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Characterisation of the Molecular Complexes that Regulate the $G_2/M$ Checkpoint of the Eukaryotic Cell Cycle

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A Thesis Submitted in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

University of Auckland, 2009
The cell cycle is one of the fundamental processes in nature, and is primarily concerned with the faithful replication of cellular contents, followed by even division to produce two identical daughter cells. It is made up of five discrete biochemical steps, comprising the interphase (G₁, S and G₂) and the mitotic phase (mitosis and cytokinesis), with two major regulatory checkpoints at G₁ and G₂. The focus of this research is the G₂ checkpoint, which ensures the successful achievement of DNA replication, prior to the initiation of mitosis. Arrest or progression is principally mediated by the CDK1/cyclin B1 complex; phosphorylation of CDK1 by wee1 kinase prevents progression to mitosis, and subsequent dephosphorylation by the CDC25 phosphatases, initially the B isoform, leads to mitotic onset.

The aim of this research was the biophysical and/or biochemical characterisation of the molecular complexes that form at the G₂ checkpoint to regulate entry into mitosis. CDK1 and cyclin B1 were separately expressed and purified from baculovirus-infected Sf9 cells. The wee1/14-3-3β complex was also expressed and purified, incorporating either full length wee1 or a truncated version from which the N-terminal domain of wee1 was deleted. Both exhibited wee1 kinase activity, at equivalent levels, p<0.001, with a 2.4 fold increase in kinase activity when wee1 is bound by 14-3-3β, p>0.001. Tryptic digestion of the complex indicated that its architecture was likely to be flexible and open, particularly within the N-terminal domain of wee1. CD analyses indicated that the wee1/14-3-3β complex was folded, with 30-40% α-helical content and 10-20% β-sheet content. Dissociation experiments were unsuccessful, however, indicating a high strength of interaction between wee1 and 14-3-3β. The empirical stoichiometry of the complex was determined as 1:1; subsequent native molecular weight determination suggested that the minimal functional unit is likely to be a 2:2 wee1/14-3-3β arrangement. It was proposed that the structural architecture of this complex may be similar to the serotonin N-acetyltransferase/14-3-3ζ complex. Experiments to determine the structure experimentally, using either TEM or x-ray crystallography, were unsuccessful, as the complex appeared to exhibit a high degree of flexibility in solution.
CDC25B was also expressed and purified, and was found to co-purify with a putative Sf9 14-3-3 protein. Consequently, it was re-cloned to co-express with 14-3-3β, and subsequent analysis of the resulting CDC25B/14-3-3β complex indicated that the empirical stoichiometry was 1:1, with the functional organization likely to be a 2:2 arrangement. It was proposed that the structural arrangement of this complex is most likely to be similar to that of the wee1/14-3-3β complex.
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<tr>
<td>14-3-3β</td>
<td>human 14-3-3 protein, β polypeptide, NCBI accession number NP_003395</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>5′-adenylyl-β,γ-imidodiphosphate, non-hydrolysable ATP analogue</td>
</tr>
<tr>
<td>amt</td>
<td>Amount</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AU</td>
<td>absorbance units</td>
</tr>
<tr>
<td>β-ME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>BG</td>
<td>background</td>
</tr>
<tr>
<td>C167S</td>
<td>mutation of cysteine residue 167 to serine</td>
</tr>
<tr>
<td>C238S</td>
<td>mutation of cysteine residue 238 to serine</td>
</tr>
<tr>
<td>C350S</td>
<td>mutation of cysteine residue 350 to serine</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CDC25B</td>
<td>human cell division cycle 25B protein, isoform 1, NCBI accession number NP_068659</td>
</tr>
<tr>
<td>CDK1</td>
<td>human cyclin dependent kinase 1, isoform 1, NCBI accession number NP_001777</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>C-terminal</td>
<td>carboxyl terminal</td>
</tr>
<tr>
<td>cyclin B1</td>
<td>human cyclin protein, isoform B1, NCBI accession number NP_114172</td>
</tr>
<tr>
<td>°C</td>
<td>degrees celsius</td>
</tr>
<tr>
<td>DEPC-treated water</td>
<td>Water treated with diethylpyrocarbonate</td>
</tr>
<tr>
<td>dephosphorylation</td>
<td>the enzymatic removal of a phosphate group from a protein</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>E183A</td>
<td>mutation of glutamic acid residue 183 to alanine</td>
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</table>
E184A mutation of glutamic acid residue 184 to alanine
EDTA ethylene diamino tetraacetic acid
FBS fetal bovine serum
G₀ an opting out gap phase of the eukaryotic cell cycle
G₁ the first gap phase of the eukaryotic cell cycle
G₂ the second gap phase of the eukaryotic cell cycle
GST glutathione-S-transferase
HeLa cells Human lymphoma-derived cell line from Helen Lane, ATCC
His-tagged polyhistidine purification tag
IEF isoelectric focussing
IMAC immobilised metal affinity chromatography
IPG immobilised pH gradient strip
IPTG isopropyl-β-D-thiogalactopyranoside
LAU laser absorbance units, for Sypro Ruby analysis
MPD 2-methyl-1,3-propanediol
MPF mitosis promotion factor
Mr molecular weight
MWCO Molecular weight cut-off point
NCBI National Centre for Biotechnology Information
NP40 nonionic P40 detergent
N-terminal amino terminal
PAGE polyacrylamide gel electrophoresis
PBS phosphate buffered saline
PCR polymerase chain reaction
PDB ID protein data bank identification number
Pfx Pyrococcus furiosus polymerase enzyme
phosphorylation the enzymatic addition of a phosphate group to a protein
pI isoelectric point
RMS root-mean-square
RNA ribonucleic acid
RNAse ribonuclease enzyme
rTEV recombinant tobacco etch virus protease
<table>
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<td>S phase</td>
<td>the synthesis phase of the eukaryotic cell cycle</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation, a measure of the variance of a dataset, calculated as the RMS deviation of the individual values from the mean value</td>
</tr>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SE</td>
<td>standard error of the mean, an unbiased estimate of the error of the population mean</td>
</tr>
<tr>
<td>Sf9</td>
<td>Spodoptera frugiperda-derived cell line, ATCC Number CRL-1711</td>
</tr>
<tr>
<td>SOC</td>
<td>superoptimal broth with catabolite repress</td>
</tr>
<tr>
<td>TAE buffer</td>
<td>tris-acetate-EDTA buffer</td>
</tr>
<tr>
<td>TE buffer</td>
<td>10 mM Tris.HCl pH 8.0, 1 mM EDTA</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N’-tetramethylethylenediamine</td>
</tr>
<tr>
<td>tr. wee1</td>
<td>truncated wee1</td>
</tr>
<tr>
<td>tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>truncated wee1</td>
<td>wee1 construct consisting of residues 215 to 646 only</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>wee1</td>
<td>human wee1 kinase, NCBI accession number NP_003381, full length construct</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside</td>
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“Double or nothing. With few exceptions, a living cell either reproduces or dies; the principle is so simple that no-one has bothered to call it a principle. A cell is born in the division of a parent cell. It then doubles in every respect: in every part, in every kind of molecules, even in the amount of water it contains. Thereafter, it divides with such equal justice that each new daughter cell is an identical copy of the parent. This doubling and halving, the cycle of growth and division, is generally known as the cell cycle...”

Dr. Daniel Mazia (1912 – 1996)

American cell biologist
Chapter 1 Introduction

The essential premise of this research is a greater understanding of the molecular interactions that allow or inhibit the onset of the mitotic phase of the eukaryotic cell cycle. The cell cycle, and cell division, is a fundamental aspect of the growth and development of all multi-cellular organisms; by necessity, it is highly regulated and tightly controlled. When those regulations and controls are lost, however, the consequences can be catastrophic. The overarching themes behind the cell cycle are well characterised; the more specialist interactions, and their context within the cell cycle, however, are still being elucidated, and it is this quest for understanding that forms the basis for this research.

This research is presented in six chapters. The introduction, Chapter 1, will discuss the eukaryotic cell cycle, from the broader themes, including the well established details of the phases of the cell cycle and regulation therein, to the known, but less characterised and understood details of the key molecules that contribute to the control of the process. The methods used for the investigation are presented in Chapter 2. The experimental results are presented in three different chapters. Chapter 3 presents the results following the expression and purification of CDK1 and cyclin B1, Chapter 4 the results of the expression, purification and characterisation of the wee1/14-3-3β complex and Chapter 5 the results relating to CDC25B. Chapter 6 aims to discuss and contextualise all of these results, with an additional discussion of the future directions for this research.

1.1: Cells: The Basic Unit of Living Organisms

In the 17th century, Robert Hooke peered through a microscope at a slice of cork and discovered that it was composed of cells. Centuries later, Rudolf Virchow set forth the cell theory, in which he proposed that the cell was the basic unit of all living organisms. This theory has since been proved correct [1].
While the cell is the basic unit of life, the process of growth and development to become a mature adult arises through the production of yet more cells; in fact, “the body of an animal can be viewed as a society or ecosystem, whose individual members are cells, [which are] organised into collaborative assemblies of tissues” [2]. The only method for an organism to achieve this growth and development, however, is through the division of those cells that already exist. In this way, all animals come from animals, all plants from plants, and all cells from cells [1], [2].

In order to accomplish this feat, an orderly sequence through which the cell is able to cycle has evolved, enabling a duplication of cellular contents followed by a physical division into two parts. This cycle of duplication and division is known as the cell cycle. The overall goal of the cell cycle is to produce two genetically identical cells from one precursor cell [2], [3].

Details of the cell cycle vary from organism to organism, with events occurring at different times during an organism's lifespan. Nevertheless, certain aspects are universal [3], as every cell must accomplish one fundamental task: to copy and pass on its genetic information to the next generation of cells. This requires the replication of the deoxyribonucleic acid (DNA) in each chromosome, followed by the accurate separation of the chromosomes into the daughter cells so that each cell receives a copy of the entire genome. Once this is accomplished, the cell physically divides to produce two identical daughter cells. Since it is dividing its contents in two, under most circumstances, cells also double their mass, which includes duplicating their cellular machinery. In this manner, the potential problem of cells becoming smaller following each instance of cell division is averted [2], [4].

The length of the cell cycle varies depending on the organism in question. The bacterial cell cycle, for example that of *Escherichia coli*, can take as little as 20 minutes to complete [5]. The cell cycle of a single-celled yeast may take 90 to 120 minutes [6]. The length of time taken for mammalian cells to progress through the cell cycle varies, but is generally approximately 24 hours, where approximately 6 – 8 hours will be spent in the S phase (Section 1.2.1.3), approximately 1 hour will be spent in the M phase (Section 1.2.2), and the remaining approximately 11 – 14 hours will be spent in one of the two G phases (Sections 1.2.1.1 and 1.2.1.2) [7], [8].
1.2: **The Eukaryotic Cell Cycle**

In 1963, Irving Leiberman suggested that, far from being an equation of cell kinetics, the mechanisms underlying the major steps in the cell cycle are a series of discrete yet interconnected biochemical events [9]. Following this realisation, research emphasis shifted towards the genetics and molecular biology underlying the overall process. This includes a deeper understanding of the formation, activation and inactivation of a series of cell cycle regulatory molecules that promote sequential progression through the different phases of the cell cycle [8], [10], [11], [12]. The human cell cycle, presented pictorially in Figure 1.1, is comprised of the interphase and the mitotic phase.

![Graphical representation of the eukaryotic cell cycle](image)

**Figure 1.1: Graphical representation of the eukaryotic cell cycle.** The eukaryotic cell cycle is made up of two gap phases, $G_1$ and $G_2$, the synthetic (S) phase and the mitotic (M) phase. This process results in two genetically identical daughter cells. An increase in cell size is associated with progression through the cell cycle, where the cell is approximately twice the size of a cell just following mitosis.

### 1.2.1: Interphase

Within the human cell cycle, the interphase will generally last at least 12 to 20 hours and can be divided into 4 steps: Gap 1 ($G_1$), Gap 0 ($G_0$), Synthesis (S) phase and Gap 2 ($G_2$).
1.2.1.1: G₀ Phase

The G₀ phase occurs only where the cell has opted out of the cell cycle, with exit from the G₁ phase. Within this stage, the cell may be biochemically active, or, more likely, in a quiescent or resting state, but does not initiate or undergo replication or division. Having entered the G₀ phase, a cell may spend its lifetime within this quiescent state [8]. This state may occur when an end stage of development has been reached and the cell will no longer divide, for example a human neuron [2], [10]. Conversely, external factors, such as mitogens, may cause the cell to re-enter the cell cycle, with entry into the G₁ phase [2], [8].

1.2.1.2: G₁ Phase

During G₁, the cell is able to grow in size, as well as undertake all necessary tissue-specific cellular and/or biochemical functions. It is during this phase that the cell must decide if it is to continue through the cell cycle; once the S phase is initiated the cell is committed to go through until the completion of cytokinesis, or undergo apoptosis [2], [10]. This decision to progress through the cell cycle, from G₁ to the S phase, is referred to as the G₁/S Checkpoint (Section 1.4). During this phase, the cell may also, in some instances, enter the G₀ phase (Section 1.2.1.1).

1.2.1.3: S Phase

During the S phase, the cell’s entire complement of DNA is faithfully replicated. There are three major steps within DNA replication: initiation, replication and termination [2], [10].

In the initiation step, several key factors are recruited to a specified region of DNA that becomes the origin of replication. These key enzymatic factors include a helicase enzyme, which is required to unwind the DNA ahead of the replication fork, a ribonucleic acid (RNA) primase enzyme, which is used to generate an RNA primer to be used in DNA replication, as well as a DNA polymerase enzyme, which performs the actual replication. A graphical
A representation of the process of DNA replication is presented in Figure 1.2. Once these factors have assembled, the DNA at the origin of replication is unwound, and the partially unwound strands form a "replication bubble", with one replication fork on either end. After the helicase unwinds the DNA, a single-strand binding protein holds the DNA strands in place. RNA primase is then bound to the starting DNA site, and the DNA polymerase enzyme is then able to initiate actual replication [2], [10].

Figure 1.2: Graphical representation of DNA replication. The leading strand is shown in red, the lagging strand in blue. Newly synthesised DNA is shown in gold and green. This process results in two identical DNA strands.

Each group of enzymes at the replication fork progresses away from the origin, unwinding and replicating the DNA strands as they move. DNA polymerase, however, can only synthesize new DNA from the 5’ to 3’ direction along the newly synthesised strand of DNA. Because of this, the DNA polymerase can only travel on one side of the original strand without any interruption. This original strand, which exhibits a 3’ to 5’ directionality, is called a leading strand. The opposing original strand, which exhibits a 5’ to 3’ directionality, is referred to as the lagging strand. Since the DNA replication on the lagging strand is not continuous, a new DNA polymerase enzyme is introduced with each instance of the helicase unwinding more DNA. As a result, the replicated DNA of the lagging strand is fragmented, referred to as Okazaki fragments. Another enzyme, DNA ligase, then connects the Okazaki fragments [2], [10].
Before the DNA replication is finally complete, enzymes proofread the sequences to ensure the nucleotides are paired up correctly. If a mistake or any damage has occurred due to the replication process, a nuclease enzyme will remove the incorrect DNA, thus allowing a DNA polymerase enzyme to fill in the gap [2], [10]. Overall, this process results in semi-conservative DNA replication, where both of the new DNA strands contain one strand of the original DNA and one wholly synthesised strand [2], [10].

1.2.1.4: G2 Phase

During G2, the cell is able to continue growing and to, again, undertake its own tissue-specific cellular and/or biochemical functions. It is also during this phase that the cell is able to ascertain if the DNA has been faithfully replicated and that the replication process itself has been completed, before undertaking mitosis and cytokinesis [2], [10], a decision-making step referred to as the G2/M Checkpoint (Section 1.4).

1.2.2: Mitotic Phase

The mitotic phase comprises mitosis and cytokinesis, lasting approximately 1 – 2 hours [8], and is concerned with the physical separation of the cell into two daughter cells.

1.2.2.1: Mitosis

Mitosis (also called karyokinesis) is the process of chromosome segregation and nuclear division. This process assures that each daughter nucleus receives a complete copy of the organism's genome. In most eukaryotes, mitosis is accompanied with a division of the cytoplasm, referred to as cytokinesis. There is also another process called meiosis, in which the daughter nuclei receive half the chromosomes of the parent. This process is involved in gamete formation and other similar processes [2], [10].
Mitosis is divided into several stages, in particular prophase, prometaphase, metaphase, anaphase, and telophase. The whole procedure is very similar among most eukaryotes, with only minor variations; a graphical overview of the process is presented in Figure 1.3. Since the genetic material has already been duplicated, there are two identical copies of each chromosome in the cell, with sister chromosomes attached to each other at the centromere. The task of mitosis is to assure that one copy (and only one copy) of each sister chromatid goes to each daughter cell after cell division [10], [13].

During prophase, the genetic material, or DNA, which normally exists in the form of chromatin, condenses into a highly ordered structure called a chromosome. The two centrioles, which serve as a type of anchor and which replicate independently of mitosis, begin recruiting microtubules to form a mitotic spindle between them. By recruiting more microtubules, therefore increasing the length of the spindle, the centrioles push apart to opposite ends of the cell nucleus. It should be noted that some eukaryotes, for instance plants, lack centrioles although the basic process is eminently similar [2], [8].
During prometaphase, the nuclear membrane dissolves in some eukaryotes, reforming later once mitosis is complete. This is called open mitosis, found in most multi-cellular forms. Many protists undergo closed mitosis, in which the nuclear membrane persists throughout. Kinetochores begin to form at the centromeres. This is a complex structure that is the attachment point by which chromosomes may be secured. Two kinetochores form on each chromosome, one for each chromatid. When the spindle grows to sufficient length, the microtubules begin searching for kinetochores on which to attach [2], [8].

As microtubules find and attach to kinetochores, they begin to line up in the middle of the cell, initiating metaphase. Complete segregation requires that every kinetochore be attached to a microtubule before separation begins. It is thought that unattached kinetochores control this process by generating a signal, referred to as the mitotic spindle checkpoint, which initiates a pause in progression before proceeding to anaphase. There are many theories as to how this is accomplished, some of them involving the generation of tension when both microtubules are attached to the kinetochore. When chromosomes are bivalently attached, they line up in the middle of the spindle, forming the metaphase plate. This does not occur in every organism; in some cases chromosomes move back and forth between the centrioles randomly, only roughly lining up along the midline [2], [8].

Anaphase is initiated when every kinetochore is attached to a microtubule and the chromosomes have lined up along the middle of the spindle. Anaphase is divided into two phases. Firstly, the proteins that bind the sister chromatids together are cleaved, allowing them to separate. The sister chromatids are pulled apart by the microtubules, towards the respective centrioles to which they are attached. Following this, the spindle axis elongates, driving the centrioles, and the set of chromosomes to which they are attached, to opposite ends of the cell. At the completion of anaphase, the cell has succeeded in separating identical copies of the genetic material into two distinct populations. During telophase, the nuclear membrane reforms around each of the newly replicated chromosomal copies and the chromosomes are unfolded back into chromatin [2], [8].
1.2.2.2: Cytokinesis

Following the exit from telophase, the cell undergoes cytokinesis, or a physical separation of the cytoplasm. During this process, the cytoskeletal fibers, in particular actin and myosin, form a contractile ring in a direction that is perpendicular to the mitotic spindle. Upon assembly, the contractile ring will continue to tighten, drawing the opposing plasma membrane ever closer. Eventually, this causes the cell to be physically pinched in half. The overall result is the formation of two distinct and identical daughter cells [2], [4].

1.3: Mitosis Promotion Factor (MPF)

The events of the cell cycle are organized into a number of sequence-dependent discrete biochemical steps. In itself, this is a process involving many different molecules, made up of a number of complicated sub-processes, which requires exquisite co-ordination across the entire cell. An integral part of this cellular co-ordination is the entry into mitosis, which is promoted by the mitosis promotion factor, or MPF [13].

The MPF is composed of a number of catalytic and regulatory proteins, complexed with histone H1 kinase. The primary catalytic subunit of this complex, CDK1, is associated with cyclin B1, which plays the role of the major regulatory subunit [13]. The activity of the MPF is regulated by a number of enzymes, including wee1 kinase and CDC25 phosphatase [2], [13]. As the catalytic subunit of the MPF, CDK1 is pivotal to mitotic entry: when this molecule is defective, mitosis fails to occur, and when CDK1 is released prematurely, mitosis will also occur prematurely [2], [13]. The ultimate activation of the MPF, however, is an exponential reaction. A positive feedback loop exists in which the presence of activated MPF is able to initiate the activation of other inactive MPF complexes. This positive feedback loop contributes to the rapid onset of mitosis [2].
1.3.1: CDK1

The kinase CDK1, also known as CDC2, plays a central role in the onset and progression of mitosis in almost all eukaryotic cells, carrying out this function within its role as the major catalytic subunit of the MPF. The regulatory control over this enzyme arises through three major mechanisms.

Firstly, partial activation of CDK1 must occur. This partial activation arises through the phosphorylation of the conserved T-loop region of CDK1, or more specifically of residue Thr-160 [8], [12], [14]. The phosphate group is conferred by the complex known as CDK1 Activating Kinase or CAK, which is composed of a complex of cyclin H, CDK7 and MAT1 [8], [14]. CAK is also involved in general transcriptional control, and for this reason, its regulatory role in the cell cycle remains somewhat controversial [14]. Secondly, following partial activation by CAK, CDK1 is functionally inactive when in its monomeric or unbound state. In order to become functionally active, it must first bind a cyclin partner. In the case of the G2/M transition, this partner is cyclin B1 [8].

Thirdly, there are a number of reversible phosphorylations that are required to control the activities of the CDK1/cyclin B1 protein complex [11], [12], [15]. Wee1 and related tyrosine kinases carry out inhibitory phosphorylations of CDK1 residues Thr-14 and Tyr-15, rendering the CDK1/cyclin B1 complex inactive. Phosphorylation at the Thr-14 binding site prevents ATP binding, and phosphorylation at the Tyr-15 site interferes with phosphate transfer to the substrate [8], [12], [15]. The inhibitory phosphates are maintained until such time as the cell is ready to progress through to mitosis and cytokinesis [8], [15], [16]. These phosphorylation events also allow a 14-3-3 regulatory molecule to bind. Due to a nuclear export signal present within the 14-3-3 molecule, this interaction results in the nuclear exclusion of the cyclin B1/CDK1/14-3-3 complex [8], [17].

Dephosphorylation of Tyr-15 is the rate limiting step for CDK1/cyclin B1 activation and mitotic entry, and is carried out by CDC25 and related tyrosine phosphatases [15], [16].
In order to promote mitosis, the cyclin B1/CDK1 complex carries out a number of roles, both within the cytoplasm and within the nucleus of the cell, and is, in fact, involved in the complete reorganisation of the cellular architecture during mitosis. The cyclin B1/CDK1 complex is thought to phosphorylate the motor protein EG5, promoting centrosome separation, and in support of this, the complex has been found to be associated with duplicated centrosomes [8], [11], [18]. The complex is involved in the breakdown of the nuclear lamina, as well as in cell rounding, and also plays a role in the fragmentation of the Golgi network [8], [11], [19], [20]. Overall, these functions are integral to the success of the mitotic division.

Following entry into mitosis, CDK1 must be completely inactivated. Without complete deactivation of CDK1 during anaphase and telophase, chromosomes are not able to re-condense, nuclear envelopes are not able to reassemble and hence cytokinesis cannot occur [21]. Cyclin proteolysis, and in particular for the G2/M checkpoint, cyclin B1 proteolysis, is the major mechanism whereby CDK1 is inactivated. CDK1 cannot function without a cyclin binding partner [13].

1.3.2: Cyclin B1

While working at Woods Hole in the summer of 1982, Tim Hunt discovered a protein in rapidly dividing sea urchin embryos that was synthesized during interphase but suddenly destroyed during cell division. The cyclic expression pattern of this protein later gave rise to the name of this family of proteins, the cyclins [21], [22].

The cyclin family of proteins is so-called due to their expression patterns – they are synthesised and destroyed during each cell cycle. All members of this family share a 150 amino acid region of homology, referred to as the cyclin box, which binds to the N-terminal region of their respective CDK binding partner(s) [8]. There exist two distinct types of cyclins. The first are G1 cyclins which are involved in promoting entry to the S phase. The second are the mitotic cyclins which are involved in entry to the mitotic phase [2].
After more than two decades of research into the eukaryotic cell cycle, the particular cyclin first characterised by Hunt has been relabelled as cyclin B, of which two isoforms exist [8]. The first, cyclin B1, functions as a mitotic cyclin, interacting with a number of regulatory elements, including CDK1, to enzymatically trigger entry into mitosis. Cyclin B1 contains a cyclin box motif at residues 202 to 233, and it is this segment that is thought to interact with the N-terminal region of CDK1 [8], [21]. The second isoform is cyclin B2. Studies have confirmed, however, that cyclin B2 is non-essential for normal growth and development; this isoform is thought to associate with the Golgi, and may play a role in the remodelling of the Golgi during mitosis [8], [23], [24]. It is cyclin B1 that is of interest in this research project.

Cyclin B1 is a 433 amino acid protein. The structure of a cyclin B1 fragment comprising residues 165 – 433 has been solved to 2.9 Å resolution with a number of mutations: C167S, E183A, E184A, C238S and C350S; these mutations were necessary to improve the stability of the construct such that crystal trials could be undertaken [25]. An image of the structure of this construct is presented in Figure 1.4. The overall architecture of cyclin B1 comprises a central hydrophobic helix, flanked by two cyclin boxes (the signature motif for this family), each of which is organized as a five-membered helix bundle. As for other members of this family, cyclin B1 contains two such cyclin boxes [25].

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**Figure 1.4: Ribbon diagram of cyclin B1.** This structure (PDB ID 2B9R, [25]) shows a construct comprising residues 165 – 433 with mutations C167S, E183A, E184A C238S and C350S, coloured from blue to red [25]. This image was generated using the program Jmol (http://www.jmol.org).
The first cyclin box is in the N-terminal part of the molecule. All known protein: protein interactions involving cyclin B1, including binding to CDK1, are thought to be mediated through interactions with this cyclin box. This cyclin box, including loops, superimposes over the N-terminal cyclin box of cyclin A (PDB ID 1VIN, [26]) with a root-mean-square difference (RMSD) in Cα positions of 0.6 Å, and over that of cyclin E (PDB ID 1KXU, [27]) with a RMSD of 0.7 Å. The most conserved of the residues in all three of these structures appear to be involved in stabilization of the molecule, by forming buried ion pair interactions, and thus the maintenance of the overall rigidity of the structure [25].

The second, C-terminal, cyclin box of cyclin B1 is similar in overall architecture to the N-terminal cyclin box, but varies in helix and loop length, as well as in the orientation of the bundle itself. The overall structure of this cyclin box did not align well with any other known cyclin family member, and it has been suggested that this second structure may contribute to cyclin B – specific functions [25].

The overall orientation of each cyclin box is held in a rigid state by a wealth of (mainly side chain – main chain) hydrogen bonds. It is postulated that this rigidity is necessary to force CDK1 into the correct conformation to allow its activation. Cyclin B1 contains an ‘RxL’ binding site, which is involved in substrate recognition. This binding motif lies at the diametrically opposite side to that of the CDK1 binding site. This binding site contains a number of conserved residues, which are contributed by helix A, with less conserved residues being contributed by a number of other helices lining the remainder of the groove. This ‘RxL’ binding site connects with the CDK1 interface via a shallow channel that extends from the CDK1 interface, over the first cyclin box and down to the ‘RxL’ binding groove [25].

The C-terminal cyclin box contains a binding groove analogous to that of the ‘RxL’ binding groove, but, instead, contains a ‘RRASK’ sequence motif. This motif is conserved in all B-type cyclins, and has been found to be important for cyclin B interactions with the kinase Myt1 and the phosphatase CDC25C. Based on molecular modelling, it is postulated that this ‘RRASK’ motif lines the cleft between cyclin B1 and CDK1, allowing access to the CDK1 active site. A number of basic or positively charged residues line this cleft, increasing the attractiveness of the site for phosphate recognition [25].
Within the construct used to model the structure of cyclin B1, the first seven N-terminal and last nine C-terminal residues were not included in the model due to poor electron density [25]. The corresponding residues in cyclin A exhibit an identical disordered nature, with poor electron density (PDB ID 1VIN) [26]. The structure of cyclin A/CDK2 complex (PDB ID 1FIN, [28]), however, reveals that this area becomes ordered and forms the end of the most N-terminal helix of cyclin A within the complex, indicating this may be the observation of a flexible region of cyclins that becomes ordered upon binding to a CDK [25], [28]. CDK1 is unable to trigger mitosis and cytokinesis without first binding cyclin B1, indicating that specificity is of the utmost importance. The exact nature of the interactions between the two is, however, as yet unknown.

During interphase, human cyclins are differentially localised within the cell, with cyclin B1, in particular, localised to the cytoplasm. This localisation is determined by a 42 amino acid sequence found within the N-terminal region of cyclin B1 [29], [30]. Deletion mutants have shown, firstly, that deletion of this region results in cyclin B1 being localised to the nucleus, and secondly, that the addition of this 42-amino acid sequence to a normally nuclear protein is enough to localise that protein to the cytoplasm [29]. This sub-cellular localisation plays an important mechanistic role, by preventing any early or otherwise inappropriate interaction between CDK1 and cyclin B1. Once CDK1 has been exported to the cytoplasm, only then is it able to interact with cyclin B1 [29], [30].

In addition to its regulatory activity, however, one of the major mechanistic functions of cyclin B1 is to ensure the directionality of cell cycle progression. Cell cycle progression is always in the forward direction, for example from event X to event Z, via event Y. Directionality is assured through the rapid degradation and/or proteolysis of target proteins, effectively both halting progression in the reverse direction and driving the cycle forward [31], [32]. This method of progression is particularly important for the regulation of human mitotic cyclins, for example cyclin B1, in which they are synthesised during interphase and rapidly proteolysed during the latter stages of mitosis and cytokinesis [22], [32] to effectively halt the mechanistic work of the MPF.
The anaphase promoting complex (or APC) is responsible for the specific proteolysis of cyclin B1. The APC, itself, is a high molecular weight complex, composed of at least eleven subunits. Within this complex set of machinery, the catalytic subunit recognises an F box sequence present within cyclin B1. This interaction allows activated ubiquitin to be assembled on the cyclin molecule, effectively forming a target for the 26S proteosome [21], [32] and hence for degradation.

### 1.3.3: Wee1 Kinase Family

Wee1 and related kinases are a family of enzymes with a highly conserved mechanism of action that are known to influence the timing of entry into mitosis. Entry to mitosis is controlled by the kinase CDK1. Wee1 and related kinases are able to delay this entry by direct inhibition of CDK1 through the phosphorylation of two highly conserved residues at the CDK1 N-terminus, Thr-14 and Tyr-15 [12], [15], [32], [33], [34], [35]. These inhibitory phosphate groups are then able to be removed by the dual specificity phosphatase CDC25, thus allowing entry to mitosis [15], [32], [33], [34], [35]. In *Saccharomyces*, when wee1 activity is lost, the cells are prompted to enter mitosis before sufficient growth has occurred, leading to the production of two abnormally small daughter cells, which suggested that wee1 is constitutively active to maintain the inhibition of CDK1. Conversely, a delay into mitosis can be seen following an increased dosage of wee1, and the daughter cell produced is abnormally large. Loss of function of wee1-related kinases in *Xenopus* and *Drosophila* also causes premature entry into mitosis. However, this same requirement for wee1, for cell size control, has not yet been demonstrated in animals [8], [34]. There are two major members of the wee1 family of kinases. The first is wee1 and the second is myt1.

#### 1.3.3.1: Wee1 Kinase

Wee1 kinase is a 646 amino acid protein comprising three domains: a large N-terminal regulatory domain, a central kinase domain, and a small C-terminal regulatory domain, [15], [36], [37], [38], [39]. It is regulated by both phosphorylation and degradation. The N-terminal domain contains two phosphorylation sites, at Ser-53 and Ser-123, which allows F-
box protein recognition and targets wee1 for ubiquitination and degradation [38], [40] in a manner similar to that of cyclin B1. This domain also contains a putative nuclear localization signal (Arg-Arg-Arg-Lys-Arg).

Although it has a strict specificity for phosphorylation of Tyr-15 in CDK1 [12], [15], [35], [41], the kinase domain of wee1 is not a typical tyrosine kinase. Sequence searches of the human genome do not place wee1 in any of the identified tyrosine kinase, or even protein kinase, subfamilies [42], but in a completely separate family [12], [43]. The structure of the catalytic domain of wee1 (residues 291 – 557) has been determined to 1.8 Å resolution (PDB ID 2IN6, [39]), and is presented in Figure 1.5. This catalytic domain displays a standard two-lobed kinase fold. The N-terminal lobe consists mainly of a five-stranded antiparallel β sheet, with a glycine-rich loop and a consensus sequence of GxGxxG found at residues 306 to 311. The C-terminal lobe contains a characteristic four-helix bundle and the two lobes are joined by a linker peptide that connects strand β5 in the N-terminal lobe to helix αD in the C-terminal lobe; this has been shown to allow relative movement of the two lobes in other kinases, for example cAMPK [15], [44].

![Figure 1.5: Ribbon diagram of the catalytic domain of wee1 kinase. An inhibitor (PD04078240) is shown bound to the active site. No electron density was evident for residues 436-455, thus these residues were omitted from the model (PDB ID 2IN6) [39]. This image was generated using the program Jmol (http://www.jmol.org).](image)

The active-site is situated in a well defined cleft with residues contributed by both the N- and C-terminal domains. The five-stranded β-sheet and glycine-rich loop from the N-terminal
domain curve over the active site from above; the hinge region defines the end of the cleft; helix αD and the catalytic segment of the C-terminal domain contribute to the other wall of the cleft; and the floor is formed by the N-terminal helix αC and the region between β8 and β9 leading into the activation segment. Many invariant or conservatively substituted residues are found in the active site, particularly in the catalytic segment [39].

The catalytic segment, residues 422 to 433, extends from strand β6 to the beginning of strand β7 and presents the essential catalytic residue, Asp-426, into the active-site cleft. This sequence of the catalytic segment, which is highly conserved, contains the active-site motif HxD and closely matches the protein kinase consensus sequence IVHxDLKPxNlx. In many kinases, the residue preceding the essential aspartate is often an arginine that is activated by phosphorylation of residues in the activation segment, referred to as RD kinases [45]. In wee1, however, the preceding residue is Met-425, which forms part of a hydrophobic cluster with Val-487 and Leu-495, which, along with Val-475, helps to anchor the activation segment [39].

The activation segment is a key functional region of protein kinases that usually extends from the back of the active site, where a conserved Asp residue (Asp-463 in wee1) coordinates the essential magnesium ion, and forms an irregular extended loop that finishes with helix αEF in the C-terminal domain. In many kinases, this loop is also able to undergo a number of conformational changes, depending on the phosphorylation state [45].

The activation segment in wee1 is 25 residues in length, and is well ordered throughout, with a conformation that corresponds very closely to the activation segments in Chk1 kinase [46], aurora kinase [47], and phosphorylase kinase [48], none of which appear to require phosphorylation for activation. Once in its active state, this segment can be stabilised by secondary structure and side chain interactions, with contributions from residues that are invariant or highly conserved in all wee1 family members and differ from the sequence patterns seen in other protein kinases. Of the 25 residues, 18 are strongly conserved in the wee1 family and 7 are unique to it [39]. Recent evidence shows that wee1 levels are also controlled by components of the circadian clock, making it a key link between the circadian clock and the cell cycle, and hence cellular proliferation [49], [50].
The C-terminal domain is able to be phosphorylated at residue Ser-642, within the consensus motif RSVSLT, generating a 14-3-3 binding site. Although it is known that Chk1 is able to phosphorylate the *Xenopus* wee1 orthologue, the exact molecule responsible for the phosphorylation of human wee1 is, thus far, unknown [33], [51]. Of all of the 14-3-3 isoforms [52], [53], it has been shown that wee1 preferentially forms a complex *in vitro* and *in vivo* with 14-3-3β. The formation of this complex leads to both an increased level of expression and an increased half-life of the wee1 molecule. In addition, wee1 in complex with 14-3-3β has been shown to exhibit approximately three times more kinase activity that that of wee1 on its own (no 14-3-3β present). This quantification of the effect of 14-3-3β has been derived from observation of cell populations following molecular co-transfection of the molecule(s) [33], and by mutagenesis, followed by immuno-precipitation [51].

**1.3.3.2: Myt1**

The second major member of the wee1 kinase family is myt1, which exhibits 35% sequence identity to human wee1 [54]. Myt1 is a 499 amino acid protein with an overall organisation similar to that of wee1 in that it contains three distinct domains: the N-terminal domain, a central kinase domain, and a small C-terminal domain. Originally isolated due to its specific threonine-targeting kinase activity, myt1 is a dual specificity kinase that is able to phosphorylate CDK1 on both Thr-14 and Tyr-15. The enzyme exhibits a higher affinity for the phosphorylation of Thr-14 than Tyr-15 [12], [35], [55], [56].

Myt1 exhibits perinuclear activity, and is localised to the endoplasmic reticulum and Golgi complex [35], [54], [55], [56], [57]. Residues 379 to 398 (GWALWQALLALLCWLWHGLA) are trans-membranous [54], [56], [58] but it is thought that the trans-membrane helices do not span the entire lipid bilayer, as both the kinase and the C-terminal domains remain cytoplasmic [56]. The C-terminal region of myt1 contains the motif RNL (residues 486 to 488), which are thought to interact with the CDK1/cyclin B1 complex. More specifically, these residues dock the cyclin binding partner of CDK1, thus allowing CDK1 to directly interact with the central kinase domain of myt1. Myt1 requires the presence of CDK1, phosphorylated at residue Thr-161, in complex with any cyclin binding partner before the requisite kinase reaction will occur [35], [55], [58].
The effect of the presence of myt1 is twofold. Firstly, the presence of myt1 is able to block the nuclear-cytoplasmic shuttling of cyclin B1. This causes a delay in cell cycle progression, and in particular a delay in progression through the G2 phase of the cell cycle [56]. The interaction between myt1 and CDK1/cyclin B1 acts to sequester the CDK1/cyclin B1 complex to the cytoplasm, pre-empting inappropriate onset of mitosis [35], [56], [57]. It has been suggested that inhibition of the CDK1/cyclin B1 complex occurs originally in the cytosol through myt1, but that exquisite control over the onset of mitosis is exhibited by wee1, within the nucleus [35], [54].

During the onset of mitosis, myt1 becomes hyper-phosphorylated [55], largely through the action of CDK1/cyclin B1 [54], [55], [56]. In addition, the MAP kinase cascade is also activated, and is responsible for a proportion of the myt1 hyper-phosphorylation through the actions of a secondary kinase (p90RSK) [35], [56], [57]. Overall, the hyper-phosphorylation of both the N- and C-terminal regions of myt1 leads to a twofold decrease in the kinase activity of the enzyme, with a concomitant decreased affinity for CDK1/cyclin B1. This decrease in affinity for CDK1/cyclin B1 has been attributed (in part) to the binding of peptidyl-prolyl isomerase (Pin1) to the phosphorylated C-terminal region of myt1 [56], [59].

1.3.4: CDC25

In order to progress through to mitosis and cytokinesis, inhibitory phosphorylations of CDK1 residues Thr-14 and Tyr-15 must be removed. This phosphate removal is catalysed by the CDC25 family of phosphatases.

In mammalian cells, there exist three isoforms of CDC25: namely CDC25A, CDC25B and CDC25C [60], [61]. Each isoform comprises two major domains, the N-terminal domain, which is involved in the regulation of the enzyme, and a C-terminal domain, which is generally catalytic in nature. For all three isoforms, the C-terminal domain is generally well conserved, but the N-terminal domain shows a higher degree of sequence variation [62]. It is CDC25B and CDC25C that are the most important isoforms for entry into mitosis [63], [64], [65].
CDC25B is a 566 amino acid protein, with the active site cysteine residue at position 473. In contrast, CDC25C is a 473 amino acid protein with its active site cysteine residue at position 377. Both molecules contain a rhodanese homology superfamily fold [66], CDC25B between residues 398 – 517, and CDC25C between residues 302 – 421. During interphase, CDC25B and CDC25C activity is suppressed through the addition of a phosphate group to residue Ser-216. Once added, this phosphate group provides a binding site for a 14-3-3 regulatory molecule which acts to reinforce the inhibition, effectively preventing CDC25 from causing premature dephosphorylation of CDK1 [67].

The CDC25B and CDC25C isoforms are known to bind different isoforms of the 14-3-3 family of regulatory molecules. This differential binding results in the sequestration of CDC25B to the cytoplasm, due to a nuclear export signal present within the 14-3-3 molecule [61], [68], [69]. 14-3-3 binding to CDC25C, however, results in the masking of CDC25C nuclear localisation signal, resulting in the CDC25C complex also being localised to the cytoplasm [70], [71], [72].

Upon entry into mitosis, the bound 14-3-3 regulatory molecule is removed through the actions of the cyclin-dependent kinase CDK2, which thus exposes the phosphate group on CDC25B and CDC25C. Dephosphorylation of the CDC25 isoforms, and therefore activation of the enzymes, is carried out by protein phosphatase 1 (PP1). The N-terminal region of CDC25B and CDC25C contains a PP1 binding motif, allowing direct recognition and interaction to occur between the two enzymes. Once they have been dephosphorylated, CDC25B and CDC25C are able to exert their effects on CDK1, and with it, the MPF [67], [72].

It is at this point that the different isoforms of CDC25 are able to exert their specific effects. Subsequent dephosphorylation of CDC25C releases the bound 14-3-3 molecule, revealing the intrinsic nuclear localisation signal of CDC25C, resulting in translocation of CDC25C to the nucleus. CDC25B, which is localised to the cytoplasm, partially activates the CDK1/cyclin B1 complex by dephosphorylating CDK1 at Thr-14. The singly dephosphorylated complex accumulates within the cytoplasm during the G2 phase. This partially activated form is then able to shuttle back to the nucleus. Complete activation of the complex is achieved through...
rapid dephosphorylation of the CDK1 residue Tyr-15, carried out by CDC25C. This latter second dephosphorylation event is the rate-limiting step for entry into mitosis [14], [61], [63], [72].

Due to their direct interaction with CDK1, the CDC25 phosphatases are able to exert a very substantial effect on a given cell population. In human cells, mutants lacking CDC25 exhibit delayed entry into mitosis and an abnormally large cell size. An increase in the gene dosage of CDC25, however, causes premature entry into mitosis and a decreased cell size [34], [61], [63].

The structure of the catalytic domain of CDC25B has been determined to 1.9 Å resolution (PDB ID 1YMK, [73]). Although CDC25B is composed of 566 amino acids, the structure that was determined was for the catalytic domain only, comprising 211 amino acids (see Figure 1.6). The overall architecture of this domain is wedge-like in shape, and exhibits an $\alpha/\beta$ motif. Overall, there are a total of six $\alpha$-helices, three strands of parallel $\beta$-sheet and large regions with poorly defined secondary structure. The longest $\alpha$-helical segment (Glu-478 to Asp-495) extends from the phosphate-binding pocket in a manner characteristic of phosphate-binding or nucleotide-binding motifs [73].

Figure 1.6: Ribbon diagram of the catalytic domain of CDC25B. This domain (PDB ID 1YMK, [73]) is comprised of an $\alpha/\beta$ motif, in a wedge-like organisation, and has a bound $\text{Cl}^-$ ion, shown as a charcoal sphere [73]. This image was generated using the program Jmol (http://www.jmol.org).
The catalytic site has, at its centre, residues His-472 to Arg-479, and contains the signature motif HCxxxxxR. Examination of the region around the catalytic site reveals a groove extending away from the catalytic site surrounded roughly by residues 427, 428, 442-448, 479, and 531-550. This cleft extends out towards the anion binding site (shown as a Cl⁻ ion, see Figure 1.4), and is thought to play a role in the substrate specificity exhibited by CDC25B [73], [74].

CDC25B has been shown to form an in vitro complex with various 14-3-3 subtypes, for example 14-3-3β, 14-3-3σ and 14-3-3ε. Only 14-3-3β, however, has been shown to affect the sub-cellular distribution and functionality of CDC25B, and hence it is believed that the in vivo binding partner of CDC25B is the β-isoform of 14-3-3 [67], [68].

1.3.5: 14-3-3 Proteins

14-3-3 proteins have been found to positively regulate several biological systems, such as cell cycle timing and response to DNA damage or stress across a range of different organisms, and have been found to associate with more than 60 proteins in vivo, including cell cycle proteins, such as CDC25B or wee1, and intracellular signalling, such as the IGF-1 receptor [53], [69], [75], [76], [77], [78], [79].

14-3-3 proteins are small acidic proteins with a molecular mass ranging from 27 to 32 kDa, with usually no recognisable catalytic domain or function [69], [76], [79]. There are seven isoforms present in mammals, all of which are highly conserved through evolution, and all of which are interchangeable within species [69], [76]. 14-3-3 proteins are predominantly dimeric in nature with the ability to form homo- or hetero-dimers with other 14-3-3 isoforms [53], [69], [76], [79].

All of the 14-3-3 proteins share a similar general structure, comprising a bundle of nine anti-parallel α-helices, which then associate to form dimers. A ribbon diagram of the structure of a typical 14-3-3 isoform is presented in Figure 1.7. The dimer forms a flattened “U” shaped
structure, with a large groove (35 Å long, 35 Å wide and 20 Å deep) running the majority of the length of each monomer [69], [75], [76], [77], [79].

![Figure 1.7: Ribbon diagram of 14-3-3σ.](image)

Figure 1.7: Ribbon diagram of 14-3-3σ. This is a representative structure of the all of the 14-3-3 isoforms (PDB ID 1YZ5, [80]). 14-3-3 molecules are α-helical, and typically form either homo- or hetero-dimers, as indicated above [80]. This image was generated using the program Jmol (http://www.jmol.org).

Dimerization is achieved through the packing of helix 1 (residues 3 to 17) of one 14-3-3 molecule with helices 3 (residues 39 to 68) and 4 (residues 75 to 107) of the second 14-3-3 protein. The remaining helices form the walls of the “U” shape [69], [75], [76], [77], [79], [80]. The groove created by dimerization has an overall acidic charge, with clustered areas that are basic or hydrophobic, resulting, overall, in a reasonably amphipathic groove. Many of the residues that are highly conserved between different members of this family are situated either within the binding interface, or within the lining of the central groove. On the other hand, the residues that are more variable across different members of this family are generally found on the outer, more exposed, surfaces of the molecule. It is postulated that these residues form the various binding interfaces for interactions with other proteins [69], [76], [79].

There are a number of mechanisms through which 14-3-3 binding is usually mediated. Firstly, binding may be facilitated by sequence-specific recognition. In this case, the target molecule usually displays one of two sequence motifs: the first is RSxpSxP, and the second is RxxxpSxP, where ‘x’ represents any amino acid and ‘pS’ represents a phosphoserine. For
example, Raf protein binds 14-3-3 and displays the first of the two above sequence motifs. Other molecules, however, do not possess either of these motifs. Instead, the phosphorylation of the target molecule tends to form a binding site for 14-3-3 [78], [79].

Structural analyses reveal that binding to phosphoserine- or phosphothreonine-containing sequence motifs involves direct interactions between the phosphate and Lys-49 and Arg-56 in helix C, and Arg-127 and Tyr-128 in helix E. These residues form a basic pocket in an otherwise acidic molecule, explaining the ability of the substrate’s serine/threonine phosphorylation to act as a molecular switch controlling ligand binding [69], [76], [79]. It has also been suggested that there may be a third as yet uncharacterised method of recognition and/or binding to 14-3-3, arising from the ability of 14-3-3 to associate with non-phosphorylated proteins that do not display either of the sequence motifs, and are not phosphorylated prior to or during 14-3-3 binding, such as the enzyme exoenzyme S [78], [79].

The effect of 14-3-3 dimerisation on the binding of the target tends to vary. In some instances, it is suggested that dimerisation does not affect target protein binding. Conversely, for other proteins, 14-3-3 dimerisation is an absolute requirement for binding. It is suggested that the mode of binding to a 14-3-3 molecule (in dimeric or monomeric form) can be described as a function of binding affinity. Those proteins that have a low binding affinity, such as those that do not display a preferred binding motif, tend to bind the dimerised 14-3-3. Similarly, those proteins that exhibit a high binding affinity for 14-3-3, such as those that display a preferred binding motif, usually bind to both the dimeric and monomeric 14-3-3. Binding targets that do not contain the binding motif usually require the presence of more than one phosphorylation site in order for 14-3-3 to bind in a stable manner [78].

The specific functions of 14-3-3 proteins are varied, but can be loosely divided into four major categories: (i) Alteration of the targets’ ability to interact with and/or bind another protein; (ii) modification of the intrinsic localisation of the target protein, by either facilitating the targets’ nuclear export via the nuclear export sequence present within the C-terminus of 14-3-3, or by masking a nuclear import sequence intrinsic to the target protein, in order to decrease the rate of nuclear import of that target; (iii) alteration, by either promotion
or inhibition, of the intrinsic catalytic activity of the target; and (iv) shielding of the target protein, to stop further modifications, for example enzymatic dephosphorylation, from occurring. It is also possible that 14-3-3 molecules can exhibit more than one functionality. For example, with the transcriptional regulator DAF-16, 14-3-3 is able to influence both the localisation and intrinsic DNA binding ability of the target protein [69], [76], [78], [79], [81].

The mode of regulation of 14-3-3 proteins is largely unknown as yet. A number of suggestions have been made, including transcriptional regulation [82], [83], or protein regulation through phosphorylation and dephosphorylation [84], [85], [86], [87]. Further investigations, however, will be required before more precise mechanisms can be elucidated.

1.4: Cell Cycle Checkpoints

Successful progression through the cell cycle is dependent on an ordered sequence of events, coordinated and regulated, in part, in eukaryotic cells, by cyclin-dependent kinase enzymes using reversible phosphorylation of effector molecules and proteins [11], [31]. This progression occurs in order to duplicate the organism’s genome and faithfully transmit that genome to the daughter cells [14]. In itself, however, this raises a fundamental question: how can it be assured that one set of events has been completed in its entirety before moving on to the next?

To ensure that fundamental events in the cell cycle both occur and occur properly, surveillance mechanisms in the form of cell cycle-associated checkpoints have evolved [11], [14]. In essence, cell cycle checkpoints can be defined as “regulatory pathways that control the orderly and timely succession of the cell cycle transitions and ensure that DNA replication and chromosome segregation are completed with high fidelity” [10]. These checkpoints monitor the genetic information to ensure that it is completely duplicated or, in the case of damage, that it is repaired correctly, and are thus able to control cell cycle progression [14]. In order for such a checkpoint to function, the transduction system must be able to accomplish three tasks: firstly, it must sense the failure of a particular event; secondly, it must be able to generate, amplify and transmit an appropriate signal to arrest the cell and
initiate an appropriate response to the failure; and thirdly, it must be able to inhibit the
downstream events that would occur later in the cell cycle [13]. Eukaryotic cells contain two
major checkpoints. The first occurs at the transition from G\textsubscript{1} to S phase. This checkpoint
ensures nutrient availability, correct cell size and the availability of all substrates required for
the S, G\textsubscript{2} and M phases. Should this checkpoint be successfully passed, the cell becomes
committed to DNA synthesis, mitosis and cytokinesis [2].

The second checkpoint occurs at the transition from G\textsubscript{2} to mitosis. Like the G\textsubscript{1}/S checkpoint,
this checkpoint ensures nutrient and substrate availability as well as correct cell size but
unlike the G\textsubscript{1}/S barrier, this latter checkpoint is also responsible for ensuring the genetic
stability of the cell prior to entering mitosis. Genetic instability is known to be one of the key
properties of malignantly transformed cells. While the genetic instability, itself, does not
contribute to a malignant phenotype, it acts as a powerful engine, to drive the activation and
inactivation of various oncogenes, ultimately leading to the malignant phenotype. Thanks to
recent advances in cell cycle research, it is thought that the genetic instability of at least some
cancer cells is thought to be a defect in the regulation of cell cycle checkpoints [2], [14]. The
G\textsubscript{2}/M checkpoint forms the basis for this research.

### 1.4.1: G\textsubscript{2}/M Checkpoint

During G\textsubscript{2}, the CDK1/cyclin B1 complex is held in an inactive state by phosphorylation of
the CDK1 residues Tyr-15 and Thr-14, a process predominantly carried out by the kinase
wee1. On the cusp of mitotic onset, the dual tyrosine and threonine phosphatases CDC25B
and CDC25C co-operatively dephosphorylate both Tyr-15 and Thr-14, resulting in rapid
activation of the CDK1/cyclin B complex, and hence the onset of mitosis [14].

Should cellular DNA become damaged or error-prone, the G\textsubscript{2}/M checkpoint becomes
activated, inducing a signal transduction pathway that functions to halt the progression of the
cell cycle until DNA repairs can be undertaken (Figure 1.8). If the checkpoint fails to
activate, cells progress through the cycle prior to the completion of the repairs, resulting in an
irreversible alteration to the genome that can have far reaching effects on co-ordinated cell
growth and development, as well as cell viability [16]. The molecular mechanisms underlying the detection of DNA damage, and the response to checkpoint arrest, are not well understood [14]; what is known is that maintenance of the inhibitory phosphorylation of the Tyr-15 residue of CDK1 inhibits the onset of mitosis, and hence maintains the G2/M checkpoint. In vertebrates, there is an additional control over the CDK1/cyclin B1 complex, namely through its interactions with 14-3-3 proteins and its resulting export to the cytoplasm [16].

Figure 1.8: Diagram of the G2/M (DNA damage) checkpoint. DNA damage ( ) is sensed through molecules such as the ATM/ATR kinases. In turn, these proteins activate (shown as ) a number of signalling molecules, such as wee1 or GADD45. These proteins aim to achieve and maintain inhibitory phosphorylations present on CDK1, and thus inhibit the cyclin B1/CDK1 complex (shown as ——). This results in the maintenance of the G2/M (DNA damage) checkpoint.

