multiple substrates. Cdc5 is therefore likely to be tightly regulated, to ensure that it phosphorylates the correct substrate at the correct time. Since there is no evidence for regulatory cofactors, Cdc5 localisation, and the consequent changes in substrate availability may be the key. Cdc5 localises predominantly to the SPBs during early mitosis, although it can also be detected in the nucleus (Cheng et al., 1998; Song et al., 2000). During late mitosis, Cdc5 relocates to the bud neck, and can be observed as a single or double ring. The polo box is required for Cdc5 localisation, and mutants which fail to localise correctly are not functional, despite having kinase activity, indicating that correct targeting of Cdc5 is essential for its cell cycle activities (Song et al., 2000).

1.8.1.5 Dbf2

Dbf2 is a protein kinase which functions in late mitosis (Johnston et al., 1990; Toyn and Johnston, 1994). Cells lacking DBF2 are viable, due to the presence of a close homologue,
DBF20, which can substitute for DBF2 if it is deleted, but dbf2Δ dbf20Δ double mutants are inviable (Toyn et al., 1991). Although DBF2 is a non-essential gene, lethal temperature-sensitive alleles have been isolated which give a telophase arrest at the restrictive temperature, consistent with Dbf2 functioning within the MEN (Johnston and Thomas, 1982). The existence of lethal temperature-sensitive mutations in a dispensable gene is unusual and is may be due to the presence of a hypothetical limiting co-factor shared by both Dbf2 and Dbf20, which is necessary for function and associates with Dbf20 when Dbf2 is deleted but not when Dbf2 is inactivated by mutation (Toyn and Johnston, 1993).

Dbf2 kinase activity is cell cycle-regulated, peaking in late anaphase/telophase. Activation of Dbf2 kinase is likely to be due to post-translational modification, although up-regulation of DBF2 transcription in mitosis may account for some of the increase (Toyn and Johnston, 1994). Dbf2 is a phosphoprotein, and it has been proposed that the de-phosphorylation of Dbf2 which occurs in late mitosis could control kinase activation. However, in vitro de-phosphorylation of Dbf2 does not affect the kinase activity (Toyn and Johnston, 1994), suggesting that de-phosphorylation may be important for other aspects of Dbf2 regulation, such as its localisation (Frenz et al., 2000).

The localisation pattern of Dbf2 throughout the cell cycle is broadly similar to that of the other MEN proteins (Frenz et al., 2000). Dbf2 is present at the SPBs for much of the cell cycle, but relocates to the bud neck in late mitosis, where it forms a double ring (Frenz et al., 2000).

Dbf2 and Dbf20 both associate with the MEN protein Mob1 (1.8.1.6), and DBF2 over-expression suppresses a mob1-77 temperature-sensitive mutation (Komarnitsky et al., 1998). However, DBF2 over-expression cannot suppress a mob1Δ deletion, suggesting that Dbf2 acts through Mob1 (Komarnitsky et al., 1998). In contrast, MOB1 over-expression suppresses the growth defects associated with both a dbf2Δ deletion and a dbf2Δ dbf20Δ double deletion, suggesting that Dbf2 assists Mob1 function, but that Mob1 can fulfil its late mitotic role independently of Dbf2 when expressed at sufficiently high levels (Komarnitsky et al., 1998).
Dbf2 is also a component of the CCR4 transcriptional regulatory complex, which includes the Ccr4 and Caf1 proteins (Liu et al., 1997). Strains deleted for CCR4 and CAF1 display some of the mitotic defects associated with a dbf2Δ deletion, and it has been proposed that in addition to its role in the MEN, Dbf2 may also regulate late mitotic gene expression through the CCR4 complex (Liu et al., 1997).

1.8.1.6 Mob1

Mob1 (Mps one binding) is a protein of unknown function, which was initially identified in a two-hybrid screen for proteins which interacted with the Mps1 protein kinase (1.6.2.2; 1.7.2.3.2) (Luca and Winey, 1998). Mob1 is a phosphoprotein, and is phosphorylated by Mps1 in vitro (Luca and Winey, 1998). However, although Mps1 is involved in SPB duplication and spindle assembly checkpoint function, temperature-sensitive mob1 alleles show no defects in these processes, but arrest in telophase at the restrictive temperature. This suggests that Mob1 is not functionally related to Mps1 (Luca and Winey, 1998). Nevertheless, some mob1 alleles do display an increase in ploidy at the restrictive temperature, a phenotype not observed in other MEN mutants, but which does occur in SPB duplication mutants. This may indicate that Mob1 does play a role in the spindle cycle (Luca and Winey, 1998), although its primary role is likely to be in mitotic exit.

Consistent with a role in mitotic exit, Mob1 interacts with Dbf2, and MOB1 over-expression suppresses the mitotic defects of dbf2 mutants (Komarnitsky et al., 1998). Interestingly, its ability to bind Dbf2 is considerably reduced in mob1 mutants, suggesting that its interaction with Dbf2 is crucial for the late mitotic function of Mob1 (Komarnitsky et al., 1998).

1.8.2 Coupling sister separation to mitotic exit

Mitotic exit and cytokinesis must not occur until after sister chromatid separation has been completed, if both the mother and daughter cells are to receive a full set of chromosomes.
Activation of mitotic exit must therefore be dependent upon the prior completion of anaphase. A number of controls have been implicated in this dependency, including the spatial regulation of Tem1 and Lte1 discussed earlier (1.8.1.2, Figure 1.3). The other mechanisms that are thought to contribute to the timing of mitotic exit are described below.

1.8.2.1 The role of APC/C<sup>Cdc20</sup>

Pds1 is not the only mitotic regulator targeted for ubiquitin-mediated proteolysis by APC/C<sup>Cdc20</sup>. Cells deleted for CDC20 arrest in metaphase due to a failure to degrade Pds1, and liberate Esp1 (Ciosk et al., 1998; Yamamoto et al., 1996). If PDS1 is also deleted, sister chromatid separation can occur, but cdc20Δ pds1Δ double mutants arrest in telophase with high Cdc28-Clb kinase activity, a phenotype associated with a failure to complete mitotic exit (Lim et al., 1998). This suggests that APC/C<sup>Cdc20</sup> also targets a negative regulator of mitotic exit for degradation. This additional substrate was shown to be the S phase cyclin Clb5 (Shirayama et al., 1999). Clb5 does not appear to be involved in regulating Cdc14 release from the nucleolus, and it has therefore been suggested that Cdc28-Clb5 opposes the function of Cdc14 directly, by counteracting the effects of its phosphatase activity (Shirayama et al., 1999). The APC/C<sup>Cdc20</sup>-dependent lowering of Cdc28-Clb5 kinase activity would therefore be a pre-requisite for mitotic exit, ensuring that mitotic exit is coupled to sister chromatid separation.

Clb5 is not the only cyclin target of APC/C<sup>Cdc20</sup>. Destruction of Clb3 is predominantly mediated by this form of the APC/C, although it is also recognised by APC/C<sup>Cdh1</sup> (Baumer et al., 2000). Interestingly, recent data suggests that APC/C<sup>Cdc20</sup> also degrades a fraction of Clb2 during anaphase (Baumer et al., 2000; Yeong et al., 2000). Proteolysis of this initial pool of Clb2, and the resultant lowering of Cdc28-Clb kinase activity, appears to be a pre-requisite for the final destruction of Clb2 mediated by APC/C<sup>Cdh1</sup> (Yeong et al., 2000). Thus it appears that Cdc20 and Cdh1 do not convey a strict substrate specificity to the APC/C, but rather control the degradation of specific cyclin fractions, which contribute to the ordered progression through mitosis. The action of APC/C<sup>Cdc20</sup> is necessary to lower Cdc28
activity to a threshold level at which Cdc14 becomes dominant, allowing it to trigger the activation of Cdh1 and Sic1, and subsequent mitotic exit (see Figure 1.5).

1.8.2.2 Pds1 as an inhibitor of mitotic exit

Although the degradation of Clb5 is not required for Cdc14 release, degradation of Pds1 is necessary (Shirayama et al., 1999). This could merely reflect the requirement of sister chromatid separation for mitotic exit. However, Pds1 also prevents Clb2 proteolysis in anaphase-arrested cells, suggesting that Pds1 may control mitotic exit directly, independently of its function as an anaphase inhibitor (see Figure 1.5) (Cohen-Fix and Koshland, 1999; Tinker-Kulberg and Morgan, 1999). It is not clear whether this role for Pds1 is Espl-dependent, since esp1-1 mutants are defective in sister chromatid separation but still complete mitotic exit (Cohen-Fix and Koshland, 1999; Shirayama et al., 1999; Tinker-Kulberg and Morgan, 1999).

1.8.2.3 The Bub2/Bfa1 spindle position checkpoint

The Saccharomyces cerevisiae BUB2 gene was initially identified in a screen for mutants which were defective in the spindle assembly checkpoint (1.7.2.3.2) (Hoyt et al., 1991). However, functional analysis suggested that the role of Bub2 in the spindle checkpoint was subtly different to that of the other Mad and Bub proteins. Low doses of benomyl, which disrupt kinetochore-microtubule interactions without damaging the mitotic spindle, induce a cell cycle delay which is dependent upon the MAD genes and BUB1/3 (1.7.2.3.2), but independent of BUB2. This suggested that Mad1-3 and Bub1/3 detect spindle kinetochore attachment whilst Bub2 senses some other aspect of spindle integrity (Wang and Burke, 1995).

Bub2 has now been shown to form a separate branch of the spindle checkpoint (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999). In the presence of spindle damage, cells containing deletions in any of the MAD and BUB genes are still able to delay the cell cycle for approximately 90 minutes. However, cells deleted for BUB2 in
combination with any other MAD or BUB gene show no cell cycle delay. Thus there are two epistasis groups within the SAC, one containing Mad1-3 and Bub1/3, and one containing Bub2, with the genes within each group functioning in two independent branches of the SAC (the Mad and Bub2 branches respectively). Either branch can exert a cell cycle delay in response to spindle damage, but both branches are required for a prolonged arrest (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999).

What are the targets of these two branches? As discussed previously, the Mad branch of the SAC pathway targets APC/C^{Cdc20}, preventing degradation of Pds1 and sister chromatid separation (1.7.2.3.2). However, deletion of BUB2 has no effect on Pds1 stability in cells arrested in the presence of the microtubule-depolymerising drug nocodazole, and the Bub2-dependent checkpoint arrest is not affected by a Mad2-resistant cdc20 allele (Alexandru et al., 1999). This suggests that Bub2 prevents progression through mitosis by some other means. Several lines of evidence suggest that Bub2 prevents mitotic exit by blocking activation of the MEN. The MEN kinase Dbf2 is down-regulated in the presence of nocodazole, indicating that it is a target of the SAC (Fesquet et al., 1999). This inhibition is dependent upon Bub2, but not Mad2 or Bub3, indicating that the Bub2 branch of the SAC controls Dbf2 (Fesquet et al., 1999). Moreover, Bub2 but not Mad2 is required to prevent degradation of Clb2 in nocodazole (Alexandru et al., 1999), supporting the idea that the two different branches of the SAC inhibit APC/C^{Cdc20} and APC/C^{Cby1} respectively, with the latter inhibition acting through the MEN.

The fission yeast homologue of Bub2, Cdc16, and another protein, Byr4, form a 2-component GTPase activating protein (GAP) for Spg1, the homologue of the MEN component Tem1 (Figure 1.4) (Furge et al., 1998). BUB2 can complement a cdc16-116 temperature-sensitive mutation, suggesting that the function of these proteins is conserved (Fankhauser et al., 1993). It has therefore been suggested that Bub2 could function as a GAP for Tem1, converting it from the GTP to GDP-bound form and consequently inactivating Tem1 and its downstream effectors (Figure 1.4, Figure 1.5). Consistent with
Figure 1.4 Bub2 and Bfa1 may form a two-component GTPase-activating protein (GAP) for Tem1. The *S. pombe* homologue of Bub2, Cdc16, forms a 2-component GAP for Spg1 with Byr4, preventing activation of Spg1 and its downstream effectors, including Cdc7. Since Spg1 is the homologue of the MEN component Tem1, and the budding yeast homologue of Byr4, Bfa1, has been shown to function in the Bub2 branch of the SAC, it has been suggested that Bub2 and Bfa1 may function as a 2-component GAP for Tem1, converting it from the GTP to the GDP-bound form. This would inactivate Tem1 and its downstream effectors, including Cdc15, the homologue of fission yeast Cdc7.
S. pombe

\[
\begin{align*}
\text{Spg1} - & \text{GDP} & \text{Cdc16, Byr4} & \text{Active} \\
\text{Inactive} & \rightarrow & \text{Spg1} - & \text{GTP} \\
& & \rightarrow & \text{Cdc7} \\
& & & \rightarrow \text{Septation}
\end{align*}
\]

S. cerevisiae

\[
\begin{align*}
\text{Tem1} - & \text{GDP} & \text{Lte1} & \text{Active} \\
\text{Inactive} & \rightarrow & \text{Tem1} - & \text{GTP} \\
& & \rightarrow & \text{Cdc15} \\
& & & \rightarrow \text{Mitotic exit} \\
& & & \rightarrow \text{Cytokinesis}
\end{align*}
\]
Figure 1.5  Coupling mitotic exit to completion of anaphase. Mitotic exit requires inactivation of mitotic CDK which is mediated by APC/C<sup>C<sub>Ctnl</sub></sup> and Sic1. APC/C<sup>C<sub>Ctnl</sub></sup> and Sic1 are activated by Cdc14 following its release from the nucleolus, which is dependent upon the MEN. Several controls ensure that activation of mitotic exit is dependent upon the prior completion of anaphase. The spatial regulation of Tem1 and Lte1 is thought to ensure that Tem1 is only activated after chromosome segregation, when the SPB<sub>daughter</sub> has entered the bud.

The Bub2/Bfa1 checkpoint pathway is also though to target Tem1, this time to negatively regulate the MEN. This pathway prevents MEN activation following spindle damage, when cells have not completed anaphase, and may form a "universal" checkpoint pathway which prevents MEN activation until anaphase has been completed.

Several APC/C<sup>C<sub>Cdc20</sub></sup> substrates also appear to inhibit MEN activation. Pds1 may negatively regulate the MEN upstream of Bub2, until it is degraded by APC/C<sup>C<sub>Cdc20</sub></sup>. In addition, mitotic CDK activity (Cdc28-Clb) opposes the function of Cdc14, and it is thought that Cdc14 only becomes dominant once Cdc28-Clb kinase activity (particularly Cdc28-Clb5) has been reduced by APC/C<sup>C<sub>Cdc20</sub></sup>-dependent proteolysis of the cyclin subunit. Thus mitotic exit cannot normally precede sister chromatid separation, since both require APC/C<sup>C<sub>Cdc20</sub></sup>-dependent proteolysis. APC/C<sup>C<sub>Cdc20</sub></sup> is also inhibited following spindle assembly checkpoint activation, generating a metaphase arrest.
Spindle assembly checkpoint activation

Metaphase

Pds1

? A

Bub2

Bfa1

Lte1

MEN

Cdc14

Cdc28 Clb

Cdc28 Clb2

APC/C

Tem1 interacts with Lte1

Anaphase

Mitotic exit

Cytokinesis
this model, the budding yeast homologue of byr4*, named BFA1/BYR4 (Alexandru et al., 1999; Li, 1999) was found to be a component of the BUB2 branch of the spindle checkpoint (Alexandru et al., 1999; Li, 1999). However, although the functions of the two proteins appear to be highly conserved, their roles and regulation may differ, since cdc16* and byr4* are both essential genes whilst their budding yeast homologues are non-essential (Cerutti and Simanis, 2000).

Although it is clear that the Mad and Bub2 branches of the SAC target different forms of the APC/C, the downstream effects of deleting components of each pathway are less well defined. Since the Mad branch of the SAC functions to inhibit APC/C^Cdc20, it is not surprising that cells lacking components of this branch are unable to delay Pds1 degradation and sister chromatid separation following spindle damage (Alexandru et al., 1999; Krishnan et al., 2000). However, cells deleted for BUB2 also begin to separate sister chromatids after 90 minutes (Krishnan et al., 2000), indicating that the Bub2 branch of the SAC is also required to prevent Pds1 degradation. Krishnan et al. (2000) suggest that the Mad branch establishes the SAC arrest, but that Bub2 is required to maintain it. Since Bub2 normally prevents activation of APC/C^Cdhl, and sister chromatid separation in bub2Δ mutant cells is dependent upon MEN function, it seems likely that APC/C^Cdhl targets Pds1 for degradation when APC/C^Cdc20 is not active. This is consistent with experiments showing that over-expression of CDH1 leads to Pds1 degradation in the absence of CDC20 (Visintin et al., 1997), and recent studies which suggest that the substrate specificity of Cdc20 and Cdhl is not as strict as previously believed (1.8.2.1) (Baumer et al., 2000; Yeong et al., 2000).

Since deletion of BUB2 is sufficient to promote MEN activation in SAC-arrested cells, despite the presence of Pds1, which functions as an inhibitor of MEN function (1.8.2.2), this implies that Pds1 exerts its negative influence on the MEN upstream of Bub2 (Figure 1.5). These experiments partly explain why both branches of the SAC are necessary for complete inhibition of mitotic progression. Inhibition of the metaphase-anaphase transition by the Mad pathway is not sufficient to prevent MEN activation in the absence of Bub2 function, and this in turn can lead to sister chromatid separation.
It has been proposed that as well as controlling two different transitions within mitosis, the two branches of the SAC monitor distinct events. Initially, this idea was based on the different cellular locations of the components of the two branches. Unlike the Mad pathway components, which localise to the kinetochore, Bub2 and Bfa1 both localise to the outer plaque of the SPB (Daum et al., 2000; Fraschini et al., 1999; Li, 1999; Pereira et al., 2000). It was therefore suggested that they might monitor the completion of anaphase, by sensing the movement of the \( \text{SPB}_{\text{daughter}} \) through the bud neck and preventing mitotic exit until this had occurred (Li, 1999; Pereira et al., 2000). Since this movement requires the completion of anaphase, and cytoplasmic microtubule function, the Bub2/Bfa1 pathway would also be induced following exposure to microtubule-depolymerising drugs such as nocodazole.

Further studies have indicated that the Bub2/Bfa1 pathway monitors spindle position, which is intimately linked with nuclear position (Adames et al., 2001; Daum et al., 2000; Pereira et al., 2000). Before anaphase is initiated, the spindle must be aligned along the mother-bud axis, and the nucleus migrates to the bud neck. Correct positioning and orientation of the nucleus is mediated by cytoplasmic microtubules and the functionally redundant \( KAR9 \) and dynein/dynactin pathways (1.6.2.3; Figure 1.1). When components of these pathways are deleted, and nuclear positioning and orientation are compromised, the cells exhibit a cell cycle delay, indicating that a checkpoint exists to monitor nuclear position. Recently, this checkpoint has been shown to be dependent on Bub2/Bfa1 (Adames et al., 2001; Daum et al., 2000; Pereira et al., 2000). Cells deleted for \( KAR9 \) or \( BIM1 \) arrested in anaphase with misorientated spindles, but if \( BUB2 \) or \( BFA1 \) were also deleted, this delay was abolished (Adames et al., 2001; Daum et al., 2000; Pereira et al., 2000). Since the cells completed anaphase without correctly aligning the spindle along the mother-bud axis, chromosome segregation often occurred within the mother cell, generating one binucleate and one anucleate cell (Adames et al., 2001; Daum et al., 2000; Pereira et al., 2000). Thus Bub2 and Bfa1 form a "spindle position checkpoint", which delays mitotic exit when the chromosomes have failed to segregate into the mother and daughter cells.
It is not clear how Bub2/Bfa1 sense successful nuclear division. Adames et al. (2001) found that mitotic exit is consistently activated within 25 minutes of SPB interaction with the bud neck. If this interaction is delayed, for example in cells lacking BIM1 or carrying a temperature-sensitive allele of dynactin (arp1"), the Bub2/Bfa1 checkpoint enforces a corresponding delay in mitotic exit. However, once the SPB interacts with the neck, the Bub2/Bfa1 checkpoint apparently ceases to function, since mitotic exit occurs even if subsequent SPB elongation through the bud neck fails (Adames et al., 2001). A role for the Bub2/Bfa1 pathway in monitoring spindle pole body movement through the neck, is supported by the finding that Bub2 interacts with the septin Cdc3 (Krishnan et al., 2000).

Since Bub2 and Bfa1 seems to monitor the SPB elongation through the bud neck (that is, completion of anaphase), and deletion of BUB2 or BFA1 is sufficient to activate mitotic exit and sister chromatid separation irrespective of APC/C^\textsuperscript{Cdc20} function, the Bub2/Bfa1 pathway might be predicted to be a "universal checkpoint pathway", necessary to establish any metaphase arrest. It is not yet clear whether this is true. Attempts to establish whether Bub2/Bfa1 function is necessary to maintain a DNA damage checkpoint arrest proved inconclusive (Krishnan et al., 2000; Wang et al., 2000). At the restrictive temperature, cells harbouring a temperature-sensitive allele of the telomere maintenance protein Cdc13 (cdc13-l) accumulate single-stranded DNA, resulting in a DNA damage checkpoint arrest. One study found that Bub2 and Bfa1 were required to maintain this arrest (Wang et al., 2000). However, using the same experimental conditions, a second study showed that Bub2/Bfa1 function was not necessary to enforce the DNA damage checkpoint arrest (Krishnan et al., 2000). The reasons for this discrepancy are not clear, and further studies are clearly necessary to clarify the role of Bub2/Bfa1 in the DNA damage checkpoint.

1.8.3 The septation initiation network (SIN)

Many components of the MEN have been identified in the fission yeast, Schizosaccharomces pombe, where they participate in an analogous signalling cascade known as the septation initiation network or SIN. Although many aspects of their regulation appear to be highly conserved, the fission yeast proteins do not control CDK
inactivation, but instead regulate the onset of cytokinesis (reviewed by McCollum and Gould, 2001).

Cytokinesis in fission yeast is accomplished by medial fission and is dependent upon an actomyosin-based contractile ring, which assembles at the medial plane of the cell following entry into mitosis. This ring contains numerous proteins, including F-actin and type-II myosin. Following mitotic spindle disassembly at the end of anaphase, the actomyosin ring constricts, and the division septum (primary septum) is deposited behind the constricting ring. Secondary septa then assemble on either side of the division septum, which is subsequently cleaved to give two daughter cells (reviewed by Balasubramanian et al., 2000).

The SIN genes regulate actomyosin ring constriction and septum formation. sin mutants proceed through multiple nuclear division cycles in the absence of septation, generating long multinucleate cells which eventually lyse (Balasubramanian et al., 1998). SIN components include the Tem1 homologue Spg1, the Cdc15 homologue Cdc7, a Dbf2 homologue named Sid2, a homologue of Mob1 (also called Mob1), and other proteins including Cdc14, Sid1, and Sid4 whose budding yeast homologues have not been identified (Table 1.1) (reviewed by McCollum and Gould, 2001).

In addition, two other proteins, Cdc16 and Byr4, have been shown to negatively regulate the SIN, since mutations in these components lead to continuous septation in the absence of nuclear division (Minet et al., 1979; Song et al., 1996). cdc16* is the homologue of the budding yeast spindle assembly checkpoint gene BUB2, and the recently identified homologue of byr4*, BFA1, is also required for SAC function (Li, 1999). Cdc16 and Byr4 have been shown to form a two-component GTPase-activating protein (GAP) for the Spg1 GTP-binding protein in vitro (Furge et al., 1998), consistent with their ability to negatively regulate the SIN. These two essential proteins are thought to act as a switch, turning off septation, and allowing cytokinesis to occur. They may also prevent premature septum formation (Le Goff et al., 1999).
Table 1.1

Comparison of mitotic exit network (MEN) and septation initiation network (SIN) components

<table>
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<tr>
<th>S. cerevisiae gene</th>
<th>S. pombe homologue</th>
<th>Protein function</th>
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<td>2-component GAP</td>
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<td>BFA1/BYR4</td>
<td>byr4</td>
<td>2-component GAP</td>
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<td>TEM1</td>
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<td>GTP-binding protein</td>
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<td>cdc7</td>
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<tr>
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<td>plo1</td>
<td>Polo-like kinase</td>
</tr>
<tr>
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<td>sid2</td>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>Unknown</td>
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<tr>
<td>Unknown</td>
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</table>


Like their budding yeast counterparts, the SIN proteins localise to the SPBs (McCollum and Gould, 2001). Spg1 localises to both SPBs throughout the cell cycle, initially in its GTP-bound state, but during anaphase B, it is converted to the GDP-bound state at one pole. The reason for this acquired asymmetry is unclear, although it appears to reflect the presence of Cdc16/Byr4 at this pole. Since Cdc7 localisation to the SPB requires Spg1 to
be GTP-bound, Cdc7 also becomes asymmetric in its localisation to the SPBs during late anaphase. In turn, the Sid1-Cdc14 complex is recruited to the Cdc7-bound pole (McCollum and Gould, 2001).

The Dbf2 homologue Sid2 and its binding partner Mob1 are constitutively localised to the SPB, but they also localise to the medial ring in late mitosis (Hou et al., 2000; Salimova et al., 2000; Sparks et al., 1999). Both the kinase activity and medial ring localisation of Sid2, are dependent upon Spg1, Cdc7, Sid1 and Cdc14, indicating that Sid2 and Mob1 function downstream of the other SIN components (Sparks et al., 1999). The movement of Sid2 to the medial ring is thought to trigger actomyosin ring contraction and septum formation, although potential substrates of Sid2 have not been identified (Sparks et al., 1999).

The role of plo1*, the fission yeast homologue of CDC5, in the SIN is likely to be complex. Although plo1* does exhibit genetic interactions with components of the SIN (Cullen et al., 2000), plo1 mutants arrest at an earlier stage of cytokinesis, before actomyosin ring assembly, and Plo1 is thought to function to determine the position of the medial ring (Bahler et al., 1998; Ohkura et al., 1995). However, Plo1 also activates septation in an Spg1-dependent manner and it has therefore been suggested that Plo1 functions upstream of Spg1 to activate the SIN (Tanaka et al., 2001).

1.8.4 Cytokinesis

Cytokinesis is the physical separation of the mother and daughter cells and is analogous to cleavage in vertebrate systems. It is dependent upon the destruction of CDK activity as cells exit mitosis (Balasubramanian et al., 2000; Field et al., 1999), and is consequently the final stage of the cell cycle.

Cytokinesis in budding yeast occurs at the bud neck, and involves two ring structures, the septin ring, and the actomyosin ring, which constricts during cytokinesis. The septins are a family of GTP-binding proteins which are conserved in eukaryotic organisms, and they form a broad, hour-glass structure at the bud neck through most of the cell cycle (Lippincott et al., 2001).
The actomyosin contractile ring covers a narrower region of the bud neck, and assembles in stages. Myosin II is recruited to the ring early in the cell cycle, immediately after the appearance of the septin ring. However, F-actin is only recruited to the ring in telophase, a process which requires the IQGAP-like protein Cyk1/Iqgl1. Recruitment of actin to the contractile ring does not occur in several MEN mutants, including \textit{dbf2}, and \textit{cdc15} (Frenz \textit{et al.}, 2000; Lippincott and Li, 1998), suggesting that MEN function may be required for this process. In contrast, loss of Tem1 function does not appear to affect actin ring formation (Lippincott \textit{et al.}, 2001), although the reasons for this discrepancy are not clear.

Just before cell division, the septin ring splits into two separate rings, and the actomyosin contractile ring, which localises between these two septin rings, undergoes contraction. Splitting of the septin rings may be a prerequisite for contraction, since the septins could provide a structural barrier to this process (Lippincott \textit{et al.}, 2001).

Due to the functional similarities between components of the mitotic exit network and the septation initiation network, it has been proposed that, in addition to their role in mitotic exit, the MEN proteins may regulate cytokinesis. There is growing evidence that this is the case. Several MEN components, including Cdc15 (Xu \textit{et al.}, 2000), Dbf2 (Frenz \textit{et al.}, 2000), Mob1 (L. Frenz, personal communication), and Cdc5 (Song \textit{et al.}, 2000) localise from the SPBs to the bud neck in late mitosis, consistent with a role in cytokinesis. Dbf2 localisation to the bud neck in late mitosis is dependent upon the septins, and the MEN proteins Cdc15, Mob1, Cdc5, and Cdc14 (Frenz \textit{et al.}, 2000). This suggests that activation of the MEN itself may trigger a feedback loop which results in the relocalisation of some of its components to the bud neck.

Experiments which bypass the requirement for the MEN proteins in mitotic exit, are beginning to reveal their roles in cytokinesis. Depletion of Tem1 in \textit{net1-1} mutant cells, which allow Cdc14 release, results in a defect in septin ring splitting and actomyosin ring contraction (Lippincott \textit{et al.}, 2001). These cells undergo cell cycle progression in the absence of cytokinesis, forming chains of cells, indicating that they complete mitotic exit,
but not cell division. A C-terminally truncated form of Cdc15 (Cdc15Δ135), which is unable to localise to the SPBs, also produces branched chains of cells (Menssen et al., 2001), consistent with an earlier study which demonstrated that an allele of CDC15 (cdc15-lyt) was defective in cytokinesis but not mitotic exit (Jimenez et al., 1998).

Cdc5 also appears to regulate cytokinesis. Over-expression of CDC5 induces the formation of multiple cytokinetic structures (Song et al., 2000), and depletion of Cdc5 causes cells to arrest in cytokinesis (Song and Lee, 2001). Furthermore, over-expression of the C-terminal domain of CDC5 (cdc5ΔN), which contains the polo box but not the kinase domain and acts as a dominant negative mutant, results in chains of cells which are undergoing cell cycle progression in the absence of cytokinesis (Song and Lee, 2001).

Thus several MEN components appear to function in both mitotic exit and cytokinesis, with these roles being separable.

1.9 Aims of the project

Mitotic exit and cytokinesis in Saccharomyces cerevisiae are regulated by the MEN. However, despite recent advances, the regulation of this complex protein network is not yet well understood. It is now clear that the Bub2/Bfa1 branch of the spindle assembly checkpoint pathway negatively regulates the MEN component Dbf2 in response to nocodazole-induced spindle damage (Fesquet et al., 1999). By analogy with the fission yeast SIN network, it has been suggested that Bub2 and Bfa1 may form a 2-component GAP for the MEN GTP-binding protein Tem1, and consequently inhibit Dbf2 indirectly. However, there is no experimental evidence that this model is correct. Moreover, Tem1 has not been shown to control Dbf2 and the other MEN proteins. The aims of the project are therefore:

• To examine the regulation of Dbf2 by Tem1 and the MEN.

• To investigate how the Bub2 spindle assembly checkpoint pathway regulates the MEN.
Chapter 2

Materials and Methods

2.1 Bacterial Techniques

2.1.1 Bacterial strains

For routine bacteriological work, *Eschericia coli (E.coli)* DH5α (F' _endA1 hsdR17 (rK mK+) supE44 thi-1 recA1 gyrA (Nal') relA1 Δ(lacZYA-argF)U169 deoR (φ80dλΔ(lacZ)M15)) was used. GST fusion proteins were produced in BL21(DE3) (F _ompT gal [dcm] [lon] hsdSb (rB-mB+; an E. coli B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene).

2.1.2 *E. coli* media and growth conditions

*E. coli* strains were grown in Luria-Bertani broth (LB) (1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl pH 7.5) or 2xYT broth (4% (w/v) bacto-tryptone, 1% (w/v) yeast extract) with the addition of 100μg/ml ampicillin for plasmid selection. Liquid cultures were grown at 37°C in a gyratory shaker [New Brunswick] at 300rpm. For solid LB media, 1.5% (w/v) Difco agar was added and the agar plates were incubated at 37°C in a constant temperature incubator. In the short-term, strains were stored at 4°C on LB agar (with ampicillin as required). For long-term storage, strains were grown to stationary phase in LB broth (with ampicillin as required) and stored at -70°C in 20% (w/v) glycerol.
2.1.3 *E. coli* transformation

To prepare chemical-competent cells, 200μl of DH5α cells grown overnight in LB medium were inoculated into 20ml fresh medium, and grown at 37°C with shaking to an O.D₆₀₀ of 0.3-0.4. The cells were cooled on ice, harvested in a Beckman centrifuge (10,000rpm, 10 min) and resuspended in 1xTSS (LB containing 10% (w/v) PEG₃₃₅₀, 50mM MgCl₂, 5% (v/v) DMSO). Competent cells were either used immediately or stored as 100μl aliquots at -70°C.

100μl of competent cells containing 1-5μl transforming DNA were incubated on ice for 10 min, then heat-shocked at 42°C for 2 min. 1ml 2xYT was added and the cells were incubated at 37°C for 1h. 100μl and 900μl aliquots were plated onto LB agar containing 100μg/ml ampicillin.

2.2 Yeast Techniques

2.2.1 Yeast strains

Details of the strains used in this study are given in Table 2.1.

2.2.2 Yeast media and growth conditions

Cells were grown in YEPD rich medium (1% (w/v) yeast extract, 2% (w/v) bacto-peptone, 2% (w/v) glucose) or, for plasmid selection, in minimal medium (0.67% (w/v) yeast nitrogen base, 2% (w/v) glucose), with the appropriate amino acid supplements at 40μg/ml. Liquid cultures were grown in a gyratory shaker [New Brunswick] at 200rpm. For solid media, 2% (w/v) Difco agar was added and the plates were incubated in a constant temperature incubator. The standard growth temperature for wild-type yeast strains was 30°C. Temperature-sensitive strains were grown at the permissive temperature of 25°C, or the restrictive temperature of 37°C, unless otherwise stated.
Table 2.1

Strains used in this study

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<th>Reference/Source</th>
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<tr>
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65
This study

SLY108  MATα bfa1Δ::KAN^ ura3 leu2 trp1 his7
This study

SLY109  MATa 3HA.BFA1 msd2-1::URA3 ura3 leu2 trp1 ade5
SLY105xJ5AB3-3a

(SL16-5b)

SLY111  MATa 3HA.BFA1 bub2Δ::LEU2 ura3 leu2 trp1 ade5
This study

SLY116  MATα 3HA.BFA1 rsi1Δ::HIS7 prsi1-8 ura3 leu2
SLY107xKTM208

(SL21-7d)  trp1 ade5

SLY117  MATα BUB2.13MYC::KAN^ bfa1Δ::KAN^ ura3
SLY104xSLY108

(SL19-14d)  leu2 trp1 ade5

SLY120  MATα 3HA.BFA1 mps1-1 rsi1Δ::HIS7 prsi1-8
SLY116xKH134

(SL31-18a)  ura3 leu2 trp1 ade5

SLY121  MATα 3HA.BFA1 clb1Δ::URA3 ura3 leu2 trp1
SLY107xCD102-1a

(SL29-1a)  ade5

SLY124  MATα 3HA.DBF2 cdc15-1 rsi1Δ::HIS7 prsi1-8
NJW007x ER1

(SL24-41c)  ura3 leu2 trp1

SLY125  MATα CDC5.3HA::URA3 rsi1Δ::HIS7 prsi1-8
This study

SLY126  MATα CDC5.3HA::URA3 tem1-3 rsi1Δ::HIS7
This study

SLY127  MATα CDC5.3HA::URA3 cdc15-1 rsi1Δ::HIS7
This study

SLY128  MATα CDC5.3HA::URA3 mob1-77 rsi1Δ::HIS7
This study

SLY129  MATα CDC5.3HA::URA3 cdc14-1 rsi1Δ::HIS7
This study

SLY130  MATα 3HA.BFA1::LEU2 bfa1Δ::KAN^ ura3
This study

SLY131  MATα 3HA.bfa1ΔCT270::LEU2 bfa1Δ::KAN^ ura3 leu2 trp1 his7
This study

SLY132  MATα 3HA.bfa1ΔCT300::LEU2 bfa1Δ::KAN^ ura3 leu2 trp1 his7
This study
This study

This study

This study

This study

This study

This study

This study

This study

This study

SLY 133  \( \text{MAT}^\alpha 3HA.bfa1\Delta NT270::LEU2\ bfa1\Delta::KAN^R \) 
  ura3 leu2 trp1 his7

SLY 134  \( \text{MAT}^\alpha 3HA.bfa1\Delta NT300::LEU2\ bfa1\Delta::KAN^R \) 
  ura3 leu2 trp1 his7

SLY 135  \( \text{MAT}^\alpha \text{TEm1.6MYC::TRP1 3HA.BFA1 ura3 leu2 trp1 ade5} \) 

SLY 136  \( \text{MAT}^\alpha \text{le1}\Delta::\text{KAN}^R\ \text{bub2}\Delta::\text{LEU2 rsi1}\Delta::\text{HIS7} \) 
  prsi1-8 ura3 leu2 trp1 his7

SLY 145  \( \text{MAT}^\alpha 3HA.bfa1-6\Delta::\text{LEU2 bfa1}\Delta::\text{KAN}^R\ \text{ura3 leu2 trp1 his7} \) 

SLY 146  \( \text{MAT}^\alpha 3HA.DBF2\ \text{bub2}\Delta::\text{LEU2 rsi1}\Delta::\text{HIS7} \) 
  prsi1-8 ura3 leu2

SL15B-1a  \( \text{MAT}^\alpha 3HA.BFA1\ mps1-1\ \text{ura3 leu2 trp1 ade5} \) 

SL18-10c  \( \text{MAT}^\alpha 3HA.BFA1\ \text{clb5}\Delta::\text{URA3 ura3 leu2 trp1 ade5} \) 

SL19-14d  \( \text{MAT}^\alpha \text{BUB2.13MYC::KAN}^R\ \text{bfa1}\Delta::\text{KAN ura3 leu2 trp1 ade5} \) 

SL20-4d  \( \text{MAT}^\alpha 3HA.BFA1\ cdc28-4\ \text{ura3 leu2 trp1 his7} \) 

SL29-1a  \( \text{MAT}^\alpha 3HA.BFA1\ \text{clb1}\Delta::\text{URA3 ura3 leu2 trp1 his3} \) 

SL31-10b  \( \text{MAT}^\alpha 3HA.BFA1\ mps1-1\ \text{rsi1}\Delta::\text{HIS7 prsi1-8} \) 
  ura3 leu2 trp1

SL35-8d  \( \text{MAT}^\alpha 3HA.BFA1\ mad2\Delta::\text{LEU2 ura3 leu2 trp1 ade5} \) 

SL36-4a  \( \text{MAT}^\alpha 3HA.BFA1\ cdc28-4\ \text{ura3 leu2 trp1 his1} \) 

SL36-1a  \( \text{MAT}^\alpha 3HA.BFA1\ cdc28-4\ \text{ura3 leu2 trp1} \) 

SL38-8a  \( \text{MAT}^\alpha 3HA.BFA1\ cdc28-4\ \text{clb1}\Delta::\text{URA3 ura3 leu2 trp1 his1} \) 

SL39-4c  \( \text{MAT}^\alpha 3HA.BFA1\ cdc28-4\ \text{clb2}\Delta::\text{LEU2 ura3 leu2 trp1} \)
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<thead>
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<th>Reference</th>
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<td>SL36-1a xCD116-8d</td>
</tr>
<tr>
<td>SL41-9d</td>
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<td>SL36-1a xK3353-7b</td>
</tr>
<tr>
<td>YD11-97</td>
<td>MATa CDC5.3HA::URA3 ura3 leu2 trp1 his3 ade2</td>
<td>D. Fesquet</td>
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<td>YDF20</td>
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<td>YDF30</td>
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<td>YLF25</td>
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<td>11C</td>
<td>MATα lel1Δ::KAN® ura3 leu2 trp1 adel his1 arg4</td>
<td>S. Jensen</td>
</tr>
</tbody>
</table>

* prsi1-8 is a CEN-based plasmid containing TRP1 and a temperature-sensitive mutation in RSII/APC2.

† msd2-l is a temperature-sensitive cdc5 allele used throughout this study.
For selective growth of kanamycin-resistant strains, 200μg/ml G-418 Geneticin sulphate [Life Technologies] was added to YEPD agar.