The first step in the induced cascade is the phosphorylation of Chk1 or Chk2 by molecules such as the ATM or ATR kinases. The activated Chk1/2 proteins are then able to directly phosphorylate, and therefore inactivate, the CDC25 phosphatases. In this inactive state, the CDC25 molecules (either the B or C isoforms) are bound by their 14-3-3 binding partners and the entire complex is exported from the nucleus. By this mechanism, the CDC25 phosphatases are not only inactivated through the addition of a phosphate group, but their
sub-cellular localisation is changed to physically segregate them from their substrate, the partially active CDK1/cyclin B1 complex [63], [72], [88], [89], [90], [91].

Overall, this ensures maintenance of the inhibitory phosphorylation of CDK1 residue Tyr-15, and hence effects a G₂ arrest [63], [72], [88], [89], [90], [91]. Wee1 has also been suggested as a phosphorylation target of Chk1, since hyper-phosphorylation of wee1 contributes to a delay in entry to mitosis [16]. It has thus been suggested that the G₂/M checkpoint uses a “double lock” mechanism to promote the cell cycle delay [13], [16], [32], [91].

The second step in the induced cascade elicits a much slower response. During this part of the cascade, p53 is phosphorylated by DNA-dependent protein kinase (DNA-PK), allowing it to dissociate from its binding partner MDM2, [90], [92], [93], [94]. This dissociation effectively activates the intrinsic DNA binding activity of p53. This ability is then further activated through acetylation by p300/PCAF, [90], [91], [92], [93], [94]. The genes that are transcriptionally activated by p53 constitute effectors of this second cascade. They include 14-3-3 regulatory molecules, which bind to the phosphorylated CDK1/cyclin B1 complex and promote export of the complex from the nucleus. GADD45 is also up-regulated, and is thought to be able to interact with and dissociate the CDK1/cyclin B1 complex [8], [17], [91].

In addition, p21Cip1 is up-regulated. This is an inhibitor of a subset of the cyclin-dependent kinases, which includes CDK1 [90], [91], [92], [93], [94]. Once this delay in progression is initiated, the cell is able to undertake repairs on the DNA that is error prone or damaged. Sensing of DNA damage is mediated by Chk1, as well as via the effectors ATM and ATR [90], [91], [92], [93].

1.5: Research Aims

The overall aim of this research was to further elucidate the molecular mechanisms underlying the control and progression of the eukaryotic cell cycle: this is a very tightly regulated biological pathway that is fundamental to eukaryotic growth and development.
The specific aim of this research was the biophysical and biochemical characterisation of the molecular complexes that form as part of the regulation of the G2/M checkpoint, in order to either promote or inhibit the onset of mitosis in eukaryotic cells. In particular, this aim included:

- expression and purification of CDK1 and cyclin B1, as well as preparation and characterisation of the complex they form;
- expression, purification and characterisation of the wee1/14-3-3β complex;
- expression and purification of one or more of the isoforms of CDC25, and characterisation of the complex it forms with 14-3-3;
- crystallisation and structural analysis of one or more of these complexes.

Overall, these goals proved to be very challenging, and thus, the main focus of the research became the wee1/14-3-3β complex.
2.1: General Methods and Materials

2.1.1: Bacterial Cell Growth

Unless otherwise stated, the bacterial cells used were *Escherichia coli* DH5α cells (Invitrogen Catalogue Number 18265-017). Cells were grown either on a Luria Broth/agar plate or as a culture in Luria Broth. Luria Broth was made up of 1% *w/v* bactopeptone, 0.5% *w/v* bactoyeast extract, 1% *w/v* NaCl. This was autoclaved for sterilization and stored at room temperature. Unless otherwise stated, once set up, all cultures were incubated with rotation at 180 rpm for 12 - 18 hours at 37 °C. Luria Broth/agar was made up of 1% *w/v* bactopeptone, 0.5% *w/v* bactoyeast extract, 1% *w/v* NaCl, 2% *w/v* agar. This was then autoclaved for sterilization. It was cooled to 50 °C, and allowed to set. The plates were then stored at 4 °C, until required. The bacterial cells were spread on to the agar plate using either a sterile glass spreader, or a sterile metal loop. The plates were then incubated for 12 - 18 hours at 37 °C.

SOC medium was made up of 2% *w/v* bactotryptone, 0.5% *w/v* bactoyeast extract, 0.5% *w/v* NaCl, 2.5 mM KCl, adjusted to pH 7, to a final volume of 97.5%. This was then autoclaved for sterilization. Just prior to use, the medium was then supplemented with 10 mM MgCl₂ and 20 mM glucose (final concentrations) to a final volume of 100%.

2.1.2: Bacterial Cell Transformation

Transformation was carried out using either electro-competent or chemically-competent cells.
Electro-competent cells were prepared using the following protocol:

- A single colony was picked from the appropriate Luria Broth/agar plate, and a 50 mL culture of the cells was grown. When the optical density at 600 nm of the culture reached 0.5 to 0.7 (mid-log phase of bacterial cell growth), the cells were centrifuged at 4,000 x g for 15 minutes at 4 °C.

- The supernatant was discarded and the pellet was re-suspended in 50 mL ice cold 10% v/v glycerol. The cells were centrifuged again (same conditions as above); the supernatant was discarded and the pellet re-suspended in 25 mL ice cold 10% v/v glycerol.

- The cells were centrifuged again (same conditions as above); the supernatant was discarded and the pellet re-suspended in 10 mL ice cold 10% v/v glycerol.

- The cells were centrifuged again (same conditions as above); the supernatant was discarded and the pellet re-suspended in 2 mL ice cold 10% v/v glycerol.

- The cell concentration was calculated to be 2 x 10^8 cells mL^{-1}. Aliquots of 50 µL of the cell suspension were then flash frozen in liquid nitrogen, and stored at -80 °C, until required.

Electro-transformation was undertaken using the following protocol:

- 3 µL of the isolated DNA was mixed with 50 µL of electro-competent cells. This cell suspension was then transferred to a 0.2 cm electroporation cuvette (Biorad).

- The cell suspension was then electroporated according to manufacturer’s instruction using an electroporation machine (Biorad).

- Immediately following electroporation, 950 µL of Luria Broth was added to the sample, which was incubated a further 60 minutes with end-over-end rotation at 180 rpm at 37 °C.

- The cell mix was then plated on a Luria Broth/agar plate containing one or more antibiotics to select for transformants.
Chemically-competent cells were prepared by the following method:

- A single colony was picked from the appropriate Luria Broth/agar plate, and a 50 mL culture of the cells was grown. When the optical density at 600 nm of the culture reached 0.5 to 0.7 (mid-log phase of bacterial cell growth), the cells were incubated for 30 - 60 minutes at 4 °C.

- Following incubation, the cells were centrifuged at 4,000 x g for 15 minutes at 4 °C.

- The supernatant was discarded and the pellet was re-suspended in 5 mL of 10% w/v polyethylene glycol 6,000, 10 mM MgSO₄, 10 mM MgCl₂, 5% v/v DMSO in Luria Broth.

- Aliquots of 300 µL of the cell suspension were then flash frozen in liquid nitrogen, and stored -80 °C.

Chemical transformation was undertaken using the following method:

- 5 µL of the isolated vector was incubated with 100 µL of chemically competent cells for 30 minutes at 4 °C.

- The cell mix was then incubated for 45 seconds at 42 °C, followed by recovery on ice for 2 minutes.

- 885 µL of Luria Broth was then added to the sample, which was incubated a further 60 minutes with end-over-end rotation at 130 rpm at 37 °C.

- The cell mix was then plated on a Luria Broth/agar plate containing one or more antibiotics to select for transformants.

**2.1.3: Vector Isolation and DNA Preparation**

Unless otherwise specified, preparation of plasmid DNA was carried out using the QiaFilter Plasmid Midi Kit (Qiagen). Preparation of PCR products was carried out using High Pure PCR Product Purification Kit (Roche). The QiaFilter Kit (Qiagen) lyases bacterial cells using a modified alkaline lysis procedure. The plasmid DNA is then bound to an anion exchange resin under low salt and pH conditions, and is eluted from the column using a high salt
buffer. Isopropanol precipitation is used to simultaneously concentrate the sample and
discard the high concentration of salt. Purification was performed according to
manufacturer's instructions.

The High Pure PCR Purification Kit (Roche) adjusts the buffer to be neutral, with a high salt
concentration, to allow binding to the silica resin of the column. The purified DNA is eluted
from the column using a low salt buffer. Purification was undertaken according to the
manufacturer's instructions.

**2.1.4: DNA Gel Electrophoresis**

50 x TAE buffer was made by mixing 242 g Tris base, 57.1 g glacial acetic acid, 100 ml 0.5
M di-Na\(^+\) EDTA with sterile, deionised water to 1 L. For DNA gel electrophoresis, a 1% w/v
gel was made up by dissolving 1 g of agarose powder in 1 x TAE buffer. This was poured
into a gel mould (Biorad), and left until almost cool to the touch, but still a liquid. An aliquot
of 2.5 µL of 10 mg mL\(^{-1}\) ethidium bromide was then mixed in, and the gel was left at room
temperature until set. Prior to loading, the sample was mixed with 1 x DNA loading dye,
where 6 x DNA loading dye is composed of 0.25% \(v/v\) bromophenol blue, 0.25% \(v/v\) xylene
cyanol FF, 15% ficoll type 4,000, 120 mM di-Na\(^+\) EDTA.

The gel, still within the mould, was submerged in a running buffer of 1 x TAE buffer and the
sample (with dye) was loaded into the wells. An electric current of 200 mA with voltage of
90 mV was then run across the gel until the dye front reached three-quarters of the way down
the gel. The resulting DNA bands were then visualized using a UV trans-illuminator.

DNA concentration was calculated according to manufacturer’s instruction, by measuring the
absorbance of the sample at 260 nm using the Nanodrop spectrophotometer N1000 (BD
Biosciences).
2.1.5: RNA Gel Electrophoresis

50 x TBE buffer was made by mixing 242 g Tris base, 275 g boric acid, 100 ml 0.5 M di-Na\(^+\) EDTA with sterile, deionised water to 1 L. For RNA gel electrophoresis, a 1\% w/v gel was made up by dissolving 1 g of agarose powder in 1 x TBE buffer. This was poured into a gel mould (Biorad), and left until almost cool to the touch, but still a liquid. An aliquot of 2.5 µL of 10 mg mL\(^{-1}\) ethidium bromide was then mixed in, and the gel was left at room temperature until set. Prior to loading, the sample was mixed with 1 x DNA loading dye (refer to Section 2.1.4 for specific composition). The gel, still within the mould, was submerged in a running buffer of 1 x TBE buffer and the sample (with dye) was loaded into the wells. An electric current of 200 mA with voltage of 90 mV was then run across the gel until the dye front reached three-quarters of the way down the gel. The resulting RNA bands were then visualized using a UV trans-illuminator.

Where appropriate, RNA concentration was calculated according to manufacturer’s instructions, by measuring the absorbance of the sample at 260 nm using the Nanodrop spectrophotometer N1000 (BD Biosciences).

2.1.6: Expression Vectors Used

Unless otherwise specified, the term “CDK1” refers to CDK1, protein isoform 1, NCBI accession number NP_001777. The DNA encoding the protein CDK1 was originally cloned into the vector pPROEx HTb. A map of this vector is provided in Figure 2.1. Expression from this vector results in a recombinant protein with a polyhistidine label fused to the N-terminus of the protein. The DNA in question was then sub-cloned into the vector pFASTBac DUAL, under the control of the p10 promoter. A map of this vector is presented in Figure 2.2. Expression from this vector under this promoter results in the expression of a native protein (no purification tag is present).
Figure 2.1: pPROEx HTb vector map. The vector contains an fl origin of replication, a trc promoter, a pUC origin and ampicillin resistance. Expression using this vector results in a recombinant protein with an N-terminal polyhistidine tag, which is potentially removable through the TEV cleavage site. This vector map was taken from the Bac-to-Bac Baculovirus Expression System Instruction Manual (Invitrogen).

Figure 2.2: pFASTBac DUAL vector map. The vector contains two multiple cloning sites. The first is under the control of the polyhedrin promoter, and the second is under the control of the p10 promoter. Expression from either of these sites results in the production of a native protein (no polyhistidine tag). This vector map was taken from the Bac-to-Bac Baculovirus Expression System Instruction Manual (Invitrogen).

Unless otherwise specified, the term “cyclin B1” refers to the cyclin protein isoform B1, NCBI accession number NP_114172. The coding sequence for cyclin B1 was cloned into the vector pFASTBac HTb. A map of this vector is provided in Figure 2.3. Unless otherwise
specified, the term “CDC25B” refers to the CDC25B phosphatase, protein isoform B3, NCBI accession number NP_068659. The pFASTBac HTb-cdc25b clone was provided by Dr. James Dickson. Expression from this vector results in the production of a recombinant protein with a polyhistidine label fused to the N-terminus of the protein.

Figure 2.3: pFASTBac HTb vector map. The vector contains an origin of replication, a polyhedrin promoter and ampicillin resistance. Expression using this vector results in a recombinant protein with an N-terminal polyhistidine tag, which is potentially removable through the TEV cleavage site. This vector map was taken from the Bac-to-Bac Baculovirus Expression System Instruction Manual (Invitrogen).

Unless otherwise specified, the term “wee1” refers to the wee1 tyrosine kinase, NCBI accession number NP_003381. Unless otherwise specified, the term “14-3-3β” refers to the 14-3-3 protein, β polypeptide, NCBI accession number NP_003395. The coding sequences for wee1, a truncated form of wee1 (covering residues 291 – 646 of wee1, Section 2.5.7) and 14-3-3β were provided by Dr. James Dickson having been cloned into the pFASTBac DUAL vector, to enable co-expression of either full length or truncated wee1 with 14-3-3β. These DNA coding sequences for wee1 and truncated wee1 were originally cloned into pFASTBac HTb, then sub-cloned into pFASTBac DUAL, such that the N-terminal polyhistidine tag was also sub-cloned along with the DNA encoding the full length or truncated wee1, under the control of the polyhedrin promoter. 14-3-3β was sub-cloned to be under the control of the p10 promoter. This same cloning rationale was also used for the co-expression of CDC25B with 14-3-3β, whereby CDC25B was sub-cloned from pFASTBac HTb to a pFASTBac
DUAL construct (containing 14-3-3β) such that the N-terminal polyhistidine tag was also sub-cloned along with the DNA encoding CDC25B.

2.1.7: Sf9 Cell Expression

Unless otherwise specified, the term 'Sf9 cells' refers to the ovarian cell line isolated from Spodoptera frugiperda, ATCC Cell Line Number CRL-1711. Uninfected Sf9 cells were maintained at a titer of 1 x 10^6 to 10 x 10^6 cells mL⁻¹, grown in SF-900II medium (Invitrogen). Unless otherwise specified, Sf9 cell cultures are grown with shaking at 130 rpm at 28 °C. Sterile conditions were maintained by undertaking all culturing and sub-culturing work in a Class II Cell Culture Hood. Unless specified otherwise, Sf9 whole cell lysate was prepared by collecting a sample of uninfected mid log phase Sf9 cells. The cells were harvested by centrifugation at 500 x g for 5 minutes at 4 °C. The supernatant was discarded and the pellet was re-suspended in 50 mM Tris.HCl pH 7.5, 0.1% v/v NP40 in such a volume as to be representative of a cell count of 12 x 10^6 cells mL⁻¹.

The sample was incubated on ice for 5 minutes, and then centrifuged at 500 x g for 5 minutes at 4 °C. The supernatant was saved, and the pellet was re-suspended in the same volume as above of 50 mM Tris.HCl pH 7.5, 300 mM NaCl, 0.1% v/v NP40. The sample was incubated on ice for an additional 5 minutes, and then re-centrifuged at 500 x g for 5 minutes at 4 °C. The pellet was discarded and the supernatant was then mixed with the supernatant from the previous step, thus forming the Sf9 whole cell lysate, at a final volume that was representative of a culture at 6 x 10^6 cells mL⁻¹.

2.1.8: Generation of Recombinant Baculovirus

Recombinant baculovirus was generated using three steps, namely transformation into E. coli DH10BAC cells, isolation of the recombinant bacmid, followed by transfection of the bacmid into Sf9 cells. Working virus stocks were then generated by viral amplification.
2.1.8.1: Transformation into E. coli DH10 BAC cells

The plasmid containing the DNA of interest was first transformed into chemically competent E. coli DH10 BAC cells (Section 2.1.2). An aliquot of 900 µL of SOC medium (Section 2.1.1) was added to the sample, which was then transferred to a round-bottom polypropylene tube and incubated for a further 4 hours with end-over-end rotation at 130 rpm at 37 °C. Following this incubation, a 200 µL aliquot and the remaining 800 µL were separately plated on to Luria Broth/agar plates containing 50 µg mL\(^{-1}\) kanamycin, 7 µg mL\(^{-1}\) gentamycin, 10 µg mL\(^{-1}\) tetracycline, 100 µg mL\(^{-1}\) X-Gal and 40 µg mL\(^{-1}\) IPTG (Section 2.1.1). The plates were then incubated for 48 hours at 37 °C.

A fresh Luria Broth/agar plate containing 50 µg mL\(^{-1}\) kanamycin, 7 µg mL\(^{-1}\) gentamycin, 10 µg mL\(^{-1}\) tetracycline, 100 µg mL\(^{-1}\) X-Gal and 40 µg mL\(^{-1}\) IPTG (Section 2.1.1) was divided into quadrants. From the original two plates, one blue colony and three white colonies were picked and streaked on to the fresh plate, one colony per quadrant. This new plate was then incubated for a further 48 hours at 37 °C, to confirm the colony phenotype.

2.1.8.2: Isolation of Recombinant Bacmid

During this second step for generating a recombinant baculovirus, an E. coli DH10 BAC colony containing the recombinant bacmid encoding the DNA sequence(s) of interest was grown as a culture in Luria Broth, and was then used to isolate the bacmid.

An isolated white colony was picked from the second plate, and was grown as a 5 mL culture in Luria Broth containing 50 µg mL\(^{-1}\) kanamycin, 7 µg mL\(^{-1}\) gentamycin, 10 µg mL\(^{-1}\) tetracycline by incubation for 12 - 15 hours with rotation at 180 rpm at 37 °C. A 3 mL sample of the culture was centrifuged at 13,000 rpm (16,000 x g) in a bench-top centrifuge for 1 min at 4 °C. The supernatant was discarded and the pellet was re-suspended in 300 µL of 15 mM Tris.HCl pH 8.0, 10 mM EDTA, 100 µg mL\(^{-1}\) RNase A. Cells were lysed by the addition of 300 µL of 0.2 M NaOH, 1% w/v SDS with incubation for 5 minutes at 18 ± 2 °C.
An aliquot of 300 µL of 3 M potassium acetate pH 5.5 was then slowly added, and the sample was incubated at 4 °C for 10 minutes. The sample was then centrifuged at 13,000 rpm (16,000 x g) in a bench-top centrifuge for 10 minutes at 4 °C.

Following centrifugation, the pellet was discarded and the supernatant (containing the bacmid) was mixed with 800 µL of isopropyl alcohol, and incubated for 10 minutes at 4 °C. The sample was then centrifuged at 13,000 rpm (16,000 x g) in a bench-top centrifuge for 20 minutes at 4 °C. The supernatant was then discarded and 200 µL of 70% v/v absolute ethanol was added, being careful not to disturb the pellet. The sample was then centrifuged at 13,000 rpm (16,000 x g) in a bench-top centrifuge for 5 minutes at 4 °C, the supernatant discarded, and the pellet allowed to air dry for 5 minutes. Once dry, the pellet was re-suspended in 20 µL of 10 mM Tris.HCl pH 8.0, 1 mM EDTA. A 1 µL aliquot of the sample was loaded on to the Nanodrop Spectrophotometer N1000 (BD Biosciences) according to manufacturer’s directions, and the concentration of DNA in the sample was calculated.

2.1.8.3: Transfection of Recombinant Bacmid

During this third step in the generation of recombinant virus, the isolated and purified recombinant bacmid was used to transfect a culture of Sf9 cells, to produce infectious baculovirus containing the coding sequences for the protein of interest.

A culture of mid-log phase Sf9 cells at 0.5 x 10⁶ cells mL⁻¹ was set up, of which 2 mL aliquots were transferred to 3 wells of a six-well cell culture plate (Nunclon). The plate was then incubated for 1 hour at 28 °C, to allow cell attachment to take place. A sample of 2 µg of isolated recombinant bacmid was mixed with SF-900II medium (Invitrogen) to give a sample with a final volume of 100 µL. Separately, 20 µL of GeneJuice (Novagen) was mixed with 180 µL of SF-900II medium (Invitrogen). A 100 µL aliquot of the GeneJuice (Novagen) sample was then transferred to the sample containing the recombinant bacmid, and 100 µL of SF-900II medium (Invitrogen) was transferred to the remaining GeneJuice (Novagen) sample. Overall, this resulted in two 200 µL samples. The two samples were
incubated for 30 minutes at 18 ± 2 °C, following which, 800 µL of SF-900II medium (Invitrogen) was added to each.

On the six-well culture plate, excess medium and unattached cells were aspirated off the culture. The 1 mL GeneJuice (Novagen) sample was gently transferred to one well of Sf9 cells. The 1 mL recombinant bacmid/GeneJuice (Novagen) sample was gently transferred to the second well of Sf9 cells, and 1 mL of SF900-II medium (Invitrogen) was then transferred to the third well, following which the plate was incubated for 4 hours at 28 °C. Following incubation, the transfection mixture was gently removed from each well. An aliquot of 2 mL of SF-900II medium (Invitrogen) was transferred to each well, and the plate was incubated for a further 72 to 96 hours at 28 °C. Following incubation, the supernatant from the well containing the recombinant bacmid was carefully aspirated, and was clarified by centrifugation at 500 x g for 10 minutes at 4 °C. The pellet was discarded and the supernatant was saved as the transfection inoculum.

2.1.8.4: Amplification of Transfected Virus

Once transfected, the virus was then amplified by mixing 1.5 mL of mid-log phase Sf9 cells at 0.5 x 10^6 cells mL^-1 with 0.5 mL of transfection inoculum, then transferred into one well of a six- well cell culture dish (Nunclon). Separately, an additional 1.5 mL of mid-log phase Sf9 cells at 0.5 x 10^6 cells mL^-1 were mixed with 0.5 mL of SF-900II medium (Invitrogen) and transferred into a second well of the same six-well cell culture dish (Nunclon). The plate was then incubated for 48 hours at 28 °C. Following incubation, the supernatant from the well that contained the transfection inoculum was carefully aspirated. This sample was clarified by centrifugation at 500 x g for 5 minutes at 4 °C. The pellet was discarded and the supernatant was saved as the primary inoculum. Unless otherwise indicated, all viral samples were supplemented with 2% v/v Fetal Bovine Serum (FBS), and stored, protected from light, at 4 °C.

A 100 mL Erlenmeyer flask was used to set up a 10 mL culture of mid log phase Sf9 cells at a titer of 1 x 10^6 cells mL^-1. A 2 µL sample of primary inoculum was added to this culture,
which was then incubated for 48 hours with shaking at 130 rpm at 28 °C. Following incubation, the culture was harvested by centrifugation at 500 x g for 5 minutes at 4 °C. The pellet was discarded, and the supernatant was saved as the secondary inoculum. A 100 mL Erlenmeyer flask was used to set up a 10 mL culture of mid log phase Sf9 cells at a titer of 1 x 10^6 cells mL^{-1}. A 2 µL sample of secondary inoculum was added to this culture, which was then incubated for 48 hours with shaking at 130 rpm at 28 °C. Following incubation, the culture was harvested by centrifugation at 500 x g for 5 minutes at 4 °C. The pellet was discarded, and the supernatant was saved as the tertiary inoculum.

2.1.9: SDS Polyacrylamide Gel Electrophoresis (SDS PAGE)

SDS polyacrylamide gel electrophoresis (SDS PAGE) was carried out using 15 x 17 cm backing plates and 16 x 17 cm glass plates separated by 0.75 mm spacers (BioRad). Unless otherwise indicated, resolving gels were composed of 22.2% acrylamide/0.6% bisacrylamide at a final concentration 12% acrylamide, 0.375 M Tris.HCl pH 8.8, 0.1% w/v SDS, 0.05% w/v ammonium persulfate and 0.0005% v/v TEMED. Stacking gels were composed of 0.125 M Tris.HCl pH 6.8, 0.1% w/v SDS, 0.05% w/v ammonium persulfate and 0.0005% v/v TEMED. The sample to be run on the gel was mixed with 4 x Quench dye (0.5 M Tris.HCl pH 6.8, 20% v/v glycerol, 10% w/v SDS, 10% v/v β-mercaptopoethanol (β-ME) and 1% w/v bromophenol blue), at an appropriate volume to give 1 x Quench dye.

The sample containing 1 x Quench was heated for 2 minutes at 95 °C. This sample was then loaded on to the gel, which was subsequently immersed in a running buffer of 0.025 M Tris, 0.192 M glycine, 1% w/v SDS. A voltage of 200 V with a current of 20 mA was passed through the gel until the dye front reached the bottom. The gel was then stained in a solution of 10% v/v ethanol, 10% v/v glacial acetic acid, 0.003% v/v coomassie blue, and subsequently de-stained in a solution of 40% v/v methanol, 10% v/v glacial acetic acid. Where appropriate, the gels were dried by being placed between two moistened sheets of cellophane, within a perspex frame and left to dry for 48 - 72 hours. Where appropriate, SDS PAGE analysis was repeated to optimise the amount of protein loaded on each gel. Where this occurred, it was ensured that the gel chosen to represent an overall result was entirely equivalent to each of the repetitions.
2.1.10: Native Polyacrylamide Gel Electrophoresis (Native-PAGE)

Native polyacrylamide gel electrophoresis was undertaken following the same protocol as for SDS PAGE, except that no SDS was added to the stacking or separating gels, the dye for the sample or the running buffer. Staining and de-staining was carried out by the same method as detailed in Section 2.1.9.

2.1.11: Immobilized Metal Affinity Chromatography (IMAC)

A 5 mL HP Chelating Column (Amersham Bioscience) was washed with 5 x column volumes of sterile deionised water. An aliquot of 3 mL of 100 mM NiCl₂ was then loaded onto the column, and washed through with a further 5 x column volumes of sterile deionised water. The column was then equilibrated by washing in 5 x column volumes of loading buffer. The sample of interest was then loaded onto the column, which was further washed with 2 – 5 x column volumes of loading buffer. The proteins that did not bind were collected as flow through. The column was then washed with wash buffer, which contained a low concentration of imidazole, to wash away as many of the contaminating proteins as possible, and the resulting fractions were collected.

The protein(s) of interest were then eluted from the column by washing in elution buffer, and the resulting fractions were collected. Where appropriate, imidazole concentrations were varied, to optimise the elution profile of the protein in question. Such optimisation included stepwise increases in imidazole concentration and/or the use of a concentration gradient to determine the optimum window of protein elution. Following elution, all fractions were analysed by SDS PAGE, to determine which fractions contained the protein(s) of interest.

2.1.12: Size Exclusion Chromatography

Size exclusion chromatography was undertaken using either one of two different columns, the first an S200 10/300 size exclusion column (Amersham Bioscience) and the second an
S200 16/600 size exclusion column (Amersham Bioscience). For each protein that was subjected to size exclusion chromatography during this research, both columns were trialled, in order to optimise preparation of the protein(s). Fractions containing the protein(s) of interest were found by SDS PAGE analysis of all fractions, followed by a more focussed analysis on those that contained the samples.

2.1.12.1: Preparation Grade Size Exclusion Chromatography

Size exclusion chromatography was undertaken using either an S200 10/300 size exclusion column (Amersham Bioscience) or an S200 16/600 size exclusion column (Amersham Bioscience). Regardless of which one of these two columns was used, the overall protocol was identical. The size exclusion column was washed with 2 x column volumes of sterile deionised water. The column was then equilibrated by washing in 2 x column volumes of sample buffer. The sample of interest was then loaded on to the column and the resulting fractions were collected.

2.1.12.2: Analytical Size Exclusion Chromatography

Analytical size exclusion chromatography was undertaken to determine the apparent molecular weight of a species in solution. An S200 10/300 size exclusion column (Amersham Bioscience) was washed with 3 x column volumes of sterile deionised water, then equilibrated in 3 x column volumes of 20 mM Tris.HCl pH 7.5, 50 mM NaCl. A sample of 100 µL of 1 mg mL⁻¹ Blue Dextran 2,000 (GE Healthcare) was then loaded on to the column, and the resulting UV trace was followed. The column was then washed with 1 x column volume of 20 mM Tris.HCl pH 7.5, 50 mM NaCl. A 100 µL sample of a solution containing 0.3 mg mL⁻¹ ferritin, 3 mg mL⁻¹ conalbumin, 3 mg mL⁻¹ carbonic anhydrase and 3 mg mL⁻¹ ribonuclease A (High Molecular Weight Standards, Mix A, GE Healthcare) was then loaded on the column, and the resulting UV trace was followed.
The following equation was then used to determine the $K_{av}$ of each of these standards:

$$K_{av} = \frac{V_e - V_o}{V_c - V_o}$$

Where:
- $V_e$ = elution volume for the respective standard
- $V_o$ = void volume for the column
- $V_c$ = total volume for the column

This information was then used to plot the $K_{av}$ v. Log [molecular weight of the species], and an equation for the resulting graph was determined. Following the analysis of the standards, the sample of interest was loaded on to the column, and was also analyzed using size exclusion chromatography, using this same protocol. The elution volume of this species was used to determine the $K_{av}$, and with it the apparent molecular weight of the species of interest.

2.1.13: Expression and Purification of rTEV Protease

Using a sterile pipette tip, a single colony from the plate containing a clone of rTEV protease in *E. coli* BL21(DE3)pARG cells was picked, and was grown up as a 5 mL Luria Broth culture containing 100 $\mu$g mL$^{-1}$ ampicillin and 25 $\mu$g mL$^{-1}$ kanamycin by incubation with rotation at 180 rpm for 12 – 18 hours at 37 °C (Section 2.1.1). This starter culture was then used to seed a 100 mL Luria Broth culture containing 100 $\mu$g mL$^{-1}$ ampicillin and 25 $\mu$g mL$^{-1}$ kanamycin (refer to Section 2.1.1). This latter culture was also incubated with rotation at 180 rpm for 12 – 18 hours at 37 °C. Following this incubation, the cells were centrifuged at 4300 rpm for 20 minutes at 4 °C. The supernatant was discarded. The pellet was re-suspended in 100 mL of Luria Broth culture containing 100 $\mu$g mL$^{-1}$ ampicillin and 25 $\mu$g mL$^{-1}$ kanamycin (Section 2.1.1). IPTG was then added to this culture at a final concentration of 1 mM, and the culture was incubated with rotation at 180 rpm for 5 hours at 37 °C.

Following this incubation, the cells were centrifuged at 4300 rpm for 20 minutes at 4 °C. The supernatant was discarded. The pellet was re-suspended in 10 mL of 25 mM Tris.HCl pH 7.5, 300 mM NaCl, 1 mM MgCl$_2$, 10% v/v glycerol, 0.05% v/v Tween-20, 2 mM $\beta$-ME.
cells were then disrupted according to manufacturer’s instruction using the Cell Disruptor (Constant Cell Disruption Systems) with a pressure setting of 18 kpi. The sample was then centrifuged at 4300 rpm for 30 minutes at 4 °C, the pellet discarded, and the supernatant saved as the whole cell lysate.

The sample was then subjected to IMAC purification, carried out as detailed in Section 2.1.12.1, with a sample buffer of 25 mM Tris.HCl pH 7.5, 300 mM NaCl, 1 mM MgCl₂, 10% v/v glycerol, 0.05% v/v Tween-20, 2 mM β-ME, a wash buffer of 25 mM Tris.HCl pH 7.5, 300 mM NaCl, 1 mM MgCl₂, 10% v/v glycerol, 0.05% v/v Tween-20, 2 mM β-ME, 200 mM imidazole and an elution buffer of 25 mM Tris.HCl pH 7.5, 300 mM NaCl, 1 mM MgCl₂, 10% v/v glycerol, 0.05% v/v Tween-20, 2 mM β-ME, 500 mM imidazole. Immediately upon elution, the fractions containing rTEV protease were mixed in a ratio of 1:1 with 25 mM Tris.HCl pH 7.5, 500 mM NaCl, 1 mM MgCl₂, 10% v/v glycerol, 1 mM β-ME, 0.5 mM EDTA. The sample was then dialyzed against 25 mM Tris.HCl pH 7.5, 500 mM NaCl, 1 mM MgCl₂, 10% v/v glycerol, 1 mM β-ME, 0.5 mM EDTA, as detailed in Section 2.1.15. Following dialysis, the sample was diluted two-fold in 50 mM Tris.HCl pH 7.0, 1 M NaCl, 20% v/v glycerol, 2 mM DTT, 1 mM EDTA, and concentrated to 2 mg mL⁻¹ using a spin concentrator (VivaSpin) with a molecular weight cut-off (MWCO) of 10 kDa. The sample was then diluted two-fold in 100% v/v glycerol, and aliquots of 200 μL of the rTEV sample were flash frozen in liquid nitrogen, or storage at -80 °C.

2.1.14: Ammonium Sulfate Precipitation

All ammonium sulfate precipitations were carried out in a total volume of 100 mL, on a magnetic stirrer, at 4 °C. Where a precipitation was carried out for concentration purposes, ammonium sulfate was slowly added to the sample over a period of 1 hour, and the sample was allowed to equilibrate for 4 hours, with continuous stirring at 4 °C. The sample was then centrifuged at 4300 rpm for 20 minutes at 4 °C, the supernatant discarded, and the pellet re-suspended in an appropriate volume of an appropriate buffer. Where a precipitation was carried out for purification purposes, an initial amount of ammonium sulfate was slowly added to the sample over a period of 1 hour. The sample was then allowed to equilibrate for 1 hour, with continuous stirring at 4 °C. The sample was then centrifuged at 4300 rpm for 20
minutes at 4 °C, the pellet discarded, and the supernatant placed back on a magnetic stirrer at 4 °C. A secondary amount of ammonium sulfate was then slowly added to the sample over a period of 1 hour. The sample was then allowed to equilibrate for 4 hours, with continuous stirring at 4 °C, and centrifuged at 4300 rpm for 20 minutes at 4 °C. The supernatant was then discarded, and the pellet was re-suspended in an appropriate volume of an appropriate buffer.

### 2.1.15: Protein Dialysis

Dialysis was carried out in Spectra/Por membrane dialysis tubing, with a MWCO of 6 - 8 kDa. The tubing was soaked in sterile, deionised water for 15 - 30 minutes, followed by a 10 minute soaking in the dialysis buffer. The other end of the tubing was then sealed with another plastic clip. The tubing containing the sample was then immersed in 1 L of dialysis buffer for 12 - 18 hours at 4 °C with stirring.

### 2.1.16: Protein Concentration

The protein concentration of a sample was determined by measuring the absorbance at 280 nm of 1 µL of the sample in question. This 1 µL was loaded on to a Nanodrop Spectrophotometer N1000 (BD Bioscience) according to manufacturer’s instruction, and the absorbance was measured. The equation to calculate the corresponding concentration is based on Beer’s Law and is as follows:

\[
\text{Concentration (mg mL}^{-1}\text{)} = \frac{M \times A_{280} \times \text{PL}}{E_{280}}
\]

where

- \( M \) = molecular weight of the protein (Da);
- \( A_{280} \) = absorbance of the protein at 280 nm;
- \( E_{280} \) = extinction coefficient of the protein (M\(^{-1}\)cm\(^{-1}\), at 280 nm), calculated using ProtParam (http://www.expasy.org/tools/protparam.html) [95];
- \( \text{PL} \) = path length (1 cm unless otherwise specified).
2.1.17: Dynamic Light Scattering (DLS)

DLS is a biophysical method that can be used to measure the hydrodynamic radius of a protein sample in solution, as well as giving an indication of the polydispersity of that protein sample in solution. Using this tool, a beam of monochromatic light, for example, that of a laser, is directed through a sample. Provided that the wavelength of the incident light is significantly smaller than the size of the particles in the sample, an interaction occurs between the light and the sample, resulting in the scattering of light. The intensity of the observed scattered light will undergo a number of fluctuations, all of which are time dependent, due to the fact that the particles in the submitted sample are undergoing Brownian motion.

The fluctuations in the intensity of light scattered by the molecules may then be analyzed by a photo diode, which sends electrical pulses to the digital signal processor to count the number of photons detected in each successive time sample. The frequency spectrum of this signal is determined by autocorrelation, whereby the similarity between the signal waveform and a slightly time delayed copy of itself is determined by multiplying the two waveforms together and then summing to give the autocorrelation function, $G(\tau)$:

$$G(\tau) = 1 + \alpha e^{-2q^2 D_T \tau}$$

Where:
- $\alpha$ = an instrument constant
- $D_T$ = translational diffusion coefficient
- $Q$ = length of the scattering vector, given by:

$$q = \frac{4\pi n}{\lambda} \sin \left( \frac{\theta}{2} \right)$$

Where:
- $n$ = refractive index of the buffer
- $\lambda$ = wavelength of the incident light, nm
- $\theta$ = scattering angle, °
Under the assumption of Brownian motion and that the molecules in solution are spheres, the Hydrodynamic Radius, $R_H$, can then be calculated using Stokes’ Equation:

$$R_H = \frac{k_b T}{6 \pi \eta D_T}$$

where $k_b$ = Boltzman’s constant  
$T$ = absolute temperature (K)  
$\eta$ = solvent viscosity

The result from this analysis is a range of biophysical data regarding the submitted sample. The data can be analyzed using two distinct methods. The first method is the cumulants analysis [96], whereby the assumption is made that the submitted sample is inherently one underlying population, and is thus reported with a single hydrodynamic radius and a single apparent molecular weight in solution, with an associated dispersity measurement. The cumulants method is the simplest and most robust method for DLS analysis of a protein sample. The second method is a regularization analysis, whereby the assumption is made that the submitted sample contains one or more distinct populations, with an estimate of the hydrodynamic radii of each subpopulation being presented, along with the associated dispersity measurement of each. Apparent molecular weight can thus be calculated using a calculator associated with the DLS software (DynaPro, Protein Solutions), which is based on a correlation between hydrodynamic radius and molecular weight of a globular protein. The regularization method of analysis can be more informative, but is inherently more complex than that of the cumulants analysis, and less well documented.

In this research, two different light scattering instruments were used for data analysis. The first was a DynaPro MSTC (Protein Solutions). The second was a DynaPro M540 Titan (Protein Solutions). As regards data analysis, there is one distinct difference between these two machines, namely that the DynaPro M540 Titan (Protein Solutions) allowed smoothing of the regularization data, whereas the DynaPro MSTC did not allow smoothing of these data. To ensure both equivalency of data, and that the data generated had not been overly smoothed or overly extrapolated, all regularization data presented are in the form of raw data, and have not been smoothed or corrected at all. Both systems, however, define polydispersity as a measure of the homogeneity of the submitted sample, and calculated polydispersity as the standard deviation of each of the $R_H$ measurements from the mean $R_H$ measurement,
expressed as a percentage. As a guideline, both systems recommended that a polydispersity of 15% or less can be considered negligible, a polydispersity of 15 – 30% can be considered moderate, and a polydispersity of 30% or more can be considered significant. An ideal situation would result in negligible polydispersity, as this would suggest that the sample can be considered homogenous, thus allowing insight into the sample as a whole. Indeed, if the sample is being subjected to crystal trials, moderate to negligible polydispersity is a requirement; significant polydispersity would suggest that heterogeneity is too high to allow crystallization to occur.

In this research, a 20 µL sample of the protein of interest (at an initial concentration of 1 mg mL\(^{-1}\)) was centrifuged at 13,000 rpm (16,000 \(x\) g) in a bench top centrifuge for 10 minutes at 4 °C. An aliquot of 12 µL of this sample was then transferred to a quartz cuvette provided by the manufacturer (DynaPro, Protein Solutions), and analysis was undertaken, using the manufacturer’s instructions/software (Dynamics, Version 5). Where necessary, samples were diluted in an appropriate buffer that had first been centrifuged as for the experimental sample.

### 2.1.18: Protein Crystallization

Two different methods were used for protein crystallization experiments, namely the sitting drop and the hanging drop vapor diffusion methods. Using the sitting drop method, crystallization experiments were undertaken using the Cartesian HoneyBee nanoliter dispensing robot (Genome Solutions) or by hand. In both cases, 96-well Intelliplates (Art Robins Instruments) were used for the crystallization experiment, and 85 µL of a solution containing buffer and/or precipitant were placed in the plate well. For experiments conducted with the robot, 0.1 µL of the protein solution was placed in a concave space adjacent to that of the well solution, and this was then mixed with 0.1 µL of the buffer and/or precipitant solution. When the experiments were undertaken by hand, 1 µL (unless otherwise specified) of the protein solution was mixed with 1 µL (unless otherwise specified) of the well solution. In both cases, the plates were the sealed using Intelliplate sealing tape.
Using the hanging drop method, crystallization trials were carried out using 24-well VDX crystallization plates (Hampton Research) with 22 x 22 mm siliconised cover slips (Hampton Research). 700 µL of a well solution containing buffer and/or a precipitant was placed in the well of the VDX crystallization plate. A 1 µL aliquot (unless otherwise specified) of the protein solution was placed on the centre of a siliconised cover slip. This drop was mixed with 1 µL (unless otherwise specified) of the well solution, and the 2 µL drop was suspended above the well. The well and cover slip were then sealed closed using Snow White petroleum jelly (Shell).
2.2: Cyclin B1 Methods and Materials

2.2.1: DNA Amplification using PCR

The coding sequence for cyclin B1 was amplified by the Polymerase Chain Reaction (PCR). A forward primer of 5’-GGCGCCATGGCGCTCCGAGTCACCAGGAACTC-3’ (Invitrogen) and a reverse primer of 5’-CCGCAGCTTTATGCATTACACCTTTGCCACAG-3’ (Invitrogen) were separately re-suspended from lyophilate in TE buffer to each give stock solutions of 1 nmol µL⁻¹. A 1 µL aliquot of this stock solution was then separately diluted in 99 µL of TE buffer to give final working solutions of 10 pmol µL⁻¹. A solution of equal volumes of 100 mM dATP, 100 mM dCTP, 100 mM dGTP and 100 mM dTTP (100 mM dNTP mixture) was also made. A 1 µL sample of Universal Human cDNA (BD Bioscience) was diluted in 9 µL of TE buffer (total volume 10 µL), to be used as amplification template.

The master mixture for this PCR reaction was compiled, and was composed of 2 x Enhancer, 2 x Pfx Polymerase Buffer, 10 mM dNTP mixture, 2 mM MgSO₄, 5 nmol of each primer (forward and reverse), adjusted to 93 µL with sterile deionised water. An aliquot of 3 µL of Pfx Polymerase was then also added. This master mixture was dispensed into five separate samples of 20 µL each, and was run through the following PCR program:

1. 94 °C 1 minute
2. 94 °C 45 seconds
3. 63 ± 5 °C 45 seconds
4. 68 °C 2 minutes, REPEAT STEPS 2 - 4 FOR 5 MORE CYCLES
5. 94 °C 45 seconds
6. 60 ± 6 °C 45 seconds
7. 68 °C 2 minutes, REPEAT STEPS 5 - 7 FOR 5 MORE CYCLES
8. 94 °C 45 seconds
9. 58 ± 6 °C 45 seconds
10. 68 °C 2 minutes, REPEAT STEPS 8 - 10 FOR 5 MORE CYCLES
11. 94 °C 45 seconds
12. 55 ± 10 °C 45 seconds
13. 68 °C 2 minutes, REPEAT STEPS 11 - 13 FOR 15 MORE CYCLES
14. 68 °C 10 minutes
15. 4 °C 10 minutes

END OF PROGRAM
After the reaction was complete, 5 µL of each of the samples was analyzed on a 1% \textit{w/v} agarose gel (refer to Section 2.1.4). The band of approximately the correct size of 1800 base pairs was cut from the gel, and purified according to manufacturer's instructions using the High Pure PCR Product Purification Kit (Roche).

\textbf{2.2.2: Cloning to Construct pFASTBac HTb-\textit{cyclin b1} Clone}

\textbf{2.2.2.1: Restriction Enzyme Digestion of the PCR Product Encoding Cyclin B1}

Following amplification, the PCR product encoding cyclin B1 was digested with the restriction enzymes NcoI (Roche) and HindIII (Roche). Within a 1.7 mL tube (Eppendorf), 30 µL of the purified PCR product was mixed with 5 µL of 10 x SuRECut Buffer H (Roche), 1 µL of NcoI enzyme (10 units, Roche), 1 µL of HindIII enzyme (10 units, Roche) and 13 µL of sterile deionised water. This reaction mixture was then incubated for 2 hours at 37 °C. After the incubation was completed, the sample was analyzed on a 1% \textit{w/v} agarose gel (refer to Section 2.1.4). The band of approximately the correct size of 1800 base pairs was cut from the gel, and purified according to manufacturer's instructions using the High Pure PCR Product Purification Kit (Roche). The resulting DNA sample was quantified using the Nanodrop spectrophotometer N1000 (refer to Section 2.1.4).

\textbf{2.2.2.2: Restriction Enzyme Digestion of pFASTBac HTb}

Laboratory stocks of the vector pFASTBac HTb were transformed into electro-competent \textit{E. coli} DH5α cells (refer to Section 2.1.2), and plated on Luria Broth/agar plates containing 100 µg mL\textsuperscript{-1} ampicillin. A single colony was picked and grown as a 5 mL culture in Luria Broth containing 100 µg mL\textsuperscript{-1} ampicillin for 16 hours with shaking at 220 rpm at 37 °C (Section 2.1.1). All 5 mL of this culture was used to purify the vector pFASTBac HTb according to manufacturer’s instruction using the Mini Plasmid Purification kit (Qiagen).
Within a 1.7 mL tube (Eppendorf), 30 µL of this DNA preparation was mixed with 5 µL of 10 × SuRECut Buffer H (Roche), 1 µL of NcoI enzyme (10 units, Roche), 1 µL of HindIII enzyme (10 units, Roche) and 13 of µL sterile deionised water (total volume of 50 µL), and incubated for 2 hours at 37 °C. As a control, the vector was also digested with each enzyme singly. Within a 1.7 mL tube (Eppendorf), 15 µL of the original DNA preparation was incubated with 2.5 µL of 10 × SureCut Buffer H (Roche), 0.5 µL of NcoI enzyme (5 units, Roche) and 7 µL of sterile deionised water for 2 hours at 37 °C. Within another 1.7 mL tube (Eppendorf), 15 µL of the original DNA preparation was incubated with 2.5 µL of 10 × SureCut Buffer H (Roche), 0.5 µL of HindIII enzyme (5 units, Roche) and 7 µL of sterile deionised water for 2 hours at 37 °C.

Following incubation, all of the samples were analysed on a 1% w/v agarose gel (refer to Section 2.1.4). The band of approximately the correct size of approximately 4900 base pairs, corresponding to that of the doubly-digested vector, was cut from the gel, and purified according to manufacturer's instructions using the High Pure PCR Product Purification Kit (Roche). The resulting DNA sample was quantified using the Nanodrop spectrophotometer N1000 (refer to Section 2.1.4).

2.2.2.3: Ligation of Cyclin B1 PCR Product into pFASTBac HTb

Using the doubly-digested and purified DNA samples, the PCR product encoding cyclin B1 was then cloned into pFASTBac HTb. In a 1.7 mL tube (Eppendorf), 20 ng of PCR product encoding cyclin B1 was incubated with 10 ng of doubly digested pFASTBac HTb, 1 x Ligase buffer (Invitrogen) and 1 µL of Ligase enzyme (5 units, Invitrogen). As controls, two additional reactions were set up. The first control contained equal amounts of identical concentrations of the doubly-digested vector, the Ligase buffer (Invitrogen) and the Ligase enzyme (Invitrogen). The second control contained equal amounts of identical concentrations of the doubly digested vector and the Ligase buffer (Invitrogen) only. All three reactions were incubated for 12 - 18 hours at 18 ± 2 °C.
3 µL of each reaction was then transformed into electro-competent *E. coli* DH5α cells using a MicroPulser (BioRad), according to manufacturer's instructions (refer to Section 2.1.2). Following incubation, the resulting three 1 mL samples of bacterial cells containing the DNA of interest were then centrifuged on a table-top centrifuge for 1 minute at 4 °C, and re-suspended in 200 µL of Luria Broth. This sample was then plated on Luria Broth/agar plates containing 100 µg mL⁻¹ ampicillin (refer to Section 2.1.1) and incubated for 12 - 18 hours at 37 °C. Using a sterile pipette tip, 6 single colonies from the plate containing the experimental reaction transformation mixture and 1 single colony from the Luria Broth/agar plate containing the first control reaction transformation mixture were picked, and each was grown up as a 5 mL Luria Broth culture containing 100 µg mL⁻¹ ampicillin (refer to Section 2.1.1). The plasmid present in each colony was purified according to manufacturer's instruction using the Mini Plasmid Preparation Kit (Qiagen).

In separate 1.7 mL tubes (Eppendorf), 20 µL of each plasmid was incubated with 3 µL of 10 x SureCut Buffer H (Roche), 1 µL NcoI enzyme (10 units, Roche), 1 µL HindIII enzyme (10 units, Roche) and 5 µL sterile deionised water for 2 hours at 37 °C. Following incubation, each sample was analyzed on a 1% w/v agarose gel (refer to Section 2.1.4). For one sample, for which an insert was indicated as being present, confirmation of the pFASTBac HTb-cyclin B1 clone was sought using dideoxy nucleotide sequencing.

### 2.2.3: Generation of Recombinant Baculoviral Encoding Cyclin B1

Recombinant Baculovirus containing the PCR product that encodes cyclin B1 was generated using the protocol detailed in Section 2.1.8. This baculovirus was then amplified, as described in Section 2.1.8.4, to give a working stock of high viral titer.

### 2.2.4: Expression Testing of Cyclin B1

The recombinant baculovirus was next tested for evidence of recombinant protein expression following Sf9 cell infection. A 100 mL Erlenmeyer flask was used to set up a 20 mL culture
of mid-log phase Sf9 cells at 1 x 10^6 cells mL⁻¹. A 2 µL sample of tertiary inoculum was added to the culture, which was then incubated for a total of 96 hours with rotation at 130 rpm at 28 °C. During this incubation, a 1 mL sample of the culture was taken at 48 hours, 72 hours and 96 hours post infection. At 96 hours post infection, the remaining 17 mL of infected culture was centrifuged at 500 x g for 5 minutes at 4 °C. The supernatant was discarded and the pellet was stored at -20 °C, until required.

For each of the above time points, the 1 mL samples were centrifuged at 500 x g for 5 minutes at 4 °C. The supernatants were discarded and each of the pellets was separately re-suspended in 350 µL of 1 x PBS, 1% w/v SDS and incubated for 5-10 minutes at 95 °C. Aliquots of 15 µL of each of these samples was separately mixed with 6 x SDS Quench dye and analyzed by SDS PAGE (refer to Section 2.1.9).

Cyclin B1 was partially purified using IMAC. The pellet from the original expression test (collected at 96 hours post infection, as above) was re-suspended in 2 mL of 50 mM Tris.HCl pH 7.5, 0.1% v/v NP40, and further incubated for 5 minutes on ice. The sample was then centrifuged at 500 x g for 5 minutes at 4 °C. The supernatant was collected and the pellet was re-suspended in 2 mL of 50 mM Tris.HCl pH 7.5, 300 mM NaCl, 0.1% v/v NP40. The sample was then incubated for a further 5 minutes on ice. Following incubation, the sample was centrifuged again at 500 x g for 5 minutes at 4 °C. The pellet was discarded and the supernatant was added to the supernatant from the previous incubation, to give a single 4 mL sample. This was the whole cell lysate containing cyclin B1.

The sample containing cyclin B1 was then purified by IMAC, following the protocol described in Section 2.1.11, using a loading buffer of 50 mM Tris.HCl pH 7.5, 150 mM NaCl, a wash buffer of 50 mM Tris.HCl pH 7.5, 150 mM NaCl, 10 mM imidazole and an elution buffer of buffer of 50 mM Tris.HCl pH 7.5, 150 mM NaCl, 40 mM imidazole. Aliquots of 15 µL of the flow through sample, and each of the eluted samples were then separately mixed with 3 µL of 6 x SDS Quench dye and, together with the whole cell lysate sample, were analyzed by SDS PAGE (refer to Section 2.1.9).
2.2.5: Large Scale Expression of Cyclin B1

A 1,000 mL Erlenmeyer flask was used to set up a 200 mL culture of mid-log phase Sf9 cells at $1 \times 10^6$ cells mL$^{-1}$. To this culture, glucose and L-glutamine were added to a final concentration of 1 mM each. A 20 µL aliquot of tertiary inoculum was added to the culture, which was then incubated for a total of 96 hours with rotation at 130 rpm at 28 °C. Following incubation, the culture was harvested by centrifugation at 500 x g for 5 minutes at 4 °C. Following centrifugation, the supernatant was discarded, and the pellet was stored at -20 °C until required.

2.2.6: IMAC Purification of Cyclin B1

Cyclin B1 was partially purified using IMAC. The pellet from the expression culture of cyclin B1 was re-suspended in 5 mL of 50 mM Tris.HCl pH 7.5, 0.1% v/v NP40, and further incubated for 5 minutes on ice. The suspension was then centrifuged at 500 x g for 5 minutes at 4 °C. The supernatant was collected and the pellet was re-suspended in 5 mL of 50 mM Tris.HCl pH 7.5, 300 mM NaCl, 0.1% v/v NP40. The sample was then incubated for a further 5 minutes on ice, then centrifuged again at 500 x g for 5 minutes at 4 °C. The pellet was discarded and the supernatant was combined with the supernatant from the previous incubation, to give a single 10 mL sample. This was the whole cell lysate containing cyclin B1.