To induce gene expression from the GAL1-10 galactose-inducible promoter, cells were grown in medium containing 2% (w/v) raffinose instead of glucose as the carbon source, and gene expression was induced by addition of 2% (w/v) galactose.

Cell cycle arrests were performed using 5μg/ml synthetic alpha factor (α-factor) [Polypeptide synthesis lab, NIMR], 0.1M hydroxyurea [Sigma] or 15μg/ml nocodazole [Sigma].

In the short-term, yeast strains were stored on YEPD or minimal agar plates at 4°C. For longer-term storage, freshly grown cells were removed from agar plates, inoculated into 1ml of storage medium (1% (w/v) yeast extract, 1% (w/v) bacto-peptone, 2% (w/v) glucose, 25% (w/v) glycerol) and stored at -70°C.

### 2.2.3 Determination of cell numbers

The cell density of yeast cultures was determined using a Casyl (model TT) particle counter [Scharfe System], according to the manufacturer's instructions. A 50μl culture sample was diluted with 10ml Casyton [Scharfe System] and sonicated using a Status 200 sonicator [Philip Harris Scientific] at 20% for 3s, to separate the cells before counting.

### 2.2.4 Growth synchronisation methods

To obtain synchronous cultures of CG378-based strains, cells were grown to mid-log phase (2-4x10^6 cells) in YEPD and arrested for 3h using 3.5μg/ml α-factor [Polypeptide synthesis lab, NIMR] unless otherwise stated. To release them from the G1/S block, the cells were filtered, washed once with saline, and resuspended in fresh YEPD. Cell cycle progression was assessed by determining the budding index (the percentage of budded cells), with %
budded cells (number of budded cells/ total number of cells) plotted as a function of time (min).

2.2.5 Re-budding assay

Spindle assembly checkpoint functionality was assessed using the re-budding assay. Cells were grown to mid-log phase (2-4x10^6 cells/ml) in YEPD and the microtubule-depolymerising agent nocodazole was added, to give a final concentration of 15\(\mu\)g/ml. Samples taken at time 0h, 1h, 2h, 3h were sonicated (2.2.3) to separate the cells, and the proportion of re-budded cells (single cells with 2 or more buds) assessed microscopically. % re-budded cells (number of re-budded cells/ total cells) was plotted as a function of time.

2.2.6 Yeast transformations

*S. cerevisiae* transformations were performed using a lithium acetate protocol (Schiestl and Gietz, 1989). To prepare competent cells, a 50ml culture was grown to mid-log phase (4x10^6-1x10^7 cells/ml), harvested (3000rpm, 1 min) and washed once with 10ml 1xTE buffer (10mM Tris-HCl, 1mM EDTA pH 7.4). The cells were harvested and resuspended at 10^8 cells/ml in freshly prepared LT buffer (1mM lithium acetate, 10mM Tris-HCl, 1mM EDTA pH 7.4). 100\(\mu\)l competent cells containing 1-10\(\mu\)g transforming DNA and 50\(\mu\)g single stranded carrier DNA, were mixed with 600\(\mu\)l LTP buffer (1mM lithium acetate, 10mM Tris-HCl, 1mM EDTA, 40% (w/v) PEG\(_{4000}\) pH 7.4). The cells were incubated at 30°C for 30 min, before being subjected to a 15 min heat shock at 42°C. Cells were harvested (13,000rpm, 5s), resuspended in 1xTE and plated directly onto selective minimal agar plates.

2.2.7 Isolation of yeast genomic DNA

To prepare yeast genomic DNA for use as a template in polymerase chain reactions (2.3.2), cells were harvested from 2ml stationary phase yeast culture (13,000rpm, 1 min), washed with 500\(\mu\)l dH\(_2\)O, harvested as before and resuspended in 200\(\mu\)l STET buffer (2% (v/v)
Triton X-100, 1% (w/v) SDS, 10mM NaCl, 10mM Tris-HCl, 1mM EDTA pH 8.0). 200μl phenol-chloroform isoamyl alcohol (25:24:1) [Gibco BRL] was added, followed by 1 volume of 0.5mm glass beads, and the cells were vortexed for 3-4 min. 200μl TE was added to the lysate, and the mixture was centrifuged (13,000rpm, 5 min). The upper, aqueous layer was transferred to 1ml 100% ethanol (AnalAR) [BDH], to precipitate the DNA, which was subsequently pelleted (13,000rpm, 2 min). The pellet was resuspended in 400μl TE containing 30μg RNase A, and incubated at 37°C for 5 minutes. The genomic DNA was precipitated by addition of 0.025 volumes 4M ammonium acetate and 2.5 volumes 100% ethanol, pelleted (13,000rpm, 2 min), and resuspended in 50μl TE.

2.3 DNA manipulation

2.3.1 Restriction endonucleases and DNA modifying enzymes

DNA was incubated with restriction endonucleases (as indicated) [New England Biolabs] in the appropriate restriction endonuclease buffer at 37°C for 90 min. Following restriction endonuclease digestion, plasmid vector DNA was incubated with calf alkaline phosphatase [Boehringer Mannheim] at 37°C for 30min. Taq polymerase [ABgene], Expand polymerase [Roche], and T4 DNA ligase [New England Biolabs] were used according to the manufacturer's instructions.

2.3.2 Polymerase chain reaction (PCR)

Polymerase chain reactions were routinely carried out using an OMN-E PCR thermal cycler [Hybaid]. Gene amplifications for cloning or PCR integration techniques were performed using the Expand High Fidelity system [Roche], in accordance with the manufacturer's instructions. Diagnostic colony PCR was performed using Taq polymerase [ABgene], in a 25μl reaction mixture containing 1xTaq polymerase reaction buffer [ABgene], 1.5mM MgCl₂ [ABgene], 0.5μM 3’ and 5’ oligonucleotides, 250μM dNTPs, 2.5u Taq polymerase and a 2mm² yeast colony. The colony PCR cycle program was 92°C (2 min) followed by 30 cycles of 92°C (30s), 52°C (30s), 72°C (2 min).
Oligonucleotides (Table 2.2) were ordered from Genosys.

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<tr>
<td>3TEM1SMA1</td>
<td>TCCCCCGGAGGGAGTACGGCGGGGTATAGTTG</td>
</tr>
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</table>

The restriction sites are underlined.
2.3.3 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in 1% (w/v) agarose gels with TAE electrophoresis buffer (40mM Tris base, 20mM glacial acetic acid, 1mM EDTA pH 8.3). DNA was loaded with 1/5th volume 6xDNA loading buffer (120mM EDTA, 40% (w/v) sucrose, 0.25% (w/v) bromophenol blue) and electrophoresed with a constant current of 60-100mA. DNA was stained using 0.05% (w/v) ethidium bromide [Life Technologies], which was added directly to the molten agarose, and visualised under short wave ultra-violet using a Uniscience TF-20M transilluminator. The molecular size of DNA fragments was determined by comparison to DNA size markers [New England Biolabs].

2.3.4 Recovery of DNA fragments from agarose gels

DNA fragments were extracted from TAE agarose gels using a BIO 101 Geneclean II glass milk extraction kit [Anachem] according to the manufacturer's instructions.

2.3.5 DNA ligations

Ligation of DNA fragments was carried out using T4 DNA ligase [New England Biolabs] in a 10μl reaction mixture containing 1x T4 DNA ligase buffer (50mM Tris-HCl pH 7.5, 10mM MgCl₂, 10mM DTT, 1mM ATP, 25μg/ml bovine serum albumin) [New England Biolabs], and 0.4U T4 DNA ligase. Ligations were incubated at room temperature for 2h or overnight at 16°C before transformation into E. coli (2.1.3).

2.3.6 Preparation of plasmid DNA

Small scale plasmid preparations from transformed E. coli cells were performed using an alkaline lysis protocol (Birnboim and Doly, 1979). DH5α cells containing plasmid DNA were grown overnight at 37°C in 4ml 2xYT containing 100μg/ml ampicillin. Cells were harvested (13,000rpm, 1 min) and resuspended in 200μl GTE buffer (50mM glucose, 25mM Tris-HCl, 10mM EDTA pH 8.0). 300μl of freshly prepared NS solution (0.2N
NaOH, 1% (w/v) SDS) was added, the solution was mixed by inversion and incubated on ice for 3 min. The solution was neutralised by adding 300μl of 3M potassium acetate (pH 4.8), and incubated on ice for a further 3 min. The precipitated SDS-protein and cell debris was pelleted (13,000rpm, 10 min) and the supernatant was transferred to a fresh tube containing 400μl chloroform. The mixture was vortexted for 30s, centrifuged (13,000rpm, 1 min) and the upper (aqueous) layer was transferred to one volume of propan-2-ol (isopropanol). Plasmid DNA was pelleted (13,000rpm, 10 min), washed with 500μl 70% ethanol, dried and resuspended in 50μl sterile dH₂O.

2.3.7 Precipitation of PCR DNA

PCR DNA for yeast transformation was precipitated from aqueous solution by adding 3M sodium acetate to a final concentration of 0.3M, followed by three volumes of 100% ethanol. The sample was cooled to -20°C for a minimum of 1h, and the precipitated DNA pelleted by centrifugation (13,000rpm, 10 min). The pellet was washed with 500μl 70% ethanol, dried, and resuspended in 10μl dH₂O.

2.4 Plasmid Construction

The plasmids used in this study are summarised in Table 2.3. Details of plasmids constructed in this study are given in sections 2.4.1-2.4.9.

2.4.1 pGAL-6His.TEM1

The wild-type TEM1 gene was amplified from genomic DNA using the 5’ oligonucleotide 5STEM1BAMH1 and the 3’ oligonucleotide 3TEM1SMA1 (Table 2.2). The resultant PCR fragment was ligated into the BamH1-Smal sites of pEMBLyex4 to generate pSL3 containing 6His-tagged Tem1p under the control of the GAL inducible promoter.
Table 2.3

Plasmids used in this study

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<tr>
<th>Plasmid name</th>
<th>Features</th>
<th>Reference/Source</th>
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<td><strong>i) E.coli</strong></td>
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<tr>
<td>pGEX-KG</td>
<td>Vector for IPTG-inducible GST-fusion protein expression. Ptac promoter, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pSL5</td>
<td>pGEX-KG containing wild-type BFA1</td>
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<td>pGEX-KG containing bfa1ΔNT270</td>
<td>This study</td>
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<td>pSM2</td>
<td>pGEX-KG containing bfa1ΔNT300</td>
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<tr>
<td>pSL15</td>
<td>pGEX-KG containing bfa1ΔCT270</td>
<td>This study</td>
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<tr>
<td>pSL16</td>
<td>pGEX-KG containing bfa1ΔCT300</td>
<td>This study</td>
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<tr>
<td><strong>ii) S.cerevisiae</strong></td>
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</tr>
<tr>
<td>pEMBLyex4</td>
<td>Vector for galactose-inducible expression of 6His-tagged genes; URA3, 2µ,</td>
<td>Lab stock</td>
</tr>
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<td>Multi-copy cloning vector; URA3; 2µ</td>
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<td>Jaspersen et al. (1998)</td>
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<td>pEMBLyex4 containing wild-type TEM1</td>
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<td>pYIplac128</td>
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</table>
2.4.2 pGAL-6His.CDC5

The wild-type CDC5 gene was amplified from genomic DNA using the 5' oligonucleotide 5CDC5BAMH1 and the 3' oligonucleotide 3CDC5SMA1 (Table 2.2). The resultant PCR fragment was ligated into the BamHI-SmaI sites of pEMBLyex4 to generate pSL20 containing 6His-tagged Cdc5 under the control of the GAL inducible promoter.

2.4.3 pGST.BFA1

The wild-type BFA1 gene was amplified from genomic DNA using the 5' oligonucleotide 5BFA1BAMH1 and the 3' oligonucleotide 3BFA1XBA1 (Table 2.2). The resultant PCR fragment was ligated into the BamHI-XbaI sites of pGEX-KG to generate pSL5 containing GST-tagged Bfa1 under the control of an IPTG-inducible promoter.

2.4.4 pGST.BFA1ΔNT270/300

Two BFA1 C-terminal fragments were amplified from genomic DNA using the 5' oligonucleotides 5BFA1ΔNT270BAMH1 and 5BFA1ΔNT300BAMH1 respectively, and the 3' oligonucleotide 3BFA1XBA1 (Table 2.2). The resultant PCR fragments were ligated into the BamHI-XbaI sites of pGEX-KG to generate pSM1 and pSM2, containing GST-tagged Bfa1 ΔNT270 and Bfa1 ΔNT300 respectively, under the control of an IPTG-inducible promoter.

2.4.5 pGST.BFA1ΔCT270/300

Two BFA1 N-terminal fragments were amplified from pSL5 plasmid DNA using the 5' oligonucleotide 5BFA1BAMH1 and the 3' oligonucleotides 3BFA1ΔCT270XBA1 and 3BFA1ΔCT300XBA1 respectively (Table 2.2). The resultant PCR fragments were ligated into the BamHI-XbaI sites of pGEX-KG to generate pSL15 and pSL16, containing GST-
tagged Bfa1 ΔCT270 and Bfa1 ΔCT300 respectively, under the control of an IPTG-inducible promoter.

2.4.6 YIplac128-3HA.BFA1

To construct an integrating plasmid containing 3HA.BFA1 under the control of its own promoter, the 3HA.BFA1 gene was amplified from SLY105 (3HA.BFA1) genomic DNA, using the 5' oligonucleotide 5BFA1-400PST1 and the 3' oligonucleotide 3BFA1XBA1 (Table 2.2). The resultant PCR fragment was ligated into the PstI-XbaI sites of YIplac128 to generate pSL10 containing 3HA.BFA1 and 400 bp of upstream sequence. This upstream sequence is sufficient for BFA1 expression, since the construct is fully functional (8.2.2), and the protein is detectable in immuno-blots (7.2.6).

2.4.7 YIplac128-3HA.bfa1ΔNT270/300

To construct integrating plasmids expressing two different 3HA-tagged C-terminal fragments of Bfa1, under the control of the Bfa1 promoter, plasmid pSL10 (YIplac128-3HA.BFA1) was digested with XhoI (which cuts between the 3HA epitope tag, and the tagged gene) and XbaI, to remove the entire BFA1 gene whilst retaining the BFA1 promoter sequence and 3HA tag sequence. Two BFA1 C-terminal fragments corresponding to those in 2.4.4 were amplified from genomic DNA using the 5' oligonucleotides 5BFA1ΔNT270XHO1 and 5BFA1ΔNT300XHO1 respectively, and the 3' oligonucleotide 3BFA1XBA1 (Table 2.2). The resultant PCR fragments were ligated into the XhoI-XbaI sites of the vector, to generate pSL13 and pSL14, containing 3HA-tagged Bfa1 ΔNT270 and Bfa1 ΔNT300 respectively, under the control of their own promoter.

2.4.8 YIplac128-3HA.bfa1ΔCT270/300

To construct integrating plasmids expressing two different 3HA-tagged Bfa1 N-terminal fragments under the control of the BFA1 promoter, two BFA1 N-terminal fragments corresponding to those in 2.4.4, were amplified from SLY105 genomic using the 5'
oligonucleotide 5BFA1-400PST1 and the 3' oligonucleotides 3BFA1ΔCT270XBA1 and 3BFA1ΔCT300XBA1 respectively (Table 2.2). The resultant PCR fragments were ligated into the PstI-XbaI sites of YIplacl28, to generate pSL11 and pSL12, containing 3HA-tagged Bfa1 ΔCT270 and Bfa1 ΔCT300 respectively, under the control of their own promoter.

2.4.9 Bfa1 site-directed mutagenesis to construct YIplacl28-3HA.bfa1-6A

The method used to construct an integrating plasmid, expressing the Bfa1-6A mutant protein (in which the six putative Cdc28 phosphorylation sites [SP or TP in single letter amino acid code] have been mutated to AP) is shown in Figure 2.1. Plasmid pSL10 (YIplacl28-3HA.BFA1) was digested with Xhol and XbaI, to remove the entire BFA1 gene, which was then inserted into the Xhol-XbaI sites of pRS315, to generate plasmid pSL17 (pRS315-BFA1). PCR mutagenesis (Brons-Poulsen et al., 1998) was then used to mutate S274, T288, T340, S454, T465 and T500 to alanine (see Figure 2.1 for mutagenesis details). The bfa1-6A mutant gene was then inserted into the Xhol-XbaI sites of pSL10 (YIplac128-3HA.BFA1; see 2.4.6, 2.4.7), to generate plasmid pSL21 (YIplac128-3HA.bfa1-6A) (Figure 2.1). Successful mutagenesis was confirmed by restriction mapping.

2.5 Strain construction

2.5.1 Tetrad dissection

Diploids were incubated on potassium sporulation medium (KSM) (1% (w/v) potassium acetate, 2% (w/v) Difco agar) at 25°C or 30°C for 3 to 5 days to allow sporulation to occur. Tetrads were digested with 5% glucylase for 30 minutes at room temperature, and dissected using a Singer Micromanipulator [Amersham].
Figure 2.1

Bfa1 site-directed mutagenesis protocol used to construct YIplac128-3HA.bfa1-6A. A fragment of the BFA1 ORF was amplified from genomic DNA using the oligonucleotides 5BFA1SDM3 and 3BFA1SDM4 (Table 2.2), which introduced mutations into the codons for S454 and T465, and T500 (step 1). The primers also introduced mutations into restriction sites flanking these codons, so that successful mutagenesis could be confirmed by restriction mapping. The resultant DNA fragment (SDM-A) was purified from a TAE agarose gel using a BIO 101 Geneclean II glass milk extraction kit [Anachem], and used as a primer in a second PCR reaction with the 3' oligonucleotide 3BFA1XBA1 (Table 2.2), generating SDM-B (step 2). SDM-B was purified and used as a primer in a third PCR reaction with the 5' oligonucleotide 5BFA1SDM5 (Table 2.2), generating SDM-C (step 3). SDM-C contains the three mutated phosphorylation sites and has a 5'HindIII restriction site and a 3' XbaI restriction site. Plasmid pSL17 (pRS315-BFA1) was digested with HindIII and XbaI to remove the corresponding fragment from the wild-type gene and SDM-C was ligated into the HindIII-XbaI sites of the vector, generating pSL18 (pRS315-bfa1-3A) (step 4). To generate bfa1-6A, a fragment of the BFA1 ORF was amplified from genomic DNA using the oligonucleotides 5BFA1SDM1 and 3BFA1SDM2 (Table 2.2), which introduced mutations into the codons for S274 and T288, and T340 (step 5). The resultant DNA fragment (SDM-D) was isolated and used as a primer in a second PCR reaction with the 5' oligonucleotide 3BFA1SDM1 (Table 2.2), generating SDM-E (step 6). SDM-E contains the three mutated phosphorylation sites and has a 5'BsgI restriction site and a 3' HindIII restriction site. pSL18 (pRS315-bfa1-3A) was digested with BsgI and HindIII to remove the corresponding wild-type fragment from the bfa1-3A gene, and SDM-E was ligated into the BsgI-HindIII sites of the vector, to generate pSL19 (pRS315-bfa1-6A) (step 7). Finally, pSL19 (pRS315-bfa1-6A) was digested with XhoI and XbaI, to remove the bfa1-6A mutant gene, which was inserted into the XhoI-XbaI sites of pSL10 (YIplac128-3HA.BFA1), to generate pSL21 (YIplac128-3HA.bfa1-6A) (step 8). Restriction mapping confirmed that the mutagenesis had been successful.
2.5.2 Plasmid integration

To construct strains SLY125 (CDC5.3HA apc2-8), SLY126 (CDC5.3HA apc2-8 tem1-3), SLY127 (CDC5.3HA apc2-8 cdc15-1), SLY128 (CDC5.3HA apc2-8 mob1-77) and SLY129 (CDC5.3HA apc2-8 tem1-3), plasmid pRS306-CDC5.3HA (URA3), containing the wild-type CDC5 gene tagged at the C-terminus with a 3HA epitope tag, was digested with Clal and transformed into strains KTM208 (apc2-8), JTAB7-5a (apc2-8 tem1-3), ER1 (apc2-8 cdc15-1), JMA1-21c (apc2-8 mob1-77) and J14AB2-12a (apc2-8 cdc4-l) respectively. Stable integrants were selected on medium lacking uracil.

To construct strain SLY136 (3HA.BFA1 TEM1.6MYC), plasmid pTEM1-6MYC (TEM1.6MYC; TRP1), containing the wild-type TEM1 gene tagged at the C-terminus with a 6MYC epitope tag, was digested with BgIII and transformed into strain SLY105 (3HA.BFA1). Stable integrants were selected on medium lacking tryptophan.

To construct strains SLY130 (3HA.BFA1), SLY131 (3HA.bfa1ΔCT270), SLY132 (3HA.bfa1ΔCT300), SLY133 (3HA.bfa1ΔNT270) and SLY134 (3HA.bfa1ΔNT300), plasmids pSL10 (3HA.BFA1), pSL11 (3HA.bfa1ΔCT270), pSL12 (3HA.bfa1ΔCT300), pSL13 (3HA.bfa1ΔNT270) and pSL14 (3HA.bfa1ΔNT300) respectively, were digested with EcoRV and transformed into strain SLY108 (bfa1Δ). Stable integrants were selected on medium lacking leucine.

To construct strain SLY145 (3HA.bfa1-6A), plasmid pSL21 (YIPlac128-3HA.bfa1-6A) was digested with EcoRV and transformed into strain SLY108 (bfa1Δ). Stable integrants were selected on medium lacking leucine.

2.5.3 Deletion of BFA1

The entire BFA1 ORF was deleted from strains YDF20 (DBF2.6MYC apc2-8) and CG379 (wild-type), generating strains SLY103 (DBF2.6MYC apc2-8 bfa1Δ) and SLY108 (bfa1Δ) respectively, using PCR-based gene disruption (Wach et al., 1994). A disruption cassette
containing a marker gene encoding resistance to genetecin sulphate (G-418) was amplified from plasmid pFA6-kanMX4 using the 5’ oligonucleotide 5BFA1KANKO and the 3’ oligonucleotide 3BFA1KANKO (Table 2.2). The PCR product was transformed into strains YDF20 or CG379, and stable integrants were selected on YEPD medium containing 200µg/ml G-418 [Life Technologies]. Integration was confirmed by colony PCR using the 5’ oligonucleotide 5BFA1KANKO and a 3’ oligonucleotide with homology to the kanamycin marker (laboratory stock).

2.5.4 C-terminal tagging of BUB2 with a 13MYC epitope

The genomic BUB2 gene was tagged at the C-terminus with a 13MYC epitope in strains CG378 (wild-type) or SLY105 (3HA.BFA1), generating strains SLY104 (BUB2.13MYC) and SLY106 (BUB2.13MYC 3HA.BFA1) respectively, using a PCR-based gene integration technique (Longtine et al., 1998). Plasmid pFA6a-13myc-kanMX6 was amplified using the 5’ oligonucleotide 5BUB2KANF2 and the 3’ oligonucleotide 3BUB2KANR1 (Table 2.2). The PCR product was transformed into strains CG378 or SLY105 and stable integrants were selected on YEPD medium containing 200µg/ml G-418. Integration was confirmed by colony PCR using the 5’ oligonucleotide 5BUB2KANF2 and the 3’ oligonucleotide 3BUB2KANR1 (Table 2.2). Re-budding assays confirmed that the spindle assembly checkpoint was fully functional in both strains, indicating that the Bub2-13MYC protein is functional (Figures 2.2 and 2.3).

2.5.5 N-terminal tagging of BFA1 with a 3HA epitope

The genomic BFA1 gene was tagged at the N-terminus with a 3HA epitope in strain CG378 (wild-type), generating strain SLY105, using a PCR-based gene integration technique (Schneider et al., 1995). Plasmid pMPY3xHA was amplified using the 5’ oligonucleotide 5BFAlPETup and the 3’ oligonucleotide 3BFAlPETdwn. The PCR product was transformed into CG378 and stable integrants selected on medium lacking uracil. Correct integration of the 3HA.URA3 cassette was confirmed by colony PCR using the 5’ oligonucleotide 5BFA1-200 and the 3’ oligonucleotide 3BFA1PETdwn (Table 2.2).
Figure 2.2  **Bub2-13MYC is fully functional.** Strain SLY104 (*BUB2.13MYC*) does not re-bud in nocodazole, indicating that the spindle assembly checkpoint is active. Compare with the wild-type (CG378) and *bub2Δ* strains.
Figure 2.3 Combining 3HA-Bfa1 and Bub2-13MYC does not affect functionality. Strain SLY106 (BUB2.13MYC 3HA.BFA1) does not re-bud in nocodazole, indicating that the spindle assembly checkpoint is active. Compare with the wild-type (CG378) and bub2Δ strains.
Figure 2.4  **3HA.Bfa1 is fully functional.** Strain SLY 105 (3HA.BFA1) does not re-bud in nocodazole, indicating that the spindle assembly checkpoint is active. Compare with the wild-type (CG378) and *bub2Δ* strains.
Integrants were streaked onto medium containing 5-Fluoroorotic acid (5-FOA) to generate 3HA.BFA1. Excision of the URA3 gene was confirmed using colony PCR as above. A re-budding assay confirmed that the spindle assembly checkpoint was fully functional in the tagged strain, indicating that the 3HA-Bfa1 protein is functional (Figure 2.4).

2.6 General Protein Techniques

2.6.1 SDS-polyacrylamide gel electrophoresis

Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The resolving gel (8%, 10% or 12% (w/v) acrylamide, 0.02% (w/v) SDS, 0.375M Tris-HCl pH 8.8, polymerised with 0.05% (w/v) APS and 0.05% (v/v) TEMED) was overlaid with stacking gel (5% w/v) acrylamide, 0.02% (w/v) SDS, 0.125M Tris pH 6.8, polymerised as before). 50µg-70µg protein extract mixed with twice the volume of Laemmli buffer (10% (w/v) glycerol, 2% (w/v) SDS, 2% (v/v) 2 β-mercaptoethanol, 0.002% (w/v) Bromophenol blue, 67.5mM Tris-HCl pH 6.8) was boiled (3 min) and loaded onto the gel. Electrophoresis was performed at either 35mA (7 x 9cm gels) or 50 mA (12 x 14cm gels) in running buffer (192mM glycine, 0.1% (w/v) SDS, 25mM Tris pH 8.2). Proteins on gels were detected using immuno-blotting, or with Coomassie Brilliant Blue (Fairbanks et al., 1971). 32P-labelled proteins were detected by developing autoradiographs from dried gels.

2.6.2 Immuno-blot (Western-blot) analysis

50µg of protein extract in Laemmli buffer was resolved using SDS-PAGE and transferred to nitrocellulose membrane [Protran] using a semi-dry transfer cell [Bio-Rad] at 1.3mA/cm². The membrane was incubated in blocking buffer (phospho-buffered saline [PBS] containing 0.05% (v/v) Tween-20, and 5% (w/v) Marvel) to block non-specific binding and then probed with primary antibodies (Table 2.4) at the appropriate dilution in blocking buffer containing 2% Marvel. The membrane was probed with the appropriate secondary antibody (anti-mouse or rabbit) conjugated to horse-radish peroxidase [Sigma],
diluted 1:10000 in blocking buffer containing 2% Marvel. Proteins were detected using chemiluminescence detection (ECL) [Amersham].

Immuno-blots were scanned using an ImageScanner with ImageMasterLabscan 3.0 software [Amersham] and band intensity was quantitated using the ImageMaster 1D Elite 3.01 software package [Amersham].

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Information</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>α- MYC (9E10)</td>
<td>mouse monoclonal, use 1:1000</td>
<td>NIMR, London</td>
</tr>
<tr>
<td>α -HA (12CA5)</td>
<td>mouse monoclonal, use 1:1000</td>
<td>NIMR, London</td>
</tr>
<tr>
<td>α -RGSHis</td>
<td>mouse monoclonal, use 1:1000</td>
<td>Qiagen</td>
</tr>
<tr>
<td>α -Dbf2</td>
<td>rabbit polyclonal, use 1:1000</td>
<td>NIMR, London</td>
</tr>
<tr>
<td>α -Clb2</td>
<td>rabbit polyclonal, use 1:3000</td>
<td>Lab stock</td>
</tr>
<tr>
<td>α -GST</td>
<td>rabbit polyclonal, use 1:20,000</td>
<td>Lab stock</td>
</tr>
</tbody>
</table>

Table 2.4

Antibodies used in this study
2.6.3 Autoradiography

Dried SDS-PAGE gels of $^{32}$P-labelled proteins were exposed to X-ray film [Kodak] in autoradiographic cassettes. Films were developed using an X-O-Graph X-ray processor. Autoradiographs were also scanned using a Storm 860 Phosphorimager, and band intensity quantitated (by integration of density over area) with the ImageQuant 5.0 software package.

5.3.1 Preparation of yeast crude extracts

Strains were grown to mid-log phase (4-5 x $10^6$ cells/ml), cells were harvested, washed twice with saline (0.9% (w/v) NaCl) and frozen on dry ice. Cell pellets (10$^7$ cells) were resuspended in 50μl lysis buffer containing 50mM Tris-HCl pH 7.5, 150mM NaCl, 15mM MgCl$_2$, 5mM EDTA, 1% (v/v) NP40, 10mM sodium fluoride, 50mM β-glycerophosphate, 1mM DTT, 1mM sodium vanadate, 1mM pNPP, 1mM glucose-1-phosphate, 10μg/ml each protease inhibitors (leupeptin, pepstatin, chymotrypsin, aprotinin, antipain) and 1mM PMSF. 10 volumes of detergent-washed glass beads were added and cells were lysed in a ribolyser [Hybaid] at 4m/s$^2$ for 8s. The lysate was separated from the beads by centrifugation, collected, and protein concentration determined using the Bradford protein assay [Bio-Rad] according to the manufacturers instructions. For co-immunoprecipitation experiments, extracts were prepared as described above but with the lysis buffer modified to contain 0.2% (v/v) NP40.

2.6.5 Co-immunoprecipitation

Protein A/G beads were washed with lysis buffer (no protease inhibitors) and incubated in 10mg/ml bovine serum albumin (BSA) [Sigma] to block non-specific binding. Beads were then washed three times with lysis buffer (no protease inhibitors) and resuspended as a 50% slurry in lysis buffer.

500μg of protein extract (2.6.4) in 100μl lysis buffer was immunoprecipitated with 2μg of anti-MYC (9E10) or anti-HA (12CA5) antibody, on a rotating wheel for 1h at 4°C. 15μl
Protein A/G slurry was added and incubated on a rotating wheel for 30 min at 4°C. Protein A/G beads-immune complex were washed three times with lysis buffer, once with 50mM Tris-HCl pH 7.5, excess liquid was removed using a drawn-out pasteur pipette, and co-precipitating proteins were released by boiling in Laemmli buffer (3 min). The supernatant was processed for immuno-blotting as described above.

2.6.6 Purification of GST-Bfa1 fusion proteins

Strain BL21(DE3) was transformed with plasmids pSL5 (GST.BFA1), pSM1 (GST.bfa1ΔNT270), pSM2 (GST.bfa1ΔNT300), pSL15 (GST.bfa1ΔCT270) or pSL16 (GST.bfa1ΔCT300) and grown overnight in 5ml LB broth containing 100µg/ml ampicillin. 500ml fresh LB broth containing 100µg/ml ampicillin was inoculated 1:100 with the overnight culture and grown at 37°C with continuous shaking to an OD500 of 0.5-0.6. Expression of the GST fusion protein was induced by addition of 0.4mM IPTG and the culture was grown at 30°C with continuous shaking for a further 2h. The cells were harvested using a Beckman centrifuge (5,000rpm, 10 min), washed in 100ml saline (0.9% (w/v) NaCl), and harvested again. The pellet was frozen on dry ice and stored at -70°C.

The pellet was resuspended in 20ml ice-cold NETN buffer (500mM NaCl, 1mM EDTA, 0.5% (v/v) NP40, 20mM Tris-HCl pH 8.0) containing protease inhibitors (10µg/ml leupeptin, pepstatin, chymotrypsin, aprotinin, antipain, and 1mM PMSF), and sonicated on ice using a Status 200 sonicator [Philip Harris Scientific] (60%, 3x10s). The lysate was spun in a Beckman centrifuge (10,000rpm, 10 min) to remove cell debris, and the supernatant transferred to a cold Falcon tube. 600µl glutathione sepharose beads (50% (v/v) slurry) [Amersham] were washed three times in NETN buffer and resuspended in NETN buffer as a 50% (v/v) slurry. 500µl glutathione sepharose beads were added to the lysate and incubated on a rotating wheel at 4°C for 1h. The beads-GST fusion protein complex was washed three times in NETN, resuspended in 200 µl elution buffer (NETN containing protease inhibitors, 10% (w/v) glycerol, and 10mM reduced glutathione), and incubated on a rotating wheel at 4°C for 1h. The eluted GST fusion protein was transferred to a fresh tube and protein concentration determined using the Bradford assay [Bio-Rad] according to
the manufacturer's instructions. GST-Bfa1 fusion proteins for use as in vitro kinase substrates were prepared fresh each time, since fusion proteins stored at -70°C were poor kinase substrates.

2.7     Kinase assays

2.7.1     Dbf2 Histone H1 kinase assays

Dbf2 was immunoprecipitated from 200µg protein extract as described (2.6.5), using 2µg of anti-MYC, anti-HA or anti-Dbf2 antibody (as indicated), and 15µl Protein A or G slurry. Protein A/G beads-immune complex were washed twice with lysis buffer (no protease inhibitors), twice with kinase buffer (25mM MOPS pH 7.2, 10mM MgCl₂) and excess liquid was removed using a drawn-out pasteur pipette. 10µl kinase buffer containing 0.5mg/ml histone H1 [Boehringer], 50mM ATP and 0.1µl [γ³²P]ATP [Amersham] was added and the reaction was incubated for 30 min at room temperature. The reaction was stopped by adding one volume of Laemmli buffer. Phosphorylated histone H1 was analysed using SDS-PAGE and autoradiography (2.6.1; 2.6.3).

2.7.2     Cdc5 and Cdc28 GST-Bfa1 kinase assays

Cdc5-3HA or Cdc28-HA was immunoprecipitated from 200µg protein extract as described (2.6.5), using 2µg of anti-HA antibody and 15µl Protein A slurry. Protein A beads-immune complex were washed twice with lysis buffer (no protease inhibitors), twice with kinase buffer, and excess liquid was removed using a drawn-out pasteur pipette. 10µl kinase buffer containing 0.5mg/ml GST-Bfa1, GST-Bfa1ACT270, GST-Bfa1ACT300, GST-Bfa1ANT270 or GST-Bfa1ANT300, 50mM ATP and 0.1µl [γ³²P]ATP was added and the reaction was incubated for 30 min at room temperature. The reaction was stopped by adding one volume of Laemmli buffer. Phosphorylated GST-Bfa1 was analysed using SDS-PAGE and autoradiography (2.6.1; 2.6.3).
2.8 Fluorescence microscopy

2.8.1 Observation of Dbf2-GFP or Tubulin-GFP-expressing cells

Cells containing integrated DBF2.GFP or TUB1.GFP were grown to mid-log phase overnight in YEPD at 23°C and washed in minimal medium prior to observation using a Photometrics CH350L liquid-cooled CCD camera, on an Olympus IX70 inverted microscope, with a 100x magnification, U-PLAN-APO 1.35 NA, oil objective lens. Images were captured and manipulated using SoftWoRx software [Applied precision Inc., Issaquah, WA], and Adobe PhotoShop version 5.5 [Adobe Systems Incorp., Mountain View, CA, USA]. Images were taken of a single focal plane, and of cells where it was possible to see all relevant fluorescence in that plane. Exposure times varied and were dependent upon the intensity of the observed fluorescence.

2.8.2 Fluorescent staining of yeast cells

For staining of actin with rhodamine-conjugated phalloidin, cells were grown to mid-log phase and fixed by adding formaldehyde directly to the medium, to a final concentration of 4%. After 1h, cells were washed with PBS containing 1mg/ml BSA, and then resuspended in PBS-BSA containing 30U/ml rhodamine-phalloidin [Molecular Probes] for 30 minutes. The cells were then washed three times in PBS-BSA, resuspended in Vectashield Mounting Medium containing 1/5mg/ml DAPI [Vector Laboratories, Inc.] and visualised.
Chapter 3

Order of function of the Mitotic Exit Network

3.1 Introduction

Exit from mitosis requires the inactivation of mitotic cyclin-dependent kinase and is
mediated by the anaphase-promoting complex (APC/C\(^{Cdh1}\)), and the CDK inhibitor Sic1.
Both Sic1 and APC/C\(^{Cdh1}\) are activated in telophase by the Cdc14 phosphatase, which is a
component of the mitotic exit network (MEN). Other MEN components include the protein
kinases Dbf2, Cdc15 and Cdc5, the G-protein Tem1, and Mob1, a protein of unknown
function. Despite much research, the regulation and order of function of the MEN
components is still poorly understood. To gain insight into MEN control, the regulation of
the kinase Dbf2, and the order in which its upstream activators function has been
investigated here.

Dbf2 is a cell cycle-regulated kinase, which is activated during mitosis. Consistent with
this, Fesquet et al., (1999) showed that cells arrested in G1 using \(\alpha\)-factor contain
negligible Dbf2 kinase activity, whilst cells arrested in metaphase using an \(apc2-8\)
temperature-sensitive mutation, contain moderately high levels of Dbf2 kinase activity.
This metaphase activity is down-regulated following spindle damage induced by the
microtubule depolymerising agent nocodazole, indicating that Dbf2 is a target of the
spindle assembly checkpoint (SAC). The inhibition depends upon Bub2, a component of
the putative Tem1 two-component GAP (Fesquet et al., 1999). In cells deleted for \(BUB2\),
Dbf2 kinase activity is no longer inhibited by nocodazole, and is present at levels even
higher than in \(apc2-8 BUB2\) cells (Fesquet et al., 1999). We believe that deletion of \(BUB2\)
allows cell cycle progression to a point where Dbf2 kinase is fully active, irrespective of
spindle assembly checkpoint function. These assay conditions provide a convenient way to
assess which components of the MEN are required for Dbf2 kinase activity. Not only do the cells exhibit maximal levels of Dbf2 kinase activity, but they are held at a uniform arrest point, ensuring that any changes in Dbf2 kinase levels are not due to further cell cycle progression (Toyn and Johnston, 1994).

3.2 Results

3.2.1 Dbf2 kinase activity is dependent upon the MEN

To determine whether any other MEN components were required for Dbf2 kinase activation, Dbf2 kinase levels were assessed in strains containing mutations in a number of MEN genes. Strains were constructed containing temperature-sensitive mutations in TEM1 (tem1-3), CDC15 (cdcl5-l), MOB1 (mob1-77), CDC5 (msd2-l) and CDC14 (cdcl4-l), in an apc2-8bub2Δ background. Each strain was grown to mid-log phase at 23°C, and then either arrested in G1 using α-factor, or in metaphase by incubating at 37°C, in the presence or absence of nocodazole. Since Dbf2 kinase activity is negligible during G1, the α-factor-arrested cells give a measure of uninduced Dbf2 kinase levels. In contrast, at 37°C, apc2-8bub2Δ cells contain maximal levels of Dbf2 kinase activity. Addition of nocodazole should not affect Dbf2 kinase activation in this strain background, but the extra assay provides a useful internal control. Dbf2 was immuno-precipitated from protein extracts using anti-Dbf2 antibody (Toyn and Johnston, 1994), and Dbf2 kinase assays performed using histone H1 as a substrate (2.7.1). As expected, the apc2-8bub2Δ strain exhibited high levels of Dbf2 kinase activity at 37°C in the presence or absence of nocodazole (Figure 3.1, panel 1). Mutating Cdc14 had no effect on Dbf2 kinase activity (Figure 3.1, panel 6). This is not surprising since it has been previously reported that Dbf2 kinase is required to release Cdc14 from the nucleolus and hence must function upstream of Cdc14 (Visintin et al., 1999). However, in the absence of functional Tem1, Cdc15, Mob1, or Cdc5, Dbf2 kinase activity was completely abolished (Figure 3.1, panels 2, 3, 4 and 5). This suggests that these proteins function upstream of, and are required for, Dbf2 kinase activation.
Figure 3.1 Tem1, Cdc15, Mob1 and Cdc5, but not Cdc14 are required for Dbf2 kinase activity. Strains containing tem1-3 (JTAB7-12d), cdc15-1 (ERN1), mob1-77 (JMAB3-12b), cdc5 (msd2-1) (J5AB3-13d) and cdc14-1 (J14AB2-20b) mutations in an apc2-8 bub2Δ background were grown to mid-log phase at 23°C. The cells were arrested with alpha-factor (α-factor) or with nocodazole at 37°C (Noco@37°C) or at 37°C (37°C) for 3h. Dbf2 was immunoprecipitated from protein extracts using anti-Dbf2 antibody (Toyn and Johnston, 1994), bound to protein A beads, and bead-bound histone H1 kinase activity was evaluated (lower panels), and quantitated by Phosphorimager. An anti-Dbf2 immunoblot was used to estimate Dbf2 protein levels. The ratios of histone H1 kinase activity/Dbf2 protein levels are plotted in the graphs (upper panels).
a-factor
Noco @ 37°C
37°C

Relative Dbf2 kinase activity
apc2-8 bub2Δ

dbf2p Kinase

apc2-8 bub2Δ
tem1

apc2-8 bub2Δ
cdc15

apc2-8 bub2Δ
mab1

apc2-8 bub2Δ
cdc5

apc2-8 bub2Δ
cdc14

H1
3.2.2 Teml acts upstream of Cdc15 to control Dbf2

Having established that Tem1, Cdc15 and Mob1 all function upstream of Dbf2, it seemed feasible to order these pathway components, by exploiting Dbf2 kinase activity. Tem1 is likely to function near the top of the pathway, since Bub2 is thought to negatively regulate the MEN by inhibiting Tem1 (Pereira et al., 2000). If Tem1 is an upstream activator of Dbf2, over-expression of Tem1 should mimic deletion of BUB2, and also lead to maximum activation of Dbf2. Strain NJW015 (3HA.DBF2 apc2-8) transformed with pSL3 (GAL-6His.TEM1; URA3; 2μ) was grown overnight at 23°C in selectable medium containing 2% raffinose before being subcultured in YEP-raffinose and grown to mid-log phase. The culture was split, and 2% galactose added to one half, to induce 6His-Tem1 expression. The cells were then arrested in G1 using α-factor, or in metaphase at 37°C, in the presence or absence of nocodazole, for 3h. Dbf2 was immunoprecipitated from protein extracts using anti-HA antibody, and H1 kinase assays performed. As expected, in the absence of Tem1 expression, Dbf2 kinase activity was down-regulated in the presence of nocodazole (Figure 3.2, panel 1). However, cells over-expressing Tem1 exhibited maximal levels of Dbf2 kinase activity, even in the presence of nocodazole and functional Bub2 (Figure 3.2, panel 2). Thus Tem1 functions upstream of Dbf2 to control its kinase activity. Moreover, these data confirm that high levels of Tem1 overcome the Bub2-dependent inhibition of the MEN (Alexandru et al., 1999).

By analogy with the fission yeast system (Schmidt et al., 1997), Tem1 is likely to act directly upstream of Cdc15. We therefore tested whether Tem1 over-expression could activate Dbf2 in the absence of functional Cdc15. Strain SLY124 (3HA.DBF2 apc2-8 cdc15-1) was transformed with pSL3 (pGAL-6His.TEM1; URA3; 2μ) and cells were cultured as described above. 2% galactose was added to induce 6His-Tem1 expression, and cells were again arrested using α-factor, or at 37°C in the presence or absence of nocodazole, prior to assaying Dbf2 kinase activity. Tem1 over-expression fails to activate Dbf2 kinase activity in the absence of functional Cdc15 (Figure 3.2, panel 3). Thus Tem1 must act upstream of Cdc15 to control Dbf2. In fact Tem1 and Cdc15 physically interact (Bardin et al., 2000), implying that Tem1 functions immediately upstream of Cdc15.
Figure 3.2  Tem1 activates Dbf2 kinase activity in a Cdc15-dependent manner. Strains NJW015 (3HA.DBF2 apc2-8) or SLY124 (3HA.DBF2 apc2-8 cdc15-1) transformed with plasmid pSL3 (GAL-6His.TEM1; 2μ), were grown overnight in selectable medium containing 2% raffinose, subcultured in YEP raffinose, and 2% galactose was added to induce 6His-Tem1 expression. The cells were arrested with alpha-factor (α-factor) or with nocodazole at 37°C (Noco@37°C) or at 37°C (37°C) for 3h. Dbf2 was immunoprecipitated from protein extracts using anti-HA monoclonal antibody (12CA5) bound to protein A beads and bead-bound histone H1 kinase activity was evaluated (upper panel), and quantitated by Phosphorimager. The upper part of the kinase assay gel was immuno-blotted and probed with anti-HA antibody to estimate Dbf2 protein levels (middle panel). Tem1 expression was confirmed by immuno-blotting (lower panel).
3.2.3 Cdc15 activates Dbf2 in a Mob1-dependent manner

If Tem1 controls Dbf2 through Cdc15, over-expression of *CDC15* should similarly activate Dbf2. To test this hypothesis, strain KTM208 (*apc2*-8) was transformed with plasmid pSJ103 (CDC15.3HA; URA3; 2µ) or pRS306 (a vector control), and grown overnight at 23°C in selectable medium before being subcultured into YEPD. Mid-log phase cells were arrested using α-factor, or at 37°C in the presence or absence of nocodazole. Dbf2 was immunoprecipitated from protein extracts using anti-Dbf2 antibody (Toyn and Johnston, 1994), and kinase assays performed. Cells transformed with a vector control were still able to down-regulate Dbf2 kinase activity in response to nocodazole (Figure 3.3, panel 1). However, in cells over-expressing Cdc15-3HA, Dbf2 kinase was activated in the presence and absence of nocodazole (Figure 3.3, panel 2). Thus Cdc15 functions upstream of Dbf2 to activate its kinase activity. This up-regulation of Dbf2 by Cdc15 was slightly less dramatic than that induced by Tem1 over-expression. This may simply reflect the lower levels of Cdc15 expression from the multi-copy vector, or, alternatively, the more pivotal role played by Tem1 in controlling the pathway.

Dbf2 and Mob1 form a heterodimer (Komarnitsky *et al.*, 1998) and Dbf2 function requires Mob1 (Figure 3.1). Mob1 is therefore likely to function directly upstream of Dbf2, and hence downstream of Cdc15. Accordingly, we investigated whether over-expression of Cdc15 could still activate Dbf2 in the absence of functional Mob1. Cdc15-3HA was expressed from a multi-copy vector in strain JMA1-21c (*apc2*-8 *mob1*-77), and cells were arrested using α-factor, or at 37°C in the presence or absence of nocodazole, prior to assaying Dbf2 kinase activity. Dbf2 kinase activity is clearly not induced by Cdc15 over-expression in an *apc2*-8 *mob1*-77 strain (Figure 3.3, panel 3). This experiment formally places Mob1 function downstream of Cdc15 and upstream of Dbf2. Thus the order in which these MEN pathway components function is Tem1-Cdc15--Mob1-Dbf2--Cdc14.
Figure 3.3  
Cdc15 activates Dbf2 kinase activity in a Mob1-dependent manner. 
Strains KTM208 (apc2-8) and JMA1-21c (apc2-8 mob1-77) were transformed with plasmid pSJ103 (CDC15.3HA; 2μ) (a gift from Sue Jaspersen, University of California), grown overnight in selectable medium, subcultured in YEPD and arrested with alpha-factor (α-factor) or with nocodazole at 37°C (Noco@37°C) or at 37°C (37°C) for 3h. Dbf2 was immunoprecipitated from protein extracts using anti-Dbf2 antibody (Toyn and Johnston, 1994), bound to protein A beads, and bead-bound histone H1 kinase activity was evaluated (lower panels), and quantitated by Phosphorimager. An anti-Dbf2 immuno-blot was used to estimate Dbf2 protein levels. The ratios of histone H1 kinase activity/Dbf2 protein levels are plotted in the graphs (upper panels).
3.2.4 Cdc5 kinase activity is not regulated by the MEN

Cdc5 is known to be a multi-functional kinase and its role within the MEN has not been established. We found that Cdc5 was required for Dbf2 kinase activity, indicating that it functions upstream of Dbf2 (Figure 3.1). However, ordering Cdc5 function within the MEN proved difficult. Like Dbf2, Cdc5 kinase activity is cell cycle-regulated (Cheng et al., 1998; Fesquet et al., 1999) and cells arrested in metaphase using an apc2-8 temperature-sensitive mutation, contain higher Cdc5 kinase activity than mid-log phase cells (Fesquet et al., 1999). However, unlike Dbf2, Cdc5 is not inhibited following spindle damage, suggesting that it may not be regulated by Tem1 (Fesquet et al., 1999).

To establish whether Cdc5 is controlled by any MEN components, Cdc5 kinase activity was assessed in an apc2-8 strain containing temperature-sensitive mutations in different MEN genes, arrested at 37°C. Strains SLY125 (CDC5.3HA apc2-8), SLY126 (CDC5.3HA apc2-8 tem1-3) SLY127 (CDC5.3HA apc2-8 cdc15-1), SLY128 (CDC5.3HA apc2-8 mob1-77), and SLY129 (CDC5.3HA apc2-8 cdc14-1) were grown to mid-log phase at 23°C. The cultures were split, and one half was shifted to the restrictive temperature of 37°C for 3h. Cdc5 was immunoprecipitated from protein extracts using anti-HA antibody (12CA5). As expected, Cdc5 kinase activity is enhanced in apc2-8 cells arrested in metaphase at 37°C relative to its levels in mid-log phase cells at 23°C (Figure 3.4, lanes 1 and 2). This increase in kinase activity at 37°C was still observed in cells that contained temperature-sensitive mutations in TEM1, CDC15, MOB1 or CDC14 (Figure 3.4). Thus mutating Tem1, Cdc15, Mob1 or Cdc14 had no effect on Cdc5 kinase activation. This indicates that, despite being required for MEN activation, Cdc5 regulation is independent of the MEN. Cdc5 is therefore not a component of the linear pathway which functions downstream of Tem1.

3.2.5 Cdc5 over-expression prevents Dbf2 kinase activation

Cdc5 could act upstream of the MEN components studied, perhaps phosphorylating an activator of Tem1 such as Lte1. Alternatively, Cdc5-dependent phosphorylation of one or more MEN components could be required for pathway activation downstream of Tem1. To
Figure 3.4  Cdc5 kinase activity is independent of the MEN. Strain SLY125 (apc2-8 CDC5.3HA) and strains containing tem1-3 (SLY126) cdc15-1 (SLY127), mob1-77 (SLY128), and cdc14-1 (SLY129) mutations in an apc2-8 CDC5.3HA background were grown to mid-log phase at 23°C. Half of each culture was shifted to the restrictive temperature of 37°C, for 3h. Cdc5 was immunoprecipitated from protein extracts using anti-HA monoclonal antibody (12CA5) bound to protein A beads and bead-bound GST-Bfa1 kinase activity (see 6.2.6) was evaluated (upper panel), and quantitated by Phosphorimager. The upper part of the kinase assay gel was immuno-blotted and probed with anti-HA antibody to estimate Cdc5 protein levels (middle panel). The ratios of GST-Bfa1 kinase activity/Cdc5 protein levels are plotted in the graphs (lower panel). Note that each mutant strain was generated by crossing, and the strain backgrounds are consequently not congenic, which may explain the differences in the relative levels of Cdc5 kinase activity.
understand how Cdc5 regulates the MEN, the effects of Cdc5 over-expression on Dbf2 kinase activity were studied. If Cdc5 over-expression activates Dbf2 kinase, it should then be possible to determine whether this activation is MEN-dependent or independent.

Strain NJW015 (3HA.DBF2 apc2-8) was transformed with pSL20 (GAL-6His.CDC5; URA3; 2μ) or pEMBLyex4 (vector control) and grown overnight at 23°C in selectable medium containing 2% raffinose before being subcultured in YEP-raffinose and grown to mid-log phase. The cultures were split, and 2% galactose added to one half to induce 6His-Cdc5 expression. The cells were then arrested in G1 using α-factor, or in metaphase at 37°C, in the presence or absence of nocodazole, for 3h. Dbf2 was immunoprecipitated from protein extracts using anti-HA (12CA5) antibody and H1 kinase assays performed. As expected, in the absence of galactose, Dbf2 kinase activity is high in cells arrested at 37°C, but low in α-factor-arrested cells, or at 37°C in the presence of nocodazole (Figure 3.5, upper panel, lanes 1-3). Surprisingly, in the presence of galactose, when Cdc5 is over-expressed, Dbf2 kinase activity in the metaphase-arrested cells is reduced by 70% (Figure 3.5, upper panel, lane 6). This effect is specific to cells that are over-expressing Cdc5, since addition of galactose had no effect on Dbf2 kinase activity in cells transformed with a vector control (Figure 3.5, lower panel). Thus over-expression of Cdc5 inhibits Dbf2 kinase activity. This unexpected result indicates that Cdc5 exerts both a negative and positive regulatory influence upon the MEN.

3.2.6 Inhibition of Dbf2 by Cdc5 is largely independent of Bub2

When Cdc5 is over-expressed in the presence of Bub2, Dbf2 kinase is inhibited (Figure 3.5). Cdc5 could prevent MEN activation through upstream regulators such as Bub2. To test this hypothesis, the effects of over-expressing Cdc5 in cells deleted for BUB2 were investigated. Strain SLY146 (3HA.DBF2 apc2-8 bub2Δ) was transformed with pSL20 (GAL-6His.CDC5; URA3; 2μ) and grown overnight at 23°C in selectable medium containing 2% raffinose before being subcultured in YEP-raffinose and grown to mid-log phase. The culture was split, and 2% galactose added to one half, to induce 6His-Cdc5 expression. The cells were arrested in G1 using α-factor, or in metaphase at 37°C, in the
Figure 3.5  **Cdc5 over-expression inhibits Dbf2 kinase activation.** Strain NJW015 (*apc2-8 3HA.DBF2*) transformed with pSL20 (*GAL-6His.CDC5; 2μ*) (upper figure), or pEMBLyex4 (an empty vector control) (lower figure) was grown overnight in selectable medium containing 2% raffinose, and subcultured in YEP raffinose. 2% galactose was added to induce 6His-Cdc5 expression and the cells were arrested with alpha-factor (αF) or with nocodazole at 37°C (Noco@37°C) or at 37°C (37°C) for 3h. Dbf2 was immunoprecipitated from extracts using anti-HA monoclonal antibody (12CA5) bound to protein A beads, and bead-bound histone H1 kinase activity was evaluated (upper panel), and quantitated by Phosphorimager. The upper part of the kinase assay gel was immuno-blotted and probed with anti-HA antibody to estimate Dbf2 protein levels (middle panel). The ratios of histone H1 kinase activity/Dbf2 protein levels are plotted in the graph (lower panel).
Dbf2 kinase

Histone H1

Relative Dbf2 kinase activity

α-HA

Dbf2

Relative Dbf2 kinase activity

apec2-8
+pGAL-6His.CDC5

Dbf2 kinase

Histone H1

α-HA

Dbf2

Relative Dbf2 kinase activity

apec2-8
+ vector
presence or absence of nocodazole, for 3h. Dbf2 was immunoprecipitated from protein extracts using anti-HA (12CA5) antibody and kinase assays performed. In the absence of galactose, Dbf2 kinase activity is low in α-factor-arrested cells, and high in cells arrested at 37°C in the presence or absence of nocodazole (Figure 3.6, lanes 1-3). Cdc5 over-expression reduced the levels of Dbf2 kinase in the metaphase-arrested cells by 60-50% (Figure 3.6, lanes 5 and 6). Thus, deletion of BUB2 reduces the Cdc5-dependent inhibition of Dbf2 by some 10-20% (from 70% to 60-50%), suggesting that Cdc5 may partially inhibit the MEN through Bub2. However, the loss of Dbf2 kinase activity observed in cells over-expressing Cdc5 is largely Bub2-independent.

3.2.7 The timing of Cdc5 over-expression determines its effects on Dbf2 kinase activity

Since Cdc5 is a multi-functional kinase, Cdc5 over-expression may interfere with an earlier cell cycle event which is important for MEN activation during mitosis. If so, over-expressing Cdc5 after cells have been arrested in metaphase, might not affect Dbf2 kinase levels. To investigate this possibility, strains NJW015 (3HA.DBF2 apc2-8) and SLY146 (3HA.DBF2 apc2-8 bub2Δ) transformed with plasmid pSL20 (GAL-6His.CDC5; URA3; 2µ) were grown overnight at 25°C, in selectable medium containing 2% raffinose as the carbon source, before being subcultured in YEP-raffinose and grown to mid-log phase. The cells were arrested in G1 using α-factor, or in metaphase at 37°C, in the presence or absence of nocodazole, for 2.5h. Each arrested culture was then split, and 2% galactose added to one half for 30 min, to induce 6His-Cdc5 expression. Dbf2 was immunoprecipitated from protein extracts using anti-HA antibody and kinase assays were performed. As expected, in the absence of galactose, the apc2-8 BUB2 strain showed high levels of Dbf2 kinase activation at 37°C, which were inhibited in the presence of nocodazole (Figure 3.7, upper panel, lanes 1-3). In contrast, again as expected, the apc2-8 bub2Δ strain showed maximal levels of Dbf2 kinase activation at 37°C in the presence or absence of nocodazole (Figure 3.7, lower panel, lanes 1-3). Galactose-induced Cdc5 over-expression had no effect on Dbf2 kinase levels (Figure 3.7, upper and lower panels, lanes 4-
Figure 3.6 Inhibition of Dbf2 kinase by Cdc5 over-expression is largely independent of Bub2. Strain SLY146 (apc2-8 bub2Δ 3HA.DBF2) transformed with pSL20 (GAL-6His.CDC5; 2µ) was grown overnight in selectable medium containing 2% raffinose, subcultured in YEP-raffinose, and 2% galactose was added to induce 6His-Cdc5 expression. The cells were arrested with alpha-factor (αF) or with nocodazole at 37°C (Noco@37°C) or at 37°C (37°C) for 3h. Dbf2 was immunoprecipitated from extracts using anti-HA monoclonal antibody (12CA5) bound to protein A beads, and bead-bound histone H1 kinase activity was evaluated (upper panel), and quantitated by Phosphorimager. The upper part of the kinase assay gel was immuno-blotted and probed with anti-HA antibody to estimate Dbf2 protein levels (middle panel). The ratios of histone H1 kinase activity/Dbf2 protein levels are plotted in the graph (lower panel).
Figure 3.7 Over-expressing Cdc5 in metaphase-arrested cells has no effect on Dbf2 kinase activity. Strains NJW015 (apc2-8 3HA.DBF2) and SLY146 (apc2-8 bub2Δ 3HA.DBF2) transformed with pSL20 (GAL-6His.CDC5; 2μ) were grown overnight in selectable medium containing 2% raffinose, subcultured in YEP-raffinose, and arrested with alpha-factor (αF) or with nocodazole at 37°C (Noco@37°C) or at 37°C (37°C) for 2.5h. 2% galactose was added for a further 30 min to induce 6His-Cdc5 expression. Dbf2 was immunoprecipitated from extracts using anti-HA monoclonal antibody (12CA5) bound to protein A beads, and bead-bound histone H1 kinase activity was evaluated (upper panel), and quantitated by Phosphorimager. The upper part of the kinase assay gel was immuno-blotted and probed with anti-HA antibody to estimate Dbf2 protein levels (middle panel). Cdc5 over-expression was confirmed by immuno-blotting (lower panel).
6). Thus Cdc5 does not affect Dbf2 kinase activation if it is over-expressed after cells have been arrested in metaphase.

3.3 Discussion

3.3.1 The order of the MEN pathway

The data presented here show that Dbf2 kinase activation is dependent upon functional Tem1, Cdc15, Cdc5, and Mob1, but independent of Cdc14, which has been reported to function downstream of Dbf2 (Visintin et al., 1999). These data are consistent with the situation in the fission yeast, Schizosaccharomyces pombe. In this organism the Dbf2 homologue Sid2 is a component of the septum initiation network (SIN) which is analogous to the MEN, and Sid2 has been shown to function downstream of Spg1 and Cdc7, the Tem1 and Cdc15 homologues respectively (Sparks et al., 1999).

By analogy with the fission yeast system, Bub2 is likely to function with Bfa1 as a 2-component GAP for Tem1 (Pereira et al., 2000). Thus the activation of Dbf2 following deletion of Bub2 is likely to be due to de-regulation of Tem1, suggesting that Tem1 functions upstream of the other MEN genes. The data presented here confirm this hypothesis. Tem1 activation of Dbf2 requires Cdc15, indicating that Tem1 is upstream of Cdc15. Since Tem1 and Cdc15 are known to interact (Asakawa et al., 2001; Bardin et al., 2000), Tem1 is likely to act directly on Cdc15. Indeed, a recent study suggests that activation of Tem1 is important for its association with Cdc15, and that this association promotes the SPB localisation of Cdc15, although it does not affect Cdc15 kinase activity (Asakawa et al., 2001). This is consistent with previous studies which showed that Cdc15 kinase activity is not cell cycle-regulated (Jaspersen et al., 1998). Presumably Tem1 activation results in Cdc15 localising to the SPB, where it phosphorylates its substrates.

What are the downstream targets of Cdc15? The data presented here indicate that Mob1 functions downstream of Cdc15. Mob1 is required for Dbf2 kinase activity, and Mob1 and Dbf2 are known to associate throughout the cell cycle (Komarnitsky et al., 1998; D.
Fesquet, unpublished observations). This suggests that Mob1 either activates Dbf2 directly, or that Mob1 and Dbf2 function together as a heterodimer. Cdc15 could thus activate Mob1 directly, leading to Dbf2 activation, or it could activate the Mob1-Dbf2 heterodimer by phosphorylating either Mob1 or Dbf2. Alternatively, Cdc15 could regulate an unidentified protein or proteins, which would in turn control Mob1 and Dbf2. Consistent with this latter model, in fission yeast the Cdc14/Sid1 heterodimer is proposed to act in the SIN pathway between Cdc7 and Sid2 (homologues of Cdc15 and Dbf2 respectively) (Guertin et al., 2000). However, homologues of cdc14+ or sid1+ have not been identified in budding yeast.

Dbf2 is known to be a phospho-protein, and it has been suggested that de-phosphorylation of Dbf2 could regulated its kinase activity, although there is no biochemical evidence for this (Toyn and Johnston, 1994). However, in apc2-8 bub2Δ cells at the restrictive temperature, when Dbf2 kinase levels are maximal, the protein is phosphorylated (Fesquet et al., 1999). Thus phosphorylation of Dbf2 by Cdc15 could promote its activation. Immunoprecipitation experiments failed to detect an interaction between Dbf2 and Cdc15 (data not shown). However, a recent study has demonstrated that Cdc15 does phosphorylate and activate Dbf2, and that this phosphorylation requires Mob1 (Mah et al., 2001). Thus the pathway can be ordered as follows: Tem1→Cdc15→(Mob1→Dbf2)→Cdc14 (Figure 3.8). The role of Cdc5 in this pathway is discussed below.

Although it is clear that Mob1 is required for Dbf2 kinase activation, and functions as its binding partner, previous studies have shown that Mob1 over-expression can bypass the lethality of a dbf2Δ dbf20Δ double mutant, suggesting that Mob1 is an effector of Dbf2 function (Komarnitsky et al., 1998). Conceivably, Mob1 activates Dbf2 and in so doing is itself activated for a downstream function. Alternatively, if Mob1 serves as a co-factor for Dbf2, when over-expressed Mob1 may confer Dbf2 function to another weakly homologous kinase.

It is not clear how exactly the release of Cdc14 from the nucleolus is regulated. Dbf2 could regulate this directly, but it is also feasible that additional proteins are involved, although these have not been identified. Once Cdc14 has been released from the nucleolus,
Figure 3.8  **Model illustrating the order of function of the MEN.** Tem1 acts directly upstream of Cdc15, which then phosphorylates Dbf2, activating the Mob1-Dbf2 heterodimer. Mob1-Dbf2 may control release of Cdc14 directly, or Dbf2 may then activate Mob1 for a downstream function (see 3.3.1). Once released, Cdc14 dephosphorylates Cdc15, apparently to promote its role in cytokinesis. The role of Cdc5 is not entirely clear, although it appears to exert a negative influence early in the cell cycle, whilst promoting MEN activation later in the cell cycle.
Spindle damage

Cdc5 → Bub2

? → Tem1 → Inactive → Tem1 → Active → Cdc15 → Mob1 → Dbf2 → Cdc14 → Mitotic exit

Cdc5 → Mitotic exit
regulation of the pathway is likely to be more complex, since Cdc14 has been shown to dephosphorylate its upstream activator Cdc15, suggesting that it forms a feedback loop involving the other MEN genes (Jaspersen and Morgan, 2000; Menssen et al., 2001). This de-phosphorylation appears to trigger a change in the SPB localisation of Cdc15, which moves from the SPB_{mother} to both SPBs (Menssen et al., 2001). This relocalisation is necessary for cytokinesis (Menssen et al., 2001), suggesting that, like the septation initiation network in fission yeast, the MEN may be involved in the regulation of cytokinesis, with this role being triggered by Cdc14.

3.3.2 The role of Cdc5 in MEN regulation

The polo-like kinase Cdc5 regulates several cell cycle events (1.8.1.4), making functional analysis difficult. The data presented here show that Cdc5 is required for Dbf2 kinase activation at the end of mitosis. This confirms that \textit{cdc5} mutants arrest in telophase due to a failure to activate the MEN, consistent with their inability to release Cdc14 from the nucleolus (Visintin et al., 1999). Cdc5 thus acts upstream of Dbf2 and Cdc14.

If Cdc5 functions within the linear pathway described here (Tem1-Cdc15-(Mob1-Dbf2)--Cdc14), then mutating the MEN components upstream of Cdc5 should abolish Cdc5 kinase activity. However, Cdc5 kinase activity was unaffected by mutations in the MEN components studied (Tem1, Cdc15, Mob1, and Cdc14), indicating that Cdc5 regulation is independent of the MEN. Although interesting, this is not surprising, since Cdc5 is not down-regulated following spindle assembly checkpoint activation, and is thus unlikely to be regulated by the Bub2/Bfa1-Tem1 pathway. The only known method of Cdc5 control identified to date is localisation (Song et al., 2000; Song and Lee, 2001).

Cdc5 could be required upstream of Tem1 for MEN activation. Alternatively, Cdc5-dependent phosphorylation of one or more MEN components could be necessary for MEN activation. The latter hypothesis implies that there are two requirements for MEN induction, namely activation of Tem1 and activation of Cdc5. To try to understand where in the MEN pathway Cdc5 acts, over-expression studies similar to those described for Tem1
and Cdc15 were performed. Unexpectedly, over-expression of Cdc5 inhibited Dbf2 kinase activity. This indicates that Cdc5 can exert both positive and negative regulation on the MEN. If the negative regulation of the MEN by Cdc5 is a genuine aspect of cell cycle control, it might act through the Bub2/Bfa1 pathway. However, deleting Bub2 only restored Dbf2 kinase activation by some 10-20%, suggesting that whilst Cdc5 over-expression may exert some negative regulation on the MEN through Bub2, the down-regulation of Dbf2 kinase activity in Cdc5 over-expressing cells is largely independent of Bub2.

Interestingly, Dbf2 kinase levels are not affected when Cdc5 over-expression is induced after cells have been arrested in metaphase. This suggests that the effects of Cdc5 over-expression on the MEN are indirect, and occur during an earlier stage of the cell cycle. One possibility is that Cdc5 can suppress the apc2-8 mutation, alleviating the metaphase arrest and hence lowering Dbf2 kinase activity. This is not the case, since Cdc5 over-expression does not allow apc2-8 mutant cells to grow at the restrictive temperature (data not shown). Alternatively, Cdc5 over-expression may interfere with the activation of one or more MEN components, for example by disrupting their SPB localisation (1.8).

Thus due to the multi-functional nature of Cdc5, it has not been possible to fully explain its role within the MEN. Since Cdc5 is required for Dbf2 kinase function, even in bub2Δ cells when Tem1 is presumably maximally activated, Cdc5 is likely to function downstream of Tem1 to activate the MEN (Figure 3.8). The most likely interpretation of the requirement for Cdc5 is that Cdc5-dependent phosphorylation of certain MEN components is necessary for their activation. However, identification of its substrates will be necessary before the role of Cdc5 at this stage in mitosis will be clearly understood.
Chapter 4

Regulation of Tem1 by Bub2 and Bfa1

4.1 Introduction

The data presented in Chapter 3 show that the mitotic exit network forms a signalling cascade, which functions in the order Tem1-Cdc15-(Mob1-Dbf2)-Cdc14. Activation of mitotic exit is therefore likely to be dependent upon the switch from the inactive, GDP-bound form of Tem1, to the active, GTP-bound form. Thus a knowledge of Tem1 regulation is the key to understanding mitotic exit.

One aspect of Tem1 control was described by Bardin et al. (2000), who showed that its putative exchange factor, Lte1, was localised to the bud throughout the cell cycle, whilst Tem1 was present at the daughter-bound SPB. The two proteins could only interact once the SPB_daugther moved through the bud neck following sister chromatid separation, ensuring that mitotic exit does not precede nuclear division (1.8.1.2; Figure 1.3).

However, the MEN is also subject to negative regulation by the Bub2 branch of the spindle assembly checkpoint (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999). It is likely that Bub2 also controls Tem1 directly (Bardin et al., 2000; Pereira et al., 2000; Shou et al., 1999), since the fission yeast homologue of Bub2, Cdc16, forms a two-component GAP with Byr4 to regulate Spg1, a homologue of Tem1 (Furge et al., 1998). Moreover, the budding yeast homologue of Byr4, Bfa1, is also part of the Bub2 branch of the SAC (Alexandru et al., 1999; Li, 1999). Here the possibility that Bub2 and Bfa1 could form a 2-component GAP for Tem1, and negatively regulate the MEN in response to spindle damage is investigated.
4.2 Results

4.2.1 Inhibition of Dbf2 kinase following spindle damage requires Bfa1

If Bfa1 and Bub2 form a two-component GAP to control Tem1, deletion of BFA1 should have the same physiological effects as deletion of BUB2. Bub2 is required to down-regulate Dbf2 kinase activity following SAC activation in metaphase-arrested cells (Fesquet et al., 1999). If Bfa1 shares this function with Bub2, deletion of BFA1 should abolish the inhibition of Dbf2 kinase observed in metaphase-arrested cells following nocodazole-induced SAC activation. The assay conditions described in Chapter 3 were used to test this hypothesis. Strains YDF20 (DBF2.6MYC apc2-8) and SLY103 (DBF2.6MYC apc2-8 bfa1A) were grown to mid-log phase at 25°C, before being arrested in G1 using α-factor, or in metaphase at 37°C, in the presence or absence of the spindle-depolymerising agent nocodazole, for 3h. Dbf2 was immunoprecipitated from protein extracts, and the levels of Dbf2 kinase activity assessed. As expected, in apc2-8 BFA1 cells, Dbf2 kinase activity was barely detectable during G1, but increased in metaphase-arrested cells (Figure 4.1, lanes 1 and 2). In the presence of nocodazole, this metaphase Dbf2 kinase activity was inhibited (Figure 4.1, compare lanes 2 and 3). In contrast, following deletion of BFA1, Dbf2 kinase activity was no longer down-regulated in the presence of nocodazole (Figure 4.1, lanes 5 and 6). Thus, like Bub2, Bfa1 is required to inhibit Dbf2 kinase following SAC activation. This is consistent with Bub2 and Bfa1 acting together to negatively regulate the MEN in response to SAC activation. Moreover, in the absence of Bfa1 function, Dbf2 kinase levels are higher than those normally observed in metaphase-arrested cells, even in the absence of nocodazole (Figure 4.1, compare lanes 3 and 6). This effect is also observed following deletion of BUB2 (Fesquet et al., 1999). This suggests that Bub2 and Bfa1 function in some capacity in metaphase-arrested cells to negatively regulate the MEN, independently of SAC activation. Loss of either protein is sufficient to allow maximal MEN activation in metaphase.
Figure 4.1  Bfa1 negatively regulates Dbf2 kinase activation. Isogenic strains YDF20 (DBF2.6MYC apc2-8) and SLY103 (DBF.6MYC apc2-8 bfa1Δ), were grown to mid-log phase at 25°C and arrested with α-factor (αF), nocodazole at 37°C (Noco @37°C) or at 37°C (37°C) for 3h. Dbf2 was immunoprecipitated from protein extracts with anti-MYC (9E10) antibody and Dbf2 kinase activity assayed (lower panel). The upper part of the kinase assay gel was immuno-blotted and probed with 9E10 antibody to control for loading.
4.2.2 Bub2 and Bfa1 physically associate

To investigate whether Bub2 and Bfa1 might function as a heterodimer, like their fission yeast counterparts (Furge et al., 1998), co-immunoprecipitation experiments were performed with epitope-tagged Bub2-13MYC and 3HA-Bfa1. The wild-type genes were tagged at the genomic locus using a PCR integration technique, to generate strain SLY106 (*BUB2.13MYC 3HA.BFA1*) (2.5.4). Functionality of the two tagged proteins was determined using a re-budding assay (2.2.5), which assesses SAC integrity. Mid-log phase cells incubated in the presence of nocodazole normally arrest in metaphase due to activation of the SAC. Loss of functionality of any SAC component (such as Bub2 or Bfa1) allows the cells to escape this arrest and re-initiate budding. Strain SLY106 did not re-bud in the presence of nocodazole (Figure 2.3) indicating that the proteins are fully functional.

To determine whether Bub2 and Bfa1 could associate, strain SLY106 was grown to mid-log phase and arrested in G1 using α-factor, or in metaphase using hydroxyurea or nocodazole (which activate the DNA damage and spindle assembly checkpoints, respectively [1.7.2.3]). When Bub2-13MYC was immunoprecipitated from protein extracts of mid-log phase cells, or G1- or metaphase-arrested cells, immuno-blotting showed that Bfa1 had co-immunoprecipitated (Figure 4.2, upper panel). Similarly, immunoprecipitation of 3HA-Bfa1 from the same protein extracts, resulted in the co-immunoprecipitation of Bub2 (Figure 4.2, lower panel). Thus a physical interaction between Bub2 and Bfa1 can be detected in mid-log phase cells, and also during G1 and metaphase. In particular, Bub2 and Bfa1 associate during a nocodazole-induced SAC metaphase arrest. This is consistent with them functioning as a heterodimer following SAC activation.

4.2.3 Bub2 and Bfa1 physically associate across the cell cycle

Figure 4.2 shows that Bub2 and Bfa1 associate during G1 and metaphase, and suggests that they may interact for much of the cell cycle. To investigate the timing of this association in more detail, co-immunoprecipitation experiments were performed with a synchronous
Figure 4.2 Bfa1 and Bub2 associate in mid-log phase cells and in G1 or metaphase-arrested cells. Strain SLY106 (BUB2.13MYC 3HA.BFA1) was grown to mid-log phase (ML), arrested with α-factor (αF), hydroxyurea (HU) or nocodazole (Noco) for 3h and harvested. 300μg protein extract was immunoprecipitated using anti-HA (12CA5) or anti-MYC (9E10) antibody (upper or lower panels respectively), analysed using SDS-PAGE, immuno-blotted and probed with anti-MYC or anti-HA antibody (upper or lower panels respectively). Lanes 1-3 show an untagged control (CG378) and two singly tagged strains. The Ig band serves as a loading control.
culture of strain SLY106 (BUB2.13MYC 3HA.BFA1). Strain SLY106 was grown to mid-log phase and arrested in G1 using α-factor. Cells were released from the G1 arrest into fresh media, and samples for protein extraction were taken at the indicated times after release. The budding index showed that good synchrony had been obtained (Figure 4.3, lower panel). Immunoprecipitation of 3HA-Bfa1 from protein extracts resulted in the co-immunoprecipitation of Bub2, as detected by immuno-blotting (Figure 4.3, upper panel). Similarly, when Bub2-13MYC was immunoprecipitated, Bfa1 was found to co-immunoprecipitate (Figure 4.3, middle panel). These data show that Bfa1 and Bub2 associate across the entire cell cycle, consistent with them functioning together as a 2-component GAP. These data also show that GAP activity is not controlled by complex formation, since the complex exists throughout the cell cycle, as well as following spindle checkpoint activation.

4.2.4 Bub2 and Bfa1 associate with Tem1 during M phase and early G1

If Bub2 and Bfa1 form a 2-component GAP which directly inhibits Tem, the three proteins presumably interact at certain stages in the cell cycle. The association of Bfa1 and Bub2 with Tem1 was initially examined by over-expressing 6His-Tem1 from a galactose-inducible promoter in strain SLY106 (BUB2.13MYC 3HA.BFA1). Strain SLY106, containing plasmid pSL3 (pGAL-6His.TEM1; URA3; 2μ) was grown overnight in selectable medium containing 2% raffinose as a carbon source, before being subcultured in YEP-raffinose and grown to mid-log phase. 6His-Tem1 expression was induced by addition of 2% galactose (as indicated), and cells were either grown for a further 2h, or grown for 45 min before being arrested in G1 by addition of α-factor, or in metaphase by addition of hydroxyurea or nocodazole, for 3h. 6His-Tem1 was precipitated from protein extracts using nickel beads, and co-precipitating proteins analysed by immunoblotting. Tem1 interacts with both Bfa1 and Bub2 in mid-log phase cells (Figure 4.4, lane 4). The association of Bfa1 and Bub2 with 6His-Tem1 was also detected in G1- and metaphase-arrested cells (Figure 4.4, lanes 5-7). This suggests that the Bub2/Bfa1/Tem1 complex may exist for much of the cell cycle.
Figure 4.3 *Bfa1 and Bub2 associate across the cell cycle.* Strain SLY106 (*BUB2.13MYC 3HA.BFA1*) was grown to mid-log phase, arrested with α-factor (αF) for 3h and released into fresh medium. Samples for protein extraction were taken at the times shown. 300μg protein extract was immunoprecipitated using anti-HA (12CA5) or anti-MYC (9E10) antibody (upper or lower panels respectively), analysed using SDS-PAGE, immuno-blotted and probed with anti-MYC or anti-HA antibody (upper or lower panels respectively). Lanes 1-3 show an untagged control (CG378) and two singly tagged strains. The Ig band serves as a loading control. The graph shows the budding index for the synchronous culture.
Figure 4.4  Bfa1 and Bub2 physically associate with Tem1 in mid-log phase cells and in G1 or metaphase-arrested cells. Plasmid pSL3 (GAL-6His.TEM1) was introduced into strain SLY106 (BUB2.13MYC 3HA.BFA1), and control strain CG378. Cells grown overnight in selectable medium containing 2% raffinose, were subcultured into YEP-raffinose and grown to mid-log phase (ML). Following the addition of 2% galactose, (as indicated), cells were grown for a further 2h to induce 6His-Tem1 expression (lanes 1-4). In addition, following a 45 min galactose-induction, strain SLY106 was arrested with α-factor (αF), hydroxyurea (HU) or nocodazole (Noco) for 3h (lanes 5-7). Protein extracts were prepared and incubated with nickel beads for 1h at 4°C, to allow binding of 6His-Tem1 and any interacting proteins. Bound proteins were washed, eluted by boiling in Laemmli buffer, and analysed using SDS-PAGE and immuno-blotting. Note that strain CG378 is congenic, not isogenic, with strain SLY106, which may account for the high level of Tem1 in lane 1.
A trivial explanation for the above data might be the artificially high levels of Tem1 expression from the GAL promoter. To eliminate this possible artefact, the co-immunoprecipitation of Bfa1 and Tem1 was examined when they were expressed at normal endogenous levels. Strain SLY135 (TEM1.6MYC 3HA.BFA1) was grown to mid-log phase, and arrested in G1 by addition of α-factor or in metaphase by addition of hydroxyurea or nocodazole. When Bfa1 was immunoprecipitated from protein extracts, immuno-blotting revealed that Tem1 co-immunoprecipitated (Figure 4.5). This association was observed in mid-log phase, and in G1- or metaphase-arrested cells. These results suggest that Tem1 forms a complex with Bfa1 and Bub2 which persists for much of the cell cycle. This is unusual, since most G-proteins only associate transiently with their GAPs (Scheffzek et al., 1998). Regulation of Tem1 by Bfa1/Bub2 is therefore unlikely to be through transient association with the GAP.