The sample was then purified using IMAC, following the protocol described in Section 2.1.11, using a loading buffer of 50 mM Tris.HCl pH 7.5, 150 mM NaCl, a wash buffer of 50 mM Tris.HCl pH 7.5, 150 mM NaCl, 10 mM imidazole and an elution buffer of buffer of 50 mM Tris.HCl pH 7.5, 150 mM NaCl, 40 mM imidazole. Aliquots of 15 µL of the flow through sample, and each of the eluted samples, were separately mixed with 3 µL of 6 x SDS Quench dye and, together with the whole cell lysate sample, were analyzed by SDS PAGE (refer to Section 2.1.9).
2.2.7: Size Exclusion Chromatography of Cyclin B1

Following IMAC, the sample was dialyzed against the buffer 20 mM Tris.HCl pH 7.5, 50 mM NaCl, following the protocol detailed in Section 2.1.15. Following dialysis, the sample was adjusted to contain 10% v/v glycerol, and was loaded into a spin concentrator with a MWCO of 30 kDa (VivaSpin) and concentrated by centrifugation to a volume of less than 5 mL. Size exclusion chromatography was then used to further purify the sample. An S200 16/600 size exclusion column (Amersham Bioscience) was used for the purification procedure, following the method described in Section 2.1.12.1, using a sample buffer of 20 mM Tris.HCl pH 7.5, 100 mM NaCl. Aliquots of 15 µL from each of the elution fractions that contained cyclin B1 were then separately mixed with 3 µL of 6 x SDS Quench dye, and were analyzed by SDS PAGE (refer to Section 2.1.9).
2.3: **CDK1 Methods and Materials**

2.3.1: **Single Stranded cDNA Library Construction**

A single stranded cDNA library was constructed and was used to isolate the cDNA encoding CDK1, following a method adapted from Chomczynski *et al.*, 1987 [97].

2.3.1.1: **Isolation of RNA**

Whole cell mRNA was isolated from the human lymphoma cell line, HeLa (ATCC Cell Line Number CCL-2). HeLa cells were cultured in Minimal Essential Medium (MEM, Invitrogen) until confluent (estimated cell titer of between 3 x 10⁶ and 5 x 10⁹ cells in total). The culture was centrifuged for 10 minutes at 1,000 x \(\text{g} \) at 4 °C. The supernatant was removed and discarded, and the pellet was gently re-suspended in 10 mL of ice cold PBS. The cell solution was re-centrifuged for 10 minutes at 1,000 x \(\text{g} \) at 4 °C, and the supernatant was removed and discarded. The pellet was re-suspended in 1 mL GITC extraction buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% \(v/v\) sodium lauroyl sarcosine, 0.1 M \(\beta\)-mercaptoethanol), and incubated for 24 hours at 4 °C.

The high molecular weight DNA present in the extract was sheared by passing through a 0.22 gauge needle approximately twenty times. A 100 µL aliquot of 2 M sodium oxaloacetate pH 4.0 was added, and the sample was swirled gently. An equal volume of saturated phenol treated with DEPC-treated water was added and mixed with gentle swirling. An aliquot of 200 µL of chloroform:isoamyl alcohol, (24:1) was added and the sample was shaken vigorously for 15 to 20 seconds. The sample was divided into two equal volumes and both were cooled on ice for 15 minutes, before being centrifuged at 10,000 x \(\text{g} \) for 20 minutes at 4 °C. The aqueous phase of each sample was transferred to fresh tubes and an equal volume of ice cold isopropanol was added to each, to precipitate the RNA. The samples were incubated for 4 hours at -20 °C.
The samples were centrifuged at 10,000 x g for 10 minutes at 4 °C and the supernatant removed and discarded. The pellets from each were washed with DEPC-treated ice cold 70% v/v ethanol, before being centrifuged at 10,000 x g for 10 minutes at 4 °C. The supernatant from each was removed and discarded, and the pellets were air-dried for 15 minutes, to remove all residual ethanol. The pellets were then separately re-suspended in 12 µL DEPC-treated water, and the two samples were recombined (total volume of 24 µL). The total amount of RNA was quantified using a Nanodrop spectrophotometer N1000 (Section 2.1.5). A 1 µL aliquot of the resulting RNA sample was analyzed on a 1% w/v TBE agarose gel (Section 2.1.5).

2.3.1.2: Reverse Transcription of RNA to Single Stranded cDNA

In two separate PCR tubes (Eppendorf), the following two reaction mixtures were combined.

<table>
<thead>
<tr>
<th>Component</th>
<th>Reaction 1:</th>
<th>Reaction 2:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primers (final concentration 50 µM)</td>
<td>Oligo d(T), 2.85 µL</td>
<td>Random Hexamers, 2.85 µL</td>
</tr>
<tr>
<td>100 mM dNTPs</td>
<td>10.00 µL</td>
<td>10.00 µL</td>
</tr>
<tr>
<td>Whole cell RNA (Section 2.3.1.1)</td>
<td>5.00 µL</td>
<td>5.00 µL</td>
</tr>
<tr>
<td>DEPC-treated water</td>
<td>2.15 µL</td>
<td>1.15 µL</td>
</tr>
</tbody>
</table>

Both reactions were incubated for 10 minutes at 65 °C, cooled on ice, and then to each, 2 µL of 10 x RT Buffer (Invitrogen), 4 µL of 25 mM MgCl2, 2 µL of 0.1 mM DTT, 1 µL of RNase OUT (40 units, Invitrogen) and 1 µL of Superscript III (200 units, Invitrogen) was added. Reaction 1 was held in ice, while reaction 2 was incubated for 10 minutes at 25 °C. Both reaction 1 and 2 were then incubated for 90 minutes at 50 °C. The Superscript III enzyme was then heat inactivated, by incubation for 5 minutes at 85 °C. All reactions were then stored at 4 °C, until required.

2.3.2: Amplification of the cDNA Encoding CDK1 using PCR

The cDNA transcript encoding CDK1 was amplified by PCR. A PCR master mixture was set up identical to that described in Section 2.3.1, but with a forward primer of 5’-GGCGCCGAATTCTTATGGAAGATTATACCAAAATAGAG-3’ (Invitrogen), a reverse
primer of 5’-CCGCGGCTGCAGCTACATCTTCTTAATCTGATTGTC-3’ (Invitrogen), and with the template for amplification being 1 µL of a 1:10 dilution of the cDNA sample prepared in Section 2.3.1.2. This master mixture was dispensed into five aliquots of 20 µL each, and run through the following PCR program:

1. 94 °C   1 minute
2. 94 °C   45 seconds
3. 63 ± 5 °C   45 seconds
4. 68 °C   2 minutes, REPEAT STEPS 2 - 4 FOR 5 MORE CYCLES
5. 94 °C   45 seconds
6. 60 ± 6 °C   45 seconds
7. 68 °C   2 minutes, REPEAT STEPS 5 - 7 FOR 5 MORE CYCLES
8. 94 °C   45 seconds
9. 58 ± 6 °C   45 seconds
10. 68 °C   2 minutes, REPEAT STEPS 8 - 10 FOR 5 MORE CYCLES
11. 94 °C   45 seconds
12. 55 ± 10 °C   45 seconds
13. 68 °C   2 minutes, REPEAT STEPS 11 - 13 FOR 15 MORE CYCLES
14. 68 °C   10 minutes
15. 4 °C   10 minutes
END OF PROGRAM

After the reaction was completed, each of the five samples was analyzed on a 1% w/v agarose gel (refer to Section 2.1.4). The band of approximately the correct size of 1300 base pairs was cut from the gel, and purified according to manufacturer’s instructions using the High Pure PCR Product Purification Kit (Roche).

2.3.3: Cloning to Construct the pPROEx HTb-cdk1 Clone

Both the PCR product encoding CDK1, isolated as in Section 2.3.2, and the vector pPROEx HTb were digested with the restriction enzymes EcoRI (Invitrogen) and PstI (Invitrogen). The PCR product encoding CDK1 was then ligated into the pPROEx HTb vector, following the protocol described in Section 2.2.2. A potential pFASTBac DUAL-cdk1 clone was then sent for confirmation by dideoxy nucleotide sequencing.
2.3.4: Sub-cloning to Construct pFASTBac DUAL-\textit{cdk1} Clone

Using the method detailed in Section 2.2.2, pFASTBac DUAL and pPROEx HTb containing the PCR product encoding CDK1 were digested with the restriction endonucleases PstI (Invitrogen) and SpeI (Invitrogen) in the presence of the appropriate REAct Buffer (Invitrogen). The doubly-digested pFASTBac DUAL and the DNA encoding CDK1 were then purified using the protocol detailed in Section 2.1.8.2. Ligation of the PCR product into this vector was undertaken using the same protocol as detailed in Section 2.2.2.3 for pFASTBac HTb, with the exceptions that 10 ng of each of the doubly-digested vector and \textit{cdk1} DNA were used, and that DNA purification was carried out using the method detailed in Section 2.1.8.2. This work was carried out by Dr. James Dickson.

2.3.5: Mutagenesis of the PCR Product Encoding CDK1 to Wild Type

Dideoxy nucleotide sequencing revealed an insertion mutation at position 108 of the PCR product for CDK1; plasmid-based PCR was used to remove this insertion. A forward primer of 5’-GTAGCCATGAAAAAAATCGACTAGAAAGTG-3’ (Invitrogen) and a reverse primer of 5’-CACTTTCTAGTCTGATTTTTTTCATGGCTAC-3’ (Invitrogen) were ordered to be cartridge-purified, and were re-suspended from lipophilate in TE buffer to each give stock solutions of 12.5 µg µL\textsuperscript{-1}. A 1 µL aliquot of this stock solution was then separately diluted in TE buffer to give final working solutions of 125 ng µL\textsuperscript{-1}. A 100 mM dNTP mixture was also prepared (Section 2.2.1).

The plasmid DNA (pFASTBac DUAL-\textit{cdk1} clone with the insertion at position 108, prepared as in Section 2.3.4) was purified from the \textit{dam}\textsuperscript{+} electro-competent \textit{E. coli} DH5\textalpha\ strain (refer to Section 2.1.1), and was used as the template for the mutagenesis reaction. The master mixture for the PCR mutagenesis reaction included 1 x Pfu Ultra II Reaction Buffer, 10 ng of the template DNA, 1 µL of each of the mutagenesis primers, 10 mM dNTPs and 1 µL of the Pfu Ultra II Fusion HS polymerase, diluted to a final volume of 50 µL using sterile deionised water. As a negative control for this reaction, an additional reaction was set up which
omitted the polymerase enzyme. Both of these reactions were then run through the following PCR program:

1. 95 °C  2 minutes
2. 95 °C  30 seconds
3. 55 °C  30 seconds
4. 72 °C  3 minutes, REPEAT STEPS 2 - 4 FOR 20 MORE CYCLES
5. 72 °C  5 minutes
6. 4 °C  10 minutes

END OF PROGRAM

The DNA samples from this PCR reaction were then separately precipitated by mixing with 5 µL 3 M sodium oxaloacetate pH 5.2 and 125 µL absolute ethanol. The samples were incubated for 30 minutes at -20 °C, and then centrifuged at 13,000 rpm (16,000 x g) in a bench-top centrifuge for 20 minutes at 4 °C. The supernatants were discarded and the DNA pellets were carefully washed in cold 70% v/v absolute ethanol, after which the samples were re-centrifuged at 13,000 rpm (16,000 x g) in a bench-top centrifuge for 20 minutes at 4 °C. The supernatants were discarded and the pellets were allowed to air dry for 5 minutes.

The pellets were next separately re-suspended in 35 µL of sterile deionised water. An aliquot of 4 µL of 10 x SuRECut Buffer A (Roche) and 1 µL of DpnI (Roche) was mixed into each sample. These were then incubated for 2 hours at 37 °C. Following incubation, 3 µL of each sample was transformed into chemically competent E. coli DH5α cells (refer to Section 2.1.2). Mutagenized recombinant plasmids were selected by colony growth under selective antibiotic.

Five single colonies from the experimental (positive) reaction were picked and were grown as a 5 mL culture in Luria Broth containing 100 µg mL⁻¹ ampicillin (refer to Section 2.1.1) and incubated for 12 - 18 hours with rotation at 180 rpm at 37 °C. The plasmid DNA present within each culture was purified according to manufacturer’s instruction using the Mini Plasmid Preparation kit (Qiagen). Confirmation that the insertion had been removed was sought using dideoxy nucleotide sequencing.
2.3.6: Generation of Recombinant Baculovirus Encoding CDK1

Recombinant baculovirus containing the PCR product that encodes CDK1 were generated using the protocol detailed in Section 2.1.8. This baculovirus was then amplified, as described in Section 2.1.8.4, to give a working stock of high viral titer.

2.3.7: Expression Testing of CDK1

The recombinant baculovirus was next tested for evidence of recombinant protein expression following Sf9 cell infection. A culture of 50 mL of mid log phase Sf9 cells at 1 x 10^6 cells mL^-1 was set up in a 250 mL Erlenmeyer flask. To this culture, 2.5 µL of the recombinant baculovirus containing the PCR product that encodes CDK1 was added. The flask was then incubated for 72 hours with shaking at 130 rpm at 28 °C. During this incubation, a 1 mL sample of culture was taken at 48 hours and 72 hours post infection. At 72 hours post infection, the remaining 48 mL of culture was centrifuged at 500 x g for 5 minutes at 4 °C. The supernatant was discarded and the pellet was stored at -20 °C, until required. Each of the two 1 mL samples was centrifuged at 500 x g for 5 minutes at 4 °C. The supernatant was discarded and the pellets were separately re-suspended in 150 µL of 50 mM Tris.HCl pH 7.5, 0.1% v/v NP40. The samples were then incubated for 5 minutes on ice, followed by centrifugation at 500 x g for 5 minutes at 4 °C. The respective supernatants were saved and the pellets were re-suspended in 150 µL of 50 mM Tris.HCl pH 7.5, 300 mM NaCl, 0.1% v/v NP40. The samples were then incubated for 5 minutes on ice, followed by centrifugation at 500 x g for 5 minutes at 4 °C. The pellets were discarded, and the respective supernatants were saved. Aliquots of 15 µL from each was then separately mixed with 3 µL of 6 x SDS Quench dye, and analyzed by SDS PAGE (refer to Section 2.1.9).

2.3.8: Large Scale Expression and Purification of CDK1

CDK1 was expressed and partially purified using a combination of IMAC, ammonium sulfate precipitation, size exclusion chromatography and ion exchange chromatography.
2.3.8.1: Expression of CDK1

A culture of 200 mL of mid log phase Sf9 cells at 1 x 10^6 cells mL\(^{-1}\) was set up in a 1,000 mL Erlenmeyer flask. Glucose and L-glutamine were added, to final concentrations of 1 mM each. To this culture, 10 µL of the quaternary baculovirus encoding CDK1 was added. The flask was then incubated for 72 hours with shaking at 130 rpm at 28 °C. Following incubation, the cells were harvested by centrifugation at 500 x \(g\) for 5 minutes at 4 °C. The supernatant was discarded, and the pellet was re-suspended in 10 mL of 50 mM Tris.HCl pH 7.5, 150 mM NaCl. The cells were then lysed using the Cell Disruptor (Constant Cell Disruption Systems), according to manufacturer's instruction, and the whole cell lysate was clarified by centrifugation at 4300 rpm for 20 minutes at 4 °C.

2.3.8.2: Ammonium Sulfate Precipitation of CDK1

The sample containing CDK1 was diluted to a total volume of 100 mL in 50 mM Tris.HCl pH 7.5, 150 mM NaCl. An ammonium sulfate precipitation was undertaken following the protocol detailed in Section 2.1.14, where proteins that precipitated in 40% saturated ammonium sulfate (addition of 22.6 g) were discarded, and proteins that precipitated between 40% and 70% saturated ammonium sulfate (addition of another 18.7 g) were collected. The pellet was re-suspended in 5 mL of 20 mM Tris.HCl pH 7.5, 50 mM NaCl, and was then dialyzed against 20 mM Tris.HCl pH 7.5, 50 mM NaCl to rid the sample of any residual ammonium sulfate, as detailed in Section 2.1.15.

2.3.8.3: Size Exclusion Chromatography of CDK1

The sample containing CDK1 was recouped from dialysis, and an S200 16/600 size exclusion column (Amersham Bioscience) was used to further purify the sample, as detailed in Section 2.1.12.1, using a sample buffer of 20 mM Tris.HCl pH 7.5, 50 mM NaCl. Aliquots of 15 µL from each of the resulting samples that contained CDK1 were then separately mixed with 3 µL of 6 x SDS Quench dye, and were analyzed by SDS PAGE (refer to Section 2.1.9).
2.3.8.4: Ion Exchange Chromatography of CDK1

Following size exclusion chromatography, the fractions containing CDK1 were pooled, and adjusted to contain 10% v/v glycerol. The sample was then concentrated to a volume of less than 5 mL using a spin concentrator with a MWCO of 10 kDa (VivaSpin). A 1 mL Mono Q ion exchange column was washed with 2 x column volumes of sterile deionised water, followed by 2 x column volumes of 2 M NaCl, and then further washed with 2 x column volumes of sterile deionised water. The column was then equilibrated in 20 mM Tris.HCl pH 7.5, 50 mM NaCl. The sample containing CDK1 was then loaded on to the equilibrated column. The non-binding proteins were washed through in 20 mM Tris.HCl pH 7.5, 50 mM NaCl. A gradient was set up to wash the column in an ever-increasing concentration of NaCl. Initially, the column was washed with 100% of 20 mM Tris.HCl pH 7.5, 50 mM NaCl. Over a period of 40 minutes, the concentration of NaCl in the wash was increased to 1,000 mM. Aliquots of 15 µL from each of the resulting fractions that contained CDK1 were then separately mixed with 3 µL of 6 x SDS Quench dye, and analyzed by SDS PAGE (refer to Section 2.1.9).

2.3.9: Crystallisation Trials of Purified Recombinant CDK1

CDK1 was expressed and purified as detailed in Section 2.3.8, concentrated to 10 mg mL⁻¹ using a spin concentrator with a MWCO of 10 kDa (VivaSpin), and the sitting drop method was used to set up crystallization experiments (Section 2.1.18), using the Cartesian HoneyBee nanolitre dispensing robot (Genome Solutions). An initial 480 conditions were screened, as five trays of 96 conditions, as described in Appendix D.
2.4: Attempt to Co-express Cyclin B1 and CDK1

2.4.1: Re-cloning of Cyclin B1 to Co-express with CDK1

The coding sequence for cyclin B1 was then re-cloned into the pFASTBac DUAL-\textit{cdk1} clone. Both the pFASTBac HTb-\textit{cyclin B1} clone and the pFASTBac DUAL-\textit{cdk1} clone were separately digested with the restriction endonucleases RsrII (Roche) and HindIII (Roche), followed by ligation of the \textit{cyclin B1} insert into the pFASTBac DUAL-\textit{cdk1} clone, using the method described in Section 2.2.2. This was undertaken by Dr. James Dickson.

2.4.2: Generation of Recombinant Baculovirus Encoding Cyclin B1 and CDK1

Recombinant baculovirus encoding both cyclin B1 and CDK1 were generated using the protocol detailed in Section 2.1.8, and were amplified as in Section 2.1.8.4 to give a working stock of high viral titer (undertaken by Dr. James Dickson).

2.4.3: Testing for Co-Expression of CDK1 and Cyclin B1

A culture of 50 mL of mid log phase Sf9 cells at 1 \times 10^6 cells mL\(^{-1}\) was set up in a 250 mL Erlenmeyer flask. To this culture, 3 \(\mu\)L of the recombinant baculovirus containing of the dual construct for CDK1 and cyclin B1 was added. The flask was then incubated for 96 hours with shaking at 130 rpm at 28 °C. The culture was harvested by centrifugation at 500 x g for 5 minutes at 4 °C, the supernatant discarded and the pellet re-suspended in 10 mL of 50 mM Tris.HCl pH 7.5, 0.1% \(\nu/\nu\) NP40. The sample was then incubated for 5 minutes at 4 °C. The cells were lysed by passage through the Cell Disruptor (Constant Cell Disruption Systems), carried out according to manufacturer's instructions, with a pressure setting of 17 kpi. Aliquots of 15 \(\mu\)L of each of the eluted fractions were separately mixed with 5 \(\mu\)L of 6 x SDS Quench dye, and analyzed by SDS PAGE (refer to Section 2.1.9).
2.5: Wee1/14-3-3β Methods and Materials

A DNA construct of full length human wee1 kinase and human 14-3-3β, cloned into pFASTBac DUAL, was prepared; unless otherwise specified, the term “wee1” refers to the full length construct. This dual expression construct was transfected into Sf9 cells using the Bac-to-Bac System (Invitrogen). This work was carried out by Dr. James Dickson. The resulting virus was then amplified for infection purposes (Section 2.1.8).

2.5.1: Expression and Purification of the Wee1/14-3-3β Complex

2.5.1.1: Initial Expression Test of the Wee1/14-3-3β Complex

A 250 mL Erlenmeyer flask was used to set up a 50 mL culture of mid log phase Sf9 cells at a titer of 1 x 10^6 cells mL⁻¹. To this culture, glucose and L-glutamine were also added to a final concentration of 1 mM each. An aliquot of 2.5 µL of the recombinant baculovirus encoding wee1 and 14-3-3β was added to the culture, which was then incubated for 72 hours with rotation at 180 rpm at 28 °C. At 72 hours post infection, a 1 mL sample of the culture was taken. The remaining 49 mL from the original culture were centrifuged at 500 x g for 5 minutes at 4 °C. The supernatant was discarded, and the pellet stored at 4 °C, until required. The 1 mL sample was centrifuged at 500 x g for 5 minutes at 4 °C. The supernatant was discarded and the pellet was re-suspended in 200 µL of 50 mM Tris.HCl pH 8.0, 0.1% v/v NP40, and incubated for 5 minutes at 4 °C. The sample was then centrifuged in a bench top centrifuge at 500 x g for 5 minutes at 4 °C. The supernatant was discarded and the pellet was re-suspended in 200 µL of 50 mM Tris.HCl pH 8.0, 300 mM NaCl, 0.1% v/v NP40, and incubated for 5 minutes at 4 °C.

Following this incubation, the sample was centrifuged in a bench top centrifuge at 500 x g for 5 minutes at 4 °C. The supernatant was carefully decanted and saved as the nuclear lysate, and the pellet discarded. Aliquots of 15 µL of the whole cell lysate was then mixed with 3 µL of 6 x SDS Quench dye, and was analyzed by SDS PAGE (refer to Section 2.1.9).
2.5.1.2: Enrichment Assay of the Wee1/14-3-3β Complex

An enrichment assay was undertaken to test for expression of the wee1/14-3-3β complex, and the extent to which the complex was able to bind to Ni$^{2+}$-charged HP Chelating resin (Amersham Bioscience). The pellet from the remaining 47 ml culture prepared earlier (Section 2.5.1.1) was dispensed in 10 aliquots, and each was re-suspended in 1 mL of one of the following buffers:

- Sample 1: The same buffer conditions/incubations/centrifugation steps were used as described in Section 2.5.1, resulting in an overall sample of 1 mL in 50 mM Tris.HCl pH 8.0, 150 mM NaCl, 0.1% v/v NP40
- Sample 2: 50 mM Tris.HCl pH 7.5, 100 mM NaCl
- Sample 3: 50 mM Tris.HCl pH 7.5, 200 mM NaCl
- Sample 4: 50 mM Tris.HCl pH 7.5, 300 mM NaCl
- Sample 5: 50 mM Tris.HCl pH 8.0, 100 mM NaCl
- Sample 6: 50 mM Tris.HCl pH 8.0, 200 mM NaCl
- Sample 7: 50 mM Tris.HCl pH 8.0, 300 mM NaCl
- Sample 8: 50 mM Tris.HCl pH 8.5, 100 mM NaCl
- Sample 9: 50 mM Tris.HCl pH 8.5, 200 mM NaCl
- Sample 10: 50 mM Tris.HCl pH 8.5, 300 mM NaCl

The cells from each of samples 2 to 10 were disrupted by passage through the Cell Disruptor (Constant Cell Disruption Systems), carried out according to manufacturer's instructions, with a pressure setting of 17 kpi. Aliquots of 500 µL of the ten whole cell lysates were separately mixed with 20 µL of HP chelating resin charged with NiCl$_2$ (Amersham Bioscience). These samples were then incubated for 60 minutes with rotation at 60 rpm at 4°C.
Following incubation, the samples were centrifuged at 13,000 rpm (16,000 x g) in a bench top centrifuge for 2 minutes at 4 °C. The supernatant was discarded, and the pellet was re-suspended in 1 mL of the same buffer as was used within each sample previously. This centrifugation and wash step was repeated a total number of three times. Following the third wash, the pellet was re-suspended in 30 µL of wash buffer. The 30 µL sample of each of the enrichment of the whole cell lysates were mixed with 5 µL of 6 x SDS Quench dye. All of these samples were then analyzed using SDS PAGE (Refer to Section 2.1.9).

2.5.1.3: Large Scale Expression and Purification of Wee1/14-3-3β Complex

2.5.1.3.1: Expression of the Wee1/14-3-3β Complex

Two 1,000 mL Erlenmeyer flasks were used to set up two 200 mL culture of mid log phase Sf9 cells at 1 x 10^6 cells mL^-1. To this culture, glucose and L-glutamine were also added to a final concentration of 1 mM each. A 10 µL aliquot of recombinant baculovirus encoding wee1 and 14-3-3β was then added to each culture. The culture was then incubated for 72 hours with rotation at 180 rpm at 28 °C.

2.5.1.3.2: IMAC Purification of the Wee1/14-3-3β Complex

At 72 hours post infection, the culture was harvested by centrifugation at 500 x g for 5 minutes at 4 °C. The supernatant was discarded and the pellet was re-suspended in 30 mL of 50 mM Tris.HCl pH 7.5, 0.1% v/v NP40, and incubated for 5 minutes at 4 °C. The sample was then adjusted to contain 300 mM NaCl, and incubated for a further 5 minutes at 4 °C. The cells were then disrupted according to manufacturer's instruction using the Cell Disruptor (Constant Cell Disruption Systems), with a pressure setting of 17 kpi.

The lysate was centrifuged at 4300 rpm for 15 minutes at 4 °C. The supernatant was saved as the whole cell lysate, and the pellet was discarded. An IMAC purification of the sample was then carried out, using the method described in Section 2.1.11, with a loading buffer of 50
mM Tris.HCl pH 7.5, 300 mM NaCl, a wash buffer of 50 mM Tris.HCl pH 7.5, 300 mM NaCl, 40 mM imidazole and an elution buffer of 50 mM Tris.HCl pH 7.5, 300 mM NaCl, 200 mM imidazole. Aliquots of 15 µL each of the lysate, the flow through and the wash in 50 mM Tris.HCl pH 7.5, 300 mM NaCl, 40 mM imidazole, as well as every second fraction from those that eluted in 50 mM Tris.HCl pH 7.5, 300 mM NaCl, 200 mM imidazole were separately mixed with 3 µL of 6 x SDS Quench dye, and were analyzed by SDS PAGE (refer to Section 2.1.9).

To confirm the identity of the respective proteins, wee1 and 14-3-3β were separately excised from an SDS PAGE analysis, and were identified using peptide mass fingerprinting. This was carried out through the Centre for Genomics and Proteomics, Centre for Molecular Biodiscovery, University of Auckland, by Dr. Christina Buchanan.

2.5.1.3.3: Size Exclusion Chromatography of the His-Tagged Wee1/14-3-3β Complex

The samples containing wee1 and 14-3-3β that were eluted from the chelating column were pooled, adjusted to contain 10% v/v glycerol and concentrated to a total volume of 0.5 mL using a spin concentrator with a MWCO of 30 kDa (VivaSpin). The sample was then subjected to size exclusion chromatography, using the protocol detailed in Section 2.1.12.1, with a sample buffer of 20 mM Tris.HCl pH 7.5, 200 mM NaCl. Aliquots of 15 µL of fractions containing the wee1/14-3-3β complex were mixed with 3 µL of 6 x SDS Quench dye, and were analyzed by SDS PAGE (refer to Section 2.1.9).

2.5.1.3.4: Small Scale Digestion of the Complex with rTEV Protease

A small sample of wee1/14-3-3β was used to test whether the recombinant Tobacco Etch Virus (rTEV) protease was able to remove the polyhistidine purification tag. The form of the enzyme used in this research was the recombinant TEV protease (rTEV protease) with an N-terminal polyhistidine purification tag. The expression and purification of this enzyme from
E. coli is further described in Section 2.1.13. The most common reaction buffer for rTEV protease is 50 mM Tris.HCl pH 8.0, 1 mM DTT, but in this research, this buffer was adjusted to include 200 mM NaCl, in order to maintain stability of the wee1/14-3-3β complex.

A 200 μL sample of 2 mg mL⁻¹ wee1/14-3-3β (expressed and purified by IMAC chromatography as detailed in Sections 2.5.1.3.1 and 2.5.1.3.2) was incubated with 10 μL of 1 mg mL⁻¹ rTEV protease in the presence of 2 mM DTT. This sample was then incubated with end-over-end rotation at 60 rpm for 12 – 18 hours at 4 °C. Following incubation, 15 μL of the sample was mixed with 3 μL of 6 x SDS Quench dye, and was analyzed by SDS PAGE (refer to Section 2.1.9).

2.5.1.3.5: Purification of Untagged Wee1/14-3-3β Complex

A sample of wee1/14-3-3β complex was expressed and partially purified using IMAC chromatography as detailed in Sections 2.5.1.3.1 and 2.5.1.3.2. Following IMAC, the sample containing wee1/14-3-3β was pooled and adjusted to contain 10% v/v glycerol, 2 mM DTT and 200 μL of 1 mg mL⁻¹ rTEV protease. The sample was then dialyzed against 20 mM Tris.HCl pH 7.5, 200 mM NaCl, 2 mM DTT, following the protocol detailed in Section 2.1.15. Following dialysis, the sample was adjusted to contain 10% v/v glycerol, and was then concentrated to a volume of less than 5 mL in total using a spin concentrator with a MWCO of 10 kDa (VivaSpin). The sample containing wee1/14-3-3β was further purified using an S200 16/60 size exclusion column, undertaken according to the method detailed in Section 2.1.12.1, using a sample buffer of 20 mM Tris.HCl pH 7.5, 200 mM NaCl. Following this analysis, 15 μL of every second fraction containing wee1/14-3-3β was separately mixed with 3 μL of 6 x SDS Quench dye, and was further analyzed by SDS PAGE (refer to Section 2.1.9). IMAC was unable to be used to purify the rTEV cleaved wee1/14-3-3β complex due to the incompatibility between the column medium and DTT at 2 mM.
2.5.2: Tryptic Analysis of Wee1/14-3-3β Complex

A trypsin digest of the wee1/14-3-3β complex was undertaken to identify exposed cleavage sites, and thus probe the overall architecture of the wee1/14-3-3β complex. The protocol for this analysis is adapted from Konigsberg, 1995 [98].

Trypsin is a serine protease enzyme that binds accessible Lys or Arg residues of a protein, and then cleaves the peptide backbone of that protein on the C-terminal side of the Lys or Arg. If the amino acid following the Lys or Arg is acidic, the rate of cleavage is reduced; if the amino acid following the Lys or Arg is Pro, cleavage is inhibited completely [99]. Trypsin will only cleave a protein where a Lys or Arg is accessible, for example within domain linker regions or within flexible regions, where the trypsin is readily able to both bind the Lys or Arg residue, and interact with the protein backbone. Where there is a highly ordered secondary structure, for example α-helices and β-sheets, this level of interaction is reduced or abrogated completely, as the enzyme is not able to gain the necessary degree of access to both the Lys or Arg, and the protein backbone. In this manner, the protein may be cleaved into fragments that lack accessible cleavage sites, either because they lack Lys or Arg residues, or because these residues are in regions that make them inaccessible, such as α-helices or β-sheets. Subsequent analysis by SDS PAGE means that the cleaved fragments can be separated from each other, allowing determination of their molecular weights, and also potentially allowing the identification the individual peptides using mass spectrometry.

A 1 mg mL⁻¹ stock solution of trypsin was made up in 1 mM HCl, 20 mM CaCl₂. A 96-well plate was used to set up two 1:4 serial dilutions of this stock solution of trypsin, whereby a 20 μL sample of the 1 mg mL⁻¹ solution of trypsin was set up, from which 5 μL was taken and diluted in 15 μL of 50 mM Tris.HCl pH 7.5, 150 mM NaCl. An aliquot of 5 μL of this second sample was taken and diluted in another 15 μL of 50 mM Tris.HCl pH 7.5, 150 mM NaCl. This was repeated until 12 samples in total had been generated (with 5 μL of the final sample discarded to maintain consistency in the volume), with final trypsin concentrations from 1 mg mL⁻¹ down to 0.2 x 10⁻⁶ mg mL⁻¹. This was set up in duplicate.
A sample of His-tagged wee1/14-3-3β complex was diluted to 4 mg mL⁻¹ in 50 mM Tris.HCl pH 7.5 150 mM NaCl. A 10 µL aliquot of this solution was added to each well containing 10 µL of the first trypsin serial dilution, which was incubated for 15 minutes at 18 ± 2 °C. An aliquot of 20 µL of the protein solution was also added to each well of the second trypsin serial dilution, which was also incubated for 15 minutes at 18 ± 2 °C.

Following this incubation, 10 µL of 10 mg mL⁻¹ PMSF in methanol was added to each reaction, and incubated a further 10 minutes at 18 ± 2 °C. Each reaction was then mixed with 1 µL of 6 x SDS Quench dye, and was analyzed by SDS PAGE (refer to Section 2.1.9). Two bioinformatics analyses were also undertaken, based on the protein sequence of human wee1, using the programs DisEMBL (http://dis.embl.de, [100]), and Phyre (http://www.sbg.bio.ic.ac.uk/phyre/html/index.html, [101]) to predict the most disordered regions of wee1.

2.5.3: Expression and Purification of the Truncated Wee1/14-3-3β Complex

A second construct of wee1, covering the kinase and C-terminal domains of wee1 (residues 215 to 646) with co-expression of human 14-3-3β, was provided by Dr. James Dickson. Unless otherwise specified, this construct is referred to as truncated wee1/14-3-3β. The viral stock provided was revived and amplified as in Section 2.1.8.4 to give a working inoculum of high viral titer.

2.5.3.1: Time course of Protein Expression of Truncated Wee1/14-3-3β

A 20 mL culture of mid-log phase Sf9 cells at a titer of 1 x 10⁶ cells mL⁻¹ was set up in a 100 mL Erlenmeyer flask. A 20 µL sample of viral inoculum was then added to this culture, which was incubated for 96 hours with rotation at 180 rpm at 28 °C. Samples of this culture (1 mL each) were taken at 48, 72 and 96 hours post infection. Each 1 mL sample was harvested by centrifugation at 500 x g for 5 minutes at 4 °C. The supernatant from each
sample was discarded, and the pellets were separately re-suspended in 200 µL of 50 mM Tris.HCl pH 7.5, 0.1% v/v NP40, and incubated for 5 minutes at 4 °C. The samples were then centrifuged again at 500 x g for 5 minutes at 4 °C. The pellets were discarded and the supernatant was saved as the whole cell lysate. Aliquots of 15 µL of each of these three samples were then separately mixed with 3 µL of 6 x SDS Quench dye and analyzed by SDS PAGE (refer to Section 2.1.9).

2.5.3.2: Large Scale Expression and Purification of the Truncated Wee1/14-3-3β Complex

2.5.3.2.1: Expression of the Truncated Wee1/14-3-3β Complex

A 1,000 mL Erlenmeyer flask was used to set up a 200 mL culture of mid-log phase Sf9 cells at a titer of 1 x 10^6 cells mL⁻¹. This culture was also supplemented with 1 mM glucose and 1 mM L-glutamine. An aliquot of 200 mL of the tertiary viral inoculum was then added to the culture, which was then incubated for 96 hours with shaking at 130 rpm at 28 °C.

2.5.3.2.2: IMAC Purification of the Truncated Wee1/14-3-3β Complex

At 96 hours post infection, the culture was centrifuged at 500 x g for 5 minutes at 4 °C. The supernatant was discarded and the pellet was re-suspended in 20 mL of 50 mM Tris.HCl pH 7.5, 0.1% v/v NP40, and incubated for 5 minutes at 4 °C. Following incubation, the sample was centrifuged at 500 x g for 5 minutes at 4 °C. The supernatant was saved as the whole cell lysate, and the pellet was discarded. The lysate sample was then adjusted to contain 150 mM NaCl.

The sample of truncated wee1/14-3-3β was subjected to IMAC purification, using the protocol detailed in Section 2.1.11, with a loading buffer of 50 mM Tris.HCl pH 7.5, 150 mM NaCl, a wash buffer of 50 mM Tris.HCl pH 7.5, 150 mM NaCl, 40 mM imidazole and an elution buffer of 50 mM Tris.HCl pH 7.5, 150 mM NaCl, 200 mM imidazole. Following
analysis, 15 µL each of the lysate, the flow through, the wash samples and the eluted proteins were separately mixed with 3 µL of 6 x SDS Quench dye, and were analyzed by SDS PAGE (Section 2.1.9).

2.5.3.2.3: Size Exclusion Chromatography of the Truncated Wee1/14-3-3β Complex

The samples eluted from the HP chelating column were pooled and concentrated to a volume of less than 5 mL using a spin concentrator with a MWCO of 10 kDa (VivaSpin). While still in the concentrator, the sample was then diluted to 20 mL in 20 mM Tris.HCl pH 7.5, 50 mM NaCl. The sample was again concentrated to be less than 5 mL in volume. These steps of dilution and concentration were repeated a total number of four times, such that the final volume of the sample was 5 mL, in 20 mM Tris.HCl pH 7.5, 50 mM NaCl.

The sample was then subjected to size exclusion chromatography using an S200 16/600 size exclusion column (Amersham Bioscience). The method followed the protocol detailed in Section 2.1.12.1, using a sample buffer of 50 mM Tris.HCl pH 7.5, 150 mM NaCl. Aliquots of 15 µL of the resulting fractions containing the truncated wee1/14-3-3β complex were mixed with 3 µL of 6 x SDS Quench dye, and were analyzed by SDS PAGE (refer to Section 2.1.9).

2.5.3.2.4: Small Scale Digestion of the Truncated Wee1/14-3-3β Complex with rTEV Protease

A small sample of truncated wee1/14-3-3β was used to test whether the protease rTEV was able to remove the polyhistidine purification tag. A sample of rTEV protease was expressed and purified as detailed in Section 2.1.13. Two 200 μL samples of truncated wee1/14-3-3β (expressed and purified by IMAC chromatography as detailed in Sections 2.5.3.2.1 and 2.5.3.2.2) were incubated with 10 µL of 1 mg mL⁻¹ rTEV protease in the presence of either 2 mM DTT or 2 mM β-ME. These samples were incubated with end-over-end rotation at 60 rpm for 12 – 18 hours at 4 °C. Following incubation, 15 µL of each sample was separately
mixed with 3 µL of 6 x SDS Quench dye, and analyzed by SDS PAGE (refer to Section 2.1.9).

2.5.4: Activity of the Full Length and Truncated Wee1/14-3-3β Complexes

2.5.4.1: Kinase Activity of the Wee1/14-3-3β Complex

The CycLex Wee1 Kinase Assay (MBL, CycLex) was undertaken to measure the kinase activity of the wee1/14-3-3β complex. This is an ELISA-based assay, using antibodies directed against phosphorylation of CDK1 residue Tyr-15. A pictorial representation of the assay is presented in Figure 2.4.

![Figure 2.4: A representation of the CycLex Wee1 Kinase assay.](image)

The assay was used to determine the kinase activity of the full length or truncated wee1/14-3-3β complex. The assay is ELISA-based, and uses primary and secondary antibodies to detect if the experimental wee1 is functional.

The assay was supplied with plate wells already coated with N-terminal CDK1 peptide. The addition of wee1 (both as a positive control and as the experimental samples) resulted in the phosphorylation of CDK1 residue Tyr-15. The presence of this additional phosphate was detected by the primary antibody, a mouse anti-phosphotyrosine monoclonal antibody. Secondary anti-mouse IgG antibody conjugated to horseradish peroxidase was used to detect the primary antibody. Horseradish peroxidase catalyses the conversion of the chromogenic substrate tetramethylbenzidine from colourless to blue; thus, where CDK1 residue Tyr-15 was phosphorylated, a blue colour developed. The addition of a stopping solution of 0.5 M H₂SO₄ halted the activity of the horseradish peroxidase, and converted the colour of the solution to yellow, commensurate with the level of primary/secondary antibody that was
present within the sample. Finally, the absorbance (at 450 nm) of the resulting samples was measured whereby the greater the yellow colour, the greater the absorbance reading, the higher the kinase activity of the wee1 enzyme.

His-tagged wee1/14-3-3β was expressed and purified as described in Sections 2.5.1.3.1 to 2.5.1.3.3, then concentrated to a total protein concentration of 1.5 mg mL⁻¹. Following SDS PAGE analysis (Figure 4.6), it is estimated that, at a minimum, 60 – 70% of this sample is the wee1/14-3-3β complex, such that the final concentration of the complex is likely to be 0.9 mg mL⁻¹ (9 μM). A Kinase Reaction Buffer was prepared by mixing 50 μL of 2 mM ATP with 950 μL of Kinase Buffer (MBL, CycLex Wee1 Kinase Assay). An ATP-negative Kinase Reaction Buffer was also prepared by mixing 950 μL of Kinase Buffer (MBL, CycLex Wee1 Kinase Assay) with 50 μL of sterile deionised water. An 8-well strip was purchased with CDK1 coated on the bottom of each of the wells (MBL, CycLex Wee1 Kinase Assay). The following reagents were added to these wells:

<table>
<thead>
<tr>
<th>Well Number</th>
<th>Kinase Reaction Buffer (μL)</th>
<th>ATP-Negative Kinase Reaction Buffer (μL)</th>
<th>Experimental wee1/14-3-3β sample (μL)</th>
<th>wee1 Positive Control (1 AU/μL, μL)</th>
<th>Buffer (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>90</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>-</td>
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<tr>
<td>3</td>
<td>-</td>
<td>90</td>
<td>10</td>
<td>-</td>
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</tr>
<tr>
<td>4</td>
<td>-</td>
<td>90</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>90</td>
<td>-</td>
<td>-</td>
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<td>10</td>
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<tr>
<td>6</td>
<td>90</td>
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<td>-</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>90</td>
<td>-</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>90</td>
<td>-</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Wells 1 and 2 represent duplicates of a positive control for the reaction, which contained recombinant wee1 expressed from *E. coli*, with an N-terminal GST fusion tag (MBL, catalogue number CY-E1172) at a concentration of 1.5 mg mL⁻¹ (13 μM). Due to its expression in a non-mammalian system, the expressed wee1 is not in complex with any other molecule, or more specifically, is not in complex with 14-3-3β. Wells 3 and 4 represent duplicates of a negative control which lacks ATP. Wee1 exhibits a kinase activity which results in the transfer of a phosphate group on to the CDK peptide that coats the wells. The donor of this phosphate group is ATP. This control is present to ensure that the donor phosphate for the reaction is the added ATP. Wells 5 and 6 represent duplicates of a negative
control which lack any wee1 enzyme. This control is present to ensure that the only activity being measured is due to the experimental sample, itself, rather than any contaminants or additives that may otherwise be present in any or all of the reagents. Wells 7 and 8 represent duplicates of the experimental sample.

Following addition of the wee1/14-3-3β sample, the strip was covered, and incubated for 60 minutes at 30 °C. The liquid was removed from each well by pipetting or decanting, and the wells were washed four times with 100 µL of 1 x Wash Buffer (MBL, CycLex Wee1 Kinase Assay). To each well, 100 µL of Anti-Phospho-Tyrosine Monoclonal Antibody PY-39 (MBL, CycLex Wee1 Kinase Assay) was then added, with the strip being re-covered and incubated for 60 minutes at 18 ± 2 °C. Following this second incubation, the liquid was removed from each well by pipetting or decanting, and the wells were washed four times with 100 µL of 1 x Wash Buffer (MBL, CycLex Wee1 Kinase Assay). To each well, 100 µL of HRP-Conjugated Anti-Mouse IgG (MBL, CycLex Wee1 Kinase Assay) was added, with the strip then being re-covered and incubated for 60 minutes at 18 ± 2 °C.

Following this third incubation, the liquid was removed from each well by pipetting or decanting, and the wells were washed four times with 100 µL of 1 x Wash Buffer (MBL, CycLex Wee1 Kinase Assay). To each well, 100 µL of Substrate Reagent (MBL, CycLex Wee1 Kinase Assay) was then added, with the strip being re-covered and incubated for 10 minutes at 18 ± 3 °C. Finally, 100 µL of Stop Reagent (MBL, CycLex Wee1 Kinase Assay) was added to each well, and a SpectraMax 340 Spectrophotometer was used to measure duplicates of the absorbance of each of the wells at a wavelength of 450 nm.

This entire protocol was repeated an additional three times, to give a total of four assays; each individual assay was undertaken with a different preparation of wee1/14-3-3β, allowing an independent comparison of each of the assays. The variation inherent in these data was assessed by calculating the standard deviation (SD) and standard error of the mean (SE), using the following equations:

\[
SD = \sqrt{\frac{\Sigma (x - \bar{X})^2}{n}}
\]

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Where: \( x \) = an individual variable within the dataset.

\[ \bar{X} \] = the population mean for the dataset.

\( n \) = total number of samples in each dataset.

\[
SE = \frac{SD}{\sqrt{n}}
\]

Where: \( n \) = total number of samples in each dataset.

The results for the activity of the positive controls and experimental samples were then subjected to a Student’s t-test, to confirm the statistical distinction between the two datasets. This test assumed statistical independence between the two datasets, with an equal sample number and an equal variance. Using the null hypothesis that there existed no difference between the two datasets, the following equation was used to calculate a t-value:

\[
t = \frac{\bar{X}_1 - \bar{X}_2}{SD_{X1X2} \times \sqrt{\frac{DF}{n}}}
\]

Where: \( \bar{X}_1 \) = Mean of the one dataset (experimental samples)

\( \bar{X}_2 \) = Mean of the other dataset (positive controls)

\( SD_{X1X2} \) = Square root of \( \frac{\text{Std Error of dataset 1} + \text{Std Error of dataset 2}}{2} \)

\( n \) = total number of samples in each dataset

The Degrees of Freedom (DF) were calculated using the following equation:

\[
DF = 2n - 2
\]

Where \( n \) = total number of samples in each dataset.

2.5.4.2: Kinase Activity of the Wee1/14-3-3β/PD0407824 Complex

The structure of the wee1 kinase domain (PDB ID 2IN6, [39]) was solved by binding PD0407824 (Figure 2.5) to the active site of wee1. This same compound was also used
within this research, in an attempt to better stabilize the kinase domain of wee1 within the wee1/14-3-3β complex, and thus potentially increase the likelihood of crystallization. To ensure that this compound inhibited the wee1/14-3-3β complex in a manner equivalent to that reported previously [102], a sample of the wee1/14-3-3β/PD0407824 complex was prepared and subjected to the CycLex Wee1 Kinase Assay (MBL, CycLex).

A sample of wee1/14-3-3β/PD0407824 was prepared by incubating untagged wee1/14-3-3β sample (Section 2.5.1.3.5) with PD0407824 at a final concentration of 200 μM for 30 minutes at 4 °C. The sample was then concentrated to a total protein concentration of 1.5 mg mL⁻¹ (using the same assumptions detailed in Section 2.5.4.1, this reflects a final wee1/14-3-3β/PD0407824 concentration of 9 μM). This sample was then subjected to the CycLex Wee1 Kinase Assay (CycLex, MBL) using the protocol detailed in Section 2.5.4.1.

### 2.5.4.3: Kinase Activity of the Truncated Wee1/14-3-3β Complex

The CycLex Wee1 Kinase Assay (MBL, CycLex) was undertaken to measure the kinase activity of the truncated wee1/14-3-3β complex. Truncated wee1/14-3-3β was expressed and purified according to the methods described in Section 2.5.3.2 and then protein from peak 2 was concentrated to 1.5 mg mL⁻¹. Following SDS PAGE analysis (Figure 4.17), it is estimated that, at a minimum, 80 – 90% of this sample is the truncated wee1/14-3-3β complex, such that the final concentration of the complex is likely to be 1.2 mg mL⁻¹ (17
μM). The assay is the same as that given in Section 2.5.4.1, and the same statistical analysis was carried out to determine whether a statistically significant difference existed between the positive controls and experimental samples from this analysis.

A second statistical analysis was then carried out to determine whether a statistically significant difference existed between the experimental samples of full length wee1/14-3-3β and truncated wee1/14-3-3β. As in Section 2.5.4.1, this Student’s t-test used assumed statistical independence between the two datasets and an equal variance, but in this case, the sample number between the two datasets differed. Using the null hypothesis that there existed no difference between the two datasets, the following equation was used to calculate a t-value:

\[
t = \frac{\bar{X}_1 - \bar{X}_2}{S_{X_{1X2}} \times \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}
\]

Where:
- \(\bar{X}_1\) = Mean of the one dataset (experimental samples)
- \(\bar{X}_2\) = Mean of the other dataset (positive controls)
- \(S_{X_{1X2}}\) = \(\sqrt{\frac{(n_1 \times SE_1^2) + (n_2 \times SE_2^2)}{n_1 + n_2}}\)
- \(SE_1\) = Standard error (Section 2.5.4.1) of dataset 1
- \(SE_2\) = Standard error (Section 2.5.4.1) of dataset 2
- \(n_1\) = total number of samples in dataset 1
- \(n_2\) = total number of samples in dataset 2

The Degrees of Freedom (DF) were calculated using the following equation:

\[
DF = n_1 + n_2 - 2
\]

Where \(n_1\) = total number of samples in Dataset 1
- \(n_2\) = total number of samples in Dataset 2
2.5.4.4: Kinase Activity of the Truncated Wee1/14-3-3β/PD0407824 Complex

PD0407824 was introduced to the truncated wee1/14-3-3β complex either by incubating the two, as in Section 2.5.4.2, or by purification, whereby the sample was prepared by incubation, then subjected to an additional size exclusion chromatography analysis, as in Section 2.5.3.2.3. The sample of purified truncated wee1/14-3-3β/PD0407824 complex was concentrated to 1.5 mg mL\(^{-1}\) (using the same assumptions as in Section 2.5.4.3, this reflects a final truncated wee1/14-3-3β/PD0407824 concentration of 17 μM). Using this sample, the CycLex Wee1 Kinase Assay (CycLex, MBL) was carried out to determine the activity of the truncated wee1 in this complex, as in Section 2.5.4.1.

2.5.5: Full Length Wee1/14-3-3β Complex Methods and Materials

2.5.5.1: Attempts to Dissociate the Wee1/14-3-3β Complex

Three attempts were made to dissociate the wee1/14-3-3β complex, in order to gauge and/or measure the strength of binding between the two molecules. For the first attempt, a HisSpinTrap column (Amersham Bioscience) was prepared according to manufacturer’s instruction. The column was equilibrated by loading 600 μL of 20 mM Tris.HCl pH 7.5, 300 mM NaCl on to the column which was then centrifuged at 1,000 rpm for 30 seconds. A sample of His-tagged wee1/14-3-3β complex was expressed and IMAC purified, as detailed in Sections 2.5.1.3.1 and 2.5.1.3.2. An aliquot of 500 μL of this sample was loaded on to the equilibrated column, which was centrifuged at 1,000 rpm for an additional 30 seconds. The flow through was then re-loaded on to the column, which was centrifuged again at 1,000 rpm for 30 seconds. The column was then washed four times by loading with 500 μL of 20 mM Tris.HCl pH 7.5, in the presence of NaCl at concentration of 1 M, 2 M, 3 M or 4 M, with centrifugation at 1,000 rpm for 30 seconds following each wash, and collection of the resulting flow through.
Following the final wash, 500 µL of 20 mM Tris.HCl pH 7.5, 200 mM NaCl, 200 mM imidazole was loaded on to the column, which was centrifuged at 1,000 rpm for a further 30 seconds. The flow through was collected, re-loaded on to the column, and centrifuged at 1,000 rpm for an additional 30 seconds. Aliquots of 15 µL of all of the collected samples were then separately mixed with 3 µL of 6 x Quench dye and analyzed by SDS PAGE (refer to Section 2.1.9).

The second attempt to dissociate the wee1/14-3-3β complex employed the same protocol as detailed above with the exception that 20 mM sodium citrate-2H2O pH 5.0 was used in place of 20 mM Tris.HCl pH 7.5, in an attempt to potentially weaken the interaction between wee1 and 14-3-3β by potentially favouring the protonation of the phosphate group that forms the primary binding site within the complex. The same washes were collected. Aliquots of 15 µL of each sample was again mixed with 3 µL of 6 x Quench dye, and all were analyzed by SDS PAGE (refer to Section 2.1.9).

The third attempt to dissociate the wee1/14-3-3β complex was then undertaken. A sample of the complex was expressed and purified (leaving the polyhistidine purification tag intact), as described in Sections 2.5.1.3.1 to 2.5.1.3.3. Following purification, the sample was concentrated to less than 0.5 mL using a spin concentrator with a MWCO of 10 kDa (VivaSpin), adjusted to contain 2 M urea and 1 mM EDTA, and was incubated with end-over-end rotation at 60 rpm for 60 minutes at 4 °C. Following incubation, the sample was analyzed by S200 10/30 size exclusion chromatography using the same method as detailed in Section 2.1.12.1, but with a sample buffer of 20 mM Tris.HCl pH 7.5, 200 mM NaCl, 2 M urea, 1 mM EDTA. Aliquots of 15 µL samples of the resulting fractions from this analysis, which contained the wee1/14-3-3β complex, were separately mixed with 3 µL of 6 x SDS Quench dye, and analyzed by SDS PAGE (refer to Section 2.1.9).

2.5.5.2: Determination of the Oligomeric State of the Wee1/14-3-3β Complex

2.5.5.2.1: Determination of Stoichiometry of the Wee1/14-3-3β Complex
An analysis of the wee1/14-3-3β complex was undertaken using the dye Sypro Ruby (Invitrogen, Molecular Probes) to determine the stoichiometry of binding between wee1 and 14-3-3β. As detailed by the manufacturer (Invitrogen Molecular Probes), Sypro Ruby is a luminescent protein stain with a fluorescence excitation maximum at 450 nm, with a lower limit of sensitivity of 0.25 to 1 ng. This stain is meant to be used in conjunction with SDS PAGE, as was the case here, since the stain then soaks into the protein to be visualized in a linear, non-sequence-dependent fashion – the greater the concentration of the protein within any region of the gel, the more stain will be taken up. When used in conjunction with sensitive laser scanning instruments, the density of the bands on the gel can be correlated with a molar amount present on a gel, thus allowing a molar ratio between two proteins to be determined.

A sample of the wee1/14-3-3β complex was expressed and purified using IMAC and size exclusion chromatography, using the methods described in Sections 2.5.1.3.1 to 2.5.1.3.3. Following purification, the sample was concentrated to a final volume of 500 µL. A four-part 1:2 serial dilution of the protein sample with 20 mM Tris.HCl pH 7.5, 50 mM NaCl in a final volume of 20 µL was set up. An aliquot of 4 µL of 6 x SDS Quench dye was mixed into each sample, which was then run on SDS PAGE (refer to Section 2.1.9). In addition, to provide an internal control on the gel, separate aliquots of 12.2 µL, 10 µL, 7.5 µL, 5 µL and 2.5 µL of PrecisionPlus Molecular Weight Standards (BioRad) were also loaded and run in parallel with the experimental samples.

Following SDS PAGE, the gel was fixed by washing with 100 mL of 50% v/v methanol, 7% v/v glacial acetic acid using gentle agitation for 30 minutes at 18 ± 2 °C. This fixing wash was repeated twice. The gel was stained with 100 mL of Sypro Ruby dye (Invitrogen, Molecular Probes) by incubation with gentle agitation for 12 - 18 hours at 18 ± 2 °C, and then washed with 100 mL of 10% v/v methanol, 7% v/v glacial acetic acid using gentle agitation for 30 minutes at 18 ± 2 °C. This final wash was repeated twice. The gel was then washed twice with 100 mL of sterile deionised water using gentle agitation for 5 minutes at 18 ± 2 °C. Finally, the gel was loaded on to a laser scanning phosphorimager (FLA 1500, FujiFilm), and scanned with a laser that emits at 437 nm. The resulting image was analyzed using MultiGauge (FujiFilm).
2.5.5.2.2: Determination of Native Molecular Weight of the Wee1/14-3-3β Complex

Three different methods were used to determine the native molecular weight of the wee1/14-3-3β complex. This was carried out with a view to gaining an insight as to the overall architecture of the wee1/14-3-3β complex.

2.5.5.2.2.1: Analytical Size Exclusion Chromatography Analysis of the Wee1/14-3-3β Complex

A sample of untagged wee1/14-3-3β was expressed and purified, using the methods described in Section 2.5.1.3.5. Following purification, the sample was concentrated to a final volume of 500 µL. The sample was then subjected to analytical size exclusion chromatography, using the method described in Section 2.1.12.2.

2.5.5.2.2.2: Non-Denaturing Gradient PAGE Analysis of the Wee1/14-3-3β Complex

A sample of untagged wee1/14-3-3β was expressed and purified, using the methods described in Section 2.5.1.3.5. Following purification, the sample was concentrated to a final volume of 500 µL. The sample was then subjected to analytical size exclusion chromatography, using the method described in Section 2.1.12.2.

A sample of untagged wee1/14-3-3β was prepared, using the protocol detailed in Section 2.5.1.3.5. An aliquot of 15 µL of this sample was then mixed with 4 x native gel dye (refer to Section 2.1.10), and was analyzed using a commercially available non-denaturing 4 – 12% acrylamide gradient gel (Biorad). A 5 µL sample of a non-denaturing protein ladder, NativeMark Molecular Weight Standards (Invitrogen), was also run in parallel on this gel. This analysis, including staining and de-staining, was carried out according to the protocol detailed in Section 2.1.9.

Following this analysis, another non-denaturing 4 – 12% acrylamide gradient gel (Biorad) was washed in sterile deionised water for 1 hour at 18 ± 2 °C. This wash was then repeated, and the gel was subsequently immersed in 175 mM β-alanine, 0.8% v/v acetic acid for 12 – 18 hours at 18 ± 2 °C. A sample of untagged wee1/14-3-3β complex was prepared, using the protocol detailed in Section 2.5.1.3.5. An aliquot of 15 µL of this sample was mixed with 2 µL of 50% v/v glycerol, 0.1% w/v Bromphenol Blue. This gel was then run following the
protocol detailed in Section 2.1.9, with a running buffer of 175 mM β-alanine, 0.8% v/v acetic acid, with a switch of the electrodes (negative to positive and \textit{vice versa}). Staining and de-staining was carried out according to the protocol detailed in Section 2.1.9.

\subsection*{2.5.5.2.2.3: DLS Analysis of the Wee1/14-3-3β Complex}

A sample of His-tagged wee1/14-3-3β was prepared, using the protocol detailed in Sections 2.5.1.3.1 to 2.5.1.3.3. This sample was then subjected to DLS analysis, following the method described in Section 2.1.17. This procedure was repeated for five individual preparations of wee1/14-3-3β complex. In collating and analyzing the data, SD and SE were derived using the equations described in Section 2.5.4.1. This same analysis was also carried out on a sample of untagged wee1/14-3-3β, prepared as described in Section 2.5.3.2, and was also repeated with five separate preparations of the complex.

\subsection*{2.5.5.2: Molecular Characterization of the Wee1/14-3-3β Complex}

\subsubsection*{2.5.5.2.1: Determination of the Isoelectric Point of the Wee1/14-3-3β Complex}

This analysis was carried out with the help of Dr. Christina Buchanan, University of Auckland Centre for Proteomics and Genomics. Isoelectric focusing (IEF) is a method that allows the separation of proteins according to their isoelectric points (pI). Proteins carry a net charge, either positive, negative or zero, with contributions of either charge from the amino acids of its primary sequence. The isoelectric point of a molecule is defined as the pH at which its net charge is zero, as determined by its migration through an electric field. The native isoelectric point of a protein is determined by the amino acid sequence of a protein in its native conformation, and the charge a protein carries is dependent upon the pH of the environment surrounding the molecule: proteins are positively charged when the pH of the surrounding solution is below their isoelectric point, and, conversely, are negatively charged when the pH of the surrounding solution is above their isoelectric point. Isoelectric focusing requires a pH gradient to be set up in the presence of the molecule(s) in question. An electric
field is then applied to that pH gradient, which induces movement of the protein to a position in the pH gradient where the overall charge is zero. Should a protein diffuse away from this point, it will immediately gain a charge (either positive or negative, depending on the specific movement involved) and thus will migrate back to its isoelectric point.

100 µg of the wee1 /14-3-3β complex (purified as detailed in Section 2.5.1.3.5) was diluted to a final volume of 185 µL in Native Rehydration Buffer (2% w/v CHAPS, 1% w/v Pharmalyte pH 3 - 10 (GE Healthcare), 50 mM DTT). The sample was vortexed vigorously for 30 seconds, then centrifuged at 10,000 x g for 3 minutes at 4 °C. Following centrifugation, the sample was pipetted evenly along the well of a Rehydration Tray (GE Healthcare). An IPG Strip (GE Healthcare) was then placed, gel-face down, on top of the sample in the rehydration tray, ensuring that the entire face of the strip was submerged in the sample. The sample was then covered in 3 mL of paraffin oil, and allowed to rehydrate for 16 hours at 18 ± 2 °C.

Following this incubation, the IPG strip was thoroughly washed with sterile deionised water, and was blotted dry on damp filter paper. The filter wicks of an IEF tray were dampened with sterile deionised water, and the IPG strip was placed gel side down in the tray, such that the ends of the strip were covering the filter wicks of the tray. The strip was covered with 3 mL of oil and placed within a Protean IEF cell. The sample was then placed within an electric field of 50 µA per strip, with a voltage gradient from 0 to 8,000 volts, for 24,000 volt-hours at 20 °C. Finally, the strip was stained and de-stained in an identical manner to that used for SDS PAGE (refer to Section 2.1.9).

2.5.5.2.2: Analysis of the Wee1/14-3-3β Complex using Circular Dichroism (CD)

CD spectroscopy was used to assess the proportions of secondary structure present in the wee1/14-3-3β complex. The protocol for this analysis is adapted from Greenfield et al. 2007 [103].
A sample of the wee1/14-3-3β complex was expressed and purified, with removal of the polyhistidine purification tag, as detailed in Section 2.5.3.5, with concentration to 2 mg mL\(^{-1}\). Following purification, the sample was dialyzed into 10 mM sodium phosphate buffer pH 7.4, as detailed in Section 2.1.15. Following dialysis, the absorbance of the dialysis buffer across the wavelength range 180 nm to 250 nm was determined, using a 0.1 cm path length quartz cuvette. The absorbance of the sample in the same cuvette and buffer was also measured across the same wavelengths. Where appropriate, the sample was diluted in dialysis buffer until its absorbance reading was less than 1.5 across all of the specific wavelengths. The final concentration of the sample was determined using the method detailed in Section 2.1.16.

A \(\pi\)-180 CD Spectrophotometer (Applied Photophysics) was used to make the CD measurements. This was set to take CD measurements over the wavelength range of 180 to 250 nm, at 0.5 nm intervals, with a time per datapoint of 10 s (such that each data point on the spectrum was a mean of 400,000 readings). The method used was as follows:

- A baseline spectrum, with no cuvette or buffer, was measured and was subtracted from all further spectra.
- A spectrum of the dialysis buffer, in the appropriate cuvette, was then taken.
- A spectrum of wee1/14-3-3β, in the dialysis buffer, in the cuvette, was then taken. The spectrum from the dialysis buffer only was subtracted from this spectrum. A Stavitski-Golay function [104] was then used to fit the data, with a window setting of 10 points and a polynomial order of 3 [103].
- The latter step was then repeated a further two times.
- The resulting data (in mdeg) were converted to Mean Residue Ellipticity (deg cm\(^2\)/dmol) using the equation:

\[
\theta = \frac{\text{mdeg} \times \text{Mean Residue Weight}}{\text{Pathlength (cm)} \times \text{Concentration (mg mL}^{-1})}
\]

Where:

\[
\text{Mean Residue Weight} = \frac{\text{Molecular weight of the protein}}{\text{Number of backbone amides}}
\]
Where appropriate, the data was also converted to $\Delta \varepsilon$ units, using the equation:

$$\Delta \varepsilon = \frac{\theta}{3298}$$

This process was then repeated with a second wee1/14-3-3$\beta$ sample, at an identical concentration and under identical conditions. Using these converted data, the programs K$_2$D$_2$ [105], [106], [107], SOMCD [108] and CDNN [109] were used to deconvolute these spectra to estimate the percentages of secondary structures adopted by the wee1/14-3-3$\beta$ complex.

The secondary structure contributions of the kinase domain of wee1 and of 14-3-3$\beta$ were also counted, using their respective crystal structures (PDB ID 2IN6, [39] and PDB ID 2BQ0, [110]). A multiple sequence alignment was carried out using the program ClustalW [111], [112] to align wee1 sequences from *Homo sapiens* (NCBI accession number NP_003381), *Mus musculus* (NCBI accession number NP_033542), *Rattus norvegicus* (NCBI accession number NP_001012760) and *Bos taurus* (NCBI accession number AAI51482). A bioinformatics analysis was then undertaken to predict the secondary structures of these same proteins, using the programs Phyre [101], PsiPred [113], [114] and JPred3 [115], [116].

2.5.5.2.3: Transmission Electron Microscopy of the Wee1/14-3-3$\beta$ Complex

This analysis was carried out with the help of Professor Alok Mitra, Dr. Sarah Greig, Mazdak Radjainia and Jae Hyun, Laboratory of Electron Microscopy, University of Auckland, with the aim of trying to visualize the wee1/14-3-3$\beta$ complex.

Three separate samples of wee1/14-3-3$\beta$ were prepared using the protocol detailed in Section 2.5.1.3.5. The first sample was diluted to 1 mg mL$^{-1}$ and 0.5 mg mL$^{-1}$ in 20 mM Tris.HCl pH 7.5, 200 mM NaCl. The second sample was diluted to 0.5 mg mL$^{-1}$ and 0.2 mg mL$^{-1}$ in 20 mM Tris.HCl pH 7.5, 200 mM NaCl. The third sample was diluted to 0.1 mg mL$^{-1}$, 0.05 mg mL$^{-1}$ and 0.03 mg mL$^{-1}$ in 20 mM Tris.HCl pH 7.5, 200 mM NaCl. A number of TEM-suitable carbon grids were prepared using well established methods [117]. Of each of the
above sample to be visualised, 10 μL was loaded on to the carbon grid, with incubation for 90 seconds. The grid was then washed three times with sterile deionised water. A 5 μL sample of 1.5% w/v uranyl acetate was then loaded on to the grid, and incubated for 45 seconds. An additional 5 μL of 1.5% w/v uranyl acetate was then loaded on to the grid, which was incubated a further 25 seconds. Each grid was then blotted dry, before being individually visualized using the FEI/Tecnai CM12 transmission electron microscope (Phillips).

2.5.5.2.4: Crystallization Trials on the Wee1/14-3-3β Complex

Following the DLS experiments (Section 2.5.5.2.2.3), samples of the wee1/14-3-3β complex were subjected to crystallization trials. These were carried out for:

- 20 mg mL\(^{-1}\) His-tagged wee1/14-3-3β complex
- 15 mg mL\(^{-1}\) of untagged wee1/14-3-3β complex
- 15 mg mL\(^{-1}\) of untagged wee1/14-3-3β complex with 200 μM PD0407824

The sitting drop method of crystallization was used to set up these crystallization experiments, using the Cartesian HoneyBee nanolitre dispensing robot (Genome Solutions). An initial 480 conditions were screened, as five trays of 96 conditions, and are further described in Appendix D.

2.5.6: Truncated wee1/14-3-3β Complex Methods and Materials

2.5.6.1: Determination of the Oligomeric State of the Truncated Wee1/14-3-3β Complex

2.5.6.1.1: Determination of Stoichiometry of the Truncated Wee1/14-3-3β Complex

An analysis of the truncated wee1/14-3-3β complex was undertaken using Sypro Ruby dye (Invitrogen, Molecular Probes) to determine the stoichiometry of binding between truncated
wee1 and 14-3-3β. A sample of the truncated wee1/14-3-3β complex purified from peak 2 was expressed and purified using IMAC and size exclusion chromatography, as in Section 2.5.3.2. Following purification, the sample was concentrated to a final volume of 500 µL. The stoichiometric analysis of the truncated wee1/14-3-3β complex was undertaken as in Section 2.5.5.2.1.

2.5.6.1.2: Determination of the Molecular Weight of the Truncated Wee1/14-3-3β Complex

Two different methods were used to determine the molecular size of the truncated wee1/14-3-3β complex. As with the full length wee1/14-3-3β complex, these analyses were undertaken to gain insight into the overall architecture of the truncated wee1/14-3-3β complex.

2.5.6.1.2.1: Analytical Size Exclusion Chromatography of the Truncated Wee1/14-3-3β Complex

Analytical size exclusion chromatography was undertaken to determine the apparent molecular weight of the truncated wee1/14-3-3β complex in solution. A sample of truncated wee1/14-3-3β was expressed and purified using IMAC and size exclusion chromatography using the methods described in Section 2.5.3.2. Following purification, the sample was concentrated to a final volume of 500 µL. This sample was then subjected to analytical size exclusion chromatography, using the method detailed in Section 2.1.12.2.

2.5.6.1.2.2: DLS Analysis of the Truncated Wee1/14-3-3β Complex

A sample of truncated wee1/14-3-3β was prepared as in Section 2.5.3.2. The purified peaks (refer to Section 4.3.3) were kept separate, and each was subjected to DLS analysis, following the method described in Section 2.1.17. This procedure was repeated for five individual preparations of peak 1 truncated wee1/14-3-3β complex, and five individual preparations of peak 2 truncated wee1/14-3-3β complex.
2.5.6.2: Molecular Characteristics of the Truncated Wee1/14-3-3β Complex

2.5.6.2.1: Analysis of the Truncated Wee1/14-3-3β Complex using CD

A sample of truncated wee1/14-3-3β was expressed and purified using IMAC and size exclusion chromatography as detailed in Section 2.5.7.2. Following purification, a sample was taken of peak 2 protein (refer to Section 4.3.3) and was subjected to analysis using CD. The method for this analysis is the same as that detailed in Section 2.5.5.2.2. This analysis was then repeated with a second sample of the truncated wee1/14-3-3β complex purified from peak 2.

2.5.6.2.2: Crystallization Trials on the Truncated Wee1/14-3-3β Complex

Following the DLS experiments detailed in Section 2.5.6.1.2.2, samples of truncated wee1/14-3-3β were subjected to a number of crystallization trials. Samples of the truncated wee1/14-3-3β complex purified from both peak 1 and peak 2 (see Section 4.2.1.3) were used in individual crystallization trials. The following preparations from each peak were tested:

- 10 mg mL\(^{-1}\) of truncated wee1/14-3-3β
- 10 mg mL\(^{-1}\) of truncated wee1/14-3-3β with 1 mM MgCl\(_2\), 1 mM AMP-PNP
- 10 mg mL\(^{-1}\) of truncated wee1/14-3-3β incubated with PD0407824 (Section 2.5.4.4)
- 10 mg mL\(^{-1}\) of truncated wee1/14-3-3β purified with PD0407824 (Section 2.5.4.4)

In all cases, the sitting drop method of crystallization was used to set up these crystallization experiments, using the Cartesian HoneyBee nanolitre dispensing robot (Genome Solutions). An initial 480 conditions were screened, as five trays of 96 conditions, and are further described in Appendix D. In addition, the Cartesian HoneyBee nanolitre dispensing robot (Genome Solutions) was used to carry out crystallization trials on 10 mg mL\(^{-1}\) truncated wee1/14-3-3β/PD0407824 (Section 2.5.7.5.2.1) using the conditions from Screen 2 (refer to Appendix D) using the hanging drop method of crystallization.
2.6: **CDC25B Methods and Materials**

The PCR product encoding CDC25B was provided by Dr. James Dickson, cloned into the vector pFASTBac HTb.

### 2.6.1: Generation of Recombinant Baculovirus Encoding CDC25B

Recombinant baculovirus encoding CDC25B were generated using the protocol detailed in Section 2.1.8. This baculovirus was then amplified as in Section 2.1.8.4 to give a high viral titer stock.

### 2.6.2: Expression Testing of CDC25B

A 250 mL Erlenmeyer flask was used to set up a 50 mL culture of mid-log phase Sf9 cells at 1 x 10^6 cells mL^-1. A 4 µL sample of tertiary inoculum was added to the culture, which was then incubated for a total of 96 hours with rotation at 180 rpm at 28 °C. During this incubation, a 1 mL sample of culture was taken at 48 hours, 72 hours and 96 hours post infection. At 96 hours post infection, the remaining 47 mL of culture was centrifuged at 500 x g for 5 minutes at 4 °C. The supernatant was discarded and the pellet was stored at -20 °C, until required.

Each of the three 1 mL samples was centrifuged at 500 x g for 5 minutes at 4 °C. The supernatant was discarded and the pellets were re-suspended in 50 mM Tris.HCl pH 9.5, 0.1% v/v NP40 in a volume representative of an original cell count of 6 x 10^6 SF9 cells per mL. The samples were then incubated for 5 minutes on ice, followed by centrifugation at 500 x g for 5 minutes at 4 °C. The respective supernatants were saved and the pellets were re-suspended in the same volume of 50 mM Tris.HCl pH 9.5, 300 mM NaCl, 0.1% v/v NP40. The samples were then incubated for 5 minutes on ice, followed by centrifugation at 500 x g for 5 minutes at 4 °C. The respective supernatants were saved and the pellets were discarded.
Aliquots of 15 µL from each of the resulting samples were then separately mixed with 3 µL of 6 x SDS Quench dye, and analyzed by SDS PAGE (refer to Section 2.1.9).

2.6.3: IMAC Purification of CDC25B

CDC25B was partially purified using IMAC. The pellet from the original expression test (Section 2.6.2) was re-suspended in 2 mL of 50 mM Tris.HCl pH 9.5, 0.1% v/v NP40, and was incubated for 5 minutes on ice. The sample was then centrifuged at 500 x g for 5 minutes at 4 °C. The supernatant was collected and the pellet was re-suspended in 2 mL of 50 mM Tris.HCl pH 9.5, 300 mM NaCl, 0.1% v/v NP40. The sample was incubated for a further 5 minutes on ice, then centrifuged again at 500 x g for 5 minutes at 4 °C. The pellet was discarded and the supernatant was added to the supernatant from the previous incubation, to give a single 4 mL whole cell lysate sample.

The sample was then subjected to IMAC chromatography, using the protocol detailed in Section 2.1.11, with a sample buffer of 50 mM Tris.HCl pH 9.5, 150 mM NaCl, a first wash buffer of 50 mM Tris.HCl pH 9.5, 150 mM NaCl, 200 mM imidazole, a second wash buffer of 50 mM Tris.HCl pH 9.5, 150 mM NaCl, 800 mM imidazole, and an elution buffer of 50 mM Tris.HCl pH 9.5, 150 mM NaCl, 1200 mM imidazole. Aliquots of 15 µL samples of each of the eluted fractions were then separately mixed with 3 µL of 6 x SDS Quench dye and, together with the whole cell lysate sample, analyzed by SDS PAGE (refer to Section 2.1.9).

2.6.4: Re-Cloning of CDC25B to Co-Express with 14-3-3β

The PCR product encoding CDC25B was re-cloned to co-express with 14-3-3β. Both the pFASTBac HTb-cdc25b clone and the pFASTBac DUAL-weel-14-3-3β clone were separately digested with the restriction enzymes RsrII (Roche) and HindIII (Roche), following the method described in Section 2.2.2. The digested coding sequence for CDC25B was then ligated into the pFASTBac DUAL plasmid, in place of the wee1 DNA insert,
following the method described in Section 2.2.2. Confirmation of the pFASTBac DUAL-cdc25b-14-3-3β clone was sought using dideoxy nucleotide sequencing.

2.6.5: Generation of Recombinant Baculovirus Encoding CDC25B and 14-3-3β

Recombinant baculovirus encoding human CDC25B and human 14-3-3β was generated using the protocol detailed in Section 2.1.8. This baculoviral stock was then amplified as in Section 2.1.8.4 to give a stock of high viral titer.

2.6.6: Expression Testing of CDC25B/14-3-3β

The recombinant baculovirus was tested for evidence of recombinant protein expression. A 250 mL Erlenmeyer flask was used to set up a 50 mL culture of mid-log phase Sf9 cells at 1 x 10^6 cells mL^{-1}. An aliquot of 1.25 µL of quaternary inoculum was added to the culture, which was then incubated for a total of 72 hours with rotation at 180 rpm at 28 °C. During this incubation, a 1 mL sample of culture was taken at 48 and 72 hours post infection. At 72 hours post infection, the remaining 48 mL of culture was centrifuged at 500 x g for 5 minutes at 4 °C. The supernatant was discarded and the pellet was stored at -20 °C, until required.

Each of the two 1 mL samples was centrifuged at 500 x g for 5 minutes at 4 °C. The supernatant was discarded and the pellets were re-suspended 50 mM Tris.HCl pH 7.5, 0.1% v/v NP40 in a volume representative of an original cell count of 6 x 10^6 SF9 cells per mL. The samples were then incubated for 5 minutes on ice, followed by centrifugation at 500 x g for 5 minutes at 4 °C. The respective supernatants were saved and the pellets were re-suspended in the same volume of 50 mM Tris.HCl pH 7.5, 300 mM NaCl, 0.1% v/v NP40. The samples were then incubated for 5 minutes on ice, followed by centrifugation at 500 x g for 5 minutes at 4 °C. The respective supernatants were saved and the pellets were discarded. Aliquots of 15 µL aliquots from each of the resulting samples were then separately mixed with 3 µL of 6 x SDS Quench dye, and were analyzed by SDS PAGE (refer to Section 2.1.9).
2.6.7: Purification of a CDC25B/14-3-3β Complex

2.6.7.1: Attempts to Use IMAC to Purify the CDC25B/14-3-3β Complex

Following expression of the CDC25B/14-3-3β complex, as detailed in Section 2.6.6, a number of conditions were tested for the purification of this complex using IMAC. The cell pellet containing the CDC25B/14-3-3β (Section 2.6.6) complex was re-suspended in 10 mL of 1 x PBS, and the sample was then divided into 10 aliquots of 1 mL each. These samples were each centrifuged at 500 x g for 5 minutes at 4 °C, their supernatants discarded and the pellets re-suspended in 2 mL of each of the following buffers:

- 50 mM Tris.HCl pH 7.5, 150 mM NaCl
- 50 mM Tris.HCl pH 7.5, 100 mM NaCl
- 50 mM Tris.HCl pH 7.5, 200 mM NaCl
- 50 mM Tris.HCl pH 7.5, 300 mM NaCl
- 50 mM Tris.HCl pH 8.5, 100 mM NaCl
- 50 mM Tris.HCl pH 8.5, 200 mM NaCl
- 50 mM Tris.HCl pH 8.5, 300 mM NaCl
- 50 mM Tris.HCl pH 9.5, 100 mM NaCl
- 50 mM Tris.HCl pH 9.5, 200 mM NaCl
- 50 mM Tris.HCl pH 9.5, 300 mM NaCl

Each sample was then lysed using the Cell Disruptor (Constant Cell Disruption Systems), with a pressure setting of 17 kpi. From each of the 10 cell lysates, 1 mL was separately mixed with 20 µL of HP chelating resin charged with NiCl₂ (Amersham Bioscience). These samples were then incubated for 60 minutes with rotation at 30 rpm at 4 °C. Following incubation, the samples were centrifuged at 13,000 rpm (16,000 x g) in a bench top centrifuge for 2 minutes at 4 °C. The supernatant was discarded, and each pellet was re-suspended in 1 mL of the same buffer as was used for that sample previously. This centrifugation and wash step was repeated a total number of three times. Following the third wash, the pellet was re-suspended in 30 µL of wash buffer. Each of these samples was mixed with 5 µL of 6 x SDS Quench dye and was analyzed using SDS PAGE (Section 2.1.9).
2.6.7.2: Cation Exchange of the CDC25B/14-3-3β Complex

A sample of the CDC25B/14-3-3β complex was expressed as detailed in Section 2.6.6. The culture was harvested by centrifugation at 500 x g for 5 minutes at 4 °C. The supernatant was discarded and the pellet was re-suspended in 5 mL of 50 mM MES pH 6.0, 0.1% v/v NP40. The sample was then incubated for 5 minutes at 4 °C, and centrifuged at 500 x g for 5 minutes at 4 °C. Following centrifugation, the pellet was discarded and NaCl was added to the supernatant to a concentration of 50 mM NaCl.

A 5 mL SP Sepharose column (Amersham) was washed with 2 column volumes of sterile deionised water, followed by 2 column volumes of 2 M NaCl, then a further 2 column volumes of sterile deionised water. The column was then equilibrated by washing with 2 column volumes of 50 mM MES pH 6.0, 50 mM NaCl. The sample containing the CDC25B/14-3-3β complex was loaded on the column, which was further washed with 50 mM MES pH 6.0, 50 mM NaCl. The proteins that did not bind were collected as flow through. Three further washes of the column were carried out, with the NaCl concentration successively increased to 200 mM, 500 mM and 750 mM, and the eluted proteins being collected.

2.6.7.3: Anion Exchange Chromatography of the CDC25B/14-3-3β Complex

The sample containing the CDC25B/14-3-3β complex was dialyzed against 20 mM CHES pH 9.5, 50 mM NaCl, as in Section 2.1.15. Following dialysis, glycerol was added to a concentration of 10% v/v, and the sample was then concentrated to a volume of less than 5 mL using a spin concentrator with a MWCO of 10 kDa (VivaSpin).

A 1 mL Mono Q ion exchange column was washed with 2 x column volumes of sterile deionised water, followed by 2 x column volumes of 2 M NaCl, and then 2 x column volumes of sterile deionised water. The column was then finally equilibrated in 20 mM CHES pH 9.5, 50 mM NaCl. The sample containing the CDC25B/14-3-3β complex was then
loaded on to the equilibrated column. The non-binding proteins were washed through in 20 mM CHES pH 9.5, 50 mM NaCl. A gradient was set up to wash the column in ever-increasing concentrations of NaCl. Initially, the column was washed with 20 mM CHES pH 9.5, 50 mM NaCl. Over a period of 30 minutes, the NaCl concentration was brought to a value of 1,000 mM. Aliquots of 15 µL from each of the resulting fractions were then separately mixed with 3 µL of 6 x SDS Quench dye, and analyzed by SDS PAGE (refer to Section 2.1.9).

2.6.7.4: Size Exclusion Chromatography of the CDC25B/14-3-3β Complex

The samples eluted from the Mono Q column (Amersham) (Section 2.6.7.4) were pooled and concentrated to a volume of less than 5 mL using a spin concentrator with a MWCO of 10 kDa (VivaSpin). While still in the concentrator, the sample was diluted to 20 mL with 20 mM Tris.HCl pH 7.5, 150 mM NaCl, then re-concentrated to be less than 5 mL in volume. This dilution and concentration was repeated a total number of four times, before a final concentration step in which the volume of the sample was reduced to 0.5 mL, in 20 mM Tris.HCl pH 7.5, 150 mM NaCl. The sample was then subjected to size exclusion chromatography, using an S200 10/300 column (Amersham Bioscience), following the protocol detailed in Section 2.1.12.1, with a sample buffer of 20 mM Tris.HCl pH 7.5, 150 mM NaCl. Aliquots of 15 µL samples of the fractions containing the CDC25B/14-3-3β complex were mixed with 3 µL of 6 x SDS Quench dye, and were analyzed by SDS PAGE (refer to Section 2.1.9).

2.6.8: Larger Scale Expression and Purification of the CDC25B/14-3-3β Complex

2.6.8.1: Large Scale Expression of CDC25B/14-3-3β

The CDC25B/14-3-3β complex was expressed and a number of approaches were trialled for the purification of the complex. A 1,000 mL Erlenmeyer flask was used to set up a 200 mL
culture of mid-log phase Sf9 cells at 1 x 10^6 cells mL^{-1}. Glucose and L-glutamine were added to the culture at final concentrations of 1 mM each. A 5 µL sample of quaternary inoculum was also added to the culture, which was then incubated for a total of 96 hours with rotation at 180 rpm at 28 °C. Following this incubation, the culture was harvested by centrifugation at 500 x g for 5 minutes at 4 °C, the supernatant discarded and the pellet stored at -20 °C.

2.6.8.2: Anion Exchange Chromatography of the CDC25B/14-3-3β Complex

The cells containing the CDC25B/14-3-3β complex were lysed and the complex was partially purified using anion exchange chromatography. The cell pellet containing the expressed CDC25B/14-3-3β was re-suspended in 20 mL of 50 mM Tris.HCl pH 7.5, 0.1% v/v NP40, and was incubated for 5 minutes at 4 °C. Following incubation, the sample was centrifuged at 500 x g for 5 minutes at 4 °C, the pellet discarded, and NaCl added to the supernatant to a final concentration of 150 mM.

The supernatant, containing CDC25B/14-3-3β, was then subjected to anion exchange chromatography using a 5 mL HiTrap Q FF ion exchange column (Amersham Bioscience), following the method described in Section 2.6.7.4, but with a sample buffer of 20 mM ethanolamine pH 9.5, 50 mM NaCl, a wash buffer of 20 mM ethanolamine pH 9.5, 250 mM NaCl and an elution buffer of 20 mM ethanolamine pH 9.5, 500 mM NaCl. Aliquots of 15 µL from every second fraction containing CDC25B/14-3-3β were then separately mixed with 3 µL of 6 x SDS Quench dye, and analyzed by SDS PAGE (refer to Section 2.1.9).

2.6.8.3: Size Exclusion Chromatography of the CDC25B/14-3-3β Complex

Following anion ion exchange chromatography, the sample containing CDC25B/14-3-3β was further purified by size exclusion chromatography. The samples eluted from the anion exchange column were pooled and concentrated to a volume of less than 5 mL using a spin concentrator with a MWCO of 10 kDa (VivaSpin). Within the same concentrator, the sample was then diluted to 20 mL with 20 mM Tris.HCl pH 7.5, 200 mM NaCl, then re-concentrated.
to be less than 5 mL in volume. This dilution and concentration step was then repeated a total of four times, after which the sample was concentrated further to a final volume of 5 mL in 20 mM Tris.HCl pH 7.5, 200 mM NaCl.

The sample, containing CDC25B/14-3-3β, was then subjected to size exclusion chromatography. This purification step was undertaken using an S200 16/600 column (Amersham Bioscience) following the protocol detailed in Section 2.1.12.1, with a sample buffer of 20 mM Tris.HCl pH 7.5, 150 mM NaCl. Aliquots of 15 µL from fractions containing the CDC25B/14-3-3β complex were mixed with 3 µL of 6 x SDS Quench dye, and were analyzed by SDS PAGE (refer to Section 2.1.9).

### 2.6.9: Sypro Ruby Analysis of the CDC25B/14-3-3β Complex

An analysis of the CDC25B/14-3-3β complex was undertaken using Sypro Ruby (Invitrogen, Molecular Probes) to determine the stoichiometry of binding between CDC25B and 14-3-3β. A small scale sample of CDC25B/14-3-3β was expressed and purified using the methods described in Sections 2.6.7.2 to 2.6.7.5. Following purification, the sample was concentrated to a final volume of 0.5 mL. The stoichiometric analysis of the sample was then undertaken using a method identical to that detailed in Section 2.1.8 for wee1/14-3-3β. This analysis was then repeated.

### 2.6.10: Analytical Size Exclusion of the CDC25B/14-3-3β Complex

Analytical size exclusion chromatography was used to determine the apparent molecular weight of the CDC25B/14-3-3β complex in solution. A small-scale sample of CDC25B/14-3-3β was expressed and purified using the methods described in Section 2.6.7, and was concentrated to a final volume of 0.5 mL. The sample of CDC25B/14-3-3β was then subjected to analytical size exclusion chromatography, following the method detailed in Section 2.1.12.2, using a buffer of 20 mM Tris.HCl pH 7.5, 150 mM NaCl.
The aims of this part of the research were the expression and purification of CDK1 and cyclin B1, as well as preparation and characterization of the complex they form. Presented in this chapter are the experimental results from these analyses, and a discussion of those results, including future directions for the research.

3.1: Cloning, Expression and Purification of Cyclin B1

3.1.1: Amplification of the DNA Coding Sequence for Cyclin B1

The human cyclin B1 coding sequence was amplified by PCR, using a commercially available cDNA library (BD Biosciences Universal Human cDNA Library) as a template. The resulting PCR products were analyzed by agarose gel electrophoresis (Section 2.2.1), and the gel is presented in Figure 3.1. PCR amplification resulted in a single band of approximately the correct size for cyclin b1 (1,300 bp).

Figure 3.1: agarose gel electrophoresis analysis of the PCR amplification of cyclin B1. Lanes 1 and 7 show DNA ladders, with relevant markers indicated. Lanes 2 – 6 show the PCR amplifications of cyclin b1.
3.1.2: Construction of the pFASTBac HTb-cyclin b1 Clone

Following PCR amplification, a pFASTBac HTb-cyclin b1 clone was prepared, transformed into *E. coli* DH5α, and grown on a Luria Broth/agar plate. Eight single colonies were picked, and grown as a 5 mL culture in Luria Broth. The plasmid DNA present in each sample was purified, digested with appropriate restriction enzymes (Section 2.2.2), and analyzed by agarose gel electrophoresis (Figure 3.2).

Figure 3.2: agarose gel analysis of the NcoI/HindIII restriction enzyme digestion of potential pFASTBac HTb-cyclin b1 clones. Lane 1 is a DNA ladder with relevant markers indicated. Lane 2 is a doubly-digested negative control (does not contain the cyclin B1 PCR product). Lanes 3 – 9 are experimental samples, doubly-digested to ascertain whether they contain the DNA coding sequence for cyclin B1.

Following this analysis, dideoxy nucleotide sequencing was used to confirm the identity of the pFASTBac HTb-cyclin b1 clone (Appendix A).

3.1.3: Expression and Purification of Cyclin B1

Using a sequence-verified pFASTBac HTb-cyclin b1 clone, a transfection was undertaken to produce recombinant baculovirus encoding cyclin B1. This was used to infect Sf9 cells, which were tested for evidence of expression of cyclin B1. Samples of the expression test were taken at 48 and 72 hours post infection, and were analyzed by SDS PAGE (Section 2.2.4). An image of the gel is presented in Figure 3.3.
The expression of cyclin B1 was not apparent, despite a number of repetitions of the analysis; SDS PAGE analysis (Figure 3.3) of the culture samples did not indicate the strong expression of a protein band at the correct molecular weight (48 kDa); in addition, western blot analysis was trialled as a higher specificity method to detect expression, with inconclusive results (His-tag expression as part of either a recombinant protein, or from a his-tagged ladder included as a positive control could not be detected, data not shown). The infection profile of the culture (cell size, gross morphology and total count following infection) suggested that expression should have occurred.

Thus, it was considered possible that the expression of cyclin B1 was low in relation to other Sf9 and/or baculoviral proteins and therefore not clearly evident by SDS PAGE analysis of the whole cell lysate. Accordingly, at 72 hours post infection, the culture was harvested, including the collection of both cytoplasmic and nuclear fractions, and an attempt to purify cyclin B1 was made using IMAC (refer to Section 2.2.4), with analysis by SDS PAGE (Figure 3.4). This showed that a His-tagged protein of the correct molecular weight for cyclin B1 was being expressed but at low levels.
3.1.4: Large-Scale Expression and Purification of Cyclin B1

A large scale expression of cyclin B1 was then undertaken. At 72 hours post infection, the Sf9 cell culture was harvested by centrifugation, and cyclin B1 was partially purified using IMAC (refer to Section 2.2.6), and analyzed by SDS PAGE (Figure 3.5). This analysis showed overloading of the lysate and flow-through samples (lanes 1 and 3, Figure 3.5), but a clear indication of cyclin B1 purification (lanes 4 to 14, Figure 3.5).
Following IMAC partial purification, the fractions containing cyclin B1 were pooled and buffer-exchanged into 20 mM Tris.HCl pH 7.5, 50 mM NaCl, 10% glycerol. The sample was then concentrated to a total volume of 5 mL, and further purified using size exclusion chromatography (Section 2.2.7). The resulting UV trace from this analysis is presented in Figure 3.6. The samples were then analyzed by SDS PAGE, as shown in Figure 3.7.

**Figure 3.6:** UV trace following size exclusion chromatography of the sample containing cyclin B1. The sample was partially purified using IMAC prior to size exclusion chromatography. Fractions corresponding to the area indicated above (fractions 11 – 22) were then analyzed by SDS PAGE.

**Figure 3.7:** SDS PAGE analysis of fractions containing cyclin B1 following IMAC and size exclusion chromatography. Lane 1 shows the infected whole cell lysate. Lane 2 shows the sample prior to size exclusion chromatography. Lane 3 shows a protein ladder with markers indicated. Lanes 4 – 15 shows the eluted samples following size exclusion chromatography (fractions 11 – 22 from Figure 3.6).
While the major protein in solution following IMAC was cyclin B1, the sample concentration sharply decreased between IMAC and size exclusion chromatography, such that the amount of cyclin B1 following size exclusion chromatography was typically only 40 – 60% of the amount of cyclin B1 which had been purified using IMAC. It was found that the molecule was very unstable; it precipitated very easily, bound to the spin concentrator membranes and degraded faster than could be purified, despite the use of broad spectrum protease inhibitors. Steps taken to stabilize recombinant cyclin B1 included:

- variations in the buffer pH (between pH 5.5 and pH 9.0);
- NaCl concentrations (between 150 mM and 500 mM);
- the use of glycerol (up to 10 – 15% w/v);
- the use of reducing agents (both 1 – 2 mM DTT and 1 – 2 mM β-ME);
- increased concentrations of broad spectrum protease inhibitors and the incorporation of EDTA in all buffers.

Unfortunately, none of these measures appeared to improve the stability of cyclin B1 (data not shown). Literature analysis revealed that this behaviour is, unfortunately, characteristic of this molecule in vitro [8], [13]. For example, the solved structure of the cyclin B1 construct (PDB ID 2B9R, [25]) was only stable after truncating the molecule to only encompass residues 165 – 433 and mutation of residues C167S, E183A, E184A, C238S and C350S, prior to crystallization trials [25]. It is possible that these mutations and truncations remove the more flexible regions of the protein, or the more reactive amino acids or segments within the protein; for example, the presence of the cysteines may result in the formation of inappropriate disulfide bridges in vitro. Indeed, cyclin B1 over-expression for these crystallisation trials resulted in protein yields of 11 mg of protein per 6 L of bacterial culture [25], a total yield that is in excess of that achieved within this research. Thus, like that of the construct for which the crystal structure was solved, it may be necessary to introduce one or a number of these alterations to the cyclin B1 construct, to improve its stability.
3.2: Cloning, Expression and Purification of CDK1

3.2.1: PCR Amplification of the DNA Encoding CDK1

A culture of HeLa cells was grown and the whole cell RNA present within this sample was isolated (Section 2.3.1.1). The RNA in this sample was then analyzed by TBE agarose gel electrophoresis (Figure 3.8).

![TBE agarose gel of 5 μL of RNA isolated from HeLa cells. This RNA was used to isolate and amplify the cDNA encoding CDK1.](image)

Figure 3.8: TBE agarose gel of 5 μL of RNA isolated from HeLa cells. This RNA was used to isolate and amplify the cDNA encoding CDK1.

The purpose behind total RNA isolation was the reverse transcription of the mRNA content for the eventual PCR amplification the coding sequence of human CDK1. The isolated RNA was analysed on a TBE agarose gel to assess the integrity of the total RNA, prior to reverse transcription to cDNA. In such a TBE gel, rRNA should appear as three distinct bands, two of equal intensity and a third of greater intensity, and mRNA should appear as a smear on the gel [118].

The gel showing total RNA that was eventually used to clone the coding sequence for human CDK1 (Figure 3.8) shows a number of bands. The largest band (corresponding to the 28S rRNA component of the sample) is, as expected, approximately twice the intensity of the other band(s). Below this, the band with the next largest size (corresponding to the 18s rRNA...
component of the sample) appears as expected. At a smaller size, however, there should appear another band (corresponding to the 5S rRNA component of the sample), of an intensity equal to that of the 18S rRNA band. Unfortunately, this latter band is not visible. The beginnings of a band, however, can be seen, but it is obscured by the presence of the loading/running dye being used. Smears may also be seen between the bands, suggesting the presence of mRNA in the total RNA sample. Overall, this suggested that the extracted total RNA was likely to be intact, and thus, was reverse transcribed to cDNA (Section 2.3.1.2). The resulting cDNA sample was diluted 1:10, and was used as a template for the PCR amplification of the DNA encoding CDK1. The resulting samples were analyzed by agarose gel electrophoresis (Figure 3.9).

Figure 3.9: agarose gel analysis showing the PCR amplification of the cDNA encoding CDK1. Lane 1 is a DNA ladder with markers indicated. Lanes 2 – 4 show the amplified PCR product, later confirmed as the coding sequence for CDK1.

3.2.2: Cloning of CDK1

Following amplification, a pPROEx HTb-\textit{cdk1} clone was prepared. The PCR product and vector were digested with complementary enzymes, ligated together, and transformed into \textit{E. coli} DH5\textalpha. Transformants were picked, grown as a culture, and the plasmid DNA present in each sample was purified, digested with appropriate restriction enzymes (Section 2.3.3), and analyzed by agarose gel electrophoresis (Figure 3.10).
Following agarose gel electrophoresis, the samples were sent for confirmation of their identity using dideoxy nucleotide sequencing (Appendix A), which indicated that the clone was pPROEx HTb-\textit{cdk1}. There was, however, an insertion at nucleotide position 108, and, following re-cloning into pFASTBac DUAL (below), mutagenesis experiments were undertaken to remove the insertion and revert the DNA sequence to that of the wild-type.

### 3.2.3: Mutagenesis and Sub-cloning of CDK1

A pFASTBac DUAL-\textit{cdk1} clone (with CDK1 expression under the control of the p10 promoter) was prepared by Dr. James Dickson (refer to Section 2.3.4).

Expression of CDK1 was carried out from the pFASTBac DUAL clone, using the Sf9 cell-based expression system, rather than from the pPROEX HT clone, using \textit{E. coli} expression, for two reasons. Firstly, it was thought that expression of this molecule within an Sf9 cell culture environment would allow for any post-translational modifications that CDK1 may require as part of its activity. Secondly, expression from the pPROEX HT vector yields a recombinant protein with an N-terminal purification tag. Expression from the pFASTBac DUAL vector, from the multiple cloning site under the control of the p10 promoter (refer to Section 2.1.6) yields a protein without an N-terminal purification tag (a native protein).
Given that the interaction of this molecule with its binding partner, cyclin B1, involves the N-terminal region of CDK1, a native construct (lacking an N-terminal fusion) is likely to be more appropriate for attempts to prepare a functional complex, and hence, research progressed using the pFASTBac DUAL-\textit{cdk1} construct. Following cloning, the DNA coding sequence for CDK1 was mutated to remove the adenosine insertion at position 108, as detailed in Section 2.3.5. The success of these two procedures was confirmed by dideoxy nucleotide sequencing, the results of which are presented in Appendix A.

### 3.2.4: Expression of CDK1

Using a sequence-verified pFASTBac DUAL-\textit{cdk1} clone, a transfection was undertaken to produce recombinant baculovirus encoding CDK1 (Section 2.3.6). Recombinant baculovirus was tested for evidence of expression of CDK1 by infection of mid-log phase Sf9 cells (Section 2.3.7). Samples were taken at 48 and 72 hours post infection, and analyzed by SDS PAGE which provided evidence that CDK1 was being expressed (Figure 3.11).

![Figure 3.11: SDS PAGE analysis showing the timeline of CDK1 expression.](image)

Lane 1 shows an uninfected whole cell lysate sample. Lanes 2 and 3 show CDK1 expression at 48 hours (Lane 2) and 72 hours (Lane 3) post infection, respectively. Lane 4 is a protein ladder with markers indicated.
3.2.5: Purification of CDK1

As recombinant CDK1 was expressed without a recombinant polyhistidine purification tag, a number of techniques were employed to purify CDK1, namely ammonium sulfate precipitation, ion exchange and size exclusion chromatography. Sf9 cells containing CDK1 were harvested by centrifugation, with the total cellular protein being collected. Both cytoplasmic and nuclear fractions were harvested in order to negate any issues regarding the sub-cellular location of the protein. The lysate was subjected to an ammonium sulfate precipitation, with collection of the pellet between 40% and 70% ammonium sulfate saturation (Section 2.3.8.2); these percentages were chosen based on a previous report of ammonium sulfate precipitation of CDK1 [119]. The sample was then dialyzed to remove residual ammonium sulfate, before being further purified using size exclusion chromatography (Section 2.3.8). The size exclusion UV trace is presented in Figure 3.12, and the resulting samples were analyzed by SDS PAGE (Figure 3.13).

![Figure 3.12: UV trace following size exclusion chromatography of CDK1. The protein was found to elute in fractions 24 – 34, which correspond to the area indicated in the above UV trace.](image-url)
Figure 3.13: SDS PAGE analysis of CDK1, following IMAC, ammonium sulfate precipitation and size exclusion chromatography. Lane 1 is a protein ladder with markers indicated. Lanes 2 – 13 are the samples eluted from the size exclusion column, fractions 23 – 34, that contained CDK1.

The fractions containing CDK1 were then pooled and were applied to a Mono Q ion exchange column (Section 2.3.8.4). A UV trace from this analysis is presented in Figure 3.14. The resulting fractions were then analyzed by SDS PAGE (Figure 3.15).

Figure 3.14: UV trace for anion exchange chromatography of CDK1. Elution from the Mono Q column was with a gradient of NaCl from 50 mM to 1 M, over a total time of 40 minutes. CDK1 was found to elute in the final peak, as shown.
The CDK1 protein was identified throughout this protocol based on its expected molecular weight; a typical yield from this purification procedure was 80 – 100 μL of CDK1 at a final concentration of 10 mg mL$^{-1}$, purified from 200 mL of Sf9 cell culture.

### 3.2.6: Crystallization of CDK1

Following CDK1 purification, the sample was subjected to crystallization trials, using the Cartesian HoneyBee nanolitre dispensing robot (Genome Solutions) (Section 2.3.9). Of all of the conditions tested, 10 – 15 % of drops were clear, which included those where a precipitate formed in the first 24 hours after the trials were set up, then dissolved. Non-uniform and heavy precipitates, which looked to exhibit a number of nucleation or focal points, were commonly formed where the crystallization conditions were of the range 15 – 30% PEG 3,350, 4,000 or 6,000, or MPEG 5,000 at a low pH of 4.2 – 5.6, and generally appeared in the first week after the trials had been set up. From 2 – 3 months after the trials were set up, phase separations were noted where the crystallization conditions were of the range 14 – 28% MPEG 5,000 or PEG 6,000 at pH 6.7 – 7.3. Future crystallization trials for CDK1 are likely to focus on further exploration of these conditions. These trials, however, did not yield any protein crystals.
In this part of the research, CDK1 was successfully expressed and purified, with a yield of 80 – 100 μL of 5 mg mL⁻¹ CDK1 protein per 200 mL of Sf9 culture (calculation based on whole sample protein concentration, using the formula detailed in Section 2.1.16), before being subjected to crystallisation trials. Future directions for the investigation of this protein include verification of the protein using a method such as peptide mass fingerprinting or Western blot analysis, followed by more extensive crystallisation trials, as well as biochemical or biophysical characterisation of the protein. With this in mind, it may prove necessary to change the purification of the protein, to include a higher affinity method. For example, this might include an immuno-precipitation step, although care should be taken as such a step is likely to require an extreme pH change or the use of chaotropic agents, which may irrevocably alter protein function and/or crystallizability. Fewer steps in the purification protocol may mean a higher yield of protein available for extensive characterisation.

Attempts to isolate cyclin B1 were less successful, because of instability, but the expression profile, solubility and level of purity achieved was on a par with, if not better than, that achieved by other groups [8], [13], [25]. It was felt, however, that the expression and purification profile of this molecule could be improved further, and thus, future directions for this protein include further optimization of purification. The focus of this improvement is likely to be either alteration of the current purification protocol, or an alteration of the expression methods. Possible changes could include an alternative high affinity step, such as an immuno-precipitation, which may allow a higher level of purity to be reached in a single step. Mutagenesis, in a similar manner to that undertaken to solve the structure of the cyclin B1 fragment, may also be useful. The subject of those crystal trials was cyclin B1 (165 – 433) with mutations of C167S, E183A, E184A, C238S and C350S. Potentially useful constructs could thus include cyclin B1 (1-433) or cyclin B1 (165-433), with or without mutagenesis of the three cysteines, and with and without mutagenesis of the two glutamic acids. Alternatively, if the protein was purified in the presence of its native binding partner, CDK1, this might achieve the desired increased stabilization of cyclin B1.
A major goal of this part of the research was the preparation of the CDK1/cyclin B1 complex, with a view towards its biophysical, biochemical or structural characterization. The \textit{in vivo} formation of the cyclin B1/CDK1 complex relies upon two events, the interaction of the proteins, and the phosphorylation of CDK1 on residue Thr-160, carried out \textit{in vivo} by CAK (Section 1.3.1). It has been shown, however, that CAK can also be provided \textit{in vitro} to induce the formation of the complex between CDK1 and cyclin B1, and that this activity is not species-specific. Indeed, CAK enzymes all share substantial similarity, such that the CAK that is present in an uninfected Sf9 cell lysate may be substituted for human CAK [8], [119]. With this in mind, a likely strategy for reconstitution of this complex would be the dual expression of cyclin B1 and CDK1, with incubation in the presence of uninfected Sf9 cell extract (containing Sf9 CAK), Mg$^{2+}$ (required for CAK activity) and ATP (as the phosphate donor), and subsequent purification using IMAC. Similar to the wee1/14-3-3β complex, the cloning rationale would include differential tag expression, whereby cyclin B1 is expressed with a polyhistidine N-terminal fusion, allowing IMAC purification of this molecule, and co-purification of CDK1. As a step towards this goal, the coding sequence for cyclin B1 was re-cloned into the pFASTBac DUAL-cdk1 construct (Section 2.4.1). The resulting pFASTBac DUAL-cyclin b1-cdk1 clone was transfected (Section 2.4.2) and tested for evidence of co-expression (Section 2.4.3), with subsequent analysis by SDS PAGE (Figure 3.16).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure3_16.png}
\caption{SDS PAGE analysis of the attempt at co-expression of CDK1 and cyclin B1. Lane 1 shows a protein ladder with markers indicated. Lane 2 shows an uninfected Sf9 cell lysate sample. Lane 3 shows a sample of Sf9 cells infected with the CDK1/cyclin B1 dual expression baculoviral sample.}
\end{figure}
Unfortunately, there was no evidence of expression of either cyclin B1 or CDK1 from the SDS PAGE analysis; Figure 3.16 shows a representative gel from this analysis, which was repeated both to compensate for overloading present in the above gel and to confirm a lack of expression of both CDK1 and cyclin B1. This may be due to low expression levels, as was seen in the expression profile of cyclin B1. Thus, the next steps regarding this complex include re-confirming the integrity of the baculovirus used to express the proteins, with subsequent trials to express and purify the complex. Verification of expression should be undertaken using a method such as western blotting analysis, to confirm the presence of expressed cyclin B1 and CDK1. Once this has been achieved, the overall aim is to undertake further biochemical and/or biophysical analysis of the complex, and to subject it to crystallization trials.
The aim of this part of the research was the expression, purification and characterization of the wee1/14-3-3β complex, as well as crystallization and structural analysis of this complex. To achieve these aims, both full length and truncated wee1 and 14-3-3β were co-expressed and purified, and a number of techniques were used to analyze the activity and integrity, oligomeric state and molecular arrangements of these complexes.