**4.2.5 Association with Tem1 requires both Bub2 and Bfa1**

To examine whether Bub2 or Bfa1 could bind to Tem1 in the absence of its partner, 6His-Tem1 was expressed from a galactose-inducible promoter in strains SLY117 (BUB2.13MYC ba1Δ) or SLY111 (3HA.BFA1 bub2Δ). Strains SLY117 and SLY111, containing plasmid pSL3 (pGAL-6His.TEM1; URA3; 2µ), were grown overnight in selectable medium containing 2% raffinose as a carbon source, before being subcultured in YEP-raffinose and grown to mid-log phase. 6His-Tem1 expression was induced by addition of 2% galactose (as indicated) for 2h. 6His-Tem1 was precipitated from protein extracts using nickel beads, and co-precipitating proteins analysed by immuno-blotting. In the SLY106 control strain (BUB2.13MYC 3HA.BFA1), both Bub2 and Bfa1 co-precipitated with Tem1 (Figure 4.6, panel 2). In contrast, in the absence of Bfa1, no interaction was detected between Tem1 and Bub2 (Figure 4.6, panel 3). Similarly, Tem1 failed to interact with Bfa1 following deletion of BUB2 (Figure 4.6, panel 4). Thus only the heterodimer binds to Tem1.
Figure 4.5  Tem1 and Bfa1 associate in mid-log phase cells and in G1- and metaphase-arrested cells. Strain SLY135 (TEM1.6MYC 3HA.BFA1) was grown to mid-log phase (ML), arrested with α-factor (αF), hydroxyurea (HU) or nocodazole (Noco) for 3h, and harvested. 300μg protein extract was immunoprecipitated using anti-HA (12CA5) antibody, analysed using SDS-PAGE, immuno-blotted and probed with anti-MYC (9E10) antibody. Lanes 1-3 show an untagged control (CG378) and two singly tagged strains. The Ig band serves as a loading control.
Figure 4.6  Bfa1 and Bub2 do not physically associate with Tem1 if either protein is absent. Plasmid pSL3 (G A L-6His.TEM1) was introduced into strains SLY117 (BUB2.13MYC bfa1Δ) and SLY111 (3HA.BFA1 bub2Δ) and control strains SLY106 (BUB2.13MYC 3HA.BFA1) and CG378. Cells grown overnight in selectable medium containing 2% raffinose, were subcultured into YEP-raffinose and grown to mid-log phase (ML). The cultures were split, and grown for a further 2h in the presence or absence of 2% galactose (+/- Gal) to induce 6His-Tem1 expression. Protein extracts were prepared and incubated with nickel beads for 1h at 4°C, to allow binding of 6His-Tem1 and any interacting proteins. Bound proteins were washed, eluted by boiling in Laemmli buffer, and analysed using SDS-PAGE and immuno-blotting. Note that the strains are congenic, not isogenic, which may account for the different levels of Tem1 expression. Low levels of 3HA-Bfa1 can be detected in panel 2, lane 1, and panel 4, lanes 1 and 2 due to a non-specific interaction between the nickel beads and the HA-epitope tag.
4.2.6 Bub2 and Bfa1 oppose the function of Lte1

The above experiments indicate that Bub2 and Bfa1 function as negative regulators of the MEN, which interact with each other and Tem1, consistent with them forming a two-component GAP for Tem1. The other known regulator of Tem1 is its exchange factor, Lte1, which is believed to activate Tem1 in late mitosis (Bardin et al., 2000). Lte1 is essential for growth at low temperatures, perhaps because the intrinsic exchange activity of Tem1 is sufficient for activation at higher temperatures. As a two-component GAP, Bub2 and Bfa1 would be predicted to antagonise the function of Lte1. To investigate the relationship between Bub2, Bfa1 and Lte1, the effects of deleting BUB2 or BFA1 in an lte1Δ strain were examined. Strains deleted for LTE1 are viable at 30°C, but unable to grow at 17°C. Interestingly, lte1Δ bublA or lte1Δ bfa1Δ double mutant strains were viable at 17°C (Figure 4.7). Thus, deletion of either BUB2 or BFA1 relieved the cold-sensitivity phenotype of the lte1Δ strain. This suggests that the essential function of Lte1 at low temperatures is to overcome the inhibitory influence of the Bub2/Bfa1 GAP.

4.2.7 Lte1 is not required for activation of Dbf2 kinase in the absence of Bub2

As discussed in Chapter 3, Dbf2 kinase activation is dependent upon the MEN. If Lte1 functions as an upstream activator of Tem1, it may be required for Dbf2 kinase function. However, the fact that bub2Δ lte1Δ cells grow well, even at low temperatures (Figure 4.7) suggests that MEN activation can occur in the absence of Lte1 function. To test this hypothesis, Dbf2 kinase activity was assessed in apc2-8 bub2Δ and apc2-8 bub2Δ lte1Δ cells arrested in metaphase, as described in Chapter 3. Strains YLF25 (apc2-8 bub2Δ) and SLY136 (apc2-8 bub2Δ lte1Δ) were grown to mid-log phase at 25°C, and arrested in G1 by addition of α-factor, or in metaphase at 37°C, in the presence or absence of nocodazole. Dbf2 was immunoprecipitated from protein extracts and H1 kinase assays were performed. As expected, in the apc2-8 bub2Δ strain, Dbf2 displayed high levels of kinase activity in the metaphase-arrested cells, in the presence or absence of nocodazole (Figure 4.8, lanes 1-3). Dbf2 kinase was activated to the same extent in the apc2-8 bub2Δ lte1Δ cells arrested in metaphase (Figure 4.8, lanes 4-6). Thus, deletion of LTE1 had no effect on Dbf2 kinase
Figure 4.7 Deletion of *BFA1* or *BUB2* relieves the cold-sensitivity of an *lte1Δ* strain. Strains SLY108 (*bfa1Δ*) and YFD30 (*bub2Δ*) were crossed with strain 11C (*lte1Δ*) to generate the *lte1Δ bfa1Δ* and *lte1Δ bub2Δ* strains. The strains were grown on YEPD agar at 30°C or 17°C to assess their cold-sensitivity.
Figure 4.8  Deletion of Lte1 has no effect on Dbf2 kinase activation at 37°C. Strains YLF25 (*apc2-8 bub2Δ*) and SLY136 (*apc2-8 bub2Δ lte1Δ*), were grown to mid-log phase at 25°C and arrested with α-factor (αF), nocodazole at 37°C (Noco @37°C) or at 37°C (37°C) for 3h. Dbf2 was immuno-precipitated from extracts with anti-Dbf2 antibody (Toyn and Johnston, 1994), and Dbf2 kinase activity assayed.
activation under these conditions. This suggests that, at least during metaphase, the negative regulation of Tem1 by Bub2/Bfa1 appears to be more important for controlling MEN activation. The positive regulation by Lte1 is clearly dispensable, at least at high temperatures. Whether Tem1 possesses alternative means of activation, or whether it is capable of intrinsic activation in the absence of Bub2/Bfa1 remains to be seen.

4.3 Discussion

4.3.1 Bub2 and Bfa1 act together to negatively regulate the MEN

The data presented here demonstrate that both Bub2 and Bfa1 are necessary for downregulation of Dbf2 kinase in response to spindle damage. This effect is unlikely to be direct, since I could detect no interaction between Bub2 and Dbf2 (data not shown). It is more likely that Bub2 and Bfa1 form a 2-component GAP for Tem1, which in turn controls the other MEN proteins (Chapter 3). Consistent with this, Bub2 and Bfa1 form a heterodimer throughout the cell cycle, and following spindle checkpoint activation. This association is likely to be essential for activity, since in the fission yeast system, both proteins are absolutely required for GAP activity (Furge et al., 1998). Moreover, in budding yeast deletion of either BUB2 or BFA1 abrogates spindle checkpoint function (Hoyt et al., 1991; Li, 1999), and disrupts the SPB localisation of the remaining protein (Pereira et al., 2000). Thus, neither protein is functional in the absence of its binding partner. In this context, it is interesting to note that the association of the two proteins is not subject to cell cycle control, suggesting that any regulation of the GAP activity is not through control of heterodimer formation.

4.3.2 Bfa1 and Bub2 bind Tem1 through the cell cycle

The ability of Bfa1/Bub2 to bind Tem1 provides further evidence that they negatively regulate the MEN through inhibition of Tem1 (Figure 4.9). The association of Tem1 with Bfa1 and Bub2 was detected in mid-log cells and was also present during G1 (α-factor-induced arrest) and metaphase (hydroxyurea or nocodazole-induced arrest). The physical
Figure 4.9  Model illustrating possible regulation of Tem1 by Bub2, Bfa1 and Lte1. Bub2 and Bfa1 associate with each other and Tem1 across the cell cycle. During metaphase (apc2-8 arrest) they negatively regulate Dbf2, presumably by inhibiting Tem1. This is consistent with Bfa1 and Bub2 forming a two-component GAP for Tem1, maintaining it in the GDP-bound (inactive) state. Lte1 is thought to form an exchange factor for Tem1, promoting exchange of GDP for GTP and activating the protein. Lte1 is essential at low temperatures, perhaps because Tem1 has a high intrinsic exchange activity at higher temperatures. Deletion of BFA1 or BUB2 relieves the cold-sensitivity of an lte1Δ deletion, consistent with their opposing functions.
association of Bub2/Bfa1 with Tem1 has also been shown by Pereira et al. (2000), but the
timing of the interaction was not addressed. Although the interaction between a G-protein
and its GAP is usually transient (Scheffzek et al., 1998), localisation studies of Tem1, Bub2
and Bfa1 support the findings described here. All three proteins localise to the SPBs
throughout the cell cycle, with the localisation of Tem1 being dependent on Bub2 and Bfa1
from G1 until late mitosis (Pereira et al., 2000), consistent with their ability to physically
associate. It is possible that Bub2 and Bfa1 could dissociate from Tem1 in late mitosis,
triggering Tem1 activation. To examine this would require a cell culture with a highly
synchronous late mitosis, and has not yet been attempted.

The interaction of Bub2/Bfa1 with Tem1 requires the presence of both proteins, again
consistent with their inability to function "solo" (4.3.1). Presumably this is because the
binding sites necessary for the interaction with Tem1 (and the SPB association), are either
shared between the two proteins, or require heterodimer formation to attain the correct
binding configuration.

4.3.3 The role of Lte1 in MEN regulation

Lte1 is thought to be an exchange factor (GEF) for Tem1 which activates the MEN
following spindle elongation in anaphase (1.8.1.2) (Bardin et al., 2000). As such, it would
antagonise the function of Bub2/Bfa1, overcoming their inhibition and promoting mitotic
exit. However, although lte1Δ strains arrest in late mitosis at low temperatures, at higher
temperatures they are viable, suggesting that Lte1 is not essential for activation of Tem1
and mitotic exit. The data presented here indicate that the cold-sensitivity of lte1Δ cells is
due to an inability to overcome the Bub2/Bfa1-mediated inhibition of Tem1, since deletion
of BUB2 or BFA1 suppresses the cold-sensitivity phenotype of an lte1Δ strain. Thus Lte1 is
required to oppose the functions of Bub2/Bfa1 at low temperatures (Figure 4.9). This,
incidentally, indicates that Bub2 and Bfa1 exert a negative influence on the MEN even in
the absence of spindle checkpoint activation, a point which will be considered in more
detail later.
Since deletion of *LTE1* has no effect on Dbf2 kinase levels in *apc2-8 bub2Δ* cells, MEN activation must be independent of Lte1 at higher temperatures. It appears that Bub2 and Bfa1 are the key regulators of Tem1, at least during metaphase, since deletion of these genes results in a considerable increase in MEN activity (Figure 4.1; Fesquet *et al.*, 1999), whilst deletion of Lte1 has no effect (Figure 4.8). Surprisingly, none of these genes are essential at normal temperatures. Moreover, the viability of *bub2Δ lte1Δ* and *bfa1Δ lte1Δ* cells indicates that none of the recognised regulators of Tem1 are necessary to control the timing of MEN function in an unperturbed cell cycle. Redundant pathways must operate to couple sister chromatid separation to mitotic exit, including the inhibition of MEN function by Clb5 and Pds1 (1.8.2), and these may contribute to the timing of MEN activation. Perhaps control of the MEN components downstream of Tem1 (for example by Cdc5, see Chapter 3), can also affect the timing of Cdc14 release. It is also possible that other regulators of Tem1 exist which have yet to be identified. Whatever the other mechanisms which control the timing of mitotic exit, it is clear that the continued inhibition of Tem1 by Bub2 and Bfa1 is essential to prevent aberrant MEN activation following spindle checkpoint activation.
Chapter 5

Bfa1 is phosphorylated in a cell cycle-dependent manner

5.1 Introduction

Bub2 and Bfa1 are evidently important negative regulators of the MEN GTP-binding protein Tem1 (Chapter 4). Tem1 controls activation of the entire mitotic exit network (Chapter 3) and it is therefore essential to understand how Bub2 and Bfa1 are regulated. In the presence of spindle damaging agents, both Bub2 and Bfa1 are required to enforce a mitotic arrest, and prevent premature mitotic exit. This suggests that they may be specifically regulated when cells are exposed to drugs such as nocodazole, which activates the spindle checkpoint. However, Bub2 and Bfa1 also seem to exert an inhibitory influence on the MEN even in the absence of nocodazole (for example see Figure 4.1), suggesting that these proteins may act every cell cycle to modulate MEN activation. A knowledge of how Bub2 and Bfa1 are regulated should therefore provide an insight into the control of the MEN by the spindle checkpoint, as well as any cell cycle functions of Bub2/Bfa1.

5.2 Results

5.2.1 Bub2 is not subject to cell cycle regulation

To examine the regulation of Bub2, the gene was tagged at the genomic locus with a 13MYC epitope using a PCR integration technique (2.5.4), to generate strain SLY104, containing BUB2.13MYC. The tagged protein was shown to be fully functional using a re-budding assay (Figure 2.2), as described previously (4.2.2). To investigate whether Bub2 was subject to regulated protein expression or post-translational modification, strain SLY104 was grown to mid-log phase and the cells were arrested in G1 using α-factor or in
metaphase using hydroxyurea (DNA damage checkpoint arrest) or nocodazole (SAC arrest). Protein extracts were analysed using SDS-PAGE and immuno-blotting. Bub2 protein is present as a single band in mid-log phase cells, as well as in G1- or metaphase-arrested cells (Figure 5.1). This indicates that Bub2 is not regulated at the level of protein stability or by post-translational modification, at least during G1 or in metaphase.

To examine the possible regulation of Bub2 during the cell cycle in more detail, Bub2 protein levels were examined in a synchronous culture of strain SLY104. Mid-log phase cells of strain SLY104 were synchronised in G1 using α-factor, and released into fresh media. Samples for protein extraction were taken at the indicated times after release. The budding curve and the periodicity of Clb2 protein levels indicated that good synchrony had been obtained. However, immuno-blotting showed that Bub2 protein levels were constant across the cell cycle, and there was no evidence of post-translational modification (Figure 5.2). This suggests that Bub2 is not a regulatory component of the putative two-component GAP.

5.2.2 Bfa1 is phosphorylated in a cell cycle-dependent manner

To investigate whether the GAP could be regulated through Bfa1, the genomic copy of BFA1 tagged at the N-terminus with a 3HA epitope using a PCR integration technique (2.5.5) to generate strain SLY105 (3HA.BFA1). The tagged 3HA-Bfa1 was shown to be fully functional using a re-budding assay (Figure 2.4), as described previously (4.2.2). To establish whether Bfa1 was regulated at the level of protein stability, or by post-translational modification, strain SLY105 was grown to mid-log phase, and the cells were arrested in G1 with α-factor or in metaphase by addition of hydroxyurea or nocodazole. Protein extracts were analysed using SDS-PAGE and immuno-blotting. Bfa1 protein levels were found to be constant in G1- and metaphase-arrested cells (Figure 5.3, lanes 1-4). However, the protein was subject to post-translational modification, since multiple bands could be distinguished using SDS-PAGE (Figure 5.3). Moreover, this post-translational modification appeared to be subject to cell cycle regulation, since the lower mobility species were more prominent in mid-log phase or metaphase-arrested cells than in G1-
Figure 5.1  Bub2 is not regulated at the level of protein stability or post-translational modification in mid-log phase cells or in cells arrested in G1 or metaphase. Strain SLY104 (BUB2.13MYC) was grown to mid-log phase and either harvested immediately or arrested using α-factor (αF), hydroxyurea (HU) or nocodazole (Noco) for 3h before harvesting. Following protein extraction, an immuno-blot was prepared and probed with anti-MYC antibody (9E10).
Figure 5.2  Bub2 is not regulated at the level of protein stability or post-translational modification during the cell cycle. Strain SLY104 (BUB2.13MYC) was grown to mid-log phase and arrested for 3h using α-factor (αF) to synchronise the cells in G1. The cells were released into fresh media and samples for protein extraction were taken every 15 minutes after release. Extracts were immuno-blotted and probed with anti-MYC (9E10) or anti-Clb2 antibodies as indicated. The lower panel shows the budding curve for the culture, whilst Clb2 protein levels serve as a marker for cell synchrony and the timing of mitosis.
Figure 5.3  Bfa1 is subject to cell cycle-dependent post-translational modification. Strain SLY105 (3HA.BFA1) was grown to mid-log phase and either harvested immediately or arrested using α-factor (αF), hydroxyurea (HU) or nocodazole (Noco) for 3h before harvesting. Following protein extraction, an immuno-blot was prepared and probed with anti-HA antibody (12CA5).
arrested cells (Figure 5.3, compare lanes 1, 3, 4 with lane 2). The post-translational modification appeared to be maximal in cells arrested following nocodazole-induced spindle checkpoint activation, suggesting that it could be important for GAP activation.

To examine whether this modification was due to phosphorylation, strain SLY105 was grown to mid-log phase, and either harvested immediately or arrested for 3h with nocodazole, to maximise the post-translational modification of Bfa1. Cell lysates were prepared in the presence or absence of phosphatase inhibitors, which are normally added to the cell lysis buffer to inhibit cellular phosphatases which might otherwise dephosphorylate phospho-proteins. The lysates were treated with calf alkaline phosphatase (CAP) and the SDS-PAGE mobility of Bfa1 was analysed by immuno-blotting. CAP treatment completely abolished the reduced mobility of Bfa1, generating a single protein band (Figure 5.4, lanes 7-8). The use of phosphatase inhibitors blocked the effect of the CAP (Figure 5.4, lanes 5 and 6). Thus the post-translational modification of Bfa1 observed in mid-log phase and nocodazole-arrested cells is due to phosphorylation.

The phosphorylated forms of Bfa1 present following α-factor-induced G1 arrest and DNA damage or SAC-induced metaphase arrest can be clearly seen in Figure 5.5, lanes 1-4. No fewer than six Bfa1 phosphorylated species are evident (Figure 5.5 inset, I-VI). These phosphorylated species are almost completely absent during the G1 arrest but are present in mid-log phase and hydroxyurea-arrested cells. In the nocodazole-treated cells certain phosphorylated species are further enhanced, most notably species VI, although species I, II and IV also increase in intensity (see also Figure 5.4, lanes 1 and 2). Since hydroxyurea and nocodazole both arrest cells in metaphase (1.7.2.3), these changes in the phosphorylation pattern may be specific to the nocodazole arrest, reflecting the SAC function of the Bub2/Bfa1 heterodimer. It should be noted that species I and II are quite faint and are not always detectable in immuno-blots, so that subsequent studies of Bfa1 phosphorylation have focussed on the changes in intensity of bands VI and IV following SAC activation.
Figure 5.4  **Bfa1 is a phospho-protein.** Strain SLY105 (3HA.BFA1) was grown to mid-log phase and either harvested immediately or following treatment with nocodazole for 3h (-/+ Noco). Protein extraction was performed as usual (2.6.4) or in lysis buffer without phosphatase inhibitors (+/- PI). 70μg extract was incubated with calf alkaline phosphatase (CAP) [Boehringer Mannheim] at 30°C for 30 min and then analysed using SDS-PAGE and immuno-blotting.
Figure 5.5  Phosphorylation of Bfa1 is dependent upon Bub2. Isogenic strains SLY105 (3HA.BFA1) and SLY111 (3HA.BFA1 bub2Δ), were grown to mid-log phase and either harvested immediately or arrested using α-factor (αF), hydroxyurea (HU) or nocodazole (Noco) for 3h before harvesting. Following protein extraction, an immuno-blot was prepared and probed with anti-HA antibody (12CA5). The different Bfa1 phosphorylated species observed in the control strain are labelled with Roman numerals (inset, I-VI).
5.2.3 **Bub2 is required for Bfa1 phosphorylation**

Since Bub2 and Bfa1 seem to be dependent on each other for function (4.3.1), the association of Bfa1 with Bub2 may be important for Bfa1 phosphorylation. To investigate this possibility, strains SLY105 (3HA.BFA1) and SLY111 (3HA.BFA1 bub2Δ) were grown to mid-log phase, and the cells arrested in G1 or metaphase by addition of α-factor, hydroxyurea or nocodazole. Protein extracts were analysed by immuno-blotting to determine Bfa1 protein mobility. In the presence of Bub2, Bfa1 was clearly subject to cell-cycle dependent phosphorylation (Figure 5.5, lanes 1-4; see previous section). Deleting *BUB2* abolished Bfa1 phosphorylation (Figure 5.5 lanes 5-8). Thus Bub2 is necessary for Bfa1 phosphorylation. This supports the idea that Bfa1 phosphorylation is important for regulation of the 2-component GAP, since in the absence of its binding partner, Bfa1 is no longer phosphorylated.

It is worth noting that in Figure 5.5, Bfa1 protein levels decrease during the α-factor arrest. Since this effect was not observed in other experiments (for example Figures 5.3 and 5.6) it is likely to be an artefact of the arrest, although it is also possible that Bfa1 protein abundance may decrease slightly during G1.

5.2.4 **The cell cycle-regulated phosphorylation of Bfa1 is prolonged in nocodazole**

The experiments described in Figures 5.3 and 5.5 suggest that Bfa1 phosphorylation is cell cycle-dependent. To investigate the cell cycle regulation of Bfa1 in more detail, a mid-log phase culture of SLY105 (3HA.BFA1) was synchronised in G1 using α-factor, before being released into fresh medium in the presence or absence of nocodazole. Samples for protein extraction were taken at the indicated times after release. The budding curve and the periodicity of Clb2 protein levels indicated that good synchrony had been obtained. Immuno-blotting revealed that Bfa1 was phosphorylated in a cell cycle-dependent manner (Figure 5.6, upper panels). Bfa1 phosphorylation is initiated about 30-40 minutes after bud emergence. However, the bulk phosphorylation becomes evident at 50 minutes, coinciding
Figure 5.6  Bfa1 undergoes cell cycle dependent phosphorylation that is protracted following nocodazole treatment. Strain SLY105 (3HA.BFA1) was grown to mid-log phase, synchronised with α-factor and released into fresh medium in the absence (upper panels) or presence (lower panels) of nocodazole. Samples for protein extraction were taken at the indicated times after release. Protein extracts were immuno-blotted and probed with anti-HA (12CA5) or anti-Clb2 as indicated. The lower panel shows budding curves for the two cultures. Clb2 protein levels serve as a marker for cell synchrony and the timing of mitosis.
The diagram shows the time course of protein expression under two conditions: with and without Nocodazole.

In the first condition, 

- α-HA and α-Clb2 proteins are expressed over time.

In the second condition, with Nocodazole:

- α-HA and α-Clb2 proteins are also expressed over time, with noticeable differences compared to the first condition.

A graph below the diagrams illustrates the percentage of budded cells over time:

- The solid line represents the control without Nocodazole, showing a peak in budded cells around 90 minutes.
- The dashed line represents the condition with Nocodazole, showing a delayed peak in budded cells compared to the control.

The x-axis represents time in minutes, ranging from 0 to 120, and the y-axis represents the percentage of budded cells, ranging from 0 to 100.
with appearance of the Clb2 mitotic cyclin near the beginning of mitosis. As the cells exit mitosis, and Clb2 is degraded, Bfa1 phosphorylation is dramatically reduced, reappearing again in the next cell cycle. Thus periodic Bfa1 phosphorylation is a normal cell cycle event.

Interestingly, the enhanced phosphorylation observed in nocodazole-arrested cells (Figure 5.5), does not appear to be a specific response to the spindle checkpoint. At around 60-70 minutes in an unperturbed cell cycle, the protein shows the same phosphorylation pattern as seen in the nocodazole-arrested culture, with the intensity of species VI and IV being enhanced (compare Figure 5.5, inset, with Figure 5.6, upper panel). Moreover, releasing the cells into nocodazole-containing medium did not alter the range of species observed (Figure 5.6, lower panels). However, in the presence of nocodazole, cells failed to exit mitosis, and neither Bfa1 phosphorylation nor Clb2 protein levels declined. Thus the SAC arrest induced by nocodazole appears to protract the normal mitotic phosphorylation of Bfa1.

5.2.5 Bfa1 phosphorylation in an apc2-8-induced metaphase arrest resembles that in a nocodazole-induced metaphase arrest

The experiments described above suggest that the phosphorylation of Bfa1 observed in nocodazole-arrested cells, is not specific to the SAC arrest. Instead, activation of the SAC leads to a protraction of the normal mitotic Bfa1 phosphorylation. To investigate whether Bfa1 undergoes specific changes in its phosphorylation status in response to SAC activation, cells were arrested in metaphase in the presence of nocodazole, or using an apc2-8 temperature-sensitive mutation, and the Bfa1 phosphorylation pattern compared. The apc2-8 mutation inactivates the APC/C, leading to a metaphase arrest similar to that caused by SAC activation.

Strains SLY105 (3HA.BFA1) and SLY116 (3HA.BFA1 apc2-8) were grown to mid-log phase at 25°C. The cultures were split, and grown for a further 3h at 25°C or 37°C in the presence or absence of nocodazole, before harvesting. Following protein extraction, the Bfa1 phosphorylation status was analysed by immuno-blotting. The wild-type strain shows
the usual mid-log phase Bfa1 phosphorylation pattern, at either 25°C or 37°C, with species III-VI being clearly detectable (Figure 5.7, lanes 1 and 3). This indicates that Bfa1 phosphorylation is not affected by heat-shock. As expected, incubation with nocodazole leads to an increase in the intensity of bands IV and VI (Figure 5.7, lanes 2 and 4). In the \textit{apc2-8} strain, there is a moderate increase in the intensity of band VI, even at 25°C (Figure 5.7, lane 5). This is not surprising, since the \textit{apc2-8} mutant is defective even at the permissive temperature. Addition of nocodazole leads to a more dramatic increase in the intensity of bands VI and IV at 25°C (Figure 5.7, lane 6). A similar enhancement of bands VI and IV is observed when cells are arrested at the restrictive temperature of 37°C. Thus the increased phosphorylation of Bfa1 observed in a nocodazole-induced metaphase arrest also occurs in an \textit{apc2-8}-induced metaphase arrest. Moreover, \textit{apc2-8} cells grown at 37°C in the presence of nocodazole show an identical phosphorylation pattern to those grown in the absence of nocodazole. So the pattern of Bfa1 phosphorylation is the same whether cells are arrested in metaphase by activation of the SAC or by mutational inactivation of the APC. This suggests that both of these arrests protract the normal mitotic phosphorylation, and hence presumably the function, of Bfa1.

5.2.6 The enhanced phosphorylation of Bfa1 during a nocodazole-induced SAC arrest is dependent on the Mad branch of the spindle checkpoint

The data presented above suggest that the protraction of Bfa1 phosphorylation observed in the presence of nocodazole, is not a specific response to SAC activation, but is a more general response to the metaphase arrest. This is consistent with the Bub2/Bfa1 pathway functioning in a general sense to couple mitotic exit to completion of anaphase (1.8.2.3). If this is true, then Bfa1 phosphorylation, and Bub2/Bfa1 pathway function in the presence of nocodazole could be dependent on the metaphase arrest established by the Mad branch of the SAC (1.7.2.3.1). It has already been shown that the Bub2/Bfa1 pathway is unable to establish a SAC arrest in the absence of Mad pathway function (Alexandru \textit{et al.}, 1999; Fesquet \textit{et al.}, 1999; Fraschini \textit{et al.}, 1999; Krishnan \textit{et al.}, 2000; Li, 1999). To investigate whether the enhanced phosphorylation of Bfa1 normally observed in nocodazole is also
Figure 5.7  Bfa1 shows the same phosphorylation pattern in cells arrested in metaphase following SAC activation or using an apc2-8 mutation. Strains SLY105 (3HA.BFAI) and SLY116 (3HA.BFAI apc2-8) were grown to mid-log phase at 25°C. The cultures were split, and grown for a further 3h at 25°C or 37°C in the presence or absence of nocodazole, before harvesting. Following protein extraction, an immuno-blot was prepared using anti-HA antibody.
dependent upon Mad checkpoint pathway function, the Bfa1 phosphorylation pattern was examined in nocodazole-arrested cells, in the presence or absence of MAD2. Strains SLY105 (3HA.BFA1) and SL35-8d (3HA.BFA1 mad2A) were grown to mid-log phase. Following addition of nocodazole, samples for protein extraction were taken at the times indicated. Immuno-blot analysis showed that the increased intensity of bands VI and IV normally associated with nocodazole-arrested cells was apparent in the wild-type strain after 60 minutes (Figure 5.8, left panel). This suggests that the Bfa1 phosphorylation changes which occur in the presence of nocodazole are not an immediate response to addition of the drug but reflect the accumulation of metaphase-arrested cells. The enhanced Bfa1 phosphorylation was still retained after 150 minutes in the wild-type cells (Figure 5.8, left panel). In contrast, addition of nocodazole had no effect on the mid-log phase Bfa1 phosphorylation pattern in the mad2A strain (Figure 5.8, right panel). The Bfa1 phospho-species VI and IV were not enhanced, and the unphosphorylated protein remained the dominant form. In conclusion, the presence of nocodazole does not lead to an immediate increase in Bfa1 phosphorylation, which might imply that the Bub2/Bfa1 checkpoint senses and responds to spindle damage directly. Instead, the enhanced Bfa1 phosphorylation appears to be a result of the metaphase delay enforced by the Mad branch of the SAC.

5.3 Discussion

5.3.1 Phosphorylation of Bfa1 may regulate Bub2/Bfa1 GAP activity

The data presented in this chapter demonstrate that Bub2 protein levels are not regulated across the cell cycle or following spindle checkpoint activation. Moreover, no post-translational modification was detected. These data are consistent with those presented by Fraschini et al., (1999), who also concluded that Bub2 is not a regulatory component of the putative Bub2/Bfa1 GAP.
Figure 5.8  The enhanced phosphorylation of Bfa1 following SAC activation is dependent on Mad2. Strains SLY105 (3HA.BFA1) and SL35-8d (3HA.BFA1 mad2Δ) were grown to mid-log phase (ML) and sampled. Nocodazole was added to each culture, and samples taken every 30 min for 150 min. Protein extracts were analysed by immunoblotting using anti-HA antibody.
Similarly, Bfa1 protein levels do not appear to be regulated during the cell cycle or in various cell cycle arrests. However, Bfa1 is phosphorylated in a cell cycle-dependent manner. Bfa1 phosphorylation is virtually absent during G1, but increases during the G2 phase to reach a maximum in late mitosis, before declining sharply as cells exit mitosis. No novel Bfa1 phosphorylated species were detected in the presence of nocodazole, but the mitotic Bfa1 phospho-forms were maintained. Thus Bfa1 is maximally phosphorylated in the presence of nocodazole, when the GAP is known to be required, suggesting that Bfa1 phosphorylation could regulate GAP activation (Figure 5.9). If so, the fact that Bfa1 is similarly phosphorylated during mitosis implies that the Bfa1/Bub2 GAP may be a normal component of late mitotic control. Treatment of cells with nocodazole and hence activation of the SAC, protracts the Bfa1 phosphorylation and, presumably, Bub2/Bfa1 function.

It is noteworthy that Bfa1 displays a slightly different phosphorylation pattern in a hydroxyurea-induced DNA damage checkpoint arrest to that observed in a nocodazole-induced spindle assembly checkpoint arrest, although both checkpoints arrest cells in metaphase by preventing Pds1 degradation. However, the mechanisms used to enforce these arrests are very different, and it is not surprising to find biochemical differences such as the Bfa1 phosphorylation status. These differences will be discussed in more detail in Chapter 10.

The fact that Bfa1 phosphorylation is dependent upon the presence of Bub2 is consistent with phosphorylation being important for GAP function, and hence checkpoint function. The requirement for Bub2 may be because the relevant kinases recognise the Bub2/Bfa1 heterodimer, although only Bfa1 is actually phosphorylated. Alternatively, the SPB localisation of Bfa1, which requires Bub2 (Pereira et al., 2000), may be necessary for Bfa1 phosphorylation. This would imply that the Bfa1 kinase is also present at the SPB.
Figure 5.9  Model illustrating how Bfa1 phosphorylation may regulate the putative Bub2/Bfa1 two-component GAP. Phosphorylation of Bfa1 during mitosis activates the GAP and prevents activation of Tem1 and the MEN. In late anaphase the Bub2/Bfa1 GAP is inactivated, presumably by activation of its phosphatase or inhibition of its kinase, leading to MEN activation, mitotic exit and cytokinesis. If cells fail to complete anaphase (for example following SAC activation or mutational inactivation of the APC/C), Bfa1 phosphorylation is protracted and Bub2/Bfa1 function is maintained.
5.3.2 Activation of the Bub2/Bfa1 pathway in metaphase-arrested cells

It is likely that the protraction of Bfa1 phosphorylation following microtubule disruption is necessary to maintain GAP activity, and prevent MEN activation. However, this enhanced phosphorylation does not appear to be a specific response to spindle damage. The data presented in this chapter show that the Bfa1 phosphorylation pattern is the same when cells are arrested in metaphase in the presence of nocodazole or using an \textit{apc2-8} mutation. This suggests that Bfa1 phosphorylation is protracted in a variety of metaphase arrests, and is consistent with the Bub2/Bfa1 pathway functioning to couple mitotic exit to completion of anaphase (1.8.2.3).

The enhanced phosphorylation of Bfa1 observed following SAC activation is dependent upon the metaphase arrest enforced by the Mad checkpoint pathway, since it is abolished following deletion of \textit{MAD2}. This suggests that the metaphase delay imposed by the Mad2 branch of the SAC is required to protract Bub2/Bfa1 pathway function following spindle damage. This is consistent with a report by Krishnan \textit{et al.}, (2000), who proposed that the Mad2 branch of the spindle checkpoint establishes a SAC delay, which the Bub2 branch is involved in maintaining. Thus both pathways may complement each other. The Mad branch initiates a delay which protracts Bfa1 phosphorylation and Bub2/Bfa1 function. In turn, the Bub2/Bfa1 checkpoint pathway ensures that the MEN does not become active, since the resultant activation of APC/C\textsuperscript{Cdh1} could lead degradation of the proteins normally targeted by APC/C\textsuperscript{Cdc20}, alleviating the Mad-dependent metaphase arrest (1.8.2.3; see also Chapter 10) (Krishnan \textit{et al.}, 2000). These data imply that rather than forming a branch of the SAC, Bub2 and Bfa1 are components of a more general checkpoint which is required to prevent MEN activation when anaphase is delayed, and as such are required to maintain a SAC arrest.
Chapter 6

Cdc5 is partially required for Bfa1 phosphorylation

6.1 Introduction

The data presented in the previous chapter show that Bfa1 is subject to cell cycle-dependent phosphorylation. Since the GAP does not appear to be controlled by other means (for example modification of Bub2 [5.2.1], or regulated heterodimer formation [4.2.5]), the phosphorylation of Bfa1 could control GAP activity. In order to investigate the role of Bfa1 phosphorylation, and to understand how this phosphorylation is controlled during mitosis and following spindle checkpoint activation, it is important to identify the kinases responsible. Since the phosphorylation of Bfa1 is maximal during mitosis, the kinase is likely to be a cell cycle-regulated kinase which is active during mitosis. Several known kinases fit these criteria, including Cdc28-Clb kinase, Cdc5, and Dbf2. In addition, since Bfa1 is a component of the spindle assembly checkpoint, and is phosphorylated following SAC activation, it could also be a substrate of Mps1, which is required for SAC function. There is some disagreement regarding the role of Mps1 in the Bub2 checkpoint pathway, since one study found that Mps1 acted upstream of both the Mad and Bub2 checkpoint pathways (Fesquet et al., 1999), whilst another study suggested that Bub2/Bfa1 were not involved in the Mps1-induced spindle arrest (Li, 1999). Any involvement of Mps1 in Bfa1 phosphorylation should shed some light on this issue.

6.2 Results

6.2.1 Mps1 is not required for Bfa1 phosphorylation

To assess whether Mps1 function is required for Bfa1 phosphorylation, Bfa1 SDS-PAGE mobility was examined in an mps1-1 temperature-sensitive strain. To ensure that any
changes in the Bfa1 phosphorylation pattern were genuinely due to the loss of Mps1 function, the cells had to be uniformly arrested in mitosis, when Bfa1 phosphorylation is maximal. Unfortunately, at the restrictive temperature, mpsl-1 mutant cells are defective in SAC function and fail to arrest in metaphase in response to nocodazole. However, since the phosphorylation pattern of Bfa1 in a nocodazole-induced SAC arrest reflects its normal phosphorylation at that stage in mitosis, cells arrested in metaphase by other means, for example using an apc2-8 temperature-sensitive mutation display the same phosphorylation pattern (5.2.5). An apc2-8 mutation was therefore introduced into a 3HA.BFA1 mpsl-1 strain, and the metaphase phosphorylation of Bfa1 in the absence of Mps1 function was examined.

Strains SLY116 (3HA.BFA1 apc2-8) and SL31-10b (3HA.BFA1 apc2-8 mpsl-1) were grown to mid-log phase at 25°C, and then grown for a further 3h at 25°C or 37°C in the presence or absence of nocodazole, before harvesting. The nocodazole-treated samples serve as a control, since Mps1 is a SAC-activated kinase, and its activity towards Bfa1 at the permissive temperature might be dependent upon SAC function. Following protein extraction, the Bfa1 phosphorylation status was analysed by immuno-blotting. In the 3HA.BFA1 apc2-8 strain, the Bfa1 phosphorylated species I-VI were present at 25°C (Figure 6.1, lanes 1-4). Unusually for mid-log phase cells, band VI was partially enhanced. This probably reflects the metaphase delay observed in apc2-8 mutant cells, even at the permissive temperature. As expected, the intensity of this band was further enhanced in the presence of nocodazole at 25°C, or following incubation at the restrictive temperature of 37°C. In the 3HA.BFA1 apc2-8 mpsl-1 strain, the Bfa1 phosphorylation pattern at 25°C or 37°C was the same as in the MPS1 control (Figure 6.1, compare lanes 5 and 6 with lanes 1 and 2). Moreover, in the presence of nocodazole, the relative proportions of the Bfa1 phosphorylated species in the mpsl-1 strain at 25°C or 37°C were identical (Figure 6.1, lanes 7 and 8), and again corresponded to those in the MPS1 control strain (Figure 6.1, compare lanes 7 and 8 with lanes 3 and 4). Thus the introduction of the mpsl-1 mutation had no effect on the Bfa1 phosphorylation pattern at the restrictive temperature, indicating that Mps1 is not required for Bfa1 phosphorylation.
Figure 6.1 Mps1 is not required for Bfa1 phosphorylation. Strains SLY116 (3HA.BFA1 apc2-8) and SL31-10b (3HA.BFA1 apc2-8 mps1-1) were grown to mid-log phase at 25°C. The cultures were split, and grown for a further 3h at 25°C or 37°C in the presence or absence of nocodazole. Following proteins extraction, the Bfa1 phosphorylation status was analysed by immuno-blotting, using anti-HA antibody (12CA5).
6.2.2 Cdc5, but not Cdc15 or Dbf2, is partly required for Bfa1 phosphorylation

To investigate whether any of the known MEN kinases act on Bfa1, the Bfa1 phosphorylation pattern was examined in strains harbouring temperature-sensitive mutations in CDC5 (cdc5 [msd2-1]), CDC15 (cdc15-1) or DBF2 (dbf2-2). Strains SLY109 (3HA.BFA1 cdc5), J15HB1-1d (3HA.BFA1 cdc15-1), and JD2HB1-56d (3HA.BFA1 dbf2-2) were grown to mid-log phase at 25°C, and then shifted to the restrictive temperature of 37°C in the presence or absence of nocodazole for 3h. The nocodazole-treated samples serve as a control, since they arrest uniformly in metaphase, ensuring that any differences between the mutants are due to a genuine biochemical effect, rather than subtle differences in their arrest points. Immuno-blotting showed that the cdc15 and dbf2 mutant strains arrested at 37°C with highly phosphorylated Bfa1 (Figure 6.2, lanes 2 and 6). Moreover, the Bfa1 phosphorylation pattern in nocodazole was identical at 25°C and 37°C (Figure 6.2, compare lanes 3 and 4 or 7 and 8). Thus mutating Cdc15 or Dbf2 has no effect on the pattern of Bfa1 phosphorylation.

In marked contrast, mutating Cdc5 had clear effects on the pattern of Bfa1 phosphorylation. At the restrictive temperature, the cdc5 mutant strain showed the same Bfa1 phosphorylation pattern as observed in the mid-log phase cells (Figure 6.3, lanes 1 and 2). Thus in the absence of functional Cdc5, the late mitotic enhancement of bands VI and IV did not occur. Furthermore, whilst Bfa1 showed the expected phosphorylation pattern in nocodazole at 25°C, with bands VI and IV enhanced relative to the mid-log phase culture, at 37°C the enhanced phosphorylation of bands VI and IV was lost (Figure 6.3, lanes 3 and 4). The intensity of bands I and II also decreased dramatically at 37°C (Figure 6.3, lanes 3 and 4). Thus Cdc5 seems to be required for the change in the Bfa1 phosphorylation pattern which occurs in late mitosis, namely the increased intensity of phosphorylated species VI and IV, and I and II.
Figure 6.2  

Cdc15 and Dbf2 are not required for Bfa1 phosphorylation. Strains J15HB1-1d (3HA.BFA1 cdc15-1) and JD2HB1-56d (3HA.BFA1 dbf2-2) were grown to mid-log phase at 25°C. The cultures were split, and grown for a further 3h at 25°C or 37°C in the presence or absence of nocodazole. The cells were harvested and subjected to protein extraction and immuno-blot analysis using anti-HA antibody (12CA5).
Figure 6.3  Cdc5 partly controls Bfa1 phosphorylation. Strain SLY109 (3HA-BFA1 cdc5'' [msd2-1]) was grown to mid-log phase at 25°C. The culture was split, and either harvested immediately, grown for a further 3h at 37°C or grown for a further 3h in the presence of nocodazole at 25°C or 37°C. The cells were harvested and subjected to protein extraction and immuno-blot analysis using anti-HA antibody (12CA5).
6.2.3 Cdc5 is required for the cell cycle-dependent phosphorylation of Bfa1

The above experiments indicate that Cdc5 is required to generate Bfa1 phosphorylated species VI and IV. To determine whether mutating Cdc5 affects the cell cycle-dependent phosphorylation of Bfa1 in a similar manner, a Bfa1 immuno-blot was prepared from a synchronous culture of strain SLY109 (3HA.BFA1 cdc5), grown at 25°C or 37°C. Strain SLY109 was grown to mid-log phase, arrested for 3h with α-factor, and released into fresh medium at 25°C. Cells were allowed to bud before shifting to the restrictive temperature, since this was found to improve the synchrony. Once budding had been initiated (30 minutes after release), the culture was split, with one half being shifted to 37°C. Samples for protein extraction were taken at the indicated times after release. Immuno-blot analysis showed that, at 25°C, Bfa1 is phosphorylated with similar kinetics to a wild-type culture, with species VI, V, IV and III present after 80 min, although the latter three species are not well defined in this experiment (Figure 6.4, upper panel). However, at 37°C, the Bfa1 phosphorylation pattern is clearly affected by the cdc5 mutation, with the unphosphorylated protein being the dominant form (Figure 6.4, middle panel). Three lower mobility species are present after 80 min, but at a relatively low intensity. Moreover, the mobility of these species is higher than those observed at 25°C, indicating that the formation of the lower mobility species has been compromised in the absence of Cdc5 function (Figure 6.4, middle panel). The highest mobility band may correspond to species VI, but, although this band is detectable at 37°C, its intensity is not enhanced in late mitosis, consistent with the result in Figure 6.3. The presence of species VI, even at the restrictive temperature, does not preclude Cdc5 being responsible for this Bfa1 phospho-form, since the msd2-1 allele of CDC5 which was used probably retains some function.

The lowest mobility species I and II were not detectable at 25°C or 37°C (Figure 6.4). Since the cdc5 allele is defective even at 25°C this may indicate that Cdc5 is also required to generate these phospho-forms. However, these bands are often quite faint, and their absence may simply reflect a failure to detect them.
Figure 6.4  **Cdc5 controls Bfa1 phosphorylation during the cell cycle.** Strain SLY109 (3HA.BFA1 cdc5) was grown to mid-log phase at 25°C, synchronised with α-factor for 3h and released into fresh media at either 25°C (upper panel) or 37°C (middle panel). Samples were removed at the times indicated and an immuno-blot prepared using anti-HA (12CA5) antibody. The lower panel shows budding curves for the two cultures.
6.2.4 Cdc5 is required to maintain complete Bfa1 phosphorylation in nocodazole-arrested cells

Cdc5 is known to function during mitosis, but has no known role in the SAC. However, Figure 6.3 indicates that the Bfa1 phosphorylation in nocodazole is compromised in the absence of Cdc5 function. To investigate whether Cdc5 is required to maintain Bfa1 phosphorylation following SAC activation, strain SLY109 (3HA.BFA1 cdc5) was grown to mid-log phase at 25°C and arrested for 3h using nocodazole. Half of the culture was then shifted to 37°C, and the effect on Bfa1 phosphorylation analysed by immuno-blotting. At the permissive temperature, species I-VI were present throughout the experiment, and the expected enhancement of species VI and IV was evident (Figure 6.5, lanes 1-4). However, once the cells had been shifted to the restrictive temperature, the intensity of the unphosphorylated protein band increased whilst that of species VI and IV decreased. In addition, species I and II were less prominent at 37°C (Figure 6.5, lanes 5-8). Thus Cdc5 function is required to retain complete Bfa1 phosphorylation during a nocodazole arrest.

As discussed in Chapter 5, the phosphorylation pattern of Bfa1 in nocodazole-arrested cells reflects its normal phosphorylation at that stage in mitosis. Consequently, cells arrested in metaphase by other means, for example using an apc2-8 temperature-sensitive mutation, display the same phosphorylation pattern (5.2.5). To examine whether this pattern was affected by mutating Cdc5, strains SLY116 (3HA.BFA1 apc2-8) and JB5A1-51C (3HA.BFA1 apc2-8 cdc5 [msd2-1]) were grown to mid-log phase at 25°C, before being arrested at 37°C in the presence or absence of nocodazole. In the presence of wild-type CDC5, the metaphase-arrested cells showed the expected range of bands, with species VI and IV being prominent (Figure 6.6, lanes 3 and 4). However, in the absence of functional Cdc5, the intensity of the unphosphorylated protein band increased, whilst that of bands VI and IV decreased (Figure 6.6, lanes 7 and 8, compare with lanes 3 and 4). Thus Cdc5 is necessary for the enhancement of bands VI and IV which occurs during these metaphase arrests, and during mitosis of an unperturbed cell cycle.
Figure 6.5  **Cdc5 is required to maintain Bfa1 phosphorylation in nocodazole-arrested cells.** Strain SLY109 (3HA.BFA1 cdc5) was grown to mid-log phase at 25°C and treated with nocodazole for 3h. The culture was sampled (0 min), and split into two halves, with one half being transferred to 37°C. Each culture was sampled at the times indicated. Following protein extraction, an immuno-blot was prepared and probed with anti-HA antibody (12CA5)
Figure 6.6  Cdc5 is partly required for Bfa1 phosphorylation in an *apc2-8*-induced metaphase arrest. Strains SLY116 (3HA*BFA1 apc2-8) and JB5A1-51C (3HA*BFA1 apc2-8 cdc5) were grown to mid-log phase at 25°C, and then grown for a further 3h at 25°C or 37°C in the presence or absence of nocodazole before harvesting. Following protein extraction, the phosphorylation status of Bfa1 was analysed by immuno-blotting, using anti-HA (12CA5) antibody.
6.2.5  *cdc5* mutants do not display high Dbf2 kinase activity in metaphase-arrested cells

If Cdc5-dependent phosphorylation of Bfa1 is important for GAP function, then *cdc5* mutant cells might be predicted to share the phenotype of *bub2Δ* or *bfa1Δ* strains. However, unlike Bub2 and Bfa1, Cdc5 is also required for mitotic exit, and the standard assays for SAC function, such as the re-budding assay (4.2.2), are consequently not suitable. One assay for Bub2/Bfa1 pathway function used extensively in this study, is the inhibition of Dbf2 kinase activity in metaphase-arrested cells treated with nocodazole (3.1, 3.2.1). Abrogation of the Bub2/Bfa1 pathway leads to maximal activation of Dbf2 kinase in the presence or absence of nocodazole. To examine whether mutations in *CDC5* also abolished the SAC-induced inhibition of Dbf2, Dbf2 kinase activity was assessed in strains KTM208 (*apc2-8*) and J5AB1-6b (*apc2-8 cdc5 [msd2-l]*), arrested at the restrictive temperature in the presence or absence of nocodazole. As expected, the *apc2-8* strain arrested in metaphase with increased levels of Dbf2 kinase relative to the α-factor control, and this activity was completely abolished in the presence of nocodazole (Figure 6.7, lanes 1-3). In the absence of functional Cdc5, Dbf2 kinase activity was completely abolished (Figure 6.7, lanes 4-6) Thus removing Cdc5 function did not lead to an increase in Dbf2 kinase levels, as is observed following loss of Bub2 or Bfa1 function (Figures 3.1, 4.1). However, this result is not unexpected, since Cdc5 is known to be required for Dbf2 kinase activation (Figure 3.1).

6.2.6  *Cdc5* phosphorylates Bfa1 *in vitro*

The experiments detailed above show that Cdc5 is required for complete phosphorylation of Bfa1. However, this effect could be indirect. To investigate whether Cdc5 phosphorylates Bfa1 directly, it was necessary to develop an *in vitro* kinase assay. GST-Bfa1 purified from *E. coli* was used as the kinase assay substrate. Purifying large quantities of the full-length fusion protein proved difficult, due to the apparent instability of the protein in *E. coli*. The final preparation of purified substrate consisted of a mixture of full-length protein (20%) and a prominent N-terminal degradation product (80%) (Figure 6.8).
Figure 6.7  

cdc5 mutants do not display high Dbf2 kinase activity in metaphase-arrested cells. Strains KTM208 (apc2-8) and J5AB1-6b (apc2-8 cdc5), were grown to mid-log phase at 25°C and arrested with α-factor (αF), nocodazole at 37°C (Noco @37°C) or at 37°C (37°C) for 3h. Dbf2 was immunoprecipitated from protein extracts with anti-Dbf2 antibody (Toyn and Johnston, 1994) and Dbf2 kinase activity assayed.
Figure 6.8  GST-Bfa1 preparation for use as a substrate in in vitro kinase assays. GST-Bfa1 was purified as described (2.6.6). 5μg eluted protein was analysed using SDS-PAGE and detected using Coomassie staining or by immuno-blotting with anti-GST antibody. The full length GST fusion protein (GST-Bfa1), and the N-terminal proteolytic fragment (*) are labelled.
Several kinases were tested for their ability to phosphorylate Bfa1 \textit{in vitro}, including Cdc5, Cdc15, and Dbf2. Cdc28 was also examined and this is considered in the next chapter. Epitope-tagged versions of the kinases, expressed at endogenous levels, were immunoprecipitated from protein extracts prepared from mid-log phase cell cultures, and kinase assays performed using GST-Bfa1 as the substrate (2.7.2). Phosphorylated GST-Bfa1 was analysed using SDS-PAGE and autoradiography. Cdc5 phosphorylates full-length Bfa1, and the N-terminal cleavage fragment (Figure 6.9, lane 4). In contrast, Cdc15 and Dbf2 do not display kinase activity towards this substrate (Figure 6.9, lanes 5 and 6). These \textit{in vitro} data correlate with the situation \textit{in vivo} (Figures 6.2, 6.3), and suggest that Cdc5 phosphorylates Bfa1 directly. Cdc5 appears to phosphorylate the N-terminal proteolytic fragment more strongly than full-length GST-Bfa1 (Figure 6.9). In fact this merely reflects the ratios of these two purification products (4:1), although it also indicates that the Cdc5 phosphorylation sites may reside in the Bfa1 N-terminus.

To confirm the ability of Cdc5 to phosphorylate Bfa1, GST-Bfa1 was used as a substrate in kinase assays performed using Cdc5 purified from baculovirus (a gift from Eliot Randle, Prolifix, Oxford, UK). Again, Cdc5 phosphorylated both full length Bfa1, and the N-terminal cleavage fragment (Figure 6.10). These data show that Cdc5 can directly phosphorylate Bfa1.

6.3 Discussion

The data presented in this chapter show that Cdc5 is partially required for Bfa1 phosphorylation. During mitosis, as Bfa1 becomes maximally phosphorylated, immunoblot analysis shows that the intensity of the band corresponding to the unphosphorylated protein decreases, whilst the intensity of bands VI and IV increases (Figure 5.6). The same changes in the Bfa1 phosphorylation pattern are observed in cells arrested in metaphase using nocodazole, or an \textit{apc2-8} temperature-sensitive mutation. However, in the absence of functional Cdc5 this enhanced phosphorylation is lost, indicating that Cdc5 is required for the mitotic phosphorylation of Bfa1. This role is specific to Cdc5, since mutating the other MEN kinases Cdc15 and Dbf2, had no effect on Bfa1 phosphorylation. Similarly, the SAC
Figure 6.9  Cdc5 and Cdc28 phosphorylate Bfa1 in vitro. Mid-log phase cultures of strains YD11-97 (CDC5.3HA), SLJ23 (CDC15.3HA), NJW007 (3HA.DBF2) and CDC28-HA (CDC28.HA), expressing endogenous levels of epitope-tagged kinase, were harvested, extracted, and the kinases immunoprecipitated using anti-HA (12CA5) antibody. Kinase assays using GST-Bfa1 as substrate were performed and analysed as described (2.7.2) (upper panel). The full length GST fusion protein (GST-Bfa1), and the N-terminal proteolytic fragment (*) are labelled. Additional kinase assays, performed using myelin basic protein as a substrate, provide a positive control (lower panel).
Figure 6.10  Purified Cdc5 phosphorylates Bfa1 in vitro. Kinase assays were performed using 6His-Cdc5 purified from baculovirus (a gift from Eliot Randle, Prolifix, Oxford, UK), and GST-Bfa1 as substrate. Phosphorylated GST-Bfa1 was analysed using SDS-PAGE and autoradiography. The full length GST fusion protein (GST-Bfa1), and the N-terminal proteolytic fragment (*) are labelled.
kinase Mps1 is not required for Bfa1 phosphorylation. Thus, although Mps1 may act
upstream of the Bub2/Bfa1 checkpoint (Fesquet et al., 1999), this effect is likely to be
indirect.

Cdc5 also phosphorylates Bfa1 in vitro, supporting the idea that Cdc5 is a Bfa1 kinase in
vivo. The in vitro phosphorylation of Bfa1 is not dependent on the presence of Bub2, which
contrasts with the situation in vivo. This does not invalidate the result, since it is rare that a
reaction in vitro displays the same specificity as the in vivo system. Moreover, the Bub2-depen-
dency of Bfa1 phosphorylation in vivo may be due to its role in localising Bfa1 to the
SPBs. Taken together, the in vivo and in vitro data suggest that Bfa1 is a substrate of Cdc5.

Cdc5 is a cell cycle-regulated kinase, which has no known function in the spindle assembly
checkpoint. However, since Bfa1 phosphorylation is a normal cell cycle event which is
protracted in nocodazole (Figure 5.6) there is no reason to suppose that its kinase(s) would
be specific to the SAC, provided their activity is maintained following SAC activation.
Intriguingly, one difference between the metaphase arrests induced by the DNA damage
checkpoint and the spindle assembly checkpoint, is that Cdc5 is down-regulated by the
former but not the latter (Fesquet et al., 1999; Sanchez et al., 1999). Consistent with this, in
hydroxyurea-arrested cells, Bfa1 does not show the enhanced phosphorylation of bands VI
and IV which is dependent on Cdc5, whereas in a nocodazole arrest, Bfa1 is maximally
phosphorylated. If Cdc5-dependent phosphorylation of Bfa1 is required for its function, this
would preclude a role for Bub2/Bfa1 in the DNA damage checkpoint (1.8.2.3).

It is important to establish the role of Bfa1 phosphorylation during the cell cycle. One way
of doing this is to find mutants which affect the phosphorylation, and study their effects on
Bub2/Bfa1 pathway function. Unfortunately, the multi-functional nature of Cdc5 makes
this difficult. One assay for Bub2/Bfa1 SAC function is the re-budding assay, which
assesses the ability of a strain to maintain a cell-cycle arrest following exposure to
nocodadazole. Strains defective in SAC components are unable to arrest, and continue to
re-bud. However, at the restrictive temperature, cdc5 mutants cannot re-bud, regardless of
SAC function, since they arrest in telophase due to a defect in mitotic exit. Another assay
used in this study to assess Bub2 pathway function is the ability of metaphase-arrested cells to down-regulate Dbf2 kinase activity in response to nocodazole. In the absence of BUB2 or BFA1, Dbf2 kinase levels are maximal in metaphase, even in the presence of nocodazole. However in the absence of Cdc5 function, Dbf2 kinase is completely abolished, since Cdc5 is required to activate Dbf2 (Figure 3.1). Mutating Cdc5 may abrogate the Bub2/Bfa1 checkpoint, but it does not lead to re-budding or increased Dbf2 kinase activation, because MEN activation also requires Cdc5 function. The best way to understand the function of Bfa1 phosphorylation by Cdc5 would be to map and mutate the Bfa1 phosphorylation sites, and analyse the phenotype of the non-phosphorylatable mutant.

Since loss of Cdc5 function only affects specific Bfa1 phospho-forms, there must be at least one additional kinase which can phosphorylate Bfa1. One good candidate is Cdc28, since the timing of Cdc28 kinase activity corresponds to that of Bfa1 phosphorylation. The role of Cdc28 in Bfa1 phosphorylation is discussed in the next chapter.
Chapter 7

Cdc28 is required for Bfa1 phosphorylation

7.1 Introduction

Chapter 6 shows that Cdc5 partly controls Bfa1 phosphorylation, in particular species IV and VI, and possibly also I and II. However, there is evidently at least one other additional kinase which acts on Bfa1. One obvious candidate could be Cdc28, since mitotic Cdc28 kinase activity (as indicated by Clb2 protein levels) increases as the phosphorylated forms of Bfa1 begin to accumulate (Figure 5.6). This chapter examines the role of Cdc28 in controlling Bfa1 phosphorylation.

7.2 Results

7.2.1 Cdc28 phosphorylates Bfa1 in vitro

The ability of Cdc28 to phosphorylate GST-Bfa1 in vitro was investigated, as described in Chapter 6. Strain CDC28-HA (CDC28.HA) was grown to mid-log phase, and Cdc28-HA was immuno-precipitated from protein extracts. Kinase assays were performed using GST-Bfa1 as the substrate (2.7.2), and phosphorylated GST-Bfa1 was analysed using SDS-PAGE and autoradiography. Cdc28 phosphorylates full-length GST-Bfa1 (Figure 6.9, lane 7), although it does not phosphorylate the N-terminal proteolytic fragment. This suggests that Cdc28 may also be a Bfa1 kinase.

7.2.2 Bfa1 phosphorylation in vivo requires Cdc28

Cdc28 is required for progression into S phase, and most conditional mutant alleles of Cdc28 consequently arrest in G1. In order to investigate whether Cdc28 is required for
Bfa1 phosphorylation during G2/M phase using a cdc28 mutant strain, it is therefore necessary to synchronise the cells, and allow them to complete S phase before shifting to the restrictive temperature. The CDC28 allele used was cdc28-4, which is known to be defective in Cdc28 kinase activity at the restrictive temperature (Reed et al., 1985). Strain SL36-4a (3HA.BFA1 cdc28-4) and control strain SLY105 (3HA.BFA1) were synchronised in G1 and released into media containing nocodazole at 25°C. The presence of nocodazole ensures that the cells will arrest at a point when Bfa1 is normally maximally phosphorylated. Passage through S phase was deemed to have taken place once the majority of cells (>80%) had produced small buds. At this stage, half of each culture was shifted to the restrictive temperature of 37°C. Once the cells (>80%) had entered mitosis, and arrested due to the presence of nocodazole, the cultures were harvested and Bfa1 phosphorylation analysed by immuno-blotting. As expected, in the control culture, Bfa1 phosphorylation was absent during the α-factor arrest, but high in the nocodazole-arrested cells at 25°C and 37°C (Figure 7.1, lanes 1-3). The phosphorylation is reduced slightly at 37°C, which may be due to a heat-shock effect, but the lower mobility species VI-III are clearly visible. In contrast, in the cdc28-4 strain at 37°C, the lower mobility species are only present as a background smear, and the unphosphorylated protein band is nearly as intense as in the α-factor-arrested sample (Figure 7.1, lanes 4-6). Thus Cdc28 function seems to be required for Bfa1 phosphorylation.

7.2.3 Cdc28 is required to maintain Bfa1 phosphorylation in nocodazole-arrested cells

Unfortunately, the cell cycle synchrony exhibited by the 3HA.BFA1 cdc28-4 strain in an α-factor block and release experiment was not good enough to examine the cell cycle-dependent phosphorylation of Bfa1 in the absence of Cdc28 function. Instead, to confirm the involvement of Cdc28 in Bfa1 phosphorylation, the effects of eliminating Cdc28 function in nocodazole-arrested cells were studied. Strain SL36-4a (3HA.BFA1 cdc28-4) was grown to mid-log phase at 25°C, sampled, and arrested using nocodazole for 3h. After sampling, half of the culture was shifted to 37°C and samples from both cultures taken every hour for a further 3h. Following protein extraction, Bfa1 protein status was examined
Figure 7.1 Cdc28 is required for Bfa1 phosphorylation. Strains SLY105 (3HA.BFA1) and SL36-4a (3HA.BFA1 cdc28-4) were grown to mid-log phase at 25°C, and synchronised in G1 by incubating with 3.5 μg/ml α-factor for 2.5h. The cultures were sampled, and released into fresh medium at 25°C, in the presence of nocodazole. When 80% of cells had initiated budding, each culture was split, and one half shifted to the restrictive temperature of 37°C. Once >80% of the cells had arrested as dumbbells, they were harvested, and subjected to protein extraction and immuno-blot analysis.
by immuno-blotting. At the permissive temperature, the 3HA.BFA1 cdc28-4 strain shows
the expected mid-log phase pattern of Bfa1 phosphorylation (Figure 7.2, lane 1). The usual
changes in this pattern were observed following the SAC-induced metaphase arrest, namely
the enhanced intensity of species VI and IV (Figure 7.2, lane 2). At the permissive
temperature, these Bfa1 phosphorylated species were present throughout the experiment
(Figure 7.2 lanes 3-7). However, when the arrested cells were shifted to the restrictive
temperature of 37°C, the phosphorylation was almost completely abolished (Figure 7.2,
lanes 6-9). Even a longer exposure failed to reveal the usual phospho-species (Figure 7.2,
lower panel) Thus the maintenance of Bfa1 phosphorylation in nocodazole is dependent on
Cdc28 function.

7.2.4 Bfa1 phosphorylation is not affected following deletion of CLB1, CLB2 or
CLB5

The data presented above suggest that Cdc28 is required for Bfa1 phosphorylation. Since
Cdc28 kinase activity is dependent upon the presence of specific cyclin subunits, deletion
of the cyclin which controls Bfa1 phosphorylation might affect both the phosphorylation
and function of Bfa1. To investigate this possibility, Bfa1 phosphorylation was examined in
cells deleted for CLB1, CLB2, or CLB5. These cyclins were prime candidates, since the
increase in Clb1 and Clb2 protein levels occurs at the time when Bfa1 phosphorylation is
initiated (Figure 5.6), whilst Clb5 has been shown to negatively regulate mitotic exit
(1.8.2.1) (Shirayama et al., 1999). Strains SLY105 (3HA.BFA1), SL29-1a (3HA.BFA1
clb1Δ), SL26-1a (3HA.BFA1 clb2Δ) and SL18-10c (3HA.BFA1 clb5Δ) were grown to mid-
log phase, harvested, and Bfa1 phosphorylation status assessed by protein extraction and
immuno-blotting. Deletion of CLB1, CLB2 or CLB5 had no effect on the Bfa1
phosphorylation (Figure 7.3). Thus, none of these proteins are individually required to
phosphorylate Bfa1 in mid-log phase cells.
Figure 7.2  Cdc28 is required to maintain Bfa1 phosphorylation in nocodazole.
Strain SL36-4a (3HA.BFA1 cdc28-4) was grown to mid-log phase at 25°C, sampled, and arrested in metaphase by addition of nocodazole. The culture was sampled, split, and one half shifted to the restrictive temperature of 37°C. Samples were taken at the indicated times, and subjected to protein extraction and immuno-blot analysis to determine the Bfa1 phosphorylation status. Two different exposures are shown (upper and lower panels).
Figure 7.3 Deleting CLB1, CLB2 or CLB5 has no effect of Bfa1 phosphorylation. Strains SLY105 (3HA.BFA1), SL29-1a (3HA.BFA1 clb1Δ), SL26-1a (3HA.BFA1 clb2Δ) and SL18-10c (3HA.BFA1 clb5Δ) were grown to mid-log phase, harvested, and subjected to protein extraction and immuno-blotting.
7.2.5 Deletion of CLB2 or CLB5 in cdc28-4 mutant cells alters the phosphorylation pattern of Bfa1

The cyclins are known to display functional redundancy, which may explain why the deletion of individual cyclins does not affect the phosphorylation of Bfa1. However, in the presence of the cdc28-4 allele, when Cdc28 kinase activity is already compromised, the effects of deleting individual cyclins are exacerbated, and can be studied (Segal et al., 1998). To try to identify the cyclin required to phosphorylate Bfa1, Bfa1 SDS-PAGE mobility was assessed in a 3HA.BFA1 cdc28-4 strain containing deletions in CLB1, CLB2, CLB3 or CLB5. Strains SL36-4a (3HA.BFA1 cdc28-4 clb1A), SL38-8a (3HA.BFA1 cdc28-4 clb2A), SL39-4c (3HA.BFA1 cdc28-4 clb3A), SL40-15d (3HA.BFA1 cdc28-4 clb3A) and SL41-9d (3HA.BFA1 cdc28-4 clb5A) were grown to mid-log phase at the permissive temperature of 25°C, harvested, and protein extracts analysed by immuno-blotting. The expected Bfa1 phosphorylated species VI-III were present in the mid-log phase culture of the cdc28-4 strain and following deletion of CLB1 or CLB3 (Figure 7.4, lanes 1, 2 and 4). Thus deletion of CLB1 or CLB3 does not affect Bfa1 phosphorylation, even when Cdc28 kinase activity is compromised.

In contrast, in the cdc28-4 clb2A strain, the Bfa1 phosphorylated species V-III could no longer be detected by immuno-blotting (Figure 7.4, lane 3). This suggests that Clb2 may be involved in promoting Bfa1 phosphorylation. However, this result should be treated with caution. Firstly, Bfa1 protein levels are slightly lower in this strain than the others, although the signal intensity should still be sufficiently high to allow detection of species V-III. Reducing the exposure time and hence the signal intensity does not prevent detection of the lower mobility species in the remaining cdc28-4 clbA strains (Figure 7.4, lower panel), suggesting that this effect is specific to the clb2A mutant. Secondly, since these cells are defective in cytokinesis, and fail to separate following sonication (data not shown), it was not possible to perform FACS analysis, which requires a single cell suspension. Therefore, the possibility that this reduction in Bfa1 phosphorylation is due to the cells being delayed in G1 cannot be eliminated. Nevertheless, since none of the other cyclins tested showed any defects in Bfa1 phosphorylation, Clb2 is the prime candidate. Unfortunately, the
Figure 7.4 Deletion of *CLB2* or *CLB5* when Cdc28 function is compromised, reduces or increases Bfa1 phosphorylation respectively. Strains SL36-4a (3HA.BFA1 cdc28-4), SL38-8a (3HA.BFA1 cdc28-4 clb1Δ), SL39-4c (3HA.BFA1 cdc28-4 clb2Δ), SL40-15d (3HA.BFA1 cdc28-4 clb3Δ) and SL41-9d (3HA.BFA1 cdc28-4 clb5Δ) were grown to mid-log phase at 25°C, harvested, and subjected to protein extraction and immuno-blotting. Two different exposures are shown (upper and lower panels).
cytokinesis defect exhibited by these cells precluded the use of re-budding assays to analyse spindle checkpoint function, and consequently any functional defects arising from the reduced phosphorylation of Bfa1 could not be assessed.

Interestingly, in the presence of the cdc28-4 allele, deletion of CLB5 led to an increase in the intensity of the Bfa1 phosphorylated species VI, and a concomitant decrease in the intensity of the unphosphorylated protein band (Figure 7.4, lane 5). Thus in the absence of CLB5 function, Bfa1 phosphorylation was enhanced. This may be due to the spindle polarity defect associated with these cells, which culminates in the translocation of the spindle and nucleus into the bud (1.6.2.3; Figure 1.1) (Segal et al., 1998). The cells subsequently arrest in metaphase, presumably due to activation of a checkpoint (Segal et al., 1998). This may account for the increased phosphorylation of Bfa1 (see Chapter 10).

7.2.6 Mutating the putative Cdc28 phosphorylation sites within Bfa1 leads to loss of the protein

Although several of the mutant strains studied proved to be defective in Bfa1 phosphorylation, the multi-functional nature of the kinases involved prevented any analysis of the phenotypic effects of this reduced phosphorylation. One method to determine the role of Bfa1 phosphorylation, is to generate a non-phosphorylatable mutant form of the protein, and analyse its functionality. Since Bfa1 phosphorylation is completely abolished in the absence of Cdc28 function, and the Cdc28 consensus phosphorylation site is known, attempts were made to mutate the putative Cdc28 phosphorylation sites in Bfa1.

Figure 7.5 shows the six putative Cdc28 phosphorylation sites within the Bfa1 amino acid sequence (SP or TP). They all reside in the C-terminus of the protein, which is consistent with the failure of Cdc28 to phosphorylate the N-terminal GST-Bfa1 proteolytic fragment in vitro (Figure 6.8). All six serine or threonine residues were mutated to alanine (SP or TP to AP), using PCR mutagenesis (2.4.9, Figure 2.1). The resulting mutant gene (bfa1-6A) was inserted into a vector derived from plasmid pSL10, to generate plasmid pSL21, containing bfa1-6A, tagged at the N-terminus with a 3HA epitope tag, and expressed under
**Figure 7.5**  
**Putative Cdc28 phosphorylation sites within the Bfa1 C-terminus.** The C-terminal 332 amino acid residues and the corresponding nucleotide sequence are shown. The phosphorylation sites (SP or TP in single letter amino acid code) are shown in bold. The nucleotides which were altered by site-directed mutagenesis (to guanine, G) to generate the *bfa1-6A* mutant are also highlighted in bold. In addition the *BsgI* and *HindIII* sites used during the PCR mutagenesis procedure are underlined, with the asterix (*) indicating the *BsgI* cleavage site (C/A). For construction details see section 2.4.9 or Figure 2.1.
the control of its own promoter. This construct, and a wild-type control, were integrated into strain SLY108 (bfa1Δ) to test the protein expression and phosphorylation levels of Bfa1-6A, and its functionality. The resultant strains SLY130 (3HA.BFA1) and SLY145 (3HA.bfa1-6A) were grown to mid-log phase and harvested. Protein extracts were subjected to SDS-PAGE and immuno-blot analysis to assess the Bfa1 protein status. The Bfa1-6A mutant protein was not detectable (Figure 7.6, lane 2). Since this cloning procedure was successful with the wild-type gene (Figure 7.6, lane 1), the most likely interpretation of this result is that the amino acid changes have disrupted the protein structure, and the misfolded protein has been targeted for proteolysis. Any functionality tests using this mutant would thus be meaningless.

7.2.7 Cdc14 is required for, but does not directly control, Bfa1 de-phosphorylation in late mitosis

The results presented in this chapter and Figure 6.8 suggest that Cdc28 directly controls Bfa1 phosphorylation. This is true of many other MEN components, including Cdc15, Sic1, Swi5 and Cdhl (1.8.1). All of these proteins are negatively regulated by Cdc28 phosphorylation, and are activated in late mitosis when they are dephosphorylated by Cdc14. Conversely, Cdc28-dependent phosphorylation is likely to activate Bfa1 function, and must therefore be removed in late mitosis to allow Tem1 activation. To test whether Bfa1 is also dephosphorylated by Cdc14, its phosphorylation status was examined in a cdc14-l mutant strain. Strain SL20-4d (3HA.BFA1 cdc14-l) was grown to mid-log phase at 25°C and then half the culture was shifted to 37°C for 3h. Bfa1 shows the normal mid-log phosphorylation pattern at 25°C, but arrests at 37°C in its maximally phosphorylated state, displaying species I-VI (Figure 7.7). Thus, like the dbf2 and cdc15 mutant strains (Figure 6.2), the cdc14 mutant arrests at 37°C with maximal levels of Bfa1 phosphorylation. This indicates that MEN function is necessary for Bfa1 de-phosphorylation and is consistent with Cdc14 controlling this de-phosphorylation.
Figure 7.6  Mutating the putative Cdc28 phosphorylation sites within Bfa1 leads to loss of Bfa1 protein. Strain SLY108 (bfa1Δ) containing integrated 3HA.BFA1 or 3HA.bfa1-6A (BFA1 with all the SP/TP sites mutated to AP, see 2.4.9) under the control of its own promoter, was grown to mid-log phase, harvested, and subjected to protein extraction and immuno-blot analysis.
Figure 7.7  Bfa1 phosphorylation is retained in the absence of functional Cdc14.

Strain SL20-4d (3HA.BFA1 cdc14-1) was grown to mid-log at 25°C, the culture was split, and grown for a further 3h at either 25°C or 37°C. The harvested cells were subjected to protein extraction and immuno-blot analysis.
To examine whether Cdc14 was indeed the Bfa1 phosphatase, MBP-Cdc14 purified from *E. coli* (a gift from G. Wells, NIMR, London, UK) was tested for its ability to dephosphorylate Bfa1 directly. Strain SLY105 (3HA.BFA1) was grown to mid-log phase, and arrested for 3h with nocodazole to ensure Bfa1 was maximally phosphorylated. A protein extract was prepared in the absence of phosphatase inhibitors, and treated with either MBP-Cdc14, MBP-Cdc14M (an inactive mutant) or calf alkaline phosphatase (CAP), which is known to dephosphorylate Bfa1 (Figure 5.4). The phosphorylation status of Bfa1 was then determined by SDS-PAGE and immuno-blot analysis. Surprisingly, the lower mobility forms of Bfa1 were still present following treatment with MBP-Cdc14 (Figure 7.8, lane 2). In contrast, CAP dephosphorylated Bfa1 efficiently, since only the unphosphorylated form of the protein was present following CAP treatment (Figure 7.8, lane 4). Thus Cdc14 failed to dephosphorylate Bfa1 *in vitro*. Since the MBP-Cdc14 is known to be active *in vitro* (G. Wells, personal communication), this suggests that Cdc14 does not dephosphorylate Bfa1 directly.

7.3 Discussion

7.3.1 Cdc28 controls Bfa1 phosphorylation

These data show that Cdc28 is required for Bfa1 phosphorylation in nocodazole-arrested cells. Since the pattern of Bfa1 phosphorylation in the nocodazole-arrested cultures is identical to that during a normal mitosis, Cdc28 almost certainly controls Bfa1 phosphorylation during mitosis. The kinase assays in Figure 6.8 indicate that Cdc28 acts directly on Bfa1 *in vitro*, and support the idea that Bfa1 is a direct substrate of Cdc28 *in vivo*. Interestingly, cells lacking Cdc28 function lose all the Bfa1 phospho-species, including those which appear later in mitosis and have been attributed to Cdc5. This suggests that Cdc28-dependent phosphorylation of Bfa1 is a prerequisite for the later Cdc5-dependent phosphorylation.
Figure 7.8  Purified Cdc14 does not dephosphorylate Bfa1 in vitro. Strain SLY105 (3HA.BFA1) was grown to mid log phase, arrested for 3h using nocodazole, and harvested. Protein extraction was performed in lysis buffer without phosphatase inhibitors. 70 μg extract was incubated with purified MBP-Cdc14, MBP-Cdc14M (an inactive mutant) (both gifts from G. Wells, NIMR, London, UK) or calf alkaline phosphatase (CAP) [Boehringer Mannheim] at 30°C for 30 min and then analysed using SDS-PAGE and immuno-blotting. The activity of the MBP-Cdc14 was confirmed by its ability to dephosphorylate Swi6 that had been phosphorylated by Cdc28 (G. Wells, personal communication).
Bfa1 phosphorylation was not affected following deletion of any of the cyclin genes tested here. This is not surprising, since the cyclins are functionally redundant. However, combining the cdc28-4 mutation with certain cyclin deletions did alter the Bfa1 phosphorylation pattern. Deleting CLB1, or CLB3 had no effect on Bfa1 phosphorylation, even when Cdc28 function was already compromised. However, in the cdc28-4 clb2Δ mutant strain, Bfa1 phosphorylation was reduced, even at the permissive temperature. This suggests that Bfa1 may be a target of the Cdc28-Clb2 kinase complex, although further experiments are necessary to confirm this (see 7.2.5). Since Clb2 is the major cyclin during mitosis, accounting for 85% of mitotic Cdc28 kinase activity within the cell (Grandin and Reed, 1993), it would not be surprising if it was the cyclin component of the CDK complex which controls Bfa1 phosphorylation. Moreover, Clb2 has been shown to localise to spindle pole bodies (Hood et al., 2001), where it could interact with Bfa1.

In contrast, the cdc28-4 clb5Δ strain showed enhanced phosphorylation of Bfa1 at 25°C, displaying the increased intensity of bands VI and IV normally associated with late mitotic cells or cells arrested in metaphase. This is probably due to the activation of a nuclear or spindle positioning checkpoint in these cells. It has been reported that cdc28-4 clb5Δ diploid cells fail to assign spindle polarity, with the result that both spindle pole bodies become "daughter-bound" and the spindle and nucleus translocate into the bud (1.6.2.3, Figure 1.1) (Segal et al., 1998). This nuclear positioning defect leads to a metaphase cell cycle arrest, which is presumably checkpoint-dependent. The phenotype is also observed in haploid cells, although with a lower penetrance (Segal et al., 1998). Thus the increased phosphorylation of Bfa1 in these cells may reflect the fact that the cells are delayed in metaphase, due to activation of a nuclear positioning checkpoint. The phosphorylation of Bfa1 also indicates that the Bub2/Bfa1 may be involved in this checkpoint arrest. This would be consistent with Bub2 and Bfa1 functioning to couple completion of anaphase to mitotic exit (5.3.2), and with the known role of the Bub2 pathway in monitoring spindle position (1.8.2.3). However, it has been suggested that the Bub2/Bfa1 pathway is inactivated once the SPB_{daughter} has translocated into the bud (Krishnan et al., 2000). Further experiments will be necessary to determine whether the nuclear positioning defect observed
in the *cdc28-4 clb5Δ* cells leads to a metaphase arrest which is dependent on the Bub2/Bfa1 pathway.