4.1: Expression and Purification of the Wee1/14-3-3β Complex

4.1.1: Wee1/14-3-3β Expression Testing and Enrichment Assay

A small-scale (50 mL) culture of Sf9 cells was set up, and was infected with recombinant baculovirus encoding wee1 and 14-3-3β (Section 2.5.1), and a 1 mL sample was taken at 72 hours post infection. The whole cell lysate sample was harvested (Section 2.5.1), then analyzed by SDS PAGE (Figure 4.1), which, despite overloading of the negative control (Lane 2 of Figure 4.1), indicated that wee1 and 14-3-3β had been successfully co-expressed, based on the projected molecular weights of the proteins.

Figure 4.1: SDS PAGE analysis of the expression of wee1 and 14-3-3β, at 72 hours post infection. Lane 1 is a protein ladder with markers indicated. Lane 2 is a sample of uninfected Sf9 whole cell lysate. Lane 3 is a sample of infected Sf9 whole cell lysate, harvested at 72 hours post infection. There appears to be doublet in Lane 3 at the expected molecular weight of 14-3-3β; purification, however, removes the higher molecular weight species.
Following this initial expression test, experiments were carried out to determine optimum lysis buffer and method conditions. The remaining culture from the initial expression test was pipetted into aliquots, and an enrichment assay was undertaken in the presence of ten different buffer conditions (refer to Section 2.5.1.2). All of these samples were analyzed by SDS PAGE (Figure 4.2). By visual comparison, it was found that the greatest level of wee1 and 14-3-3β, in the presence of the fewest contaminants, was where lysis was undertaken in the presence of 50 mM Tris.HCl pH 7.5, 300 mM NaCl.

Figure 4.2: SDS PAGE analysis of enrichment assays across 10 different buffers to determine optimum conditions for cell lysis for wee1/14-3-3β purification. Lane 1 is a protein ladder with markers indicated. Lane 2 is the infected whole cell lysate. Lanes 3 – 12 are the enrichment assays carried out in the presence of hypotonic lysis (Lane 3), 50 mM Tris.HCl pH 7.5, 100 – 300 mM NaCl (Lanes 4 – 6), 50 mM Tris.HCl pH 8.5, 100 – 300 mM NaCl (Lanes 7 – 9), 50 mM Tris.HCl pH 9.5, 100 – 300 mM NaCl (Lanes 10 – 12).

4.1.2: Large Scale Expression and Purification of the Full Length Wee1/14-3-3β Complex

4.1.2.1: Large Scale Expression and Purification of the His-Tagged Wee1/14-3-3β Complex

A large-scale (400 mL) culture of Sf9 cells was grown and infected with baculovirus encoding the wee1 and 14-3-3β proteins, harvested by centrifugation and lysed by cell
disruption (Section 2.5.3.2.1). Following protein harvest, the wee1/14-3-3β complex was partially purified using an IMAC column, with elution of the complex in 50 mM Tris.HCl pH 7.5, 300 mM NaCl, 200 mM imidazole (Section 2.5.3.2.2). The resulting fractions were analyzed by SDS PAGE (Figure 4.3).

![Figure 4.3: SDS PAGE analysis of the IMAC purification of the wee1/14-3-3β complex. Lane 1 is the infected whole cell lysate. Lane 2 shows the flow through proteins that did not bind the column. Lane 3 shows proteins that were washed out of the column in 40 mM imidazole. Lane 4 is a protein ladder with markers indicated. Lanes 5 – 10 are the eluted fractions containing wee1/14-3-3β complex.]

To confirm that the major proteins being purified were wee1 and 14-3-3β, the respective bands were excised from an SDS PAGE, and the proteins were identified using peptide mass fingerprinting (Figure 4.4), carried out through the Centre for Genomics and Proteomics, University of Auckland (Section 2.5.3.2.2).

![Figure 4.4: Peptide mass fingerprinting results for the wee1/14-3-3β complex following IMAC purification and SDS PAGE analysis. This was carried out by the Centre for Proteomics and Genomics, University of Auckland. These results confirmed that the purified proteins are human wee1 and human 14-3-3β.]

Chapter 4
Experimental Results: Wee1/14-3-3β

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Trypsin is predicted to cleave wee1 into up to 80 fragments (Appendix C); these results matched 21 trypsin digested fragments from the expressed wee1 to the coding sequence of wee1. Trypsin is predicted to cleave 14-3-3β into up to 30 fragments (Appendix C); these results matched 13 trypsin digested fragments from the expressed 14-3-3β to the coding sequence of 14-3-3β. These results confirm that the proteins being purified are human wee1 and human 14-3-3β.

Following partial IMAC purification of the complex, buffer exchange was undertaken to reduce the amount of imidazole present, and the wee1/14-3-3β sample was then concentrated to a total volume of 0.5 mL, before being further purified using an S200 10/30 size exclusion column (Amersham Biosciences). The UV trace from this column is presented in Figure 4.5. The eluted fractions were then analyzed by SDS PAGE (Figure 4.6).

![Figure 4.5: UV trace of the further purification of the wee1/14-3-3β complex using size exclusion chromatography.](image-url)

The wee1/14-3-3β complex was eluted in the void volume, in the fractions indicated. Size exclusion columns with different pore sizes were trialled, but none were successful in moving this protein from the void peak, to a resolved peak (data not shown).
4.1.2.2: Small Scale Digestion of the Wee1/14-3-3β Complex with the rTEV Protease

A sample of purified wee1/14-3-3β was incubated with purified rTEV protease in the presence of DTT (Section 2.5.1.3.4) to determine whether this enzyme could be used to remove the polyhistidine purification tag. Following incubation, the sample was analyzed by SDS PAGE (Figure 4.7).
This analysis indicated that under these conditions, a proportion of wee1 was able to be digested by the rTEV protease, to remove the polyhistidine purification tag. Thus, steps were taken to purify the untagged wee1/14-3-3β complex.

4.1.2.3: Large Scale Expression and Purification of the Un tagged Wee1/14-3-3β Complex

Purification of wee1/14-3-3β was then undertaken to include cleavage of the polyhistidine purification tag, to give an untagged wee1/14-3-3β complex. A sample of wee1/14-3-3β was expressed and purified by IMAC, as in Sections 2.5.1.3.1 and 2.5.1.3.2. The fractions containing wee1/14-3-3β were pooled and incubated with rTEV, in conjunction with dialysis to reduce the imidazole concentration (Section 2.5.1.3.5). Untagged wee1/14-3-3β was further purified by size exclusion chromatography (Section 2.5.1.3.3). The UV trace from this analysis is presented in Figure 4.8. Every second fraction from this column was then analyzed by SDS PAGE (Figure 4.9).
During its purification, the His-tagged wee1/14-3-3β complex consistently eluted in the excluded (void) volume of the size exclusion chromatography column. In contrast, the untagged wee1/14-3-3β complex was able to be purified as a separate peak, following size exclusion chromatography analysis. Overall, this analysis indicated that the untagged wee1/14-3-3β complex was able to be purified.

### 4.2: Tryptic Analysis of the Wee1/14-3-3β Complex

The wee1/14-3-3β complex was subjected to trypsin digestion (refer to Section 2.5.2), whereby either 10 μL or 20 μL of the wee1/14-3-3β complex (4 mg mL⁻¹) was incubated with a serial dilution of trypsin (final concentrations of 1 mg mL⁻¹ to 0.2 x 10⁻⁶ mg mL⁻¹) and the resulting samples were analyzed by SDS PAGE (Figure 4.10).
Figure 4.10: SDS PAGE analysis of the trypsin digest of the wee1/14-3-3β complex. Lane 1 shows the sample prior to trypsin digest. Lane 2 is a protein ladder, with markers indicated. Lanes 3 – 6 show the last four samples of the serial dilution following trypsin digestion (final concentrations of 15 x 10⁻⁶, 3.8 x 10⁻⁶, 0.95 x 10⁻⁶ and 0.24 x 10⁻⁶ mg mL⁻¹) at the lower concentration of wee1/14-3-3β. Lanes 7 – 10 show the last four samples of the serial dilution following trypsin digestion (concentrations as above) at the higher concentration of wee1/14-3-3β.

This trypsin digestion revealed two aspects of the complex:

- the molecular weight of the 14-3-3β component of the complex did not appear to change, no matter what the concentration of trypsin, which suggested that the majority of the 14-3-3β molecule is trypsin-resistant, as the SDS PAGE resolution would have revealed a change in molecular weight of more 1 – 2 kDa;

- the molecular weight of the wee1 component of the complex dropped from 75 – 80 kDa (Lane 1 Figure 4.10) to a species of 40 – 50 kDa (Lanes 4, 5, 7 and 8, Figure 4.10) and then was proteolysed completely (Lane 1 and, to a lesser extent, Lane 2, Figure 4.10), which suggested that a 40 – 50 kDa portion of the wee1 protein was reasonably proteolytically stable.

For comparison, predictions of the trypsin cleavage sites in both wee1 and 14-3-3β are presented in Appendix C. Following this analysis, bioinformatics analyses were undertaken using the programs DisEMBL [100] (Figure 4.11) and Phyre [101] (Figure 4.12) to predict probability of disorder of the wee1 molecule (refer to Section 2.5.2).
This analysis predicted that wee1, particularly the N-terminal domain, is likely to be relatively disordered. A Probability of Disorder of 1.0 represents a high probability of that residue (denoted by the horizontal axis) will be disordered; conversely a Probability of Disorder of 0.0 represents a low probability of that residue being disordered.

This predictive tool also indicated that the wee1 protein is likely to be relatively disordered, in particular as regards the N-terminal domain. The ‘Index’ numbers refer to the residue number for the wee1 protein. The ‘Disopred’ ‘d’ indicates a residue that is predicted as being disordered. The ‘Disopred’ ‘0’ indicates a residue that is predicted to be ordered. The ‘Diso_prob’ numbers indicate a probability that the disorder prediction is likely to be correct, 0 being a low probability of disorder and 9 being a high probability of disorder.

Both of these analyses predicted that the N-terminal domain of wee1 is likely to be relatively disordered, with only two or three segments that are ordered (approximately residues 120 –
140, 180 – 200 and 240 – 260 according to DisEMBL and approximately residues 15 – 30 and 90 – 100, according to Phyre); both DisEMBL and Phyre predicted the remainder of the 298 residues would be disordered. The central region of wee1 appears reasonably well ordered in both predictions. This corresponds to the kinase domain of wee1, which is, indeed, well ordered, as shown in the crystal structure of the domain (PDB ID 2IN6, [39]). The C-terminal domain of wee1 is also predicted to be relatively disordered; DisEMBL predicted that this domain would be more disordered than ordered, whereas Phyre predicted that approximately half of the domain would be disordered.

Analysis of the sequence of wee1 indicated that the region including the kinase domain and extending to the C-terminal domain has a molecular weight of 42 kDa, and might, therefore, correspond to the proteolytically stable fragment found in the trypsin digest experiment. This portion also corresponds to the most rigid section of the molecule, as indicated in the above disorder probabilities (Figures 4.11 and 4.12, respectively). Consequently, a truncated version of wee1, comprising the kinase and C-terminal domains was considered a better candidate for analysis. A construct comprising wee1 residues 291 – 646 (Mr 42 kDa) with co-expression of 14-3-3β (Mr 28 kDa) had previously been constructed and was available.

### 4.3: Expression and Purification of Truncated Wee1/14-3-3β

#### 4.3.1: Expression Testing of the Truncated Wee1/14-3-3β Complex

The recombinant baculovirus encoding truncated wee1 and 14-3-3β was thawed, and was tested for evidence of expression of both proteins, by using SDS PAGE to analyze culture samples taken at 48, 72 and 96 hours post infection (Section 2.5.3.1). The resulting gel is presented in Figure 4.13.
Figure 4.13: SDS PAGE analysis of the timeline of the expression of the truncated wee1/14-3-3β complex. Lane 1 is the uninfected whole cell lysate. Lanes 2 – 4 are the infected cell lysates at 48 (Lane 2), 72 (Lane 3) and 96 (Lane 4) hours post infection. Lane 5 is a protein ladder with markers indicated.

4.3.2: IMAC Purification of the Truncated Wee1/14-3-3β Complex

A larger scale culture of truncated wee1/14-3-3β was grown, with subsequent purification of the complex using IMAC, described in Sections 2.5.3.2.1 and 2.5.3.2.2. The purification fractions were then analyzed by SDS PAGE, presented in Figure 4.14.

This analysis indicated that both truncated wee1 and 14-3-3β had been expressed and could be purified by IMAC.
Figure 4.14: SDS PAGE analysis of fractions following IMAC purification of the truncated wee1/14-3-3β complex. Lane 1 shows the infected whole cell lysate. Lane 2 shows the proteins that did not bind the IMAC column. Lane 3 is the proteins that were washed out in 40 mM imidazole. Lane 4 is a protein ladder with markers indicated. Lanes 5 – 12 are the eluted fractions that contain the truncated wee1/14-3-3β complex, eluted in 200 mM imidazole.

To confirm that these proteins were, indeed, truncated wee1 and 14-3-3β, respectively, the individual bands were excised from the SDS PAGE gel, and were sent for identification by peptide mass fingerprinting, carried out by the Centre for Proteomics and Genomic, University of Auckland. The results from this identification process are presented in Figure 4.15. Trypsin is predicted to cleave truncated wee1 into up to 46 fragments (Appendix C); these results matched 20 trypsin digested fragments from the expressed truncated wee1 to the coding sequence of wee1. Trypsin is predicted to cleave 14-3-3β into up to 30 fragments (Appendix C); these results matched 13 trypsin digested fragments from the expressed 14-3-3β to the coding sequence of 14-3-3β. These results confirm that the proteins being purified are human wee1 and human 14-3-3β.
4.3.3: Size Exclusion Chromatography of the Truncated Wee1/14-3-3β Complex

Following IMAC purification, the truncated wee1/14-3-3β complex was subjected to further purification using an S200 16/60 size exclusion column (Amersham Bioscience), as in Section 2.5.3.2.3. The UV trace from this purification is presented in Figure 4.16, and the SDS PAGE analysis of the eluted fractions is presented in Figure 4.17.
Size exclusion chromatography of the truncated wee1/14-3-3β complex resulted in the purification of the complex as two separate purification peaks; peak 1 is of larger apparent molecular weight and peak 2 is of smaller apparent molecular weight. Unless otherwise indicated, these peaks were maintained separately throughout further analyses.
4.3.4: Attempt to Cleave the Truncated Wee1/14-3-3β Complex using rTEV Protease

A sample of IMAC purified truncated wee1/14-3-3β complex was incubated with the rTEV protease, in an attempt to cleave the N-terminal polyhistidine purification tag (Section 2.5.3.2.4). Following incubation, the sample was analyzed by SDS PAGE, presented in Figure 4.18.

This analysis indicated that the rTEV protease was unable to cleave the His tag from the truncated wee1/14-3-3β complex, as indicated by no size difference before and after incubation with the rTEV protease. In addition, the rTEV protease used was tested using a His tagged protein that is known to be accessible to the protease, and was found to be functional (data not shown).

Figure 4.18: SDS PAGE analysis of an attempt to use the rTEV protease to cleave the N-terminal polyhistidine purification tag from the truncated wee1/14-3-3β complex. Lane 1 shows a protein ladder with markers indicated. Lane 2 shows the sample of truncated wee1/14-3-3β complex prior to the addition of the rTEV protease. Lane 3 shows the sample of truncated wee1/14-3-3β complex following incubation with rTEV protease and 2 mM DTT. Lane 4 shows the sample of truncated wee1/14-3-3β complex following incubation with rTEV protease and 2 mM β-ME.
4.4: Kinase Activity of the Full Length and Truncated Wee1/14-3-3β Complexes

4.4.1: Kinase Activity of the Wee1/14-3-3β Complex

The activity of the wee1/14-3-3β complex was determined using a CycLex Wee1 Kinase Assay (MBL, CycLex) following the method described in Section 2.5.4.1. Overall, four individual preparations of wee1/14-3-3β were used to undertake this activity assay; the preparations analysed were aliquots taken from pooled samples following purification as in Section 2.5.1.3.3. An image of one of the resulting 8-well strips is presented in Figure 4.19. Following this analysis, the absorbance of each of the wells at a wavelength of 450 nm was measured, as presented in Table 4.1.

![Figure 4.19: An image of the 8-well strip used to determine the activity of the wee1/14-3-3β complex. Wells 1 and 2 are duplicates of the positive control. Wells 3 and 4 are duplicates of the ATP-deficient negative controls. Wells 5 and 6 are duplicates of the enzyme-deficient negative controls. Wells 7 and 8 are duplicates of the experimental samples.](image)

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<td>Positive Control 1</td>
<td>1.22</td>
<td>1.20</td>
<td>1.13</td>
<td>1.16</td>
</tr>
<tr>
<td>Positive Control 2</td>
<td>1.64</td>
<td>1.60</td>
<td>1.71</td>
<td>1.74</td>
</tr>
<tr>
<td>ATP-Deficient Control 1</td>
<td>0.21</td>
<td>0.21</td>
<td>0.14</td>
<td>0.14</td>
</tr>
<tr>
<td>ATP-Deficient Control 2</td>
<td>0.11</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Wee1-Deficient Control 1</td>
<td>0.07</td>
<td>0.07</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td>Wee1-Deficient Control 2</td>
<td>0.13</td>
<td>0.12</td>
<td>0.17</td>
<td>0.16</td>
</tr>
<tr>
<td>Experimental Sample 1</td>
<td>3.37</td>
<td>3.38</td>
<td>3.45</td>
<td>3.51</td>
</tr>
<tr>
<td>Experimental Sample 2</td>
<td>3.47</td>
<td>3.50</td>
<td>3.04</td>
<td>3.14</td>
</tr>
</tbody>
</table>

Table 4.1: Absorbance readings following analysis of the wee1/14-3-3β complex using the CycLex Wee1 Kinase Assay. Readings indicate that the experimental sample (expressed wee1/14-3-3β) consistently resulted in a higher absorbance at 450 nm than the positive control.
Using the data from Table 4.1, and the equations for calculating standard deviation (SD) and standard error (SE) described in Section 2.5.4.1, the mean values for each of the controls and the experimental samples were then determined, as follows:

- The positive controls exhibited a mean absorbance of 1.39 AU, which reflects a molar activity of 0.11 AU mol\(^{-1}\) L;
- The ATP-deficient negative controls exhibited a mean absorbance of 0.16 AU, which reflects a molar activity of 0.02 AU mol\(^{-1}\) L;
- The enzyme-deficient negative controls exhibited a mean absorbance of 0.13 AU, which reflects a molar activity of 0.01 AU mol\(^{-1}\) L;
- The experimental samples exhibited a mean absorbance of 3.35 AU, which reflects a molar activity of 0.37 AU mol\(^{-1}\) L.

A graphical analysis of this data is presented in Figure 4.20, in which the data have been normalized to the individual positive controls for each of the individual assays, including the standard errors for these data.

Figure 4.20: Graphical representation of the molar activities of the wee1/14-3-3β complex samples following wee1 kinase activity assay. Data generated from four individual and separate functional analyses of wee1/14-3-3β, followed by a graphical representation of the mean dataset from this analysis. These data have been normalized to the individual positive controls for each of the separate assays, including the standard deviations for the data.
Direct comparison of the molar activities of the positive control and experimental samples indicated that the experimental wee1/14-3-3β sample exceeded that of the positive control by a factor of 3.5. The data from these assays (the absorbancy measurements for the positive controls and experimental samples, normalised for molar activity) were then subjected to a Student’s t-test. Using the null hypothesis that there existed no difference between the two datasets, the t-value was calculated to be 11.7 (to 1 decimal place), and the degrees of freedom were determined to be 30. The calculated t-value and the degrees of freedom were used to consult a standard t-distribution table, which indicated that for this level of degrees of freedom, at p < 0.001, the critical t-value is 3.7 (to 1 decimal place); as the calculated t-value exceeded that of the tabulated t-value, it was, therefore, possible to reject the null hypothesis, indicating there is a statistically significant difference between the activity of the positive control (wee1 only) and the experimental sample (the wee1/14-3-3β complex), p < 0.001. Taken as a whole, this analysis indicated that the wee1/14-3-3β complex exhibited wee1 kinase activity. Furthermore, the wee1/14-3-3β kinase activity exceeded the kinase activity of the wee1 positive control by a factor of 3.5, a difference confirmed as significant using the Student’s t-test.

4.4.2: Kinase Activity of the Wee1/14-3-3β/PD0407824 Complex

Following preparation of the wee1/14-3-3β/PD0407824 complex by incubation (Section 2.5.6.4.1), the activity of the complex was determined using a CycLex Wee1 Kinase Assay (MBL, CycLex), as in Section 2.5.4.2. This analysis used a preparation of the wee1/14-3-3β complex that was equivalent in apparent purity and in vitro behaviour as had been encountered with all previous preparations of the complex. An image of the resulting 8-well strip is presented in Figure 4.21, and the absorbance results are presented in Table 4.2.

**Figure 4.21:** An image of the 8-well strip used to determine the activity of the wee1/14-3-3β/PD0407824 complex. Lanes 1 and 2 are duplicates of the positive controls. Lanes 3 and 4 are duplicates of the ATP-deficient negative controls. Lanes 5 and 6 are duplicates of the enzyme-deficient negative controls. Lanes 7 and 8 are duplicates of the experimental wee1/14-3-3β/PD0407824 samples.
Table 4.2: Absorbance readings following testing of the activity of the wee1/14-3-3β/PD0407824 complex using the CycLex Wee1 Kinase Assay. Readings indicate that the wee1/14-3-3β complex is inhibited by PD0407824.

Overall, this analysis indicated that the wee1/14-3-3β/PD0407824 complex does not exhibit any measurable wee1 kinase activity.

4.4.3: Kinase Activity of the Truncated Wee1/14-3-3β Complex

The kinase activity of three different preparations of the truncated wee1/14-3-3β complex (aliquots taken from pooled samples from peak 2, Section 4.3.3) was determined using a CycLex Wee1 Kinase Assay (MBL, CycLex), following the method described in Section 2.5.7.3.1. An image of the resulting 8-well strip is presented in Figure 4.22, and Table 4.3 shows the absorbance of each of the wells.

Figure 4.22: An image of the 8-well strip used to determine the kinase activity of the truncated wee1/14-3-3β complex. Wells 1 and 2 are duplicates of the positive control. Wells 3 and 4 are duplicates of the ATP-deficient negative controls. Wells 5 and 6 are duplicates of the enzyme-deficient negative controls. Wells 7 and 8 are duplicates of the experimental sample, truncated wee1/14-3-3β.
Table 4.3: Absorbance readings for the truncated wee1/14-3-3β complex following the CycLex Wee1 Kinase Assay. Readings indicated that the experimental sample, truncated wee1/14-3-3β, consistently gave a higher absorbance, and therefore a higher relative activity, than the (wee1 only) positive control.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Assay 1</th>
<th>Assay 2</th>
<th>Assay 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Control 1</td>
<td>1.09</td>
<td>1.10</td>
<td>0.77</td>
</tr>
<tr>
<td>Positive Control 2</td>
<td>1.15</td>
<td>1.15</td>
<td>0.73</td>
</tr>
<tr>
<td>ATP-Deficient Control 1</td>
<td>0.22</td>
<td>0.23</td>
<td>0.24</td>
</tr>
<tr>
<td>ATP-Deficient Control 2</td>
<td>0.21</td>
<td>0.21</td>
<td>0.12</td>
</tr>
<tr>
<td>Wee1-Deficient Control 1</td>
<td>0.24</td>
<td>0.24</td>
<td>0.29</td>
</tr>
<tr>
<td>Wee1-Deficient Control 2</td>
<td>0.25</td>
<td>0.25</td>
<td>0.16</td>
</tr>
<tr>
<td>Experimental Sample 1</td>
<td>3.25</td>
<td>3.25</td>
<td>2.97</td>
</tr>
<tr>
<td>Experimental Sample 2</td>
<td>3.21</td>
<td>3.21</td>
<td>3.16</td>
</tr>
</tbody>
</table>

The mean values for each of the controls and the experimental samples were then determined as follows:

- The positive control exhibited a mean absorbance value of 1.15 AU, which reflected a molar activity of 0.10 AU mol⁻¹ L⁻¹;
- The ATP-deficient negative control exhibited a mean absorbance value of 0.18 AU, which reflected a molar activity of 0.01 AU mol⁻¹ L⁻¹;
- The enzyme deficient negative control exhibited a mean absorbance value of 0.19 AU, which reflected a molar activity of 0.01 AU mol⁻¹ L⁻¹;
- The experimental samples exhibited a mean absorbance value of 3.20 AU, which reflected a molar activity of 0.19 AU mol⁻¹ L⁻¹.

A graphical analysis of these data is presented in Figure 4.23, where the data have been normalized to the individual positive controls for each of the individual assays, including the standard errors for this data.
Overall, the data indicated that the activity of the experimental truncated wee1/14-3-3β exceeds that of the positive control by a factor of 2.1. The data were then subjected to a Student’s t-test (described for the full length wee1/14-3-3β complex, Section 2.5.4.1) to determine if a statistically significant difference existed between the positive control and the experimental sample. Using the null hypothesis that there existed no difference between the positive controls and the experimental samples, the calculated t-value was 3.91 (to 1 decimal place). The degrees of freedom were determined to be 22. A standard t-distribution table indicated that at this level of degrees of freedom, at p < 0.001, the critical t-value is 3.8 (to 1 decimal place); as the calculated t-value exceeded that of the tabulated t-value, it was, therefore, possible to reject the null hypothesis. This analysis confirms a statistically significant difference between the positive control (wee1 only) and the experimental sample (the truncated wee1/14-3-3β complex), p < 0.001.

The activity data corresponding to both full length wee1/14-3-3β and truncated wee1/14-3-3β were then compared using a Student’s t-test (Section 2.5.4.3). Using the null hypothesis that there existed no difference between the two datasets, the t-value was calculated to be 0.32 (to 2 decimal places). The degrees of freedom were determined to be 26. A standard t-distribution table indicated that for this level of degrees of freedom, at p < 0.001, the critical
t-value is 3.7 (to 1 decimal place); as the calculated t-value did not exceed that of the tabulated t-value, it was, therefore, possible to accept the null hypothesis, which indicated that there is no statistically significant difference between the kinase activity of the full length wee1/14-3-3\(\beta\) complex and the kinase activity of the truncated wee1/14-3-3\(\beta\) complex.

Overall, this analysis indicated that the truncated wee1/14-3-3\(\beta\) complex exhibits wee1 kinase activity. Furthermore, the truncated wee1/14-3-3\(\beta\) kinase activity exceeded the kinase activity of the positive control by a factor of 2.1, a difference confirmed as significant using the Student’s t-test. An additional Student’s t-test confirmed that the wee1 kinase activity of the truncated wee1/14-3-3\(\beta\) complex was equivalent to that of the wee1/14-3-3\(\beta\) complex.

### 4.4.4: Kinase Activity of the Truncated Wee1/14-3-3\(\beta\)/PD0407824 Complex

The activity of the truncated wee1/14-3-3\(\beta\)/PD0407824 complex, purified using size exclusion chromatography, was determined using a CycLex Wee1 Kinase Assay (MBL, CycLex), as in Section 2.5.4.4. An image of the resulting 8-well strip is presented in Figure 4.24. The absorbance of the resulting wells is presented in Table 4.4.

#### Figure 4.24: An image of the 8-well strip used to determine the activity of the truncated wee1/14-3-3\(\beta\)/PD0407824 complex, purified using size exclusion chromatography. Lanes 1 and 2 are duplicates of the positive control. Lanes 3 and 4 are duplicates of the ATP-deficient negative control. Lanes 5 and 6 are duplicates of the enzyme-deficient negative control. Lanes 7 and 8 are duplicates of the experimental wee1/14-3-3\(\beta\)/PD0407824 samples.
<table>
<thead>
<tr>
<th>Well</th>
<th>Abs. (450 nm, AU)</th>
<th>Average Abs. (450 nm, AU)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.15</td>
<td>1.12</td>
<td>Positive Control</td>
</tr>
<tr>
<td>3</td>
<td>0.22</td>
<td>0.22</td>
<td>ATP-deficient Negative Control</td>
</tr>
<tr>
<td>4</td>
<td>0.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.24</td>
<td>0.24</td>
<td>Enzyme Deficient Negative Control</td>
</tr>
<tr>
<td>6</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.25</td>
<td>0.23</td>
<td>Experimental Sample</td>
</tr>
<tr>
<td>8</td>
<td>0.21</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.4: Absorbance readings following testing of the activity of the truncated wee1/14-3-3β/PD0407824 complex using the CycLex Wee1 Kinase Assay. Readings indicate that the truncated wee1/14-3-3β complex is inhibited in the presence of PD0407824.

This analysis indicated that the purified truncated wee1/14-3-3β/PD0407824 complex did not show any wee1 kinase activity, and that the PD0407824 compound did inhibit the complex.

4.5: Full Length Wee1/14-3-3β Experimental Results

4.5.1: Attempts to Dissociate the Wee1/14-3-3β Complex

Three separate experimental approaches were made to dissociate the wee1/14-3-3β complex. Firstly, a sample of wee1/14-3-3β was immobilized on an IMAC column at pH 7.4, and was washed with buffer containing a sequentially increasing concentration of NaCl (up to 4 M), with subsequent elution of the sample with 200 mM imidazole (Section 2.5.5.1). Samples from all of the washes, including the eluted sample were analyzed by SDS PAGE (Figure 4.25).

While a sample from each NaCl wash (1 – 4 M) was analyzed on the SDS PAGE gel, there appeared to be little to no protein present in these samples, as no protein bands were present in the corresponding gel lanes (Lanes 3 – 7 of Figure 4.25). This indicated that a high ionic strength buffer of up to 4 M NaCl at pH 7.5 is of insufficient strength to dissociate the wee1/14-3-3β complex.
Figure 4.25: SDS PAGE analysis of an attempt to disrupt the binding between wee1 and 14-3-3 β using up to 4 M NaCl at pH 7.5. Lane 1 is the sample prior to the analysis. Lane 2 is a protein ladder with markers indicated. Samples of the flow-through, and washes with 1 M, 2 M, 3 M and 4 M NaCl were loaded into Lanes 3 - 7, but the samples did not contain protein, and hence are not visible. Lane 8 shows the eluted sample containing wee1/14-3-3 β, which suggested that an ionic strength of 4 M NaCl at pH 7.5 is not enough to disrupt the interaction between wee1 and 14-3-3β.

In the second approach, the same protocol was then repeated but at pH 5.0, and the resulting samples were again analyzed by SDS PAGE (refer to Section 2.5.5.1). The resulting gel analysis is identical to that presented in Figure 4.25. This indicated that a high ionic strength buffer of up to 4 M NaCl at pH 5.0 is also of insufficient strength to dissociate the wee1/14-3-3β complex.

A third approach to dissociate wee1 and 14-3-3β was undertaken by incubating the wee1/14-3-3β complex with 2 M urea and 1 mM EDTA, followed by analysis using size exclusion chromatography (refer to Section 2.5.5.1). This resulted in the UV trace presented in Figure 4.26, and appropriate fractions were analyzed by SDS PAGE (Figure 4.27).
Following size exclusion chromatography in the presence of 2 M urea and 1 mM EDTA, SDS PAGE analysis indicated that the wee1/14-3-3β complex was intact (as judged by the absence of these proteins in any of the remaining fractions collected and analysed, data not shown). This result suggested that the interaction between wee1 and 14-3-3β is strong enough to withstand the moderately denaturing condition of 2 M urea and 1 mM EDTA.

Figure 4.26: UV trace for size exclusion chromatography of the wee1/14-3-3β complex in the presence of 2 M urea and 1 mM EDTA, undertaken in an attempt to dissociate the complex.

Figure 4.27: SDS PAGE analysis of an attempt to use size exclusion chromatography in the presence of urea and EDTA to dissociate wee1 and 14-3-3β. Lane 1 shows the sample prior to the analysis. Lane 2 is a protein ladder, with markers indicated. Lanes 3 – 7 show Fractions 6 – 10, containing the wee1/14-3-3β complex, following size exclusion chromatography. Protein bands corresponding to Wee1 and 14-3-3β were absent in any of the other fractions, following analysis by size exclusion chromatography (as judged by SDS PAGE, data not shown), suggesting that the complex was intact after incubation in 2 M urea/1 mM EDTA.
4.5.2: Determination of the Oligomeric State of the Wee1/14-3-3β Complex

4.5.2.1: Determination of Stoichiometry of the Wee1/14-3-3β Complex

A sample of the wee1/14-3-3β complex was expressed and partially purified, and an aliquot from the pooled sample was used to prepare a 1:2 dilution series that was analyzed by SDS PAGE, in conjunction with five aliquots of protein standards (Section 2.5.5.2.1). The resulting gel was fixed, stained with Sypro Ruby stain (Invitrogen, Molecular Probes), destained, and washed with sterile de-ionized water, before being subjected to densitometry analysis (Section 2.5.5.2.1), and is shown in Figure 4.28.

![Figure 4.28: SDS PAGE analysis of the wee1/14-3-3β complex stained with Sypro Ruby and analyzed using MultiGauge. Lanes 1 – 4 show a 1:2 dilution series of the wee1/14-3-3β complex. Lanes 5 – 9 show the protein ladder with markers indicated, which were used to construct a calibration curve.](image)

Protein standards that had been run in parallel with the wee1/14-3-3β sample (refer to Section 2.5.5.2.1) were analyzed using MultiGauge (Fujifilm). The relative densities of the 50 kDa and 75 kDa protein bands from all of the aliquots were determined; this was measured as the Laser Absorbance Units (LAU) of each protein band, from which the background reading (BG) of each was subtracted, resulting in the measurement of LAU-BG (see Table 4.5). In conjunction with the known amounts of the protein standards that were loaded onto the gel,
the corrected relative densities of the respective protein bands (reported as the LAU-BG values) were used to construct a calibration curve (Figure 4.29).

<table>
<thead>
<tr>
<th>Gel Lane</th>
<th>LAU</th>
<th>BG</th>
<th>LAU-BG</th>
<th>Loaded (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1283.42</td>
<td>133.99</td>
<td>1149.43</td>
<td>180.0</td>
</tr>
<tr>
<td>6</td>
<td>1075.32</td>
<td>107.21</td>
<td>968.11</td>
<td>150.0</td>
</tr>
<tr>
<td>7</td>
<td>796.3</td>
<td>87.61</td>
<td>708.7</td>
<td>112.5</td>
</tr>
<tr>
<td>8</td>
<td>651.1</td>
<td>79.92</td>
<td>571.18</td>
<td>75.0</td>
</tr>
<tr>
<td>9</td>
<td>407.46</td>
<td>63.91</td>
<td>343.55</td>
<td>37.5</td>
</tr>
<tr>
<td>5</td>
<td>4425.48</td>
<td>337.21</td>
<td>4088.27</td>
<td>900.0</td>
</tr>
<tr>
<td>6</td>
<td>2980.99</td>
<td>248.53</td>
<td>2732.47</td>
<td>750.0</td>
</tr>
<tr>
<td>7</td>
<td>2711.73</td>
<td>268.56</td>
<td>2443.17</td>
<td>562.5</td>
</tr>
<tr>
<td>8</td>
<td>1922.36</td>
<td>186.56</td>
<td>1735.81</td>
<td>375.0</td>
</tr>
<tr>
<td>9</td>
<td>1217.09</td>
<td>141.75</td>
<td>1075.34</td>
<td>187.5</td>
</tr>
</tbody>
</table>

Table 4.5: Density measurements of the 75 kDa and 50 kDa protein bands from the PrecisionPlus Molecular Weight standards, following SDS PAGE and Sypro Ruby staining. The Laser Absorbance Units (LAU) for each protein band was measured, with subtraction of the background (BG) readings. These density values (LAU-BG) were used, along with the known amount of protein loaded, to construct a calibration curve.

Figure 4.29: Calibration curve for Sypro Ruby analysis, constructed using densitometry analysis of the 75 kDa and 50 kDa protein bands of the PrecisionPlus Molecular Weight standards.

The wee1/14-3-3β complex samples were then also analyzed to determine protein band density (each lane individually), also with MultiGauge (FujiFilm), using the same process as for the protein standards, above. The calibration curve (Figure 4.29) was used to convert
these density (LAU-BG) measurements to a quantity of protein within the gel (in ng), which was used to calculate the molar amount of protein present (nmol), and thus the molar ratio of wee1 to 14-3-3β in the complex. This analysis is presented in Table 4.6.

<table>
<thead>
<tr>
<th>Gel Lane</th>
<th>Molecule</th>
<th>LAU</th>
<th>BG</th>
<th>LAU-BG</th>
<th>Relative Amt. (ng)</th>
<th>Molar Amount (nmol)</th>
<th>Molar Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wee1</td>
<td>2886.07</td>
<td>328.37</td>
<td>3057.7</td>
<td>8282.19</td>
<td>115.68</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>14-3-3β</td>
<td>1928.18</td>
<td>196.36</td>
<td>1331.82</td>
<td>3501.36</td>
<td>124.68</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Wee1</td>
<td>2422.89</td>
<td>393.09</td>
<td>2029.8</td>
<td>5434.82</td>
<td>75.91</td>
<td>1.01</td>
</tr>
<tr>
<td></td>
<td>14-3-3β</td>
<td>1928.18</td>
<td>196.36</td>
<td>1331.82</td>
<td>3501.36</td>
<td>124.68</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Wee1</td>
<td>1432.41</td>
<td>322.31</td>
<td>1110.1</td>
<td>2887.17</td>
<td>40.33</td>
<td>1.04</td>
</tr>
<tr>
<td></td>
<td>14-3-3β</td>
<td>592.46</td>
<td>132.93</td>
<td>459.53</td>
<td>1085.04</td>
<td>38.64</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Wee1</td>
<td>584.12</td>
<td>239.39</td>
<td>344.73</td>
<td>767.04</td>
<td>10.71</td>
<td>1.13</td>
</tr>
<tr>
<td></td>
<td>14-3-3β</td>
<td>234.79</td>
<td>70.97</td>
<td>163.82</td>
<td>265.90</td>
<td>9.47</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.6: Protein band density measurements and calculation of molar ratio between wee1 and 14-3-3β, following analysis by SDS PAGE and Sypro Ruby stain. The density (Laser Absorbance Units, LAU, with subtraction of background readings, BG), for each protein band was measured, and the previously constructed calibration curve (Figure 4.14) was used to convert this measurement into a protein amount, and a molar ratio between wee1 and 14-3-3β.

Overall, this analysis indicated an empirical stoichiometric ratio of 1 wee1 molecule binding to 1 14-3-3β molecule, within the complex. This analysis was carried out on three separate occasions, using protein independently prepared, and all three of the analyses yielded the same empirical stoichiometry of 1:1 in the wee1/14-3-3β complex (Appendix B).

4.5.2.2: Determination of Molecular Weight of the Wee1/14-3-3β Complex

Analytical size exclusion chromatography, native PAGE analysis and DLS were used to determine the native molecular weight of the wee1/14-3-3β complex.

4.5.2.2.1: Analytical Size Exclusion Chromatography of the Wee1/14-3-3β Complex

Analytical size exclusion chromatography was used to measure the apparent molecular weight of the wee1/14-3-3β complex. High Molecular Weight Standards (GE Healthcare) were subjected to size exclusion chromatography (Section 2.1.12.2), to first determine the
characteristics of the column used. The standards included are Blue Dextran 2,000 (Figure 4.30), Ferritin, Conalbumin, Carbonic Anhydrase and Ribonuclease A (Figure 4.31).

**Figure 4.30: UV trace of the size exclusion chromatography of Blue Dextran 2,000.** Due to the large size of this molecule, it is excluded from the column, and thus is useful in measuring the void volume of the column.

These standards were normalized using the following equation:

$$K_{AV} = \frac{Ve-Vo}{Vc-Vo}$$

Where:

- $Ve$ = elution volume of the species in solution
- $Vo$ = exclusion volume of the column
- $Vc$ = total volume of the column

The results of this normalization (Table 4.7) were then used to construct a calibration curve, shown in Figure 4.32.
Table 4.7: Analysis of High Molecular Weight standards following analytical size exclusion chromatography.

<table>
<thead>
<tr>
<th>Standard</th>
<th>Vₐ (mL)</th>
<th>KₐV</th>
<th>Mr (kDa)</th>
<th>LOG(Mr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue Dextran 2,000</td>
<td>8.54</td>
<td>-</td>
<td>2,000</td>
<td>6.30</td>
</tr>
<tr>
<td>Ferritin</td>
<td>10.47</td>
<td>0.13</td>
<td>440</td>
<td>5.64</td>
</tr>
<tr>
<td>Conalbumin</td>
<td>14.42</td>
<td>0.39</td>
<td>75</td>
<td>4.88</td>
</tr>
<tr>
<td>Carbonic Anhydrase</td>
<td>16.50</td>
<td>0.53</td>
<td>29</td>
<td>4.46</td>
</tr>
<tr>
<td>Ribonuclease A</td>
<td>18.45</td>
<td>0.66</td>
<td>13.7</td>
<td>4.14</td>
</tr>
</tbody>
</table>

Figure 4.31: UV trace of the size exclusion chromatography of High Molecular Weight Standards. This mixture of four protein standards of known molecular weight were normalized then used to construct a calibration curve.

Figure 4.32: Calibration curve constructed following analytical size exclusion chromatography, using High Molecular Weight standards.
Untagged wee1/14-3-3β complex was purified and analyzed by analytical size exclusion chromatography (as in Section 2.5.5.2.2.1). A UV trace of this analysis is presented in Figure 4.33.

The elution volume for this sample was measured as 8.57 mL. Using the normalizing equation detailed above, this complex is calculated as exhibiting a $K_{AV}$ of 0.034. This corresponds to the wee1/14-3-3β complex exhibiting an apparent molecular weight of 803 kDa (to 3 significant figures).

4.5.2.2.2: Linear Gradient Native Gel Analysis of the Wee1/14-3-3β Complex

Non-denaturing PAGE, with a linear gradient from 4 – 12% (at pH 8.8), was used to analyze the both the wee1/14-3-3β complex and a number of protein standards (refer to Section 2.5.5.2.2.2), as shown in Figure 4.34. The protein standards were visible but, unexpectedly, the wee1/14-3-3β complex was not visible.
This non-denaturing PAGE analysis was then repeated, but under acidic conditions (Section 2.5.5.2.2). This analysis omitted the molecular weight standards as standards that would be visible under acidic conditions could not be found. The gel analysis is shown in Figure 4.35.

The latter acidic gel shows a single band of reasonable resolution, suggesting that the wee1/14-3-3β complex presents as one species in solution, and that the complex is relatively basic in nature; attempts to determine the molecular weight using this method, however, were inconclusive. Following this analysis, an IPG strip (GE Healthcare) was used to experimentally determine the isoelectric point of the wee1/14-3-3β complex, the results of which are presented in Section 4.1.4.1.
4.5.2.2.3: DLS Analysis of the Wee1/14-3-3β Complex

(1) His-tagged Wee1/14-3-3β Complex

Five separate preparations of His-tagged wee1/14-3-3β were purified and individually subjected to DLS experiments (DynaPro, Protein Solutions) (refer to Section 2.5.5.2.2.3). A representative dataset from one of these analyses is presented in Figure 4.36.

<table>
<thead>
<tr>
<th>Cumulants Analysis Results</th>
<th>Regularisation Analysis Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>![Cumulants Graph]</td>
<td>![Regularisation Graph]</td>
</tr>
</tbody>
</table>

Figure 4.36: Representative results from one analysis of wee1/14-3-3β using DLS. This analysis was undertaken using five separate samples of wee1/14-3-3β, all with similar results.

The cumulants method for analysis of these samples (Table 4.8), assuming a single species in solution, indicated that the sample was very large, with a molecular weight of 26,800 ± 3780 kDa, with a polydispersity of 25 ± 3%.

The regularisation method for analysis of these samples (Table 4.9), assuming one or more species in solution, indicated that the sample comprised a protein species with a molecular weight of 33,300 ± 94.9 kDa, and a polydispersity of 20 ± 4%, when calculations are based on intensity peaks, or a molecular weight of 11,200 ± 72.2 kDa, with a polydispersity of 11 ± 1%, when calculations are based on mass peaks.
### Table 4.8: Results of the DLS analysis of the His-tagged wee1/14-3-3β complex, using the cumulants method of analysis. All values are quoted to the nearest whole number.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hydrodynamic Radius (nm)</th>
<th>Polydispersity (%)</th>
<th>Mr (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>49</td>
<td>30</td>
<td>30901</td>
</tr>
<tr>
<td>2</td>
<td>48</td>
<td>27</td>
<td>29120</td>
</tr>
<tr>
<td>3</td>
<td>33</td>
<td>32</td>
<td>11890</td>
</tr>
<tr>
<td>4</td>
<td>51</td>
<td>15</td>
<td>32740</td>
</tr>
<tr>
<td>5</td>
<td>48</td>
<td>21</td>
<td>29460</td>
</tr>
<tr>
<td>MEAN</td>
<td>46</td>
<td>25</td>
<td>26822</td>
</tr>
<tr>
<td>SD</td>
<td>7</td>
<td>7</td>
<td>8469</td>
</tr>
<tr>
<td>SE</td>
<td>3</td>
<td>3</td>
<td>3787</td>
</tr>
</tbody>
</table>

### Table 4.9: Results of the DLS analysis of the His-tagged wee1/14-3-3β complex, using the regularisation method of analysis. Molecular weights were determined from hydrodynamic radii measurements, using a manufacturer-supplied calculator (DynaPro, Protein Solutions). All values are quoted to the nearest whole number.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Radius due to Intensity Peaks</th>
<th>Radius due to Mass Peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount of Sample (%)</td>
<td>Hydrodynamic Radius (nm)</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>52</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>62</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>37</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>55</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>52</td>
</tr>
<tr>
<td>MEAN</td>
<td>100</td>
<td>52</td>
</tr>
<tr>
<td>SD</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>SE</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

All of the measurements for the apparent molecular weight of the His-tagged wee1/14-3-3β complex, whether derived through the cumulants or regularisation analyses, are very large. A likely reason for such large measurements is soluble aggregation, mediated via either the proteins of the complex, or the His-tag. Because of this, however, these figures are not likely to be an accurate measurement of the molecular weight of the complex, and hence have been disregarded.
Five separate preparations of untagged wee1/14-3-3β were purified, and were individually subjected to DLS experiments (DynaPro, Protein Solutions) (refer to Section 2.5.5.2.2.3). A representative dataset from one of these analyses is presented in Figure 4.37.

The cumulants method for analysis of these samples (Table 4.10), assuming a single species in solution, indicated that the sample exhibited a mean molecular weight of 69,900.00 ± 39,300 kDa, and a polydispersity of 101 ± 17%. The regularisation method for analysis of these samples (Table 4.11), assuming one or more species in solution, indicated that, on average, the submitted sample was comprised of a three separate species in solution:

- the first (contributing 83 ± 12% of the mass, but only 36 ± 12% of the light scattering intensity) had a molecular weight of 617 ± 29 kDa, with an associated polydispersity of 25 ± 7%;
- the second (contributing 5 ± 4% of the mass, but 54 ± 12% of the light scattering intensity) had a molecular weight of 166,000 ± 88,200 kDa, with an associated polydispersity of 45 ± 17%;

- the third (contributing 13 ± 9% of the mass and 21 ± 6% of the light scattering intensity) had a molecular weight of 2.01 x 10^9 ± 1.71 x 10^9 kDa, with an associated polydispersity of 33 ± 5%.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hydrodynamic Radius (nm)</th>
<th>Polydispersity (%)</th>
<th>Mr (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>201</td>
<td>95</td>
<td>4140</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>101</td>
<td>5142</td>
</tr>
<tr>
<td>3</td>
<td>31</td>
<td>113</td>
<td>10479</td>
</tr>
<tr>
<td>4</td>
<td>84</td>
<td>150</td>
<td>146219</td>
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<tr>
<td>5</td>
<td>101</td>
<td>48</td>
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</tr>
<tr>
<td>SD</td>
<td>38</td>
<td>37</td>
<td>87827</td>
</tr>
<tr>
<td>SE</td>
<td>17</td>
<td>16</td>
<td>39278</td>
</tr>
</tbody>
</table>

Table 4.10: Results of DLS analysis of the untagged wee1/14-3-3β complex, using the cumulants method of analysis. All values are quoted to the nearest whole number.

The untagged wee1/14-3-3β complex is able to be purified using size exclusion chromatography (Section 4.1.2.3); thus, the cumulants analysis-derived apparent molecular weight for the complex of 69,900.00 ± 39,300 kDa is not likely to be an accurate reflection of the actual molecular weight of the wee1/14-3-3β complex. Analysis of these results using the regularisation method indicates that there is likely to be more than one species in solution, one of which is excessively large. It seems likely therefore, that results from the cumulants analysis are more reflective of this other very large protein species, rather than reflecting the likely in vivo molecular weight of the wee1/14-3-3β complex.

Following size exclusion chromatography purification, the wee1/14-3-3β complex was analysed by SDS PAGE (Section 4.1.2.3, Figure 4.8), which indicated that the predominant species in solution was, indeed, the wee1/14-3-3β complex. Thus, in interpreting the regularization analysis-derived DLS data (Table 4.8), it seemed logical to assume that the measured species that contributed the greatest mass to the sample was likely to represent that
of the wee1/14-3-3β complex. This suggested that the DLS-measured molecular weight of the wee1/14-3-3β complex was 617 ± 29 kDa, with an associated polydispersity of 25 ± 7%.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Intensity Contribution (%)</th>
<th>Mass Contribution (%)</th>
<th>Hydrodynamic Radius (nm)</th>
<th>Mr (kDa)</th>
<th>Polydispersity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>45</td>
<td>99</td>
<td>9</td>
<td>547</td>
<td>23.40</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>1</td>
<td>53</td>
<td>36917</td>
<td>26.50</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>0.1</td>
<td>504</td>
<td>7 x 10⁶</td>
<td>26.20</td>
</tr>
<tr>
<td>2</td>
<td>41</td>
<td>99</td>
<td>10</td>
<td>688</td>
<td>46.70</td>
</tr>
<tr>
<td></td>
<td>47</td>
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<td>54</td>
<td>38536</td>
<td>44.70</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>0.2</td>
<td>2621</td>
<td>3 x 10⁹</td>
<td>37.80</td>
</tr>
<tr>
<td>3</td>
<td>73</td>
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<td>10</td>
<td>679</td>
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<tr>
<td></td>
<td>27</td>
<td>0.2</td>
<td>1225</td>
<td>57 x 10⁶</td>
<td>39.50</td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>82</td>
<td>9</td>
<td>606</td>
<td>20.08</td>
</tr>
<tr>
<td></td>
<td>43</td>
<td>0.2</td>
<td>104</td>
<td>0.2 x 10⁹</td>
<td>15.30</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>17</td>
<td>10593</td>
<td>8810 x 10⁶</td>
<td>17.90</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>36</td>
<td>9</td>
<td>564</td>
<td>28.60</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>18</td>
<td>164</td>
<td>0.4 x 10⁹</td>
<td>93.60</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>46</td>
<td>3893</td>
<td>847 x 10⁶</td>
<td>46.70</td>
</tr>
</tbody>
</table>

|            | MEAN | 83 | 9 | 617 | 25.38 |
|            | SD   | 28 | 0.4 | 65 | 18.06 |
|            | SE   | 12 | 0.2 | 29 | 7.49 |
|            | MEAN | 54 | 94 | 166306 | 45.03 |
|            | SD   | 24 | 52 | 176385 | 34.58 |
|            | SE   | 12 | 26 | 88193 | 16.92 |
|            | MEAN | 20 | 3767 | 2 x 10⁹ | 33.62 |
|            | SD   | 13 | 4032 | 3 x 10⁹ | 11.46 |
|            | SE   | 6  | 1803 | 2 x 10⁹ | 5.12 |

Table 4.11: Results of the DLS analysis of the untagged wee1/14-3-3β complex, using the regularisation method of analysis. Either two or three different species (coloured blue, red or green) were present in each sample (numbered 1 - 5). Molecular weights were determined from hydrodynamic radii measurements, using a manufacturer-supplied calculator (DynaPro, Protein Solutions). All values are quoted to the nearest whole number, or significant figure, as appropriate.
4.5.3: Molecular Characterization of the Wee1/14-3-3β Complex

4.5.3.1: Determination of the Isoelectric Point of the Wee1/14-3-3β Complex

Linear gradient native gel analysis of the wee1/14-3-3β complex indicated that the native isoelectric point of the complex was likely to be at a high pH, as the wee1/14-3-3β complex was only able to be visualized using this method under acidic conditions (Section 4.5.2.2.2, Figures 4.34 and 4.35). Thus, an IPG strip (GE Healthcare) was used to determine the native isoelectric point of the complex (Section 2.5.5.2.1). An image of this strip is presented in Figure 4.38, and suggested that the pH of the complex, under non-reducing and non-denaturing conditions, was pH 9.0 – 9.5.