To try to understand the role of phosphorylation in regulating the function of Bfa1, a mutant version of the protein was constructed, in which all of the putative Cdc28 phosphorylation sites (SP/TP) had been mutated. Since loss of Cdc28 abolishes Bfa1 phosphorylation completely, a mutant which was no longer a Cdc28 substrate should be completely unphosphorylated. Immuno-blotting showed that this mutant protein, Bfa1-6A, was not expressed. It is likely that mutating the phosphorylation sites interferes with the structure of the protein, causing mis-folding and subsequent proteolysis. This may be because of the structural differences between the serine or threonine residues and the alanine residue with which they were replaced, or because the phosphorylation itself may be required for stability. This latter possibility is unlikely, because the unphosphorylated protein is detectable during the cell cycle. However, in the absence of Cdc28 function, Bfa1 protein levels do decline, suggesting that phosphorylation may contribute to stability.

The lack of Bfa1-6A expression means that functionality studies to examine the role of Bfa1 phosphorylation cannot be performed using this mutant. It is possible that mutating individual phosphorylation sites would generate a mutant which was both stable, and non-phosphorylatable. However, finding a suitable combination of mutant sites would be very time-consuming. Alternatively, peptide mapping the Bfa1 phosphorylation sites and then mutating them might provide the key to the function of Bfa1 phosphorylation, another time-consuming approach. These studies would be interesting avenues to explore in the future.

### 7.3.2 De-phosphorylation of Bfa1

Many substrates of Cdc28 which are involved in regulating mitotic exit are activated in late mitosis following de-phosphorylation by the phosphatase Cdc14. However, Bfa1 does not seem to be a Cdc14 substrate. Although Cdc14 function is required for Bfa1 de-phosphorylation, Cdc14 does not dephosphorylate Bfa1 directly *in vitro.*
Although Bfa1 is dephosphorylated abruptly in late mitosis in an MEN-dependent manner, this does not necessarily mean that the phosphatase responsible is activated in late mitosis. Figure 5.9 illustrates the equilibrium which probably exists between the phosphorylated and unphosphorylated forms of Bfa1. De-phosphorylation of Bfa1 could therefore be achieved through either activation of its phosphatase(s), or inactivation of its kinases. The two kinases which act on Bfa1, Cdc28 and Cdc5, are both inactivated in late mitosis by APC/C\(^{Cdh1}\), which targets both Cdc5, and Clb1 and Clb2, for ubiquitin-mediated proteolysis. The data presented in this chapter and Chapter 6 show that continued function of Cdc28 and Cdc5 is necessary to maintain complete Bfa1 phosphorylation. Thus the APC/C\(^{Cdh1}\)-mediated decrease in their kinase activities is sufficient to explain the de-phosphorylation of Bfa1 in late mitosis, without invoking a cell cycle-regulated phosphatase (Figure 7.9). This model also explains why Cdc14 function is required for the loss of Bfa1 phosphorylation, since APC/C\(^{Cdh1}\) is not activated in a cdc14 arrest. The dependency of Bfa1 de-phosphorylation on MEN activation indicates that a feedback loop exists (Figure 7.9).

If Bfa1 de-phosphorylation is dependent upon MEN activation, then the initial inactivation of Bub2/Bfa1 in late anaphase must be controlled by some other means. One possibility is that the interaction of the Tem1/Bub2/Bfa1 complex with Lte1 which occurs as the SPB\(_{daugter}\) enters the bud (Figure 1.3), overcomes Bub2/Bfa1 function and leads to Tem1 activation. Alternatively, the APC/C\(^{Cdc20}\)-dependent decrease in Cdc28-Clb kinase activity which occurs as cells enter anaphase may be sufficient to promote loss of Bfa1 phosphorylation, which is then accelerated as APC/C\(^{Cdh1}\) becomes activated. The mechanisms which may contribute to Bub2/Bfa1 inactivation in late mitosis will be discussed in more detail in Chapter 10.
Figure 7.9  Regulation of Bfa1 by Cdc28 and Cdc5. Cdc28 (possibly bound to Clb2) phosphorylates Bfa1 from early mitosis, as Clb2 protein levels rise (see Figure 5.6). This phosphorylation may be a pre-requisite for the Cdc5-dependent phosphorylation of Bfa1 which also occurs during mitosis, slightly after the Cdc28-dependent phosphorylation. Phosphorylation presumably activates the GAP during mitosis, but following activation of APC/C^{Cdh1}, Cdc28 and Cdc5 kinase activities decline, and Bfa1 is dephosphorylated. Thus a feedback loop exists, ensuring that once APC/C^{Cdh1} is activated, Bub2/Bfa1 are inactivated until the following cell cycle. It is unclear how this switch is triggered, but it is likely that several factors control activation of mitotic exit (see text).
Phosphatase?

Bub2 → Bfa1 → Bub2

Active

Cdc28

MEN

Cdc5

Clb2

Phosphatase?

Bfa1

Inactive

Bub2

MEN

Cdc28

Cdc5

Clb2

APC/C

Cdh1
Chapter 8

Functional analysis of Bfa1 N-terminal and C-terminal fragments

8.1 Introduction

The data presented in the previous two chapters indicate that Bfa1 is subject to Cdc28- and Cdc5-dependent phosphorylation, which is likely to control Bub2/Bfa1 activation. However, in order to establish the role of Bfa1 phosphorylation, it will be necessary to generate mutant forms of the protein which are no longer phosphorylated. This in turn, requires identification and subsequent mutation of the Bfa1 amino acid residues which are targeted by Cdc28 and Cdc5. Attempts to generate a non-phosphorylatable mutant form of Bfa1 by mutating the putative Cdc28 sites were not successful (Chapter 7). A more targeted mutagenesis approach might prove more informative, but this requires identification of the Bfa1 phosphorylation sites by phospho-peptide mapping. Unfortunately, producing large quantities of full-length purified Bfa1 for this procedure proved difficult, due to the instability of the protein when expressed in *E. coli* (Figure 6.8). One way to overcome this is to express smaller fragments of the protein, which may be more stable *in vitro* and therefore more suitable for the peptide mapping procedure. In addition, analysis of these protein fragments might narrow down the location of the potential phosphorylation sites. Finally, identification of functional domains of the protein which can be expressed easily in *E. coli* would also prove useful for setting up an *in vitro* Bub2/Bfa1 GAP assay within the laboratory.
8.2 Results

8.2.1 Expression of the Bfa1 N-terminus and C-terminus in vivo

To study the different functional properties of the Bfa1 C-terminus and N-terminus in vivo, plasmids were constructed containing 3HA.BFA1, 3HA.bfa1ΔNT270, 3HA.bfa1ΔNT300, 3HA.bfa1ΔCT270 and 3HA.bfa1ΔCT300 under the control of the BFA1 promoter (see 2.4.6-2.4.8 for construction details). The four different BFA1 deletion fragments lacked 270 or 300 amino acid residues at the N- or C-termini (Figure 8.1). Each plasmid was integrated into a bfa1Δ strain (SLY108) to generate strains SLY130 (3HA.BFA1), SLY131 (3HA.bfa1ΔCT270), SLY132 (3HA.bfa1ΔCT300), SLY133 (3HA.bfa1ΔNT270) and SLY134 (3HA.bfa1ΔNT300). To verify that the Bfa1 fragments were expressed in vivo, strains SLY130, SLY131, SLY132, SLY133 and SLY134 were grown to mid-log phase, harvested, and subjected to protein extraction and immuno-blot analysis. Figure 8.2 shows the expression levels and SDS-PAGE mobility of the different Bfa1 fragments. All four Bfa1 fragments are expressed in vivo. The C-terminal fragments (ΔNT270 or 300) have an apparent molecular weight (MW) of ≈46kDa, approximately 15kDa lower than the predicted MW (Figure 8.2, lanes 1 and 2). This may indicate that the C-terminal fragments are unstable, and subject to cleavage. In contrast, the N-terminal fragments display the predicted molecular weight (Figure 8.2, lanes 3 and 4).

Lower mobility forms of at least three of the fragments, 3HA-Bfa1ΔNT300, 3HA-Bfa1ΔNT270 and 3HA-Bfa1ΔCT300, can be detected in the immuno-blot (Figure 8.2, lanes 1, 2 and 3). This suggests that these fragments are phosphorylated in vivo. However, the larger N-terminal fragment (3HA-Bfa1ΔCT270) does not seem to be phosphorylated at all (Figure 8.2, lane 4).

8.2.2 Functionality of the Bfa1 N-terminus and C-terminus in vivo

Having established that all four constructs were expressed in vivo, the ability of these protein fragments to complement a bfa1Δ deletion was investigated. Strains deleted for
Figure 8.1  Cartoon showing Bfa1 N-terminal and C-terminal deletion fragments.

The putative Cdc28 phosphorylation sites are confined to the C-terminus of the protein (Figure 7.5), whilst Cdc5 is able to phosphorylate the Bfa1 N-terminus (Figure 6.8).
Figure 8.2  Expression and SDS-PAGE mobility of the Bfa1 N-terminal and C-terminal deletion fragments. Strains SLY131 (3HA.bfa1ΔCT270), SLY132 (3HA.bfa1ΔCT300), SLY133 (3HA.bfa1ΔNT270) and SLY134 (3HA.bfa1ΔNT300) were grown to mid-log phase and harvested. Protein extracts were analysed by immuno-blotting with anti-HA (12CA5) antibody.
BFA1 are defective in the SAC-induced arrest following nocodazole treatment, and continue to progress through the cell cycle rather than arresting in metaphase. This cell cycle progression can be assessed by a re-budding assay, which monitors bud formation in a sonicated culture. Control strains CG378 (wild-type), SLY108 (bfa1Δ) and SLY130 (bfa1Δ::3HA.BFA1), and strains SLY131 (bfa1Δ::3HA.bfa1ΔCT270), SLY132 (bfa1Δ::3HA.bfa1ΔCT300), SLY133 (bfa1Δ::3HA.bfa1ΔNT270) and SLY134 (bfa1Δ::3HA.bfa1ΔNT300) were grown to mid-log phase. Following addition of nocodazole, new bud formation was assessed microscopically every hour for 4h, and % re-budding plotted as a function of time. As expected, CG378 shows negligible levels of re-budding (up to 6%), whilst the bfa1Δ strain achieves levels of re-budding approaching 60% (Figure 8.3). Integrating a wild-type 3HA.BFA1 construct into the bfa1Δ strain restores the SAC response (Figure 8.3). However, integration of the Bfa1 N-terminus (ΔCT270 or 300) does not complement the deletion, since the 3HA.bfa1ΔCT270 and 3HA.bfa1ΔCT300 strains display levels of re-budding of approximately 60% (Figure 8.3). Thus the Bfa1 N-terminus is not functional. In contrast, integration of the C-terminal Bfa1 constructs (ΔNT 270 or 300) is sufficient to prevent re-budding in the presence of nocodazole (Figure 8.3). This shows that the C-terminus of Bfa1 is fully functional, and that the N-terminal 300 amino acids, at least, are dispensable for SAC function.

8.2.3 Phosphorylation of the Bfa1 N-terminus and C-terminus in vitro

The kinase assays in Figure 6.9, show that Cdc5 is capable of phosphorylating both full-length Bfa1, and an N-terminal proteolytic fragment, indicating that some Cdc5 phosphorylation sites must lie in the Bfa1 N-terminus. In contrast, Cdc28 only phosphorylates the full-length protein in vitro, and an analysis of the Bfa1 amino acid sequence (Figure 7.5), suggests that Cdc28 phosphorylates the Bfa1 C-terminus. To investigate where its Cdc28 and Cdc5 phosphorylation sites are situated, the four different deletion fragments of Bfa1, bfa1ΔNT270, bfa1ΔNT300, bfa1ΔCT270 and bfa1ΔCT300, were cloned into a GST expression vector (2.4.4, 2.4.5) and the corresponding fusion proteins were purified from E. coli (2.6.6). 5μg of each purified protein preparation was subjected to SDS-PAGE analysis and detected by Coommassie staining or immuno-blotting.
Figure 8.3 Re-budding assay to assess functionality of the Bfa1 N-terminal and C-terminal deletion fragments. Strains CG378, SLY108 (bfa1Δ) and SLY130, SLY131, SLY132, SLY133 and SLY134 (SLY108 containing integrated 3HA.BFA1, 3HA.bfa1ΔCT270, 3HA.bfa1ΔCT300, 3HA.bfa1ΔNT270, or 3HA.bfa1ΔNT300 respectively) (2.5.2), were grown to mid-log phase at 30°C. Following addition of nocodazole, the percentage of re-budded cells was assessed microscopically and plotted as a function of time (h).
with anti-GST antibody (Figure 8.4). All four fusion proteins were successfully purified. Like their in vivo counterparts, the C-terminal fragments showed a lower molecular weight in SDS-PAGE than predicted, indicating that proteolysis may be occurring (Figure 8.4, upper panels). In addition, the proteins co-purified with a 66kDa protein, probably a chaperone (C, Figure 8.4), again suggesting that the protein is unable to fold properly in E. coli. The C-terminal fragments showed the expected molecular weight, but, as observed with full-length GST-Bfa1 (Figure 6.8), an N-terminal proteolytic fragment was present in the preparation (Figure 8.4, lower panels). However, this only accounts for 50% of total protein (compared with 80% for full-length GST-Bfa1, Figure 6.8). This suggests that the N-terminal fragments are more stable than the full-length fusion protein.

Having purified the Bfa1 deletion fragments, the ability of Cdc5 and Cdc28 to phosphorylate the N- and C-termini was investigated. First, Cdc5 kinases assays were performed using the Bfa1 N-terminal fragments. Strain YD11-97 (CDC5.3HA) was grown to mid-log phase, and harvested. Cdc5 was immuno-precipitated from protein extracts using anti-HA antibody (12CA5) and kinase assays were performed using GST-Bfa1ΔCT270 or GST-Bfa1ΔCT300 as substrates. Cdc5 strongly phosphorylates both Bfa1 N-terminal fragments (Figure 8.5, lanes 3 and 4). The phosphorylation is more efficient than that of the N-terminal proteolytic fragment (*) or the full-length protein (Figure 8.5). This suggests that the Cdc5 phosphorylation sites reside in the N-terminus, but that some are missing from the proteolytic fragment. In the full-length protein, the C-terminus may overlap the Cdc5 phosphorylation sites, and partly prevent it phosphorylating the N-terminus.

The ability of Cdc28 to phosphorylate the N-terminus was not tested, because both fragments retain at least one Cdc28 consensus site (Figure 8.1), and are likely to give an uninformative positive result. However, both Cdc28 and Cdc5 were tested for their ability to phosphorylate the Bfa1 C-terminus in vitro. In addition, since Clb2 may be the cyclin subunit which co-operates with Cdc28 to phosphorylate Bfa1 (7.2.5), the ability of Clb2-associated Cdc28 kinase to phosphorylate Bfa1 was examined. Since both C-terminal fragments are known to be functional (Figure 8.3) only the larger fragment, containing all
Figure 8.4  Purification of GST-Bfa1 deletion fragments for use as *in vitro* kinase assay substrates. The GST-Bfa1ΔCT or ΔNT fragments were purified as described (2.6.6). 5µg eluted protein was analysed using SDS-PAGE and detected using Coomassie staining or by immuno-blotting with anti-GST antibody. The GST fusion protein, co-purifying chaperone (C) and the N-terminal proteolytic fragment (*) are labelled. Note that the C-terminal fragments (ΔNT) have an apparent molecular weight of ≈55KDa, 15KDa less than expected (see text).


Figure 8.5  **Cdc5 phosphorylates the N-terminus of Bfa1 in vitro.** Strain YD11-97 (CDC5.3HA) was grown to mid-log phase and harvested. Following protein extraction, Cdc5 was immuno-precipitated using anti-HA (12CA5) antibody bound to protein A beads, and *in vitro* kinase assays performed using GST-Bfa1, GST-Bfa1ΔCT270 and GST-Bfa1ΔCT300 as substrates. Phosphorylated GST-Bfa1 was analysed using SDS-PAGE and autoradiography.
the putative Cdc28 consensus sites, was tested. Strains YD11-97 (CDC5.3HA), CDC28-HA (CDC28.HA) and CLB2(T) (CLB2.HA) were grown to mid-log phase and harvested. Cdc28, Cdc5 and Clb2 were immunoprecipitated from protein extracts using anti-HA (12CA5) antibody and in vitro kinase assays performed using GST-Bfa1ΔNT270 as substrate. Phosphorylated Bfa1ΔNT270 was analysed using SDS-PAGE and autoradiography. Cdc5 did not phosphorylate the C-terminal fragment of Bfa1 (Figure 8.6, lane 1). However, both Cdc28 and Cdc28-Clb2 were able to phosphorylate GST-Bfa1ΔNT270 in vitro (Figure 8.6, lanes 2 and 3). This shows that Cdc5 recognises phosphorylation sites within the N-terminus of Bfa1, but not the C-terminus. Cdc28 phosphorylates the C-terminus, which is not surprising, since all its consensus sites are located in this half of the protein. Consistent with Bfa1 being a substrate of Clb2, the Bfa1 C-terminus is also specifically phosphorylated by Cdc28-Clb2.

8.3 Discussion

The data presented in this chapter show that the C-terminus of Bfa1 is necessary and sufficient for its spindle assembly checkpoint function in vivo. This is a surprising result, particularly since immuno-blot analysis suggests that the C-terminal protein fragments examined here may be cleaved in vivo, generating even smaller fragments. The fact that such a small section of the protein is sufficient for activity, raises a question mark over the role of the N-terminus. This may act as a regulatory component of the protein in vivo, modifying the activity of the C-terminus. Structural studies of the protein are likely to be necessary before its function and regulation will be fully understood.

Both the C-terminal and N-terminal fragments of Bfa1 are still phosphorylated in vivo. This suggests the C- and N-terminal sections of Bfa1 may fold into distinct domains which are still recognised by their respective kinases when produced independently of each other. The C-terminus of Bfa1 is phosphorylated in vitro by Cdc28, but not Cdc5. This is consistent with analysis of the protein sequence which shows that all the putative Cdc28 phosphorylation sites are situated in the C-terminus. These data also show that Cdc5 does not phosphorylate the same consensus sites as Cdc28, as has been suggested previously.
Figure 8.6  Cdc28 and Cdc28-Clb2 phosphorylate the C-terminus of Bfa1 \textit{in vitro}.

Strains YD11-97 (\textit{CDC5.3HA}), CDC28-HA (\textit{CDC28.HA}) and CLB2(T) (\textit{CLB2.HA}) were grown to mid-log phase and harvested. Following protein extraction, Cdc5, Cdc28 or Clb2-Cdc28 were immuno-precipitated using anti-HA (12CA5) antibody bound to protein A beads, and \textit{in vitro} kinase assays performed using GST-Bfa1\Delta NT270 as a substrate. Phosphorylated GST-Bfa1\Delta NT270 was analysed using SDS-PAGE and autoradiography (upper panel) and quantitated by Phosphorimager. Cdc5, Cdc28 and Clb2 protein levels were estimated by immuno-blotting (middle panel). The ratios of GST-Bfa1\Delta NT270 kinase activity/kinase protein levels are plotted in the graph (lower panel).
(Rudner and Murray, 2000). In fact, Cdc5 phosphorylates the Bfa1 N-terminal fragments, indicating that its consensus sites must reside within this section of the protein. Since the Bfa1 N-terminus is not functional in vivo, the role of its phosphorylation by Cdc5 remains enigmatic.

Clb2-associated Cdc28 kinase phosphorylates the C-terminus of Bfa1 as strongly as the total Cdc28 fraction in vitro. Whilst these data do not prove that Clb2 is the Bfa1-specific cyclin, they are certainly consistent with this notion. Taken with the in vivo data in Chapter 7, it seems likely that Cdc28-Clb2 is involved in Bfa1 phosphorylation.

Finally, these studies show that the Bfa1 C- and N-termini are both stable when expressed in vivo and more stable in vitro than full length GST-Bfa1. Since the functionality and in vitro kinase specificity of these protein fragments is also known, they should be useful tools for either peptide mapping or in vitro GAP assays.
Chapter 9

Bub2 is essential to prevent mitotic exit in \textit{apc2-8} metaphase-arrested cells

9.1 Introduction

As discussed in the previous chapters, and in the Introduction, the Bub2/Bfa1 checkpoint pathway seems to monitor the movement of the daughter-bound spindle pole body (SPB) through the bud neck, and prevents activation of the mitotic exit network until after this event has occurred. In an unperturbed cell cycle, this checkpoint pathway is dispensable, because the timing of late mitotic events ensures that the insertion of the SPB through the neck will precede mitotic exit and subsequent cytokinesis. However, if cells are delayed in metaphase, for example following activation of the Mad branch of the SAC, the Bub2/Bfa1 pathway is required to enforce a corresponding delay in mitotic exit. In the absence of Bub2/Bfa1 function, the MEN is able to trigger activation of APC/C\textsuperscript{C\textsubscript{Cbl}} and Sic1, leading to loss of Cdc28-Clb kinase activity, spindle disassembly and cytokinesis.

The data presented in previous chapters suggest that activation of the Bub2/Bfa1 GAP may be regulated by Bfa1 phosphorylation. If so, GAP function is unlikely to be solely required during a spindle checkpoint arrest, since the pattern of Bfa1 phosphorylation in nocodazole is identical to that in mitosis of an unperturbed cell cycle and in an \textit{apc2-8}-induced metaphase arrest. To investigate whether Bub2/Bfa1 could be part of an universal checkpoint pathway which prevents mitotic exit in metaphase-arrested cells, the effects of deleting \textit{BUB2} in \textit{apc2-8} metaphase-arrested cells was investigated.

At the restrictive temperature, \textit{apc2-8} cells arrest in metaphase as dumbbells with short spindles located near the bud neck (Figure 9.1) (Kramer \textit{et al.}, 1998). If this arrest is
dependent upon the Bub2/Bfa1-mediated inhibition of the MEN, deleting BUB2 or BFA1 should allow these cells to exit mitosis and initiate subsequent rounds of cell division.

9.2 Results

9.2.1 Bub2 restrains mitotic exit in metaphase-arrested cells

To investigate the role of BUB2 in maintaining an apc2-8 metaphase arrest, strains SJY123 (GFP.TUB1 apc2-8) and SJY124 (GFP.TUB1 apc2-8 bub2Δ) were constructed. In addition to the apc2-8 mutation, these strains contained integrated GFP-Tubulin (GFP.TUB1), which allows visualisation of the spindles and spindle pole bodies using fluorescence microscopy. The cells were grown to mid-log phase at 23°C, before being shifted to the restrictive temperature of 37°C. The proportion of metaphase-arrested dumbbells and re-budded cells was assessed microscopically and plotted as a function of time. As expected, the apc2-8 control cells arrested at the restrictive temperature as dumbbells (Figure 9.1, upper panel). Even after incubation at 37°C for 6h, none of the apc2-8 cells had begun to re-bud (Figure 9.1, upper panel). In contrast, whilst 80% of the apc2-8 bub2Δ cells also arrested as dumbbells after 2h, thereafter the proportion of metaphase-arrested cells began to fall, with a concomitant increase in re-budded cells (Figure 9.1, upper panel). This confirms that deletion of BUB2 enables apc2-8 metaphase-arrested cells to initiate subsequent rounds of budding and suggests that the Bub2/Bfa1 pathway is indeed required to prevent mitotic exit during an apc2-8-induced metaphase arrest.

Samples for fluorescence microscopy were taken after incubation at the restrictive temperature for 2h and 6h, to assess the status of the spindles in the two strains. After 2h at 37°C, both the apc2-8 and apc2-8 bub2Δ cells arrested as dumbbells, with short spindles situated in the mother cell, near the neck (Figure 9.1, lower panels). However, after 6h, whilst the apc2-8 cells are still arrested as dumbbells with a short spindle present at the neck (Figure 9.1, upper and lower panels), a significant proportion (>30%) of the apc2-8 bub2Δ cells have clearly re-budded (Figure 9.1, upper and lower panels). In these re-budded
Figure 9.1  Bub2 restraints mitotic exit and initiation of the next cell cycle in \textit{apc2-8} metaphase-arrested cells. Strains SJY123 (\textit{GFP.TUB1 apc2-8}) and SJY124 (\textit{GFP.TUB1 apc2-8 bub2A}) were grown to mid-log phase at 23°C, shifted to 37°C and sampled at 2h intervals. Re-budding was assessed microscopically following sonication, with % re-buddled cells plotted as a function of time (h) (upper panel). SPB re-duplication and spindle status were examined by fluorescent microscopy (lower panel).

Work done in collaboration with Sanne Jensen
cells, the mitotic spindle is still present near the neck of the mother cell, but is visualised as a triangular structure rather than a linear bipolar spindle (Figure 9.1, lower panels). These images indicate that SPB duplication has occurred, and that the spindle microtubules are now organised by at least three spindle pole bodies. This provides further evidence that deletion of Bub2 allows mitotic exit, which is followed by initiation of events of the subsequent cell cycle, including re-budding and SPB re-duplication. However, the mitotic spindle is still present in these cells, suggesting that mitotic exit per se is not sufficient to allow spindle disassembly to occur.

9.2.2 Actin ring formation is controlled by the MEN and restrained by Bub2 in metaphase-arrested cells

In addition to controlling loss of CDK activity, the MEN is believed to play a role in controlling cytokinesis. However, although apc2-8 bub2Δ cells re-bud following a prolonged arrest, they do not complete cytokinesis, suggesting that MEN activation alone is not sufficient to activate cell division. One of the early stages of cytokinesis in budding yeast is the formation of the actomyosin-based contractile ring at the bud neck, which occurs in late mitosis (1.8.4) (Lippincott and Li, 1998). To investigate the role of Bub2 and the MEN in controlling cytokinesis, actin ring formation was examined in apc2-8, apc2-8 bub2Δ cells, and apc2-8 bub2Δ cells harbouring mutations in different MEN genes. Strains KTM208 (apc2-8), YLF25 (apc2-8 bub2Δ) and strains containing temI-3 (JTAB7-12D), cdc15-1 (ERN1), mobl-77 (JMB3-12B), cdc5 (msd2-1) (J5AB3-13D) and cdc14-1 (J14AB2-20B) mutations in an apc2-8 bub2Δ background were grown to mid-log phase at 23°C. The cells were arrested at 37°C for 3h, stained with phalloidin to detect actin, and visualised using fluorescence microscopy. The actin ring is not detectable in apc2-8-arrested cells (Figure 9.2). This indicates that actin ring formation must normally occur after metaphase. However, in the apc2-8 bub2Δ strain, an actin ring was detectable in up to 60% of cells (Figure 9.2, panel 2, arrow). Thus in the absence of Bub2 function, actin ring formation occurs in metaphase-arrested cells, indicating that Bub2 normally restrains this event. This suggests that actin ring formation may be dependent upon MEN activation. Consistent with this hypothesis, the actin ring is no longer detectable in apc2-8 bub2Δ cells
Figure 9.2  Bub2 prevents premature actin ring formation in metaphase-arrested cells by restraining MEN function. Strains KTM208 (apc2-8), YLF25 (apc2-8 bub2Δ), JTAB7-12D (apc2-8 bub2Δ tem1-3), ERN1 (apc2-8 bub2Δ cdc15-1), JMAB3-12B (apc2-8 bub2Δ mob1-77), J5AB3-13D (apc2-8 bub2Δ cdc5) and J14AB2-20B (apc2-8 bub2Δ cdc14-1) were grown to mid-log phase at 25°C. Cells were arrested at 37°C for 3h and stained for DNA with DAPI and for actin with rhodamine-phalloidin (2.8.2) (Frenz et al., 2000). The actin ring is indicated with an arrow. Some 60% of apc2-8 bub2Δ cells eventually accumulate with a detectable actin ring.

Work done in collaboration with Lisa Frenz
containing mutations in the MEN genes TEM1, CDC15, MOB1, CDC5 or CDC14. This suggests that the MEN controls actin ring formation.

9.2.3 Dbf2 does not re-localise to the bud neck in apc2-8 bub2A cells

Although deletion of BUB2 leads to actin ring formation in apc2-8 metaphase-arrested cells, cell division is not completed. This suggests that MEN activation is sufficient to trigger the early stages of cytokinesis, but that additional steps are necessary to complete it. At least one of these additional requirements may be the re-localisation of certain MEN proteins, including Cdc15, Cdc5, and Dbf2 to the bud neck (Frenz et al., 2000; Song et al., 2000; Song and Lee, 2001; Xu et al., 2000). Detailed studies of the Dbf2 localisation have shown that this movement is dependent upon MEN function (Frenz et al., 2000). To investigate whether deletion of BUB2 allowed relocalisation of Dbf2 to the bud neck in apc2-8 metaphase-arrested cells, strains CLF7-4f (DBF2.GFP apc2-8), CLF13-2d (DBF2.GFP apc2-8 bub2Δ) and SJY125 (DBF2.GFP apc2-8 mad2Δ), expressing Dbf2-GFP which can be visualised using fluorescence microscopy, were grown to mid-log phase at 23°C before being arrested at 37°C for 3h. Cells were visualised using fluorescence microscopy to determine the Dbf2-GFP localisation, and representative cells were photographed. At 23°C, Dbf2 showed the expected cell cycle localisation in all three strains. Dbf2 was present on the SPBs for much of the cell cycle (Figure 9.3 and data not shown), before re-localising to the bud neck in late mitosis (Figure 9.3). Just prior to cytokinesis, Dbf2 was no longer detected at the SPBs but formed a double ring at the neck (for example Figure 9.3, apc2-8 bub2Δ strain). After incubation at 37°C all three strains arrested in metaphase with Dbf2 localised to the SPBs. Dbf2 was never observed at the bud neck in the apc2-8 or apc2-8 mad2Δ strains, which is not surprising since these cells maintain the metaphase arrest. However, Dbf2 also retained its SPB localisation in apc2-8 bub2Δ cells (Figure 9.3), which are known to activate the MEN (9.2.1, 9.2.2). It is important to note that the combination of the DBF2.GFP allele, which is slightly temperature-sensitive, and the apc2-8 mutation means that these cells are prone to lysis following prolonged (>3h) incubation at the restrictive temperature. This lysis is particularly prevalent in re-budded cells, and consequently it was not possible to examine
Figure 9.3  Dbf2 does not re-localise to the bud neck in \textit{apc2-8 bub2Δ} metaphase-arrested cells. Strains CLF7-4f (\textit{DBF2.GFP apc2-8}), CLF13-2d (\textit{DBF2.GFP apc2-8 bub2Δ}) and SJY125 (\textit{DBF2.GFP apc2-8 mad2Δ}) were grown to mid-log phase at 23°C before being arrested at 37°C for 3h and photographed.

Work done in collaboration with Sanne Jensen
the localisation of Dbf2 in cells which have completed mitotic exit. It is possible that deletion of *BUB2* in *apc2-8* cells does enable Dbf2 to re-localise to the bud neck, but that the movement was not detected due to experimental limitations. However, the failure to detect Dbf2 at the neck in any of the cells observed (out of approximately 100 examined), when actin ring formation can be observed in up to 60% of cells arrested as dumbbells (Figure 9.2), suggests that this result is likely to be correct, and that deletion of *BUB2* is not sufficient to allow Dbf2 relocalisation to the bud neck in these cells.

9.3 Discussion

9.3.1 Control of Mitotic exit by the Bub2 checkpoint pathway

Exit from mitosis in budding yeast requires the activation of the MEN and involves the inactivation of mitotic CDK, spindle disassembly and cytokinesis. Destruction of mitotic kinase is believed to act as a licensing event for several cell cycle events, including budding, SPB duplication and DNA replication (1.8). Cells harbouring conditional mutations in components of the APC/C, for example *apc2-8*, are unable to initiate Pds1 degradation and subsequent sister chromatid separation, and arrest in metaphase at the restrictive temperature. Since APC/C^Cdc20-mediated proteolysis of Pds1, Clb5, Clb3 and Clb2 is thought to trigger activation of the MEN and mitotic exit, the metaphase arrest which results from inactivation of the APC/C might be predicted to be sufficient to prevent mitotic exit. However, the failure of the Mad2 SAC pathway to maintain a metaphase arrest in the absence of Bub2, suggests that an additional level of control is necessary.

Here we show that Bub2 is required to restrain MEN activation and prevent mitotic exit in *apc2-8* metaphase-arrested cells. At the restrictive temperature *apc2-8* cells arrest indefinitely (> 6h) in metaphase, as dumbbells with short bipolar spindles. In contrast, whilst *apc2-8 bub2A* cells initially arrest as dumbbells, following prolonged incubation (3-4h) the cells begin to re-bud, and re-duplicate their spindle pole bodies. Since licensing of bud formation and SPB duplication is normally dependent upon CDK inactivation, deletion of Bub2 alone is sufficient to allow these cells to complete mitotic exit. It was not possible
to establish whether these cells had also re-replicated their DNA, due to high levels of cell lysis in the culture, which prevented FACS analysis, but since re-initiation of DNA synthesis relies on the same licensing event it is likely that DNA re-replication also occurs. These data are consistent with Bub2/Bfa1 playing a universal role in regulating mitotic exit in metaphase-arrested cells. An exception to this rule may be during the DNA damage-induced metaphase arrest (see Chapter 10).

How does deletion of BUB2 promote mitotic exit in apc2-8-arrested cells? As discussed in previous chapters, Bub2 almost certainly prevents Tem1 activation. In the absence of Bub2, Tem1 is able to trigger a signalling cascade involving Cdc15, Cdc5, Mob1 and Dbf2, which culminates in the release of Cdc14. Although it was not possible to monitor Cdc14 release in the apc2-8 or apc2-8 bub2Δ strains due to cell lysis during the fixation procedure, by monitoring downstream events such as bud formation it is reasonable to infer that release of Cdc14 must occur in the latter strain. Cdc14 normally activates both APC/C\(^\text{Cdb1}\) and Sic1, which co-operate to inactivate Cdc28-Clb kinase. However, in the apc2-8 bub2Δ strain, the APC/C is non-functional, and loss of Cdc28-Clb kinase activity must occur solely through Sic1-mediated inhibition of the CDK complex. This is evidently sufficient to trigger mitotic exit, albeit following a delay of two or more hours. The delay probably reflects the time it takes for Sic1 to accumulate to a level high enough to eliminate Cdc28 kinase activity. These data contradict models which suggest that APC/C\(^\text{Cdc20}\)-dependent destruction of Clb2 and Clb5 is a pre-requisite for mitotic exit, and show that Sic1 will eventually trigger CDK inactivation independently of APC/C function. However, destruction of Clb2 and Clb5 is probably important for the rapid and efficient progression through mitosis which occurs in an unperturbed cell cycle.

Although deletion of BUB2 leads to the initiation of subsequent cell cycle events such as bud emergence and SPB duplication in apc2-8 cells, spindle elongation, spindle disintegration and cytokinesis do not occur. Thus, whilst MEN activation and the presumed CDK inactivation allow initiation of the next cell cycle, they are not sufficient to allow completion of the final stages of the previous one. One likely explanation for these observations is that these events require APC/C function. Spindle elongation is dependent
on APC/C^{Cdc20}-mediated Pds1 degradation which releases Esp1 (Jensen et al., 2001), whilst a key step in spindle disassembly is thought to be the proteolysis of Ase1 by APC/C^{Cdh1} (Zachariae and Nasmyth, 1999). The failure of apc2-8 bub2Δ cells to elongate and disassemble their spindles in consistent with these models.

9.3.2 The role of the MEN in regulating cytokinesis

The inability of apc2-8 bub2Δ cells to complete cytokinesis is even more intriguing than their failure to disassemble the mitotic spindle. The data presented here show that Bub2 and the MEN do regulate actin ring formation. Deletion of BUB2 leads to premature actin ring formation in apc2-8-arrested cells, which is abolished by loss of function mutations in the MEN components Tem1, Cdc15, Cdc5, Mob1, and Cdc14. Whether formation of the actin ring is controlled by the MEN directly, or is dependent on a downstream event such as CDK inactivation remains to be seen, although the requirement for Cdc14 suggests that CDK inactivation may be the key.

Although they do form an actomyosin ring, apc2-8 bub2Δ cells fail to complete cytokinesis. A trivial explanation is that the actin ring is defective in some way. However, it is more likely that the final stages of cytokinesis are dependent upon events downstream of the MEN which are blocked in these cells. One event which is likely to be important for cytokinesis is the re-localisation of certain MEN components from the SPBs to the bud neck in late mitosis. It is becoming clear that after the MEN proteins have triggered CDK inactivation, they are also involved in directly promoting cytokinesis, a function which seems to controlled by their localisation (1.8.4). Unlike, Cdc5 and Cdc15, Dbf2 kinase has not been formally shown to control cytokinesis directly, but it is likely that this will prove to be the case. The re-localisation of Dbf2 to the bud neck is known to be MEN-dependent (Frenz et al., 2000), implying that a feedback loop exists within the MEN. However, the data presented here show that Dbf2 is situated at the SPBs in apc2-8 bub2Δ cells. This indicates that activation of the MEN is necessary but not sufficient for re-localisation of Dbf2, and suggests that APC/C function may be important for this process. If, as is likely, re-localisation of MEN components such as Dbf2 to the bud neck in late mitosis is required
to trigger cytokinesis, it is tempting to speculate that the failure of *apc2-8 bub2Δ* cells to re-localise these proteins may account for their cytokinesis defect. APC/C function may be required for this re-localisation, either directly (for example to degrade proteins which localise the MEN components to the SPBs), or indirectly (for example to disassemble the spindle which could inhibit MEN re-localisation, or another final stage of cytokinesis). However, other interpretations are also possible and further work is needed to clarify the role of the MEN and the APC/C in control of cytokinesis.

These experiments show that deletion of *BUB2* is sufficient to allow MEN activation, independently of the interaction between Tem1 and Lte1, which cannot occur in these cells since the spindle fails to elongate through the bud neck. Moreover, in the absence of Bub2 mitotic exit occurs independently of APC/C^Cdc20^-mediated destruction of the anaphase inhibitors Pds1 and Clb5. This emphasises the importance of the Bub2/Bfa1 pathway in regulating mitotic exit during a metaphase arrest. These experiments also provide a convenient way to distinguish between events which are solely dependent upon MEN activation or CDK inactivation, and which can occur in the absence of APC/C function through Sic1 (actin ring formation, bud formation, SPB duplication and DNA replication), and those which require an additional step, which is almost certainly APC/C-dependent (spindle elongation, spindle disassembly, Dbf2 re-localisation and cytokinesis). This is illustrated in Figure 9.4.
Figure 9.4  Control of late mitotic events by the MEN, Sic1 and the APC/C. Deletion of Bub2 triggers MEN activation and subsequent downstream events in *apc2-8* metaphase-arrested cells, suggesting that the Bub2/Bfa1 pathway is essential to restrain MEN function during any metaphase arrest. MEN activation leads to release of Cdc14, which in turn triggers activation of APC/C\(^{C\text{dh1}}\) and Sic1. In the absence of APC/C function, MEN activation is sufficient to trigger actin ring formation and mitotic exit (which leads to re-budding and SPB re-duplication). As shown in the figure, both of these events are probably a result of Sic1-mediated CDK inactivation, although it is possible that the MEN controls actin ring formation directly.