![Image of IPG Strip](image-url)

Figure 4.38: An image of the IPG Strip used to measure the isoelectric point of the wee1/14-3-3β complex. This IPG Strip (GE Healthcare) indicates that this species had an isoelectric point at pH 9 – 9.5

4.5.3.2: Analysis of the Wee1/14-3-3β Complex using CD

Two separate samples of the wee1/14-3-3β complex were purified and subjected to CD analysis in triplicate (refer to Section 2.5.6.2.1). The spectral data resulting from the first analysis were then converted to molar residue ellipticity (refer to Section 2.5.6.2.1), with a graph of these data being presented in Figure 4.39.
Figure 4.39: CD spectrum of the first analysis of the wee1/14-3-3β complex, plotted in terms of molar ellipticity. The analysis was carried out in triplicate (Analysis 1 – 3). These data were used to predict the secondary structure content of the complex.

Three different programs (K$_2$D$_2$, CDNN and SOMCD) were then used to analyze the resulting spectra, and convert the data into a prediction of secondary structure content (Section 2.5.5.2.2). This is presented in Table 4.12.

<table>
<thead>
<tr>
<th>Program</th>
<th>Secondary Structure (%)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-Helix</td>
<td>β-Sheet</td>
<td>Random Coil</td>
<td></td>
</tr>
<tr>
<td>K$_2$D$_2$</td>
<td>Analysis 1</td>
<td>38</td>
<td>12</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Analysis 2</td>
<td>29</td>
<td>14</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Analysis 3</td>
<td>37</td>
<td>12</td>
<td>51</td>
</tr>
<tr>
<td>SOMCD</td>
<td>Analysis 1</td>
<td>42</td>
<td>21</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Analysis 2</td>
<td>42</td>
<td>21</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Analysis 3</td>
<td>42</td>
<td>21</td>
<td>37</td>
</tr>
<tr>
<td>CDNN</td>
<td>Analysis 1</td>
<td>37</td>
<td>16</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>Analysis 2</td>
<td>38</td>
<td>15</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>Analysis 3</td>
<td>39</td>
<td>14</td>
<td>46</td>
</tr>
<tr>
<td>MEAN</td>
<td>38</td>
<td>16</td>
<td>46</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.12: Secondary structure content for the first sample of wee1/14-3-3β, following CD analysis. The analysis was performed in triplicate, and the values for secondary structure content were predicted using three different computer programs. All data are presented to the nearest whole percentage.

These analyses indicated that the secondary structure content of the wee1/14-3-3β complex was 38% α-helix and 16% β-sheet, with the remaining 46% not adopting a regular secondary structure.
The spectral data resulting from the second analysis were then converted to molar residue ellipticity (refer to Section 2.5.6.2.1), with a graph of these data being presented in Figure 4.40.

![CD Spectrum of Wee1/14-3-3β](image)

**Figure 4.40:** CD spectrum of the second analysis of the wee1/14-3-3β complex, plotted in terms of molar ellipticity. The analysis was carried out in triplicate (Analysis 1 – 3). These data were also used to predict the secondary structure content of the complex.

The same three different programs (K2D2, CDNN and SOMCD) were then used to analyze the resulting spectra, and, again, convert the data into a prediction of secondary structure content (Table 4.13).

<table>
<thead>
<tr>
<th>Program</th>
<th>Secondary Structure (%)</th>
<th>α-Helix</th>
<th>β-Sheet</th>
<th>Random Coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>K2D2</td>
<td>Analysis 1</td>
<td>31</td>
<td>13</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Analysis 2</td>
<td>28</td>
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<td>55</td>
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<td></td>
<td>Analysis 3</td>
<td>30</td>
<td>15</td>
<td>55</td>
</tr>
<tr>
<td>SOMCD</td>
<td>Analysis 1</td>
<td>53</td>
<td>16</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Analysis 2</td>
<td>53</td>
<td>16</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Analysis 3</td>
<td>53</td>
<td>16</td>
<td>31</td>
</tr>
<tr>
<td>CDNN</td>
<td>Analysis 1</td>
<td>33</td>
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<td></td>
<td>Analysis 2</td>
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<td></td>
<td>Analysis 3</td>
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<tr>
<td>MEAN</td>
<td></td>
<td>36</td>
<td>16</td>
<td>48</td>
</tr>
</tbody>
</table>

**Table 4.13:** Secondary structure content for the second sample of wee1/14-3-3β, following CD analysis. The analysis was performed in triplicate, and the values for secondary structure content were predicted using three different computer programs. All data are presented to the nearest whole percentage.
These analyses indicated that the secondary structure content of the wee1/14-3-3β complex was 36% α-helix and 16% β-sheet with the remaining 48% of the complex not adopting a regular secondary structure. These percentages are both consistent with each other, and consistent with the previous analysis.

Using these two datasets, the mean percentages of secondary structure were calculated and, assuming a 1:1 stoichiometry (Section 4.5.2.1), were converted to numbers of residues within the wee1/14-3-3β complex. This analysis indicated:

- 37% of the complex, or 330 residues, adopt an α-helical conformation;
- 16% of the complex, or 143 residues, adopt a β-sheet secondary structure;
- 47% of the complex, or 419 residues, do not adopt a regular secondary structure (random coil).

Focus then turned to a broad assignment of these secondary structural elements to areas or regions of wee1 or 14-3-3β. The structures of the kinase domain of wee1 (PDB ID 2IN6) [39] and of the 14-3-3β dimer (PDB ID 2BQ0) [110] were analyzed to determine their contributions to the secondary structure of the complex (Table 4.14).

<table>
<thead>
<tr>
<th>Wee1</th>
<th>Number of Residues</th>
<th>Secondary Structures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>α-Helix</td>
</tr>
<tr>
<td>N-Terminal Domain</td>
<td>298</td>
<td>-</td>
</tr>
<tr>
<td>Kinase Domain</td>
<td>268</td>
<td>102</td>
</tr>
<tr>
<td>C-Terminal Domain</td>
<td>80</td>
<td>-</td>
</tr>
<tr>
<td>TOTAL</td>
<td>892</td>
<td>288</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>14-3-3β</th>
<th>Number of Residues</th>
<th>Secondary Structures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>α-Helix</td>
</tr>
<tr>
<td></td>
<td>246</td>
<td>186</td>
</tr>
</tbody>
</table>

Table 4.14: Secondary structure content of the components of the wee1/14-3-3β complex, from the known crystal structures. This secondary structure information is taken from the structures of the wee1 kinase domain (PDB ID 2IN6) [39] and the 14-3-3β dimer (PDB ID 2BQ0) [110].
In order to correlate these experimental measures of secondary structure with sequence data, a protein sequence consensus was sought across a number of homologues of wee1, as this is likely to provide a more reliable prediction of secondary structures than if the prediction was undertaken from any single sequence. Thus, the program ClustalW [111], [112] was used to undertake a multiple sequence alignment of wee1 from *H. sapiens*, *M. musculus*, *R. norvegicus* and *B. taurus* (Section 2.5.6.2.1), and the results are shown in Figure 4.41.

This alignment indicates a very high conservation of residues across these proteins, suggesting that a secondary structure prediction for wee1 based on all of these sequences is likely to be reasonably insightful. Three different programs (Phyre, PsiPred, and JPred3) were used to predict the secondary structures of these same proteins, and the alignments are presented in Figures 4.42, 4.43 and 4.44, respectively.
Figure 4.41: ClustalW multiple sequence alignment of wee1 protein sequences from *H. sapiens*, *M. musculus*, *R. norvegicus* and *B. taurus*. Invariant residues are denoted by ‘*’*, conserved substitutions by ‘*:’ and semi-conserved by ‘.’. The wee1 protein sequences from these species show a very high level of sequence identity.
Figure 4.42: Alignment of secondary structure predictions for the wee1 protein sequences from \textit{H. sapiens}, \textit{M. musculus}, \textit{R. norvegicus} and \textit{B. taurus} using Phyre. The wee1 protein sequence from \textit{H. sapiens} is shown above predictions. A residue in an \(\alpha\)-helical conformation is denoted by ‘H’, a residue in a \(\beta\)-sheet conformation by ‘E’, with a ‘-’ representing the prediction of a non-regular secondary structure. Approximate positions of the N-terminal, kinase and C-terminal domains are bracketed and labelled as appropriate.
Figure 4.43: Alignment of secondary structure predictions of the wee1 protein sequences from *H. sapiens*, *M. musculus*, *R. norvegicus* and *B. taurus* using PsiPred. The wee1 protein sequence from *H. sapiens* is shown above predictions. A residue in an α-helical conformation is denoted by ‘H’, a residue in a β-sheet conformation by ‘E’, with a ‘-’ representing the prediction of a non-regular secondary structure. Approximate positions of the N-terminal, kinase and C-terminal domains are bracketed and labelled as appropriate.
Chapter 4  

Experimental Results: Weel1/14-3-3β

Figure 4.44: Alignment of secondary structure predictions for the wee1 protein sequences from *H. sapiens*, *M. musculus*, *R. norvegicus* and *B. taurus* using JPred3. The wee1 protein sequence from *H. sapiens* is shown above predictions. A residue in an α-helical conformation is denoted by ‘H’, a residue in a β-sheet conformation by ‘E’, with non-regular secondary structure denoted by ‘-’. Approximate positions of the N-terminal, kinase and C-terminal domains are bracketed and labelled as appropriate.
These alignments suggested that the N-terminal domain of wee1 is likely to contain one to two $\alpha$-helices at the N-terminal end of the domain and perhaps a short $\beta$-sheet region at the C-terminal end of the domain, with consistent predictions of no secondary structure in between. The kinase domain predictions across all three programs broadly matched the known secondary structural elements of this domain, including a prediction of no regular secondary structure around residues 435 to 456, for which there was no electron density in the crystal structure (PDB ID 2IN6, [39]). The C-terminal domain predictions indicated a consensus of a long $\alpha$-helical region from the end of the kinase domain, extending through most of the C-terminal domain, then a short $\beta$-sheet at the C-terminal end of this domain.

4.5.3.3: Transmission Electron Microscopy of the Wee1/14-3-3$\beta$ Complex

Samples of the wee1/14-3-3$\beta$ complex were prepared, diluted to concentrations of 1 – 0.03 mg mL$^{-1}$, loaded on to carbon grids, and were visualized using the FEI/Tecnai CM12 transmission electron microscope (Phillips) (refer to Section 2.5.5.2.3). Representative images taken of wee1/14-3-3$\beta$ at 0.2 mg mL$^{-1}$, at two different magnifications, are presented in Figure 4.45.

![Figure 4.45: TEM images of the wee1/14-3-3$\beta$ complex at a concentration of 0.2 mg mL$^{-1}$. These images were taken at two different magnifications, as indicated by the scale bars on the bottom left hand corners of the images.](image-url)
These images suggest that the samples of the wee1/14-3-3β complex are reasonably homogeneous. Closer inspection, however, revealed that the overall shape of the complex appeared to be quite varied, no matter what the concentration of the starting sample. This suggested an inherent difficulty in distinguishing objects from artefacts, and *vice versa*, and hence single particle analysis was not likely to be useful in imaging the wee1/14-3-3β complex.

4.5.3.4: Crystallization Trials on the Wee1/14-3-3β Complex

A number of preparations of the wee1/14-3-3β complex were subjected to crystallization trials, including preparations of wee1/14-3-3β with and without the polyhistidine purification tag, with and without an inhibitor present and at various concentrations, as detailed in Section 2.5.5.2.4. The predominant feature of the crystallization drops trialled for the His-tagged wee1/14-3-3β complex was a uniform precipitate, which tended to appear between 24 and 72 hours after the trials were set up, with little to no further changes. Conditions that contained 20 – 30% w/v PEG Mr 3350 or Mr 4,000 and a sulfate salt at 0.2 M, for example Li₂SO₄, (NH₄)₂SO₄ or K₂SO₄, were the most promising, and tended to form non-uniform precipitates, usually during the first week after being set up. In fact, spherulites of the His-tagged wee1/14-3-3β complex appeared in 20% w/v PEG Mr 3350, 0.2 M K₂SO₄, but were unable to be replicated, even with further screening of PEG Mr 3350 at concentrations of 5 – 35% w/v and K₂SO₄ at concentrations of 0.05 – 0.35 M. The subsequent dissolution of the spherulites meant that seeding was unable to be used to replicate the growth. With this single exception, however, none of the conditions were able to be developed further, into phase separations, spherulites or, indeed, crystals. Results following crystallization trials on the untagged wee1/14-3-3β complex, or the wee1/14-3-3β/PD0407824 complex mirrored the results described, with the exception that unbound PD0407824 tended to form mushroom-like structures within the first 48 hours of a crystallization trial that developed into spiked ball-like structures over the subsequent two to three weeks. All attempts at crystallization of this complex were, however, unsuccessful.
4.6: *Truncated Wee1/14-3-3β Experimental Results*

4.6.1: Determination of the Oligomeric State of the Truncated Wee1/14-3-3β Complex

4.6.1.1: Determination of Stoichiometry of the Truncated Wee1/14-3-3β Complex

A sample of the truncated wee1/14-3-3β complex (peak 2, Section 4.3.3) was expressed and purified before being used to prepare a 1:2 dilution series that was analyzed by SDS PAGE, in conjunction with five aliquots of protein standards (Section 2.5.6.1.1). The resulting gel was fixed, stained with Sypro Ruby stain (Invitrogen, Molecular Probes), de-stained, and washed with sterile de-ionized water, before being subjected to densitometry analysis (described for the stoichiometric analysis of the full length wee1/14-3-3β complex, Section 2.5.5.2.1), and is shown in Figure 4.46.

![Figure 4.46: SDS PAGE analysis of truncated wee1 and 14-3-3β stained with Sypro Ruby stain, and analyzed using MultiGauge. Lanes 1 – 5 are the protein ladder with markers indicated, which were used to construct a calibration curve. Lanes 6 – 10 show a 1:2 dilution series of wee1/14-3-3β, the density of which were measured to determine binding stoichiometry.](image-url)
Protein standards that had been run in parallel with the truncated wee1/14-3-3β sample (refer to Section 2.5.6.1.1) were analyzed using MultiGauge (Fujifilm). The relative densities of the 50 kDa and 75 kDa protein bands from all of the aliquots were determined; this was measured as the Laser Absorbance Units (LAU) of each protein band, from which the background reading (BG) of each was subtracted, resulting in the measurement of LAU-BG (see Table 4.15). In conjunction with the known amounts of the protein standards that were loaded onto the gel, the corrected relative densities of the respective protein bands (reported as the LAU-BG values) were used to construct a calibration curve (Figure 4.47).

<table>
<thead>
<tr>
<th>Gel Lane</th>
<th>LAU</th>
<th>BG</th>
<th>LAU-BG</th>
<th>Amount Loaded (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>75 kDa band</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>543.61</td>
<td>136.28</td>
<td>407.33</td>
<td>180.0</td>
</tr>
<tr>
<td>2</td>
<td>498.66</td>
<td>156.22</td>
<td>342.44</td>
<td>150.0</td>
</tr>
<tr>
<td>3</td>
<td>340.63</td>
<td>126.91</td>
<td>213.72</td>
<td>112.5</td>
</tr>
<tr>
<td>4</td>
<td>200.60</td>
<td>82.49</td>
<td>118.10</td>
<td>75.0</td>
</tr>
<tr>
<td>5</td>
<td>145.87</td>
<td>89.44</td>
<td>56.42</td>
<td>37.5</td>
</tr>
<tr>
<td>50 kDa band</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1995.97</td>
<td>201.25</td>
<td>1794.73</td>
<td>900.0</td>
</tr>
<tr>
<td>2</td>
<td>1810.87</td>
<td>228.14</td>
<td>1582.73</td>
<td>750.0</td>
</tr>
<tr>
<td>3</td>
<td>1245.83</td>
<td>164.68</td>
<td>1081.14</td>
<td>562.5</td>
</tr>
<tr>
<td>4</td>
<td>735.70</td>
<td>106.06</td>
<td>629.64</td>
<td>375.0</td>
</tr>
<tr>
<td>5</td>
<td>500.63</td>
<td>103.34</td>
<td>397.29</td>
<td>187.5</td>
</tr>
</tbody>
</table>

Table 4.15: Density measurements of the 75 kDa and 50 kDa protein bands from the PrecisionPlus Molecular Weight standards, following SDS PAGE and Sypro Ruby staining. The Laser Absorbance Units (LAU) for each protein band was measured, with subtraction of the background (BG) readings. These density values (LAU-BG) were used, along with the known amount of protein loaded, to construct a calibration curve.

![Sypro Ruby Calibration Curve](image)

Figure 4.47: Calibration curve of Sypro Ruby analysis, constructed using densitometry analysis of the 75 kDa and 50 kDa protein bands of the PrecisionPlus Molecular Weight standards.
The truncated wee1/14-3-3β complex samples were then also analyzed to determine protein band density (each lane individually), also with MultiGauge (FujiFilm), using the same process as for the protein standards, above. The calibration curve (Figure 4.47) was used to convert these density (LAU-BG) measurements to a quantity of protein within the gel (in ng), which was used to calculate the molar amount of protein present (nmol), and thus the molar ratio of truncated wee1 to 14-3-3β. This analysis is presented in Table 4.16.

<table>
<thead>
<tr>
<th>Gel Lane</th>
<th>Molecule</th>
<th>LAU</th>
<th>BG</th>
<th>LAU-BG</th>
<th>Relative Amt (ng)</th>
<th>Molar Amt (nmol)</th>
<th>Molar Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Truncated wee1</td>
<td>12436.10</td>
<td>547.54</td>
<td>11888.56</td>
<td>6169.38</td>
<td>141.39</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>14-3-3β</td>
<td>8321.60</td>
<td>378.32</td>
<td>7943.28</td>
<td>4129.78</td>
<td>147.06</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Truncated wee1</td>
<td>7205.02</td>
<td>392.83</td>
<td>6518.20</td>
<td>3576.27</td>
<td>81.96</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>14-3-3β</td>
<td>5080.05</td>
<td>301.57</td>
<td>4778.48</td>
<td>2522.90</td>
<td>59.84</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Truncated wee1</td>
<td>4154.26</td>
<td>283.74</td>
<td>3870.52</td>
<td>2063.98</td>
<td>47.30</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>14-3-3β</td>
<td>2793.65</td>
<td>215.75</td>
<td>4979.20</td>
<td>2589.60</td>
<td>59.84</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Truncated wee1</td>
<td>2129.67</td>
<td>196.11</td>
<td>1933.65</td>
<td>1060.36</td>
<td>24.30</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>14-3-3β</td>
<td>1429.61</td>
<td>171.33</td>
<td>1258.28</td>
<td>713.33</td>
<td>16.84</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Truncated wee1</td>
<td>1138.76</td>
<td>152.60</td>
<td>986.16</td>
<td>569.16</td>
<td>13.04</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>14-3-3β</td>
<td>792.99</td>
<td>146.25</td>
<td>646.73</td>
<td>397.75</td>
<td>9.84</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.16: Protein band density measurements and calculation of molar ratio between truncated wee1 and 14-3-3β, following analysis by SDS PAGE and Sypro Ruby stain. The density of each protein band was measured (Laser Absorbance Units, LAU, with subtraction of background readings, BG), and the previously constructed calibration curve (Figure 4.47) was then used to convert this measurement into a protein amount, ultimately allowing calculation of a molar ratio between truncated wee1 and 14-3-3β.

This analysis indicated a stoichiometric ratio of 1 truncated wee1 molecule binding to 1 14-3-3β molecule, (1:1) within the complex. This analysis was carried out on three separate occasions, using protein independently prepared, and all three of the analyses yielded the same empirical stoichiometry of 1:1 in the truncated wee1/14-3-3β complex (Appendix B).

4.6.1.2: Determination of Molecular Weight of the Truncated Wee1/14-3-3β Complex

Two different methods were used to assess the native molecular weight of the Wee/14-3-3β complex, namely analytical size exclusion chromatography and DLS.
4.6.1.2.1: Analytical Size Exclusion Chromatography of the Truncated Wee1/14-3-3β Complex

High Molecular Weight Standards (GE Healthcare) were analyzed by analytical size exclusion chromatography and were used to construct a calibration curve (Section 4.1.3.2.1). Truncated wee1/14-3-3β complex was then also analyzed by analytical size exclusion chromatography, as in Section 2.5.6.1.2.1. A UV trace of this analysis is presented in Figure 4.48.

The elution volume for this sample was measured as 9.58 mL and 12.69 mL, respectively, enabling the use of the normalizing equation detailed in Section 2.1.12.2. The resulting analysis is presented in Table 4.17.

<table>
<thead>
<tr>
<th></th>
<th>$V_e$ (mL)</th>
<th>$K_{AV}$</th>
<th>Mr (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Truncated wee1/14-3-3β, Peak 1</td>
<td>9.58</td>
<td>0.10</td>
<td>523</td>
</tr>
<tr>
<td>Truncated wee1/14-3-3β, Peak 2</td>
<td>12.76</td>
<td>0.30</td>
<td>140</td>
</tr>
</tbody>
</table>

Table 4.17: Analytical size exclusion chromatography results for the truncated wee1/14-3-3β complex. The elution volume of the truncated wee1/14-3-3β complex was measured for both peak 1 and peak 2, enabling the calculation of the $K_{AV}$ for each purified peak of this complex. Molecular weights are quoted to 3 significant figures.
Overall, the molecular weight of the species in solution was calculated to be 523 kDa (peak 1) or 140 kDa (peak 2), respectively.

4.6.1.2.2: DLS Analysis of the Truncated Wee1/14-3-3β Complex

Five separate preparations of the truncated wee1/14-3-3β complex were purified, and each peak was individually subjected to DLS experiments (DynaPro, Protein Solutions) (refer to Section 2.5.6.1.2.2).

(1) Peak 1 of the Truncated wee1/14-3-3β Complex

Initial analysis centred on peak 1 of the truncated wee1/14-3-3β complex (Section 2.5.6.1.2.2), and a representative dataset from one of these analyses is presented in Figure 4.49.

The cumulants method for analysis of these samples (Table 4.18), assuming a single species in solution, indicated that the species in solution was very large, with a molecular weight of 10,400 ± 1,310 kDa, with a polydispersity of 34 ± 4%.
The regularisation method for analysis of these samples (Table 4.19), assuming one or more species in solution, indicated that the sample comprised a single species in solution with a molecular weight of 7,790 ± 138 kDa, with a polydispersity of 14 ± 3%, when calculations are based on intensity peaks, or a molecular weight of 2,880 ± 55 kDa, with a polydispersity of 7 ± 1%, when calculations are based on mass peaks.

DLS Cumulants Analysis of Peak 1 of the Truncated wee1/14-3-3β Complex:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hydrodynamic Radius (nm)</th>
<th>Polydispersity (%)</th>
<th>MR (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>33</td>
<td>23</td>
<td>12300</td>
</tr>
<tr>
<td>2</td>
<td>33</td>
<td>40</td>
<td>11710</td>
</tr>
<tr>
<td>3</td>
<td>33</td>
<td>37</td>
<td>11890</td>
</tr>
<tr>
<td>4</td>
<td>32</td>
<td>28</td>
<td>11050</td>
</tr>
<tr>
<td>5</td>
<td>23</td>
<td>43</td>
<td>5257</td>
</tr>
<tr>
<td>MEAN</td>
<td>31</td>
<td>34</td>
<td>10441</td>
</tr>
<tr>
<td>SD</td>
<td>4</td>
<td>9</td>
<td>2933</td>
</tr>
<tr>
<td>SE</td>
<td>2</td>
<td>4</td>
<td>1312</td>
</tr>
</tbody>
</table>

Table 4.18: Results of the DLS analysis of peak 1 of the truncated wee1/14-3-3β complex, using the cumulants method of analysis. All values are quoted to the nearest whole number.

DLS Regularisation of Peak 1 of the Truncated wee1/14-3-3β Complex:

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Radius due to Intensity Peaks</th>
<th>Radius due to Mass Peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount of Sample (%)</td>
<td>Hydrodynamic Radius (nm)</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>34</td>
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<tr>
<td>2</td>
<td>100</td>
<td>41</td>
</tr>
<tr>
<td>3</td>
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<td>13</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>35</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
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<td>100</td>
<td>30</td>
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<tr>
<td>SD</td>
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<td>7</td>
</tr>
<tr>
<td>SE</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 4.19: Results of the DLS analysis of peak 1 of the truncated wee1/14-3-3β complex, using the regularisation method of analysis. Molecular weights were determined from hydrodynamic radii measurements, using a manufacturer-supplied calculator (DynaPro, Protein Solutions). All values are quoted to the nearest whole number.
The measured apparent molecular weights of peak 1 of the truncated wee1/14-3-3β complex, whether derived through the cumulants or the regularization methods of analysis, were very large in solution, and were all in excess of 2800 kDa. This is more likely to be an artefact of the *in vitro* expression of the recombinant complex, rather than an accurate reflection of the *in vivo* molecular weight of the truncated wee1/14-3-3β complex, and thus these measurements have been disregarded.

(2) Peak 2 of the Truncated wee1/14-3-3β Complex

Five separate preparations of peak 2 of the truncated wee1/14-3-3β complex were separately subjected to DLS experiments (DynaPro, Protein Solutions) (Section 2.5.6.1.2.2). A representative dataset from one of these analyses is presented in Figure 4.50.

<table>
<thead>
<tr>
<th>Cumulants Analysis</th>
<th>Regularisation Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Cumulants Analysis" /></td>
<td><img src="image2" alt="Regularisation Analysis" /></td>
</tr>
</tbody>
</table>

*Figure 4.50: Representative results following the DLS analysis of peak 2 of the truncated wee1/14-3-3β complex. This analysis was undertaken using five separate samples, all with similar results.*

The cumulants method for analysis of these samples (Table 4.20), assuming a single species in solution, indicated that the sample exhibited a mean molecular weight of 4133 ± 399 kDa, and a polydispersity of 57 ± 2%.
The regularisation method for analysis of these samples (Table 4.21), assuming one or more species in solution, indicated that, on average, the submitted sample consisted of two separate species in solution:

- the first (contributing $95 \pm 1\%$ of the mass, but only $17 \pm 2\%$ of the light scattering intensity) had an apparent molecular weight of $168 \pm 0.01 \text{kDa}$, with an associated polydispersity of $1 \pm 0.3\%$, when calculations were based on intensity peaks, and an apparent molecular weight of $163 \pm 0.1 \text{kDa}$, with an associated polydispersity of $1 \pm 0.3\%$, when calculations were based on mass peaks;

- the second (contributing $5 \pm 1\%$ of the mass but $83 \pm 2\%$ of the light scattering intensity) had an apparent molecular weight of $6,800 \pm 4 \text{kDa}$, with an associated polydispersity of $9 \pm 2\%$, when calculations were based on intensity peaks, or an apparent molecular weight of $3,600 \pm 4 \text{kDa}$, with an associated polydispersity of $7 \pm 0.4\%$, when calculations were based on mass peaks.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hydrodynamic Radius (nm)</th>
<th>Polydispersity (%)</th>
<th>MR (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19</td>
<td>61</td>
<td>3174</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
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<td>4550</td>
</tr>
<tr>
<td>3</td>
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<td>55</td>
<td>3831</td>
</tr>
<tr>
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<td>23</td>
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</tr>
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<td>5</td>
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<td>SE</td>
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<td>399</td>
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</tbody>
</table>

Table 4.20: Results of the DLS analysis of peak 2 of the truncated wee1/14-3-3β complex, using the cumulants method of analysis. All values are quoted to the nearest whole number.
### Table 4.21: Results of the DLS analysis of peak 2 of the truncated wee1/14-3-3β complex, using regularisation analysis.

Two different species (coloured blue or red) were present in each sample (numbered 1 - 5). Molecular weights were determined from hydrodynamic radii measurements, using a manufacturer-supplied calculator (DynaPro, Protein Solutions). All values are quoted to the nearest whole number, or significant figure, as appropriate.

Cumulants analysis indicated that the apparent molecular weight of protein from peak 2 of the truncated wee1/14-3-3β complex was 4,100 ± 400 kDa. This is a measurement far in excess of expectation, given that the molecular weight of a complex of 1:1 stoichiometry of binding is 70 kDa (calculated using ProtParam [http://www.expasy.org/tools/protparam.html][95]). Thus, this method of analysis is likely to have over-simplified the analysis for the determination of the actual molecular weight of the complex, and was disregarded as to its contribution to this measurement.

Following size exclusion chromatography of the truncated wee1/14-3-3β complex, the protein from peak 2 was analysed by SDS PAGE, which indicated that the predominant species in solution was that of the truncated wee1/14-3-3β complex (Figure 4.17, Section C h a p t e r  4  E x p e r i m e n t a l  R e s u l t s :  W e e 1 / 1 4 - 3 - 3β - 173 -).
Therefore, in interpreting the regularization analysis-derived DLS data, it seemed logical to assume that the measured species that contributed 95% mass to the sample (species 1, Table 4.21) was likely to be representative of the truncated wee/14-3-3β complex. In addition, the manufacturer (DynaPro, Protein Solutions) recommends the use of intensity peak data to measure native molecular weight. Taken as a whole, this suggested that the DLS-measured molecular weight of peak 2 protein of the truncated wee1/14-3-3β complex was 168 ± 0.01 kDa, with an associated polydispersity of 1 ± 0.3%.

4.6.2: Molecular Characterization of the Truncated Wee1/14-3-3β Complex

4.6.2.1: Analysis of the Truncated Wee1/14-3-3β Complex using CD

Two separate samples of the truncated wee1/14-3-3β complex (peak 2, Section 4.2.1.3) were purified and subjected to CD analysis (refer to Section 2.5.6.2.1). The spectral data resulting from the first analysis, in triplicate, were then converted to molar residue ellipticity (refer to Section 2.5.6.2.1), with a graph of these data being presented in Figure 4.51.

![CD Spectrum of Truncated Wee1/14-3-3β](image)

Figure 4.51: CD spectrum of the first analysis of the truncated wee1/14-3-3β complex, plotted in terms of molar ellipticity. This analysis was carried out in triplicate (Analysis 1 – 3). These data were also used to predict the secondary structure content of the complex.
Three different programs (K$_2$D$_2$, CDNN and SOMCD) were then used to analyze the resulting spectra, and predict the secondary structure content of the truncated wee1/14-3-3β complex (Table 4.22).

<table>
<thead>
<tr>
<th>Program</th>
<th>Secondary Structure (%)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>α-Helix</td>
<td>β-Sheet</td>
<td>Random Coil</td>
</tr>
<tr>
<td>K$_2$D$_2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Analysis 1</td>
<td></td>
<td>48</td>
<td>8</td>
<td>44</td>
</tr>
<tr>
<td>Analysis 2</td>
<td></td>
<td>48</td>
<td>8</td>
<td>44</td>
</tr>
<tr>
<td>Analysis 3</td>
<td></td>
<td>48</td>
<td>8</td>
<td>44</td>
</tr>
<tr>
<td>SOMCD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Analysis 1</td>
<td></td>
<td>47</td>
<td>17</td>
<td>36</td>
</tr>
<tr>
<td>Analysis 2</td>
<td></td>
<td>46</td>
<td>22</td>
<td>32</td>
</tr>
<tr>
<td>Analysis 3</td>
<td></td>
<td>46</td>
<td>22</td>
<td>32</td>
</tr>
<tr>
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<tr>
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<td>40</td>
</tr>
<tr>
<td>Analysis 2</td>
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<td>48</td>
<td>11</td>
<td>41</td>
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<tr>
<td>Analysis 3</td>
<td></td>
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<td>10</td>
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</tr>
<tr>
<td>MEAN</td>
<td></td>
<td>47</td>
<td>13</td>
<td>39</td>
</tr>
</tbody>
</table>

Table 4.22: Secondary structure content for the first sample of truncated wee1/14-3-3β complex, following CD analysis. The analysis was performed in triplicate and the values for secondary structure content were predicted using three different programs. All data are presented to the nearest whole percentage.

The secondary structure predictions from this analysis were reasonably consistent with each other, and indicated that the secondary structure content of the truncated wee1/14-3-3β complex was 47% α-helix, 13% β-sheet, with the remaining 39% not adopting a regular secondary structure.

The spectral data resulting from the second analysis, in triplicate, were then converted to molar residue ellipticity (refer to Section 2.5.6.2.1), with a graph of these data being presented in Figure 4.52.
The same three different programs (K$_2$D$_2$, SOMCD and CDNN) were then used to analyze the resulting spectra and, again, predict the secondary structure content of the truncated wee1/14-3-3β complex (see Table 4.23).

<table>
<thead>
<tr>
<th>Program</th>
<th>Secondary Structure (%)</th>
<th>α-Helix</th>
<th>β-Sheet</th>
<th>Random Coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_2$D$_2$</td>
<td>Analysis 1</td>
<td>48</td>
<td>8</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>Analysis 2</td>
<td>48</td>
<td>8</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>Analysis 3</td>
<td>48</td>
<td>8</td>
<td>44</td>
</tr>
<tr>
<td>SOMCD</td>
<td>Analysis 1</td>
<td>46</td>
<td>22</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Analysis 2</td>
<td>46</td>
<td>22</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Analysis 3</td>
<td>46</td>
<td>22</td>
<td>32</td>
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<td>CDNN</td>
<td>Analysis 1</td>
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<td>10</td>
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<td></td>
<td>Analysis 2</td>
<td>51</td>
<td>11</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Analysis 3</td>
<td>52</td>
<td>10</td>
<td>38</td>
</tr>
<tr>
<td>MEAN</td>
<td></td>
<td>49</td>
<td>13</td>
<td>38</td>
</tr>
</tbody>
</table>

Table 4.23: Secondary structure content for the second sample of the truncated wee1/14-3-3β complex, following CD analysis. The analysis was performed in triplicate, and the values for secondary structure content were predicted using three different computer programs. All data are presented to the nearest whole percentage.

The secondary structure predictions from this (second) analysis indicated that the secondary structure content of the truncated wee1/14-3-3β complex was 49% α-helix, 13% β-sheet, with
the remaining 38% not adopting a regular secondary structure. These predictions are both consistent with each other and consistent with the previous (first) analysis.

Using these two datasets, the mean percentages of secondary structure were calculated and, assuming a 1:1 stoichiometry (Section 4.6.1.1), were converted to number of residues in the truncated wee1/14-3-3β complex. This analysis indicated:

- 49% of the complex, or 291 residues, adopt an α-helical secondary structure;
- 13% of the complex, or 77 residues, adopt a β-sheet secondary structure;
- 38% of the complex, or 226 residues, do not adopt a regular secondary structure (random coil).

4.6.2.2: Crystallization Trials on the Truncated Wee1/14-3-3β Complex

The truncated wee1/14-3-3β complex was subjected to a number of crystallization trials, including the two different protein peaks obtained from size exclusion chromatography, with and without the polyhistidine purification tag, with and without the presence of PD0407824, at various protein concentrations, as in Section 2.5.6.2.2. As in regards to crystallization trials on the wee1/14-3-3β complex, the predominant result from crystallization trials on the truncated wee1/14-3-3β complex, purified from either peak 1 or peak 2, was uniform protein precipitation. In addition, truncated wee1/14-3-3β protein, purified from peak 1, had a tendency to show non-uniform and heavy precipitates where the crystallization condition was 7 – 28% w/v PEG Mr 6,000 or MPEG Mr 5,000 at pH 8.5 or 9.1. These results were not able to be improved through the addition of PD0407824. Truncated wee1/14-3-3β purified from peak 2, however, was apparently more reactive than protein purified from peak 1, and had a tendency to show similar non-uniform precipitates under conditions described above, as well as in the presence of 20 – 30% w/v PEG Mr 3350 or 20 – 30% w/v MPEG Mr 5,000 or PEG Mr 6,000, the latter at the lower pH of 5.0 – 6.0. The addition of alcohols, such as MDP or isopropanol, also appeared to promote non-uniform protein precipitation. Fine screening around all these conditions, though, was not able to promote the growth protein crystals (a number of crystals were grown, but all were proved to be either salt or re-crystallized PEG). Overall, all attempts to crystallize the truncated wee1/14-3-3β complex were unsuccessful.
The aim of this part of the research was to express and purify one or more of the isoforms of CDC25, and to characterise the complex it forms with 14-3-3. To this end, the clone of pFASTBac HTb-cdc25B was provided by Dr. James Dickson. Presented here are the results and discussion of this work, including the future directions. Based on these results, a comparison could also be made between the CDC25B/14-3-3β complex and the wee1/14-3-3β complex, and is presented in Chapter 6.

5.1: Expression Testing of CDC25B

Using the pFASTBac HTb-cdc25B clone provided by Dr. James Dickson, a transfection was undertaken to produce recombinant baculovirus encoding CDC25B. This recombinant virus was amplified and was used to undertake expression testing of CDC25B (Section 2.6.1).

A small-scale (50 mL) culture of mid-log phase Sf9 cells was set up, and infected with a baculovirus encoding CDC25B. Samples of the culture (1 mL each) were taken at 48, 72 and 96 hours post infection, with the remainder of the culture being harvested by centrifugation at 96 hours post infection (Section 2.6.2). The 1 mL samples were individually harvested by centrifugation, and were lysed (Section 2.6.2), before being analyzed by SDS PAGE (Figure 5.1). Despite some overloading present in the representative gel presented (Figure 5.1), this analysis indicated that CDC25B was being expressed, based on the projected molecular weight of CDC25B. Comparison of the cytoplasmic and nuclear fractions (Lanes 6 and 7 of Figure 5.1) also indicated that this expression was localized to the cytoplasm only.

While CDC25B does not contain a known nuclear export signal, the in vivo mitotic promoting activity of the CDC25B/14-3-3β complex is known to occur within the cytoplasm (Section 1.3.4). Thus, the in vitro expression profile exhibited by CDC25B/14-3-3β in Sf9 Cells is consistent with its known in vivo cytoplasmic role.
Figure 5.1: SDS PAGE analysis indicating the timeline of CDC25B expression. Lane 1 shows an uninfected whole cell lysate sample. Samples of the infected culture were taken at 48 (Lane 2), 72 (Lane 3) and 96 (Lane 4) hours post infection. Lane 5 is a protein ladder with markers indicated. Lane 6 shows the cytoplasmic lysate sample, and Lane 7 shows the nuclear lysate sample. Expression of this protein, CDC25B, is evident in Lane 6 and not Lane 7, indicating that expression is localized to the cytoplasm only.

5.2: Purification of CDC25B

The remaining 47 mL of expression culture were then used to undertake purification of CDC25B. The cells were lysed, and the resulting protein sample was subjected to IMAC purification (Section 2.6.3), and analyzed by SDS PAGE, as shown in Figure 5.2.

Figure 5.2: SDS PAGE analysis of the IMAC purification of CDC25B. Lane 1 represents the cell lysate prior to purification. Lane 2 is a protein ladder, with markers indicated. Lane 3 shows the proteins that did not bind the column. Lane 4 shows a wash with buffer containing 200 mM imidazole, and Lane 5 with 800 mM imidazole, with elution of CDC25B in 1.2 M imidazole (Lanes 6 – 11).
The SDS PAGE analysis of the IMAC purification (Figure 5.2) demonstrates a clear band at approximately 65 kDa, corresponding to CDC25B, and also shows that it co-eluted with another molecule of approximately 30 - 35 kDa. It was suspected that this was an insect cell 14-3-3 protein, since CDC25B is most stable when in complex with a 14-3-3 partner [67]. Indeed, there is evidence that CDC25B can form an *in vitro* complex with a number of 14-3-3 isoforms [68], [70]. When in complex exclusively with the β isoform, the *in vitro* sub-cellular distribution and activity profiles of CDC25B changed, to more closely reflect the measured *in vivo* expression, distribution and activity profile of CDC25B [68]. Thus, CDC25B was re-cloned to co-express with its most likely binding partner, human 14-3-3β.

### 5.3: Re-cloning of the DNA Encoding CDC25B into pFASTBac DUAL

The DNA encoding CDC25B was re-cloned into the pFASTBac DUAL plasmid, already containing the coding sequence for human 14-3-3β, in order to undertake CDC25B/14-3-3β co-expression. The pFASTBac HTb-cdc25b clone was transformed into *E. coli* DH5α. A single colony was picked and was grown as a culture in Luria Broth, containing the appropriate antibiotics. The plasmid DNA was purified, digested with appropriate restriction enzymes (Section 2.6.4), and then analyzed on an agarose gel (Figure 5.3). From this gel, the digested CDC25B insert was excised and purified (Section 2.6.4).

![Figure 5.3: agarose gel analysis following the restriction enzyme digestion of the pFASTBac HTb-cdc25b clone. Lane 1 is a DNA ladder, with markers indicated. Lane 2 is an undigested vector control. Lane 3 is the doubly-digested clone. The band indicated was purified and re-cloned for co-expression with 14-3-3β.](image-url)
In parallel, the pFASTBac DUAL-weel-14-3-3β clone was transformed into *E. coli* DH5α, and a single colony was picked and was grown as a culture in Luria Broth, containing the appropriate antibiotics. The plasmid DNA from this culture was purified, digested with the appropriate restriction enzymes to remove the wee1 coding sequence (Section 2.6.4), then analyzed on an agarose gel (Figure 5.4).

![Figure 5.4: agarose gel analysis following the restriction enzyme digestion of the pFASTBac DUAL-weel-14-3-3β clone. Lane 1 is a DNA ladder, with markers indicated. Lane 2 is an undigested control for the reaction. Lane 3 is the doubly-digested clone retaining the coding sequence for 14-3-3β, which was purified from this gel and then used in a ligation reaction to generate the pFASTBac DUAL-cdc25b-14-3-3β clone.](image)

The coding sequence for CDC25B, previously agarose gel purified, was ligated into the doubly-digested pFASTBac DUAL-14-3-3β plasmid. The resulting sample, as well as the two additional controls, was then transformed into *E. coli* DH5α (Section 2.6.4). Nine single colonies were picked and grown as 5 mL cultures in LB (with appropriate antibiotics). The plasmid DNA from each sample was purified, digested with appropriate restriction enzymes (Section 2.6.4), and analyzed on an agarose gel (Figure 5.5).
This latter analysis indicated a number of potential pFASTBac DUAL-cdc25b-14-3-3β clones. Correct sub-cloning was confirmed by dideoxy nucleotide sequencing (Appendix A).

5.4: Expression of CDC25B/14-3-3β

Using the sequence-verified pFASTBac DUAL-cdc25b-14-3-3β clone, a transfection was undertaken to produce recombinant baculovirus for the co-expression of CDC25B and 14-3-3β (Section 2.6.5). A small-scale (50 mL) culture of mid-log phase Sf9 cells was set up, and infected with this baculovirus. At 48 and 72 hours post infection, a 1 mL sample of infected culture was taken. These cells were harvested by centrifugation, lysed (Section 2.6.6) and analyzed by SDS PAGE (Figure 5.6). The remaining Sf9 cells of the 72 hour post infection culture were harvested by centrifugation at 72 hours post infection.
Both CDC25B and 14-3-3β were shown to express, and comparison of the cytoplasmic and nuclear lysate samples (Lanes 6 and 7 of Figure 5.6) indicated that both were localized to the cytoplasm, consistent with the *in vivo* profile of the CDC25B/14-3-3β complex.

### 5.5: Purification of CDC25B/14-3-3β Complex

#### 5.5.1: IMAC Purification of CDC25B/14-3-3β

Initial attempts to purify CDC25B/14-3-3β centred on the use of IMAC. The buffer conditions were varied, but none resulted in the retention of this complex to the IMAC resin. This was in marked contrast to expressed CDC25B (no 14-3-3β present), where elution of CDC25B was only achieved through washing with 1.2 M imidazole (Section 5.2). The binding between CDC25B and 14-3-3β is thought to be mediated via phosphorylation of Ser-216, present in the N-terminal domain of CDC25B [68]. The construct being expressed contained a recombinant N-terminal polyhistidine purification tag, but the failure of IMAC
raises the possibility that the bound 14-3-3β may have occluded the tag. Without the advantage of IMAC, a variety of protocols were used to purify the complex.

5.5.2: Purification of CDC25B/14-3-3β using Alternative Chromatographic Methods

Purification of the CDC25B/14-3-3β complex was then carried out using a combination of ion exchange and size exclusion chromatography. Sf9 cells containing co-expressed CDC25B/14-3-3β complex were lysed under low pH/low salt conditions, loaded on to an SP Sepharose column (Amersham), and washed with buffer containing increasing salt concentrations, up to 750 mM NaCl (Section 2.6.7.2). All eluted samples were collected, and analyzed by SDS PAGE, as shown in Figure 5.7.

![Figure 5.7: SDS PAGE analysis of the SP Sepharose chromatography of the CDC25B/14-3-3β complex. Lane 1 shows cytoplasmic lysate. Lane 2 shows a protein ladder with markers indicated. Lane 3 shows the proteins that did not bind the column. Lane 4 shows the proteins that were eluted in 200 mM NaCl. Lane 5 is the proteins that were eluted in 500 mM NaCl. Lane 6 is the proteins that were eluted in 750 mM NaCl.](image)

Following cation exchange chromatography, protein not retained by the column was pooled and dialysed overnight against 20 mM CHES pH 9.5, 50 mM NaCl (Section 2.6.7.3). Following dialysis, the sample was loaded on to a Mono Q anion exchanger (Amersham). A NaCl gradient was run over this column, from 50 mM to 1 M, with collection of the resulting fractions (Section 2.6.7.3). The UV trace from this analysis is presented in Figure 5.8.
Fractions 16 – 23 from this analysis were then analysed by SDS PAGE, as shown in Figure 5.9.

**Figure 5.8:** UV trace following Mono Q anion exchange chromatography of the sample containing CDC25B/14-3-3β. The green line shows the NaCl gradient, from 50 mM to 1 M. The complex was found to elute in fractions 19 and 20, in the peak indicated.

**Figure 5.9:** SDS PAGE analysis of the Mono Q anion exchange chromatography of the CDC25B/14-3-3β complex, following SP Sepharose chromatography of the sample. Lane 1 is the cytoplasmic lysate. Lane 2 is the sample following cation exchange. Lane 3 is a protein ladder with markers indicated. Lane 4 – 12 shows the eluted fractions from this analysis, Fractions 16 – 23, including the CDC25B/14-3-3β complex.

The fractions containing the CDC25B/14-3-3β complex were pooled, and dialysed into a Tris.HCl buffer at pH 7.5. The sample was concentrated to 0.5 mL, and analyzed by size exclusion chromatography (Section 2.6.7.4). The UV trace from this analysis is presented in
Figure 5.10. Analysis by SDS PAGE indicated Fractions 8 – 13 contained the CDC25B/14-3-3β complex (shown in Figure 5.11), and that the remainder of the fractions contained low molecular weight protein contaminants (data not shown).

**Figure 5.10:** UV trace following size exclusion chromatography of the CDC25B/14-3-3β sample, following SP Sepharose and Mono Q chromatography. The CDC25B/14-3-3β complex was found to elute in the fractions 8 – 13, as shown.

**Figure 5.11:** SDS PAGE analysis following sequential SP Sepharose, Mono Q chromatography and size exclusion chromatography of the CDC25B/14-3-3β complex. Lane 1 is the cytoplasmic lysate. Lane 2 is the sample following cation exchange chromatography. Lane 3 is the sample following Mono Q ion exchange. Lane 4 is a protein ladder with markers indicated. Lanes 5 – 10 are the eluted fractions from size exclusion chromatography, Fractions 8 – 13, containing the CDC25B/14-3-3β complex.
5.6: Large Scale Expression and Purification of CDC25B/14-3-3β

An attempt at larger-scale purification of the CDC25B/14-3-3β complex was carried out by replicating the small-scale purification. A sample of CDC25B/14-3-3β was expressed and the cells harvested by centrifugation (Section 2.6.8.1). Following cell lysis, the lysate was subjected to anion exchange purification, using a HiTrap Q FF column (Amersham Bioscience), as in Section 2.6.8.2, and analysed by SDS PAGE (see Figure 5.12).

Following anion exchange chromatography, the partially purified complex was buffer exchanged to pH 7.5 and further purified by size exclusion chromatography (Section 2.6.8.3). The UV trace from this purification step is presented in Figure 5.13, and an SDS PAGE analysis of every second fraction is presented in Figure 5.14. The presence or absence of CDC25B was followed throughout this protocol based on the molecular weight of the species in solution. It was found that this scaled-up protocol was not useful in the purification of the CDC25B/14-3-3β complex.
Figure 5.13: UV trace of the size exclusion chromatography of the CDC25B/14-3-3β complex, following anion exchange chromatography. The CDC25B/14-3-3β complex was found to elute in Fractions 34 – 38, as indicated by the two black lines.

Figure 5.14: SDS PAGE analysis of every second fraction following size exclusion chromatography of the partially purified CDC25B/14-3-3β complex following Q FF anion exchange chromatography. Lane 1 shows the sample following anion exchange chromatography, prior to size exclusion chromatography. Lane 2 is a protein ladder with markers indicated. Lanes 3 – 12 show every second of fractions 20 – 38 eluted from the S200 16/600 column. The CDC25B/14-3-3β complex was found to elute in fractions 34 - 38.

The small scale protocol routinely gave consistent results for the purification of the CDC25B/14-3-3β complex (as measured by SDS PAGE analysis). It was anticipated that a larger-volume purification (50 – 100 mL to 100 mL and above) may give results of similar consistency, at similar or improved purity or better, but with a greater amount of
CDC25B/14-3-3β complex present. This relied on the assumption that the major purification step should have favoured the enrichment of the CDC25B/14-3-3β complex, over the other impurities. Instead, it was found that anion exchange chromatography uniformly enriched both the CDC25B/14-3-3β complex and other impurities. This suggests that the use of these alternative chromatographic methods is not sufficient to adequately purify the CDC25B/14-3-3β complex.

5.7: Sypro Ruby Analysis of the CDC25B/14-3-3β Complex

SDS PAGE and Sypro Ruby were used in combination to determine the stoichiometry of binding of the CDC25B/14-3-3β complex. In general terms, this technique couples SDS PAGE analysis with Sypro Ruby stain (Invitrogen Molecular Probes) and measurement of band density using a phosphorimager (expressed as LAU-BG). PrecisionPlus Molecular Weight Standards (Biorad) are used to assemble a calibration curve, allowing correlation between band density (LAU-BG) and the amount of protein loaded (in ng). A more detailed discussion of this technique is presented in Section 6.1.4.1. When adjusted to account for molecular weight, this allows calculation of the molar ratio between CDC25B and 14-3-3β, and hence the stoichiometry of binding between the two molecules.

The CDC25B/14-3-3β complex was expressed and purified before being used to prepare a 1:2 dilution series that was analyzed by SDS PAGE, in conjunction with five aliquots of protein standards (Section 2.6.9). The resulting gel (Figure 5.15) was fixed, stained with Sypro Ruby stain (Invitrogen, Molecular Probes), de-stained, and washed with sterile de-ionized water, before being subjected to densitometry analysis (Section 2.6.9).

Protein standards run in parallel with the CDC25B/14-3-3β complex (refer to Section 2.5.5.1) were analyzed using MultiGauge (Fujifilm). The relative densities of the 50 kDa and 75 kDa protein bands from all of the aliquots were determined; this was measured as the Laser Absorbance Units (LAU) of each protein band, from which the background reading (BG) of each was subtracted, resulting in the measurement of LAU-BG (see Table 5.1).
Figure 5.15: SDS PAGE analysis of the CDC25B/14-3-3β complex stained with Sypro Ruby and analyzed using MultiGauge. Lanes 1 – 5 show a 1:2 dilution series of the CDC25B/14-3-3β complex. Lanes 6 – 9 show the protein ladder with markers indicated, which were used to construct a calibration curve.

Table 5.1: Density measurements of the 75 kDa and 50 kDa protein bands from the PrecisionPlus Molecular Weight standards, following SDS PAGE analysis and Sypro Ruby staining. The Laser Absorbance Units (LAU) for each protein band was measured, with subtraction of the background (BG) readings. These density values (LAU-BG) were used, along with the known amount of protein loaded, to construct a calibration curve.

In conjunction with the known amounts of the protein standards that were loaded onto the gel, the corrected relative densities of the respective protein bands (reported as the LAU-BG values) were used to construct a calibration curve (Figure 5.16).
The CDC25B/14-3-3β complex samples were then also analyzed to determine protein band density (each lane individually) using the same process as for the protein standards. The calibration curve (Figure 5.16) was used to convert these density (LAU-BG) measurements to a quantity of protein within the gel (in ng), and to calculate the molar amount of protein present (nmol), allowing the molar ratio of CDC25B to 14-3-3β in the complex to be assessed. This analysis is presented in Table 5.2.

Table 5.2: Protein band density measurements and calculation of molar ratio between CDC25B and 14-3-3β, following analysis by SDS PAGE and Sypro Ruby stain. The density (Laser Absorbance Units, LAU, with subtraction of background readings, BG), for each protein band was measured, and the previously constructed calibration curve (Figure 5.16) was used to convert this measurement into a protein amount, and a molar ratio between CDC25B and 14-3-3β.
Overall, the analysis indicates an empirical stoichiometric ratio of one CDC25B molecule binding to one 14-3-3β molecule, within the complex. The analysis was carried out on a total of two separate occasions, using protein independently prepared. For each analysis, the empirical stoichiometry between the two molecules was determined as is 1:1; the second analysis is presented in Appendix B.

While this result is consistent for both analyses, it is preliminary, and should be re-confirmed using a verified and further purified sample of CDC25B/14-3-3β.

14-3-3 proteins are able to bind their substrates as either monomers or (homo- or hetero-) dimers, with the latter being the most common and only visualized arrangement for these molecules [69], [76], [79] (discussed further in regards to the wee1/14-3-3β complex in Section 6.1.4.1). An overall empirical stoichiometry of 1:1 suggests that the functional arrangement (in vivo) is likely to be either 1 CDC25B molecule in complex with 1 14-3-3β molecule, or 2 CDC25B molecules in complex with 2 14-3-3β molecules (a functional stoichiometry of 1:1 or 2:2). As in the case of the wee1/14-3-3β complex (discussed in Section 6.1.4.1), this method is unable to determine which arrangement is likely to be correct. Consequently, the native molecular weight of the CDC25B/14-3-3β complex was assessed using size exclusion chromatography, in an attempt to ascertain which arrangement is more likely to be present.

5.8: Analytical Size Exclusion of the CDC25B/14-3-3β Complex

The CDC25B/14-3-3β complex was expressed and purified as detailed in Section 2.6.7 and subjected to analytical size exclusion chromatography (Section 2.6.10). The UV trace of this analysis is presented in Figure 5.17.

The elution volume for the CDC25B/14-3-3β sample was measured as 8.68 mL, enabling the calculation of the $K_{AV}$ for this species as 0.041 (using equations detailed in Section 4.1.3.2.1). Thus, the overall apparent molecular weight in solution for this species was calculated to be 767 kDa (to 3 significant figures).
Figure 5.17: UV trace of analytical size exclusion chromatography of the CDC25B/14-3-3β complex. The protein complex eluted in the peak shown, resulting in an elution volume of 8.68 mL for this sample.

ProtParam (http://www.expasy.org/tools/protparam.html [95]) predicts the molecular weight of a complex of CDC25B (65 kDa) and 14-3-3β (28 kDa), assuming a 1:1 empirical stoichiometry of binding, would be 93 kDa; thus, this apparent molecular weight likely reflects 8 CDC25B molecules in complex with 8 14-3-3β molecules. Given the likely binding modes of 14-3-3β (discussed Section 5.7), this may represent an arrangement of aggregated heterodimers of 1:1 stoichiometry, or aggregated heterotetramers of 2:2 stoichiometry.

5.9: Future Directions for CDC25B/14-3-3β

CDC25B was able to be expressed and partially purified by IMAC. Co-purification with a suspected Sf9-derived 14-3-3 molecule led to re-cloning CDC25B to co-express with human 14-3-3β. Purification of this CDC25B/14-3-3β complex was problematic, and future directions for the purification of the complex will involve a re-design of the expression construct, to allow for the inclusion of an effective affinity purification step. For example, this might include introducing a C-terminal polyhistidine purification tag, or an immuno-
precipitation affinity purification step. The introduction of such a step would hopefully lead to improved protein purity and yield, and with it, greater opportunities for further characterization.
Chapter 6 Discussion

The overall aims of this project were to characterise the molecular complexes that form during the eukaryotic cell cycle to either activate or inhibit the onset of mitosis at the G2/M checkpoint. While it is known which molecules are involved in these processes, (refer to Chapter 1), the exact nature of their interactions was largely unknown. To understand the regulation of the cell cycle is to understand one of the most fundamental processes of eukaryotic cellular growth and development. An enhanced understanding of the molecular interactions that control one key step in the cell cycle – the transition to mitosis – formed the primary objective of this research.

The research focussed on the expression and purification of cyclin B1 and CDK1, with a view towards the reconstitution of the cyclin B1/CDK1 complex, the biophysical and biochemical investigation of the wee1/14-3-3β complex, which inhibits the onset of mitosis, as well as the investigation of CDC25B, which promotes the onset of mitosis. Cyclin B1 and CDK1 experimental results are presented and discussed in Chapter 3. The experimental results for wee1/14-3-3β complex, which became the major focus of this thesis, are presented Chapter 4, and the experimental results for CDC25B are presented and discussed in Chapter 5. Presented in this chapter is a discussion of the wee1/14-3-3β results and their wider context, as well as a comparison between the wee1/14-3-3β complex and the CDC25B/14-3-3β complex.
6.1: Analysis of the Wee1/14-3-3β Complex

6.1.1: Expression of Full Length and Truncated Wee1/14-3-3β Complexes

6.1.1.1: Expression of the Full Length Wee1/14-3-3β Complex

Co-expression of wee1 and 14-3-3β was undertaken using the Bac-to-Bac Baculovirus Expression System (Invitrogen) to generate infectious recombinant baculovirus containing coding sequences for both proteins. Expression of the wee1/14-3-3β complex was judged by comparing a change in the SDS PAGE banding pattern between uninfected and infected Sf9 culture samples. The measured molecular weight of 14-3-3β from SDS PAGE was 25 – 30 kDa (Figure 4.5, Section 4.1.2.1), matching the expected molecular weight of 28.0824 kDa (calculated using ProtParam, http://www.expasy.org/tools/protparam.html [95]). The expected molecular weight of wee1 was 71.5974 kDa and that of the His-tagged wee1 was 75.0221 kDa (also calculated using ProtParam, http://www.expasy.org/tools/protparam.html [95]). SDS PAGE gave an apparent molecular weight of 85 – 90 kDa for His-tagged wee1 (Figure 4.5, Section 4.1.2.1), and 75 – 80 kDa for untagged wee1 (Figure 4.9, Section 4.1.2.3). This observed difference (the expected molecular weight of 71.5974 kDa vs. the measured molecular weight of 75 – 80 kDa) may be due to post translational modification of the wee1 kinase.

Wee1 is known to exhibit phosphorylation sites at residues Ser-53 and Ser-123 [38], [40]. In addition, the formation of a complex between wee1 and 14-3-3β depends upon the phosphorylation of wee1 residue Ser-642 [8], [33], [51]. The exact molecule that is responsible for the in vivo phosphorylation of wee1 is, as yet, unknown. However, there is enough redundancy between human and Sf9 cell systems that the Sf9-derived cell cycle machinery is able to phosphorylate the expressed human wee1, allowing formation of the complex. The addition of a phosphate group alters the actual molecular weight of a protein by a minimal amount, but is known to alter the mass/charge ratio of a protein, to the point where that protein can exhibit an altered migration pattern following SDS PAGE analysis [120], [121], [122]. It is possible that the expressed wee1 has been phosphorylated, due to its
ability to form a complex with 14-3-3β, which has altered the mass/charge ratio for the protein, thus increasing the apparent molecular weight of wee1 to the observed 75 – 80 kDa.

6.1.1.2: Tryptic Analysis of the Wee1/14-3-3β Complex

The wee1/14-3-3β complex was subjected to trypsin digestion in order to find stable fragments of either wee1 or 14-3-3β that were protected from proteolysis either by their location in the individual proteins or by complex formation. This analysis (Figure 4.10, Section 4.2) showed that, at increasing concentration of trypsin, the molecular weight of the wee1 component of the complex dropped from 75 – 80 kDa to a species of 40 – 50 kDa and then was proteolysed completely. Conversely, the molecular weight of 14-3-3β did not change, regardless of trypsin concentration.

Analysis of the trypsin digestion of wee1 suggested that a significant part of the full length molecule (25 – 40 kDa, leaving a contiguous 40 – 50 kDa intact) is readily accessible to rapid proteolysis. Based on protein sequence alone, trypsin is predicted to be able to cleave wee1 into up to 80 fragments (refer to Appendix C), many of which are one to two amino acids alone. Given that trypsin is a globular enzyme, and therefore requires space to interact with both the side chain and peptide backbone of the target amino acid, this trypsin digestion result suggested that the 25 – 40 kDa segment(s) of wee1 exhibited an open architecture, consistent with little ordered secondary structure. It also suggested that the wee1 protein contains a domain or region of approximately 40 – 50 kDa that is more tightly folded, with ordered secondary structure. The complete proteolysis of this intermediate degradation product at higher trypsin concentrations suggests that the secondary structure of this domain or region must also contain some flexible regions or loops.

In order to map these results on to the wee1 structure, a probability of disorder analysis was carried out using DisEMBL [100] and Phyre [101] (Section 4.2). These analyses predicted that the N-terminal domain of wee1 (30 kDa, calculated using ProtParam [95]) is likely to be disordered and hence, would be readily accessible to trypsin proteolysis. It seemed likely, therefore, that incubation of the full length wee1 with trypsin led, initially, to the degradation
of this 30 kDa N-terminal domain, and the appearance of the 40 – 50 kDa wee1 intermediate degradation product. The DisEMBL [100] and Phyre [101] analyses further indicated that this shorter wee1 construct, from the beginning of the kinase domain to the C-terminus, was likely to be more stable, and would retain the ability to form a complex with 14-3-3β (due to the inclusion of wee1 residue Ser-642, refer to Section 1.3.3.1). This fragment would have a molecular weight of 42 kDa, consistent with the 40 – 50 kDa species of the proteolysis.

The second observation from the proteolytic digestion of wee1/14-3-3β complex was the lack of digestion of 14-3-3β; at all trypsin concentrations, this species appeared to remain intact. Based on protein sequence alone, trypsin is predicted to be able to cleave 14-3-3β into up to 30 fragments (refer to Appendix C), many of which, like that of wee1, are one to two amino acid stretches only. The structure of 14-3-3β (PDB ID 2BQ0, [110]) indicates that residues Lys-11, Arg-20, Arg-43, Arg-57, Arg-58, Lys-76, Arg-85, Lys-87, Lys-117, Lys-124, Lys-122, Arg-129, Lys-159 and Lys-169 are not surface accessible, and hence are not likely to be susceptible to trypsin proteolysis. On the other hand, 14-3-3β residues Lys-5, Lys-13, Lys-29, Lys-51, Arg-62, Lys-70, Arg-73, Lys-77, Lys-82, Lys-105, Lys-140, Lys-160, Lys-189, Lys-195, Lys-214 and Arg-224 are all surface accessible, and would normally be expected to be open to trypsin proteolysis. The fact that none of these sites were cut (as determined by protein denaturation and subsequent SDS PAGE analysis) indicates that these residues have been protected from digestion. A potential exclusion to this is residue Arg-73: this lies in a flexible loop region, and would be an ideal candidate for tryptic digestion, if not for the fact that the next residue is Glu-72. The presence of this acidic residue could have slowed the rate of digestion to the point where no digestion was seen at all.

Protection of these residues may arise either from the geometry of the micro-environment surrounding the residue in question, or from the presence of another domain or molecule that provides protection. The most likely scenario is the former one, whereby the geometry of the micro-environment surrounding these residues is not favourable for trypsin binding and subsequent proteolytic digestion. This is evidenced by that fact that even when wee1 has been completely digested by trypsin (Lane 3, Figure 4.10, Section 4.2), 14-3-3β persists in an intact state. It is possible that some protection of 14-3-3β does come from its complexation with wee1, but this seems not to be the main factor.
Overall, this trypsin digestion experiment led to three conclusions:

- firstly, the overall architecture of the wee1 molecule apparently contains segments that are flexible or unfolded;

- secondly, the molecular shape of the wee1 kinase domain to C-terminal domain in complex with 14-3-3β is likely to be compact in nature;

- thirdly, under \textit{in vitro} conditions, the shorter construct, truncated wee1/14-3-3β, is likely to be very useful: it is more stable, and potentially more crystallisable than the full length construct, while still retaining both the kinase activity and the ability to form a complex with 14-3-3β.

It was this third observation that led to the expression and purification of truncated wee1 in complex with 14-3-3β.

\textbf{6.1.1.3: Expression of the Truncated Wee1/14-3-3β Complex}

The second construct analysed as part of this research included an N-terminally truncated version of wee1, extending from the kinase domain to the C-terminus (residues 215 to 692), also in complex with 14-3-3β. It lacks the N-terminal region of the molecule, which is the wee1 domain that DisEMBL [100] and Phyre [101] predicted would be the most disordered portion of the protein (Figures 4.11 and 4.12, respectively, Section 4.2). \textit{In vivo}, this N-terminal region is also involved in the regulation of wee1, and contains the nuclear localisation signal required for the wee1/14-3-3β complex to be directed into the nucleus. The lack of a nuclear localisation signal is reflected within the \textit{in vitro} expression profile of this truncation mutant, which exhibited expression in the cytoplasm only; hence it was only the cytoplasmic cell fraction that was collected and used when undertaking purification.
6.1.2: Purification of the Full Length and Truncated Wee1/14-3-3β Complexes

Purification of either the full length or truncated wee1/14-3-3β complex was undertaken using a combination of IMAC and size exclusion chromatography. IMAC purification of the full length wee1/14-3-3β complex or the truncated wee1/14-3-3β complex was performed using differential affinity purification tag expression. The wee1 species (either truncated or full length) was expressed with an affinity purification tag, whereas the 14-3-3β component had no affinity purification tag. This meant that the wee1 component of the complex was purified by IMAC, and the 14-3-3β component of the complex was co-purified due to its interaction with wee1. A major benefit of such differential tag expression is that any unbound expressed 14-3-3β will not be purified by IMAC.

This raises the possibility, however, that not all wee1 molecules being purified are complexed with 14-3-3β. Wee1 on its own is known to be unstable, and is rapidly degraded by intracellular proteosomes; the presence of 14-3-3β bound to wee1 is known to substantially increase the in vitro and in vivo half life of wee1 [33]. This makes it probable that any unbound wee1 in the sample is more liable to be degraded, rather than remain in the cell fraction to be purified. The strong binding between wee1 and 14-3-3β (which is further discussed in Section 6.1.3.2) in combination with the increased half life of wee1 in the presence of 14-3-3β suggests that the more stable wee1/14-3-3β complex should be favoured in the cell.

The initial construct expressed and purified was that of the full length wee1 in complex with 14-3-3β. Following size exclusion chromatography of this sample, the complex consistently appeared in the void volume of the size exclusion column. There are two potential reasons for this. The first is that the molecular size of the complex may be much greater than the pore size of the column being used. A 1:1 stoichiometry of binding would yield a complex of molecular weight 100 kDa (given that wee1 has a molecular weight of 72 kDa and 14-3-3β has a molecular weight of 28 kDa). Hence, exclusion from the size exclusion column may arise through the complex being composed of many wee1 molecules in complex with many
14-3-3β molecules, with the high molecular weight species being too great for the purification of the sample using this size exclusion column.

The second potential reason for the exclusion of this species from the size exclusion column is that the individual complexes are forming high molecular weight oligomeric aggregates, again increasing their molecular weight (apparent or otherwise) to a point where the species become excluded. For this reason, the presence of reducing agents, such as DTT and β-ME, and variations of NaCl concentrations were tested for their effect on the complex, but none were able to improve the elution profile of this complex to a resolved peak following size exclusion (data not shown). Aggregation of this complex may also be enhanced by the polyhistidine purification tag – the tags from adjacent complexes may have formed non-covalent cross links, leading to an overall increase in the molecular weight of the native species.

Following cleavage of the polyhistidine purification tag, the wee1/14-3-3β species was further purified using size exclusion chromatography. With the purification tag present, the native complex was consistently excluded from the column, and appeared in the void volume. With cleavage of the polyhistidine purification tag, however, the native complex was resolved by the column into a single peak, albeit with an apparent molecular weight that was larger than expected. The inference from this result (purification of the complex as a single resolved peak) is that the complex exists in a distinct multimeric state. Due to the apparent separation of a discrete species following rTEV cleavage, it seems likely that polyhistidine tag-mediated aggregated oligomers may have formed in the case of the tagged complex, thus pushing the species into the excluded volume of the size exclusion column.

Upon size exclusion chromatography of the truncated wee1/14-3-3β species, the complex was consistently purified in two distinct peaks. No amount of buffer variation was able to resolve that sample into one peak or the other; variation of buffer pH, NaCl concentration and the presence or absence of a reducing agent all resulted in two peaks, with some, but limited, variation in the amounts of protein in each peak. Overall, buffer at pH 7.5, with 50 – 150 mM NaCl in the absence of a reducing agent was found to give the majority of protein in the second peak containing protein of a smaller apparent molecular weight, with a lesser amount
of protein in the first peak, containing protein of a larger apparent molecular weight. Analytical size exclusion and DLS were used to determine the molecular weight of the proteins from these peaks, and the results are discussed in Section 6.1.4.2.

A number of attempts were also made to cleave the polyhistidine purification tag from the wee1 N-terminus in the truncated wee1/14-3-3β complex. Both the full length wee1/14-3-3β complex (this work) and the isolated wee1 kinase domain [39] contained a cleavage site that was accessible to the rTEV protease. The truncated wee1/14-3-3β complex cleavage site was not accessible to the rTEV protease, however. The inference behind this observation is that the presence either of the C-terminus of wee1 or of 14-3-3β in the complex leads to an occlusion of the cleavage site, with either or both of these latter two domains interacting with the N-terminal region of the kinase domain.

The presence of 14-3-3β has been shown to alter the inherent activity of the wee1 molecule [33], [51]; thus, it is most likely that this molecule interacts with the kinase domain of the wee1 molecule, and also provides protection against rTEV cleavage of the polyhistidine purification tag. Structurally, this implies that while the specific coupling between wee1 and 14-3-3β occurs via the wee1 C-terminal phosphoserine, either an interaction exists between 14-3-3β and the N-terminal end of the wee1 kinase domain, or the presence of 14-3-3β alters the conformation of the kinase domain such that the rTEV cleavage site is no longer accessible.

Truncated wee1/14-3-3β was also purified in the presence of PD0407824. This compound (Figure 2.5, Section 2.5.4.2) was introduced in an attempt to decrease the flexibility of the wee1 constructs and thereby enhance the likelihood of crystallization, in the same manner as crystallisation of the kinase domain was achieved [39]. This compound was initially added as for the wee1 kinase domain construct, by incubation of PD0407824 with the already purified full length or truncated wee1/14-3-3β complex, but subsequent crystallisation trials were unsuccessful; thus it was felt that further purification of the truncated wee1/14-3-3β/PD0407824 complex could further increase homogeneity, and with it, the probability of crystallisation.
Mass spectrometry was used to confirm that the expressed proteins were, in fact, human wee1, a truncated human wee1 and human 14-3-3β. For this purpose, an in-gel trypsin digestion of the corresponding SDS PAGE bands was undertaken, with the peptides being identified using mass spectrometry. The peptides were then the subject of a BLAST search against a database representing known human, baculoviral and Sf9 peptides. The match returned for these samples was, in each case, the expected proteins, confirming that the proteins being expressed and purified were, in fact, those of human wee1, a truncated human wee1 and human 14-3-3β, respectively (results presented in Sections 4.1.2.1 and 4.3.2). This work was carried out with the help of Dr. Christina Buchanan, in the Centre for Proteomics and Genomics, at the University of Auckland.

It was thought possible that the 14-3-3 species that was co-purified (or a proportion of the co-purified 14-3-3β) could actually be an endogenous insect cell 14-3-3 molecule; if so, however, the proportion of insect cell derived 14-3-3 bound to wee1 is below the level of detection by mass spectrometry, and can therefore be considered negligible.

### 6.1.3: Activity and Integrity of the Wee1/14-3-3β Complex

#### 6.1.3.1: Kinase Activity of the Wee1/14-3-3β Complex

The wee1 kinase activity of the full length and truncated wee1/14-3-3β complex was tested using the CycLex Wee1 Kinase Assay (CycLex, MBL). This method uses an Enzyme Linked Immunosorbant Assay (ELISA) based method to test the kinase activity of wee1 (described in Section 2.5.4.1).

Each functional assay was undertaken using a separate preparation of the experimental sample. The experimental samples used for these assays comprised the two different wee1/14-3-3β constructs (full length and truncated wee1); the majority were undertaken using just the complex of interest, but with one additional assay for each construct being undertaken with PD0407824 bound. The functional assay for full length wee1/14-3-3β was
repeated a total number of four times, with each assay being undertaken using a separate preparation of the complex. Each assay also included a duplicate of each of the samples (both positive and negative controls, as well as the experimental samples), with the absorbance of each well also being read in duplicate (results presented in Section 4.4.1).

These results showed that the wee1/14-3-3β complex exhibited wee1 kinase activity, and that the level of molar activity was 3.5-fold higher than that of the positive control (Section 4.4.1). The activity difference between the positive control and the experimental sample was also shown to be statistically significant, p<0.001 (Section 4.4.1). The same assay, under the same conditions, was used to examine expressed and purified truncated wee1/14-3-3β (results presented in Section 4.4.3). These assays confirmed that the complex formed with truncated wee1 still had full wee1 kinase activity. In fact, the molar activity of the truncated wee1/14-3-3β sample again exceeded that of the wee1 positive control, this time by a factor of 2.1 (Section 4.4.3) Statistical analysis confirmed the significance of this increase in activity relative to that of wee1 alone, p<0.001 (Section 4.4.3), and also confirmed that there was no significant difference between the full length and truncated wee1/14-3-3β complexes, in terms of wee1 kinase activity.

Overall, these data indicate that, within this research, the wee1/14-3-3β complex exhibits, at a minimum, a 2.1-fold increase in molar activity over the wee1 only positive control. Two previous reports have quantified the effects of 14-3-3β on the kinase activity of wee1, the first using co-transfection and blotting in conjunction with immuno-precipitation [33], and the second by mutation of the C-terminus of wee1, also in conjunction with immuno-precipitation [51]. Both of these groups found that the presence of 14-3-3β increased the intrinsic kinase activity of wee1 by a factor of 2.4 and 3, respectively. This suggests an equivalency between the wee1/14-3-3β complex prepared as part of this research and that prepared by those other groups [33], [51].

The addition of PD0407824 (Figure 2.5) to both full length and truncated wee1 was also shown to abolish the kinase activity of their respective complexes with 14-3-3β, confirming that it acts as a wee1 kinase inhibitor in the context of complexation with 14-3-3β,
presumably by binding the active site of the respective wee1 proteins as with the previous wee1 structure [39].

A limitation of this work was that while the kinase activity of wee1/14-3-3β and truncated wee1/14-3-3β purified from peak 2 were measured, the kinase activity of truncated wee1/14-3-3β purified from peak 1 was not measured, and might exhibit an altered kinase activity profile than that of the protein purified from peak 2. As such, future research regarding this complex and its activity might include an analysis of the kinase activity of protein purified from this peak.

6.1.3.2: Attempts to Dissociate the Wee1/14-3-3β Complex

The wee1/14-3-3β complex was formed by co-expression of its two components. A number of attempts were made to separate wee1 and 14-3-3β, in order to measure the strength of binding between them (Section 2.5.5.1). These attempts exploited the fact that the recombinant 14-3-3β did not carry a polyhistidine purification tag, and its retention on an IMAC column is, therefore, dependent on its interaction with wee1. The complex was bound to the column, then washed with an increasing concentration of NaCl (up to 4 M), such that the ionic strength of buffer might overcome the strength of binding between wee1 and 14-3-3β. This would have led to the collection of 14-3-3β in the flow through. Instead, it was found that the interaction between wee1 and 14-3-3β was strong enough to withstand washing with 4 M NaCl (Section 4.5.1).

A second attempt was then undertaken, this time at a lower pH (pH 5.0 instead of pH 7.5). As described in Section 1.3.3.1, phosphorylation of the wee1 residue Ser-642 forms the primary binding site for 14-3-3β, with further stabilisation being achieved through other (secondary) interactions along the binding interface. It was anticipated that the decrease in pH to acidic conditions (pH 5.0) would potentially partially protonate the oxygen(s) of the phosphate group, thereby weakening the binding between wee1 and 14-3-3β. A series of washes was undertaken at this lower pH, again sequentially increasing the NaCl concentration to 4 M with the aim of disrupting the interaction between wee1 and 14-3-3β,
thereby washing out 14-3-3β. This resulted, however, in the elution of the intact complex, testament, as above, to the high affinity of binding between wee1 and 14-3-3β.

A third attempt to separate the two molecules was then undertaken, using 2 M urea ((NH₂)₂CO). Urea is often used as a protein denaturant, due to its ability to disrupt non-covalent interactions within proteins. Urea is able to form extensive hydrogen bonds with water (up to six per molecule) and, accordingly, exhibits a very high solubility, resulting in the potential use of urea in protein stability and folding analyses at concentrations of up to 10 M. The use of urea at such concentrations, however, usually results in the complete denaturation of proteins. Lesser concentrations of urea usually result in only partial denaturation of a reasonably well ordered/stable protein; in fact, literature suggests that up to a concentration of 2 – 3 M urea, 80 – 100% of a typical protein will retain its native conformation, although it is stripped of any non-covalently interacting co-factors [123].

At these concentrations, it was thought that urea might be able to disrupt the non-covalent interaction between wee1 and 14-3-3β (results presented Section 4.5.1). To ensure that any separation that occurred was not immediately reversible, 1 mM EDTA was also introduced. The interaction between wee1 and 14-3-3β occurs through phosphorylation of the wee1 C-terminal Serine, Ser-642, which is thought to be mediated via a magnesium ion [33]. Removal of this ion, using a chelating agent such as EDTA, should assist in the complete dissociation of the complex. Analysis by size exclusion chromatography failed to reveal any evidence of dissociation, however. Only one protein peak, representing the intact wee1/14-3-3β complex, was found, with no additional peaks for the individual wee1 and 14-3-3β components.

The presence of 14-3-3β increases the activity of the wee1 kinase by a minimum factor of 2.1 (Section 6.1.3.1). The binding between wee1 and 14-3-3β is strong enough to withstand up to 4 M NaCl at pH 7.5 or 5.0, or up to 2 M urea/1 mM EDTA, which are not likely physiological conditions. Following previous indications that wee1 not complexed with 14-3-3β exhibits a substantially decreased in vitro and in vivo half life [33], this tight binding in conjunction with the increased enzymatic activity suggests that a highly active wee1 kinase is favoured, within the eukaryotic cell cycle (Section 1.2).
6.1.4: Determination of the Oligomeric State of the Wee1/14-3-3β Complex

6.1.4.1: Determination of the Stoichiometry of the Wee1/14-3-3β Complex

The stoichiometry of binding between wee1 and 14-3-3β was determined by densitometry, using Sypro Ruby (Invitrogen Molecular Probes) staining in combination with SDS PAGE analysis (Sections 2.5.5.2.1 and 2.5.6.1.1). Both wee1/14-3-3β and truncated wee1/14-3-3β were analyzed, and, as detailed in Sections 4.5.2.1 and 4.6.1.1, both analyses gave the same empirical stoichiometry: a 1:1 ratio of wee1 (or truncated wee1) to 14-3-3β. This result was obtained consistently in three separate analyses for each of the full length and truncated wee1/14-3-3β complexes.

14-3-3 proteins are able to bind their substrates as either monomers or (homo- or hetero-) dimers, but all experimentally determined 14-3-3β structures are dimeric [69], [76], [79]. An empirical stoichiometry of 1:1 means, therefore, that the functional arrangement is likely to be either 1 (full length or truncated) wee1 molecule in complex with 1 14-3-3β molecule, or 2 (full length or truncated) wee1 molecules in complex with 2 14-3-3β molecules (a functional stoichiometry of either 1:1 or 2:2). The Sypro Ruby analysis is method is unable to determine which arrangement is likely to be correct. Thus, a number of methods were used to determine the native molecular weight of the (full length or truncated) wee1/14-3-3β complex.

6.1.4.2: Determination of the Native Molecular Weight of the Wee1/14-3-3β Complex

6.1.4.2.1: Analytical Size Exclusion Chromatography of the Wee1/14-3-3β Complex

The first method employed to determine the native molecular weight of the wee1/14-3-3β complex was analytical size exclusion chromatography, as in Section 2.5.5.2.2.1. Both
untagged wee1/14-3-3β and truncated wee1/14-3-3β were analysed using this method, but the His-tagged wee1/14-3-3β could not be similarly analysed, as it eluted in the excluded volume of the column. As described in Section 4.5.2.2.1, untagged wee1/14-3-3β exhibited an apparent molecular weight of 803 kDa. With a 1:1 empirical stoichiometry (Section 6.1.4.1), this apparent molecular weight likely reflects a complex of 8 wee1 molecules and 8 14-3-3β molecules, given that the combined molecular weights of wee1 (72 kDa) and 14-3-3β (28 kDa) is 100 kDa.

Analysis of the truncated wee1/14-3-3β complex by analytical size exclusion chromatography (Section 4.6.1.2.1) gave two different apparent molecular weights. Truncated wee1/14-3-3β complex purified from peak 1 exhibited an apparent molecular weight of 523 kDa, which, assuming 1:1 empirical stoichiometry, corresponded to 7 truncated wee1 molecules in complex with 7 14-3-3β molecules, given that the combined molecular weights of truncated wee1 (42 kDa) and 14-3-3β (28 kDa) is 70 kDa. Truncated wee1/14-3-3β complex purified from peak 2 exhibited an apparent molecular weight of 140 kDa, which, assuming 1:1 empirical stoichiometry, likely reflected 2 truncated wee1 in complex with 2 14-3-3β molecules.

The apparent molecular weight of truncated wee1/14-3-3β protein purified from peak 2 is consistent with the assumption that the underlying functional stoichiometry if 1:1 or 2:2. The apparent molecular weight of truncated wee1/14-3-3β protein purified from peak 1, however, is less consistent with this assumption. The major disadvantage of this method for the determination of apparent native molecular weight is that it is shape dependent. If the native species is elongated, or if there is a flexible linker, giving rise to domain movement in solution, the native species can be excluded from the matrix of the size exclusion column. This would result in an earlier elution profile, and an apparent molecular weight larger than would otherwise be expected. Conversely, if the native species exhibits a tertiary structure that is compact in relation to its primary sequence, the species in question will be eluted from the column later than would otherwise be expected, and would be attributed with an apparent molecular weight that smaller than expected.
Thus, as regards the apparent molecular weight of the truncated wee1/14-3-3β complex, purified from peak 1, the measured 7:7 arrangement may reflect the native molecular arrangement of the complex. On the other hand, it may be that the native size of the truncated wee1/14-3-3β complex in solution is smaller or greater than expected, and may therefore represent either a 6:6 arrangement, or an 8:8 arrangement. Equally, this peak may also represent an intermediate that would normally only be transient, or may also represent an irregular array of molecular interactions and/or aggregations between the two molecules. If the latter is the case, this arrangement of molecules could have formed due to the high concentration of the molecules in vitro, compared to their likely relative concentrations in vivo.

To validate these results, however, especially in light of the unlikely but possible heptameric arrangement, a second technique for determination of native molecular weight was sought.

6.1.4.2.2: Non-Denaturing Gradient PAGE Analysis of the Wee1/14-3-3β Complex

A second method used in attempts to determine the native molecular weight of the complex was non-denaturing polyacrylamide gel electrophoresis. Migration of a species through an acrylamide gel matrix depends upon both the mass and charge of the protein in question. SDS PAGE analysis includes heat denaturation, which removes secondary, tertiary and quaternary protein structures, leaving single amino acid chains and any covalently bound factors. For example, this technique dissociates the wee1/14-3-3β complex to wee1 and 14-3-3β, with migration through the gel reliant on the mass and charge of each amino acid chain.

Non-denaturing PAGE, however, leaves the secondary, tertiary and quaternary structures intact, meaning that gel migration is due to the mass/charge ratio of native proteins (or protein complexes), including any associated factors. For example, this technique would leave the wee1/14-3-3β complex intact, with gel migration reliant on the mass/charge ratio of the native complex. Non-denaturing gradient PAGE analysis also leaves the sample intact, but it is run through a gel matrix containing ever decreasing amounts of acrylamide, and thus with ever decreasing pore sizes, which eventually physically restrict gel migration, such that
migration is dependent on the mass of the sample [124], [125], [126]. For example, this technique leaves the wee1/14-3-3β complex intact, with minimal contribution from native charge (provided that the protein is reasonably anionic), and separation predominantly based on the mass of the sample.

Attempts to visualise the wee1/14-3-3β complex by this method were unsuccessful, however, despite a number of attempts at various concentration (refer to Figure 4.34, Section 4.5.2.2.2). One potential reason for the lack of visualisation of this complex could be that it is highly cationic. Given that a standard gel is cast at pH 6.8 for the stacking gel and pH 8.8 for the resolving gel (Section 2.1.9), a basic protein would be repelled by the charge of the gel, and may never enter the gel. Thus, this analysis was repeated under acidic conditions (Section 2.5.5.2.2.2), which enabled visualisation of the wee1/14-3-3β complex (Figure 4.35, Section 4.5.2.2.2), indicating that the complex is cationic. Protein standards that would be visible under non-denaturing acidic conditions, however, could not be found, and hence this method was not able to contribute to the native molecular weight determination of the complex. Confirmation of the cationic nature of the wee1/14-3-3β was also sought, using an IPG strip (GE Healthcare) to determine its isoelectric point, discussed in Section 6.1.5.1.

6.1.4.2.3: DLS Analysis of the Wee1/14-3-3β Complex

Dynamic light scattering (DLS) (DynaPro, Protein Solutions) was used both to estimate the molecular weight of the wee1/14-3-3β complex, and to measure the polydispersity of samples in solution. DLS analysis of five separate and independent samples of the untagged wee1/14-3-3β complex gave an apparent molecular weight for the species of 617 ± 29 kDa, with an associated polydispersity of 25 ± 7% (Section 4.5.2.2.3). Assuming a 1:1 empirical stoichiometry, this would correspond to an arrangement of 6 wee1 molecules in complex with 6 14-3-3β molecules. DLS analysis of five separate samples of the truncated wee1/14-3-3β complex, purified from peak 2, indicated an apparent molecular weight of 168 ± 0.01 kDa, with an associated polydispersity of 1 ± 0.3% (Section 4.6.1.2.2). Assuming an empirical stoichiometry of 1:1, this apparent molecular weight likely reflected 2 truncated wee1 molecules in complex with 2 14-3-3β molecules.
Molecular weight estimates from analytical size exclusion chromatography and DLS suggest that the wee1/14-3-3β complex comprises either 8 wee1 proteins in complex with 8 14-3-3β proteins or 6 wee1 proteins in complex with 6 14-3-3β proteins, respectively. For the truncated wee1/14-3-3β complex, however, both DLS and analytical size exclusion chromatography gave estimated native molecular weights corresponding to 2 truncated wee1 proteins in complex with 2 14-3-3β proteins.

Given that the latter truncated construct is the smallest construct used, and is predicted by DisEMBL and Phyre to exhibit less disorder (discussed in Section 6.1.1.2), the consistency of the measurements across both DLS and analytical size exclusion chromatography strongly points to this 2:2 arrangement as the most relevant species. The molecular weights measured for the wee1/14-3-3β complex are likely to represent *in vitro* aggregates of this empirical heterotetrameric arrangement. Whether the 2:2 stoichiometric arrangement is more correctly described as a dimer of heterodimers (where the empirical unit is 1 wee1 in complex with 1 14-3-3β) or a true heterotetramer (where the empirical unit is 2 wee1 molecules in complex with 2 14-3-3β molecules), however, can only be determined by more in-depth analysis.

Consideration of the preferred modes of binding by 14-3-3 proteins, though, favours the latter true heterotetrameric arrangement, rather than a dimer of heterodimers. 14-3-3 molecules are known to associate with their binding partners as either monomers or dimers, but most commonly as dimers [69], [76], [79]. The structure of the 14-3-3 dimer contains a large amphipathic groove, of dimensions 35 Å wide, 35 Å long and 20 Å deep [75]. Liu *et al.*, 1995 [75] and Marais *et al.*, 1995 [76] independently suggested that the contribution of each monomer to this groove would be large enough to accommodate a range of residue conformations, from an extended structure to an amphipathic helix, and also suggested that this would be an attractive mode by which a 14-3-3 dimer might bind its substrate. This would form a quaternary structure whereby the empirical unit is composed of four proteins. Two structures which support this hypothesis have since been determined.
The first is a complex between 2 serotonin N-acetyltransferases and 2 14-3-3\(\zeta\) molecules (PDB ID 1IB1, [127]), shown in Figure 6.1. In this complex, the serotonin N-acetyltransferase contains the N-terminal motif RRHTLP (residues 28-33), within which the phosphorylation of Thr-33 produces a 14-3-3\(\zeta\) binding site and the residues surrounding Thr-33 adopt an extended conformation to line the 14-3-3 monomer groove [127]. This allows extensive contacts to be made between each serotonin N-acetyltransferase N-terminal region (residues 18-39, including the phosphorylated Thr-33) and its bound 14-3-3\(\zeta\) monomer, in a manner previously described by Rittinger et al., 1999 [128]. In this structure, one 14-3-3\(\zeta\) molecule binds one N-acetyltransferase, with dimerisation mediated by the 14-3-3\(\zeta\). The empirical unit is 2 N-acetyltransferases in complex with 2 14-3-3\(\zeta\) molecules. The serotonin N-acetyltransferase does not bridge the 14-3-3\(\zeta\) dimer, and in fact there is no significant contact between the two opposing serotonin N-acetyltransferase molecules. Each serotonin N-acetyltransferase is oriented such that the catalytic segment of the protein protrudes from the 14-3-3 dimer in a direction parallel with the 14-3-3 binding groove, thus creating a central cavity (View B Figure 6.4) [127].

![Figure 6.1: Ribbon diagrams of the serotonin N-acetyltransferase/14-3-3\(\zeta\) complex. View A of this complex (PDB ID 1IB1, [127]) shows the dimerized 14-3-3\(\zeta\), shown in yellow and red, with two serotonin N-acetyltransferase proteins bound, shown in blue and green. View B is rotated 90 ° about the horizontal axis, with the same monomer colours, and shows the N-terminal domains of the serotonin N-acetyltransferase proteins lining each of the 14-3-3\(\zeta\) monomer grooves [127]. Image generated using Jmol (http://www.jmol.org).](image-url)
Overall, this complex is a heterotetramer with an empirical stoichiometry of 1:1. The contacts between the serotonin N-acetyltransferase and its associated 14-3-3ζ monomer are not restricted to the residues in direct proximity to the phosphothreonine binding site, but also extend to include contact between all of the 14-3-3ζ helices on the interior concave surface of each 14-3-3ζ and the serotonin binding domain of the serotonin N-acetyltransferase. These contacts lead to a significant increase in the serotonin binding ability of the serotonin N-acetyltransferase [127].

The second structure (PDB ID 2O98, [129]) is complex of a 2 plant P-type H⁺ ATPase peptides (residues 905-956) in complex with 2 14-3-3 molecules (each lacking its C-terminal 18 residues), and is shown in Figure 6.2.

**Figure 6.2: Ribbon diagrams of an H⁺ ATPase domain/14-3-3 complex.** View A of this complex (PDB ID 2O98, [129]) shows the dimerized 14-3-3, shown in pale green and grey, with two H⁺ ATPase domains bound, shown in dark green and mid green. View B is rotated 90 ° about the horizontal axis, with the same monomer colours, and shows the C-terminal regions of the H⁺ ATPase domains lining each of the 14-3-3 monomer grooves [129]. Image generated using Jmol (http://www.jmol.org).

The 14-3-3 binding site in the H⁺ ATPase peptide is created by phosphorylation of the C-terminal residue Tyr-954, with this C-terminal segment then adopting an extended conformation, similar to the above structure, to line the 14-3-3 binding groove. Extensive
contacts are formed between this segment and each 14-3-3 monomer, to stabilise this interaction. As with the serotonin N-acetyltransferase complex, each H⁺ ATPase peptide binds one 14-3-3 molecule, with dimerisation of the 14-3-3 then leading to the formation of a heterotetramer; overall, there is 1:1 empirical stoichiometry and 2:2 functional stoichiometry in this H⁺ ATPase peptide/14-3-3 complex. In contrast to the serotonin N-acetyltransferase complex, the two binding partners, the H⁺ ATPase domains, are able to interact with each other, and extend from the centre of the 14-3-3 dimerisation domain in a direction perpendicular to the 14-3-3 binding groove. The interaction with 14-3-3 does not directly affect the primary activity of the enzyme (building/maintaining an electrochemical proton gradient), but rather the presence of a bound 14-3-3 molecule stops the autoinhibitory activity of the enzyme [129].

The two most significant differences between the above two models (Figures 6.1 and 6.2) are:

- the orientation of the catalytic components of the complexes, relative to 14-3-3: serotonin N-acetyltransferase protrudes from the 14-3-3 dimer in the same plane as the 14-3-3 binding groove, whereas the H⁺ ATPase peptide extends from the centre of the 14-3-3 dimer interface perpendicular to the 14-3-3 binding groove;
- the effect of 14-3-3 binding on the activity of the catalytic component of the complex: 14-3-3 binding to serotonin N-acetyltransferase significantly increases the serotonin binding ability of the enzyme, whereas 14-3-3 binding to the H⁺ ATPase removes the self-regulating activity of the enzyme, but does not affect its activity.

In terms of relating these structures back to the wee1/14-3-3β complex, it is likely that the latter complex adopts a structure that more closely resembles the serotonin N-acetyltransferase/14-3-3ζ complex, for reasons discussed below. A schematic diagram of such an arrangement is presented in Figure 6.3.

An arrangement such as this would satisfy a number of biochemical and biophysical observations. Firstly, wee1 binds to 14-3-3β via its C-terminal phosphoserine (Ser-642) [33], [51]. Previous analyses reveal that this mode of binding involves direct interactions between the phosphate and Lys-49 and Arg-56 in helix C, and Arg-127 and Tyr-128 in helix E of 14-3-3β [69], [76]. This suggested arrangement also allows for this interaction to occur.
Secondary structure predictions for the C-terminal domain of wee1 by Phyre, PsiPred, and JPred3 indicate the presence of a conserved helix (Section 4.5.2.2, discussed Section 6.1.5.2). This conserved helix would be in a prime position to line the 14-3-3β monomer groove, thus forming the interface between wee1 and 14-3-3β, or could act as a linker from the 14-3-3β monomer to the catalytic segments of wee1.

![Figure 6.3: Schematic diagram of the proposed heterotetrameric arrangement of the wee1/14-3-3β complex.](image)

It is proposed that the C-terminal domain of wee1 (shown in green) binds the amphipathic groove of the 14-3-3β dimer (shown in gold). This allows the wee1 kinase domain (shown in blue) to still be able to access its substrate, the cyclin B1/CDK1 complex, and could explain the increase in wee1 kinase activity in the presence of 14-3-3β. It potentially also allows for the N-terminal domain of wee1 (shown in red) to be accessible, for regulation of the complex.

Secondly, the activity of wee1 is increased by a factor of 2.1 when bound to 14-3-3β (Section 6.1.3.1); this proposed model not only potentially allows for a direct interaction to occur between 14-3-3β and the wee1 kinase domain, but the model itself is based on a known example (serotonin N-acetyltransferase) for which activity is also increased in the presence of its 14-3-3 binding partner [127]. The catalytic activity of the wee1/14-3-3β complex involves the phosphorylation of CDK1 residue Tyr-15 in the cyclin B1/CDK1 complex (Section 1.3.3.1). A model based on the structure of the serotonin N-acetyltransferase/14-3-3ζ complex [127] allows enough space for the cyclin B1/CDK complex to physically interact with the wee1 kinase component of this complex.

Lastly, this mode of association of wee1 and 14-3-3β would satisfy biophysical experimental results. The model provides a heterotetrameric arrangement of 1:1 empirical stoichiometry...
between wee1 and 14-3-3β, which is consistent with the results seen here (Sections 6.1.4.1 and 6.1.4.2). It also allows for the formation of the 14-3-3 dimer, the preferred oligomeric state of 14-3-3 [75] [76]. In addition, it should allow full accessibility of the N-terminal domain of wee1, which is the domain responsible for the subcellular profile of the complex, and well as for the ubiquitination of wee1 for targeted degradation (Section 1.3.3.1).

### 6.1.5: Molecular Characterisation of the Wee1/14-3-3β Complex

#### 6.1.5.1: Determination of the Isoelectric Point of the Wee1/14-3-3β Complex

Non-denaturing PAGE analysis, discussed in Section 6.1.4.2.2, indicated that the wee1/14-3-3β complex is cationic, as shown by the requirement for acidic conditions for visualisation of the complex. Subsequent IEF analysis indicated that the isoelectric point of the native, functional complex is approximately 9.0 – 9.5 (Section 4.5.3.1), which is consistent with the non-denaturing PAGE analysis. This highly cationic value was unexpected, however, given that ProtParam [95] predicted that the denatured isoelectric point of wee1 was 6.3 and the denatured isoelectric point of 14-3-3β was 4.8. This experimental result implies that acidic residues are buried by complex formation.

The intracellular pH is known to be approximately 7.4 in the cytoplasm, and 6.8 in the nucleus [2], [130], [131], with the drop most likely due to the presence of (negatively charged) nuclear DNA. The isoelectric point of the wee1/14-3-3β complex is 9.0 – 9.5. Once formed, the wee1/14-3-3β complex is known to be localised to the nucleus (Section 1.3.3.1), where the microenvironment is likely to be at pH 6.8, suggesting that the complex will still be cationic.

#### 6.1.5.2: Analysis of the Wee1/14-3-3β Complex using CD

Untagged wee1/14-3-3β and truncated wee1/14-3-3β were also analysed by CD, to determine secondary structure content, using a method adapted from Greenfield et al., 2007 [103].
CD analyses of the full length and truncated wee1/14-3-3β complexes (Sections 4.5.3.2 and 4.6.2.1) indicated the complexes were folded and gave the following secondary structure contents:

<table>
<thead>
<tr>
<th></th>
<th>wee1/14-3-3β:</th>
<th>Truncated wee1/14-3-3β:</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-helical structure:</td>
<td>330 residues</td>
<td>291 residues</td>
</tr>
<tr>
<td>β-sheet structure:</td>
<td>143 residues</td>
<td>77 residues</td>
</tr>
<tr>
<td>Non-regular structure:</td>
<td>419 residues</td>
<td>226 residues</td>
</tr>
</tbody>
</table>

This analysis indicated that both full length and truncated wee1/14-3-3β complexes are folded. Focus then turned to the broad assignment of secondary structure content to the domains of wee1 and 14-3-3β. To aid in putting these assignments into perspective, the secondary structure contributions of the wee1 kinase domain and of 14-3-3β are shown in Table 4.14 (Section 4.5.3.2), and Figure 6.4 shows a domain organisation diagram of wee1 and 14-3-3β. The difference between the two structures analysed by CD (full length vs. truncated wee1, each in complex with 14-3-3β) is due solely to the N-terminal region of wee1; the differences between the two CD datasets should, therefore, correspond to the secondary structure contributions of the N-terminal domain of wee1.

If taken literally, these figures suggest that within the N-terminal domain of wee1:

- 39 residues adopt an α-helical secondary structure;
- 66 residues adopt a β-sheet secondary structure;
- 193 residues do not adopt a regular secondary structure (random coil).

Rather than being an absolute representation of the secondary structure content of this domain, however, these values indicate that the N-terminal domain of wee1 likely contains a small α-helical content, a slightly larger β-sheet content, and is predominantly of an irregular structure. This is consistent with the DisEMBL [100] and Phyre [101] protein disorder predictions (presented Section 4.2, discussed Section 6.1.1.2).
A sequence alignment of wee1 homologues was undertaken to determine the more conserved regions of the protein (Figure 4.41), and multiple sequence alignment in combination with three structure prediction methods (using Phyre, PsiPred and JPred3) were used to try to estimate predicted secondary structures for comparison with the CD results. These results are presented in Section 4.5.3.2, Figures 4.42, 4.43 and 4.44. The consensus prediction suggests that the N-terminal domain contains one or two α-helices at the N-terminal end of this domain and perhaps a β-sheet at the C-terminal end on this domain, with the remainder of the domain not adopting a regular secondary structure (random coil). These predictions are broadly consistent with the above CD-measured secondary structure contributions.

The same secondary structure alignments described above (Section 4.5.3.2, Figures 4.42, 4.43 and 4.44) were then used in an attempt to predict the secondary structure content of the C-terminal domain of wee1. The predictions were very consistent, and the consensus suggested that the C-terminals domain is likely to exhibit an α-helix at the N-terminal end of this domain, and a β-sheet at the C-terminal end of this domain. These secondary structure predictions are broadly consistent with both the known secondary structures of the kinase domain of wee1 and the CD results for the N-terminal domain of wee1, lending weight to the overall consensus.
6.1.5.3: Transmission Electron Microscopy of the Wee1/14-3-3β Complex

Attempts to visualise the wee1/14-3-3β complex by transmission electron microscopy (Section 4.5.3.3) suggested that while the overall sample of the complex appears reasonably homogeneous, closer inspection indicates significant variability in solution, consistent with DLS results. In terms of the potential use for single particle analysis, this means that there is no definable starting point for three dimensional reconstruction. There are two possible reasons why this may be the case. Firstly, the sample may simply be at too high a concentration to be able to discern individual species in solution. Prediction of how a protein species will behave on a carbon grid is a difficult task, and determination of the appropriate starting concentration may, and often does, require a trial–and–error approach. Thus, a number of different protein concentrations were used (1 - 0.03 mg mL⁻¹, Section 2.5.5.2.3), but none appeared to improve the image of the wee1/14-3-3β complex.

Secondly, the wee1/14-3-3β complex may be inherently flexible in solution, with domain movements potentially obscuring the overall architecture of the complex. Given the DLS-measured polydispersities of this sample (Section 4.5.2.2.3), this latter possibility is supported. Indeed, a moderate degree of movement in solution was evident even with a wee1/14-3-3β sample at a concentration of 0.03 mg mL⁻¹.

Overall, these results, and the likely reasons behind them, suggested that single particle analysis is not useful in defining the shape or architecture of the wee1/14-3-3β complex.

6.1.5.4: Crystallisation Trials of the Wee1/14-3-3β Complex

Crystallisation trials were undertaken on both the full length and truncated wee1/14-3-3β complexes, at concentrations of 10 – 20 mg mL⁻¹, in the presence or absence of inhibitors, in the presence or absence of the polyhistidine purification tag (where appropriate), using either the hanging or sitting drop techniques (where appropriate), as detailed in Sections 2.5.5.2.4 and 2.5.6.2.2, respectively.
Initial crystallisation attempts on the full length wee1/14-3-3β complex (both untagged and His-tagged) were not successful. To potentially improve crystallizability, the wee1/14-3-3β construct was re-designed on the basis of trypsin digestion of the wee1/14-3-3β complex (Section 6.1.1.2), which identified the N-terminal domain as likely to be substantially disordered. Attempts to crystallise the complex formed with this truncated wee1 were, however, also unsuccessful. Attempts were made to stabilise the structure with a wee1-specific inhibitor, PD0407824, previously used in the crystallisation of the isolate wee1 kinase domain [39], but were also unsuccessful.

Attempts to use TEM to visualise the wee1/14-3-3β complex were hampered by apparent variability in solution. This is consistent with DLS-measured polydispersities, which, using the cumulants analysis, were 25% and 101% for the His-tagged and untagged full length complexes, respectively, and 34% and 57% for the truncated wee1/14-3-3β complex, purified from peak 1 or 2 respectively (Sections 4.5.2.2.3 and 4.6.1.2.2). Such variability may also be the reason for the unsuccessful crystallisation of the complex. Future strategies for the crystallisation of the complex are, therefore, likely to focus on areas that reduce this variability, for example surface entropy reduction, cross linking reagents or mutagenesis or construct redesign to limit the variability.

### 6.1.6: Future Directions for the Investigation of the Wee1/14-3-3β Complex

Overall, the analyses of the truncated and full length wee1/14-3-3β complexes led to the following conclusions:

1. Wee1 and 14-3-3β expressed in Sf9 cells have been shown to form a stable complex that retains the kinase activity of wee1. In fact, the presence of 14-3-3β increases the activity of wee1 kinase by a factor of 2.4. In terms of activity, the wee1 kinase shows equal activity with and without the N-terminal domain of the protein being present.

2. The association between wee1 and 14-3-3β is very strong, and can withstand an ionic strength of up to 4 M NaCl, or up to 2 M urea/1 mM EDTA.

3. The empirical stoichiometry of binding between wee1 and 14-3-3β is 1:1.
4. Various methods were used to determine the native molecular weight of the complex, with the consensus favouring an arrangement consisting of 2 wee1 molecules in complex with 2 14-3-3β molecules.

5. An overall architecture was suggested, modelled on the serotonin N-acetyltransferase/14-3-3ζ complex, whereby the C-terminal domain of wee1 bound to a surface groove of the 14-3-3β monomer, thus forming the primary interface between the two molecules, forming a heterotetramer of wee1/14-3-3β.

6. The wee1/14-3-3β complex is a cationic protein species, with a native isoelectric point of 9.0 – 9.5.

7. CD, used to measure the secondary structure content of the complexes, indicated that both are folded with the full length wee1/14-3-3β complex having approximately 37% α-helical content, 16% β-sheet content and 47% non-regular structure, and the truncated wee1/14-3-3β complex having approximately 49% α-helical content, 13% β-sheet content and 38% non-regular structure.

8. In line with CD results, the N-terminus of wee1 was predicted to be relatively unstructured, but with suggestions that this domain was likely to contain an α-helix followed by a β-strand or sheet at the N-terminal end of the domain;

9. In addition, multiple alignments of secondary structure predictions of wee1 homologues led to the consensus prediction that the C-terminal domain of wee1 was likely to contain an α-helix at the N-terminal end of the domain, and a β-strand or sheet at the C-terminal end of the domain.

10. Attempts were made to view the wee1/14-3-3β complex using both TEM and crystallisation. The apparent inherent flexibility of the complex and/or other potential variations in it meant that these attempts were unsuccessful.

11. Tryptic digestion indicated that the overall architecture of the wee1 molecule was flexible and/or open enough to allow complete digestion by trypsin, with the N-terminal domain of wee1 being the most susceptible. Conversely, 14-3-3β, even in complex with wee1, is trypsin-impervious, suggesting a compact and reasonably enclosed tertiary structure (as confirmed by the structure of the molecule).

Future directions for the analysis of the complex could include further manipulation of the construct(s) to give a more tractable form for structural analysis by either TEM or x-ray
crystallography. For the latter case, such modifications could include further purification to improve the homogeneity of the sample, the use of alternative constructs deleting flexible sections from one or both components of the complex, or other modifications such as surface entropy reduction, mutagenesis or surface residue modification. Small angle x-ray scattering (SAXS) may also be useful in determining a molecular envelope for the complex, through which the protein sequences may be threaded, although the apparent flexibility of the complex may, like that of TEM (Section 6.1.5.3), be somewhat of an issue.