However, MEN activation is not sufficient to trigger spindle elongation, spindle disassembly, Dbf2 re-localisation or cytokinesis when the APC/C is inactive, suggesting that these steps require APC/C function. Spindle elongation and spindle disassembly are probably controlled directly by the APC/C, through proteolysis of Pds1 and Ase1 respectively. Dbf2 re-localisation and cytokinesis may require a combination of CDK inactivation and APC/C function. In fact, since cytokinesis is dependent upon the presence of an actin ring, this ensures that it occurs after CDK inactivation.
Chapter 10

Discussion

10.1 Order of function of the mitotic exit network

Activation of mitotic exit and cytokinesis in budding yeast is dependent upon the mitotic exit network (1.8.1). Although many proteins have been shown to function within the MEN, including Tem1, Cdc15, Cdc5, Dbf2, Mob1 and Cdc14 (Johnston et al., 1990; Kitada et al., 1993; Shirayama et al., 1994; Toyn and Johnston, 1994), their regulation and order of function remained enigmatic. By studying the activation of Dbf2 kinase I have shown that the MEN functions in the order Tem1-Cdc15-Mob1-Dbf2-Cdc14 (Chapter 3). These results, combined with recently published data, have provided a detailed model of MEN pathway function. Activation of Tem1 promotes its association with Cdc15, and recruits Cdc15 to the SPB (Asakawa et al., 2001). Cdc15 then phosphorylates and activates Dbf2 (Mah et al., 2001), which is also present at the SPBs (Frenz et al., 2000). Dbf2 kinase activity is also dependent upon its interaction with Mob1 (Chapter 3), but since Dbf2 and Mob1 associate throughout the cell cycle (G. Wells, personal communication), activation of Dbf2 in late mitosis is probably controlled by Cdc15. Thus activation of Tem1 triggers a signalling cascade which culminates in Dbf2 kinase activation.

Activation of the Mob1-Dbf2 complex presumably promotes the release of Cdc14 from the nucleolus, although it is not clear whether Mob1 and Dbf2 directly control the release or whether additional proteins are involved. With the exception of Cdc14, none of the MEN genes identified to date were found to act downstream of Dbf2, suggesting that Dbf2 may indeed control Cdc14 release directly. Dbf2 has not been detected in the nucleolus, but detailed studies of Dbf2 localisation also failed to detect the protein concentrated in the nucleus (Frenz et al., 2000), although Dbf2 is known to be a component of the CCR4
transcriptional complex (Liu et al., 1997). Dbf2 could feasibly be present at low levels in the nucleolus, at least during late mitosis.

The only MEN component studied which was not ordered within the MEN pathway was Cdc5. Cdc5 is required for Dbf2 kinase activation, and must therefore be involved in activation of the MEN. However, Cdc5 kinase is not controlled by any of the MEN components studied (Tem1, Cdc15, Mob1, Cdc14). This is similar to the situation in S. pombe, where Plol kinase activation is independent of the SIN (Tanaka et al., 2001). Thus Cdc5 is not a component of the linear pathway outlined above. Cdc5 could promote MEN function upstream of Tem1, either by activating Lte1, or inactivating Bub2 through Bfa1. However, there are two reasons why this is unlikely to be the case. Firstly, Cdc5 is still required to activate Dbf2 kinase in cells lacking BUB2, where any upstream regulation of Tem1 is likely to have been removed. Secondly, Cdc5-dependent phosphorylation of Bfa1 and Lte1 is likely to inhibit Tem1 (see later) (Lee et al., 2001) indicating that the Cdc5-dependent activation of the MEN must occur downstream of Tem1. Thus the independent activation of both Tem1 and Cdc5 is necessary to promote MEN signal transduction (Figure 10.1). Presumably Cdc5-dependent phosphorylation is required to activate one or more MEN components. Several of the MEN components are known to be phosphoproteins, including Cdc15 (Jaspersen and Morgan, 2000; Menssen et al., 2001; Xu et al., 2000), Dbf2 (Toyn and Johnston, 1994) and Mob1 (D. Fesquet, personal communication), and are therefore potential Cdc5 substrates. Investigating the phosphorylation status of these proteins in cdc5 mutants may help to clarify the role of Cdc5 in controlling the MEN.

10.2 Control of Tem1 by Bub2/Bfa1

As discussed in Chapter 3, Tem1 functions at the top of the MEN signalling pathway, and its activation appears to trigger activation of the entire MEN. It is therefore critical that Tem1 is tightly regulated, since premature MEN activation could lead to premature mitotic exit and cytokinesis, generating anucleate and binucleate cells.
Figure 10.1 Model illustrating the control of mitotic exit in budding yeast. Activation of the MEN requires the activation of both Tem1 and Cdc5. The mechanisms controlling Cdc5 activation are not well understood, but Tem1 is subject to negative regulation by Bub2/Bfa1, and positive regulation by Lte1. Bub2 and Bfa1 act during metaphase to ensure that Tem1 activation does not occur. Bub2/Bfa1 GAP function is likely to be dependent upon Cdc28- and Cdc5-dependent phosphorylation of Bfa1. Cdc5 therefore functions at least twice within the MEN, initially acting upstream of Bub2/Bfa1 to prevent Tem1 activation. Once Tem1 is active, Cdc5 is required for MEN activation.
Metaphase → Anaphase → Mitotic exit

Cytokinesis

APC/C^{Cdc20} → APC/C^{Cdh1}

Pds1

Cdc28
Clb2

Cdc28
Cdc5
Bfa1
Bub2
Lte1
Tem1

MEN

Active

Cdc5
10.2.1 Bub2/Bfa1 appear to form a two-component GAP for Tem1

Bub2 and Bfa1 are thought to form a two-component GAP which negatively regulates Tem1. I have therefore investigated the regulation of Tem1 by Bub2 and Bfa1. Using Dbf2 kinase activity as a measure of MEN pathway activation, I have shown that, like Bub2, Bfa1 is required to inhibit MEN activation in the presence of spindle damage. Thus Bub2 and Bfa1 both act as negative regulators of the MEN following activation of the SAC. Bub2 and Bfa1 physically associate throughout the cell cycle, consistent with them functioning as a two-component GAP for Tem1. Moreover, both Bub2 and Bfa1 interact with Tem1, and this interaction may exist for much of the cell cycle, since it is detectable during G1 and metaphase.

10.2.2 Bfa1 phosphorylation is likely to regulate GAP activity

Since the Bub2/Bfa1 heterodimer exists throughout the cell cycle, GAP activation cannot be regulated by heterodimer formation, and must therefore be controlled by some other means. Bub2 protein levels are constant throughout the cell cycle and the protein is not subject to post-translational modification, indicating that Bub2 is not a regulatory component of the GAP. However, Bfa1 is subject to cell-cycle dependent phosphorylation which is maximal during mitosis, declining as cells exit mitosis and enter G1 phase. Consistent with a role in promoting Bub2/Bfa1 GAP activity towards Tem1, this phosphorylation is maintained following activation of the spindle assembly checkpoint.

Bfa1 phosphorylation is unlikely to regulate the association of the Bub2/Bfa1 heterodimer with Tem1, since all three proteins are present as a complex during G1 phase, when Bfa1 is unphosphorylated. Similarly, phosphorylation is unlikely to control the localisation of Bub2 and Bfa1, since both proteins are present at the SPB throughout the cell cycle (Fraschini et al., 1999; Pereira et al., 2000). The simplest interpretation is that Bfa1 phosphorylation promotes Bub2/Bfa1 GAP activity towards Tem1.
Unfortunately I have been unable to definitively prove that phosphorylation of Bfa1 is required for Bub2/Bfa1 function. Mutant kinases which affect Bfa1 phosphorylation (see below) also lead to a cell cycle arrest, rendering the standard assays for Bub2 pathway functionality (re-budding assay, Dbf2 kinase inhibition assay) useless. Attempts to construct a non-phosphorylatable mutant form of Bfa1 by site-directed mutagenesis were unsuccessful, since the mutant form of the protein was not detectable in vivo (presumably due to degradation). In the future, identification of the Bfa1 phosphorylation sites by peptide mapping may aid the construction of a non-phosphorylatable mutant. However, this is beyond the scope of this project.

10.2.3 Bfa1 is phosphorylated by Cdc28 and Cdc5

Two cell cycle-regulated kinases were found to be required for Bfa1 phosphorylation, namely Cdc28 and Cdc5 (Figure 10.1). Initiation of the Cdc28-dependent phosphorylation coincides with the increase in Clb2 protein levels in G2 phase, and data presented in Chapters 7 and 8 indicate that Cdc28-Clb2 may control Bfa1 phosphorylation. The Cdc28-dependent phosphorylation precedes that by Cdc5, and is probably necessary for the Cdc5-dependent phosphorylation. Cdc5 controls the changes in Bfa1 phosphorylation which occur later in mitosis, namely the increased intensity of phosphorylated species IV and VI (Chapter 6). This pattern of phosphorylation is observed in cells arrested in metaphase in the presence of nocodazole, or using an apc2-8 temperature-sensitive mutation, when the GAP is known to be active. This suggests that the Cdc5-dependent phosphorylation may be even more important for regulation than that of Cdc28. Unfortunately the Cdc5 phosphorylation sites have not been mapped, so like that of Cdc28, the function of the Cdc5-dependent Bfa1 phosphorylation remains enigmatic.

The presumed activation of Bub2/Bfa1 GAP activity by Cdc5-dependent phosphorylation of Bfa1 implies that Cdc5 negatively regulates the MEN pathway upstream of Tem1. This contrasts with the situation in Schizosaccharomyces pombe, where the homologue of CDC5, plol+, has been suggested to act as an upstream activator of the TEM1 homologue, spg1+ (Tanaka et al., 2001). However, the authors state that their data is also consistent
with Plo1 acting in concert with the SIN to induce downstream events (namely septation). This latter interpretation is consistent with the role of Cdc5 in the MEN discussed above (10.1), and it therefore seems likely that the roles of Plo1 and Cdc5 in activating the SIN and the MEN are conserved, and occur downstream of Spg1/Tem1. Whether Plo1 also negatively regulates the SIN by phosphorylating Byr4 remains to be seen, although Byr4 is also thought to be a phospho-protein (Song et al., 1996).

### 10.3 Bub2 and Bfa1 enforce a dependency relationship between spindle elongation through the bud neck, and mitotic exit

*BUB2* and *BFA1* formally appear to function as a branch of the spindle assembly checkpoint pathway (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999). However, cells arrested in metaphase following SAC activation display the same Bfa1 phosphorylation pattern as that observed during mitosis of an unperturbed cell cycle or in an *apc2-8*-induced metaphase arrest. This suggests that Bub2/Bfa1 checkpoint function is not confined to the SAC, but fulfils a more general mitotic role.

The data presented in this thesis show that the Bub2 checkpoint pathway is also required to prevent mitotic exit when cells are arrested in metaphase using an *apc2-8* temperature-sensitive mutation (Chapter 9). In the absence of Bub2 function, *apc2-8* cells arrested at the restrictive temperature begin to initiate events of the subsequent cell cycle, namely SPB duplication and bud formation. Pereira et al., (2000) similarly showed that cells arrested in metaphase following deletion of another APC/C subunit, *CDC26*, were unable to maintain a metaphase arrest in the absence of *BUB2*. This suggests that the Bub2 pathway is required to restrain mitotic exit during a variety of metaphase arrests. This is consistent with data published by Adames et al., (2001) who propose that Bub2 and Bfa1 maintain Tem1 and the MEN in an inactive state until the SPB<sub>daughter</sub> has entered the bud during anaphase. The movement of the SPB<sub>daughter</sub> through the bud neck inactivates Bub2 and Bfa1, allowing mitotic exit to proceed. Bub2 and Bfa1 therefore enforce a dependency relationship between spindle elongation through the bud neck and initiation of mitotic exit. Thus the Bub2 pathway is likely to be necessary to delay mitotic exit during any cell cycle arrest in...
which spindle elongation through the bud neck fails to take place. The spindle assembly checkpoint, nuclear positioning checkpoint and mutational inactivation of the APC/C all lead to cells arresting with the nucleus in the mother cell, and therefore require Bub2 and Bfa1 to prevent mitotic exit by maintaining Tem1 in its inactive form.

10.4 Inactivation of Bub2/Bfa1 in late mitosis

The Bub2/Bfa1-mediated inhibition of Tem1 is only essential for viability when progression through mitosis is delayed, for example following SAC activation (Hoyt et al., 1991; Krishnan et al., 2000; Li, 1999) or spindle misalignment (Daum et al., 2000; Pereira et al., 2000). However, the Bub2 pathway is likely to function every cell cycle to ensure that mitotic exit occurs after spindle elongation. The cell cycle-dependent phosphorylation of Bfa1 is consistent with the GAP functioning every cell cycle (10.3). Activation of Tem1 in late mitosis is therefore dependent upon the loss of Bub2/Bfa1 function. This is likely to be controlled by redundant pathways.

10.4.1 Lte1 overcomes the Bub2/Bfa1-mediated inhibition of Tem1

Bardin et al., (2000) proposed that Tem1 activation was dependent upon the SPB\textsubscript{daughter} entering bud neck and contacting Lte1 (1.8.1.2; Figure 1.3). This model is evidently an over-simplification, since LTE1 is a non-essential gene, at least at high temperatures. However, Lte1 is involved in activating Tem1 \textit{in vivo}. \textit{lte1Δ} strains are cold-sensitive, and arrest in telophase at low temperatures, indicating that Lte1 is essential for mitotic exit under these conditions. The data presented here show that deletion of \textit{BUB2} or \textit{BFA1} relieves the cold-sensitivity of an \textit{lte1Δ} strain, indicating that the essential mitotic function of Lte1 is to overcome the negative regulation of Tem1 imposed by Bub2 or Bfa1. Since G-protein's GAP and GEF often share similar binding sites (Parrini et al., 1997), Lte1 may displace Bub2/Bfa1 from Tem1. Interestingly, although the localisation of Tem1 to the SPB is dependent on Bub2 and Bfa1 for much of the cell cycle, in late mitosis this dependency is lost (Pereira et al., 2000), suggesting that the nature of the Tem1-Bub2/Bfa1 interaction may indeed change at this stage in the cell cycle.
10.4.2 Bub2/Bfa1 are inactivated once the SPB\textsubscript{daughter} interacts with the bud neck

In the absence of Lte1, activation of Tem1 in late mitosis must be achieved by some other means. Bardin \textit{et al.}, (2000) suggest that Tem1 activation does not require Lte1 at higher temperatures because Tem1 has a high intrinsic exchange activity. Whilst this is likely to be true, it is only part of the story. In metaphase-arrested cells, when both SPBs are situated in the mother cell, Bub2/Bfa1 prevent Tem1 activation. Thus Bub2/Bfa1 can clearly inhibit any intrinsic exchange activity of Tem1. Deletion of \textit{BUB2} or \textit{BFAL} in these cells leads to activation of the MEN (Chapter 4; Fesquet \textit{et al.}, 1999). This suggests that whilst Tem1 is likely to possess a high intrinsic exchange activity, which enables it to become active independently of Lte1, this "self-activation" can only occur in the absence of Bub2 and Bfa1. This implies that activation of Tem1 only occurs once the negative regulation mediated by Bub2/Bfa1 has been removed.

Bub2 and Bfa1 must therefore be inactivated before mitotic exit can occur. Loss of Bub2/Bfa1 function is partly controlled by Lte1 (see above) but there is clearly an additional, Lte1-independent mechanism which operates in \textit{lte1\Delta} cells. It has been suggested that the Bub2 checkpoint is inactivated as the SPB\textsubscript{daughter} passes through the bud neck (Figure 10.2) (Daum \textit{et al.}, 2000). The mechanism for this is not clear, although it may involve interactions with components of the septin ring (Krishnan \textit{et al.}, 2000). Alternatively, Bub2/Bfa1 may sense changes in cytoplasmic microtubule dynamics which take place as the SPB\textsubscript{daughter} interacts with the bud neck (Daum \textit{et al.}, 2000). It is worth noting that the Bub2 checkpoint is not infallible, since Bub2/Bfa1 are inactivated once the SPB\textsubscript{daughter} interacts with the bud neck. If the SPB\textsubscript{daughter} subsequently fails to enter the daughter cell, mitotic exit still occurs, generating one anucleate and one binucleate cell (Adames \textit{et al.}, 2001; Daum \textit{et al.}, 2000).
Figure 10.2  Speculative model illustrating how the activation of Tem1 in late mitosis may be regulated. Bub2, Bfa1 and Tem1 all localise to the SPB. During metaphase Bub2 and Bfa1 maintain Tem1 in its inactive, GDP-bound state. Cdc28- and Cdc5-dependent phosphorylation of Bfa1 probably promotes Bub2/Bfa1 GAP activity (red lines). Pds1 may also act upstream of Bub2/Bfa1 to inhibit the MEN (Krishnan et al., 2000).

APC/C\(^{Cdc20}\) activation at the metaphase-anaphase transition promotes Pds1 degradation and loss of sister chromatid cohesion. APC/C\(^{Cdc20}\) also targets Clb3, Clb5 and Clb2 for ubiquitin-mediated proteolysis, which may lead to a reduction in Bfa1 phosphorylation and a decrease in GAP activity. Following sister chromatid segregation, the spindle elongates and the SPB\(_{\text{daughter}}\) interacts with the bud neck, inactivating the Bub2/Bfa1 pathway and allowing Tem1 to become active. As the SPB\(_{\text{daughter}}\) enters the daughter cell, Tem1 interacts with Lte1. Although not essential for Tem1 activation at high temperatures, this interaction probably enhances the activity of Tem1, and is indeed essential for Tem1 activation below 17°C.

Tem1 activation triggers the MEN signalling cascade which culminates in activation of APC/C\(^{Cld1}\). APC/C\(^{Cld1}\)-mediated proteolysis of Clb2 and Cdc5 leads to a loss of Bfa1 phosphorylation. This triggers a positive feedback loop, and ensuring that Bub2/Bfa1 remain inactive until the subsequent cell cycle.
Metaphase

- Spindle
- Lte1 localises to the bud
- Tem1, Bub2 and Bfa1 localise to the SPB

Anaphase

- Bub2/Bfa1 interact with the bud neck
- Tem1 interacts with Lte1

Bfa1 - Bub2 - Lte1

Tem1

MEN

APC/C^{Cdc20} → Pds1

APC/C^{Cdh1} → Cdc28, Clb2, Cdc5
10.4.3 Loss of Bfa1 phosphorylation may contribute to Bub2/Bfa1 inactivation

The knowledge that Bfa1 is subject to Cdc28- and Cdc5-dependent phosphorylation introduces a further dimension to the regulation of Bub2/Bfa1 GAP activity. If phosphorylation is required for GAP function, then Bfa1 de-phosphorylation could inactivate the GAP. Although Bfa1 does not seem to be a substrate of Cdc14, de-phosphorylation of Bfa1 in late mitosis is dependent upon MEN function. Since the MEN activates APC/C^{Cdh1}, which targets Clb2 and Cdc5 for ubiquitin-mediated proteolysis, it seems likely that a feedback loop operates whereby APC/C^{Cdh1} activation leads to destruction of Cdc28-Clb2 and Cdc5, and loss of Bfa1 phosphorylation (Figure 10.2).

Whilst this model explains why Bfa1 phosphorylation declines rapidly following MEN activation, it does not explain how Bub2/Bfa1 activity is reduced sufficiently to allow MEN activation in this first place. Whilst this is likely to be primarily dependent upon the spatial regulation described above, the APC/C^{Cdc20}-dependent reduction in Cdc28-Clb2 kinase activity which occurs as cells enter anaphase (Yeong et al., 2000) may also reduce Bub2/Bfa1 activity sufficiently to allow initiation of MEN signalling. This would then be rapidly amplified as APC/C^{Cdh1} became active (Figure 10.2).

In summary, a number of pathways probably control activation of Tem1 in late mitosis, by promoting the removal of Bub2/Bfa1 activity (Figure 10.2). APC/C^{Cdc20}-mediated proteolysis of Clb2 may lead to a loss of Bfa1 phosphorylation, reducing Bub2/Bfa1 activity. Proteolysis of Pds1 by APC/C^{Cdc20} also promotes MEN activation, by a mechanism which is thought to act upstream of the Bub2 pathway (Krishnan et al., 2000). Inactivation of Bub2/Bfa1 is also regulated spatially. As the spindle elongates, the SPB_{daughter} interacts with the bud neck, leading to inactivation of Bub2/Bfa1 by an unknown mechanism. In addition, spindle elongation through the bud neck brings Tem1 into contact with Lte1, which overcomes any remaining Bub2/Bfa1-mediated inhibition of Tem1. Activation of APC/C^{Cdh1} by the MEN leads to proteolysis of Cdc5 and Clb2, triggering a feedback loop which ensures that Bub2/Bfa1 remain inactive until the following cell cycle.
It is worth noting that spindle elongation is of course also dependent upon APC/C<sup>Cdc20</sup> function, and activation of APC/C<sup>Cdc20</sup> would therefore normally lead to inactivation of Bub2/Bfa1 and initiation of mitotic exit. The only occasion where Bub2/Bfa1-mediated inhibition continues to operate following APC/C<sup>Cdc20</sup> activation is during the nuclear positioning checkpoint, when the SPB<sub>daughter</sub> fails to interact with the bud neck due to spindle misalignment.

### 10.5 The requirement for the Bub2/Bfa1 pathway in metaphase-arrested cells

Since Bub2 and Bfa1 function in a general sense to couple spindle elongation to initiation of mitotic exit, it is inaccurate to refer to the Bub2 pathway as a branch of the SAC. It is in fact a separate checkpoint, more accurately described as a spindle positioning checkpoint. However, since APC/C<sup>Cdc20</sup> function and subsequent spindle elongation are required to inactivate the Bub2 pathway, any cell cycle arrest which prevents either APC/C<sup>Cdc20</sup> activation, or spindle elongation through the neck will protract the function of Bub2/Bfa1. Deletion of BUB2 or BFA1 in these cells allows MEN activation, leading to mitotic exit and initiation of subsequent cell cycle events.

#### 10.5.1 The role of Bub2/Bfa1 in SAC-arrested cells

Interestingly, deletion of BUB2 or BFA1 in cells arrested in metaphase following SAC activation not only leads to activation of mitotic exit, but also enables cells to segregate sister chromatids, despite the fact that the Mad checkpoint pathway is still functional, and APC/C<sup>Cdc20</sup> is therefore inactive. MEN activation therefore not only leads to activation of mitotic exit, but also promotes the metaphase/anaphase transition, independently of APC/C<sup>Cdc20</sup>. The most likely explanation for this is that suggested by Krishnan <em>et al.</em>, (2000), and is based upon the growing conviction that the substrate specificity of APC/C<sup>Cdc20</sup> and APC/C<sup>Cdh1</sup> is not as great as was previously thought (Yeong <em>et al.</em>, 2000). In essence, even if APC/C<sup>Cdc20</sup> function has been inhibited, activation of APC/C<sup>Cdh1</sup> will lead to degradation of both its own substrates and those normally targeted by APC/C<sup>Cdc20</sup>. Thus in
order to prevent progression through mitosis, activation of both \( \text{APC/C}^{\text{Cdc20}} \) and \( \text{APC/C}^{\text{Cdh1}} \) must be prevented (Figure 10.3).

This model explains why both the Mad and Bub2 checkpoint pathways are required to enforce a SAC arrest. If cells are incubated in the presence of the microtubule depolymerising agent nocodazole, in the absence of the Mad checkpoint pathway, \( \text{APC/C}^{\text{Cdc20}} \) remains active and anaphase is initiated. Since spindle elongation through the bud neck is compromised in the absence of the spindle, Bub2/Bfa1 exert a short anaphase delay, but as soon as one of the SPBs interacts with the bud neck, Bub2 pathway function is lost and MEN activation occurs (Figure 10.4, lower panel). Conversely, cells with a functional Mad checkpoint pathway, but lacking Bub2 or Bfa1 are able to inhibit \( \text{APC/C}^{\text{Cdc20}} \) in the presence of nocodazole. However, without the Bub2/Bfa1-dependent inhibition of Tem1, the MEN is activated, leading to activation of \( \text{APC/C}^{\text{Cdh1}} \) which substitutes for \( \text{APC/C}^{\text{Cdc20}} \) (Figure 10.4, lower panel). In either case, the net effect is loss of the arrest, leading to loss of sister chromatid segregation, and mitotic exit.

### 10.5.2 Bub2/Bfa1 and the DNA damage checkpoint pathway

If, as suggested above, it is necessary to simultaneously prevent the function of both \( \text{APC/C}^{\text{Cdc20}} \) and \( \text{APC/C}^{\text{Cdh1}} \) in order to block progression through mitosis, then it is critical that checkpoints which arrest cells in metaphase inhibit both these forms of the APC/C. The spindle assembly checkpoint prevents activation of \( \text{APC/C}^{\text{Cdc20}} \) by sequestering Cdc20 (see above). This automatically protracts the function of Bub2/Bfa1 and prevents activation of \( \text{APC/C}^{\text{Cdh1}} \) by inhibiting Tem1 (Figure 10.5, upper panel).

In contrast, the DNA damage checkpoint may inhibit \( \text{APC/C}^{\text{Cdc20}} \) and \( \text{APC/C}^{\text{Cdh1}} \) by very different means. Following activation of the DNA damage checkpoint, Chk1-dependent phosphorylation of Pds1 prevents its degradation by \( \text{APC/C}^{\text{Cdc20}} \), leading to a metaphase arrest. In addition, the checkpoint leads to inhibition of Cdc5. If Cdc5-dependent phosphorylation of Bfa1 is required for Bub2/Bfa1 function, its inhibition would compromise the Bub2/Bfa1-dependent inhibition of Tem1. However, since Cdc5 is
Figure 10.3  Model illustrating the role of the Bub2/Bfa1 checkpoint pathway following activation of the spindle assembly checkpoint. Spindle damage leads to activation of the Mad checkpoint pathway which inhibits APC/C\textsuperscript{Cdc20}, generating a metaphase delay. This arrest prevents spindle elongation and therefore protracts the function of the Bub2/Bfa1 checkpoint pathway. The pathways which operate to enforce the spindle checkpoint arrest are shown in red.
Spindle damage

Bfa1
Bub2
Lte1

MEN

APC/C

Pds1

APC/C

Cdc28
Clb2

Metaphase → Anaphase → Mitotic exit

Cytokinesis
Model illustrating why both the Mad and Bub2 checkpoint pathways are required to induce a spindle assembly checkpoint arrest. Following spindle damage, the Mad checkpoint pathway inhibits APC/C<sup>Cdc20</sup>, generating a metaphase arrest. However, in the absence of Bub2/Bfa1 function, the intrinsic exchange activity of Tem1 is sufficient to trigger activation of the MEN. This in turn leads to activation of APC/C<sup>Cdc11</sup>. APC/C<sup>Cdc11</sup> normally targets Cdc5, Clb2, Cdc20 and Ase1 for ubiquitin-mediated proteolysis. However, in the absence of APC/C<sup>Cdc20</sup> function, APC/C<sup>Cdc11</sup> also promotes the degradation of Pds1, Clb3, and Clb5. Thus activation of the MEN bypasses the metaphase-anaphase delay, as well as the delay in mitotic exit.

Conversely, if the Mad checkpoint pathway is disrupted, cells fail to arrest in metaphase following spindle damage. Sister chromatid separation proceeds as normal, but spindle positioning and elongation are perturbed due to the disruption of cytoplasmic and spindle microtubules. The Bub2/Bfa1 checkpoint pathway therefore enforces an anaphase delay. However, the movement of the nucleus within the mother cell means that the SPBs are likely to collide with the bud neck, and once this interaction occurs, the Bub2/Bfa1 pathway is inactivated, allowing the cell to escape the arrest and initiate mitotic exit.
**Metaphase**

- APC/C
- Cdc20
- Pds1

- Mps1
- Mad1/2/3
- Bub1/3

**Anaphase**

- APC/C
- Cdc20
- Pds1

- Cdc28
- Clb2

**Mitotic exit**

- APC/C
- Cdh1

**Cytokinesis**

- Bfa1
- Bub2

- MEN
Figure 10.5  Model illustrating the possible differences between the mechanisms employed by the spindle assembly and DNA damage checkpoints to enforce a metaphase arrest. The SAC inhibits APC/C\(^{Cdc20}\), enforcing a metaphase delay which protracts the function of the Bub2/Bfa1 checkpoint pathway. Bub2 and Bfa1 are absolutely required to inhibit the MEN and prevent mitotic exit.

The DNA damage checkpoint promotes the phosphorylation of Pds1, which prevents its degradation by APC/C\(^{Cdc20}\). In addition, this checkpoint leads to the inhibition of Cdc5. This is likely to further inhibit APC/C\(^{Cdc20}\) function, since Cdc5-dependent phosphorylation of the APC/C is thought to promote its activity. Since Cdc5 is required for Bfa1 phosphorylation, inhibition of Cdc5 is likely to abrogate Bub2/Bfa1 pathway function and lead to Tem1 activation. However, even following Tem1 activation, MEN activation cannot occur in the absence of Cdc5 (Chapter 3). By inhibiting Cdc5, the DNA damage checkpoint may therefore be able to prevent mitotic exit independently of Bub2/Bfa1.
Spindle assembly checkpoint

\[ \text{APC/C}^{Cdc20} \]
\[ \text{Pds1} \]
Metaphase \( \rightarrow \) Anaphase \( \rightarrow \) Mitotic exit

Cytokinesis

DNA damage checkpoint

\[ \text{APC/C}^{Cdc20} \]
\[ \text{Pds1} \]
Metaphase \( \rightarrow \) Anaphase \( \rightarrow \) Mitotic exit

Cytokinesis
required downstream of Tem1 to activate the MEN (10.1) inhibition of Cdc5 would itself prevent MEN activation. In addition, Cdc5-dependent phosphorylation of the APC/C is likely to be required for its activity (1.7. 2.1) (Figure 10.5, lower panel). Thus the spindle assembly and DNA damage checkpoints both prevent activation of both forms of the APC/C. The crucial difference is that the former protracts Cdc5 activity and hence Bub2/Bfa1 function, whilst the latter inhibits Cdc5, and therefore probably abrogates Bub2/Bfa1 function. This is consistent with Bub2 and Bfa1 being dispensable for a DNA damage checkpoint-induced metaphase arrest, as reported by Krishnan et al., (2000).

10.5.3 Metaphase arrests which may be independent of the Bub2 pathway

There are two metaphase arrests where Bub2 and Bfa1 have not yet been proven to play a role. The first is the DNA damage checkpoint-induced metaphase arrest (10.5.2). The second metaphase arrest where the involvement of Bub2 and Bfa1 has not yet been shown is that induced by the nuclear positioning defect in cdc28-4 clb5Δ mutant cells, when the undivided nucleus translocates into the bud. The latter arrest is presumably checkpoint-dependent, but the components of this putative checkpoint have not been identified. Since the Bub2 checkpoint is thought to be inactivated as the SPB_{daughter} enters the bud, it might seem improbable that Bub2 and Bfa1 are involved in this checkpoint arrest. However this has not been confirmed experimentally.

Alternatively, Bub2 and Bfa1 function could be retained in these cells, preventing MEN activation. Consistent with the latter model, Bfa1 phosphorylation is enhanced in cdc28-4 clb5Δ cells, suggesting that GAP activity is maintained. This would imply that under certain circumstances Bub2/Bfa1 activity can be maintained even if the SPB_{daughter} has passed through the bud neck. It is worth noting that the presumed inactivation of Bub2/Bfa1 which occurs as the SPB_{daughter} enters the bud, creates an asymmetry between the two SPBs, with active Tem1 being present at the SPB_{daughter}. This situation is similar to that in S. pombe, where Cdc16 and Byr4 (homologues of Bub2 and Bfa1) inactivate Spgl (the Tem1 homologue) at one pole. This asymmetry is important for activation of the SIN pathway and cytokinesis. The same may be true in S. cerevisiae. If so, when the nucleus
translocates into the daughter cell in the \textit{cdc28-4 clb5A} mutant, the loss of SPB asymmetry (and presumed activation of Tem1 at both poles) could interfere with MEN activation.

10.6 Regulation of cytokinesis by Bub2/Bfa1 and the MEN

Deletion of Bub2/Bfa1 leads to activation of mitotic exit and initiation of events of the subsequent cell cycle in \textit{apc2-8}-arrested cells (Chapter 9). In the absence of APC/C function, CDK inactivation is presumably due to direct inhibition of the Cdc28-Clb complex by Sic1. This is sufficient to allow new bud formation and SPB duplication (and presumably also DNA replication). However, these cells do not complete cytokinesis. Deletion of Bub2 leads to formation of the acto-myosin contractile ring, which is MEN-dependent (although it is not clear whether the MEN components regulate actin ring formation directly, or indirectly through CDK inactivation). However, the failure to complete cytokinesis suggests that APC/C function is also required. The lack of cell division may be due to the failure of the MEN components to re-locate to the bud neck, a step which could be APC/C-dependent. Alternatively, spindle disassembly, which requires the APC/C-dependent proteolysis of Ase1 and therefore fails to occur in these cells, could be necessary for cytokinesis. Whatever the additional requirements, it is clear that whilst CDK inactivation is necessary and sufficient for initiation of the subsequent cell cycle, consistent with previously published data (Noton and Diffley, 2000), CDK inactivation is necessary but not sufficient for cytokinesis.

10.7 The role of the Bub2/Bfa1 pathway in higher eukaryotes

The fact that the \textit{Saccharomyces cerevisiae} MEN and \textit{Schizosaccharomyces pombe} SIN pathways are so highly conserved suggests that they will also exist in higher eukaryotes. However, since the roles of these two protein signalling networks are subtly different, it is difficult to predict their functions in more complex systems. Two possible models are discussed below, based on the roles of Bub2/Bfa1 and Cdc16/Byr4 in the two yeasts.
Although dispensable in yeast, checkpoint pathways are often an essential aspect of cell cycle control in mammalian systems, and this may well be true for the Bub2 pathway. There is good evidence that the genetic instability associated with tumour development in mammalian is due to loss of checkpoint regulation. One characteristic of human tumour cells is that they are aneuploid, often containing more than a 2C DNA content. Based on this, it has been suggested that during tumour development, cells periodically undergo chromosome duplication in the absence of cell division, doubling their DNA content. They then gradually lose chromosomes (often tumour suppressors) whilst retaining others (often oncogenes), generating a more malignant tumour through a process of natural selection for rapidly dividing cells. This loss and gain of entire chromosomes contributes to the process of clonal expansion.

In budding yeast Bub2 and Bfa1 appear to monitor the completion of sister chromatid segregation, preventing initiation of mitotic exit and cytokinesis until anaphase has been completed. Loss of the Bub2 checkpoint can lead to cell division occurring before nuclear segregation, generating anucleate and binucleate cells. If the Bub2 pathway is conserved in humans, mutations in this pathway could similarly lead to the production of binucleate cells, initiating the clonal expansion described above.

Alternatively, the role of the Bub2/Bfa1 pathway in higher eukaryotes may be more similar to that in S. pombe. In this organism, Cdc16 and Byr4 negatively regulate septation, and loss of function mutations lead to the formation of multiple septa in the absence of cytokinesis. If this function is conserved in humans, then up-regulation of Cdc16 and Byr4 could also lead to aneuploidy, by preventing cell division in cells which have replicated their chromosomes.
References


232


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Publications


The Dbf2 protein kinase functions as part of the mitotic-exit network (MEN), which controls the inactivation of the Cdc28-Cib2 kinase in late mitosis [1]. The MEN includes the Tem1 GTP binding protein; the kinases Cdc15 and Cdc6; Mob1, a protein of unknown function; and the phosphatase Cdc5 [2]. Here we have used Dbf2 kinase activity to investigate the regulation and order of function of the MEN. We find that Tem1 acts at the top of the pathway, upstream of Cdc15, which in turn functions upstream of Mob1 and Dbf2. The Cdc5 Polo-like kinase impinges at least twice on the MEN since it negatively regulates the network, probably upstream of Tem1, and is also required again for Dbf2 kinase activation. Furthermore, we find that regulation of Dbf2 kinase activity and actin ring formation at the bud neck are causally linked. In metaphase-arrested cells, the MEN inhibitor Bub2 represses both Dbf2 kinase activity [3] and actin ring formation [4]. We find that the MEN proteins that are required for Dbf2 kinase activity are also required for actin ring formation. Thus, the MEN is crucial for the regulation of cytokinesis, as well as mitotic exit.

Results and discussion

The budding-yeast mitotic-exit network (MEN) is extremely complex, and the regulation of its numerous components is poorly understood. Here we have investigated the regulation of the MEN kinase Dbf2 and the order in which its upstream activators function.

We have previously shown that cells arrested in metaphase by the use of an *apc2-8* temperature-sensitive mutation contain high Dbf2 kinase activity [3]. This activity is downregulated following nocodazole-induced spindle damage, and this finding indicates that Dbf2 is a target of the spindle assembly checkpoint (SAC). This inhibition depends upon the putative Tem1 GTPase-activating protein (GAP), which consists of Bub2 and Bfa1. Cells deleted for *BUB2* or *BFA1* are unable to arrest the cell cycle in response to spindle damage and contain high levels of Dbf2 kinase activity in an *apc2-8* arrest [3, 4]. These assay conditions provide a convenient way to assess which components of the MEN are required for Dbf2 kinase activity. Not only do the cells arrest with high Dbf2 kinase activity, but they are held at a uniform arrest point. This ensures that any observed changes in Dbf2 kinase activity are not due to further cell cycle progression [5].

We constructed strains containing temperature-sensitive mutations of the MEN genes *TEM1* (tem1-3), *CDC15* (cdc15-1), *MOB1* (mob1-77), *CDC5* (msd2-1), and *CDC14* (cdc14-1) in an *apc2-8 bub2Δ* background. We arrested the cells in G1 by using α-factor or in metaphase by incubating them at 37°C in the presence or absence of nocodazole, and we performed Dbf2 kinase assays. As expected, the *apc2-8 bub2Δ* strain exhibited high levels of Dbf2 kinase activity at 37°C in the absence of nocodazole (Figure 1, panel 1). Mutating Cdc14 had no effect on Dbf2 kinase activity (Figure 1, panel 6). This is not surprising since it has been previously reported that Dbf2 kinase is required to release Cdc14 from the nucleolus and hence must function upstream of Cdc14 [6]. However, in the absence of functional Tem1, Cdc15, Mob1, or Cdc5, Dbf2 kinase activity was completely abolished (Figure 1, panels 2, 3, 4, and 5). To ensure that this data was not influenced by the *apc2-8 bub2Δ* genetic background, we also assayed Dbf2 kinase activity at 37°C in the single *cdc5, cdc15, mob1*, and *cdc14* mutants. As expected, in the first three mutants Dbf2 kinase activity was effectively abolished, while *cdc14* was without effect (data not shown). Nud1 was recently shown to be part of the MEN [7], and the *nud1-44* mutation also abolished Dbf2 kinase activation at 37°C (data not shown). This suggests that these proteins function upstream of, and are required for, Dbf2 kinase activation. These data are consistent with the situation in the fission yeast, *Schizosaccharomyces pombe*. In this organism the Dbf2 homolog Sid2 is a component of the septum initiation network (SIN), which is analogous to the MEN, and Sid2 has been shown to function down-
the latter two lineages separated. This, along with new mutations in the human-chimpanzee common ancestor, accounts for the higher genetic relatedness of chimpanzees and humans that appears to encompass the majority of the genome [1, 3, 4]. However, at positions in the genome where allelism was maintained throughout the period of existence of the human-chimpanzee common ancestor, some of the same alleles that became fixed in the gorilla lineage may also have been fixed in only one of the human or chimpanzee lineages. The HERV-K-GC1 provirus provides a compelling piece of evidence for such a model, as it is the clearest example to date of a specific locus within the genome where chimpanzees and gorillas are more closely related to each other than any of them is to humans. Moreover, since neutral alleles are maintained in a population for only a limited time that depends on the size of the population [4, 19-24], the data presented here imply that the separation of the Homo, Pan, and Gorilla lineages occurred within a period of time that was sufficiently short for such allelism to be maintained. The significance of the work presented here is the demonstration of the utility of HERV-K as a marker for studying human evolution, the conclusion that HERV-K was active at about the time that the three lineages were evolutionarily separating, and the very strong experimental evidence that, in some fraction of the genome, chimpanzees, bonobos, and gorillas are more closely related to each other than any of them is to humans. HERV-K and other retrotransposable elements should contribute to determining what that fraction is.

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References

Tem1, Cdc15, Mob1, and Cdc5, but not Cdc14, are required for Dbf2 kinase activity. Strains containing tem1-3 (JTAB7-12D), cdc15-1 (ERN1), mob1-77 (JUBA3-12B), cdc5 (msd2-1) (JUBA3-13D), and cdc14-1 (J14AB2-20B) mutations in an apc2-8 bub2A background were grown to mid-log at 25°C. The cells were arrested with α-factor (α-factor), with nocodazole at 37°C (Noco@37°C), or at the same temperature without nocodazole (37°C) for 3 hr. Dbf2 was immunoprecipitated from extracts with anti-Dbf2 antibody [5], and HI kinase assays were performed (lower panels). Relative kinase activity was assessed with a phosphorimager and plotted against Dbf2 protein levels (upper panels).

stream of Spg1 and Cdc7, the respective Tem1 and Cdc15 homologs [8].

Having established that Tem1, Cdc15, and Mob1 all function upstream of Dbf2, we decided to order these pathway components by exploiting Dbf2 kinase activity. It seemed likely that Tem1 would be near the top of the pathway since Bub2 is presumed to act directly on Tem1; indeed, Bub2 physically associates with Tem1 ([9]; data not shown). Because Bub2 is a putative GAP, its deletion would presumably hyperactivate Tem1 and account for the high Dbf2 kinase activity in bub2A cells. Conversely, the overexpression of Tem1 should mimic the deletion of BUB2 and also hyperactivate Dbf2. The overexpression of Tem1 from a galactose-inducible promoter in apc2-8 cells arrested at 37°C did indeed hyperactivate Dbf2, even in the presence of nocodazole and functional Bub2 and Bfa1 (Figure 2a, panel 2). This confirms that Tem1 functions upstream of Dbf2 to activate its kinase activity.

We used the effect of Tem1 overexpression on Dbf2 kinase to show that Cdc15 acts downstream of Tem1 to control Dbf2. In an apc2-8 cdc15-1 strain arrested at 37°C, Tem1 overexpression fails to activate Dbf2 kinase activity (Figure 2a, panel 3). Thus, Tem1 must act upstream of Cdc15 to control Dbf2. In fact, Tem1 and Cdc15 physically interact [10], and this interaction implies that Tem1 functions immediately upstream of Cdc15.

If Tem1 controls Dbf2 through Cdc15, overexpression of the latter should similarly activate Dbf2 kinase. When Cdc15-3HA was expressed from a multi-copy plasmid in an apc2-8 strain, activation of Dbf2 kinase occurred in the presence and absence of nocodazole (Figure 2b, panel 2). This activation of Dbf2 by Cdc15 was slightly less dramatic than that induced by Tem1 overexpression. This may simply reflect the lower levels of Cdc15 overexpression from the multi-copy vector or, alternatively, the more pivotal role played by Tem1 in controlling the pathway.

Dbf2 and Mob1 form a heterodimer [11], and Dbf2 function requires Mob1 (Figure 1). Mob1 should therefore function downstream of Cdc15. Accordingly, we investigated whether overexpression of Cdc15 could still activate Dbf2 in the absence of functional Mob1. Dbf2 kinase activity is clearly not induced by Cdc15 overexpression in an apc2-8 mob1-77 strain (Figure 2b, panel 3). This experiment formally places Mob1 function downstream of Cdc15 and upstream of Dbf2. However, overexpression of Mob1 bypasses the essential requirement for Dbf2/Mob2 function in mitotic exit [11], and this finding suggests that Mob1 is an effector of Dbf2 function. Conceivably, Mob1 activates Dbf2 and, in so doing, is itself activated for a downstream function.

Thus, the order in which the MEN pathway components function is Tem1-Cdc15-Mob1-Dbf2-Cdc14. Once Cdc14 has been released from the nucleolus, regulation of the pathway is likely to be more complex since Cdc14 has been shown to dephosphorylate its upstream activator Cdc15. This suggests that it forms a feedback loop involv-
Dbf2p Kinase

\( \alpha \)-HA

- Gal

Nocodazole

- 3°C

\( \alpha \)-factor

- 3°C

\( \alpha \)-HA

- Gal

- Histone H1

(a) Tem1 activates Dbf2p kinase activity in a Cdc15-dependent manner. Strains NJW015 (3HA-DBF2 apc2-8) or SLY124 (3HA-DBF2 apc2-8 cdc15-1) transformed with plasmid pSL3 (GAL-6His-TEM1; 2 \( \mu \)g) were grown overnight in selectable medium containing 2% raffinose. They were then subcultured in YEP raffinose, and for the induction of βGAL expression, 2% galactose was added. The cells were arrested as described (Figure 1). Extracts were subjected to immunoprecipitation and kinase assays (upper panel). Immunoblotting the upper part of the kinase assay gel and probed with anti-HA antibody controlled for the even immunoprecipitation ing the other MEN genes [12, 13]. This may be important for control of cytokinesis [14].

Cdc5 is known to be a multifunctional kinase, and its role within the MEN is complex. Mutating Tem1, Cdc15, Mob1, and Cdc14 had no effect on Cdc5 kinase activity (data not shown). So, while not regulated by these MEN proteins, Cdc5 is required for Dbf2 kinase activity (Figure 1). Surprisingly, overexpression of Cdc5 inhibits Dbf2 kinase activity by some 70% (Figure 2c). We have observed that Bfa1 is phosphorylated in a Cdc5-dependent manner early in mitosis [4]. Thus, Cdc5 could act upstream of Tem1 to negatively regulate the MEN while also being required downstream for Dbf2 kinase activation. Indeed, deletion of \( BUB2 \) partially alleviated the inhibitory effect of Cdc5 on Dbf2 (data not shown). Note that in Figure 1 Tem1 is already activated by the deletion of \( BUB2 \), so only the downstream, activating role of Cdc5 is observed. Whether this latter control is a direct effect on any MEN proteins is uncertain, but we note that Cdc15, Mob1, and Dbf2 are phosphoproteins [5, 12]; our unpublished data).

We have proposed that the MEN functions in cytokinesis [15]. Interestingly, we have observed a strong correlation between mutations that abolish Dbf2 kinase activity and mutations that prevent formation of the actomyosin contractile ring. In \( apc2-8 \) arrested cells, the actin ring is not present (Figure 3; [4]). However, if \( BUB2 \) is deleted, these cells form an actin ring prematurely (Figure 3; [4]). Thus Bub2 restrains actin ring formation in metaphase-arrested cells. Biochemically, Bub2 also restrains Dbf2 activity in metaphase-arrested cells [3], and this finding implies that actin ring formation may be regulated by the same mechanisms as Dbf2 kinase, namely Bub2 and the MEN. To investigate this, we determined whether actin ring formation in \( apc2-8 \) \( b u b 2 \Delta \) cells was dependent upon the MEN. Actin ring formation was prevented in \( apc2-8 \) \( b u b 2 \Delta \) cells containing mutations in the MEN genes Tem1, Cdc15, Mob1, or Cdc5 (Figure 3). These MEN genes therefore control both Dbf2 kinase activation and actin ring formation. Interestingly, although Cdc14 is not required for

of Dbf2p (middle panel). Tem1 expression was confirmed by immunoblotting (lower panel). (b) Cdc15 activates Dbf2p kinase activity in a Mob1-dependent manner. Strains KTM206 (3HA-DBF2 apc2-8) and IAM309-12B (3HA-DBF2 apc2-8 mob1-77) were transformed with plasmid pSL103 (pRS426-CDC15-3HA; 2 \( \mu \)g), grown overnight in selective medium, subcultured in YEP glucose, and arrested as described (Figure 1). Extracts were subjected to immunoprecipitation and kinase assays (lower panel). Relative kinase activity was assessed with a phosphorimager and plotted against Dbf2p protein levels (upper panel). (c) Cdc5 overexpression inhibits Dbf2p kinase activation. Strain NJW015 transformed with pSL20 (GAL-6His-CDC5; 2 \( \mu \)g) was induced with galactose and arrested at 37°C (see above). Kinase assays, immunoblotting, and quantitation of the kinase activity were also carried out as described above.
Brief Communication

787

Figure 3

Actin ring formation in apc2-8 bub2Δ cells is dependent upon MEN function. Strains KTM208 (apc2-8), YLF25 (apc2-8 bub2Δ), JTAB7-12D (apc2-8 bub2Δ tem1-3), ERN1 (apc2-8 bub2Δ cdc15-1), JIMAB3-12B (apc2-8 bub2Δ mob1-77), J5AB3-13D (apc2-8 bub2Δ cdc5) and J14AB2-20B (apc2-8 bub2Δ cdc14-1) were grown to mid-log at 25°C. Cells were arrested at 37°C for 3 hr and stained for DNA with DAPI and for actin with rhodamine-phalloidin [15]. The actin ring is indicated with an arrow. Some 60% of apc2-8 bub2Δ cells eventually accumulate with a detectable actin ring.

Dbf2 kinase activation, it is necessary for actin ring formation (Figure 3). Thus, actin ring formation may require the activity of proteins downstream of Dbf2. Significantly, Cdc15, Dbf2, and Cdc5 also form a ring at the bud neck during cytokinesis [13, 15, 16].

In summary, we have exploited Dbf2 kinase activity to establish that the MEN functions in the order Tem1-Cdc15-Mob1-Dbf2-Cdc14. Cdc5 is not controlled by the MEN but may interact with it at two levels. It may negatively regulate Tem1 function through Bub2/Bfa1, although this needs corroboration, and in addition Cdc5 is required for Dbf2 activation (Figure 1). Our data show that the MEN is also required for, and regulates, actin ring formation, an early step in cytokinesis.

Materials and methods

Strains and media

Relevant yeast strains and their genotypes are shown in Table 1. Strains are derived from CG378 and CG379, essentially isogenic wild types. Yeast cell culture and genetic techniques were carried out as described previously [3]. To generate strain NW0015, we tagged Dbf2 at the N terminus with a 3HA epitope tag by PGR integration [17]. Correct integration was confirmed by colony PCR.

Plasmids

We constructed plasmids pSL3 (pGAL-6His-TEM1; URA3) and pSL20 (pGAL-6His-CDC5; URA3) by amplifying the wild-type TEM1 and CDC5 genes, respectively, from genomic DNA with primers that inserted 5’ BamHI and 3’ Smal sites. We ligated the resultant PCR fragment into the BamHI-Smal sites of pEMBLyex4 to generate pSL3 and pSL20, respectively.

Overexpression of Tem1 and Cdc15

To overexpress Tem1 or Cdc5, we transformed cells with pSL3 or pSL20, as appropriate, and grew them overnight in selectable medium containing 2% raffinose. Cells were subcultured in YEP raffinose for 4–5 hr, and 6His-Tem1 expression was induced by the addition of 2% galactose. To overexpress Cdc15, we transformed cells with plasmid pSJ103 (pRS426-CDC15-3HA; URA3) (kindly provided by Sue Jasper sen), grew them overnight in selectable medium, and subcultured them in YEP glucose.

Protein analysis

Cell pellets (107 cells) were resuspended in 50 μl lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 15 mM MgCl2, 5 mM EDTA, 1% NP40, 10 mM NaF, 50 mM β-glycerophosphate, 1 mM DTT, 1 mM Na3VO4, 1 mM pNPP, 1 mM glucose-1-phosphate, 10 μg/ml each protease inhibitors (leupeptin, pepstatin, chymotrypsin, aprotinin, antipain), and 1 mM PMSF. Cells were disrupted with glass beads.

For immunoblot analysis, 50 μg of protein extract in Laemmli buffer was resolved by SDS-PAGE, transferred to Protran nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany), and detected by chemiluminescence detection (ECL) (Amersham) with primary antibodies anti-HA (12GA5), anti-Dbf2 [5], or anti-RGS-HIS (Qiagen).

For Dbf2 kinase assay measurement, 200 μg of total protein extract in Laemmli buffer was resolved by SDS-PAGE, transferred to Protran nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany), and detected by chemiluminescence detection (ECL) (Amersham) with primary antibodies anti-HA (12CA5), anti-Dbf2 [5], or anti-RGS-HIS (Qiagen).

For Dbf2 kinase assay measurement, 200 μg of total protein extract was immunoprecipitated by incubation with 2 μg of anti-Dbf2 [5] or anti-HA (12CA5) antibodies on a rotating wheel for 1 hr at 4°C. Protein A beads were added, and the incubation continued for 30 min. The protein A beads/immune complex was washed three times with lysis buffer and twice with kinase buffer (25 mM MOPS [pH 7.2], 10 mM MgCl2) and assayed as described previously [3].
### Table 1

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* prsi1-8 is a CEN-based plasmid containing TRP1 and a temperature-sensitive mutation in RS11/APC2.

**Fluorescent staining and observation of yeast cells.**

Actin was stained with rhodamine-conjugated phalloidin and visualized as described previously [15].

**Acknowledgements**

We thank Sue Jaspersen for reagents and Sanne Jensen for advice during manuscript preparation.

**References**

The Bub2-dependent mitotic pathway in yeast acts every cell cycle and regulates cytokinesis

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INTRODUCTION

In eukaryotic cells, inactivation of mitotic kinase, made up of a cyclin-dependent kinase and a B-type cyclin, is required for exit from mitosis. Ubiquitin-mediated proteolysis of the cyclin is principally responsible for the inactivation. An E3 ubiquitin ligase, the anaphase-promoting complex (APC) selects the cyclin for proteolysis. Additional associated regulatory factors are necessary for specificity of target selection, such as Cdc20 at metaphase and Cdh1/Hct1 at anaphase for proteolysis of the mitotic cyclin. Furthermore, in budding yeast, activation of APC requires the Polo-like kinase Cdc5 (Charles et al., 1998) and another group of proteins known as the mitotic exit network (MEN) (reviewed by Morgan, 1999). This includes three protein kinases, Dbf2, Dbf20 and Cdc15, as well as Tem1, a small G-protein, and Ltel, the probable exchange factor for Tem1 (Shirayama et al., 1994). The genes constituting the MEN show many interactions (e.g. Jaspersen et al., 1998; Kitada et al., 1993; Shirayama et al., 1994). This, together with the participation of multiple protein kinases as well as a G-protein, indicates a regulatory network. The late mitotic events controlled by the MEN are likely to include APC activation for its anaphase function (Alexandru et al., 1999; Fesquet et al., 1999; Jaspersen et al., 1998) and also cytokinesis (Frenz et al., 2000 and references therein).

SUMMARY

In eukaryotes an abnormal spindle activates a conserved checkpoint consisting of the MAD and BUB genes that results in mitotic arrest at metaphase. Recently, we and others identified a novel Bub2-dependent branch to this checkpoint that blocks mitotic exit. This cell-cycle arrest depends upon inhibition of the G-protein Tem1 that appears to be regulated by Bfa1/Bub2, a two-component GTPase-activating protein, and the exchange factor Ltel. Here, we find that Bub2 and Bfa1 physically associate across the entire cell cycle and bind to Tem1 during mitosis and early G1. Bfa1 is multiply phosphorylated in a cell-cycle-dependent manner with the major phosphorylation occurring in mitosis. This Bfa1 phosphorylation is Bub2-dependent. Cdc5, but not Cdc15 or Dbf2, partly controls the phosphorylation of Bfa1 and Ltel. Following spindle checkpoint activation, the cell cycle phosphorylation of Bfa1 and Ltel is protracted and some species are accentuated. Thus, the Bub2-dependent pathway is active every cell cycle and the effect of spindle damage is simply to protract its normal function. Indeed, function of the Bub2 pathway is also prolonged during metaphase arrests imposed by means other than checkpoint activation. In metaphase cells Bub2 is crucial to restrain downstream events such as actin ring formation, emphasising the importance of the Bub2 pathway in the regulation of cytokinesis. Our data is consistent with Bub2/Bfa1 being a rate-limiting negative regulator of downstream events during metaphase.

Key words: Yeast, Bfa1, Bub2, Ltel, Cdc5, Spindle checkpoint, Metaphase

Another regulatory pathway acting in late mitosis and controlling APC function is the spindle assembly checkpoint (SAC) (Amon, 1999; Gardner and Burke, 2000). This is activated by a damaged spindle or unattached kinetochores, and was originally envisaged to be a single pathway resulting in a cell-cycle arrest at metaphase. Recently, a second branch to the SAC was discovered that results in inhibition of mitotic exit (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999). This is controlled by Bub2 specifically and the MEN protein kinase Dbf2 was found to be a target of the Bub2 pathway (Fesquet et al., 1999). Taking the data on Bub2 collectively, together with recent work on mitotic exit (Bardin et al., 2000; Pereira et al., 2000; Shou et al., 1999), it seems most likely that Bub2 is controlling the G-protein Tem1. Bub2 has homology to GTPase-activating proteins (GAPs) that inhibit G-protein function. The fission yeast homologue of Bub2, Cdc16, forms a two-component GAP with Byr4 to regulate Spg1, a homologue of Tem1 (Furge et al., 1998). Significantly, Spg1 controls the septum initiation network that is analogous to the MEN (reviewed by Gould and Simanis, 1997; Le Goff et al., 1999). Moreover, there is a budding yeast homologue of Byr4, named Bfa1 (Li, 1999), which is part of the SAC, specifically of the Bub2 branch (Alexandru et al., 1999; Li, 1999). All of this points to Bub2/Bfa1 functioning as a two-component GAP to control
Tem1, Lte1, the exchange factor, is also likely to be controlling Tem1. Lte1 is phosphorylated in a cell-cycle-dependent manner and localises to the bud cortex where it is involved in spatial control of MEN activation (Bardin et al., 2000; Pereira et al., 2000).

The mechanism by which mitotic exit is restrained during metaphase arrest is unclear. Tem1 is almost certainly the key to this (Alexandru et al., 1999). Complete inhibition of Tem1 should prevent activation of the MEN and hence mitotic exit. To fully inactivate Tem1 might require stimulation of the Bub2/Bfa1 GAP activity as well as inhibition of Lte1. Accordingly, we have investigated the regulation of these proteins. Both Lte1 and Bfa1, but not Bub2, are phosphorylated in the cell cycle and following SAC activation. Spindle damage, or metaphase arrest caused by other means, simply provokes the normal mitotic phosphorylation of Bfa1. Cdc5 partly controls the cell-cycle phosphorylation of Bfa1, including the phosphorylation seen in metaphase arrest. During metaphase arrest in bub2Δ cells, downstream events associated with cytokinesis take place. Our data strongly suggests that the Bub2 pathway acts every cell cycle, and that Bub2 does indeed restrain MEN function in metaphase.

MATERIALS AND METHODS

Strains and media

Relevant yeast strain genotypes are indicated in Table 1. All yeast strains were in a CG378 or congenic CG379 background. Growth of yeast strains and transformations have been described previously (Fesquet et al., 1999). Cell-cycle arrests were performed using 3.5 μg/ml α-factor, 0.1 M hydroxyurea or 15 μg/ml nocodazole unless otherwise stated.

The genomic BUB2 gene was tagged at the C-terminus with a 13MYC epitope by a PCR-based gene integration (Longtine et al., 1998). BFA1 was tagged at the N-terminus with a 3HA epitope using a PCR-based gene integration technique (Schneider et al., 1995). Tagging was confirmed by PCR. Benomyl sensitivity assays showed that the tagged Bfa1 and Bub2 proteins were fully functional.

Plasmids and DNA manipulations

The TEM1 open reading frame was subcloned as a BamHI-Smal PCR fragment into pEMBLex4 (pGAL, 2 μm, URA3) to generate plasmid pSL3, containing 6His-TEM1 under the control of the inducible GAL promoter.

The Lte1HA3-pTRP1 plasmid was used to tag the endogenous Lte1 protein with 3HA epitopes at the C-terminus. The integrating pTRP1 plasmid carries the sequence for 3 HA epitopes, which can be fused to a protein of interest at the NotI site (Mondesert et al., 1997). A Sall/NotI fragment spanning the last 500 bases of the LTE1 gene was cloned into pTRP1. The plasmid was linearised with Aalll and integrants selected.

Over-expression of Tem1

Strain SLY106 containing a galactose-inducible TEM1 construct (pSL3) was grown overnight in minimal medium containing 2% raffinose, then subcultured in yeast extract-peptone (YPE) 2% raffinose for 4.5 hours. 6His-Tem1 expression was induced by addition of galactose to 2% final concentration. Cells were either harvested at 90 minutes after galactose induction, or were induced for 45 minutes prior to a 3 hour incubation period with α-factor, hydroxyurea (HU) or nocodazole, to give a cell-cycle arrest. Samples were taken for protein extraction and nickel precipitation (see below).

Preparation of yeast crude extracts and protein analysis

Cell pellets (10^7 cells) were resuspended in 50 μl lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 15 mM MgCl₂, 5 mM EDTA, 1% NP40, 10 mM NaF, 50 mM glycophosphate, 1 mM DTT, 1 mM NaF, 1 mM pNPP, 1 mM glucose-1-phosphate, 10 μg/ml each of the protease inhibitors leupeptin, pepstatin,
RESULTS

Bfa1 and Bub2 physically associate across the cell cycle

If Bfa1 and Bub2 form a two-component GAP to control Tem1, mutation of either the BFA1 or BUB2 genes should have similar effects. Bub2 is needed for down-regulation of Dbf2 kinase activity following SAC activation (Fesquet et al., 1999) and Bfa1 was found to have an identical function (Fig. 1A). That is, strain SLY103 (Dbf2-6Myc bfa1 Δ apc2-8) displays high Dbf2 kinase activity in the presence of nocodazole. This is consistent with the two proteins having the same function and interacting at some level.

A direct interaction between Bub2 and Bfa1 was established by co-immunoprecipitation of the two proteins (data not shown), confirming recent observations (Pereira et al., 2000). Moreover, we found that Bfa1 and Bub2 associated across the entire cell cycle (Fig. 1B) as well as in the presence of nocodazole (not shown). Tem1 control by Bfa1/Bub2 therefore does not occur by means of transient protein association of the GAP.
Fig. 4. Bfa1 undergoes cell-cycle-dependent phosphorylation that is enhanced following nocodazole treatment. Strain SLY105 (3HA-BFA1) was synchronised with α-factor and released into fresh medium in the absence (A) or presence (B) of nocodazole. Samples for protein extraction were taken at the indicated times after release. Extracts were immunoblotted and probed with anti-HA or anti-Clb2 as indicated. The right panel shows budding curves for the two cultures.

**Bfa1 and Bub2 associate with Tem1 during M phase and early G1**

The association of Bfa1 and Bub2 with Tem1 was initially examined by expressing GAL-G-6His-Tem1 in strain SLY106 (Bub2-13Myc 3HA-Bfa1). 6His-Tem1 was precipitated using nickel beads and immunoblotting revealed an interaction with Bfa1 and Bub2 (Fig. 2, lane 4). Surprisingly, the Bfa1/Bub2 association with 6His-Tem1 also occurred in various cell-cycle arrests, including α-factor, hydroxyurea and nocodazole (Fig. 2, lanes 5-7). This data was confirmed by co-immunoprecipitation of Bfa1, Bub2 and Tem1 expressed at normal endogenous levels (data not shown). The complex is therefore likely to persist for much of the cell cycle, unlike most G-proteins and their regulatory factors. Regulation of Tem1 by Bfa1/Bub2 does not therefore happen through transient association. While this manuscript was in preparation, Pereira et al. presented similar data showing the physical interaction of Bfa1 and Bub2 with Tem1 but without addressing the duration of the interaction in the cell cycle (Pereira et al., 2000). Furthermore, we find that in absence of either Bfa1 or Bub2 the remaining protein no longer binds to Tem1 (data not shown).

**Table 1. Yeast strains used in the present study to investigate the mitotic pathway**

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*ppc2-8 is a CEN-based plasmid containing TRP1 and a temperature-sensitive mutation in RSH/APC2.*
Bfa1 and Lte1 are phosphorylated in a cell-cycle-dependent manner and in response to nocodazole

Immunoblotting showed the stability of both Bub2 and Bfa1 to be constant in the cell cycle (data not shown). Bub2 was also not phosphorylated; however, Bfa1 is clearly a phosphoprotein (Fig. 3A, B). When the BUB2 gene was deleted, all phosphorylation of Bfa1 was abolished (Fig. 3C). So the heterodimer may be the kinase substrate with the phosphorylations being confined to Bfa1. Alternatively, it was recently shown that Bfa1 localisation to the spindle pole body (SPB) requires Bub2 (Pereira et al., 2000) and this may account for the Bub2-dependence of Bfa1 phosphorylation. The relevant kinase may itself be located on the SPB.

No fewer than six separate Bfa1 phosphorylated species are evident (Fig. 3A, I-VI; see also Figs 4, 6, 7A). There are clear changes in the Bfa1 phosphorylation patterns observed in different cell-cycle arrests (Fig. 3A). The most striking is the almost complete loss of phosphorylation in the α-factor arrest, but there is also a marked change in the nocodazole-treated cells where the phosphorylated band IV is increased slightly (see also Figs 6A, 7A) and band VI is obviously accentuated. The phosphorylated state of Bfa1 therefore changes according to the physiological state of the cells. This was confirmed in a synchronous culture (Fig. 4A), where the Bfa1 phosphorylated species detected corresponded to the six observed in the mid-log sample (Fig. 3A). These phosphorylations start at about 30-40 minutes after bud emergence. However, the bulk phosphorylation becomes evident at 50 minutes coinciding with the appearance of the Clb2 mitotic cyclin near the beginning of mitosis. Much of the Bfa1 phosphorylation is then lost as Clb2 is degraded and it reappears in the next cell cycle.

In a synchronised culture treated with nocodazole, the same range of retarded species was observed (Fig. 4B; see also Fig. 3A), the most prominent differences being an increase in the phosphorylated species VI.

The cell-cycle-dependent phosphorylation of Bfa1 implies that the Bfa1/Bub2 GAP is a normal component of late mitotic control. Consistent with this, the proposed exchange factor for Tem1, Lte1, is also highly phosphorylated in the cell cycle with a similar timing to Bfa1 (Fig. 5A). Similar data regarding Lte1 phosphorylation was recently described by Bardin et al. (Bardin et al., 2000). However, in addition we investigated the phosphorylation profile of Lte1 after SAC activation. When nocodazole was added to half of our synchronous culture, the great majority of Lte1 shifted to the upper phosphorylated species but no additional phosphorylations were observed (Fig. 5B).

This cell-cycle-dependent phosphorylation of Bfa1 and Lte1 shows that they must have a physiological function every cell cycle starting in early mitosis. Treatment of cells with nocodazole, and hence activation of the SAC, protracts the phosphorylation and, presumably, their function. As we are principally interested in Bub2 control, we have concentrated on the regulation of Bub2/Bfa1 for the remainder of this work.

Cdc5 partly controls phosphorylation of Bfa1

Identification of the protein kinases that control phosphorylation of Bfa1 is crucial to understanding regulation of Bub2/Bfa1. Of various mitotic kinases examined, including Mps1, Cdc15 and Dbf2, only the Polo-like kinase Cdc5 affected some of the phosphorylated forms of Bfa1. Transfer of strain SLY 109 (3HA-Bfa1 cdc5 (msd2-1)) to 37°C with and without nocodazole revealed significant changes in the Bfa1 phosphorylated species (Fig. 6A; see also Fig. 7A). The two phosphorylated bands IV and VI are slightly reduced in the 37°C cells without nocodazole. Both these bands are clearly accentuated following nocodazole treatment at 25°C and are significantly reduced in level at 37°C indicating that Cdc5 is required for Bfa1 phosphorylation in the presence of nocodazole. In synchronised cultures transferred to an environment at 37°C, bands I and II are absent and are not evident even on longer exposures, so these may also be
controlled by Cdc5. Bands IV and VI are essentially absent at 37°C (Fig. 6B). Cdc5 therefore clearly controls some of the Bfa1 phosphorylation events.

Interestingly, the hyper-phosphorylated species of Ltel observed in metaphase-arrested wild-type cells (Fig. 6D, lane 4) are not detectable in nocodazole-treated cdc5 cells grown at the semi-permissive temperature of 30°C (Fig. 6D, lane 2). It is therefore likely that Cdc5 also regulates the mitotic phosphorylation of Ltel.

Continued function of Cdc5 is necessary for the nocodazole-induced changes in Bfa1 phosphorylation. A culture of strain SLY 109 was treated with nocodazole for 3 hours and divided: one half was incubated further at 25°C, whereas the other half was placed at 37°C. At 25°C the expected enhancement of phosphorylated species IV and VI was observed (Fig. 6C). Incubation of one half of the culture was continued at this temperature, whereas the other half was transferred to 37°C. After sampling, an immunoblot was prepared from the cells. The lower panel shows budding curves for the two cultures. (C) Mid-log cells of strain SLY 109 were synchronised with α-factor and released at each 25°C or 37°C. Samples were removed at the times indicated and an immunoblot prepared. The lower panel shows budding curves for the two cultures. (D) Strains SJY121 and 122 were grown to mid-log phase at 25°C. The cultures were split and split at 30°C, cells were harvested and an immunoblot prepared from the extract. Note that longer exposure did not reveal further phosphorylations in lanes 1 and 2.

a trivial explanation for the above data. cdc5 arrest may simply occur at a stage when Bfa1 is experiencing dephosphorylation. A number of experiments rule this out. First, nocodazole induces arrest in metaphase and the effect of cdc5 was apparent in nocodazole-treated cells (Fig. 6A). Second, in the cdc5-synchronised cells incubated at 37°C, species IV does not appear at all but is incubated with nocodazole. Following 3 hours incubation at 30°C, cells were harvested and an immunoblot prepared from the extract. Note that longer exposure did not reveal further phosphorylations in lanes 1 and 2.

Bfa1 phosphorylation in an apc2-8-induced metaphase arrest resembles that in a nocodazole-induced metaphase arrest

The phosphorylation of Bfa1 and Ltel in normal cycling cells
shows that these proteins are physiologically active during every cell cycle rather than just in response to spindle damage. Moreover, there are similarities in the pattern of Bfa1 phosphorylation in the cell cycle and in nocodazole-induced metaphase arrest. This may therefore indicate a protraction of events operating in a normal cell cycle. If correct, metaphase arrest induced by means other than nocodazole treatment should induce similar changes in the pattern of Bfa1 phosphorylation. To examine this, an apc2-8 strain was transferred to 37°C, which inactivates APC and causes a metaphase arrest like that due to nocodazole treatment.

Cultures of a wild type and strain SLY116 (apc2-8 3HA-Bfa1) were grown at 25°C and half were transferred to 37°C. The 25°C and 37°C cultures were further divided and nocodazole was then added to one of each pair of cultures. The wild type showed the expected Bfa1 phosphorylations at mid-
log phase and after nocodazole treatment (Fig. 7A, lanes 1-4). With the apc2-8 strain the significant result is the similar Bfa1 phosphorylations in the culture incubated at 37°C without nocodazole and those cultures incubated with the drug (compare lane 7 with lanes 2, 4 and 6). So the pattern of Bfa1 phosphorylation is the same whether cells are arrested in metaphase by activation of the SAC or by mutational inactivation of the APC.

Incubation of a cdc5 mutant at 37°C affected Bfa1-phosphorylated bands IV and VI (Fig. 6). If the apc2-8 arrest physiologically resembles nocodazole-induced arrest, cdc5 should affect Bfa1 phosphorylation in a similar way. Strain JB5A1-51C (apc2-8 cdc5 3HA-Bfa1) was transferred to 37°C with or without nocodazole and phosphorylated species IV and VI were once again reduced in intensity (Fig. 7A, compare lanes 11 and 7, lanes 12 and 8). The pattern of Bfa1 phosphorylation during metaphase arrest is therefore not specific to nocodazole treatment. Hence Bub2/Bfa1 is part of an intrinsic mitotic control that operates during metaphase.

Bub2 and Bfa1 would be expected to have a negative effect on events controlled by Tem1, as we have demonstrated for Dbf2 kinase activity (Fesquet et al., 1999; Fig. 1A). If the apc2-8 arrest is physiologically equivalent to that induced by nocodazole, deletion of BUB2 should similarly permit downstream cell-cycle events prevented by the protracted activation of the Bub2/Bfa1-dependent pathway. In the apc2-8-induced metaphase arrest, a normal cortical distribution of actin patches occurred without formation of an actin ring at the bud neck (Fig. 7B, top panel). However, when BUB2 was deleted from this strain, the actin ring was formed in metaphase during apc2-8 incubation at 37°C. Deletion of MAD2 from the apc2-8 strain was without effect. This result was not simply due to secondary effects caused by mutational inactivation of APC. When metaphase arrest was achieved by over-expressing inductestructible Pds1 (Cohen-Fix et al., 1996), the same result was obtained. That is, following Pds1Dbf over-expression in a wild type, actin patches were cortical as expected for a metaphase arrest, whereas overexpression in a bub2A strain resulted in the inappropriate formation of an actin ring (Fig. 7B, bottom two panels). Thus the Bub2-dependent pathway is activated during metaphase to inhibit downstream events. The formation of the actin ring, which is one of the initial events of cytokinesis, is probably a reflection of Tem1 control of cytokinesis through regulation of the MEN (Frenz et al., 2000).

To explore further the events controlled by Bub2, in a similar experiment we monitored the localisation of Dbf2-GFP in apc2-8, apc2-8 bub2Δ and apc2-8 mad2Δ strains. Dbf2 localised solely to the SPBs (data not shown) and no bud neck staining was evident (Frenz et al., 2000). Thus Bub2 does not control localisation of Dbf2 and, presumably, other MEN proteins. However, upon protracted incubation of a bub2Δ strain, the apc2-8 bub2Δ strain showed some 40% rebudding (Fig. 7C). Moreover, SPB duplication was also observed in these cells (Fig. 7D). Because these events do not occur in the apc2-8 strain, deletion of BUB2 must allow mitotic exit in a proportion of the apc2-8 bub2Δ-arrested cells. Presumably, Cdc14 is released leading to Cdc28 inactivation by Sic1 (Shou et al., 1999). However, Cdc14 release is transient and we were unable to observe this. Of particular interest is the absence of cytokinesis in the rebudded cells. Although Bub2 controls some aspects of cytokinesis, such as actin ring formation, additional controls are clearly necessary for later events, including relocation of MEN proteins such as Dbf2.

DISCUSSION

Our data support the notion that Bfa1 is a regulatory subunit for Bub2. The two proteins physically interact but Bfa1 alone shows cell-cycle-dependent phosphorylations. Because Bub2 has sequence homology to GAP proteins, Bfa1 presumably modulates this GAP activity. Consistent with Bfa1/Bub2 regulating Tem1 to block MEN function, both proteins physically associate with it, presumably leading to inactivation of Tem1 as appropriate. This interpretation is entirely consistent with the comparable system in fission yeast. The Bfa1/Bub2 homologues Byr4 and Cdc16 are known to form a
two-component GAP (Furge et al., 1998). Cdc16 contains GAP homology and, like Bfa1, Byr4 shows electrophoretic mobility shifts, although it was not shown to be a phosphoprotein (Song et al., 1996). Moreover, Cdc16 and Byr4 also interact directly with the G-protein Spg1 (Gardner and Burke, 2000; Jwa et al., 1998; Song et al., 2000). Finally, Spg1 is a key regulator of the septum initiation network, which is analogous to the MEN (see Introduction).

A GAP activity, of course, acts negatively and we find that deletion of Bub2 or Bfa1 alone is sufficient to allow downstream events during metaphase arrest: Dbf2 kinase activation (Fig. 1A; Fesquet et al., 1999), actin ring formation and ultimately mitotic exit. Regarding actin ring formation, in apc2-8-induced metaphase arrest actin patches are cortical but in an apc2-8 bub2A double mutant, the absence of active Bfa1/Bub2 permits actin ring formation at the bud neck. This was not an aberration due to mutational inactivation of APC as the same effect was observed in cells over-expressing an indestructible Pds1. This is a particularly striking observation as actin ring formation is one of the initial steps in cytokinesis. Its occurrence during metaphase arrest in bub2A cells shows clearly that Bub2 is a regulator of cytokinesis. Interestingly, in apc2-8 bub2A-arrested cells Dbf2 does not relocate from the SPBs to the bud neck and in those cells in which mitotic exit (re budding) occurs, cytokinesis is not completed. Hence, additional controls are required for the later steps of cytokinesis.

A critical question with regard to Bfa1/Bub2, and Lte1, is how their activation is regulated. Control of Bfa1/Bub2 does not occur through their transient association with one another or with Tem1. The interaction with Tem1 is long-lived and appears to persist at least for much of mitosis and into G1 (t-factor arrest). This is unusual for a GAP activity that normally binds only briefly to its substrate. At present, the only significant regulatory feature detected for Bfa1/Bub2 and Lte1 is phosphorylation. To characterise this fully requires mapping the individual sites and assessing their relevance in an in vitro GTPase assay for Tem1, which is clearly beyond the scope of this study.

The simplest interpretation of the Bfa1 phosphorylation is that it is important for activation of the GAP activity and hence inactivation of Tem1 (Fig. 8A). In a normal cell cycle this would tend to inactivate Tem1 for much of mitosis presumably to inhibit mitotic exit and cytokinesis. Then at the appropriate time, Tem1 would be activated at least partly by dephosphorylation of Bfa1. By contrast, Lte1 phosphorylation would be inactivating and its dephosphorylation late in mitosis would stimulate the exchange factor contributing further to the rapid activation of Tem1 in anaphase.

During metaphase arrest caused by either nocodazole treatment or apc2-8 arrest at 37°C, the same Bfa1-phosphorylated species are apparent as seen in the cell cycle. However, there are changes in the ratio of various species and, importantly, these are the same in both types of arrest. The Bfa1 phosphorylation changes are therefore not specific to SAC activation but may occur in any metaphase arrest. Most likely, metaphase arrests simply protract the normal physiological role of Bfa1/Bub2 in order to shut down Tem1 and block cytokinesis.

The phosphorylation of Bfa1 and Lte1 suggests that there may be intracellular signalling pathways impinging upon these Tem1 regulatory proteins (Fig. 8A). The only protein kinase we detected with any effect on the pattern of Bfa1 phosphorylation is Cdc5. Incubation of a cdc5 temperature-sensitive mutant at 37°C resulted in changes to the pattern of Bfa1 phosphorylation. Continued Cdc5 activity is also necessary for maintaining the nocodazole-induced Bfa1 phosphorylation changes. However, whether Cdc5 directly phosphorylates Bfa1 in vivo is not yet clear. In in vitro kinase assays, full length Bfa1 was a poor substrate for Cdc5, although an N-terminal fragment of the protein was a particularly good substrate (data not shown). In addition, we found Lte1 phosphorylation to be under Cdc5 control. Unfortunately Cdc5 phosphorylation sites have not been characterised. Further work is therefore clearly necessary, once again entailing definition of Bfa1 and Lte1 phosphorylation sites, to establish whether they are direct Cdc5 substrates. Interestingly, Plo1, the fission yeast homologue of Cdc5, has recently been shown to act upstream of the septation initiation network, which is analogous to the MEN (Tanaka et al., 2001).

One final implication of our data concerns the intracellular localisation of Tem1. In metaphase arrested cells deletion of Bub2 or Bfa1 is sufficient to allow downstream events to occur (see above). In such cells Tem1 is located on SPBs (Pereira et al., 2000; S.J., unpublished), which in metaphase mostly lie in the mother cell. Thus Bfa1/Bub2 are clearly the rate-limiting controls operating on Tem1 to prevent mitotic exit and cytokinesis in metaphase. This conclusion is also implicit in the data of Bardin et al. (2000) who showed that Bub2 prevents mitotic exit when nuclear division takes place in the mother cell. In metaphase, Lte1 is confined to the daughter cell (Bardin et al., 2000; Pereira et al., 2000) so upon movement of one SPB into the daughter, Tem1 encounters Lte1 for the first time, which presumably triggers Tem1 activation. Formally, Bfa1/Bub2 are therefore negative regulators of Tem1 in metaphase and prevent downstream events (Fig. 8B). By contrast, Lte1 may be a positive activator of Tem1 functioning in anaphase to promote mitotic exit. Hence, for much of mitosis, Bfa1/Bub2 and Lte1 impose their control over different mitotic phases and are temporally distinct. When Tem1 enters the daughter, the balance between Lte1 and Bub2 control ofTem1 must shift in favour of Lte1 as part of the commitment to mitotic exit. In agreement with these antagonistic effects on Tem1 activity, we find that deletion of Bfa1 or Bub2 is sufficient to rescue the cold-sensitivity of lte1A mutants (data not shown), consistent with the model in Fig. 8.

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REFERENCES