In terms of better defining the interface between wee1 and 14-3-3β, future experiments could include the use of cross-linking agents or surface residue markers, either of which, in combination with the use of mass spectrometry, would better define the regions involved in the interactions between the two proteins. In terms of a better understanding of the complex, future experiments may also include residue specific mutagenesis, for either a better understanding of the interface between the two proteins, or to measure the effect of such mutations on the biochemical activity of the wee1/14-3-3β complex.
6.2: Analysis of CDC25B

CDC25B was expressed and purified, and was shown to co-purify with a molecule thought to be an Sf9-derived 14-3-3 protein (Section 5.2), and was subsequently sub-cloned to co-express with 14-3-3β (Section 5.3). The CDC25B/14-3-3β complex was shown to express, but purification was problematic (Section 5.5). Despite it having been sub-cloned to include an N-terminal His-tag fusion, the CDC25B/14-3-3β complex was unable to be retained by an IMAC resin. Other chromatographic methods were used to purify the complex, which were reasonably successful for cultures of less than 50 to 100 mL, but scale-up to cultures above this volume did not result in protein of adequate purity for ongoing characterisation of the complex. Small-scale purification, however, yielded protein for preliminary characterisation, including determination of the stoichiometry of binding (Section 5.7) and native molecular weight (Section 5.8). Following this initial characterisation, the CDC25B/14-3-3β and wee1/14-3-3β complexes were compared, with a view to gaining insight into the CDC25B/14-3-3β complex.

6.2.1: Overall Insights into the CDC25B/14-3-3β Complex

Although the analysis of the CDC25B/14-3-3β complex was less comprehensive than that of the wee1/14-3-3β complex, a number of comparisons between the two complexes can be made. Wee1 and CDC25B exhibit opposing cellular functions: Wee1 functions to inhibit the cyclin B1/CDK1 complex (Section 1.3.3.1), whereas CDC25B functions to activate this same complex (Section 1.3.4). The molecular architectures of the two are also opposites: wee1 has a regulatory N-terminal domain, a central active domain, and a 14-3-3-binding C-terminal domain, whereas CDC25B has a 14-3-3-binding and regulatory N-terminal domain, followed by a C-terminal catalytic domain. Both molecules form stable complexes with 14-3-3β, in both cases through a phosphoserine residue; the wee1 phosphoserine is located in its C-terminal domain (Ser-642), whereas that in CDC25B (Ser-216) is located in its the N-terminal domain. These domain structures are shown in Figure 6.5. For both proteins, the binding of 14-3-3β alters the subcellular location of the respective molecule; export to the nucleus for wee1, and retention in the cytoplasm for CDC25B.
For both wee1/14-3-3β (Section 6.1.4.1) and CDC25B/14-3-3β (Section 5.7), the empirical stoichiometry of the respective complex is 1:1. Native molecular weight analysis indicated that the molecular weight of both complexes was consistent with 8 wee1 molecules or CDC25B molecules in complex with 8 14-3-3β molecules. Given the likelihood of 14-3-3β dimerisation (Section 1.3.5), and that the likely functional organisation of the wee1/14-3-3β complex is a 2:2 heterotetramer (Section 6.1.4.3), the CDC25B/14-3-3β complex is also likely to be a 2:2 heterotetramer. Further discussion of this assembly, with a focus on the wee1/14-3-3β complex, is presented in Section 6.1.4.3.

While these parallels are interesting, however, they need to be further confirmed. This requires, though, that the CDC25B/14-3-3β complex be expressed and purified on a larger scale than that achieved within this research; the future directions for achieving this are discussed in Section 5.9.
6.3: Summary of Research and Future Directions

The focus of this research was to better understand the specific interactions, biochemical and biophysical, which occur between key molecules that regulate the G2/M checkpoint of the eukaryotic cell cycle. To this end, a number of analyses were undertaken.

The key proteins that for the catalytic arm of the mitosis promotion factor, CDK1 and cyclin B1, were expressed and purified, with a view to characterisation of the cyclin B1/CDK1 complex. Future directions for this complex include optimisation of expression and purification, for ongoing analysis.

The wee1/14-3-3β complex was expressed and purified, and was shown to exhibit wee1 kinase activity. Attempts to dissociate the wee1/14-3-3β complex were unsuccessful, testament to the strength of binding within the complex. Digestion of the complex with trypsin indicated that the architecture of the complex was likely to be flexible and open, particularly within the N-terminal domain. CD analyses indicated the wee1/14-3-3β complex exhibited approximately 37% α-helical secondary structure, 16% β-sheet secondary structure, with the remaining 47% not adopting a regular secondary structure. The empirical stoichiometry of the complex was determined as 1:1, with the minimal functional unit likely to be a 2:2 arrangement. The structural architecture of this complex may be similar to the serotonin N-acetyltransferase/14-3-3ζ complex. Attempts were made to determine the structure experimentally, using either TEM or x-ray crystallography, but the complex appeared to exhibit too much flexibility or movement in solution for single particle analysis, and the same factors may also have prevented crystallisation. Future directions for the analysis of this complex include ongoing biochemical investigation of the complex, and further attempts to improve the crystallizability of the sample.

CDC25B was expressed and purified, and was found to co-purify with a putative Sf9 14-3-3 protein. Thus, it was re-cloned to co-express with 14-3-3β. Subsequent analysis of this CDC25B/14-3-3β complex indicated that the empirical stoichiometry within the complex was 1:1, with the functional organization likely to be an aggregate of a 2:2 arrangement. The
structural arrangement of this complex is most likely to be similar to that described for the wee1/14-3-3β complex. Future directions for the analysis of this complex include re-cloning of the construct, to allow a more specific purification method to be used, allowing further analysis of the complex.

Future directions for the overall project include re-cloning these proteins to co-express as large multimeric complexes, for example, the cyclin B1/CDK1/wee1/14-3-3β complex or the cyclin B1/CDK1/CDC25B/14-3-3β complex, using the MultiBac system [132] which allows heterologous protein expression, from the same expression cassette, using a baculoviral expression system. Such an undertaking will, however, require careful planning, as these proteins are likely to be entirely functional when expressed in Sf9 cells [8], [13], and thus are likely to form complexes only transiently, as occurs in vivo during the cell cycle. Following investigation of the individual protein complexes described above, analysis of this larger complex could provide further insight into how these complexes interact, to promote or inhibit the onset of mitosis.
References


References


[82] Aitken, A., Howell, S., Jones, D., Madrazo, J. and Patel, Y. 14-3-3 α and δ are the Phosphorylated Forms of raf-Activating 14-3-3 β and ζ in vivo: Stoichiometric Phosphorylation in Brain at a Ser-Pro-Glu-Lys Motif. *Journal of Biological Chemistry* 1995; 270:5706 - 5709.


Appendix A  Sequencing Results

Unless otherwise specified, final sequencing results indicate the sequence of the CDK1 clone matches that of CDK1, protein isoform 1, NCBI accession number NP_001777, the sequence of the cyclin B1 clone matches that of the cyclin protein isoform B1, NCBI accession number NP_114172, and the sequence of CDC25B matches that of CDC25B phosphatase, protein isoform B3, NCBI accession number NP_068659.

A.1  Cyclin B1 Sequencing (Forward Strand)
A.2 Cyclin B1 Sequencing (Reverse Strand)
A.3 CDK1 Sequencing (Forward Strand)
A.4 CDK1 Sequencing (Reverse Strand)
A.5 CDC25B Sequencing for Re-cloning into pFASTBac DUAL (Forward Strand)
Appendix B  Sypro Ruby Analyses

B.1 Wee1/14-3-3β Sypro Ruby Analyses

Three samples of the wee1/14-3-3β complex were expressed and purified, and an aliquot from the pooled sample of each was used to prepare three 1:2 dilution series that were separately analyzed by SDS PAGE, in conjunction with five aliquots of protein standards (Section 2.5.5.1). The resulting gels were fixed, stained with Sypro Ruby stain (Invitrogen, Molecular Probes) and washed, before being subjected to densitometry analysis (Section 2.5.5.1). The 50 kDa and 75 kDa bands from the protein standards that had been run in parallel with each wee1/14-3-3β sample were analyzed using MultiGauge (Fujifilm), and in conjunction with the known amounts of the protein standards that were loaded onto the gel, were used to construct three calibration curves (Section 2.5.5.1). A representative set of these results is presented in Section 4.5.2.1.

The wee1/14-3-3β complex samples were then also subjected to densitometry analysis. The respective calibration curve from each analysis was used to convert these density (LAU-BG) measurements to a quantity of protein within each gel (in ng), which was used to calculate the molar amount of protein present (nmol), and thus the molar ratio of wee1 to 14-3-3β in the complex. These analyses are presented in Table B.1.

Overall, these analyses consistently indicated an empirical stoichiometric ratio of 1 wee1 molecule binding to 1 14-3-3β molecule, within the complex.
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Table B.1: Protein band density measurements and calculation of molar ratio between wee1 and 14-3-3β, following analysis by SDS PAGE and Sypro Ruby stain. Three analyses were carried out, whereby the density (Laser Absorbance Units, LAU, with subtraction of background readings, BG), for each protein band was measured, and the respective previously constructed calibration curve was used to convert this measurement into a protein amount, and a molar ratio between wee1 and 14-3-3β.

**B.2 Truncated Wee1/14-3-3β Sypro Ruby Analyses**

Three samples of the truncated wee1/14-3-3β complex were expressed and purified before being used to prepare three 1:2 dilution series’ that were separately analyzed by SDS PAGE,
in conjunction with five aliquots of protein standards (Section 2.5.7.4.1). The resulting gels were fixed, stained with Sypro Ruby stain (Invitrogen, Molecular Probes) and washed, before being subjected to densitometry analysis (Section 2.5.7.4.1). The 50 kDa and 75 kDa bands from the protein standards that had been run in parallel with each truncated wee1/14-3-3β sample were analyzed using MultiGauge (Fujifilm), and in conjunction with the known amounts of the protein standards that were loaded onto the gel, were used to construct three calibration curves (Section 2.5.7.4.1). A representative set of these results is presented in Section 4.6.1.1. The truncated wee1/14-3-3β complex samples were then also subjected to densitometry analysis. The respective calibration curve from each analysis was used to convert these density (LAU-BG) measurements to a quantity of protein within each gel (in ng), which was used to calculate the molar amount of protein present (nmol), and thus the molar ratio of truncated wee1 to 14-3-3β in the complex. These analyses are presented in Table B.2. Overall, these analyses consistently indicated an empirical stoichiometric ratio of 1 truncated wee1 molecule binding to 1 14-3-3β molecule, within the complex.

**B.3 CDC25B/14-3-3β Sypro Ruby Analyses**

Two samples of the CDC25B/14-3-3β complex were expressed and purified before being used to prepare three 1:2 dilution series’ that were separately analyzed by SDS PAGE, in conjunction with five aliquots of protein standards (Section 2.6.9). The resulting gels were fixed, stained with Sypro Ruby stain (Invitrogen, Molecular Probes) and washed, before being subjected to densitometry analysis (Section 2.6.9). The 50 kDa and 75 kDa bands from the protein standards that had been run in parallel with each CDC25B/14-3-3β sample were analyzed using MultiGauge (Fujifilm), and in conjunction with the known amounts of the protein standards that were loaded onto the gel, were used to construct three calibration curves (Section 2.6.9). A representative set of these results is presented in Section 5.7.
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Table B.2: Protein band density measurements and calculation of molar ratio between truncated wee1 and 14-3-3β, following analysis by SDS PAGE and Sypro Ruby stain. Three analyses were carried out, whereby the density (Laser Absorbance Units, LAU, with subtraction of background readings, BG), for each protein band was measured, and the respective previously constructed calibration curve was used to convert this measurement into a protein amount, and a molar ratio between truncated wee1 and 14-3-3β.

The CDC25B/14-3-3β complex samples were then also subjected to densitometry analysis. The respective calibration curve from each analysis was used to convert these density (LAU-BG) measurements to a quantity of protein within each gel (in ng), which was used to calculate the molar amount of protein present (nmol), and thus the molar ratio of CDC25B to 14-3-3β in the complex. These analyses are presented in Table B.3. Overall, these analyses consistently indicated an empirical stoichiometric ratio of 1 CDC25B molecule binding to 1 14-3-3β molecule, within the complex.
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<td>1089.75</td>
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<td>355.48</td>
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<td>41.63</td>
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<td>256.68</td>
<td>128.69</td>
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<td>967.25</td>
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Table B.3: Protein band density measurements and calculation of molar ratio between CDC25B and 14-3-3β, following analysis by SDS PAGE and Sypro Ruby stain. Two analyses were carried out, whereby the density (Laser Absorbance Units, LAU, with subtraction of background readings, BG), for each protein band was measured, and the respective previously constructed calibration curve was used to convert this measurement into a protein amount, and a molar ratio between CDC25B and 14-3-3β.
Appendix C  Predicted Trypsin Cleavage Fragments of Wee1 and 14-3-3β

To assist in analysis of trypsin digestions of wee1, truncated wee1 and 14-3-3β (peptide mass fingerprinting results Section 4.1.2 and 4.3.2, respectively and trypsin digestion of the wee1/14-3-3β complex, Section 4.2), the bioinformatics program PeptideCutter (http://www.expasy.ch/tools/peptidecutter) was used to predict the potential cleavage fragments of wee1 and 14-3-3β following trypsin digestion.

The result of this prediction indicated that trypsin could potentially cleave wee1 at the following residues:

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<tr>
<th>6</th>
<th>13</th>
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<th>22</th>
<th>24</th>
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</table>

This prediction also indicated that trypsin could potentially cleave 14-3-3β at the following residues:

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<tr>
<th>5</th>
<th>11</th>
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<tr>
<td>1</td>
<td>0.2 M cadmium chloride, 40% (v/v) MPD</td>
<td>0.2 M potassium fluoride, 40% (v/v) MPD</td>
<td>0.2 M ammonium fluoride, 40% (v/v) MPD</td>
<td>0.2 M lithium chloride, 40% (v/v) MPD</td>
<td>0.2 M magnesium chloride, 40% (v/v) MPD</td>
<td>0.2 M sodium chloride, 40% (v/v) MPD</td>
<td>0.2 M calcium chloride, 40% (v/v) MPD</td>
<td>0.2 M magnesium chloride, 40% (v/v) MPD</td>
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<tr>
<td>2</td>
<td>0.2 M ammonium chloride, 40% (v/v) MPD</td>
<td>0.2 M sodium iodide, 40% (v/v) MPD</td>
<td>0.2 M potassium iodide, 40% (v/v) MPD</td>
<td>0.2 M sodium thiocyanate, 40% (v/v) MPD</td>
<td>0.2 M potassium thiocyanate, 40% (v/v) MPD</td>
<td>0.2 M lithium nitrate, 40% (v/v) MPD</td>
<td>0.2 M magnesium nitrate, 40% (v/v) MPD</td>
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<td>3</td>
<td>0.2 M sodium nitrate, 40% (v/v) MPD</td>
<td>0.2 M ammonium nitrate, 40% (v/v) MPD</td>
<td>0.2 M potassium nitrate, 40% (v/v) MPD</td>
<td>0.2 M sodium formate, 40% (v/v) MPD</td>
<td>0.2 M potassium formate, 40% (v/v) MPD</td>
<td>0.2 M ammonium formate, 40% (v/v) MPD</td>
<td>0.2 M lithium formate, 40% (v/v) MPD</td>
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<tr>
<td>4</td>
<td>0.2 M magnesium acetate, 40% (v/v) MPD</td>
<td>0.2 M sodium malonate, 40% (v/v) MPD</td>
<td>0.2 M sodium acetae, 40% (v/v) MPD</td>
<td>0.2 M calcium acetae, 40% (v/v) MPD</td>
<td>0.2 M potassium acetae, 40% (v/v) MPD</td>
<td>0.2 M ammonium acetae, 40% (v/v) MPD</td>
<td>0.2 M lithium acetae, 40% (v/v) MPD</td>
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<tr>
<td>5</td>
<td>0.2 M cesium chloride, 40% (v/v) MPD</td>
<td>0.2 M ferric chloride, 40% (v/v) MPD</td>
<td>0.2 M ammonium tartrate, 40% (v/v) MPD</td>
<td>0.2 M potassium tartrate, 40% (v/v) MPD</td>
<td>0.2 M di-ammonium tartrate, 40% (v/v) MPD</td>
<td>0.2 M di-ammonium citrate, 40% (v/v) MPD</td>
<td>0.2 M tri-ammonium citrate, 40% (v/v) MPD</td>
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<tr>
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<td>0.2 M sodium bromide, 40% (v/v) MPD</td>
<td>0.2 M K$_2$HPO$_4$, 40% (v/v) MPD</td>
<td>0.2 M (NH$_4$)$_2$HPO$_4$, 40% (v/v) MPD</td>
<td>0.2 M tri-lithium citrate, 40% (v/v) MPD</td>
<td>0.2 M tri-sodium citrate, 40% (v/v) MPD</td>
<td>0.2 M potassium citrate, 40% (v/v) MPD</td>
<td>0.2 M di-ammonium hydrogen citrate, 40% (v/v) MPD</td>
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<td>0.1 M citric acid pH 4.0, 10% (v/v) MPD</td>
<td>0.1 M sodium acetate pH 5.0, 10% (v/v) MPD</td>
<td>0.1 M MES pH 6.0, 10% (v/v) MPD</td>
<td>0.1 M HEPES pH 7.0, 10% (v/v) MPD</td>
<td>0.1 M Tris pH 9.0, 10% (v/v) MPD</td>
<td>0.1 M bicine pH 9.0, 10% (v/v) MPD</td>
<td>0.1 M citric acid pH 4.0, 20% (v/v) MPD</td>
<td>0.1 M sodium acetate pH 5.0, 20% (v/v) MPD</td>
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<tr>
<td>8</td>
<td>0.1 M MES pH 6.0, 20% (v/v) MPD</td>
<td>0.1 M HEPES pH 7.0, 20% (v/v) MPD</td>
<td>0.1 M Tris pH 8.0, 20% (v/v) MPD</td>
<td>0.1 M bicine pH 9.0, 20% (v/v) MPD</td>
<td>0.1 M citric pH 4.0, 40% (v/v) MPD</td>
<td>0.1 M sodium acetate pH 5.0, 40% (v/v) MPD</td>
<td>0.1 M MES pH 6.0, 40% (v/v) MPD</td>
<td>0.1 M HEPES pH 7.0, 40% (v/v) MPD</td>
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<td>0.1 M Tris pH 8.0, 40% (v/v) MPD</td>
<td>0.1 M bicine pH 9.0, 40% (v/v) MPD</td>
<td>0.1 M citric acid pH 4.0, 65% (v/v) MPD</td>
<td>0.1 M sodium acetate pH 5.0, 65% (v/v) MPD</td>
<td>0.1 M MES pH 6.0, 65% (v/v) MPD</td>
<td>0.1 M HEPES pH 7.0, 65% (v/v) MPD</td>
<td>0.1 M Tris pH 8.0, 65% (v/v) MPD</td>
<td>0.1 M bicine pH 9.0, 65% (v/v) MPD</td>
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<tr>
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<td>0.02 M CaCl$_2$, 0.1 M Na acetate pH 4.6, 15% (v/v) MPD</td>
<td>0.1 M imidazole-HCl pH 8.0, 15% (v/v) MPD, 5% (w/v) PEG 4000</td>
<td>0.2 M ammonium acetate, 0.1 M Na citrate pH 5.6, 15% (v/v) MPD</td>
<td>0.2 M Mg acetate, 0.1 M MES pH 6.5, 15% (v/v) MPD</td>
<td>0.2 M trisodium citrate, 0.1 M HEPES pH 7.5, 15% (v/v) MPD</td>
<td>0.1 M tri-sodium citrate, 0.1 M HEPES pH 7.5, 20% (v/v) MPD</td>
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<tr>
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<td>0.2 M NaCl, 20% (v/v) MPD, 4% (w/v) glycerol</td>
<td>0.02 M CaCl$_2$, 0.1 M Na acetate pH 4.6, 30% (v/v) MPD</td>
<td>0.2 M ammonium acetate, 0.1 M Na citrate pH 5.6, 30% (v/v) MPD</td>
<td>0.2 M Mg acetate, 0.1 M MES pH 6.5, 30% (w/v) MPD</td>
<td>0.5 M sodium acetate, 0.1 M HEPES pH 7.5, 30% (v/v) MPD</td>
<td>0.2 M trisodium citrate, 0.1 M HEPES pH 7.5, 30% (v/v) MPD</td>
<td>0.1 M HEPES pH 7.5, 30% (w/v) PEG 4000</td>
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<td>30% (w/v) MPD, 20% (w/v) ethanol</td>
<td>0.1 M imidazole pH 8.0, 35% (w/v) MPD</td>
<td>0.1 M Tris pH 8.5, 40% (w/v) MPD</td>
<td>0.1 M HEPES pH 7.5, 47% (w/v) MPD</td>
<td>47% (w/v) MPD, 2% (w/v) tert-butanol</td>
<td>50% (w/v) MPD</td>
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<td>0.2 M NaH₂PO₄, 20% PEG 3350</td>
<td>0.2 M K₂HPO₄, 20% PEG 3350</td>
<td>0.2 M NH₄H₂PO₄, 20% PEG 3350</td>
<td>0.2 M tri-lithium citrate, 20% PEG 3350</td>
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<td>2</td>
<td>0.2 M tri-sodium citrate, 20% PEG 3350</td>
<td>0.02 M CaCl₂, 0.1 M Na acetate pH 4.6, 30% (v/v) 2-methyl-1,4-pentanediol</td>
<td>0.4 M potassium sodium tartrate</td>
<td>0.4 M (NH₄)₂HPO₄</td>
<td>0.1 M Tris pH 8.5, 2.0 M ammonium sulfate</td>
<td>0.2 M Tris pH 8.5, 30% (v/v) MDP</td>
<td>0.2 M MgCl₂, 0.1 M Tris pH 8.5, 30% (v/v) PEG 4000</td>
<td>0.1 M Na cacodylate pH 6.5, 1.4 M Na acetate</td>
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<tr>
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<td>0.2 M tri-sodium citrate, 0.1 M cacodylate pH 5.6, 30% (v/v) isopropanol</td>
<td>0.2 M ammonium acetate, 0.1 M tri-sodium citrate pH 5.6, 30% (v/v) PEG 4000</td>
<td>0.2 M ammonium acetate, 0.1 M Na acetate pH 4.6, 30% (v/v) PEG 4000</td>
<td>0.2 M ammonium acetate, 0.1 M Na acetate pH 4.6, 30% (v/v) PEG 4000</td>
<td>0.2 M MgCl₂, 0.1 M HEPES pH 7.5, 30% (v/v) isopropanol</td>
<td>0.2 M MgCl₂, 0.1 M HEPES pH 7.5, 30% (v/v) PEG 4000</td>
<td>0.2 M MgCl₂, 0.1 M HEPES pH 7.5, 28% (v/v) MPD</td>
<td>0.2 M Na cacodylate pH 6.5, 30% (v/v) PEG 8000</td>
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<tr>
<td>4</td>
<td>0.1 M HEPES pH 7.5, 1.5 M LiSO₄</td>
<td>0.2 M Mg, 0.1 M Tris pH 5.6, 30% PEG 4000</td>
<td>0.2 M ammonium acetate, 0.1 M Na cacodylate pH 6.5, 30% (v/v) isopropanol</td>
<td>0.2 M ammonium acetate, 0.1 M Na cacodylate pH 6.5, 30% (v/v) isopropanol</td>
<td>0.2 M ammonium acetate, 0.1 M Na cacodylate pH 6.5, 30% (v/v) isopropanol</td>
<td>0.2 M MgCl₂, 0.1 M NaH₂PO₄, 0.5 M LiSO₄</td>
<td>0.2 M MgCl₂, 0.1 M NaH₂PO₄, 0.5 M LiSO₄</td>
<td>0.2 M MgCl₂, 0.1 M NaH₂PO₄, 0.5 M LiSO₄</td>
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<td>5</td>
<td>0.2 M CaCl₂, 0.1 M Na acetate pH 4.6, 20% (v/v) isopropanol</td>
<td>0.2 M ammonium acetate, 0.1 M tri-sodium citrate pH 5.6, 30% (v/v) MPD</td>
<td>0.2 M tri-sodium citrate, 0.1 M HEPES pH 7.5, 20% (v/v) MPD</td>
<td>0.2 M Na acetate, 0.1 M NaH₂PO₄</td>
<td>0.2 M ammonium sulfate, 0.3% (v/v) PEG 8000</td>
<td>0.2 M ammonium sulfate, 0.3% (v/v) PEG 8000</td>
<td>0.2 M ammonium sulfate, 0.3% (v/v) PEG 8000</td>
<td>0.2 M ammonium sulfate, 0.3% (v/v) PEG 8000</td>
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<td>6</td>
<td>4.0 M sodium formate</td>
<td>0.1 M Na acetate pH 4.6, 2.0 M Na formate</td>
<td>0.1 M Tris pH 8.5, 6.0% (v/v) PEG 8000</td>
<td>0.1 M HEPES pH 7.5, 1.4 M tri-sodium citrate</td>
<td>0.1 M HEPES pH 7.5, 2% (v/v) PEG 400, 0.2 M ammonium sulfate</td>
<td>0.1 M tri-sodium citrate, 0.1 M HEPES pH 7.5, 20% (v/v) isopropanol</td>
<td>0.1 M Na acetate pH 4.6, 20% (v/v) PEG 8000</td>
<td>0.2 M Na acetate, 0.1 M NaH₂PO₄, 0.1 M HEPES pH 7.5, 0.5% (v/v) MPD</td>
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<td>7</td>
<td>30% (w/v) PEG 1500</td>
<td>0.2 M magnesium formate</td>
<td>0.2 M Zn acetate, 0.1 M Na cacodylate pH 6.5, 18% (v/v) PEG 8000</td>
<td>0.2 M Ca acetate, 0.1 M Na cacodylate pH 6.5, 18% (v/v) PEG 8000</td>
<td>0.1 M Tris pH 8.5, 2.0 M (NH₄)₂HPO₄</td>
<td>1.0 M NaH₂PO₄, 0.5% (v/v) PEG 8000</td>
<td>0.5 M LiSO₄, 1.5% (v/v) PEG 8000</td>
<td>0.05 M K₂HPO₄, 20% (v/v) PEG 8000</td>
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<td>8</td>
<td>25% (v/v) ethylene glycol</td>
<td>35% (v/v) dioxane</td>
<td>2.0 M ammonium sulfate, 5% (v/v) isopropanol</td>
<td>1.0 M imidazole pH 7.0</td>
<td>1.5 M NaCl, 10% (v/v) ethanol</td>
<td>0.1 M Na acetate pH 4.6, 2.0 M NaCl</td>
<td>0.2 M NaCl, 0.1 M Na acetate pH 4.6, 30% (v/v) MPD</td>
<td>0.01 M CoCl₂, 0.1 M Na acetate pH 4.6, 1.0 M Li hexanediol</td>
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<tr>
<td>9</td>
<td>0.1 M cadmium chloride, 0.1 M acetate pH 4.6, 30% (v/v) PEG 400</td>
<td>0.2 M ammonium sulfate, 0.1 M Na acetate pH 4.6, 30% (v/v) MPD</td>
<td>0.2 M potassium sodium tartrate, 0.1 M tri-sodium citrate pH 5.6, 2.0 M ammonium sulfate</td>
<td>0.2 M potassium sodium tartrate, 0.1 M tri-sodium citrate pH 5.6, 2.0 M ammonium sulfate</td>
<td>0.1 M amonium sulfate, 0.1 M Na acetate phosphate pH 5.6, 2% (v/v) MPD</td>
<td>0.1 M tri-sodium citrate pH 5.6, 10% (v/v) Jettanine M-600</td>
<td>0.1 M tri-sodium citrate pH 5.6, 30% (v/v) MPD</td>
<td>0.1 M tri-sodium citrate pH 5.6, 30% (v/v) MPD</td>
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<tr>
<td>10</td>
<td>0.1 M NaH₂PO₄, 0.1 M K₂HPO₄, 0.1 M MES pH 6.5, 2.0 M NaCl</td>
<td>0.1 M MES pH 6.5, 12% (v/v) PEG 20000</td>
<td>1.6 M ammonium sulfate, 0.1 M MES pH 6.5, 10% (v/v) dioxane</td>
<td>0.01 M CoCl₂, 0.1 M MES pH 6.5, 1.8 M ammonium sulfate</td>
<td>0.2 M ammonium sulfate, 0.1 M MESH pH 6.5, 30% (v/v) PEGME 5000</td>
<td>0.1 M HEPES pH 7.5, 10% (v/v) PEG 8000</td>
<td>0.5 M ammonium sulfate, 0.1 M MES pH 6.5, 30% (v/v) PEGME 5000</td>
<td>0.1 M HEPES pH 7.5, 10% (v/v) PEG 8000</td>
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<tr>
<td>11</td>
<td>0.1 M NaCl, 0.1 M HEPES pH 7.5, 1.6 M ammonium sulfate</td>
<td>0.1 M HEPES pH 7.5, 2.0 M ammonium formate</td>
<td>0.05 M cadmium chloride, 0.1 M HEPES pH 7.5, 1.0 M Na acetate</td>
<td>0.1 M HEPES pH 7.5, 4.3 M NaCl</td>
<td>0.1 M HEPES pH 7.5, 30% (v/v) PEG 10000</td>
<td>0.2 M MgCl₂, 0.1 M Tris pH 8.5, 3.4 M Li hexanediol</td>
<td>0.1 M Tris pH 8.5, 25% (v/v) tert-butanol</td>
<td>0.01 M Nickel (II) chloride, 0.1 M Tris pH 8.5, 1.0 M LiSO₄</td>
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<tr>
<td>12</td>
<td>1.5 M ammonium sulfate, 0.1 M Tris pH 8.5, 32% (v/v) glycerol</td>
<td>0.01 M Nickel (II) chloride, 0.1 M Tris pH 8.5, 20% (v/v) PEGME 550</td>
<td>0.1 M NaCl, 0.1 M bicine pH 9.0, 20% (v/v) PEGME 550</td>
<td>0.1 M bicine pH 9.0, 2.0 M MgCl₂</td>
<td>3.2 M ammonium sulfate pH 6.0</td>
<td>3.2 M ammonium sulfate pH 7.0</td>
<td>3.2 M ammonium sulfate pH 8.0</td>
<td>DII Initial Crystallisation Screens</td>
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<td>1</td>
<td>7% (w/v) PEG 6000, 0.2 M citric acid pH 4.9</td>
<td>14% (w/v) PEG 6000, 0.2 M acetic acid pH 4.9</td>
<td>21% (w/v) PEG 6000, 0.2 M citric acid pH 4.9</td>
<td>28% (w/v) PEG 6000, 0.2 M acetic acid pH 4.9</td>
<td>7% (w/v) MPEG 5000, 0.2 M citric acid pH 4.9</td>
<td>14% (w/v) MPEG 5000, 0.2 M acetic acid pH 4.9</td>
<td>21% (w/v) MPEG 5000, 0.2 M citric acid pH 4.9</td>
<td>28% (w/v) MPEG 5000, 0.2 M acetic acid pH 4.9</td>
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<td>2</td>
<td>7% (w/v) PEG 6000, 0.2 M succinic acid/KOH pH 5.5</td>
<td>14% (w/v) PEG 6000, 0.2 M succinic acid/KOH pH 5.5</td>
<td>21% (w/v) PEG 6000, 0.2 M succinic acid/KOH pH 5.5</td>
<td>28% (w/v) PEG 6000, 0.2 M succinic acid/KOH pH 5.5</td>
<td>7% (w/v) MPEG 5000, 0.2 M succinic acid/KOH pH 5.5</td>
<td>14% (w/v) MPEG 5000, 0.2 M succinic acid/KOH pH 5.5</td>
<td>21% (w/v) MPEG 5000, 0.2 M succinic acid/KOH pH 5.5</td>
<td>28% (w/v) MPEG 5000, 0.2 M succinic acid/KOH pH 5.5</td>
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<tr>
<td>3</td>
<td>7% (w/v) PEG 6000, 0.2 M malate pH 8.5</td>
<td>14% (w/v) PEG 6000, 0.2 M malic acid/KOH pH 5.5</td>
<td>21% (w/v) PEG 6000, 0.2 M malic acid/KOH pH 5.5</td>
<td>28% (w/v) PEG 6000, 0.2 M malic acid/KOH pH 5.5</td>
<td>7% (w/v) MPEG 5000, 0.2 M malic acid/KOH pH 5.5</td>
<td>14% (w/v) MPEG 5000, 0.2 M malic acid/KOH pH 5.5</td>
<td>21% (w/v) MPEG 5000, 0.2 M malic acid/KOH pH 5.5</td>
<td>28% (w/v) MPEG 5000, 0.2 M malic acid/KOH pH 5.5</td>
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<td>4</td>
<td>7% (w/v) PEG 6000, 0.2 M BTP/HCl pH 6.7</td>
<td>14% (w/v) PEG 6000, 0.2 M BTP/HCl pH 6.7</td>
<td>21% (w/v) PEG 6000, 0.2 M BTP/HCl pH 6.7</td>
<td>28% (w/v) PEG 6000, 0.2 M BTP/HCl pH 6.7</td>
<td>7% (w/v) MPEG 5000, 0.2 M BTP/HCl pH 6.7</td>
<td>14% (w/v) MPEG 5000, 0.2 M BTP/HCl pH 6.7</td>
<td>21% (w/v) MPEG 5000, 0.2 M BTP/HCl pH 6.7</td>
<td>28% (w/v) MPEG 5000, 0.2 M BTP/HCl pH 6.7</td>
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<td>7% (w/v) PEG 6000, 0.2 M HEPES/KOH pH 7.3</td>
<td>14% (w/v) PEG 6000, 0.2 M HEPES/KOH pH 7.3</td>
<td>21% (w/v) PEG 6000, 0.2 M HEPES/KOH pH 7.3</td>
<td>28% (w/v) PEG 6000, 0.2 M HEPES/KOH pH 7.3</td>
<td>7% (w/v) MPEG 5000, 0.2 M HEPES/KOH pH 7.3</td>
<td>14% (w/v) MPEG 5000, 0.2 M HEPES/KOH pH 7.3</td>
<td>21% (w/v) MPEG 5000, 0.2 M HEPES/KOH pH 7.3</td>
<td>28% (w/v) MPEG 5000, 0.2 M HEPES/KOH pH 7.3</td>
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<td>6</td>
<td>7% (w/v) PEG 6000, 0.2 M Tris/HCl pH 7.9</td>
<td>14% (w/v) PEG 6000, 0.2 M Tris/HCl pH 7.9</td>
<td>21% (w/v) PEG 6000, 0.2 M Tris/HCl pH 7.9</td>
<td>28% (w/v) PEG 6000, 0.2 M Tris/HCl pH 7.9</td>
<td>7% (w/v) MPEG 5000, 0.2 M Tris/HCl pH 7.9</td>
<td>14% (w/v) MPEG 5000, 0.2 M Tris/HCl pH 7.9</td>
<td>21% (w/v) MPEG 5000, 0.2 M Tris/HCl pH 7.9</td>
<td>28% (w/v) MPEG 5000, 0.2 M Tris/HCl pH 7.9</td>
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<tr>
<td>7</td>
<td>7% (w/v) PEG 6000, 0.2 M BTP/HCl pH 8.5</td>
<td>14% (w/v) PEG 6000, 0.2 M BTP/HCl pH 8.5</td>
<td>21% (w/v) PEG 6000, 0.2 M BTP/HCl pH 8.5</td>
<td>28% (w/v) PEG 6000, 0.2 M BTP/HCl pH 8.5</td>
<td>7% (w/v) MPEG 5000, 0.2 M BTP/HCl pH 8.5</td>
<td>14% (w/v) MPEG 5000, 0.2 M BTP/HCl pH 8.5</td>
<td>21% (w/v) MPEG 5000, 0.2 M BTP/HCl pH 8.5</td>
<td>28% (w/v) MPEG 5000, 0.2 M BTP/HCl pH 8.5</td>
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<td>7% (w/v) PEG 6000, 0.2 M MPEG 5000, 0.2 M AMP/PSO/KOH pH 9.1</td>
<td>14% (w/v) PEG 6000, 0.2 M AMP/PSO/KOH pH 9.1</td>
<td>21% (w/v) PEG 6000, 0.2 M AMP/PSO/KOH pH 9.1</td>
<td>28% (w/v) PEG 6000, 0.2 M AMP/PSO/KOH pH 9.1</td>
<td>7% (w/v) MPEG 5000, 0.2 M AMP/PSO/KOH pH 9.1</td>
<td>14% (w/v) MPEG 5000, 0.2 M AMP/PSO/KOH pH 9.1</td>
<td>21% (w/v) MPEG 5000, 0.2 M AMP/PSO/KOH pH 9.1</td>
<td>28% (w/v) MPEG 5000, 0.2 M AMP/PSO/KOH pH 9.1</td>
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<td>9</td>
<td>15% PEG 600, 0.2 M imidazole malate pH 5.5</td>
<td>10% PEG 4000, 0.2 M imidazole malate pH 7.0</td>
<td>7.5% PEG 10000, 0.2 M imidazole malate pH 8.5</td>
<td>0.75 M ammonium sulfate, 0.15 M Na citrate pH 5.5</td>
<td>0.8 M NaHPO4/K-HPO4 Ov pH 7.0</td>
<td>0.75 M trisodium citrate, 10 mM Na borate pH 8.5</td>
<td>24% PEG 600, 0.2 M imidazole malate pH 5.5</td>
<td>15% PEG 4000, 0.2 M imidazole malate pH 7.0</td>
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<td>12.5% PEG 10000, 0.2 M malate pH 8.5</td>
<td>1.0 M ammonium sulfate, 0.15 M Na citrate pH 5.5</td>
<td>1.32 M NaHPO4/K-HPO4 Ov pH 7.0</td>
<td>1.0 M trisodium citrate, 10 mM Na borate pH 8.5</td>
<td>33% PEG 600, 0.2 M imidazole malate pH 5.5</td>
<td>20% PEG 4000, 0.2 M imidazole malate pH 7.0</td>
<td>17.5% PEG 10000, 0.2 M imidazole malate pH 5.5</td>
<td>1.5 M ammonium sulfate, 0.15 M Na citrate pH 5.5</td>
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<td>11</td>
<td>1.6 M NaHPO4/K-HPO4 Ov pH 7.0</td>
<td>1.2 M trisodium citrate, 10 mM Na borate pH 8.5</td>
<td>42% PEG 600, 0.2 M imidazole malate pH 5.5</td>
<td>25% PEG 4000, 0.2 M imidazole malate pH 7.0</td>
<td>22.5% PEG 10000, 0.2 M imidazole malate pH 5.5</td>
<td>2.0 M ammonium sulfate, 0.15 M Na citrate pH 5.5</td>
<td>2.0 M NaHPO4/K-HPO4 Ov pH 7.0</td>
<td>1.5 M trisodium citrate, 10 mM Na borate pH 8.5</td>
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<td>3.2 M ammonium sulfate pH 9.0</td>
<td>2.4 M ammonium sulfate pH 9.0</td>
<td>2.4 M ammonium sulfate pH 9.0</td>
<td>2.4 M ammonium sulfate pH 9.0</td>
<td>2.4 M ammonium sulfate pH 9.0</td>
<td>2.4 M ammonium sulfate pH 9.0</td>
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<td>1.6 M ammonium sulfate pH 6.0</td>
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Appendix D

DIII Initial Crystallisation Screens
### D.4: Screen 4

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</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2 M cadmium chloride, 40% (v/v) MPD</td>
<td>0.2 M potassium fluoride, 40% (v/v) MPD</td>
<td>0.2 M ammonium fluoride, 40% (v/v) MPD</td>
<td>0.2 M lithium chloride, 40% (v/v) MPD</td>
<td>0.2 M magnesium chloride, 40% (v/v) MPD</td>
<td>0.2 M sodium chloride, 40% (v/v) MPD</td>
<td>0.2 M calcium chloride, 40% (v/v) MPD</td>
<td>0.2 M potassium chloride, 40% (v/v) MPD</td>
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<td>0.2 M ammonium chloride, 40% (v/v) MPD</td>
<td>0.2 M sodium iodide, 40% (v/v) MPD</td>
<td>0.2 M ammonium iodide, 40% (v/v) MPD</td>
<td>0.2 M sodium thiocyanate, 40% (v/v) MPD</td>
<td>0.2 M lithium nitrate, 40% (v/v) MPD</td>
<td>0.2 M magnesium nitrate, 40% (v/v) MPD</td>
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<td>0.2 M sodium nitrate, 40% (v/v) MPD</td>
<td>0.2 M potassium nitrate, 40% (v/v) MPD</td>
<td>0.2 M ammonium nitrate, 40% (v/v) MPD</td>
<td>0.2 M sodium formate, 40% (v/v) MPD</td>
<td>0.2 M ammonium formate, 40% (v/v) MPD</td>
<td>0.2 M lithium formate, 40% (v/v) MPD</td>
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<td>0.2 M magnesium acetate, 40% (v/v) MPD</td>
<td>0.2 M sodium malonate, 40% (v/v) MPD</td>
<td>0.2 M sodium acetate, 40% (v/v) MPD</td>
<td>0.2 M calcium acetate, 40% (v/v) MPD</td>
<td>0.2 M ammonium acetate, 40% (v/v) MPD</td>
<td>0.2 M lithium sulfate, 40% (v/v) MPD</td>
<td>0.2 M magnesium sulfate, 40% (v/v) MPD</td>
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<td>0.2 M cesium chloride, 40% (v/v) MPD</td>
<td>0.2 M ferric chloride, 40% (v/v) MPD</td>
<td>0.2 M ammonium sulfate, 40% (v/v) MPD</td>
<td>0.2 M di-sodium tetratrate, 40% (v/v) MPD</td>
<td>0.2 M potassium tetratrate, 40% (v/v) MPD</td>
<td>0.2 M di-ammonium tetratrate, 40% (v/v) MPD</td>
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<td>0.2 M sodium bromide, 40% (v/v) MPD</td>
<td>0.2 M K2HPO4, 40% (v/v) MPD</td>
<td>0.2 M (NH4)2HPO4, 40% (v/v) MPD</td>
<td>0.2 M tri-lithium citrate, 40% (v/v) MPD</td>
<td>0.2 M tri-sodium citrate, 40% (v/v) MPD</td>
<td>0.2 M tri-potassium citrate, 40% (v/v) MPD</td>
<td>0.2 M di-ammonium hydrogen citrate, 40% (v/v) MPD</td>
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<td>0.1 M citric acid pH 4.0, 10% (v/v) MPD</td>
<td>0.1 M sodium acetate pH 5.0, 10% (v/v) MPD</td>
<td>0.1 M MES pH 6.0, 10% (v/v) MPD</td>
<td>0.1 M HEPES pH 7.0, 10% (v/v) MPD</td>
<td>0.1 M Tris pH 8.0, 10% (v/v) MPD</td>
<td>0.1 M bicine pH 9.0, 10% (v/v) MPD</td>
<td>0.1 M citric acid pH 4.0, 20% (v/v) MPD</td>
<td>0.1 M sodium acetate pH 5.0, 20% (v/v) MPD</td>
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<td>0.1 M MES pH 6.0, 20% (v/v) MPD</td>
<td>0.1 M HEPES pH 7.0, 20% (v/v) MPD</td>
<td>0.1 M Tris pH 8.0, 20% (v/v) MPD</td>
<td>0.1 M bicine pH 9.0, 20% (v/v) MPD</td>
<td>0.1 M citric acid pH 4.0, 40% (v/v) MPD</td>
<td>0.1 M sodium acetate pH 5.0, 40% (v/v) MPD</td>
<td>0.1 M MES pH 6.0, 40% (v/v) MPD</td>
<td>0.1 M HEPES pH 7.0, 40% (v/v) MPD</td>
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<td>9</td>
<td>0.1 M Tris pH 8.0, 40% (v/v) MPD</td>
<td>0.1 M bicine pH 9.0, 40% (v/v) MPD</td>
<td>0.1 M citric acid pH 4.0, 65% (v/v) MPD</td>
<td>0.1 M sodium acetate pH 5.0, 65% (v/v) MPD</td>
<td>0.1 M citric acid pH 4.0, 60% (v/v) MPD</td>
<td>0.1 M MES pH 7.0, 65% (v/v) MPD</td>
<td>0.1 M Tris pH 8.0, 65% (v/v) MPD</td>
<td>0.1 M bicine pH 9.0, 65% (v/v) MPD</td>
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<td>10</td>
<td>0.1 M Tris pH 8.0, 65% (v/v) MPD</td>
<td>0.1 M MES pH 7.0, 65% (v/v) MPD</td>
<td>0.1 M sodium pH 6.0, 65% (v/v) MPD</td>
<td>0.1 M Tris pH 8.0, 65% (v/v) MPD</td>
<td>0.1 M HEPES pH 7.0, 65% (v/v) MPD</td>
<td>0.1 M Tris pH 8.0, 65% (v/v) MPD</td>
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<td>11</td>
<td>0.1 M tri-sodium citrate, 0.1 M HEPES pH 7.5, 10% (v/v) MPD</td>
<td>0.05 M MgCl2, 0.1 M Tris pH 8.5, 12% (v/v) MPD</td>
<td>0.02 M CaCl2, 0.1 M Na acetate pH 4.6, 15% (v/v) MPD</td>
<td>0.1 M lithium citrate, 0.1 M HEPES pH 7.5, 20% (v/v) MPD</td>
<td>0.2 M ammonium acetate, 0.1 M Na citrate pH 5.6, 15% (v/v) MPD</td>
<td>0.2 M Mg acetate, 0.1 M MES pH 6.5, 15% (v/v) MPD</td>
<td>0.1 M potassium acetate, 0.1 M HEPES pH 7.5, 30% (v/v) MPD</td>
<td>0.2 M tri-sodium citrate, 0.1 M HEPES pH 7.5, 15% (v/v) MPD</td>
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<tr>
<td>12</td>
<td>0.1 M imidazole pH 8.0, 20% (v/v) MPD, 10% (v/v) PEG 4000</td>
<td>0.1 M NaCl, 20% (v/v) MPD, 4% (v/v) PEG 4000</td>
<td>0.02 M CaCl2, 0.1 Na acetate pH 4.6, 30% (v/v) MPD</td>
<td>0.2 M ammonium acetate, 0.1 M Na citrate pH 5.6, 30% (v/v) MPD</td>
<td>0.2 M Mg acetate, 0.1 MES pH 6.5, 30% (v/v) MPD</td>
<td>0.5 M ammonium sulfate, 0.1 MES pH 7.5, 30% (v/v) MPD</td>
<td>0.5 M ammonium sulfate, 0.1 MES pH 7.5, 20% (v/v) MPD</td>
<td>0.1 M imidazole pH 8.0, 30% (v/v) MPD, 4% (v/v) PEG 4000</td>
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### D.5: Screen 5

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<tr>
<td>1</td>
<td>2 M AmSO₄, 2% PEG 400, 0.1 M Na Acetate pH 5.5</td>
<td>2 M AmSO₄, 10% glyceraldehyde, 0.1 M MgSO₄, 0.1 M imidazole pH 6.5</td>
<td>2 M AmSO₄, 5% PEG 400, 0.1 M MgSO₄, 0.1 M Tris pH 7.5</td>
<td>2 M NaCl, 5% PEG 400, 0.1 M MgCl₂, 0.1 M Na acetate pH 5.5</td>
<td>3 M NaCl, 5% isopropanol, 0.1 M Na acetate pH 5.5</td>
<td>2.5 M NaKPO₄, 5% isopropanol, 0.1 M Na acetate pH 5.5</td>
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<tr>
<td>2</td>
<td>2 M NaKPO₄, 2% PEG 400, 0.1 M imidazole pH 6.5</td>
<td>2.5 M NaKPO₄, 20% glyceraldehyde, 0.1 M HEPES pH 7.5</td>
<td>1 M NaKPO₄, 5% PEG 400, 0.1 M Na acetate pH 5.5</td>
<td>2 M Am citrate, 1% PEG 400, 0.1 M Na acetate pH 5.5</td>
<td>2 M Am citrate, 5% isopropanol, 0.1 M isopropanol, 0.1 M imidazole pH 6.5</td>
<td>2 M LiSO₄, 5% isopropanol, 0.1 M Na acetate pH 5.5</td>
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<td>3</td>
<td>2 M LiSO₄, 8% PEG 2000 MME</td>
<td>1 M LiSO₄, 15% PEG 400, 0.1 M Na acetate pH 6.5</td>
<td>0.75 M Am Citrate, 25% PEG 400, 0.1 M Na acetate pH 4.5</td>
<td>1.5 M AmSO₄, 12% isopropanol, 0.1 M Na acetate pH 6.5</td>
<td>2 M Am citrate, 5% PEG 400, 0.1 M HEPES pH 7.5</td>
<td>2 M LiSO₄, 5% isopropanol, 0.1 M Na acetate pH 5.5</td>
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<td>4</td>
<td>1 M Am Citrate, 15% isopropanol, 0.1 M Tris pH 8.5</td>
<td>2 M Na Formate, 25% PEG 3350, 0.1 M Na acetate pH 4.5</td>
<td>25% PEG 1500, 30% MDP, 0.1 M Na acetate pH 7.5</td>
<td>15% PEG 3350, 30% isopropanol, 0.1 M ammonium citrate, 0.1 M HEPES pH 7.5</td>
<td>10% PEG 3350, 30% MDP, 0.2 M ammonium citrate, 0.1 M sodium acetate pH 5.5</td>
<td>4% PEG 1500, 30% MDP, 0.1 M Na acetate pH 7.5</td>
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<td>5</td>
<td>10% PEG 1500, 30% isopropanol, 0.2 M LiSO₄, 0.1 M Na acetate pH 5.5</td>
<td>15% PEG 8000, 4% PEG 400, 0.1 M Na acetate pH 5.5</td>
<td>30% PEG 1500, 0% isopropanol, 0.1 M M Na acetate pH 7.5</td>
<td>20% PEG 1500, 30% MDP, 0.2 M isopropanol, 0.1 M HEPES pH 7.5</td>
<td>8% NaCl, 10% PEG 400, 0.1 M Na acetate pH 7.5</td>
<td>0.8% NaKPO₄, 20% PEG 400, 0.1 M HEPES pH 7.5</td>
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<td>6</td>
<td>20% PEG 3350, 25% PEG 400, 0.1 M NaCl, 0.1 M Tris pH 5.5</td>
<td>20% PEG 8000, 10% PEG 400, 0.5 M NaCl, 0.1 M Na acetate pH 5.5</td>
<td>20% PEG 1500, 10% isopropanol, 0.1 M Na acetate pH 7.5</td>
<td>20% PEG 8000, 1% PEG 400, 0.1 M Na acetate pH 7.5</td>
<td>1.4 M NaKPO₄, 10% PEG 400, 0.1 M Na acetate pH 4.5</td>
<td>1 M ammonium citrate, 1% PEG 400, 0.1 M Na acetate pH 4.5</td>
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<td>7</td>
<td>25% PEG 3350, 4% isopropanol, 0.1 M CaCl₂, 0.1 M HEPES pH 7.5</td>
<td>20% PEG 8000, 10% PEG 400, 0.5 M NaCl, 0.1 M Na acetate pH 5.5</td>
<td>20% PEG 2000, 10% isopropanol, 0.1 M M Na acetate pH 7.5</td>
<td>20% PEG 8000, 20% isopropanol, 0.1 M Na acetate pH 7.5</td>
<td>3 M Sodium Formate, 4% PEG 8000, 0.1 M Na acetate pH 7.5</td>
<td>0.8% ammonium citrate, 2% PEG 4000, 0.1 M Tris pH 8.5</td>
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<td>8</td>
<td>2 M NaCl, 12% PEG 1500, 1.5% PEG 400, 0.1 M Na acetate pH 5.5</td>
<td>3 M Sodium Formate, 0.5% PEG 400, 0.1 M Na acetate pH 7.5</td>
<td>1 M NaKPO₄, 0.5% PEG 400, 0.1 M HEPES pH 7.5</td>
<td>3 M Sodium Formate, 20% PEG 3350, 0.5% isopropanol, 0.1 M Na acetate pH 4.5</td>
<td>2 M NaCl, 5% PEG 4000, 0.1 M Na acetate pH 7.5</td>
<td>0.5 M NaCl, 15% PEG 8000, 0.1 M Na acetate pH 5.5</td>
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<td>9</td>
<td>0.3 M Na acetate, 25% PEG 2000 MME</td>
<td>0.2 M LiSO₄, 25% PEG 2000 MME</td>
<td>0.2 M MgCl₂, 25% PEG 2000 MME</td>
<td>0.2 M KBr, 25% PEG 2000 MME</td>
<td>2 M KSCN, 25% PEG 2000 MME</td>
<td>0.8 M Na acetate, 15% PEG 4000</td>
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<tr>
<td>10</td>
<td>0.2 M MgCl₂, 15% PEG 4000</td>
<td>0.2 M KBr, 15% PEG 4000</td>
<td>0.2 M KSCN, 15% PEG 4000</td>
<td>0.2 M KBr, 25% PEG 2000 MME</td>
<td>0.8 M Na acetate, 10% PEG 8000, 0.4% PEG 550 MME</td>
<td>0.8 M Na acetate, 15% PEG 4000</td>
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<td>11</td>
<td>0.2 M KSCN, 10% PEG 8000, 10% PEG 1000</td>
<td>0.8 M Na formate, 10% PEG 8000, 10% PEG 1000</td>
<td>0.3 M Na acetate, 8% PEG 20000, 8% PEG 550 MME</td>
<td>0.2 M LiSO₄, 8% PEG 20000, 8% PEG 550 MME</td>
<td>0.2 M KBr, 8% PEG 20000, 8% PEG 550 MME</td>
<td>0.8% Na formate, 8% PEG 20000, 8% PEG 550 MME</td>
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<td>12</td>
<td>1.6 M ammonium sulfate pH 8.0</td>
<td>1.6 M ammonium sulfate pH 8.0</td>
<td>1.6 M ammonium sulfate pH 9.0</td>
<td>1.6 M ammonium sulfate pH 9.0</td>
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